INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book. These are also available as one exposure on a standard 35mm slide or as a 17” x 23” black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6” x 9” black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600
Optimization and characterization of an in vitro bovine mammary cell culture system to study regulation of milk protein synthesis and mammary differentiation

Talhouk, Rabih Shakib, Ph.D.
The Ohio State University, 1988

Copyright ©1989 by Talhouk, Rabih Shakib. All rights reserved.
OPTIMIZATION AND CHARACTERIZATION OF AN IN VITRO BOVINE MAMMARY CELL CULTURE SYSTEM TO STUDY REGULATION OF MILK PROTEIN SYNTHESIS AND MAMMARY DIFFERENTIATION.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

Rabih S. Talhouk, B.S., M.S.

* * * * *

The Ohio State University


Dissertation Committee:

F. L. Schanbacher
D. N. Foster
G. H. Hines
J. L. Pate

Approved by:

Floyd Schanbacher
Adviser
Department of Dairy Science
DEDICATION

To my parents and my homeland
ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Floyd Schanbacher for his careful guidance and input throughout the course of these studies. His trust and patience allowed me the freedom of work and thought, his friendship and constructive criticism of the dissertation made me a better scientist. Thanks are due to members of my advisory committee, Drs. Douglas N. Foster, Charles H. Hines, and Joy L. Pate for their support and critical reading of the dissertation.

I am indebted for Ron Neiswander for his help in all the seemingly never ending ELISA assays, for teaching me proper and exact methodology and analytical techniques, and most of all for his friendship.

The friendship and support of Rick Goodman, Peggy Hiltner, Lois Palin, Pam Pattanjitivali, Vince Frazzini and Gamini Wickramatilake made my stay in Wooster a pleasant experience.

I am grateful for Barb Gerard for her expert help in typing the dissertation, and for the constant encouragement she provided. The services of Elke Kretzschmar and Frances Butts at the electron microscopy laboratory, and those of Margaret Latta at the photography
laboratory for preparing the electron and light photomicrographs are greatly appreciated.

I would like to extend my appreciation for the late Dr. A. El-Zein his enthusiasm, dedication and hope for a better future for Lebanon will always be cherished and remembered.

I am blessed by the never ending support and love of my parents, without them none of this would have been possible. The constant support and the frequent calls "to make sure everything is in place" of my brother Akram were a definite plus.

Finally, I am thankful for all the precious times I spent with my wife, Salma, her understanding, encouragement, and her seeing the good in all circumstances were the light at the end of the road. We got there.
VITA

February 20, 1959 ..................................... Born - Beirut, Lebanon

1981 .............................................................. B.S., American University of
Beirut, Beirut, Lebanon

1981-1983 ...................................................... M.S., American University of
Beirut, Beirut, Lebanon

1983-present ............................................. Graduate student, Ohio
Agriculture Research and
Development Center, The
Ohio State University,
Wooster, Ohio

PUBLICATIONS

by substratum and hormones of milk protein secretion in lactating

1988. Growth factor activity in secretions from the developing

from lactating bovine mammary cells grown in vitro of a partial

Secretion of lactoferrin and casein by bovine mammary cells


FIELD OF STUDY

Major field: Lactational Physiology
Minor field: Cell Biology
# TABLE OF CONTENTS

DEDICATION................................................. 11  
ACKNOWLEDGMENTS...................................... iii  
VITA............................................................... v  
LIST OF TABLES............................................. xi  
LIST OF FIGURES.......................................... xii  

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION .............................. 1</td>
<td></td>
</tr>
<tr>
<td>List of References ....................... 19</td>
<td></td>
</tr>
<tr>
<td>II. AN IN VITRO SYSTEM FOR LONG TERM CULTURE OF BOVINE MAMMARY CELLS: SYNTHESIS AND SECRETION OF CASEIN AND LACTOFERRIN ........... 26</td>
<td></td>
</tr>
<tr>
<td>Introduction ................................. 26</td>
<td></td>
</tr>
<tr>
<td>Materials and Methods.................. 28</td>
<td></td>
</tr>
<tr>
<td>Materials ............................... 28</td>
<td></td>
</tr>
<tr>
<td>Collection of mammary tissue and cell preparation .. 29</td>
<td></td>
</tr>
<tr>
<td>Cell freezing ........................... 32</td>
<td></td>
</tr>
<tr>
<td>Cell plating .............................. 33</td>
<td></td>
</tr>
<tr>
<td>Preparation of collagen gels .......... 34</td>
<td></td>
</tr>
<tr>
<td>Nuclear count method ................. 35</td>
<td></td>
</tr>
<tr>
<td>Media sampling and immunoprecipitation of radio-labelled milk protein .......... 36</td>
<td></td>
</tr>
<tr>
<td>ELISA assay for milk proteins .......... 38</td>
<td></td>
</tr>
<tr>
<td>Scanning electron microscopy .......... 40</td>
<td></td>
</tr>
<tr>
<td>Results ......................................... 41</td>
<td></td>
</tr>
<tr>
<td>Characteristics of primary culture preparations ........ 41</td>
<td></td>
</tr>
<tr>
<td>Growth and behavior of mammary cells on type I collagen ....... 42</td>
<td></td>
</tr>
<tr>
<td>Synthesis and secretion of milk proteins ........ 56</td>
<td></td>
</tr>
<tr>
<td>Effect of FCS on milk protein secretion in low density cultures .......... 58</td>
<td></td>
</tr>
<tr>
<td>Effect of plating density on $a_2,1$-casein and LF secretion .......... 58</td>
<td></td>
</tr>
</tbody>
</table>

vii
III. REGULATION OF MILK PROTEIN SYNTHESIS AND SECRETION IN BOVINE MAMMARY CELL CULTURE: EFFECT OF CELL SUBSTRATUM, AND STAGE OF THE MAMMARY DEVELOPMENT

Introduction ................................................................. 94
Materials and Methods ....................................................... 97
Preparation of bovine mammary cell culture .................. 98
Preparation of substrata .............................................. 99
Cell number determination ........................................ 100
Media sampling and analysis of 35S-Radio-labelled proteins ........................................ 102
Enzyme linked immunosassay ...................................... 104
Transmission Electron Microscopy .............................. 104
Results ........................................................................... 105
Performance of cell culture on plastic or on collagen. .......... 105
Growth behavior and total protein synthesis and secretion ........................................ 105
SDS-PAGE electrophoresis ......................................... 113
LF secretion and intracellular accumulation .................. 118
aS, l-casein secretion and intracellular accumulation .......... 121
Ultrastructural features .............................................. 128
Performance of cells embedded within a collagen gel
matrix or plated on Matrigel ........................................ 134
Growth behavior and total protein synthesis and secretion ........................................ 134
SDS-PAGE electrophoresis ......................................... 144
Regulation of LF secretion ........................................ 144
aS, l-casein secretion and intracellular/acinar accumulation ........................................ 151
Ultrastructural features .............................................. 157
Effect of cell stage, substratum, and FCS on LF secreted per cell ........................................ 166
Discussion .................................................................... 169
Morphogenesis .......................................................... 169
Regulation of total protein synthesis secretion .............. 178
Regulation of specific milk protein synthesis and secretion ........................................ 180
Lactoferrin .............................................................. 180
aS, l-casein ............................................................ 186
**IV. MORPHOLOGICAL AND FUNCTIONAL DIFFERENTIATION OF BOVINE MAMMARY CELLS ON FLOATING TYPE I COLLAGEN GELS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>198</td>
</tr>
<tr>
<td>Materials and Methods.</td>
<td>201</td>
</tr>
<tr>
<td>Materials</td>
<td>201</td>
</tr>
<tr>
<td>Preparation of bovine mammary cell culture</td>
<td>202</td>
</tr>
<tr>
<td>Responsiveness to prolactin.</td>
<td>203</td>
</tr>
<tr>
<td>Cell number determination.</td>
<td>204</td>
</tr>
<tr>
<td>Media sampling and analysis of $^{35}$S-Radiolabelled proteins</td>
<td>205</td>
</tr>
<tr>
<td>Enzyme linked immunoassay.</td>
<td>207</td>
</tr>
<tr>
<td>Transmission Electron Microscopy</td>
<td>208</td>
</tr>
<tr>
<td>Results</td>
<td>209</td>
</tr>
<tr>
<td>Morphology and total protein synthesis</td>
<td>209</td>
</tr>
<tr>
<td>LF and $\alpha_\beta_1$-casein synthesis and secretion in cells from a developing mammary gland</td>
<td>222</td>
</tr>
<tr>
<td>LF and $\alpha_\beta_1$-casein in mammary cell cultures from a lactating gland</td>
<td>233</td>
</tr>
<tr>
<td>Prolactin regulation of $\alpha_\beta_1$-casein and LF in lactating cells on floating collagen gels.</td>
<td>242</td>
</tr>
<tr>
<td>Discussion</td>
<td>247</td>
</tr>
<tr>
<td>List of References</td>
<td>264</td>
</tr>
</tbody>
</table>

**V. PARTIAL CHARACTERIZATION AND PURIFICATION OF GROWTH FACTORS IN PRIMIGRAVIDA BOVINE MAMMARY SECRETIONS FROM A DEVELOPING MAMMARY GLAND.**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>269</td>
</tr>
<tr>
<td>Materials and Methods.</td>
<td>272</td>
</tr>
<tr>
<td>Materials</td>
<td>272</td>
</tr>
<tr>
<td>Collection of mammary secretion.</td>
<td>273</td>
</tr>
<tr>
<td>Cell cultures.</td>
<td>274</td>
</tr>
<tr>
<td>Mitogen assay.</td>
<td>275</td>
</tr>
<tr>
<td>Liquid chromatography.</td>
<td>276</td>
</tr>
<tr>
<td>Enzyme linked immunoassay.</td>
<td>277</td>
</tr>
<tr>
<td>Radio-receptor competitive binding assay</td>
<td>277</td>
</tr>
<tr>
<td>Results</td>
<td>279</td>
</tr>
<tr>
<td>Growth factor activity at different stages of mammary development.</td>
<td>279</td>
</tr>
<tr>
<td>Fractionation of growth factors in mammary secretions</td>
<td>283</td>
</tr>
<tr>
<td>Competitive displacement of $^{125}$I-mEGF.</td>
<td>292</td>
</tr>
<tr>
<td>Physical and chemical characterization</td>
<td>292</td>
</tr>
</tbody>
</table>
Growth factor profiles in bovine colostrum 
and milk .................................................. 297
GF in bovine mammary cell culture conditioned 
medium .................................................. 300
Discussion .............................................. 304
List of References ................................. 318

VI. CONCLUSION ................................. 325

List of references ................................. 342

BIBLIOGRAPHY ................................. 345
<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effect of the substratum, stage of mammary development, and FCS on LF secretion on per cell basis by bovine mammary cells in culture</td>
<td>176</td>
</tr>
<tr>
<td>2. Sensitivity of growth promoting activities in decaseinated mammary secretions from a primi-gravida heifer at 7 month gestation, partially purified bMDGF-1 (from DEAE-Sepharose, Fig. 59B), and GF detected in mammary cell culture media to heat and trypsin/chymotrypsin treatment</td>
<td>295</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Photomicrograph of freshly prepared mammary acini minutes after plating.</td>
<td>43</td>
</tr>
<tr>
<td>2. Cell morphology and growth of cryopreserved bovine mammary cells on 100ul collagen.</td>
<td>45</td>
</tr>
<tr>
<td>Scanning electron microscopy (SEM). Day 2.</td>
<td></td>
</tr>
<tr>
<td>3. Cell morphology and growth of cryopreserved bovine mammary cells on 100ul collagen.</td>
<td>47</td>
</tr>
<tr>
<td>SEM on day 4 of culture.</td>
<td></td>
</tr>
<tr>
<td>4. Cell morphology and growth of cryopreserved bovine mammary cells on 100ul collagen.</td>
<td>49</td>
</tr>
<tr>
<td>SEM on day 10 of culture.</td>
<td></td>
</tr>
<tr>
<td>5. Photomicrograph of cryopreserved bovine mammary cells from a lactating mammary gland</td>
<td>52</td>
</tr>
<tr>
<td>cultured on 100ul collagen. Day 2.</td>
<td></td>
</tr>
<tr>
<td>6. Photomicrograph of cryopreserved bovine mammary cells from a lactating mammary gland</td>
<td>54</td>
</tr>
<tr>
<td>cultured on 100ul collagen. Day 4.</td>
<td></td>
</tr>
<tr>
<td>7. SDS-PAGE Autoradiograms of protein A-Sepharose immuno-</td>
<td>57</td>
</tr>
<tr>
<td>precipitated LF, αβ1-casein and β-lactoglobulin secreted by lactating bovine mammary cells.</td>
<td></td>
</tr>
<tr>
<td>8. Effect of FGS on milk protein secretion of</td>
<td>59</td>
</tr>
<tr>
<td>cryopreserved bovine mammary cells from a lactating gland</td>
<td></td>
</tr>
<tr>
<td>9. Effect of FGS on growth of cryopreserved bovine mammary cells from a lactating gland</td>
<td>60</td>
</tr>
<tr>
<td>cultured on 100ul collagen. Serum free medium.</td>
<td></td>
</tr>
<tr>
<td>10. Effect of FGS on growth of cryopreserved bovine mammary cells from a lactating gland</td>
<td>62</td>
</tr>
<tr>
<td>cultured on 100ul collagen. Medium supplemented with 15% FGS</td>
<td></td>
</tr>
<tr>
<td>(compare to Fig. 9)</td>
<td></td>
</tr>
</tbody>
</table>

xii
11. Effect of cell plating density and FCS on protein secretion by cryopreserved lactating bovine mammary cells grown on 100ul collagen .......... 65

12. Effect of the development stage of the mammary gland, cryopreservation and storage in liquid nitrogen on LF secretion into the media .......... 67

13. Effect of enzymatic digestion of bovine mammary tissue on mammary cell growth in culture (optimal digestion) .... 68

14. Effect of enzymatic over-digestion of bovine mammary tissue on mammary cell growth in culture ........ 70

15. Effect of enzymatic over-digestion of bovine mammary tissue on protein secretion in culture ........ 73

16. Photomicrograph of cryopreserved bovine mammary cells from a lactating gland on day 6 of culture on 100 ul layer of collagen ........................................ 107

17. Photomicrograph of cryopreserved bovine mammary cells from a lactating gland on day 6 of culture on 500 ul layer of collagen ........................................ 109

18. Effect of the substratum, mammary cell stage, and FCS on total TCA-precipitable proteins secreted by mammary cells in culture .......... 111

19. SDS-PAGE electrophoresis of total $^{35}$S-radiolabelled proteins secreted into the medium by bovine mammary cells from a lactating gland grown on plastic, 100 ul, and 500 ul collagen .............. 114

20. SDS-PAGE electrophoresis of total $^{35}$S-radiolabelled proteins secreted into the medium by bovine mammary cells from a developing gland grown on plastic, 100 ul, and 500 ul collagen .............. 116

21. Lactoferrin secretion by lactating and developing bovine mammary cells in culture in response to different extracellular matrices and FCS .......... 119

22. $\alpha_{s,1}$-casein secretion by bovine mammary cells in culture as affected by substratum, stage of mammary development, and FCS .......... 122
23. Total, secreted, and intracellular αs1-casein in cultures of lactating and developing mammary cells in response to different substrata and FCS........124

24. Transmission electron photomicrograph of mammary tissue from a lactating mammary gland at day 10 in culture.........................129

25. Transmission electron photomicrograph of mammary tissue from a developing mammary gland at day 10 in culture......................131

26. Photomicrograph of bovine mammary cells at day 6 in culture on Matrigel-4.................................................................135

27. Photomicrograph of bovine mammary cells at day 6 in culture on Matrigel-37.................................................................137

28. Photomicrograph of bovine mammary cells at day 6 in culture embedded within collagen..........................139

29. Cell population increase for bovine mammary cells embedded in collagen, cultured on 500 ul collagen, and on Matrigel-4..............142

30. Levels of TCA-precipitable 35S-labelled proteins in media of bovine mammary cells, as influenced by the substratum, FCS, and stage of mammary development.........................143

31. Profile of [35S] methionine labelled protein secreted into the media by lactating and developing bovine mammary cells grown in FCS free media on Matrigel...............................145

32. LF secretion by bovine mammary cells in culture as influenced by substratum (Matrigel or embedded in collagen), stage of mammary development, and FCS.................................147

33. Effect of substratum (Matrigel), stage of mammary development, and FCS on the intracellular/acinar accumulation of LF in cultures of bovine mammary cells.................................149
34. $\alpha_{s,1}$-Casein secretion by bovine mammary cells from a developing or a lactating mammary gland cultured (+/- FCS) on Matrigel or embedded within collagen. .................................................. 152

35. Intracellular/acinar accumulation of $\alpha_{s,1}$-casein in bovine mammary cells cultured on Matrigel. ...................... 155

36. Photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture, embedded within a collagen matrix. Two um cross sections. .......................................................... 158

37. Transmission electron photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture, embedded within a collagen matrix. .................................................... 160

38. Photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture on Matrigel-4 ............................................. 162

39. Transmission electron photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture on Matrigel-4 ............................................. 164

40. Effect of detachment and thickness of collagen substratum, mammary cell stage, and FCS on total TCA-precipitable $^{35}$S-methionine labelled proteins secreted by mammary cells in culture ................................................. 212

41. SDS-PAGE profile of $^{35}$S-labelled secreted proteins by bovine mammary cells on attached and detached collagen. .................................................. 215

42. Photomicrograph of bovine mammary cells from a developing gland on day 12 in culture on top of a 500 ul layer collagen. 2 um cross section. ............................................. 216

43. Photomicrograph of bovine mammary cells from a developing gland on day 12 in culture on top of a 500 ul layer collagen. 2 um cross section. ............................................. 218

44. Transmission electron photomicrograph of bovine mammary cells from a developing gland on day 12 in culture on top of a 500 ul layer collagen. ......................... 220
45. Photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. (X 1000) ............ 223

46. Photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. (X 400) ............ 225

47. Transmission electron photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. (X 9,000) ................................................................................ 227

48. Transmission electron photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. (X 21,000) ............................................................... 229

49. Regulation of LF secretion by bovine mammary cells from a developing gland in culture, in response to substratum detachment .......................... 232

50. Effect of detachment of the substratum on the regulation of αs,1-casein secretion by bovine mammary cells from a developing gland in culture .... 234

51. αs,1-Casein intracellular accumulation in cultures of bovine mammary cells from a developing gland in response to substratum detachment ......................... 235

52. Regulation of LF secretion by bovine mammary cells from a lactating gland in culture in response to substratum detachment. .......................... 237

53. Effect of detachment of the substratum on the regulation of αs,1-casein secretion by bovine mammary cells from a lactating gland in culture. .... 238

54. Intracellular levels of αs,1-casein in bovine mammary cultures from a lactating mammary gland. Effect of substratum detachment. .................................................... 239

55. α-Lactalbumin secretion by mammary cells from a lactating gland on 500 ul collagen in FCS free medium .............................. 241
56. Effect of Prl on α₁,1-casen, and LF secretion by bovine mammary cells on a 500 ul collagen, detached on day 6 of culture .......................... 245

57. Effect of mammary secretions, FCS, and mEGF on [³H]-thymidine incorporation into AKR-2B cells .................. 281

58. The mitogenic activity of skimmed mammary secretions assayed at 1% concentration taken at different stages of bovine mammary gland development ......................................................... 284

59. Ion-exchange chromatography of decaseinated mammary secretions from a 7 months primigravida heifer ........................................................................................................ 287

60. Gel-filtration chromatography of pooled fractions containing growth promoting activity (bMDGF-1 and bMDGF-2) eluting from CM-Sepharose .............................................. 290

61. Inhibition of [¹²⁵I]-mEGF binding to AKR-2B cells by skimmed mammary secretions from a 7 month primigravida heifer, partially purified bMDGF-1 (from DEAE ion exchange chromatography Fig. 59), and C) Receptor grade mEGF .............................................. 293

62. CM-Sepharose cation exchange chromatography of decaseinated mammary secretions: from colostrum milk ........................................................................................................ 298

63. Secretion of growth promoting activity and lactoferrin into the media by cultured bovine mammary cells cultured on different substrata ................................. 301
CHAPTER I

INTRODUCTION

The mechanisms controlling development of the mammary gland are complex. Development of the mammary gland starts in fetal life and undergoes structural, compositional, and functional changes during the lifetime of an individual. Anderson (1985) described the different stages of bovine mammary gland development and growth from fetal life up to puberty and through gestation. Briefly, in bovidae, the gland of a neonatal female is characterized by a prominent fat pad with limited branching of primary and secondary ducts, and an immature teat structure. During the period between birth and puberty, the gland grows at the same rate (isometric) as that of the rest of the body. At puberty, estrogen and progesterone synthesis and release from the ovaries initiates mammary ductular growth into the fat pad and stromal tissue of the mammary gland. This leads to a complex network of branching ductular processes, with a limited amount of lobulo-alveolar development. The major portion of mammary growth takes place during pregnancy, and is characterized by further ductular branching and extensive lobulo-alveolar development. Growth of the mammary gland and increase in secretory cell number continues through early stages of
lactation (Henderson et al., 1985). Involution, the regression of the mammary gland triggered by cessation of milk removal or weaning, is accomplished by loss of mammary epithelial cells and loss of alveoli and ductular termini. During involution, adipose cells are formed, and fat tissue replaces degenerating alveoli. The extent of degeneration varies between species; however, it has been suggested that secretory epithelial cells degenerate, while myoepithelial cells remain viable (Lascelles and Lee, 1978) and act as a framework that maintains the organization of the acinar structure and the remaining viable cells. Smith and Medina (1987) suggested the presence of a mammary stem cell population that initiated replenishment of mammary secretory cells during the recurring pregnancy and lactation (for reviews, see Forsyth, 1982; Knight and Peaker, 1982; Russo and Russo, 1987).

The development and differentiation of the mammary gland is an intricate process, occurring through combined interactions of several factors, including hormones, polypeptide growth factors (GF), and stromal/cell interactions (Forsyth, 1983; Dembinski and Shiu, 1987; Bissell and Hall, 1987). Lobulo-alveolar growth and increase in epithelial secretory cell number occurs during the latter part of pregnancy (Cowie, 1971), when extensive remodeling of the extracellular matrix is taking place. This increase in the number of mammary secretory epithelial cells during pregnancy is a major determinant of milk yield in lactation (Tucker, 1981).
Several hormones are involved in the development of the mammary gland. In fact, the mammary gland possesses receptors for estrogen, progesterone, prolactin, corticosteroids, and insulin (Knight and Peaker, 1982; Gloeckler et al., 1986). In vitro studies have shown that the presence of 3 major lactogenic hormones, prolactin, cortisol, and insulin in the cell culture media was essential for the survival and differentiation of the mammary cells in culture (Liotta et al., 1979; McGrath, 1987; Emerman and Pitelka, 1977).

The involvement of extracellular matrix in growth and survival of the mammary epithelium and the importance of this matrix in maintaining efficient milk protein synthesis and secretion by mammary epithelial cells was demonstrated by Wicha et al. (1979) and Wicha et al. (1982). Recently, numerous reports have identified GF in mammary tissue extracts (Kidwell et al., 1984) or secretions (Brown and Blakeley, 1983a; Noda et al., 1984) with some being unique to the mammary gland (Kidwell and Salomon, 1987). However, few reports address the question of the role these GF play in the development of the rodent mammary gland, while none relate to their role in bovine mammary development.

It is clear from the aforementioned that factors affecting basement membrane deposition are important in the regulation and maintenance of mammary epithelia. Bano et al. (1985) and Kidwell et al. (1984) showed
that GF can regulate basement membrane synthesis and deposition in the mammary gland, and that this, in turn, would be one mode of regulation for the mammary epithelial cells.

The responsiveness of the mammary gland to GF has been well documented. Turkington (1969) first demonstrated that epidermal growth factor (EGF), like insulin, stimulated DNA synthesis in mouse mammary epithelial cells in organ culture. Takotani and Oka (1983 a,b) reported that while EGF inhibited the hormonal effect of insulin, cortisol, and prolactin on the induction of casein and α-lactalbumin synthesis, it stimulated thymidine incorporation into DNA in five day mammary organ culture. They further demonstrated that the inhibitory effect of EGF on casein production was less pronounced in cultured mammary cells from mice at less advanced stages of pregnancy; however, the mitogenic effect was manifested equally in all respective stages. Thus, they suggested that the mitogenic activity of EGF on mammary cells and the inhibitory effect of EGF on casein production were not related. In an earlier study, Tonelli and Sorof (1980) demonstrated the requirement of EGF for lobulo-alveolar development of a completely regressed mouse mammary gland in whole organ culture. Moreover, Vonderhaar et al. (1983) reported that estrogen/progesterone priming of Balb/c mice stimulated production of an EGF-like GF by the mammary gland. This GF may in turn act synergistically with EGF produced by the submaxillary gland to promote lobulo-alveolar development in the mammary gland in vitro.
Dembinski and Shiu (1987) suggested that the insulin-like growth factor (IGF) family (including relaxin) and fibroblast growth factors (FGF) are involved in the development of the mammary gland.

The in vivo effects of GF on mammary gland development have not been as extensively studied as the in vitro effects. Ances (1973) reported that the plasma levels of EGF increased during pregnancy in humans. Van Noorden et al. (1977) and Byyny et al. (1974) found that the submandibular gland of lactating mice became enlarged, and contained increased concentrations of EGF. Indeed, the salivary glands are a major source of EGF in mice (Cohen and Savage, 1974). Okamoto and Oka (1984) demonstrated that sialoadenectomized virgin mice underwent normal pregnancy and delivery, but their mammary gland was 20-30% smaller than normal mice, produced less milk, and failed to support survival of their pups. The decreased mammary growth was manifested during the latter part of pregnancy. In contrast, EGF treated sialoadenectomized mice had mammary glands of normal size and normal pup survival (Okamoto and Oka, 1984). The above suggested that the submandibular gland product, presumably EGF, is important for lactogenesis and mammary development in mice. Whether EGF acts alone or coordinately with other GF synthesized by the mammary gland (Vonderhaar et al., 1983) is not known. Several reports have demonstrated that EGF is transported from the blood to the milk via transcellular or pericellular pathways (Blakeley et al., 1982; Gresik et al., 1984). Thus, the origin or the source of the GF in
mammary secretion appears to be from plasma as well as from the mammary gland itself. In fact, Rall et al. (1985) and Kidwell and Salomon (1987) identified mRNA in normal mammary tissue for transforming growth factor (TGF)-beta, TGF-alpha, and EGF.

From the preceding, it is clear that the mammary gland is a target organ for GF. Spitzer and Grosser (1987) purified EGF receptors from bovine mammary glands of lactating and non-lactating pregnant animals. The binding of EGF to its receptors was enhanced in pregnant animals. This increased binding in pregnant animals paralleled the stage of mammary epithelial cell proliferation during the latter part of pregnancy, as previously described by Cowie (1971).

Survival and growth of mammary epithelium in vitro is dependent upon its contact with its extracellular matrix substratum. Wicha et al. (1979) reported that rat mammary epithelia fail to survive when plated on a type I collagen substratum, but with an inhibitor (cis-hydroxyproline) to collagen deposition in the medium. Providing the appropriate cell substratum or extracellular matrix is an important factor during mammary development; it functions by controlling morphogenetic movements and tissue-tissue interactions (Bernfield et al., 1973; Vracko, 1974). In parallel, Wicha et al. (1980) and Liotta et al. (1979) reported that mammary gland involution is preceded by degeneration of the extracellular substratum (basement membrane). Thus,
factors affecting extracellular matrix deposition are critical for the viability of mammary epithelial cells.

The importance of the extracellular matrix and its relationship to the growth response triggered by EGF was demonstrated by Lembach (1976); serum requirement for the in vitro growth of human fibroblasts was replaced by ascorbic acid, mEGF, and mEGF binding arginine esterase (normally complexed with mEGF in vivo) in the medium. Ascorbic acid caused no growth response, but it was essential to enhance the production of hydroxyproline in collagen, necessary for extracellular matrix formation. In a later study, the role of EGF was further clarified. Primary cultures of rat mammary epithelia that were grown on type I collagen and stimulated by EGF and glucocorticoids synthesized basement membrane collagen (type IV) during proliferation. EGF stimulated amino acid incorporation into type IV collagen, while glucocorticoids suppressed the elaboration of type IV collagenolytic activity by the cells. However, the growth response to EGF or glucocorticoids depends on the substratum provided for the cells. Cells grown on plastic or type I collagen required EGF and dexamethasone for growth and attachment, while those grown on type IV collagen substratum showed little or no such dependence. This change in requirement is not a result of changes in the receptor number or affinity to EGF (Salomon et al., 1981). Similarly, Bano et al. (1985) isolated a mammary derived growth factor (MDGF1) from human milk, and showed that it also induced
collagen synthesis in mouse mammary epithelial cells in culture, and that this effect was attenuated when those cells were provided with their own matrix components. Mohanam et al. (1988) showed that EGF binding receptors on mammary cells are modulated by the extracellular matrix. As mammary cells invaded into new stroma, they made EGF, TGF-alpha, or other mammary derived growth factors that stimulated synthesis of basement components. Thus, the authors suggested that overproduction of basement membrane was regulated by a feedback loop wherein the mammary epithelial cells down regulated the number of their GF receptors and limited stimulation of the mammary cells by the GF.

Studies on the role of GF on bovine mammary development are limited to defining growth entities in bovine colostrum and milk. Brown and Blakeley (1983 a,b) identified a human platelet derived growth factor (hPDGF)-like in goat colostrum and suggested the presence of a similar activity in bovine colostrum. Campbell and Baumrucker (1988), Francis et al. (1986), and Francis et al. (1988) reported the presence of IGF's and their binding proteins in bovine colostrum and milk. Reports on the role or presence of GF in secretion from a developing bovine mammary gland prior to colostrum formation are lacking. Kidwell et al. (1984) reported the presence of a collagen stimulating activity in extract from bovine mammary tissue taken during the peak of rapid development (day 11-14) induced by an estrogen/progesterone treatment (Smith and Schanbacher, 1973). This period (day 11-14) is characterized by rapid
growth of the gland and by lobulo-alveolar development, and precedes colostrum formation. Similarly, Spitzer and Grosse (1987) demonstrated that levels of EGF binding receptors on bovine mammary cells were elevated at a similar period of normal bovine mammary development during gestation. Thus, the probable involvement of GF in affecting proliferation of bovine mammary cells is evident. Furthermore, the modulation of GF receptors on mammary cells during mammary development, and the effect of GF on remodeling the extracellular matrix, is strong evidence that both GF and the extracellular matrix are important elements that regulate development of the mammary gland.

The realization that the extracellular matrix is important in inducing and maintaining a differentiated state of mammary cells in culture was first reported by Emerman and Plitelka (1977). The authors demonstrated that mammary cells cultured on a floated type I collagen matrix maintained a long term differentiated function as measured by casein synthesis and secretion. In addition these cells assumed a cuboidal cellular morphology with ultrastructural characteristics typical of lactating secretory cells. In follow-up studies, Burwen and Pitelka (1980) deduced that the interaction of cells with collagen was the important determinant of a differentiated function. Since that first report, others (Wicha et al., 1982, and Parry et al., 1987) have utilized different types of matrices and have established (mostly for
rodent species) long term cultures capable of synthesizing and secreting milk proteins.

Reports in the literature stress the importance of the extracellular matrix on gene regulation in cultures of rodent mammary cells; however, different theories on the mechanisms by which the extracellular matrix regulates gene expression are proposed. Regulation of milk protein synthesis and secretion by mammary epithelial cells plated on floating collagen was demonstrated by Lee et al. (1984), Lee et al. (1985), and Rocha et al. (1985). The need for a flexible collagen substratum that allows the contraction of the gel, and thereby allows morphological changes in cell shape which trigger differentiation and casein secretion was demonstrated by Lee et al. (1984), and Haeuptle et al. (1983). In contrast, Wicha et al. (1982) utilized an inflexible extracellular matrix (prepared from rat mammary tissue) containing essential components found in vivo, and demonstrated successful long term culture of rat mammary cells with maintenance of a differentiated state characterized by casein, α-Lactalbumin, and lactose synthesis and secretion. α-Lactalbumin levels were 5 fold higher than that produced on a floating type I collagen. Later, Blum et al. (1987) and Li et al. (1987) demonstrated the superiority of an attached matrix, Matrigel, (a commercial preparation of extracellular matrix prepared from the Engelbreth-Holm-Swarm, "EHS", transplantable mouse tumor, Kleinman et al., 1986) over any other individual component of the Matrigel, i.e.,
laminin and heparan sulfate proteoglycan. In an attempt to clarify the above two observations, Parry et al. (1987) grew COMMA-D1 mammary cells on filter membrane well inserts, and showed that cells cultured on inflexible but floated filter membranes, without any extracellular components provided as substratum were able to synthesize their own extracellular matrix. They proposed that the free access of nutrients to the basal side of the cells allowed them to synthesize membrane components and differentiate in vitro. It should be noted that these authors used a mammary cell line; nonetheless, the idea that cells on floated collagen have access to nutrients from their basal side and thereby can polarize, synthesize their own basement membrane, and hence differentiate in culture, is appealing. In parallel, cells on an inflexible matrix, but provided with basement membrane components, could also differentiate and secrete caseins in vitro. The results imply that synthesis or availability of the proper matrix components is the limiting factor in achieving a differentiated culture, and not the contractility of the matrix per se. This further emphasizes the importance of the extracellular matrix on mammary cell function.

In addition to the regulation of casein gene expression at the transcriptional level by stromal substratum (Lee et al., 1985), Lee et al. (1987) demonstrated that mouse mammary epithelial cells prepared from a mid-pregnant gland synthesized and secreted transferrin. Furthermore, Blum et al. (1987) and Chen and Bissell (1987) demonstrated
that in mouse mammary epithelial cells transferrin synthesis and secretion is regulated at the transcriptional and post transcriptional level by the extracellular matrix. While Parry et al. (1987) suggested that transferrin is secreted from the basal as well as the apical side, contrary to casein, which is only secreted apically.

The effect of the extracellular matrix on mammary cells extends beyond regulation of the protein synthetic capability and ultrastructural organization of rodent mammary cells in vitro. The extracellular matrix also affects the multicellular organization of the mammary cells in culture; i.e., whether they are embedded in a type I collagen matrix (Yang et al., 1986), plated on Matrigel (Barcellos-Hoff et al., 1987; Blum et al., 1987), or on top of a type I collagen layer (Emerman and Pitelka, 1977; Neville, 1987). Hamamoto et al. (1988) cultured mouse mammary epithelial cells inside a type I collagen gel, and demonstrated that the cells/acini assume a three dimensional branching duct like structure and secrete casein into a formed lumen when exposed to lactogenic hormones. Similarly, Barcellos-Hoff et al. (1987) demonstrated that cells maintain their acinar structure and vectorially secrete casein into a formed lumen when mouse mammary epithelia are cultured on matrigel. However, cells on matrigel do not form branching duct-like structures like those embedded in collagen (Richards et al., 1982). In contrast to the three dimensional morphology described above, culturing cells on top of a type I collagen
layer induces restructuring of the acini with migration of cells to form a two dimensional confluent sheath-like layer of cells. These cells are able to synthesize and secrete casein when the matrix is floated in the presence of lactogenic hormones (Rocha et al., 1985; Neville, 1987).

It is evident that multicellular organization and mammary cell differentiation are regulated by the extracellular matrix, but are the two events mutually exclusive? Wiens et al. (1987) cultivated mouse mammary epithelial cells on 3T3 adipocytes and induced ductular morphogenesis, irrespective of whether milk proteins were synthesized or not, thus indicating that morphogenesis is not necessarily related to a functionally differentiated culture.

Efforts to successfully cultivate bovine mammary cells are minimal with no reports of long term maintenance of bovine cultures expressing normal mammary cell secretory functions. The first attempts to culture bovine mammary cells by Ebner et al. (1961), and later efforts by Anderson and Larson (1970) utilized glass as the substrate. The growth of the cells in culture was characterized by fibroblast overgrowth and loss of differentiated function and protein secretory ability after few days. More recently, however, MacKenzie et al. (1982) and MacKenzie et al. (1985) successfully cultured acini from the developing but not lactating bovine mammary gland. Growth patterns showed acinar restructuring into sheath-like layers when cultured on top of a type I
collagen matrix. Ultrastructural studies revealed the epithelial nature of these cells even after 24 days in culture. Cryopreservation did not favor the viability of fibroblastic cells over epithelial cells, and ultrastructural organization of cryopreserved cells was similar to that for cultures of fresh cell preparations. However, while neither preparation showed secretory vesicles and casein micelles, $\alpha$-lactalbumin secretion into the medium was evident in fresh preparations of mammary cells, but only after detachment of the type I collagen matrix. $\alpha$-Lactalbumin secretion was lost in preparations of cryopreserved cells.

Cryopreservation of primary bovine mammary cells is an advantage in the culture of these cells because of 1) abundance of mammary tissue, and hence, the ability to prepare and functionally define a large quantity of cells from a single animal, avoiding variability between different preparations, and 2) difficulty and economic impracticality of sacrificing dairy cows at desired stages of gestation or lactation. McGrath (1987) and MacKenzie et al. (1982) reported successful cryopreservation of mammary cells from a developing bovine mammary gland.

Recently, Shamay and Gertler (1986) and McGrath (1987) cultivated bovine mammary cells from a developing mammary gland within a collagen matrix. The cells in culture were responsive to insulin stimulation, and the authors proposed that the system was suitable to study growth
and development of bovine mammary cells in culture. However, neither author reported secretion of milk proteins by their cultures. Similar to mouse (Richards et al., 1982) and rat mammary epithelial cells (Yang et al., 1986), bovine mammary cells embedded in collagen form a three dimensional branching duct-like structure (McGrath, 1987). In contrast, bovine mammary cells cultured on glass (Ebner et al., 1961) or on type I collagen (MacKenzie et al., 1982) restructured their acinar structure and spread into a two dimensional sheath-like morphology. This suggested that similar to rodent mammary cells in vitro, bovine mammary cells in culture respond to extracellular matrix, at least in terms of their multicellular organization.

Larson (1976) introduced an important concept in the regulation of lactogenesis. He showed that cultures of bovine mammary cells produce $\beta$-casein, $\beta$-lactoglobulin, and orotic acid in the first 2-4 days in culture. In that early stage of culture $\beta$-lactoglobulin, but not orotic acid is feedback regulated, and its secretion is inhibited by addition of exogenous $\beta$-lactoglobulin into the medium. The concept of feedback regulation of milk protein synthesis or secretion was reintroduced later by Henderson et al. (1985) to account for the higher milk yield in thrice daily milking of goats. They suggested that the higher milk yield was due to more frequent removal of a feed-back inhibitor of milk secretion and/or growth of the mammary gland. Later the same group partially purified an inhibitor that accumulates in
milk and thereby depresses milk protein synthesis and secretion (Wilde et al., 1987). The inhibitor proved to be a 10,000-30,000 mol. wt. peptide and was not one of the normal milk proteins. It is not known whether the feedback regulation proposed by Larson (1976) and that of Wilde et al. (1987), involve either the same inhibitor or cellular regulation mechanisms of milk protein synthesis. Nevertheless, the idea that milk protein synthesis and secretion are feedback regulated by other proteins in the mammary secretions, and that milk proteins are coordinately expressed and secreted in vivo is very intriguing, and warrants further investigation as a major regulatory mechanism for the mammary gland. An in vitro model of ruminant mammary cell culture that would allow such studies is needed. Blum et al. (1987) and Lee et al. (1984) showed that at least the synthesis and secretion of different classes of milk caseins and α-lactalbumin are not coordinately regulated in cultures of rodent mammary cells.

Identification of the mammary secretory proteins and their function at the different stages of bovine mammary development, provides a firm basis of knowledge towards optimizing a culture system that will duplicate normal mammary function in vivo. Schanbacher and Smith (1975) and Hoppe (1976) determined the milk protein profiles at different stages of bovine mammary development. The authors concluded that an involuted gland is a "functional organ", and that mammary secretions are regulated according to the stage of mammary development. Lactoferrin,
an iron binding protein with a bacteriostatic function, is synthesized by bovine mammary cells, and is specifically expressed during periods of mammary involution, or pregnancy. On the other hand, α-lactalbumin, glycosyltransferase, and micellar casein synthesis and secretion increase during lactogenesis.

The knowledge available about the growth and development of the mammary gland, especially bovine, as well as the recent advances in rodent mammary cell culture systems suggest that establishing an *in vitro* model for bovine mammary cell culture is achievable. Such a system, if developed, would allow studies at the molecular and cellular levels not possible in whole animals. This will improve our understanding of critical steps in bovine mammary development and differentiation and the factors affecting milk protein secretion.

Thus, the objectives of this dissertation are:

1. To develop and characterize a bovine mammary cell culture system that is able to maintain long term normal mammary cell function (i.e., cell proliferation and milk protein synthesis and secretion).

2. To study and compare the effect of different cell substrata on the performance of cultured bovine mammary cells derived from a developing and/or lactating gland, in terms of:
   a. Regulation of milk protein synthesis and secretion.
b. Growth morphology and multicellular organization.

c. Ultrastructural morphology.

3. To test for the presence of growth promoting activity in mammary secretions during different stages of mammary development throughout gestation, and if such an activity exists:

   a. Determine its temporal pattern throughout gestation.

   b. Correlate the growth of the mammary gland to the determined pattern of the growth promoting activity.

   c. Attempt to partially purify and characterize the growth promoting activity in pre-colostric secretions from a mid-late developing mammary gland.
REFERENCES


CHAPTER II

AN IN VITRO SYSTEM FOR LONG TERM CULTURE
OF BOVINE MAMMARY CELLS:
SYNTHESIS AND SECRETION OF CASEIN AND LACTOFERRIN.

INTRODUCTION

The culture of ruminant mammary cells, especially bovine, has been more difficult than mouse or other rodents because of expense, difficulty of physiological staging, and quality and health of tissue available for collection. Cells from a lactating bovine mammary gland were first cultured on glass substrate by Ebner et al. (1961), and later by Groves and Larson (1965), and by Larson (1976). These early efforts to culture lactating bovine mammary cells on glass or plastic failed to maintain cellular function beyond 3-4 days in vitro and were unable to show regulated synthesis and secretion of specific milk proteins after that time. The culture of bovine mammary cells from a developing mammary gland, but not from a lactating one, was done on type I collagen by MacKenzie et al. (1982), and within a collagen matrix by McGrath (1987). Even with collagen substrates, these workers reported an inability to grow lactating bovine mammary cells with any significant...
retention of function. In contrast, Emerman and Pitelka (1977) showed that mouse mammary cells cultured on floated type I collagen assume a cuboidal shape and synthesize and secrete normal milk proteins. From this, the importance of extracellular matrix on the regulation of morphology and function of the mammary cell, as well as its effect on the expression of milk protein genes, has become apparent (reviewed by Bissel and Hall, 1987).

We describe here a method for the preparation and cryopreservation of bovine mammary cells from both lactating and developing mammary glands. Such cryopreserved bovine mammary cells prepared and grown on a thin (100 ul/1.0 cm well) layer of type I collagen in serum free medium, maintained the capacity for strong milk protein secretion for at least 14 days in culture. Cryopreserved bovine mammary cells secreted lactoferrin (LF) into the medium at levels comparable to LF secreted by a fresh preparation of the same cells. Mammary cells from a developing gland secreted more LF than those from lactating glands, as would be expected from the relative LF concentrations in secretions from the two developmental states of the mammary gland in vivo. Casein, but not LF secretion, from cryopreserved lactating cells was shown to be dependent on a critical cell density, below which no casein secretion was noted.
MATERIALS AND METHODS

Materials

Medium M199 with EBSS (Earl’s Balanced Salt Solution) and L-glutamine, bovine insulin (I), ovine prolactin (Prl, 31 I.U./mg), alpha-chymotrypsin (from bovine pancreas, type II, 40-60 units/mg), elastase (porcine pancreas, type IV, 70 units/mg), hyaluronidase (from bovine testes, type I-S, 300 units/mg), soybean trypsin inhibitor (STI, type I-S), and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical Company (St. Louis, MO). Cell culture plates were from Bellco (Vineland, N.J.) and fungizone from Gibco (Grand Island, N.Y.). Gentamycin and bovine fetal calf serum (FCS) were from M.A. Bioproducts (Walkersville, MD). Laminin was purchased from Collaborative Research Incorporated (Bedford, MA). Cortisol (F), progesterone and estrogen were from Steraloids Incorporated (Wilton, NH), and collagenase type III from Worthington Biochemicals (Freehold, NJ). L-\(^{35}\)S-methionine (sp. act. approx. 1100 Ci/m mole, in 2 umoles 2-mercaptoethanol/ml) was obtained from New England Nuclear (Boston, MA), and CL-4B sepharose Protein-A from Pharmacia Fine Chemicals (Uppsala, Sweden).
Collection of mammary tissue and cell preparation

Primary bovine mammary cells were prepared for culture by modifications of the procedures of Kraehenbuhl (1977) and Ebner et al. (1961). Mammary parenchymal tissue from Holstein dairy cows was obtained aseptically, within 5-10 minutes after slaughter, from either peak lactation or at mid-development on day 14-15 after hormonal induction of mammary gland development (Smith and Schanbacher, 1973). The tissue was immediately cut into 0.5-1 cm thick slices of approximately 10 gms each and immersed in sterile Hank's Balanced Salt Solution (HBSS) with gentamycin (50 ug/ml) and fungizone (2.5 ug/ml) at room temperature (to avoid solidification of lipids as recommended by MacKenzie et al., 1982) for transport to the laboratory.

Tissue selected for digestion (100-200 gms) was washed 2-3 times in fresh sterile HBSS with antibiotics as above, sectioned into 1-2 gm pieces, 15-20 gm/100 mm dish, and then minced into 1 mm³ pieces by slicing with opposing scalpel blades. The mincing of the tissue was done as rapidly as possible with care to prevent crushing. Typically, 100 gms of tissue was minced within 20-30 min. Prolonged periods of mincing has been shown detrimental to the eventual yield of functional cells.
Immediately upon completion of mincing, each dish of tissue (15-20 gms/dish) was transferred into a trypsinizing flask (Bellco Glass, Inc. N.J.) at room temperature, containing HBSS (5 mls/gm of tissue) with gentamycin as above. The minced tissue in the digestion flask was gently stirred until the mincing of the remaining tissue was complete. Typically, 100 gms tissue was minced for each digestion. The minced tissue was washed with HBSS by gentle stirring for 10 minutes, allowed to settle, and the supernatant discarded. The washing was repeated 2-3 times or until a clear, non-milky suspension was obtained. Gentamycin was supplemented at 50 ug/ml through all the washes and across the digestion interval. The mixture of enzymes used for tissue digestion was prepared in HBSS without Ca++ and Mg++ (5 mls/gm of original tissue weight) as follows: 3.6 mg (2000 units) collagenase, 10 mg hyaluronidase, 10 mg chymotrypsin, 0.2 mg elastase, 0.05 mg STI and 100 mg BSA (fraction V) per gm of tissue. The enzyme solution was added to the minced and washed tissue and incubated at 37°C for 90-120 minutes, on a temperature controlled rotary shaker (Lab. Line Environmental Shaker). For digestion, a spinner flask was used in our early studies with digestion performed in a water bath with constant gentle magnetic stirring for the same period of time; however, cell yield and uniformity of preparation was enhanced with rotary shaking in a trypsinizing flask.
The time of digestion was critical for good cell yield and function, but also depended on the stage of the gland from which the tissue was obtained. Care must be taken to avoid over-digestion of tissue. Visible criteria were the best indicators for monitoring the progress of digestion. Essentially, digestion was allowed to continue as long as "wisps" of digested tissue were still noticed to string from the larger pieces of tissue. After 10-20 minutes of digestion, the minced tissue fragments swelled and assumed a "grape cluster" appearance due to swelling and separation of lobules and acini. Soon thereafter, acini separated into thread-like structures (usually within the first 60-90 minutes). After most of the tissue dissociated into thread-like acinar aggregates, the digestion mix became turbid. At this point, digestion was interrupted and the digesta filtered through a sterile stainless steel sieve (200-500 um opening). The retentate was forced through the sieve with a glass pestle to facilitate the dispersion of the collagenous undigested material and the release of cells therefrom. The retentate was then recombined with the filtrate and digestion continued for another 15-30 minutes. At this stage, the digesta was monitored carefully by light microscopy for completeness of digestion, acinar integrity, and cell yield. Whenever the majority of the acini consisted of clumps of approximately 50-100 cells as estimated by microscopy, digestion was stopped by filtering again through the stainless steel sieves. The undigested retentate was discarded. The filtrate with acini, acinar fragments, and single cells was centrifuged at 100xg, 5
min, 4°C. The supernatant was discarded and the cells were washed 3-4 times by centrifugation at 100xg, 5min, 4°C, with M199 (1 cm³ of packed cell volume/10 ml of M199) supplemented with 5-10% FCS to insure complete removal of collagenase and other enzymes. Even minute amounts of residual collagenase will partially digest the type I collagen matrix upon which the cells are plated, and also degrade plated acini and acinar fragments into single cells, thus impairing attachment efficiency of the cell preparation. Finally, the cells were either plated as freshly prepared tissue in M199 supplemented with hormones (5 ug insulin/ml, 5 ug cortisol/ml and 8 ug prolactin/ml), laminin (1-2 ug/cm² growth area), gentamycin (50 ug/ml), and 15% FCS, or prepared for cryopreservation in liquid nitrogen for later use.

**Cell freezing**

Washed cells and acini were prepared for cryopreservation by resuspension in cryogenic medium (M199 with EBSS, 20% FCS, and 10% dimethyl sulfoxide, DMSO). Cells were frozen in liquid nitrogen at -196°C, at a density of 1-1.5x10⁷ in 1 ml/vial (Sarstedt, N.J.). Cell number was determined according to the procedure described by Ethier (1985), as outlined below. Because of the time consuming nature of this approach, it is recommended that cell number be determined during the washing steps on an aliquot of HBSS with cells taken after the first wash to allow determination of volume of cryopreservative medium needed.
later after washing. This avoids prolonged incubation at high cell densities which has proven detrimental to cell function. In another approach, cell number was conveniently estimated as follows: 1 ml of packed cells, centrifuged at 100 x g for 5 minutes, contained approximately 4-6x10^7 cells. Each ml of packed cells was resuspended in 4 ml of cryogenic medium.

The cells were suspended in cryogenic medium on ice with gentle stirring (sufficient to avoid injury but yet keep the cells suspended) and dispensed into 2 ml cryogenic vials as fast as possible. Incubation for long periods at 4°C with DMSO and high cell density was determined to be detrimental to bovine mammary cells. Vials with cells were placed in a -80°C freezer for 1-2 hrs and then transferred to liquid nitrogen for prolonged storage (MacKenzie et al., 1982).

**Cell plating**

For plating, each vial of cells was thawed rapidly in a 37°C water bath until free of ice crystals. Bovine mammary cells were sensitive to DMSO, and incubation at 37°C for longer than needed was detrimental. The cells were resuspended and washed in 25-30 ml of cold M199 with 10% FCS and then centrifuged at 100 x g, 5 min, 4°C. After a repeat wash, cells were resuspended in M199 supplemented with hormones, 15% FCS, laminin (1-2 ug/cm^2 of growth area), and gentamycin (50 ug/ml). Prior
to plating cells, nuclei were counted to confirm cell number. Typically, cells were plated at $2 \times 10^5$ cells/1.0cm well, unless otherwise indicated. FCS and laminin were provided to the culture only upon plating to facilitate cell attachment. FCS was then either depleted on day 2 at the first medium change (FCS free medium) or supplemented to the medium (FCS supplemented medium) according to the treatment desired. The medium was changed at 2 day intervals after that.

**Preparation of collagen gels**

The procedure for the preparation of rat tail type I collagen was essentially as described by Bornstien (1958) and as used for mouse mammary cell culture by Emerman and Pitelka (1977), and for bovine mammary gland acinar culture (Baumrucker, 1985). Rat tail tendons were cut into 2 mm pieces and dissolved by suspension in 17.5 mM acetic acid (20 mls per gm of original rat tail weight), for 48 hrs, 4°C, with gentle continuous stirring.

After centrifugation at 5000 x g, 30 minutes, 4°C, the pellet of undissolved tendon fragments was discarded. The supernatant collagen solution was recentrifuged at 5000 x g, 60 minutes, 4°C. The upper 1/3 of the supernatant collagen solution (excessively diluted) was discarded, while the lower 2/3 was saved for matrix preparation. This solution was stored at 4°C with gentamycin at 100 ug/ml. The protein
content of the final collagen solution preparation should be 1.0-1.5 mg/ml, as measured by the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL.). Concentrations lower than this resulted in poor gelation, while higher concentrations resulted in an optically opaque matrix. The solution could be used for up to 6 months. For gel formation, 105 uls of 10X HBSS, and 90 ul of 0.3N NaOH were added to 1.0 ml of type I collagen. The solution was kept on ice to avoid premature gelling of neutralized collagen solution. Neutralized collagen solution (100 ul/1.0 cm well; 24 well tissue culture plate) was dispensed with gentle swirling to allow uniform coverage of the bottom surface. After incubation for 30 minutes, 37°C to allow gelation, 1.0 ml of M199 supplemented with gentamycin was added to each well and incubated at 4°C overnight. The volume of 10X HBSS required to produce a firm matrix varied slightly with different batches of collagen. It must be adjusted for each batch accordingly.

**Cell count method**

The number of bovine mammary cells was determined by the method used with rodent mammary cells, described by Ethier (1985), for counting nuclei after detergent lysis of the cell. Briefly, prior to plating or cryopreservation, cells were pelleted from a known volume of medium by centrifugation (100xg, 4°C, 5 minutes), resuspended with vortexing in 2 mls of swelling buffer (1.5 mM MgCl₂, 0.01M hepes; PH 7.4), for 10
minutes, at room temperature. Two hundred ul of Ethylhexadecyl dimethyl ammonium bromide (Eastman Kodak Co., Rochester N.Y.) was added to lyse cells, but not their nuclei. The suspension was vortexed vigorously and incubated for 10 minutes, at room temperature. Nuclei were counted with a hemocytometer.

This method also allowed quantitation of cells plated in 24 well plates (1.0 cm wells) after release of cells from collagen. To do so, collagen with attached cells was rimmed from the sides of the well, digested in situ with 0.5 ml of M199 containing 1% collagenase, and STI (1 mg/ml) with gentle shaking for 30 minutes, 37°C. Well contents were aspirated and the well was washed with an equal volume of HBSS to remove any cells that remained attached. The wash was combined with the cells and centrifuged at 100 x g, 5 minutes, 4°C. The cells were then swollen, lysed, and free nuclei were counted.

Media sampling and immunoprecipitation of radiolabelled milk proteins

Medium was sampled at 2 day intervals by gentle aspiration with a Pasteur pipet and dispensed into plastic tubes containing leupeptin (1 ug), pepstatin (1 ug), and aprotinin (200 KIU) per ml of medium. Care was taken not to allow the pipet to touch the collagen layer since uncontrolled detachment of the collagen layer might occur. The aspirated
medium was centrifuged at 500 x g, 5 minutes, 4°C, to remove cell debris or unattached cells. The supernatant was then transferred to fresh tubes for storage at -20°C until analyzed. Each sample collected was a pool of the medium from three replicate wells, and each treatment was run in duplicate. Standard deviation was calculated for duplicates.

Synthesis and secretion of specific milk proteins was determined after radiolabelling with $^{35}$S-methionine and immunoprecipitation of radiolabelled proteins using the protocols of Suard et al. (1983) and Simmons et al. (1984). Prior to radiolabelling, the cells were washed twice with M199 with hormones (1 ml each), and then incubated in methionine free minimum essential medium (MEM, Sigma Chemical Co.) supplemented with hormones for 1 hr. at 37°C. Radiolabelling was initiated by the addition of $^{35}$S-methionine (50 uCi/0.5ml/well) for a period of 16 hrs. Labelling was stopped by the addition of 50 uls unlabelled methionine (4 mg/ml). The medium was then harvested, proteinase inhibitors added as before, centrifuged (500 x g, 5 min, 4°C), and stored at -80°C.

For immunoprecipitation of radiolabelled proteins, 300 uls H$_2$O and 200 uls 5X RIPA (10mM Tris, 0.15M NaCl, 5mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate and 0.1% SDS; PH 8.5) were added to each 0.5 ml of medium or cell lysate to obtain a 1X RIPA solution. To this, 20-40 ug of purified IgG fractions of antisera prepared in rabbits against LF, $\alpha_s$, $\alpha_l$-casein or
\( \beta \)-lactoglobulin was added and mixed by rocking for 3 hrs. at room temperature. Protein-A Sepharose beads (50 ul of a 1:1 dilution with \( \text{H}_2\text{O} \)) were then added, and mixing continued for another hour. Protein-A Sepharose was washed by microcentrifugation 6 times with 1 ml of 1X RIPA each, followed by 1 wash with 1 ml of 1X RIPA (without SDS and Na-Deoxycholate). For SDS-PAGE electrophoresis and autoradiography of radiolabelled proteins, Protein-A Sepharose beads were resuspended in 50 ul of 1X SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 0.2% SDS, 10% glycerol, and 2.5% mercaptoethanol) boiled for 2 minutes, and then 40 ul were loaded onto a Laemelli gel (3% polyacrylamide stacking gel, and 17.5% polyacrylamide separating gel) and resolved by electrophoresis. The gel was stained in coomassie blue, soaked in a minimal volume of Enhance (New England Nuclear, MA) for 1-2 hrs., followed by a 30 minute wash in water, dried, and exposed to X-ray film (XRP-1; Kodak, Rochester, N.Y.) at -80\(^\circ\)C.

**ELISA assay for milk proteins**

The concentrations of milk protein (\( \alpha_s,1 \)-casein, and LF) secreted into the culture medium, were quantitated by specific Enzyme-Linked Immunosorbent Assay (ELISA) with biotin/avidin/peroxidase coupling and color detection, essentially as described by Kendall et al. (1983) and Guesden et al. (1979), and using commercial avidin/biotin/peroxidase complex (ABC complex, Vector Laboratories, CA). These ELISA systems
allowed specific quantitation of the milk proteins LF, and $\alpha_{s,1}$-casein in cell culture medium, with a typical minimum detection limit of 10 ng/ml. $\alpha_{s,1}$-Casein and LF were routinely monitored by ELISA to determine culture performance.

Before ELISA assay, aliquots of culture medium were diluted with a 5X diluent optimized for each milk protein. Diluents (5X) for each of the protein ELISAs were: casein - 0.5 M Tris-HCl, 8.0 M urea, 4.2% NaCl, 0.1% NaN$_3$, 1% Dextran T-70, and 5% heat treated rabbit serum (HTRS), adjusted to pH 7.4; LF same as for casein, but with no urea or Dextran P-70. ELISA protein standards were prepared (10X diluent) in 20 mM Tris, 0.85% NaCl, 1% HTRS, 0.02% NaN$_3$, pH 7.4. For experiments with FCS in the culture medium, the diluent used for the standards was supplemented with FCS so that FCS final concentration was equal to that of FCS in the diluted samples. Both sample and standard diluent were stored at -20°C. For the ELISA assays, wells were coated by addition of 50 ul of the appropriate antibody dilution (10 ug IgG prepared from sheep/ml 0.05 M NaHCO$_3$, pH 9.5), with incubation for 1 hr., at room temperature. The well contents were then aspirated, and a blocking solution of 1% HTRS in TBS (20 mM Tris, 0.85% NaCl, pH 7.4) was added to the wells and incubated for 10 minutes, at room temperature. The wells were then washed 3 X with TBS. Dilutions of standards or sample (75 ul/well) were added to duplicate wells and incubated at room temperature, for 1 hr. for LF, or overnight for $\alpha_{s,1}$-casein. To each
well, after washing (3X with TBS) was added 75 ul of biotinylated IgG antibody (6 ug IgG/ml for LF, and 24 ug IgG/ml for casein) with incubation for 30 minutes (90 minutes for caseins), at room temperature. After washing (3X with TBS), freshly prepared ABC reagent was added (60 ul/well) and incubated for 15 minutes. The wells were washed again (5 X with TBS) to insure removal of excess and unbound ABC complex. Then, 100 ul of ABTS/peroxide (Sigma Chemical Co., MO) substrate was added to each well and incubated at room temperature until color developed, usually 15-90 minutes, depending on protein assayed. Absorbance was read at 405 nm (Bio-Tek EL310 ELISA plate reader, Burlington, VT.) once the desired color level was reached.

**Scanning electron microscopy**

Cells cultured on a 100ul type I collagen in a 1.0cm well were fixed in a solution of 3% glutaraldehyde, 2% paraformaldehyde, and 1.5% Acrolein, pH 7.3. The specimens were dehydrated, vacuum dried, and prepared for observation and analysis according to Liebens and Dehaven (1978).
RESULTS

Characteristics of primary culture preparation

From adaptations of procedures already described in the literature for primary cultures of mammary tissue from bovine and murine species, we have developed a procedure that yields reproducible amounts of acini, and for the first time, that synthesize and secrete milk proteins in culture for at least 14 days. Variations in cell yields between different cows were overcome by uniform handling of the tissue during the primary culture preparations, and careful monitoring of the stage and extent of tissue digestion.

Immediately after plating, the majority of the acini ranged in size from 50 to 100 μm (fresh or frozen preparation), and appeared as clumps of cells surrounded by a continuous membrane-like structure, with few of the acini lacking this membrane. This type of unbound clumps consisted of aggregates of cells, with the individual boundaries of each cell clearly evident (Fig. 1). A small percent of the cell population consisted of single cells scattered across the growth area provided. Mostly, cells that were surrounded by the membrane-like structure were
the ones that attached, synthesized and secreted milk proteins in culture. Clumps of cells lacking this membrane rarely attached and were washed out by day 2 of culture. Likewise, single cells had a poor attachment efficiency, and if they attached, they did not divide, and cytoplasmic granulation was followed by severe vacuolation and cell death.

**Growth and behavior of mammary cells on type I collagen gel**

The first indications of cell attachment and growth were noted within 24-48 hrs. after cell plating. The acini attached to the collagen matrix via projections sent out by the cell clumps that made contact with the matrix and anchored the cells in place (Fig. 2; scanning EM). Between day 2-6, depending on the cell preparation, cell spreading and formation of a single layer of cells surrounding the acini was noted. The diameter of this cell layer increased until a uniform layer of cells was evident between day 6-10 (Fig. 3 and 4). As cell spreading and migration proceeded, the original clump of cells (acinar structure) was reduced in diameter and virtually disappeared. The initial site of attachment remained evident, as a dense, darker area of cell growth. It appears that cell migration, in addition to cell proliferation, was involved in the morphological restructuring of acini during culture.
Figure 1. Photomicrograph of freshly prepared mammary acini minutes after plating. Note clumps of cells bound by membrane (+), and those lacking (−) the membrane. (100x).
Figure 1.
Figure 2. Cell morphology and growth of cryopreserved bovine mammary cells on 100ul collagen. Scanning electron microscopy (SEM) of bovine mammary cells on day 2 of culture. Note attachment and initiation of cell spreading from the original clump of cells (250x).
Figure 3. Cell morphology and growth of cryopreserved bovine mammary cells on 100ul collagen. SEM on day 4 of culture. Flattening of the original cell clump in Fig. 2, and cell spreading (250x).
Figure 4. Cell morphology and growth of cryopreserved bovine mammary cells on 100ul collagen. SEM on day 10 of culture. Cell clumps in Fig. 2 and 3 are not evident, and cells spread into a sheet layer (250x).
Figure 4.
During this restructuring of the acini distinct cell types were observed. The cell spreading from the acini, was initiated by a large polygonal type of cells (Fig. 5 and 6) that formed a single layer and spread to cover the growth surface. As cell spreading occurred this single initial layer was overlaid by another type of cells with spindle shaped fibroblast-like morphology. This top layer of spindle shaped cells appeared to migrate along with the underlying layer, however it did not overgrow it until contact was made with a neighboring population of spreading cells. At day 8-10 in culture (Fig. 13) a dense layer of spindle-like cells tightly packed together formed. At this stage, the large polygonal cells were not seen.

Spreading cells covered all the growth area, however the type I collagen (as cast in the wells) formed a curved meniscus at the outer boundaries frequently producing a thick layer of collagen at the periphery of this growth area. The growth and spreading of cells rarely covered that area of the well, and the few acini which attached there, had a pattern of growth that differed from the central growth area in the well. In addition to acinar remodeling and cell migration cell division was evident and cell population increased 2-3 fold across the culture period as determined by nuclei count. Typically, cryopreserved cells were plated at 2 x10^5 cells/well. The cell number at day 4 of an
Figure 5. Photomicrograph of cryopreserved bovine mammary cells from a lactating mammary gland cultured on 100 ul collagen in M199 with I, Frl. and F. 15% FCS was present in medium through the first medium change at day 2. Note cells spreading away from the cell clump on day 2 of culture. Large polygonal (P) type of cells are the first to migrate. (x 100).
Figure 5.
Figure 6. Photomicrograph of cryopreserved bovine mammary cells from a lactating mammary gland cultured on 100μl collagen in M199 with I, Prl. and F. 15% FCS was present in medium through the first medium change at day 2. Cells on day 4 of culture. Note migration of cells away (arrow head) from original cell acinus to overlay the polygonal type cells noted in Figure 5. (x 100).
FCS free culture was in the range of 0.5-1.0 \times 10^5 \text{ cells/well, and by day 14 this number increased up to 2-3 } \times 10^5 \text{ cells/well.}

**Synthesis and secretion of milk proteins**

Cryopreserved lactating bovine mammary cells synthesized and secreted major milk proteins when plated on 100 \mu l type I collagen gel at 2 \times 10^5 \text{cells/well in an FCS free medium. Immunoprecipitation of } ^{35}S\text{-methionine radiolabelled milk proteins in the medium at days 2, 4 and 6 of culture demonstrated the ability of these cells to synthesize and secrete LF, } \alpha_s,1\text{-casein and } \beta\text{-lactoglobulin (Fig. 7). Proteins secreted during the 14 day culture period showed increasing levels of LF synthesis, and a gradual loss of ability to secrete } \alpha_s,1\text{-casein beyond day 2. The drop in secreted } \alpha_s,1\text{-casein and } \beta\text{-lactoglobulin was not due to a release of entrapped intracellular pools of this protein, since the SDS-PAGE of immunoprecipitated } ^{35}S\text{-methionine radiolabelled cultures at day 2 and 4, showed that } \alpha_s,1\text{-casein and } \beta\text{-lactoglobulin were being synthesized and secreted (Fig. 7B and 7C). The decline in } \alpha_s,1\text{-casein levels secreted into the medium, when increasing amounts of LF were secreted throughout the culture period showed that secretory mammary epithelial cells in culture were viable, and able to maintain milk protein synthesis and for extended periods in culture.}
<table>
<thead>
<tr>
<th>Mol. Wt. (10^-3)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>92.5</td>
<td>LF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>66.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31.0</td>
<td></td>
<td>αs1-Cas</td>
<td></td>
</tr>
<tr>
<td>21.5</td>
<td></td>
<td></td>
<td>β-Lac.</td>
</tr>
<tr>
<td>14.4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Experimental Day**

6 2 4 2 4

Figure 7. SDS-PAGE Autoradiograms of protein A-Sepharose immunoprecipitated LF, αs1-casein and β-lactoglobulin secreted into medium by cultured bovine mammary cells (cryopreserved) from a lactating gland, in FCS free culture. Medium (M199 with I, Prl. and F) was used for immunoprecipitation of each protein. Panels: (A) Lactoferrin immunoprecipitated from 800 μl of media on day 6, (B) αs1-casein, from 200μl, on days 2 and 4 and (C) β-lactoglobulin, from 200 μl, on days 2 and 4.
Effect of FCS on milk protein secretion in low density cultures

Losing the ability to secrete $\alpha_s,1$-casein coincided with the withdrawal of FCS from the cultures. Hence, we studied the effect of FCS on the performance of cryopreserved cells from a lactating bovine mammary gland in culture. Figure 8 (A and B) shows that FCS in the medium increased LF secretion across the culture period, compared to LF secreted by cells in FCS depleted medium. Interestingly, FCS prevented the decline of $\alpha_s,1$-casein secretion that was noted when the cells were grown in an FCS depleted medium (Fig. 8B). Examining the differences in cell morphology, Fig. 9 and 10 show a more dense cell population with the fibroblast-like cells being the predominant population in day 8 cells grown with FCS (Fig. 9) vs. cells grown without FCS (Fig. 10).

Effect of plating density on $\alpha_s,1$-casein and LF secretion

The cell population density on day 6, in an FCS free culture (Fig. 10) was markedly different from the cell density in cultures with 15% FCS (Fig. 9). Thus we studied the effect of cell population density on the performance of the mammary cells in an FCS free culture. Cells were plated at the same density as in the above studies, i.e., at $2 \times 10^5$ cells per well (1x) and at $1 \times 10^5$, $4 \times 10^5$ and $8 \times 10^5$ cells/well. FCS was supplied upon plating and withdrawn on day 2, medium was sampled at 2 day
Figure 8. Effect of FCS on milk protein secretion of cryopreserved bovine mammary cells from a lactating gland. Cells were plated at 2×10⁵ cells/0.5ml/well on 100μl collagen (1.0 cm well) in M199 supplemented with lactogenic hormones (I, Prl. and F). 15% FCS was added up to day 2 (O), or up to day 10 (•). Concentration of (A) LF and (B) αs₁-casein secreted into the medium are shown as determined by ELISA.
Figure 9. Effect of FCS on growth of cryopreserved bovine mammary cells from a lactating gland. Cells were plated at $2 \times 10^5$ cells/0.5ml/well on 100ul collagen (1.0 cm well) in M199 supplemented with lactogenic hormones (I, Prl. and F). Photomicrograph showing the morphology of cells on day 6 of culture in FCS depleted medium.
Figure 9.
Figure 10. Effect of FCS on growth of cryopreserved bovine mammary cells from a lactating gland. Cells were plated at 2x10^5 cells/0.5ml/well on 100ul collagen (1.0 cm well) in M199 supplemented with lactogenic hormones (I, Prl. and F). Photomicrograph showing the morphology of cells on day 6 of culture in medium supplemented with 15% FCS up to day 10. Note abundance of spindle shaped cells and dense cell population (compare to Fig. 9).
Figure 10.
intervals, and αs,1-casein and LF were monitored by the ELISA. LF (Fig. 11A) synthesis and secretion seemed to increase in proportion to the increased cell plating density. In contrast, αs,1-casein secretion (Fig. 11B) was not detected in the cultures in an FCS depleted environment until the plating density exceeded 4x10⁵ cells/well. When FCS was supplemented to cultures with cell plating density exceeding 4 x10⁵ cells/well, αs,1-casein secretion was not affected (Fig. 11C).

**Effect of cryopreservation and cell stage on milk protein secretion**

The attachment efficiency of cryopreserved mammary cells from a lactating gland decreased by 40-50% compared to freshly prepared cells. It is the case for many cryopreserved cells to have reduced viability and/or plating efficiency. Freshly isolated mammary cells from a lactating gland plated on 100 ul type I collagen in FCS depleted medium maintained high levels of αs,1-casein secretion (>1 μg/ml) for at least 8 days in culture, and approximately 2-3 μg/ml of LF (data not shown). Whether the decrease of protein levels (casein and LF) in cryopreserved cells was a result of lower number of cell population, or a loss of protein synthetic capability on a per cell basis remains to be determined.

Freezing mammary cells and maintaining their normal mammary function after prolonged periods of storage is an advantage that allows the study
Figure 11. Effect of cell plating density and FCS on protein secretion by cryopreserved lactating bovine mammary cells grown on 100ul collagen. Medium consisted of M199 supplemented with lactogenic hormones (I, Prl. and F). ELISA assays were used to determine (Panel A) LF and (Panel B) \( \alpha_5 \)-casein concentration secreted in the medium of bovine mammary cells plated at \( 1 \times 10^5 \) (△), \( 2 \times 10^5 \) (●), \( 4 \times 10^5 \) (○), and \( 8 \times 10^5 \) (☐) cells/0.5 ml/well. Panel (C); \( \alpha_5 \)-casein concentration in the medium of cells plated at > \( 4 \times 10^5 \) cells/well in FCS free medium (○) or in 15% FCS medium (□).
of factors affecting mammary development and differentiation using the same source of cells. Even after periods of more than 1 year of storage in liquid nitrogen, the secretory function of the cells in culture was maintained. Figure 12 (A and B) shows that mammary cells maintained the ability to secrete LF after 16 months of storage in liquid nitrogen for cells from a lactating gland, and 5 months of storage for cells from a developing gland. In addition, the cells from a lactating mammary gland were able to secrete αs,1-casein as shown in Fig. 8 and 11 even after storage in liquid nitrogen for approximately 18 months. Comparing the performance of frozen cells from developing and lactating mammary gland at the same plating density, we note that cells from developing mammary gland secreted higher levels of LF than did cells from a lactating gland; the same pattern was also noted for freshly plated cells (Fig. 12A and 12B).

Remarks on the preparation of primary cultures:

Enzymatic dissociation of bovine mammary tissue: The interval of tissue dissociation differed significantly for tissue from different cows, and 10-15 minutes of over-digestion in the second hour of dissociation typically resulted in a majority of individual cells or small clumps of 5-15 cells that, although viable and able to attach, failed to synthesize and secrete milk proteins. Figure 13 and 14, show the different morphology of cells in culture at day 6. Cells from a
Figure 12. Effect of the development stage of the mammary gland, cryopreservation and storage in liquid nitrogen on LF secretion into the medium. Cells cultured in an FCS free medium (M199 with I, Prl. and F) on 100ul collagen at 5x10^5 cells/0.5ml/well. Cells from a lactating (Panel A) or a developing (panel B) mammary gland plated fresh ( ■ ■ ), after 1-2 months storage ( □□□□□ ) or after prolonged (> 5 months) ( □□□□□□□□□ ) storage in liquid nitrogen. LF concentration in the medium was determined by ELISA.
Figure 13. Effect of enzymatic over-digestion of bovine mammary tissue on mammary cell growth in culture. Cryopreserved bovine mammary cells from a lactating gland were plated at $5 \times 10^5$ cells/well on 100ul collagen and cultured in M199 with hormones (I, Prl. and F). FCS (15%) was supplemented to the medium up to day 2 only. Phase contrast micrograph of Cells on day 8 of culture from an optimally digested tissue. Note spindle shape morphology of cell population. (Compare to cell growth and morphology in Fig. 14).
Figure 14. Effect of enzymatic over-digestion of bovine mammary tissue on mammary cell growth in culture. Cryopreserved bovine mammary cells from a lactating gland were plated at $5 \times 10^5$ cells/well on 100μl collagen and cultured in M199 with hormones (I, Prl. and F). FCS (15%) was supplemented to the medium up to day 2 only. Phase contrast micrograph of cells on day 8 of culture from an over-digested tissue at day 8 of culture. The majority of cells are polygonal in shape. (Compare to cell growth and morphology in Fig. 13).
Figure 14.
properly digested mammary tissue (Fig. 13) have a spindle shape
morphology with multilayering of cells. On the other hand, Fig. 14
shows cells from an over digested mammary tissue preparation. The cells
typically appeared as polygonal and epithelial-like, with large nuclei.
However, their milk protein synthetic capability was impaired as shown
for LF in Fig. 15. These epithelial-like cells were a non functional
population which did not modulate their LF synthesis or secretion in
response to substrata, nor did these cells synthesize or secrete caseins
(results not shown).

Requirements for cell attachment: Attempts to plate the cells in
FCS depleted medium were unsuccessful. Supplementation of the plating
medium with fibronectin or transferrin (instead of FCS) at different
concentrations and combinations failed to induce attachment. In
contrast, 3.75 mg of BSA (fraction V)/ml of medium induced attachment of
mammary cells to the collagen matrix; the attachment efficiency was
improved when the cultures were supplemented with fibronectin (5 ug/cm²
growth area) and/or transferrin (50 ug/ml). Nonetheless, those cells
failed to secrete αS1-casein, and only secreted 10-20% (200-300 ng/ml)
of the LF secreted by cells cultured in an FCS free environment which
had been exposed to FCS upon plating.
Figure 15. Effect of enzymatic over-digestion of bovine mammary tissue on protein secretion in culture. Cryopreserved bovine mammary cells from a lactating gland were plated at $5 \times 10^5$ cells/well on 100ul collagen and cultured in M199 with hormones (I, Prl. and F). FCS (15%) was supplemented to the medium up to day 2 only. Lactoferrin secretion in the medium from bovine mammary cells from a (O) properly digested tissue (as in Fig. 13), or from an (•) over-digested tissue (as in Fig. 14).
DISCUSSION

The results obtained showed that mammary tissue from a lactating and a developing mammary gland can be successfully dissociated into acini and individual cells, and cultured on type I collagen. The acini and cells could be cultured either as freshly prepared or after cryopreservation (for more than 1.5 yrs.) without losing the capacity to synthesize and secrete milk protein. The dissociation procedure was a modification of procedures used by Ebner et al. (1961), and Krachenbuhl (1977). The first attempts to culture bovine mammary cells was reported by Ebner et al. (1961) who noted the existence of several types of cell growth; an observation similar to our findings, and that of Richards and Nandi (1978) and MacKenzie et al. (1985). An important difference between early cultures and current mammary culture methods is the substratum used for cell attachment. Ebner et al. (1961) utilized glass as a cell substrate. Surprisingly, their description of cellular attachment and spreading on glass was similar to what was more recently noted for bovine mammary cells (MacKenzie et al., 1982), and/or mouse mammary epithelial cells (Neville, 1987) cultured on type I collagen. Later studies by Groves and Larson (1965) and Larson (1976) demonstrated synthesis of major milk proteins by cultured bovine mammary cells from a lactating gland cultured on a glass substrate. However, these studies resulted in loss of milk protein synthetic capability after only 2-3
days of culture, suggesting that the mammary cells either reverted to a less differentiated state, or the cultures were overgrown by stromal cells.

The importance of the extracellular matrix/cell interactions in the development of the mammary gland was recognized by Daniel and DeOme (1965). But it was not until Emerman and Pitelka (1977) cultured mouse mammary epithelial cells on type I collagen, and floated the matrix, that a mammary epithelial cell culture able to maintain a differentiated function was achieved. Since the first demonstration of the role and importance of the extracellular matrix for the growth and maintenance of normal mammary function, several laboratories have utilized different types of extracellular matrices and demonstrated profound effects on function and morphology of rodent mammary epithelial cells in culture (Hamamoto et al., 1988; Blum et al., 1987; Levine and Stockdale, 1985; Parry et al., 1987).

In contrast, work on bovine mammary culture has been limited, and mostly involves mammary explant cultures (Goodman et al., 1983; Shamay et al., 1987). Explant cultures of bovine mammary cells, although easy to prepare, have a short life and carry-over of mammary effectors such as hormones, which make regulatory studies difficult. Anderson and Larson (1970), in a comparative study, showed that dispersed cell cultures of bovine mammary cells are superior to explant cultures for
studying milk protein synthesis and responsiveness of the cells to hormones.

MacKenzie et al. (1982) and MacKenzie et al. (1985), described the successful long term cultivation of bovine mammary cells from pregnant and non-pregnant heifers. They also reported the cryopreservation of bovine mammary cells. Both fresh and cryopreserved cells were cultured on top of a type I collagen layer. The authors demonstrated low levels of α-lactalbumin synthesis and secretion only in non-frozen cells, while they failed to demonstrate evidence for casein synthesis or micelles in ultrastructural studies. However, they did show that cryopreserved and fresh cells possessed microvillus structures, tight junctions and cellular morphologies and ultrastructures typical for epithelial and myoepithelial cells. Their efforts to culture cells from a lactating mammary gland were not successful. Similarly, by embedding cells within a type I collagen, McGrath (1987) reported successful long term culture (14 days) of fresh and cryopreserved mammary cells from pregnant, non-lactating dairy cattle. When embedded, the cells grew in a 3 dimensional branching duct-like morphology and stained positive for keratin, indicating their epithelial nature. However, no evidence was presented to demonstrate milk synthetic capabilities. Similar to MacKenzie et al. (1982), attempts to culture cells from lactating bovine mammary tissue (McGrath, 1987) were unsuccessful. MacKenzie et al. (1982) and McGrath (1987) suggested that this could be due to long
digestion intervals of more than 2 hrs. (up to 18 hrs.), and the sensitivity of lactating cells to cryopreservation. In our studies and others (Larson et al., 1965; Baumrucker, 1985) for cell preparations from lactating tissue, the digestion interval did not exceed two hours.

Attempts to digest lactating tissue preparations for periods appreciably longer than 2 hrs (i.e., 5-6 hrs or overnight) resulted in single cells or small clumps (3 or 4 cells) that had poor attachment efficiency. If the cells attached, they did not remain viable for more than 2 days. Similar observations were reported by Park et al. (1979). Henceforth, for obtaining viable cultures of lactating bovine mammary cells, we have restricted our digestion to no longer than 2 hrs at 37°C.

Other critical and limiting steps noted in our primary culture preparation method were: 1) The extent of tissue mincing prior to digestion. Pieces larger than 1 mm³ resulted in nonuniform dissociation. Stringing of the tissue commenced from the outside inwards, subjecting only released clumps to prolonged exposure to enzymes. This resulted in a high percentage of single cells, and reduced the yield of functional viable cells. 2) The use of $1 \times 10^{-6}$M EDTA for 2-3 minutes towards the end of digestion increased the number of single cells, at the expense of healthy viable acini. A similar observation was reported by MacKenzie et al. (1982). 3) Washing the cells during the culture with the use of centrifugal forces exceeding
200xg was detrimental. Centrifugation at 40-100xg was sufficient to harvest the total cell population. 4) During digestion, a gelatinous mass of tissue and cellular debris formed to varying degrees in different preparations, and trapped within it appreciable amounts of undigested tissue, affecting the final cell yield. This effect (also described by Park et al., 1979) was reduced with the use of a fluted tissue digestion flask instead of spinner flask for digestion. 5) Due to the difficulty and cost in obtaining functional and healthy mammary tissue from bovine sources, we adopted the use of digestion enzymes as described by Kraehenbuhl (1977) and MacKenzie et al. (1982), and we have not optimized the amounts or need of each of the enzymes used. However, limited and preliminary data indicated that the use of collagenase and BSA for dissociation of mammary tissue, as described in the Materials and Methods section, yielded viable acini that maintained their milk protein synthetic capability.

Cell yield per 100 gms of original wet weight of bovine mammary tissue varied between 8-16 x10^8 cells. Similar yields were obtained by McGrath (1987). The cell yield depended on stage of development of the mammary gland, site of tissue sample within the gland, and lactational status of animal. Gertler et al. (1982) and Shamay and Gertler (1986) reported wide variability between different animals for the response of bovine mammary explants to hormones and their ability to synthesize casein, but not between replicate cultures from the same mammary gland.
They attributed the variation to genetic differences or differing stages of mammary development. We have noted similar variations in our cultures. Protein secretion, namely $\alpha_\text{s,1}$-casein but not LF, differed substantially in mammary tissue obtained from different animals, but showed less variation within the same culture preparation. We have no evidence to claim that this variation is attributed to the differences between animals; however, we suggest that in addition to the possible variability among tissue from different animals, the size of tissue mincing prior to digestion and the extent of digestion played a critical role in the variability noted (Fig. 1, 13, 14, and 15).

The convenience of handling 100-200 gms of mammary tissue per digestion and then storing the cells in liquid nitrogen allowed a large number of experimental permutations using the same preparation of cells. The effect of cryopreservation on the mammary cells has not been examined in detail. MacKenzie et al. (1985) reported $\alpha$-lactalbumin secretion by fresh bovine mammary cells from a developing gland cultured on floated type I collagen. Cryopreserved cells, on the other hand, lost their ability to secrete $\alpha$-lactalbumin into the medium. In another study, Hansen et al. (1986), used short term cultures (4-8 hrs) of goat mammary acini and demonstrated the synthesis of specific milk fatty acids and the ability of the cells to respond to hormones, whether they were fresh or frozen. Viability of cryopreserved/rethawed cells was 60%, and the incorporation of labelled acetate into cellular lipids on a
per cell basis remained unchanged. In our studies, only 50-60% of cryopreserved cells attached as compared to fresh preparations of cells. Their protein synthetic capability in terms of LF secretion was 50-60% of that of the fresh cells (Fig. 12), suggesting no significant loss in the cellular synthetic capacity. We have also shown that cryopreserved cells can synthesize αs,1-casein for a period of at least 10 days (Figs. 8B and 11B); however, this was found to be a complex response dependent on a critical cell density in the culture. This observation confirmed the importance of cell/cell interactions required to initiate and maintain casein synthesis in rodent mammary culture as proposed by Levine and Stockdale (1985).

Although several types of extracellular matrices have been used for rodent mammary cultures (Bissell and Barcellos-Hoff, 1987; Parry et al., 1987; Wiens et al., 1987; Hamamoto et al., 1988), bovine mammary cultures have been limited to growing the cells on a type I collagen layer (Baumrucker, 1985; MacKenzie et al., 1982), glass (Groves and Larson 1965), or embedded within type I collagen (Shamay and Gertler, 1986; McGrath, 1987). Shamay and Gertler (1986) reported efforts to culture bovine cells on different substrata to assess cell growth. Their efforts were met with little success, and no description of the work was provided. Embedding of mammary cells in a type I collagen, whether from bovine (McGrath, 1987) or rodent species (McGrath et al., 1985), maintained the three dimensional structure, and duct-like
processes developed within a few days of culture. In contrast, plating the cells on top of a type I collagen substratum induced restructuring of the acini into a two dimensional sheet, that formed confluent multilayers of cells (MacKenzie et al., 1982). Nevertheless, Baumrucker (1985) reported that acini from lactating bovine mammary gland cultured on top of a type I collagen layer, maintained their spherical acinar structure, and remained intact irrespective of media treatment, for a period of at least 7 days in culture. Studies by Enami et al. (1985) showed that gel strength regulated the growth rate of mammary tumor cells cultured within a collagen matrix. This suggested that matrices of the same composition, but of different consistency, might regulate cell morphology; as do different extracellular substrata (Talhouk et al., 1989).

The effect of the extracellular matrix on the form and function of the mammary gland has been discussed by Bissell and Hall (1987) and Bissell et al. (1982). They hypothesized that the influence of the extracellular matrix is mediated through the organization of cytoskeletal components and interaction with the nuclear matrix of the mammary cell, and thereby modulates gene expression, intracellular protein trafficking and secretion, and perhaps multicellular organization. It is now clear from a number of studies that extracellular matrix affects gene expression for casein and α-lactalbumin, and their synthesis and secretion in murine mammary systems.
(Emerman and Pitelka, 1977; Blum et al., 1987). In addition, Weins et al. (1987) demonstrated that cytodifferentiation of murine mammary cells and the ability to respond to lactogenic hormones, was dependent on specific cell and/or matrix interaction, whereas growth and formation of ductular outgrowth was hormone independent. Recently, Chen and Bissell (1987) reported that transferrin, an iron binding protein synthesized by murine mammary cells (Lee et al., 1987), was always secreted into the medium irrespective of the cell substrata; however, the relative levels of transferrin secreted were regulated by the substrata.

LF, an iron binding protein synthesized and secreted by bovine mammary cells, is developmentally regulated, and is important in the defense of the mammary gland against microbial pathogens, especially during pregnancy or early involution of the mammary gland. LF concentrations in secretions from developing or involuting bovine mammary gland are 10-100x that for milk. Furthermore, for the bovine mammary gland and its secretions, high LF concentrations in the secretion usually indicate a nonlactating, undifferentiated, or injured mammary gland (for review, see Schanbacher and Smith 1975).

We have previously shown (Talhouk et al., 1986) that LF was regulated in cultures of bovine mammary cells by substratum and serum. The latter has been found to increase LF in the medium, whereas preparation of extracellular matrix from bovine tissue (Wicha et al.,
mixed with type I collagen (1:10) was found to suppress LF secretion when compared to the same cells plated on type I collagen. We also found that secretion of LF by cultures of mammary cells from a developing mammary gland was substantially higher than that of cells from a lactating gland, similar to in vivo mammary secretions obtained at the two different stages.

At a plating density of $2 \times 10^5$ cells/well on 100 ul type I collagen, bovine mammary cells from a lactating gland secreted LF into the medium for at least 2-3 weeks. This indicated that mammary secretory epithelial cells were viable and functional for the entire experimental period. The declining pattern of $\alpha_s1$-casein secretion into the medium resembled patterns noted by Groves and Larson (1965) when the cells were plated on glass.

The ability to maintain secretion of LF throughout the culture, but not $\alpha_s1$-casein, suggested that the multilayering of spindle-shaped cell types in cellular sheets was an indication of dedifferentiation of mammary epithelial cells and not a sign of stromal cell overgrowth and loss of functional epithelia. Plating of bovine mammary cell preparations which were predominately epithelial-like polygonal cells, as shown in Fig. 14, resulted in very low levels of LF secretion and no casein secretion. Cells were not viable in these cultures beyond day 7-10, and severe vacuolation was noted. This suggested (Levine and
Stockdale, 1985) the importance of maintaining different cell types in cultures of mammary cells to maintain their protein synthesis and secretion capabilities.

FCS in mammary cultures plated at low density induced $\alpha_{s,1}$-casein synthesis and secretion, in addition to increasing secretion of LF. The possibility that FCS induced $\alpha_{s,1}$-casein secretion due to the presence of lactogenic hormones (I, Prl. and F) is improbable, since hormone levels supplied in the culture medium were at least 100 fold greater than that contributed by 15% FCS in the medium (Hyclone Laboratories, Inc., Logan, UT). Growth factors in FCS are possible effectors of mammary cells; however, contradictory reports as to their involvement in mammary development and differentiation has been reported (Taketani and Oka, 1983; Silberstein and Daniel, 1987). Nevertheless, this possibility can not be ruled out, and further studies are required. An indirect effect of growth factors could be depicted for initiating casein secretion. Earlier studies by Ceriani (1976), Schwartz and Bissell (1977), and Bissell and Hall (1987) reported that an increase in the cell number of mouse mammary culture, COMMA-D1 cells or tendon cells, modulated production of extracellular matrix. In addition, Park et al. (1976 a,b) and Larson (1976) reported a linear relationship of the amount of milk protein synthesized per cell and the cell density in cultures of rat mammary cells. Thus, it is possible that growth factors and other mitogens in FCS could induce an increase in cell population,
and hence, modulate the production of extracellular matrix components. This could induce casein synthesis and secretion. Our studies clearly indicated that an increase in cell plating density (Fig. 11B and 11C) was able to alleviate the need for FCS in the culture except for days 0-2 (due to the requirement for FCS for cell attachment). In addition, maintaining FCS in the medium of low plating density (2 x 10^5 cells/well) cultures resulted in a more densely populated culture on day 6, as shown by Fig. 9. It was those densely populated cells that maintained their α_{s,1}-casein secretion. Thus, the ability of lactating bovine mammary cells to secrete α_{s,1}-casein in a completely serum free medium was dependent on a critical cell density below which the cells did not maintain α_{s,1}-casein secretion. Interestingly, while a threshold of cell density was required for α_{s,1}-casein secretion, a linear relation existed between cell plating density and LF secretion within the range studied. In addition, the cell number across the culture period (0-14 days) increased by 2-3 fold, while casein and LF levels varied independently of that pattern of cell growth. This indicated that the cells underwent a transition state in the culture that initiated LF secretion, α_{s,1}-casein secretion or the loss of it.

Efforts to eliminate FCS even at plating showed that bovine mammary cells in the absence of FCS did not attach. However, plating of cells without FCS, but with BSA (3.75 mg/ml), transferrin (50 ug/ml), and fibronectin (5 ug/cm^2), resulted in successful attachment, limited cell
spreading, and showed no signs of a stressed cell population, such as cytoplasmic vacuolation and/or granulation. These cells secreted no casein and only 200-300 ng of LF/ml of medium. The reason for the BSA (Fraction V) requirement is unknown, but may be attributable to impurities such as fatty acids or other factors which facilitate growth and attachment (Levay-Young et al., 1987).

Goodman et al. (1988) showed that bovine LF shares a 62% homology with the human transferrin gene in amino acid sequence derived from a partial cDNA clone reported to contain portions of an iron binding site. In addition, Adrian et al. (1986) reported that human transferrin gene contains a conserved sequence regulated by acute phase reactions. The spreading and migration of mammary cells on type I collagen and the remodeling of the acinar structure in vitro was initiated by a "serum spreading factor" (Neville, 1987). Thus, it is intriguing to speculate that the spreading and remodeling of the acinar structure induced LF synthesis and secretion by the bovine mammary cells, perhaps similar to in vivo situations, where the bovine mammary gland responds to injury by increasing the LF concentration in post-injury mammary secretions (Harmon et al., 1976).

In conclusion, we have demonstrated that $\alpha_{s,1}$-casein synthesis and secretion in cultures of lactating bovine mammary cells was dependent on a critical cell density, below which no $\alpha_{s,1}$-casein secretion was noted.
In contrast, LF secretion was not dependent on a critical cell density; instead, LF secretion increased linearly with the increase in cell plating. We have also established a bovine mammary cell culture system from a lactating gland on 100 ul type I collagen that maintained synthesis and secretion of α\textsubscript{\text{S-1}}-casein and LF for at least 10 days in a completely FCS free (except between day 0-2) defined medium. Such a culture system will allow studies of mammary development and differentiation to be done at the molecular and cellular levels that are not addressable in vivo. It is apparent that other major factors such as the different substrata, serum, and developmental state of mammary cell (lactating vs. developing) dramatically modulate cell performance and morphology. Hence, we are now able to prepare and culture cryopreserved lactating bovine mammary cells in large scale, and successfully culture these cells under conditions which permit synthesis and secretion of major milk proteins for at least two weeks. Thus, optimization of the culture will require detailed study of the effect of different cell substrata, serum, and developmental state of the cells.
REFERENCES


CHAPTER III

REGULATION OF MILK PROTEIN SYNTHESIS AND SECRETION IN BOVINE MAMMARY CELL CULTURE: EFFECT OF CELL SUBSTRATUM, AND STAGE OF THE MAMMARY DEVELOPMENT.

INTRODUCTION

The development and morphogenesis of the mammary gland, and the acquired response of the mammary epithelium to hormones is brought about by the interaction of the mammary cells with the surrounding mammary mesenchyme (Daniel and Silberstein, 1987, and Sakakura, 1987). The importance of the interaction of the mammary cell with its substratum in vitro was recognized by the pioneering work of Emerman and Pitelka (1977) on rodent mammary cells. Later work on the rodent system showed the effect of different substrata on milk protein gene expression (Li et al., 1987, and Blum et al., 1987) and mammary cell morphology (Hamamoto et al., 1988, and Bissell and Hall, 1987). Most of the work on the rodent mammary culture system utilized mammary cells from mid-pregnant animals; however, early work on bovine mammary cell culture utilized cells from a lactating bovine mammary gland (Ebner et al., 1961 and Larson, 1976). In contrast, the recent studies on bovine mammary cell
culture focused on the growth regulation of bovine mammary cells obtained from a mid-pregnant animal and embedded in collagen (McGrath, 1987), or grown on collagen (MacKenzie et al., 1982). Neither of the mammary bovine culture systems demonstrate successful long term maintenance of milk protein synthesis and secretion, nor is there a comparative study demonstrating the performance of bovine mammary cells on different substrata, and from different stages of pregnancy and lactation; hence this study.

We have previously described methods for the serum free culture of bovine mammary cells using cryopreserved cells prepared from developing and lactating mammary tissue. When plated on collagen, cells from a lactating gland maintained synthesis and secretion of $\alpha_{s,1}$-casein and LF for 10-14 days or longer. $\alpha_{s,1}$-Casein secretion but not LF required a minimum threshold of cell density, below which $\alpha_{s,1}$-casein secretion was lost in 2-4 days in serum free culture. However, serum prevented the loss of $\alpha_{s,1}$-casein secretion in mammary cells plated at low density. In contrast, serum increased the levels of LF secretion by these cells (Talhouk et al., 1989). In this report we describe the performance of cryopreserved cells prepared from lactating and developing bovine mammary gland and grown on different substrata: plastic, Matrigel, embedded within and cultured on collagen. The results showed that cryopreserved bovine mammary cells from both a developing and lactating mammary gland can be successfully cultivated in culture. Synthesis and
secretion of milk proteins as well as cell morphology were regulated by the substratum. Two distinctive patterns of cell growth were noted. Mammary acini on plastic or collagen spread and formed confluent cell sheets, while those embedded within collagen or on Matrigel maintained their acinar structure with no evidence of lumen formation. Mammary cells from developing and lactating gland secreted LF under all substratum conditions, but the relative amounts secreted were regulated by the substratum. Serum induced higher LF secretion. In addition cells from a developing gland secreted higher LF than did cells from a lactating gland.

In contrast, αs,1-casein was secreted into the medium only by mammary cells cultured on collagen, and was selectively induced in cells from a lactating gland. These data also suggest different pathways of secretion of LF and αs,1-casein.
MATERIALS AND METHODS

Materials

Medium M199 with EBSS (Earl's Balanced Salt Solution) and L-glutamine, bovine insulin (I), ovine prolactin (Prl, 31 I.U./mg), alpha-chymotrypsin (from bovine pancreas, type II, 40-60 units/mg), elastase (porcine pancreas, type IV, 70 units/mg), hyaluronidase (from bovine testes, type I-S, 300 units/mg), soybean trypsin inhibitor (STI, type I-S), and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical company (St. Louis, MO). Cell culture plates were from Belco (Vineland, N.J.) fungizone from Cibco (Grand Island, N.Y.). Gentamycin and bovine fetal calf serum (FCS) were from M.A. Bioproducts (Walkersville, MD). Laminin and Basement Membrane Matrigel were purchased from Collaborative Research Incorporated (Bedford, MA). Cortisol (F), progesterone and estrogen were purchased from Steraloids Incorporated (Wilton, NH), and collagenase type III from Worthington Biochemicals (Freehold, NJ). Trans-35S (sp. act. approx. 1100 Ci/m mole, in 10 mM 2-mercaptoethanol/ml) was obtained from ICN (Irvine, CA).
Preparation of primary bovine mammary cell culture

Primary cultures of bovine mammary cells were prepared (Talhouk et al., 1986; Talhouk et al., 1989, manuscript in preparation) by modifications of the procedures of Kraehenbuhl (1977) and Ebner et al. (1961). Briefly, acini, acinar fragments, and cells were prepared from 100-200 gms of lactating bovine mammary tissue (lactating cells) or from a mid-developing bovine mammary tissue (developing cells), obtained after hormonal induction (day 14-15) of mammary gland (Smith and Schanbacher, 1973), by enzymatic dissociation. The mixture of enzymes used for tissue digestion was prepared in HBSS without Ca** and Mg** (5 mls/gm of original tissue weight) and included the following enzymes: 3.6 mg (2000 units) collagenase, 10 mg hyaluronidase, 10 mg chymotrypsin, 0.2 mg elastase, 0.05 mg STI and 100 mg BSA (fraction V) per gm of tissue. After dissociation (90-120 minutes) was complete the cells were washed with HBSS and cryopreserved in liquid nitrogen at 1-1.5 x10^7 cells/ml/vial (Sarstedt, N.J.). Prior to the start of culture, cryopreserved cells were thawed at 37°C and were plated at 5-6 x10^5 cells/1.0 cm well in 0.5 ml M199 with insulin (5 ug/ml), cortisol (5 ug/ml), prolactin (8 ug/ml), and gentamycin (50 ug/ml) and supplemented with 15% FCS and laminin (1-2 ug/cm^2 of growth area). Laminin and FCS were supplemented upon plating to facilitate cell attachment. FCS was either withdrawn from the medium on day 2 (FCS free cultures) or
maintained through the entire experimental period (FCS supplemented medium). The resulting bovine mammary cells and acini were plated on the appropriate substratum as per each experiment.

**Preparation of substrata**

The cells were grown in 24 well plates on the following substrata: 1) Plastic, 2) 100 ul type I collagen, 3) 500ul type I collagen, 4) Embedded within type I collagen, 5) Matrigel, gelled at 37°C and incubated overnight at 4°C (Matrigel-4), 6) Matrigel gelled at 37°C, and cells plated on top directly after gelling (Matrigel-37). All of the above substrata except for 6) were prepared a day prior to plating and were incubated overnight at 4°C with 1ml of M199, and gentamycin per well (1cm² growth area). Before use, the culture plates were incubated for 30-60 minutes at 37°C, and then the medium was aspirated before plating the cells.

The procedure for rat tail type I collagen preparation was essentially as described by Bornstien (1958) and as used for mouse mammary cell culture by Emerman and Pitelka (1977), and for bovine mammary gland cell culture (Talhouk et al., 1989). Neutralized type I collagen solution (100 ul or 500 ul/1.0 cm well; 24 well tissue culture plate) was dispensed with gentle swirling to allow uniform coverage of the bottom surface. After incubation for 30 minutes, 37°C to allow
gelation, 1.0 ml of M199 supplemented with gentamycin was added to each well with incubation at 4°C overnight.

For cells embedded in collagen, only 200 ul of collagen was gelled and incubated overnight at 4°C, and then upon plating, 5-6 X10^5 cells were resuspended in 300 ul collagen, and added on top of the already gelled 200 ul of collagen. The added collagen (with the cells in it) was allowed to gel at 37°C for 15-30 minutes in a 5% CO_2 incubator, before 0.5 ml of medium was added on top.

Matrigel was prepared according to the manufacturer's procedure (Collaborative Research incorp., Wilton, NH) and dispensed at 100 ul/1.0 cm well to allow uniform coverage of the bottom surface.

Cell number determination

The number of bovine mammary cells was determined by the method used with rodent mammary cells (Ethier 1985). Briefly, prior to plating or cryopreservation, cells were pelleted from a known volume of medium by centrifugation (100xg, 4°C, 5 minutes), resuspended with vortexing in 2 mls of swelling buffer (1.5 mM MgCl_2, 0.01M hapes; pH 7.4), 10 minutes, at room temperature. Two hundred ul of Ethylhexadecyl dimethyl ammonium bromide (Eastman Kodak Co., Rochester N.Y.) was added to lyse cells but not their nuclei. The suspension was vortexed vigorously and incubated
for 10 minutes, at room temperature. Nuclei were counted with a
hemocytometer.

This method also allowed quantitation of cells plated in 24 well
plates (1.0 cm wells) after release of cells from collagen. To do so,
collagen with attached cells was rimmed from the sides of the well,
digested in situ with 0.5 ml of M199 containing 1% collagenase, and STI
(1 mg/ml) with gentle shaking for 30 minutes, 37°C. Well contents were
aspirated and the well was washed with an equal volume of HBSS to remove
any cells that remained attached. The wash was combined with the cells
and centrifuged at 100 x g, 5 minutes, 4°C. The cells from one well
were then resuspended in 3.0 ml of 10 mM Tris, 1 mM EDTA, and 0.1 M
NaCl, pH 7.4 (TEN), washed by centrifugation (100 x g, 5 min, 4°C), and
resuspended in 1.5 ml of TEN. One ml was aliquoted and stored at -20°C
with proteinase inhibitors (leupeptin 1 ug/ml, pepstatin 1 ug/ml, and
aprotinin 200 KIU per ml of TEN, for analysis of specific milk proteins
by ELISA; the remaining 0.5 ml was recentrifuged (100 x g, 5 min, 4°C),
the supernatant decanted and then the cells were swollen, lysed, and the
free nuclei counted. Two aliquotes from each sample were counted. Cell
number was determined from duplicate wells on days 4, 10, and 14.
MamA sampling and analysis of $^{35}$S-radiolabelled proteins

Medium was sampled at 2 day intervals by gentle aspiration with a Pasteur pipet and dispensed into plastic tubes containing leupeptin (1 ug), pepstatin (1 ug), and aprotinin (200 KIU) per ml of medium. The aspirated medium was centrifuged at 500 x g, 5 minutes, 4°C, to remove cell debris or unattached cells. The supernatant was then transferred to tubes for storage at -20°C until analyzed by ELISA. Each medium sample collected was a pool of the medium from four replicate wells, and each treatment was run in duplicate. Standard deviation was calculated for duplicates. Samples for analysis of intracellular milk proteins were collected in duplicates and stored as described in the section describing the cell count method. Prior to the analysis of the intracellular milk proteins the cells were sonicated twice for 15 seconds each, to insure rupture of cells and release of intracellular proteins.

Synthesis and secretion of proteins was determined after radiolabelling with Trans-$^{35}$S ($^{35}$S-methionine and $^{35}$S-cysteine) and by using protocols described by Suard et al. (1983). Prior to radiolabelling on day 10, the cells were washed twice with M199 with hormones (1 ml each), and then incubated in methionine-free minimum essential medium (MEM, Sigma Chemical Co.) supplemented with hormones for 1 hr. at 37°C. Radiolabelling was initiated by the addition of
$^{35}$S-methionine (50 uCi/0.5ml/well) for a period of 16 hrs. Labelling was stopped by the addition of 50 uls unlabelled methionine (4 mg/ml). The medium was then harvested, proteinase inhibitors added as before, centrifuged (500 x g, 5 min, 4°C), and stored at -80°C.

For analysis of $^{35}$S-radiolabelled proteins, equal volumes of medium were mixed 1:1 with 2X SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 0.2% SDS, 10% glycerol, and 2.5% mercaptoethanol) then boiled for 2 minutes. Forty ul samples were loaded onto a Laemmlli gel (3% polyacrylamide stacking gel, and 17.5% polyacrylamide separating gel) and resolved by electrophoresis. The gel was stained in coomassie blue, soaked in a minimal volume of Enhance (New England Nuclear, MA) for 1-2 hrs., followed by a 30 minute wash in water, dried, and exposed at -80°C.

The relative amount of $^{35}$S-radiolabelled proteins were determined by the total TCA precipitable protein method. Briefly, 20 ul of medium was spotted on a Whatmann 3MM filter paper (1 cm dia.). Then, an equivalent of 15% FCS or M199 was spotted on the filter paper to standardize the total amount of proteins between FCS free samples vs. FCS supplemented samples. The filters were air dried, washed with 3 mls of 10% cold TCA, and boiled for 5 minutes. This was followed by two 3 ml washes of cold TCA, one wash with 95% ethanol (3 ml), one wash with acetone (3 ml) and finally the filters were rinsed with 1 ml of ether. The ether was aspirated, and the filters were air dried. Seven hundred ul of protosol...
"dihydroxy acetone" (NEN, Boston, MA) was added, the samples heated at 55°C for 20 minutes, and 20 ul of glacial acetic acid was added, followed by 3.5 ml of scintillation cocktail. The 35S-radiolabelled proteins levels were determined by counting on a liquid scintillation counter.

Enzyme Linked Immunoassay

Specific milk proteins were determined in medium and cell lysates of bovine mammary cell cultures with biotin-avidin-horseradish peroxidase enzyme linked immunoassay (ELISA) using biotinylated antibody monospecific for bovine lactoferrin, a-casein and ß-lactalbumin (Talhouk et al., 1989; Cuesdon et al., 1979; Kendall et al., 1983).

Transmission electron Microscopy

Cells cultured on 500 ul collagen, embedded within collagen or plated on Matrigel-4 in 1.0 cm wells were fixed in a solution of 3% glutaraldehyde, 2% paraformaldehyde, and 1.5% Acrolein, in 0.1M phosphate buffer, pH 7.3. The specimens were washed with buffer and post-fixed in 2% osmium tetroxide in the same buffer for 3 hrs, and washed and stained overnight in 1% uranyl acetate. Samples were dehydrated and embedded according to Spurr, (1969), post-stained with 0.5% uranyl acetate and 0.1% lead and prepared for observation.
RESULTS

Mammary cells cultured on the different substrata showed two distinctive growth patterns: Those grown on plastic or on collagen gel restructured their acinar morphology by spreading to form a confluent sheet of cells (Fig. 16). In contrast, cells grown within (embedded) a collagen matrix, or on top of a Matrigel basement membrane maintained their acinar structure across the culture period (Fig. 26, 27, and 28).

The results describe the growth morphology and regulation of protein synthesis and secretion of cryopreserved mammary cells on A) substrata that cause loss of acinar morphology and formation of cell sheets (i.e., on plastic or on collagen surfaces; and B) on Matrigel basement membrane gel surfaces or embedded within a collagen gel.

Performance of cells cultured on plastic or on collagen

Growth behavior and total protein synthesis and secretion. Cells from a developing or lactating mammary gland grown on plastic or on a thin layer of collagen (100 ul/1.0 cm well), attached within the first 48 hrs. By day 2, cells migrated from the acini as single sheet of large cells that was overlayered by spindle shaped fibroblast-like
cells. This cell migration was accompanied by flattening of the acini until a multilayered sheet of spindle shaped cells was formed (Fig. 16) between days 6-8 (Talhouk et al., 1989). Similarly, cells grown on 500 ul collagen/1.0 cm well restructured their acinar morphology into sheets of cells (Fig. 17). However, at later stages in the culture (beyond day 6), growth of cells into the underlying collagen was observed.

Cell plating density on all substrata was 5-6 x 10^5 cells/0.5 ml/1.0 cm well. Typically, the attachment efficiency after cryopreservation and thawing of the primary preparation of cells was between 20-30% for cells cultured on 100 ul or 500 ul collagen. The attachment efficiency on plastic was lower and ranged between 15-20% (data not shown). The cell population increased 2-3 fold between days 4-14 in culture in an FCS free medium; whereas, for medium supplemented with 15% FCS, the increase ranged between 2-5 fold.

Total TCA-precipitable ^{35}S-labelled proteins secreted were virtually the same for cells from either a developing or a lactating mammary gland cultured in an FCS free medium on either plastic, 100 ul or 500 ul collagen. Cells supplemented with 15% FCS from a developing or a lactating mammary gland had higher levels of TCA precipitable ^{35}S-labelled secreted proteins than their FCS depleted counterparts (Fig. 18A). This increase in TCA precipitable ^{35}S-labelled proteins in the
Figure 16. Photomicrograph of cryopreserved bovine mammary cells from a lactating gland on day 6 of culture on 100 ul layer of collagen. Cells were plated at $5 \times 10^3$ cells/well/0.5 ml of M199 with Prl, I, and F.
Figure 17. Photomicrograph of cryopreserved bovine mammary cells from a lactating gland on day 6 of culture on 500 ul layer of collagen. Cells were plated at 5 x 10^5 cells/well/0.5 ml of M199 with Prl, I, and F. Note the difference in growth morphology between cells on 100 ul collagen (Fig. 16), and the predominance of spindle-shaped cells on the 500 ul collagen layer that appear to grow into the collagen.
Figure 18. Effect of the substratum, mammary cell stage, and FCS on total TCA-precipitable proteins secreted by mammary cells in culture. Lactating and developing bovine mammary epithelial cells labelled on day 10 of culture for 16 hrs, and the TCA precipitated medium was counted by liquid scintillation counting. A) TCA precipitable values of secreted proteins by cells from lactating or developing mammary gland cultured on plastic, 100 ul and 500 ul collagen. Medium consisted of M199 with Prl, I, and F with 15% FCS (■■■), or 0% FCS (□□□). B) Increase in TCA precipitable values of secreted proteins in the medium relative to the increase in cell number as a result of supplementary FCS to the medium. The histograms represent the fold increase in cell number ([□□□]) to that of TCA precipitable proteins in the medium ([■■■]) for developing or lactating mammary cells cultured on plastic, 100 ul or 500 ul collagen.
Figure 18.
medium was not closely correlated with the increase in cell number (Fig. 18B). This indicated that FCS enhanced the secretory ability on a per cell basis. On the other hand, for cells grown in medium supplemented with 15% FCS, those on plastic had higher levels of total TCA precipitable $^{35}$S-labelled proteins secreted in their medium than cells grown on 100 ul or 500 ul collagen.

**SDS-PAGE electrophoresis.** Equal volumes of medium from cultures on each substratum were loaded on SDS-PAGE gels. After electrophoresis several protein bands were prominent; however, the bands corresponding to the mol wt of the specific milk proteins (LF, $\alpha_s$-casein, $\alpha$-lactalbumin, and $\beta$-lactoglobulin) were minor or absent. Cells from a lactating bovine mammary gland (Fig. 19) showed different SDS-PAGE profiles in proteins from medium of cells on plastic than cells on 100 ul or 500 ul collagen. A 45,000 mol wt band was common to cells cultured on the three different substrata; however, it was mostly evident in the medium of cells on plastic. In contrast, two bands of mol wt 77,000 and 86,000 were expressed and secreted by cells on 100 ul and 500 ul collagen, but not by cells on plastic. On the other hand, the SDS-PAGE profile showed no marked differences in proteins of medium from cells from a developing mammary gland grown on plastic or 100 ul or 500 ul collagen gel (Fig. 20), with two major bands appearing at 45,000, and 77,000 mol wt. Among the specific milk proteins, the 77,000 mol wt band is of a similar mol wt to LF (77,000). As mentioned, this band was
Figure 19. SDS-PAGE electrophoresis of total $^{35}$S-radiolabelled proteins secreted into the medium by bovine mammary cells from a lactating gland grown on plastic, 100 ul, and 500 ul collagen. Cells were radiolabelled on day 10 in culture for 16 hrs with $^{35}$S-methionine, and the proteins of the culture medium resolved on 17.5% developing mammary cells and grown on the different substrata.
LACTATING

Figure 19.
Figure 20. SDS-PAGE electrophoresis of total $^{35}$S-radiolabelled proteins secreted into the medium by bovine mammary cells from a developing gland grown on plastic, 100 ul, and 500 ul collagen. Cells were radiolabelled on day 10 in culture for 16 hrs with $^{35}$S-methionine, and the proteins of the culture medium resolved on 17.5% developing mammary cells and grown on the different substrata.
Figure 20.

DEVELOPING

Plastic 100 µl 500 µl Collagen
evident in SDS-PAGE patterns of medium from cells derived from either developing or lactating glands and grown on 100 ul, 500 ul and plastic. No such band was noted from medium of lactating cells cultured on plastic. No bands with a mol wt similar to $\alpha_{s,1}$-casein, $\alpha$-lactalbumin or $\beta$-lactoglobulin were noted in the SDS-PAGE profile of medium from cells of a lactating or a developing gland.

**LF secretion and intracellular accumulation.** Lactating bovine mammary cells cultured in an FCS free medium secreted LF (Fig. 21A and 21B). Cells cultured on plastic secreted less LF than cells on 100 ul collagen (thin layer) with the highest LF levels secreted by cells on 500 ul collagen (thick layer) (Fig. 21A). This pattern of regulation did not change when the medium was supplemented with 15% FCS (Fig. 21B). FCS in the medium increased levels of LF secreted in the medium for each substratum provided for the lactating cells (Fig. 21A vs 21B).

Developing bovine mammary cells secreted higher levels of LF than lactating bovine mammary cells when cultured on the same substratum (Fig. 21). In addition, the regulation of LF secretion by developing mammary cells was different from that of lactating mammary cells. In FCS free cultures, developing mammary cells on plastic secreted less LF than cells on collagen. No significant difference was noted in levels of secreted LF by cells on 100 ul or 500 ul collagen (Fig. 21C). On the other hand, when 15% FCS was supplemented to the medium, the effect
Figure 21. Lactoferrin secretion by lactating and developing bovine mammary cells in culture in response to different extracellular matrices and FCS. Bovine mammary cells were cultured on plastic (O), 100 ul (●), and 500 ul (△) collagen, in M199 supplemented with lactogenic hormones Prl, I, and F. Medium was changed every 2 days, and assayed for LF concentration by ELISA. LF secretion by lactating cells grown in A) FCS free medium, B) grown with 15% FCS in the medium. C) LF secretion by developing cells in FCS free medium, or D) with 15% FCS in the medium.
Figure 21.
of the substratum on the regulation of LF secretion was abrogated (Fig. 21D). Nevertheless, cells plated on the same matrix secreted higher levels of LF in FCS supplemented medium than in FCS depleted medium.

Intracellular accumulation of LF in cultured mammary cells was minimal and rarely exceeded 150 ng/ml. This was irrespective of the levels of LF secreted into the medium. Intracellular levels of LF were not dependent on, nor responsive to the extracellular matrix (plastic vs. collagen), the stage of the cell (developing vs. lactating), or the presence or absence of FCS. From the comparison of secreted vs intracellular LF, almost all (>90%) of the LF synthesized was secreted by the cells in culture.

\( \alpha_s,1\text{-casein secretion and intracellular accumulation} \). Concentrations of secreted and intracellular \( \alpha_s,1\text{-casein} \) were determined throughout the culture period for lactating and developing mammary epithelial cells (Fig. 22 and 23). For lactating cells (Fig. 22A and 22B) \( \alpha_s,1\text{-casein} \) regulation was similar to that of LF with the lowest \( \alpha_s,1\text{-casein} \) levels secreted by mammary cells on plastic, and the highest by mammary cells on 500 ul collagen. FCS slightly enhanced \( \alpha_s,1\text{-casein} \) secretion by mammary cells on plastic and on 100 ul collagen. No effect was noted for cells on 500 ul collagen. In marked contrast to LF, casein secretion by lactating mammary cells declined during the first 4-6 days of culture, irrespective of the substratum on which the cells
Figure 22. $\alpha_s\beta$-casein secretion by bovine mammary cells in culture is affected by substratum, stage of mammary development, and FCS. Medium from mammary cells on plastic (O), 100 ul (●), and 500 ul (△) collagen were assayed for $\alpha_s\beta$-casein by ELISA, at 2 day intervals. Medium consisted of M199 with Prl, I, and F. A) Lactating cells in FCS free medium, or B) supplemented with 15% FCS. C) Developing cells in FCS free medium, and D) in medium supplemented with 15% FCS. Note differences in scale of A) and B) vs C) and D).
Figure 22.
Figure 23. Total, secreted, and intracellular $\alpha_s$-casein in cultures of lactating bovine mammary cells (A and B) or developing (C and D) mammary cells in response to different substrata and FCS. Cells and medium were collected on days 4, 10, and 14 for analysis of $\alpha_s$-casein concentration by ELISA. Lactating mammary cells grown in medium either A) FCS free or B) supplemented with 15% FCS. Developing mammary cells grown in medium in either C) FCS free, or D) supplemented with 15% FCS. $\square$, secreted $\alpha_s$-casein; $\square$, total $\alpha_s$-casein (secreted + intracellular).
Figure 23.
were grown. After this period, $\alpha_s,1$-casein levels either continued to decline (Fig. 22A, plastic), or by days 6-8 of culture, the cells again began to synthesize and secrete $\alpha_s,1$-casein (Fig. 22A and 22B). At this stage in culture, the ability to re-initiate $\alpha_s,1$-casein synthesis and secretion was dependent on the substratum. The period between day 6-8 correlated with the formation of a uniform sheet of cells and nearly complete restructuring of the acini. LF, on the other hand, was secreted in increasing amounts during the culture period and, in most cases, plateaued at day 10-14 (Fig. 21), regardless of the substratum and/or developmental stage of the cells.

Developing bovine mammary cells (Fig. 22C and 22D) secreted $\alpha_s,1$-casein in culture. In contrast to lactating mammary cells, $\alpha_s,1$-casein secretion was not influenced by the substratum on which the cells were grown. The levels of $\alpha_s,1$-casein in the medium peaked at day 2 in culture, with a gradual decline in $\alpha_s,1$-casein secretion thereafter to only 10-50 ng/ml at day 14 in FCS free culture (Fig. 22C). Supplementing the medium with 15% FCS did not change the pattern of $\alpha_s,1$-casein secretion into the medium, however, higher $\alpha_s,1$-casein secretion was maintained across the culture period for all the substrata (Fig. 22D).

Intracellular levels of $\alpha_s,1$-casein in cultures of lactating and developing cells, were determined on days 4, 10 and 14. In contrast to LF, $\alpha_s,1$-casein accumulated intracellularly (Fig. 23). Lactating
mammary cells cultured on plastic showed low total (intracellular + secreted) and intracellular $\alpha_s,1$-casein compared to cells cultured on collagen (Fig. 23A and 23B). Hence, low secretion of $\alpha_s,1$-casein by these cells was not due to failure to export synthesized casein. Similar to $\alpha_s,1$-casein accumulation in the medium, lactating cells on collagen accumulated intracellular levels of $\alpha_s,1$-casein and in increasing amounts between day 4 and the later stages of the culture (day 10-14). There were no clear differences in the regulation of intracellular $\alpha_s,1$-casein between lactating cells on 100 ul and 500 ul collagen, or in the presence or absence of FCS in the medium. Further analysis is required to clarify this issue.

FCS free cultures of mammary cells from a developing gland showed low accumulation of $\alpha_s,1$-casein intracellularly irrespective of the substratum. In contrast, cells on collagen and supplemented with 15% FCS in their medium showed increased intracellular levels of $\alpha_s,1$-casein throughout the culture period. At the same time, a decrease in the levels of $\alpha_s,1$-casein secreted in the medium was evident. This implies that $\alpha_s,1$-casein secretion, and not $\alpha_s,1$-casein synthesis, was impaired in cultures of mammary cells from a developing mammary gland supplemented with 15% FCS. No change in intracellular accumulation of $\alpha_s,1$-casein across the 14 day experimental period (Fig. 23C and 23D) was noted when the same cells were cultured on plastic, whereas secreted $\alpha_s,1$-casein in medium decreased between day 4 and days 10-14. This
suggested that cells from a developing mammary gland cultured on plastic in 15% FCS medium gradually lost their ability to synthesize $\alpha_{s,1}$-casein. No attempts were made to determine if the regulation of $\alpha_{s,1}$-casein synthesis was at the pre- or post-transcriptional level.

**Ultrastructural features.** Transmission electron microscopy studies revealed the presence of different cell types in day 12 FCS free cultures on 500 ul collagen, whether the cells were derived from a developing or a lactating gland. Cultures of lactating bovine mammary cells were multilayered, usually 2-3 layers of cells, and characterized by a mostly columnar type cell that overlaid a squamous type cell. In contrast, cultures of developing cells consisted of 3-4 layers of cells deep, and the columnar cells overlaid several sheets of cells that consisted of columnar and squamous type cells, largely reflecting a disorganized cell population.

Figures 24 and 25 show sections of cells from day 12 cultures obtained from a lactating and developing gland, respectively. Interdigitation and desmosomes join neighboring cells, and no dilated areas between the cells was noted as previously reported for developing cells by MacKenzie et al. (1985). In addition, cells were joined at their apical ends with tight junctions, and cells from the lactating gland maintained an ordered array of tight junctional complex (Fig. 24).
Figure 24. Mammary tissue from a lactating mammary gland at day 10 in culture. Cells were cultured on 500 μl collagen. Medium consisted of M199 with Prl, I, and F with no FCS. Note microvilli (MV), tight junctions (TJ), and desmosomes (D). X 13,500.
Figure 24.
Figure 25. Mammary tissue from a developing mammary gland at day 10 in culture. Cells were cultured on 500 ul collagen. Medium consisted of M199 with Prl, I, and F with no FCS. Note microvilli (MV), tight junctions (TJ), and desmosomes (D). X 21,000.
Figure 25.
This was in contrast to the mammary cells from a developing gland which possessed loose, relatively disordered tight junctional complexes (Fig. 25).

As shown by the ELISA, lactating but not developing mammary cells on 500 ul collagen in FCS free culture secreted $\alpha_s,1$-casein into the medium (Fig. 22A) Both preparations showed more than 1 ug of LF/ml of medium (Fig. 21A and 21C). However, transmission electron microscopy studies of both preparations showed no casein micelles, secretory vesicles, or fat droplets (Fig. 2A and 25). Generally, bovine mammary cells cultured for 12 days appeared undevoloped, regardless of whether from lactating or developing gland. Ultrastructural features in cultures of lactating and developing cells included a network of organized RER, and an extensive number of mitochondria. A high nuclear:cytoplasmic ratio was evident, and several of the nuclei had indentations.

The top layer of cells possessed elongated microvilli. In certain sections, lactating or developing mammary cells showed short and stubby microvilli. Cells in the underlying layers also possessed microvillar-like structures.
Performance of cells embedded within a collagen gel matrix or plated on top of a Matrigel basement membrane

Growth behavior and total protein synthesis and secretion. Bovine mammary cells cultured within collagen or on Matrigel maintained their three dimensional structure, with differences in growth patterns evident within each of the substrata. Plating the cells on Matrigel-4 prevented spreading of cells from the acini (Fig. 26). At stages beyond day 2 or 4, most of the acini developed fibrillar processes projecting into the area surrounding the acini. As the culture progressed, acini increased in size sufficient to become visible to the naked eye, and were scattered across the well. In contrast, the same cells assumed a different morphology when grown on Matrigel-37. The cells spread from the acini as early as day 4, and this progressed across the culture until a confluent sheet was evident. A notable difference from cells plated on collagen was that cells on Matrigel-37 maintained the acinar structure within the cell sheet. These acinar-like structures grew in size, in addition to spreading of cells away from them (Fig. 27).

Growth of bovine mammary cells embedded in collagen was similar to that described by other workers for bovine (McGrath, 1987) and for murine (Hamamoto et al., 1980) mammary cell cultures. Briefly, the acini embedded in collagen sent outgrowths of ductular-like processes into the collagen, and assumed a three-dimensional structure, similar to
Figure 26. Photomicrograph of bovine mammary cells at day 6 in culture on Matrigel-4. Medium consisted of M199 with Prl, I, and F with no FCS. X 100.
Figure 26.
Figure 27. Photomicrograph of bovine mammary cells at day 6 in culture on Matrigel-37. Medium consisted of M199 with Prl, I, and F with no FCS. X 100.
Figure 27.
Figure 28. Photomicrograph of bovine mammary cells at day 6 in culture embedded within collagen. Medium consisted of M199 with Prl, I, and F with no FCS. X100.
Figure 28.
In vivo morphology (Fig. 28). In general, developing cells were easily maintained within collagen, and the presence of FCS enhanced their growth considerably. Lactating cells were harder to grow within collagen, and in a few instances they failed to survive past day 6 of culture as observed by loss of $^{35}$S-labelled protein synthesis and secretion (not shown).

Figure 29 shows representative profiles for the in vitro growth of lactating cells (Fig. 29A) and developing cells (Fig. 29B) cultured on different substrata in medium supplemented with 15% FCS. Typically, cells on Matrigel-4 and Matrigel-37 attached better than did cells on collagen, and also maintained a higher cell population throughout the culture. Cells embedded in collagen had a slower growth rate than cell populations on other substrata. This profile was consistent for lactating and developing cells grown in an FCS free medium. An exception to the above was that lactating cells in FCS free medium on Matrigel-4 and Matrigel-37 grew at the same rate as cells on collagen.

Total TCA precipitable $^{35}$S-radiolabelled protein secreted into the medium was determined for cells cultured on Matrigel or embedded within collagen. Similar to our previous data (Fig. 18A and 18B) for cells on plastic, 100 ul and 500 ul collagen (i.e., substrata that induce restructuring of the acini), FCS increased the total levels of TCA precipitable $^{35}$S-labelled proteins by 3-4 fold (Fig. 30). This
Figure 29. Cell population for bovine mammary cells embedded in collagen (●), cultured on 500 ul collagen (○), and on Matrigel-4, gelled at 37°C, 30 min. and then incubated at 4°C overnight in 1 ml of M199 (▲). Cells were maintained in M199 with Prl, I, F, and 15% FCS. Cell number was determined by counting nuclei released after selective cell lysis on days 4, 10, and 14 for A) lactating cells and B) developing cells.
Figure 30. Levels of TCA-precipitable $^{35}$S-labelled proteins in medium of bovine mammary cells, as influenced by the substratum, FCS, and stage of mammary development. Cells from lactating and developing mammary gland were maintained up to 10 days in culture, labelled for 16 hrs, TCA-precipitated the $^{35}$S-labelled proteins in the medium, and counted by liquid scintillation counting. Medium consisted of M199 with Prl, I, and F supplemented with 15% FCS (■■■), or without FCS (□□□).
increase in TCA precipitable proteins did not correlate with the increase in cell number (1.5-2 fold) for cells plated on Matrigel or embedded in collagen. In addition, cells embedded in collagen showed significantly lower levels of TCA precipitable \(^{35}\text{S}\)-labelled proteins secreted into the medium than cells on Matrigel. This effect was more pronounced in cultures with 15\% FCS in the medium for either lactating or developing mammary cells.

**SDS-PAGE electrophoresis.** The SDS-PAGE electrophoretic profile of proteins in FCS free medium (Fig. 31) showed that \(^{35}\text{S}\)-labelled proteins of similar size were secreted by developing or lactating mammary cells in culture. The method used for preparing Matrigel (Matrigel-4 vs Matrigel-37) had no influence on the profile of secreted proteins. A 45,000 mol wt and a 33,000 mol wt band were prominent. The former was evident in all media of cultured bovine mammary cells, regardless of the substratum on which the cells were cultured. The 33,000 mol wt band was observed only in medium from cells cultured on Matrigel. SDS-PAGE analysis of the \(^{35}\text{S}\)-labelled proteins secreted by cells cultured within collagen was not performed.

**Regulation of LF secretion.** Cells grown on Matrigel or embedded in collagen showed modulation of LF secretion by the different substrata, similar to our previous results for cells on plastic or 100 ul or 500 ul collagen.
Figure 31. Profile of $[^{35}\text{S}]$-methionine labelled protein secreted into the media by lactating (A) and developing (B) bovine mammary cells. Cells grown in FCS free media on Matrigel with lactogenic hormones for 10 days in culture were labelled for 16 hrs and their media resolved on 17.5% SDS-PAGE. Levels 1 and 3 and media samples from mammary cells plated Matrigel-37 that was gelled at 37°C, 30 min, and the cells plated on it immediately after that. Lanes 2 and 4 are media samples from mammary cells plated on Matrigel-4 after gelling at 37°C, 30 min followed by overnight incubation at 4°C in 1 ml of M199.
More LF was secreted into the medium by developing mammary cells (Fig. 32C and 32D) than by lactating mammary cells (Fig. 32A and 32B) cultured on the same substratum. Similarly, FCS dramatically increased LF secretion within each substratum tested (Fig. 32A and C vs 32B and D). The above confirmed our earlier results noted for mammary cells cultured on collagen or plastic (Fig. 21).

Comparing LF secreted into the medium within each of the substrata permutations (Matrigel-4, Matrigel-37, and embedding in collagen), stage of cell and the presence or absence of 15% FCS as a supplement to the medium, a general pattern of LF secretion was noted. Figure 32 shows that LF was secreted into the medium in increasing concentrations up to day 8-10 and then plateaued. The highest LF levels secreted into the medium were by cells on Matrigel-37, while the lowest LF level was secreted by cells embedded in collagen. Cells on Matrigel-4 consistently showed lower LF in the medium, than cells on Matrigel-37.

No intracellular accumulation of LF was noted in cells embedded in collagen. In contrast, LF accumulated intracellularly or was trapped within the acini (intracellular/acinar) only in cells cultured on Matrigel (Fig. 33). No other substrata caused similar marked intracellular/acinar accumulation of LF. Interestingly, intracellular/acinar accumulation of LF for cells on Matrigel (Fig. 33)
Figure 32. LF secretion by bovine mammary cells in culture as influenced by substratum, stage of mammary development, and FCS. Medium and sampling intervals as for Fig. 9. Mammary cells were cultured on Matrigel, after gelation at 37°C, 30 min (●), or after gelation at 37°C, 30 min, and incubation at 4°C, overnight (○), or embedding within collagen (△). Panel A, lactating mammary cells in an FCS free medium, or Panel B, in medium supplemented with 15% FCS. Panel C, developing mammary cells in FCS free medium, or Panel D, with 15% FCS.
Figure 32.
Figure 33. Effect of substratum, stage of mammary development, and FCS on the intracellular/acinar accumulation of LF in cultures of bovine mammary cells. Samples collected on days 4, 10, and 14 and assayed by ELISA. A) Lactating mammary cells in FCS free medium, B) with 15% FCS supplemented to the medium. C) Developing mammary cells maintained in FCS free medium or D) supplemented with 15% FCS. (O), Matrigel-4, gelled at 37°C, 30 min and incubated at 4°C, overnight; (Theta), Matrigel-37, gelled at 37°C, 30 min, and (Delta) embedded within collagen.
Figure 33.
was the opposite that of LF accumulation in the medium (Fig. 32). Intracellular/acinar levels of LF for cells cultured on Matrigel-37 were lower than intracellular/acinar LF levels of cells cultured on Matrigel-4. This was noted under all experimental permutations and across the entire experimental period.

LF secretion and intracellular/acinar accumulation correlated with the growth morphology of the cells cultured on Matrigel. Lactating or developing cells cultured on Matrigel-4 did not spread but maintained acinar-like structures throughout the culture period (Fig. 26). Retention of the acinar structure on Matrigel-4 was also associated with higher levels of intracellular/acinar LF. It should be pointed out that the method used to harvest and lyse cells for analysis of intracellular milk proteins could not distinguish between intracellular levels or levels trapped within the acini. On the other hand, cells cultured on Matrigel-37 partially restructured the acini, and formed sheets of cells extending beyond the remnant acini (Fig. 27). Such cells consistently secreted higher LF into the medium and had less intracellular/acinar LF than cells that refrained from spreading (Matrigel-4).

\( \alpha_s,1 \)-Casein secretion and intracellular/acinar accumulation. \( \alpha_s,1 \)-Casein levels secreted into the medium declined progressively after day 2 to less than 10 ng/ml by day 10 of FCS-free cultures for all substrata tested (Fig. 34). Addition of 15% FCS to the medium did not prevent the
Figure 34. α_{s,1}-Casein secretion by bovine mammary cells from a developing or a lactating mammary gland cultured on Matrigel gelled at 37°C, 30 min and incubated with 4°C overnight (O), Matrigel gelled at 37°C, 30 min (●), or embedded within collagen (Δ). Medium sampled and assayed as Fig. 12. Lactating mammary cells in A) FCS free medium, or B) 15% FCS supplemented medium. Developing cells in C) FCS free medium, or D) in medium supplemented with 15% FCS.
Figure 34.
loss of $\alpha_s, \lambda$-casein secretion, however, slightly higher (20-100 ng of $\alpha_s, \lambda$-casein/ml medium) secretion was maintained.

Intracellular/acinar levels of $\alpha_s, \lambda$-casein were determined on days 4, 10 and 14. Developing mammary cells cultured on Matrigel did not accumulate intracellular/acinar levels of $\alpha_s, \lambda$-casein (Fig. 35C and 35D), whereas lactating mammary cells did (Fig. 35A and 35B). Cells on Matrigel-4 which retained their acinar structure, progressively accumulated $\alpha_s, \lambda$-casein intracellularly/acinar during the culture, regardless of the presence or absence of FCS in the medium. In addition, intracellular/acinar $\alpha_s, \lambda$-casein concentrations were significantly higher in those cells on Matrigel-4, as compared to cells on Matrigel-37 which spread and partially restructured their acini. Limited data for lactating or developing cells embedded within collagen showed no accumulation of $\alpha_s, \lambda$-casein intracellularly/acinar; however, more studies are needed to confirm this observation.

These studies showed that for cells cultured on Matrigel, whether from a lactating or a developing mammary gland, LF was secreted into the medium, and accumulated intracellularly/acinar. In contrast $\alpha_s, \lambda$-casein also accumulated intracellular/acinar, but was not secreted in comparable levels into the medium in lactating cells cultured on Matrigel. This raised the question of whether $\alpha_s, \lambda$-casein was secreted unidirectionally to a formed lumen within the acini, whereas LF was
Figure 35. Intracellular/acinar accumulation of αs,1-casein in bovine mammary cells cultured on Matrigel. Cells were collected by collagenase digestion of the matrix, and resuspended in 0.5 ml of Tris buffer per well. A) Lactating mammary cells in FCS free medium, or B) with 15% FCS. C) Developing mammary cells in FCS free medium or D) supplemented with 15% FCS. (O), Matrigel-4, gelled at 37°C, 30 min. and incubated 4°C overnight; (ø), Matrigel-37, gelled at 37°C, 30 min.
Figure 35.
secreted bi-directionally to both apical and basal destinations (Parry et al., 1987). Hence we examined the ultrastructure of cells and acini cultured on Matrigel or embedded in collagen.

**Ultrastructural features.** Light and transmission electron microscopy revealed that neither lactating or developing cells cultured on Matrigel or embedded within collagen showed cell polarization or lumen formation. A typical cross section of acinar structures embedded in collagen (Fig. 36) or cultured on Matrigel-4 (Fig. 38) are represented by these FCS free cultures of developing cells. Transmission electron microscopy of these same cells showed no evidence of polarization for cells comprising the acinar-like structure (Fig. 37 and 39). A loose filament structure surrounding the nucleus was noted in a high proportion of the cells. No tight junctions were evident; however, desmosomes were frequent between neighboring cells. In addition, a disorganized microvillar-like array surrounded cell peripheries to form intercellular spaces within the acini (Fig. 37 and 39) which may be the site of intra-acinar accumulation of secreted proteins.

On rare occasions (Fig. 37), cells embedded in collagen formed a lumen lined with small microvillar-like projections. The cells surrounding the lumen-like structure had a high nuclear/cytoplasm ratio and no secretory vesicles.
Figure 36. Photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture, embedded within a collagen matrix. Two um cross sections, at X 400 magnification. No cell organization around a lumen is noted.
Figure 37. Transmission electron photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture, embedded within a collagen matrix. Note microvilli-like structures between cells (arrow head), desmosomes (D), bundles of filaments (FIL) surrounding nucleus (N), and a lumen-like structure (X). X 9,000.
Figure 38. Photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture on Matrigel-4. No cell organization around a lumen is evident. (see Fig. 39). Two um cross sections, at X 400 magnification.
Figure 38.
Figure 39. Transmission electron photomicrograph of bovine mammary cells from a developing gland at day 10 in FCS free culture on Matrigel-4. Note microvilli-like structures between cells (arrow head), desmosomes (D), and bundles of filaments (FIL) surrounding nucleus (N). X 9,000.
Effect of cell stage, substratum, and FCS on LF secreted per cell

Table 1 shows that the regulation of LF secretion as affected by the developmental state of the mammary cell, FCS and substratum provided did not change when compared on a per cell basis. Indeed, on similar substrata, cells from a developing gland secreted more LF per cell compared to cells from a lactating gland. In addition, FCS increased LF levels on a per cell basis.

For lactating mammary cells, those cultured on a 500 ul collagen secreted the highest levels of LF, with cells cultured on 100 ul collagen were next highest. Cells on plastic, Matrigel, or embedded within collagen down-regulated their LF secretion on a per cell basis. Developing mammary cells exhibited a pattern of regulation similar to lactating cells. Specifically, cells cultured on Matrigel, or embedded within collagen, showed depressed LF secretion per cell compared to cells on either 100 ul or 500 ul collagen. Cells on plastic had reduced LF secretion per cell if grown in the absence of FCS.

The regulation of LF secreted per cell did not differ from the regulation of total LF secreted into the medium, with the exception of cells embedded in collagen compared to cells on Matrigel (Table 1). The LF secreted per cell in cultures embedded in collagen was higher than LF secreted per cell by those on Matrigel. This pattern was the opposite
Table 1. Effect of the substratum, stage of mammary development, and FCS on LF secretion on a per cell basis, by bovine mammary cells in culture.

<table>
<thead>
<tr>
<th></th>
<th>FCS in medium:</th>
<th>0% FCS</th>
<th>15% FCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. day: 4 10 14</td>
<td>4 10 14</td>
<td></td>
</tr>
<tr>
<td>Lactating cells</td>
<td>(LF pg/cell)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plastic</td>
<td>0.56 0.65 0.55</td>
<td>0.80</td>
<td>1.60 2.20</td>
</tr>
<tr>
<td>100 ul Collagen</td>
<td>0.60 2.05 1.90</td>
<td>0.60</td>
<td>1.90 3.09</td>
</tr>
<tr>
<td>500 ul Collagen</td>
<td>0.58 2.44 4.10</td>
<td>0.50</td>
<td>4.30 3.75</td>
</tr>
<tr>
<td>Embedded</td>
<td>ND ND ND</td>
<td>0.32</td>
<td>1.80 1.40</td>
</tr>
<tr>
<td>Matrigel-4</td>
<td>0.63 0.34 0.55</td>
<td>0.55</td>
<td>1.40 1.70</td>
</tr>
<tr>
<td>Matrigel-37</td>
<td>0.82 0.95 1.10</td>
<td>0.47</td>
<td>1.40 1.70</td>
</tr>
<tr>
<td>Developing cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plastic</td>
<td>2.60 2.30 1.90</td>
<td>5.30</td>
<td>9.40 8.60</td>
</tr>
<tr>
<td>100 ul Collagen</td>
<td>2.60 6.20 5.60</td>
<td>2.20</td>
<td>9.90 8.10</td>
</tr>
<tr>
<td>500 ul Collagen</td>
<td>2.20 7.30 5.60</td>
<td>2.20</td>
<td>8.90 6.60</td>
</tr>
<tr>
<td>Embedded</td>
<td>0.62 4.20 2.21</td>
<td>2.30</td>
<td>4.20 3.79</td>
</tr>
<tr>
<td>Matrigel-4</td>
<td>1.30 0.76 0.85</td>
<td>1.06</td>
<td>1.60 1.80</td>
</tr>
<tr>
<td>Matrigel-37</td>
<td>1.04 1.09 1.50</td>
<td>1.43</td>
<td>3.60 2.10</td>
</tr>
</tbody>
</table>

* Concentrations were obtained by dividing the total amount of LF secreted into the medium by the total cell # under each of the substratum conditions and stage of cell.
for total levels of LF secreted into the medium (Fig. 32). However, considering that cells on Matrigel accumulated high levels of intracellularly/acinar LF, the differences noted on a per cell basis would be less evident if the total amounts (in medium + intracellular/acinar) of LF synthesized were considered.
DISCUSSION

Morphogenesis

While tissue specific gene expression and structural morphogenesis are assumed to be modulated by the substratum, the mechanisms that govern such interactions are still speculative. However, several levels have been documented at which the extracellular matrix affects gene expression (Bissell and Hall, 1987; Bissell et al., 1982). The mammary gland is one of the extensively studied systems, in terms of its development and interaction with its extracellular environment (Russo and Russo, 1987, Daniel and Silberstein, 1987; Blum et al., 1987). While most of the in vitro studies have utilized the mammary gland of rodent species, in vitro studies on bovine mammary development are limited. Previously, we demonstrated that cryopreserved cells from lactating bovine mammary gland maintained long term culture on 100 ul collagen. The cells synthesized and secreted LF and $\alpha_s, \lambda$-casein for at least 10 days in culture (Talhouk et al., 1989). In this study, we cultured cryopreserved bovine mammary cells derived from a developing and a lactating gland and examined the effect of different substrata on cellular morphology and regulation of milk protein synthesis and secretion.
Regulation of protein synthesis and secretion, and the multicellular organization of the bovine mammary cells were dramatically affected by the different substrata utilized. Cryopreserved bovine mammary cells cultured on plastic or on top of a collagen gel formed a sheet of cells that covered the entire growth area. Restructuring of the acini was evident as early as day 2-4, and the formation of a sheet of cells at day 6-8 for lactating and developing cells. At the transmission electron microscopy level, cells at day 12 in FCS free culture on 500 ul collagen showed polarized epithelial mammary cells with microvilli at the apical surface. However, developing cells showed less maturity of the tight junction complex than did lactating mammary cells. Pitelka and Hamamoto (1977) showed that the tight junctions between neighboring cells at the apical side of the cell consist of a loose network of filaments of variable length and width during mid-pregnancy. At parturition, the tight junction network rearranges with compaction into a highly ordered and narrow structure. This maturity of the tight junction is a hallmark of mammary cell differentiation during lactogenesis.

Similar to our observations, other studies utilizing bovine (MacKenzie et al., 1982; Larson, 1976) or rodent mammary cells (Lee et al., 1984; Rocha et al., 1985) cultured on plastic or collagen showed restructuring of the acini and formation of a cell sheet. However,
Baumrucker (1985) reported that lactating bovine mammary cells maintained their acinar structure when cultured on collagen gels for at least 7 days. Similarly, our early efforts (unpublished observation), to culture lactating bovine mammary cells on 500 ul collagen routinely resulted in cells maintaining acinar structure for periods up to 2 weeks. In contrast, restructuring of the acini was noted when these same cells were cultured on top of 100 ul collagen. We were not successful in replicating this observation with other preparations of collagen but with the same lot of cryopreserved cells. Studies by Coriani et al. (1976) suggested that the strength and consistency of the collagen determined the growth rate of mammary tumor cells in culture. Thus, it is likely that the plating of cells on a less firm but thick collagen allowed the acinar structures to invade into the collagen and assume the morphology and functions of embedded cells grown within collagen. All of our subsequent studies, including those described in this report, have consistently shown restructuring of acini when mammary cells were digested and plated as described by Talhouk et al. (1989) on top of 100 ul or 500 ul collagen.

Slight differences were observed in the morphology at the light microscopy level between cells plated on 100 ul and 500 ul collagen. In the latter, a population of spindle-like cells penetrated the thick layer of collagen and formed three dimensional processes. This was not seen in cells grown on 100 ul collagen. Parry et al. (1987) reported
that a thin layer of PFMR-9 matrices attached on plastic impeded the access of hormones and nutrients to the basolateral surface of COMMA-D1 cells and prevented differentiation events. This effect was abrogated when the PFMR-9 matrices were attached on millicel well inserts. This allowed free access of hormones and nutrients to the basolateral surface of the cells, and induced differentiation. Aside from allowing access of the nutrients to the basolateral surface, an attached thick (500 ul) layer of collagen is more flexible and contractile than a thin (100 ul) layer. This should facilitate differentiation of mammary cells in culture (Haeuptle et al., 1983).

Emerman and Pitolka (1977) reported the first successful long term culture of mouse mammary epithelial cells on top of collagen gels. Full induction of casein secretion occurred only after detaching and floating of the collagen which allowed the layer of flattened cells to contract the collagen and assume a cuboidal morphology and also gain access of nutrients and hormones to basolateral surface of the cells. Later studies (Wicha et al., 1982) showed that coating an inflexible plastic surface with extracellular matrix components can induce differentiation events and specific gene expression in rat mammary cells. All cases of differentiation in the above models, required multicellular reorganization and extensive restructuring of the alveoli.
On the other hand, embedding murine mammary cells or plating them on Matrigel conserved their three-dimensional structure (Hamamoto et al., 1988; Barcellos-Hoff et al., 1987). The embedding of bovine mammary cells in collagen from a non-pregnant or a pregnant heifer was reported by Shamay and Gortler (1986) and McGrath (1987) to cause three-dimensional growth ductular-like processes from the acini, prolonged maintenance of cells in culture, and their responsiveness to insulin. No evidence of milk protein synthesis and secretion was shown. Lumen formation was evident by day 12 of culture, and the colonies consisted mostly of epithelial cells as shown by immunocytochemistry using anti-human keratin antibody. Mammary cells from other species behave similarly when embedded within collagen gels. Deoks et al. (1988) reported cultivation of rat mammary cells within collagen and showed their dependence on insulin and IGF-1 in serum-free medium. Yang et al. (1987) cultured human breast epithelial cells derived from various sources (fibrodenoma, reduction mammoplasty, or mastectomy tissue) within collagen and showed the formation of lumina surrounded by a single layer of epithelial cells, which was, in turn, surrounded by a basal layer of myoepithelial cells. Recently, Hamamoto et al. (1988) established a serum-free culture of mammary epithelial cells from adult virgin mice by embedding in collagen. The cells responded to prolactin, and acquired polarization with lumen formation similar to that in vivo. Junctional complexes and secretory vesicles containing casein were evident.
In our hands, lactating or developing bovine mammary cells embedded within collagen maintained their three-dimensional structure with branching duct-like processes. Cell growth was either maintained in a quiescent state with a 1-2 fold increase in the number of lactating cells or increased several fold (5-6) for developing cells supplemented with FCS (Fig. 29B). In contrast to growth in the cell population of normal rat or mouse mammary epithelial cells (McGrath, 1987) embedded in collagen, we showed that bovine mammary cells on plastic or on collagen had an enhanced rate of growth (Fig. 29), with the collagen being the most suitable substratum for growth among the above mentioned substrata. It is important to note that the actual growth and increase in cell population in a two-dimensional culture vs. a three-dimensional growth pattern within a collagen matrix cannot be compared directly due to differences in attachment efficiencies in each of the above conditions (Yang et al., 1986).

A marked difference between our system for bovine mammary cells embedded in collagen and others (McGrath 1987) was that ultrastructural examination of lactating and developing mammary cells showed no lumen formation except in rare instances. No secretory vesicles were noted, and a lack of cell polarization and orientation towards a lumen was evident. In contrast, McGrath (1987) showed that developing bovine mammary cells embedded in collagen had a lumen surrounded by epithelial cells.
Finally, bovine cells embedded in collagen consisted of different mammary cell types. Secretory mammary epithelial cells remained viable throughout the entire experimental period (14 days), as indicated by their ability to secrete LF and regulate its secretion in response to FCS and the stage of the mammary cell.

The apparent discrepancies between the bovine and the murine mammary cells embedded in collagen could be attributed to species differences, cryopreservation of bovine cell preparation, or suboptimal hormonal supplements. Hamamoto et al. (1988) showed that attainment of a secretory morphology was highly dependent on the presence of optimal hormonal stimuli.

Similar differences in the performance of bovine and rodent mammary cells were noted when those cells were cultured on the Matrigel basement membrane. Bissell and Barcellos-Hoff (1987) and Barcellos-Hoff et al. (1987) established a culture of mammary epithelial cells from mid-pregnant mice and plated on Matrigel. Such cells on Matrigel grew as globular cell masses (termed "mammospheres") with little spreading, and lumen formation was evident by day 6, with apical surfaces of the epithelia oriented towards the lumen and joined by tight junctions. Secretory vesicles and fat droplets were vectorially secreted into the lumen. In contrast, ultrastructural studies of our bovine mammary cells
on Matrigel showed no such organization, with no lumen or cell polarization.

Lactating and developing bovine mammary cells cultured on Matrigel grew in globular clumps and retained their acinar structure throughout the culture period. However, such bovine cells on Matrigel showed two different patterns of growth on Matrigel which were dependent on whether Matrigel was incubated at 4°C after gelation but before plating of cells. In contrast to the growth pattern described by Barcellos-Hoff et al. (1987) and Blum et al. (1987), bovine cells plated on Matrigel-37 partially restructured their acini, with the formation of a circumscribing layer of cells which migrated outward from the acini. The acini, in turn, grew in size with formation of a large sphere of cells resembling (Fig. 27) their original acinar-like structure at the point of attachment. However, when bovine cells were grown on Matrigel-4, the globular clumps of cells grew in size but did not spread, and maintained their acinar-like morphology (Fig. 26). No significant differences in cell number was noted between the two patterns of growth during the culture period (data not shown).

Basement membrane is largely (80-85%) composed of laminin, type IV collagen, entactin, nidogen, and heparan sulfate proteoglycan (Kleinman et al., 1986), all of which are part of the extracellular matrix of the mammary gland in vivo as well (Warburton, 1982, 1984; Silberstein and
Daniel, 1982). However, the critical component(s) of the Matrigel responsible for the maintenance of the mammary cell acinar morphology are not determined. However, Blum et al. (1987) and Li et al. (1987) showed that no one component can substitute for the complete Matrigel in affecting cell morphology, shape, and differentiation. Saunders and Bernfield (1987) identified a proteoglycan on mouse mammary epithelial cell surface that binds to the extracellular matrix and not to type IV collagen. They further suggested that this proteoglycan is involved in stabilizing linkage of the cells to extracellular matrix via fibronectin molecules in that matrix. The loss of this proteoglycan due to injury in vivo, or due to their suspension for in vitro culture, would loosen their anchorage and allow for epithelial spreading on a thin matrix.

From the above, it could be argued that the gelling of Matrigel for 30 minutes at 37°C allowed for only partial gelation and loss of glycoproteins that are integral components of the Matrigel. This could induce partial restructuring of the acini. In contrast, gelation of Matrigel for 3-4 hours at 37°C (Bissell and Barcellos-Hoff, personal communication) or overnight incubation at 4°C after gelation, may provide a more complete gel with firm incorporation of all components which influence cell behavior, and hence, stabilize the acinar morphology of mammary cells in culture. On the other hand, Neville (1987) attributed the "non-spreading behavior" of mammary cells on Matrigel compared to that on collagen to the semifluid properties of the
Matrigel, which allowed the cells to embed within the Matrigel and maintain their acinar-like morphology.

### Regulation of total protein synthesis and secretion

Synthesis and secretion of specific milk proteins in cultures of mammary cells is regulated at the transcriptional, translational, and post-translational level by the extracellular matrix and hormones (Wicha et al., 1982; Blum et al., 1987; Wiens et al., 1987).

SDS-PAGE electrophoretic profiles showed that bovine mammary cells cultured on different substrata secreted a number of proteins that are not normal constituents of milk, nor were specific milk proteins any of the major bands shifted by growth on different substrata. One major protein secreted by bovine mammary cells, regardless of the substratum provided and stage of the cells (except for lactating cells on plastic), was an unidentified 45,000 mol wt protein. A protein of similar size was noted in all intracellular extracts as well (data not shown). Ben-Ze'ev (1987) reported a cytokeratin of a similar mol wt in cell extracts of dense cultures of bovine mammary cells. In addition, Asch and Asch (1985) showed that primary cultures of mouse mammary epithelial cells exhibited a loss or a reduction in their basic polypeptide cytoskeletal keratins, while a prominent acidic 46,000 mol wt protein was induced.
Whether the 45,000 mol wt protein seen in bovine mammary cultures is a cytoskeletal protein remains to be determined.

Similar to our observations, Suard et al. (1983) and Lee et al. (1984) reported that several proteins not found in milk were synthesized and secreted into the medium by cultured rodent mammary cells. The latter authors demonstrated that these proteins were secreted by either mammary myoepithelial or epithelial cells, but not by mammary fibroblasts. They further suggested that these non-milk proteins were secreted in vivo through the basal surface instead of the apical surface, as are milk proteins. This possibility could not be determined under our experimental conditions. Further studies are needed to characterize and define the non-milk secreted proteins.

The regulation of specific milk proteins was influenced by the substratum. However, cells in FCS depleted medium from different stages of mammary development, and cultured on the different substrata, showed comparable levels of secreted TCA precipitable $^{35}$S labelled proteins. Similar results were noted for cells cultured in FCS supplemented medium. However, cells in FCS supplemented medium had higher $^{35}$S labelled secreted proteins than cells in FCS depleted medium. This suggested that regulation of milk protein synthesis and secretion was specific and did not affect, nor was it affected by the total protein synthetic capability of the mammary cells. Note that cells embedded in collagen
showed a 50-90% decrease of the total TCA precipitable $^{35}$S-labelled secreted proteins compared with other substrata. Another exception was the higher TCA precipitable secreted $^{35}$S-labelled proteins for cells cultured on Matrigel in an FCS supplemented medium.

**Regulation of specific milk protein synthesis and secretion**

Similar to the rodent system (Wicha et al., 1982; Emerman and Pitelka, 1977), we found that $\alpha_5\beta_1$-casein synthesis and secretion in bovine mammary cell cultures was influenced by the substratum and FCS. In accordance to our previous results (Talhouk et al., 1986), we also found that the regulation of LF synthesis and secretion was influenced by the substratum and FCS.

**Lactoferrin.** LF, an iron-binding protein first purified from bovine milk by Groves (1960), is similar to ovotransferrin and serum transferrin. Mason (1970) showed histologically that LF was synthesized by mammary alveolar epithelial cells in vivo, while Talhouk et al., (1986), recently showed LF synthesis and secretion by bovine mammary cells in culture. Recently, LF was shown to be synthesized by mouse mammary cells in culture (Bissell and Barcellos-Hoff, 1988, personal communication). Transferrin in other species has been shown to be synthesized and secreted by cultured cells derived from rabbit (Suard et al., 1983), rat (Blum et al., 1987), and mouse mammary gland (Lee et
al., 1984). Synthesis of transferrin by bovine mammary cells has not been demonstrated and its low concentration in bovine milk results from slight serum transudation (Schnbacher and Smith, 1975).

Whether LF functions in the growth and development of the bovine mammary gland is not known. Its presence in milk is assumed to act as an iron carrier from the mother to the neonate (Aisen and Leibman, 1982). LF concentration in mammary secretion from a developing gland was several fold higher than that in normal milk (Schanbacher and Smith, 1975). Hence, high concentrations of LF in the mammary secretion were suggested to mark the involuted or developing bovine mammary gland. Later, Harmon et al. (1976) reported that LF synthesis and secretion in the bovine mammary gland can be induced in response to microbial injury of the gland without affecting the synthesis and secretion of other milk proteins. Since lactogenic hormones are not known to change during lactation or after mastitis, this suggested that LF regulation is independent of lactogenic hormone concentrations. The physiological role of LF in other tissues has been mainly linked to immunoregulatory functions (Baggiolini et al., 1970; Richie et al., 1987).

The function of transferrin in the direct regulation of rodent mammary growth and development is not determined. Suard et al. (1983) and Barcellos-Hoff and Bissell (1987) reported that transferrin synthesis in cultures of rabbit and mouse mammary cells was not
responsive to lactogenic hormones, but regulated by the substratum. The latter authors suggested transferrin as a possible autocrine regulator of mammary growth and development, since it has been implicated in cell growth regulation and proliferation (McClelland et al., 1984; Li et al., 1981; Laskey et al., 1988). During pregnancy and mammary development transferrin mRNA level in mammary tissue is higher than that in virgin or lactating mouse mammary tissue (Chen and Bissell, 1987).

For bovine mammary cells in culture, LF secretion per cell is highest from developing cells or cells grown with serum, regardless of the substratum on which they are grown. Higher LF secretion by developing cells in culture is consistent with earlier observations that mammary secretions from developing bovine glands have higher LF concentration than those from lactating glands (Schanbacher and Smith, 1975). Hence, cultures of developing mammary cells receiving a full complement of lactogenic hormones maintained their predisposition to secrete higher LF than cells from a lactating gland. This difference in LF concentration in the medium between cells from a developing and a lactating gland did not reflect an increase in the cells' ability to synthesize and secrete total proteins, since the total TCA $^{35}$S-labelled proteins in the medium were comparable within each substratum.

The effect of FCS on increasing LF secretion in vitro has parallels in vivo. Upon injury of the gland, the mammary cell is exposed to serum...
components from the blood, through increased pericellular transudation, with a coincident increase of LF in milk (Harmon et al., 1976).

Transferrin synthesis and secretion by mouse mammary cells in vitro are regulated by the substrata at the transcriptional and translational levels (Suard et al., 1983; Blum et al., 1987; Chen and Bissell, 1987). Transferrin was synthesized and secreted by cells cultured on plastic, collagen, Matrigel, or laminin. Cells on plastic secreted less transferrin than cells on other substrata. This was not due to a decrease in total protein synthesis, or due to different distribution of cell types on plastic vs. the other substrata as determined by a fluorescent luminal-epithelial marker (Blum et al. 1987).

In our system, LF regulation is influenced by the substratum, and lactating or developing cells cultured on plastic secreted less LF than when cultured on collagen (Fig. 21), although their total TCA precipitable $^{35}$S-labeled proteins were similar (Fig. 18A). An exception to the above was developing cells in 15% FCS medium.

Interestingly, lactating and developing mammary cells that maintained their acinar structure in culture (i.e., cells embedded in collagen and grown on Matrigel-4 and Matrigel-37) secreted less LF into the medium than when cultured on collagen. In addition, cells on
Matrigel-37 secreted higher LF in the medium than mammary cells on substrata that prevented cell spreading, and favored growth of the mammary cells in clumps (i.e., embedding in collagen, or Matrigel-4.) The increase in LF levels coincided with the initiation of cell spreading from the acini, around day 6 of culture (Fig. 32). This confirmed our observation that cell sheets on collagen secreted higher LF into the medium than cells that did not spread into sheets. Thus the increase in LF secretion into the medium associated with restructuring of the acini into sheets of cells, may be attributed to: 1) cells cultured on collagen in FCS free medium were exposed to FCS upon plating in order to facilitate cell attachment, which may induce cell spreading by a spreading factor in FCS. This spreading and exposure to FCS could mimic the situation of mammary injury in vivo, when the gland responds by secreting higher LF for prolonged periods (Harmon et al., 1976). However, this is modulated by substratum since both FCS and an appropriate substratum were needed to initiate the spreading of acinar mammary cells into sheets; i.e. cells plated on Matrigel-4 or embedded in collagen do not spread. 2) Cells that maintain their acinar structure (Matrigel-4, Matrigel-37) accumulate intracellular/acinar LF and prevent its release into the medium (Fig. 34 and Fig. 35). A precedent for this was reported by Bissell and Barcellos-Hoff (1987) and Barcellos-Hoff et al. (1987). Mouse mammary cells on Matrigel vectorially secreted milk proteins into a formed lumen. A 70,000 mol wt protein was secreted apically into the lumen, and into the medium
presumably through the basolateral surface. In addition, Parry et al. (1987) reported for cultured COMMA-D1 cells on Millicell well inserts, that casein secretion was unidirectional through the apical surface, while transferrin was secreted through both the apical and basal surfaces of a polarized mammary cell.

We examined the possibility of intracellular/acinar accumulation of LF. Regardless of the matrix, cell stage, and FCS, no intracellular accumulation of LF was noted at any stage of the culture, except for cells cultured on Matrigel-4 and Matrigel-37. Cells on Matrigel showed increasing levels of intracellular/acinar LF that paralleled the accumulation in the medium, and at times, exceeded it by several fold (Fig. 34 and 35). In contrast to LF concentration in the medium, cells on Matrigel-4 (which do not spread) accumulated more intracellular/acinar LF than cells plated on Matrigel-37 (Fig. 35). This suggested the entrapment of LF within the acini. A similar pattern was noted for intracellular/acinar entrapment of $\alpha_{s,1}$-casein from lactating cells on Matrigel-4 and Matrigel-37. $\alpha_{s,1}$-Casein was secreted exclusively into the acini with no levels of casein secreted into the medium. It is important to note that ultrastructural examination of the cells on Matrigel revealed no lumen or cell polarization; instead, interstitial channels occupied by microvillus-like projections were evident between cells. Hence, this pattern of LF and casein secretion and entrapment within the acinar-like structure cannot be explained as
bi-directional secretion of LF and unidirectional secretion of caseins. No accumulation of LF was noted in cells embedded within collagen, although morphologically, embedded cells were similar to those plated on top of a Matrigel-4.

\( \alpha_s, \beta\)-casein. Regulation of casein synthesis and secretion in mammary cells has been studied by many workers. The first successful induction of casein synthesis and secretion was for cells plated on collagen gel, with subsequent detachment of the collagen to float in culture (Pitelka and Emorman, 1977). The cells contracted the collagen and thereby assumed their cuboidal morphology. The contractility of the gel allowed changes in cell shape, which induced cytodifferentiation (Suard et al, 1983; Haeuptle et al., 1983). Alternatively, Wicha et al. (1982) and Blum et al. (1987) reported that providing the cells with proper matrix components on a non contractile substratum also induced cytodifferentiation. From our results on the regulation of casein synthesis and secretion in the bovine mammary cell culture system, we propose that substratum type and form is critical for casein synthesis and secretion. The substratum should provide proper contractility and/or access of nutrients to the basolateral surface. It should also permit restructuring of the acini to allow release of secretory proteins through the apical surface.
αs,1-Casein synthesis and secretion was regulated by the substratum in cultures derived from a lactating mammary gland. Restructuring of the acini into cell sheets was not sufficient to initiate casein synthesis and secretion, since the same cells cultured on plastic did not secrete nor accumulate intracellular levels of casein. However, we cannot rule out that casein was not synthesized and rapidly degraded on plastic as shown by Blum et al. (1987) and Lee et al. (1985). Providing a contractile substratum which also permits access of nutrients to the basolateral surface of bovine mammary cells in culture seems important to induce casein synthesis and secretion. This was noted when lactating cells cultured on a thick (500 ul) layer of collagen secreted more casein into the medium than cells cultured on a thin (100 ul) layer of collagen or on plastic (Fig. 22A and 22B). In addition, detaching the matrix for lactating cells cultured on 500 ul collagen, specifically increased the levels of casein secreted into the medium by 10 fold, without affecting the levels of LF secreted in the medium (Talhouk et al., 1988).

Cryopreserved cells from a lactating mammary gland cultured on Matrigel or embedded within collagen failed to secrete detectable amounts of αs,1-casein into the medium beyond day 4-6 of culture. Interestingly, cells on Matrigel-4 had high levels of intracellular/acinar αs,1-casein (~150 ng/ml) compared to the levels of secreted αs,1-casein (6 ng/ml). We were not able to determine whether
the intracellular/acinar levels of $\alpha_s,1$-casein were intracellular pools (not secreted) or trapped within the intracellular spaces of the acini after secretion from the cells. In either case, the Matrigel appears to be unsuitable for protein secretion studies in bovine mammary cells, although it facilitates high attachment efficiency. The lack of a definite cellular organization and polarization of the mammary cells on Matrigel, or embedded within collagen, could explain their low casein synthetic abilities.

$\alpha_s,1$-Casein accumulated intracellularly and extracellularly (secreted) in cultures of lactating cells on collagen, whereas LF did not accumulate intracellularly but was secreted. This suggested that $\alpha_s,1$-casein and LF were secreted via different routes or mechanisms, with different regulations for each paths (Burgess and Kelly, 1987).

In earlier studies, we showed that $\alpha_s,1$-casein secretion by lactating bovine mammary cells plated at low density on collagen was dependent on supplementing medium with FCS (Talhouk et al., 1989). The effect of FCS was attributed to an increase in cell population, suggesting that casein synthesis was dependent on cell density. In the current study, we noted no effect of FCS on $\alpha_s,1$-casein secretion by high density cultures of lactating bovine mammary cells. In fact, a slight decrease in $\alpha_s,1$-casein level in medium was noted by cells cultured on 500 ul collagen. This effect was more evident when the collagen matrix was
detached (Talhouk et al. 1988), suggesting that for optimal casein synthesis and secretion, a high cell density and removal of FCS was necessary.

Developing cells showed declining $\alpha_{s,1}$-casein secretion into the medium with time in culture, irrespective of the substratum or FCS in the medium. However, these cells whether cultured on thin (100 ul) or thick (500 ul) collagen accumulated intracellular levels of $\alpha_{s,1}$-casein demonstrating their ability to synthesize $\alpha_{s,1}$-casein, but not secrete it into the medium.

Similar to Mackenzie et al. (1985), ultrastructural studies of cells from a lactating mammary gland cultured on a thick layer of collagen in an FCS depleted medium, showed no evidence for casein micelles or secretory vesicles, however $\alpha_{s,1}$-casein was secreted into the medium. Hence, for these, and perhaps other in vitro conditions, $\alpha_{s,1}$-casein may be secreted as a non-micellar soluble casein, as commonly occurs in secretions from undifferentiated or involuted bovine mammary glands (unpublished observation).

**Conclusion.** In conclusion, we have demonstrated that bovine mammary cells in culture can actively synthesize and secrete LF and $\alpha_{s,1}$-casein. The regulation of both proteins is influenced by the developmental stage of the mammary cells used for culture and the substratum on which they
are grown. In addition, LF and $\alpha_s,1$-casein are independently regulated in vitro and possibly secreted via different routes and/or mechanisms.

Finally, the parallel increase in synthesis and secretion of LF (a marker of involution or dedifferentiation of the bovine mammary gland) and $\alpha_s,1$-casein (a marker of the differentiated mammary gland) in cultures of lactating bovine mammary cells is an interesting concept, because of the reciprocal regulation and expression of the two proteins in vivo, except in injured glands (Harmon et al., 1976). The reports that transferrin is a growth regulator in several tissues (Ekbolm and Thesloff, 1985; Beach et al., 1983) could imply a similar role for LF in mammary development. Nonetheless, if this was the case, we would expect at a certain point in culture, when the cells fully differentiate into lactating cells, for LF to down-regulate and casein to up-regulate, similar to the in vivo situation. This reciprocal pattern for the two proteins was not evident in our cultures so far. Nonetheless, the comparative regulation of LF and casein synthesis, and their use as markers for cell status in culture, are valuable aids to our understanding of the factors affecting mammary growth, development, and differentiation.
REFERENCES


CHAPTER IV

MORPHOLOGICAL AND FUNCTIONAL DIFFERENTIATION OF BOVINE
MAMMARY CELLS ON FLOATING TYPE I COLLAGEN GELS

INTRODUCTION

The effect of the substratum and cell shape on the expression of milk proteins by mammary cells in cultures has been established at the transcriptional and translational levels by several workers (Lee et al., 1985; Wicha et al., 1982; Suard et al., 1983). We have previously shown that synthesis and secretion of milk proteins, lactoferrin (LF) and $\alpha_s\beta_\text{-casein}$, by bovine mammary cells in culture was regulated by the substratum. Seemingly simplistic, $\alpha_s\beta_\text{-casein}$ was not detected in the medium of the bovine mammary cells in culture unless the acini or cell clumps initially plated restructured, and formed confluent sheets of cells (Talhouk et al., 1989a). The early work of Emerman and Pitelka (1977) demonstrated that mammary cells from mid-pregnant mice cultured on a floating collagen substratum formed confluent sheets and synthesized and secreted higher levels of casein than their counterparts on attached collagen and/or plastic. No reports are available in the
literature that describe cryopreservation of dissociated rodent mammary cells, or the performance of lactating rodent mammary cells on floated collagen. MacKenzie et al. (1985) showed that cells from mid-pregnant but not lactating bovine mammary gland secreted α-lactalbumin into the medium when plated as fresh cells on a detached collagen. Those same cells lost their ability to express α-lactalbumin upon cryopreservation, and the authors failed to demonstrate any ultrastructural evidence for casein synthesis.

Detachment of the substratum allows the mammary cells to contract the gel and assume a cuboidal morphology, which eventually induces differentiation of the mammary cells as indicated by higher casein synthesis and secretion (Haauptle et al., 1983). In this study we report that cryopreserved bovine mammary cells from developing and lactating gland specifically regulated their milk protein synthesis and secretion upon detachment of the collagen substratum. The collagen was detached on day 2 prior to cell spreading into sheets, or on day 6 after the cells had spread and formed confluent multilayered sheets. Detachment on day 2 did not prevent cell spreading or contraction of the substratum. As shown by monitoring the intracellular and secreted concentrations of the milk proteins (LF, αs,1-casein, and α-lactalbumin) by ELISA, cells from a developing mammary gland on detached collagen secreted less LF than cells on attached collagen with no evidence of enhanced αs,1-casein secretion. In contrast, detaching the collagen
in serum free cultures of bovine mammary cells from a lactating gland on
day 2 or 6 did not affect LF synthesis and secretion, yet it
specifically induced αs,1-casein and α-lactalbumin synthesis and
secretion. Cells on collagen (detached on day 6) secreted 10 fold the
αs,1-casein secreted by cells on attached collagen and 2-3 fold that
secreted by cells on collagen detached on day 2. Ultrastructural
studies showed that cells from a lactating gland on collagen (detached
on day 6) contained fat droplets and secretory vesicles with casein
micelles. Our results also confirmed previous findings (Talhouk et al.,
1989a) that serum increased LF secretion, and that cultured bovine
mammary cells from a developing gland secreted more LF than cells from a
lactating gland.

Furthermore, we demonstrated that cryopreserved bovine mammary cells
from a lactating gland under serum free conditions, on a day 6 detached
collagen modulated their αs,1-casein synthesis in response to prolactin.
Cells receiving prolactin throughout the culture period efficiently
synthesized and secreted αs,1-casein. In addition, prolactin was
required to initiate, but not maintain αs,1-casein secretion into the
medium. No αs,1-casein was detected when prolactin was depleted from
the medium throughout the experimental period. Other studies suggested
that mammary cells acquired sensitivity to prolactin between day 4-8 in
culture, a period coincident with the formation of confluent sheets of
cells.
MATERIALS AND METHODS

Materials

Medium M199 with EBSS (Earl's Balanced Salt Solution) and L-glutamine, bovine insulin (I), ovine prolactin (Prl, 31 I.U./mg), alpha-chymotrypsin (from bovine pancreas, type II, 40-60 units/mg), elastase (porcine pancreas, type IV, 70 units/mg), hyaluronidase (from bovine testes, type I-S, 300 units/mg), soybean trypsin inhibitor (STI, type I-S), and bovine serum albumin (BSA, fraction V) were purchased from Sigma Chemical Company (St. Louis, MO). Cell culture plates were purchased from Belco (Vineland, N.J.) and fungizone was from Gibco (Grand Island, N.Y.). Gentamycin and bovine fetal calf serum (FCS) were from M.A. Bioproducts (Walkersville, MD). Laminin was purchased from Collaborative Research Incorporated (Bedford, MA). Cortisol (F), progesterone and estrogen were purchased from Steraloids Incorporated (Wilton, NH), and collagenase type III from Worthington Biochemicals (Freehold, NJ). Trans-$^{35}$S (sp. act. approx. 1100 Ci/m mole, in 10 mM 2-mercaptoethanol/ml) was obtained from ICN (Irvine, CA).
Preparation of primary bovine mammary cell culture

Primary cultures of bovine mammary cells were prepared (Talhouk et al., 1986; Talhouk et al., 1989b, manuscript in preparation) by modifications of the procedures of Kraehenbuhl (1977) and Ebner et al. (1961). Briefly, acini, acinar fragments, and cells were prepared from 100-200 gms of lactating bovine mammary tissue (lactating cells) or from a mid-developing bovine mammary tissue (developing cells), obtained after hormonal induction (day 14-15) of mammary gland (Smith and Schanbacher, 1973), by enzymatic dissociation. The mixture of enzymes used for tissue digestion was prepared in HBSS without Ca++ and Mg++ (5 mls/gm of original tissue weight) and included the following enzymes: 3.6 mg (2000 units) collagenase, 10 mg hyaluronidase, 10 mg chymotrypsin, 0.2 mg elastase, 0.05 mg STI and 100 mg BSA (fraction V) per gm of tissue. After dissociation (90-120 minutes) was complete, the cells were washed with HBSS and cryopreserved in liquid nitrogen at 1-1.5 x10^7 cells/ml/vial (Sarstedt, N.J.). Prior to the start of culture, cryopreserved cells were thawed at 37°C and were plated at 5-6 x10^5 cells/1.0 cm well in 0.5 ml M199 with insulin (5 ug/ml), cortisol (5 ug/ml), prolactin (8 ug/ml), and gentamycin (50 ug/ml) and supplemented with 15% FCS and laminin (1-2 ug/cm^2 of growth area). Laminin and FCS were supplemented upon plating to facilitate cell attachment. FCS was either withdrawn from the medium on day 2 (FCS free cultures) or maintained through the entire experimental period (FCS supplemented
medium). The resulting bovine mammary cells were plated on type I collagen prepared from rat tail tendons (Talhouk et al., 1989b) as modified from Bornstein (1958). The collagen was either maintained attached throughout the experiment, or detached by gently rimming the gel with a pasteur pipet along the side of the well, on day 2 prior to cell spreading, or on day 6 when the cells had spread and formed cell sheets.

**Responsiveness to prolactin**

Lactating bovine mammary cells on collagen (detached day 6) in an FCS free medium were used for this experiment. The control treatment consisted of cells receiving M199 with I (5 ug/ml), F (5 ug/ml) and prolactin (8 ug/ml) with gentamycin (50 ug/ml) for the entire experimental period. Media for other treatments were similar to the control medium, except for prolactin which was supplemented or depleted according to experimental set up. Treatments consisted of the following: prolactin supplied from day 0-8 and then withdrawn from the medium, or supplied from day 8-14 of culture. The negative control did not receive any prolactin throughout the entire experimental period. In the treatment when prolactin was withdrawn from the medium on day 8, the cells were given three 1 ml washes, with M199 with hormones (I, and F), prior to the addition of the prolactin depleted medium.
Cell number determination

The number of bovine mammary cells was determined by the method described by Ethier (1985). Briefly, prior to plating or cryopreservation, cells were pelleted from a known volume of medium by centrifugation (100xg, 4°C, 5 minutes), resuspended with vortexing in 2 mls of swelling buffer (1.5 mM MgCl₂, 0.01M hepes; pH 7.4), 10 minutes, room temperature. Two hundred ul of Ethylhexadecyl dimethyl ammonium bromide (Eastman Kodak Co., Rochester N.Y.) was added to lyse cells but not their nuclei. The suspension was vortexed vigorously and incubated for 10 minutes, room temperature. Nuclei were counted with a hemocytometer.

This method also allowed quantification of cells plated in 24 well plates (1.0 cm wells) after release of cells from collagen. To do so, collagen with attached cells was rimmed from the sides of the well, digested in situ with 0.5 ml of M199 containing 1% collagenase, and STI (1 mg/ml) with gentle shaking for 30 minutes, 37°C. Well contents were aspirated and the well was washed with an equal volume of HBSS to remove any cells remaining attached. The wash was combined with the cells and centrifuged at 100 x g, 5 minutes, 4°C. The cells from 1 well were then resuspended in 3.0 ml of 10 mM Tris, 1 mM EDTA, and 0.1 M NaCl, pH 7.4 (TEN), washed by centrifugation (100 x g, 5 min, 4°C), and resuspended in 1.5 ml of TEN. One ml was aliquoted and stored at -20°C with
proteinsase inhibitors (leupeptin 1 ug/ml, pepstatin 1 ug/ml, and aprotinin 200 KIU per ml of TEN, for analysis of specific milk proteins by ELISA. The remaining 0.5 ml was recentrifuged (100 x g, 5 min, 4°C), the supernatant decanted and then the cells were swollen, lysed, and the free nuclei were counted. Two aliquotes from each sample were counted. Cell number was determined from duplicate wells on days 4, 10, and 14.

Media sampling and analysis of $^{35}$S-radiolabelled proteins

Medium was sampled at 2 day intervals by gentle aspiration with a Pasteur pipet and dispensed into plastic tubes containing leupeptin (1 ug), pepstatin (1 ug), and aprotinin (200 KIU) per ml of medium. The aspirated medium was centrifuged at 500 x g, 5 minutes, 4°C, to remove cell debris or unattached cells. The supernatant was then transferred to tubes for storage at -20°C until analyzed by ELISA. Each medium sample collected was a pool of the medium from four replicate wells, and each treatment was run in duplicate. Standard deviation was calculated for duplicates. Samples for analysis of intracellular milk proteins were collected and stored as described in the section describing the nuclear count method. Prior to analysis of the intracellular milk proteins the cells were sonicated twice for 15 sec, to insure rupture of cells and release of intracellular proteins.
Synthesis and secretion of proteins was determined after radiolabelling with Trans-\(^{35}\)S (\(^{35}\)S-methionine and \(^{35}\)S-cysteine) and by protocols described by Suard et al. (1983). Prior to radiolabelling on day 10, the cells were washed twice with 1 ml of H199 with hormones and then incubated in methionine free minimum essential medium (MEM, Sigma Chemical Co.) supplemented with hormones for 1 hr. at 37\(^{\circ}\)C. Radiolabelling was initiated by the addition of \(^{35}\)S-methionine (50 \(\mu\)Ci/0.5ml/well) for a period of 16 hrs. Labelling was stopped by the addition of 50 uls unlabelled methionine (4 mg/ml). The medium was then harvested, proteinase inhibitors added as before, centrifuged (500 \(\times\) g, 5 min, 4\(^{\circ}\)C), and stored at -80\(^{\circ}\)C.

For analysis of \(^{35}\)S-radiolabelled proteins, equal volumes of medium were mixed 1:1 with 2X SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 0.2% SDS, 10% glycerol, and 2.5% mercaptoethanol) then boiled for 2 minutes. Forty ul samples were loaded onto a Laemelli gel (3% polyacrylamide stacking gel, and 17.5% polyacrylamide separating gel) and resolved by electrophoresis. The gel was stained in coomasie blue, soaked in minimal volume of Enhance (New England Nuclear, MA) for 1-2 hrs., followed by a 30 minutes wash in water, dried, and exposed at -80\(^{\circ}\)C.

The relative amount of \(^{35}\)S-radiolabelled proteins was determined by the total TCA precipitable protein method. Briefly, 20 ul of medium were spotted on a (circular, 1cm dia.) 3MM Whatmann filter paper. An
equivalent of 15%FCS or M199 was added to the filter paper to standardize the total amount of proteins between FCS free samples vs. FCS supplemented samples. The filters were air dried, washed with 3 mls of 10% cold TCA and brought to a boil for 5 minutes. This was followed by two 3 ml washes of cold TCA, one 3 ml wash with 95% ethanol, one 3 ml wash acetone, and finally the filters were rinsed with 1 ml of ether, that was aspirated, and then the filters were air dried. Seven hundred ul of protosol "dihydroxy acetone" (NEN Research Products, Boston, MA.) was added, the samples heated at 55°C for 20 minutes, then 20 uls of glacial acetic acid was added followed by 3.5 ml of scintillation cocktail. Levels of $^{35}$S-radiolabelled proteins were determined by counting on a liquid scinillation counter.

**Enzyme Linked Immunoassay**

Specific milk proteins were determined in media and cell lysates of bovine mammary cell cultures with biotin-avidin-horseradish peroxidase enzyme linked immunoassay (ELISA) using biotinylated antibody monospecific for bovine lactoferrin, $\alpha_s,1$-casein and $\alpha$-lactalbumin (Talhouk et al., 1989b).
**Transmission electron microscopy**

Cells cultured on a 500 ul collagen, embedded within collagen or plated on Matrigel-4 in 1.0 cm well were fixed in a solution of 3% glutaraldehyde, 2% paraformaldehyde, and 1.5% Acrolein, in 0.1M phosphate buffer, pH 7.3. The specimens were washed with buffer and post-fixed in 2% osmium tetroxide in the same buffer for 3 hrs, washed and stained overnight in 1% uranyl acetate. Samples were dehydrated and embedded according to Spurr (1969), post-stained with 0.5% uranyl acetate and 0.1% lead and prepared for observation.
RESULTS

Morphology and total protein synthesis

Cells from lactating or developing mammary gland plated at 5-6 X 10^5 cells/0.5 ml/1.0 cm well attached within the first 24-48 hours in the presence of 15% FCS which was then either removed from the medium on day 2, or maintained throughout the culture period. Cell spreading and formation of sheets was evident in both FCS depleted or supplemented cultures. The cells formed a confluent sheet by day 6 of culture. Previous studies (Larson, 1976; Talhouk et al., 1989a,b) suggested that substrata that cause spreading of the cells and formation of confluent sheets were better models to use in studying protein synthesis and secretion in bovine mammary cell cultures.

After detachment and floatation on day 2 or 6 of the collagen gel, as described by Emerman and Pitelka (1977), contraction of the gels began within 4-8 hrs after floating, with the diameter of the gel reduced to half its original size within 24-48 hours. Contraction then continued slowly until the termination of the culture at 14 days. The detached substratum diameter was reduced to about 1/3 its original size. Detachment of thin collagen gels (100 ul collagen/1.0 cm well) often
resulted in either tearing of the collagen gel layer or only partial detachment, with the central portion of the gel remaining attached to the plastic. This caused the circumference of the gel to curl inward toward the center of the well so as to fold onto itself. This curling created (within 2-4 days after detachment) a clump of cells entrapped within the folded collagen gel. Such wells with cells so entrapped failed to secrete either LF or \( a_{s,1} \)-casein into the medium and appeared non-functional (data not shown). Such difficulties were not encountered for cells plated on 500 ul (thick) collagen gels/1.0 cm well. Proper detachment of the collagen substratum contracted the gel into a cup-like structure, and induced regulation of synthesis and secretion of specific milk proteins in mammary cell cultures from either developing or lactating mammary glands (see later, Fig. 49 to Fig. 54).

The cell population was not affected by detachment of the collagen gel substratum compared to that on attached gels. The number of nuclei indicated that mammary cells continued to replicate in the presence or absence of FCS, with a 2-4 fold increase in cell number between days 4 (250,000 +/- 50,000 cells/well) and 14 (525,000 +/- 100,000 cells/well) in culture. For our bovine mammary cells, detachment of the substratum caused no significant decrease in cell number and did not affect cell spreading and migration away from the acini, in contrast to the effect of detachment of substratum reported for bovine mammary cultures (MacKenzie et al., 1982). Mammary cells from a lactating or a
developing mammary gland cultured on 100 ul or 500 ul collagen gel showed no differences in their total $^{35}$S-labelled proteins secreted into the medium. Furthermore, detachment of the substratum did not affect the total amount of $^{35}$S-labelled proteins. This same effect was noted whether the cultures were depleted from, or maintained in 15% FCS. However, the total $^{35}$S-labelled proteins secreted by cells in FCS supplemented medium were substantially higher than those receiving an FCS depleted medium (Fig. 40). This increase in protein synthetic ability did not only reflect an increase in cell number, but also the cell capacity to synthesize and secrete protein. On the other hand, the increase in protein synthesis need not be due to synthesis and secretion of specific milk proteins, as will be shown later.

$^{35}$S-labeled proteins secreted by cells from developing or lactating mammary gland and cultured on attached or floated substratum were analyzed by SDS-PAGE fluorography. Equal volumes of medium from FCS free cultures were loaded onto the SDS-PAGE gel. Several bands were noted, mostly corresponding to non-milk $^{35}$S-labelled proteins that are not known to exist in mammary secretions. The general profile of total $^{35}$S-labelled secreted proteins by cells cultured on 100 ul vs. 500 ul collagen gels showed no differences, regardless of the stage of the mammary gland from which cells were obtained. Although the $^{35}$S-labelled proteins secreted into the medium showed no differences upon detachment and between cell development
Figure 40. Effect of detachment and thickness of collagen substratum, mammary cell stage, and FCS on total TCA-precipitable $^{35}$S-methionine labelled proteins secreted by mammary cells in culture. Bovine mammary epithelial cells from lactating (top panel) or a developing (bottom panel) gland were labelled on day 10 of culture for 16 hrs, and the TCA precipitated medium was counted by liquid scintillation counting. TCA precipitable $^{35}$S-labelled secreted proteins by mammary cells cultured on attached (□), a detached collagen on day 2 (■■) or day 6 (■■■). Thickness of the substratum was 100 ul or 500 ul/1.0 cm well. Medium consisted of M199 with Prl, I, and F with 15% FCS, or 0% FCS.
Figure 40.
stages, the synthesis and secretion of specific milk proteins (LF and α_{s,1}-casein) were dramatically altered (Fig. 49 to Fig. 54). With lactating cells on 500 ul collagen gels, detachment induced the secretion of a protein with a mol wt similar to that of α_{s,1}-casein, while the other secreted proteins remained unchanged (Fig. 41, arrowhead). This indicated specific induction of secretion of this protein under detachment conditions.

Mammary cells from developing and lactating glands cultured on 500 ul collagen gel under FCS free conditions and detached on day 6 of culture were analyzed by light and transmission electron microscopy. Thick (2 μm) histological cross section of cultured cells from a developing gland showed that the cells stack in multilayers with no particular orientation, and no fat droplets within the cells (Figs. 42 and 43). Ultrastructural studies on those same cells further confirmed that the cell sheets were multilayers of cells, with the top layer being epithelial cells with apical microvilli, tight junctions, and lateral membrane interdigitation. A limited RER network was evident, but poorly organized, with no evidence of secretory vesicles (Fig. 44).

In contrast, thick cross sections (2 μm) of cells from a lactating gland cultured on collagen gels and detached on day 6 in FCS free medium showed an organized and uniform cell sheet of 2-3 cells thick. The top layer appeared as cuboidal epithelial cells with localized areas showing
Figure 41. Protein synthesis and secretion by bovine mammary cells on attached and detached type I collagen. Bovine mammary cells from a lactating gland were labelled on day 10 in an FCS free culture for 16 hrs, and the culture medium was resolved on 17.5% SDS-PAGE. The labelled proteins were visualized by fluorography. Profile of $^{35}$S-labelled secreted proteins by mammary cells on 500 ul layer of collagen. Lane 1 represents profiles of proteins secreted by cells attached substratum; Lane 2, detached substratum on day 2 of culture; Lane 3, detached substratum on day 6 of culture.
Figure 42. Photomicrograph of bovine mammary cells from a developing gland on day 12 in culture on top of a 500 μl layer collagen. Cells were plated at 5 x 10^5 cells/well/0.5 ml of M199 with Prl, I, and F. Note multiple cell layers, and lack of orientation. 2 um cross section, x400.
Figure 42.
Figure 43. Photomicrograph of bovine mammary cells from a developing gland on day 12 in culture on top of a 500 ul layer collagen. Cells were plated at 5 x 10^5 cells/well/0.5 ml of M199 with Prl, I, and F. Note multiple cell layers, lack of orientation, and growth of cells into the collagen matrix. 2 um cross section, x400.
Figure 43.
Figure 44. transmission electron photomicrograph of bovine mammary cells from a developing gland on day 12 in culture on top of a 500 ul layer collagen. Cells were plated at 5 x 10^3 cells/well/0.5 ml of M199 with Prl, I, and F. Cells in the top layer of the cell sheet (see Fig. 42 and 43). Microvilli (MV) are present on the apical surface of the cells. Tight junctions (TJ) join neighboring cells. Disorganized RER (arrowhead), and numerous mitochondria are evident. X 21,000.
fat droplets in their cytoplasm. The underlying layer of cells consisted mainly of a flattened cell type oriented parallel to the collagen substratum (Fig. 45 and 46). Transmission electron microscopy also showed two distinct cell types (Fig. 47 and 48). The underlying layer of cells was specifically characterized by microfilament-like structures surrounding the nucleus. These cells, although in contact with the collagen layer, had microvillar-like structures surrounding them. Hemidesmosomes were evident and numerous between this type of cell and the overlying epithelial cells. The epithelial top layer of cells had an extensive network of organized RER and Golgi apparatus. A tight junctional complex and numerous microvilli were located at the apical surface of the cell. Secretory activity was evident in the epithelial cells ultrastructurally with numerous fat droplets in the cytoplasm and close to the apical membrane, with some (Fig. 46) protruding through the apical membrane, as in secretion. Secretory vesicles with casein micelles were numerous in the cytoplasm and closely surrounding fat droplets (Fig. 48).

**LF and α₅,₁-casein synthesis and secretion in cells from a developing mammary gland**

Detachment of the 100 ul collagen, in FCS supplemented cultures of developing and lactating cells, to study regulation of the specific milk proteins as affected by detachment of the substratum caused the curling
Figure 45. Photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. Medium and plating density as Figure 42. Note cellular organization and layering of cells. Top layer consist of cuboidal cells with their axes parallel to each other. An underlying flattened layer of cells (arrowhead) separate the collagen from the cuboidal type cells (X 1,000).
Figure 46. Photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. Medium and plating density as Figure 42. Numerous fat droplets (F) are evident in different areas of the culture (X 400).
Figure 47. Transmission electron photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. Medium and plating density as Figure 42. Note cellular organization and layering of cells. Top layer consist of secretory cells with numerous fat droplet (F) evident. Microvilli (MV) and tight junctions (TJ) are also noted. Underlying the epithelial secretory cells is another layer of myoepithelia cell (M), showing microfilaments (arrowhead) oriented in parallel to the nuclei axes (X 9,000).
Figure 47.
Figure 48. Transmission electron photomicrograph of bovine mammary cells from a lactating gland on day 12 of culture. Cells were plated on a 500 ul layer of collagen. Medium and plating density as Figure 42. Secretory cells show an organized colgi (G), extensive network of rough endoplasmic reticulum (RER), secretory vesicles containing casein (SV), fat droplet (F) and microvilli (MV). Tight junctions (TJ) are also evident. (X 21,000).
of the collagen with the cells onto themselves, and inconsistent results were obtained. Hence, studies on detachment of 100 ul collagen in FCS supplemented cultures were not addressed.

Analysis of secreted milk proteins (LF, $\alpha_s,1$-casein, and $\alpha$-lactalbumin), as quantified by an ELISA assay, confirmed the above findings. Intracellular levels of LF and $\alpha_s,1$-casein were also determined by ELISA assay. LF secretion into the medium by cells from a developing mammary gland cultured on collagen gels was decreased when the gel was detached and allowed to float. The ability of FCS to increase the secretion of LF into the medium, as shown in our earlier studies (Talhouk et al., 1986), was not as evident when the substratum was detached and allowed to float. Typically, cells on floated collagen gels down-regulated LF secretion by 50-75% compared to attached cultures under the same conditions (Fig. 49). Cells on detached substratum continued to secrete 1-3 ug of LF/ml from day 6 through day 14. This decrease in LF secretion was not due to intracellular accumulation and failure to export LF, since intracellular pools of LF did not increase. Total intracellular concentration of LF did not exceed 150 ng/ml and was not correlated to whether the substratum was floated or maintained attached. In addition, this decline in secreted LF did not reflect a loss of the cellular ability to secrete proteins since the total $^{35}$S-labelled secreted proteins did not differ significantly for floated vs. attached cultures. Hence, detachment of
Figure 49. Regulation of LF secretion by bovine mammary cells from a developing gland in culture, in response to substratum detachment. Bovine mammary cells were cultured on an attached (O), or a detached collagen on day 2 (A), or day 6 (Δ). Medium consisted of M199 supplemented with lactogenic hormones Prl, I, and F. Medium was changed every 2 days, and assayed for LF concentration by ELISA. A) Mammary cells on 100 ul type I collagen in an FCS free medium. B) Mammary cells on 500 ul type I collagen in an FCS free medium, or C) with 15% FCS supplemented to the medium.
the collagen substratum appears to specifically affect LF synthesis without affecting its subsequent secretion.

Detachment of the substratum alone did not enhance the cells' ability to secrete \( \alpha_s,1 \)-casein, and concentration of \( \alpha_s,1 \)-casein in the medium continued to decline beyond day 2-4 of culture (Fig. 50) in cells on detached or attached collagen substrata. Interestingly, cells on attached collagen gels supplemented with FCS synthesized but did not efficiently secrete \( \alpha_s,1 \)-casein into the medium (Fig. 51C vs Fig. 50C). Floating of the substratum did not relieve this block on secretion, since \( \alpha_s,1 \)-casein levels in the medium did not increase upon detachment of the collagen; however, substratum detachment did prevent the accumulation of intracellular \( \alpha_s,1 \)-casein (Fig. 51C). This suggests that upon detachment, intracellular pools of \( \alpha_s,1 \)-casein were either diverted to lysosomal degradation which prevented their intracellular accumulation, or that \( \alpha_{s1} \)-casein was not synthesized. \( \alpha \)-lactalbumin was not detected in the medium under any of the above conditions for cells from a developing mammary gland.

**LF and \( \alpha_s,1 \)-casein in mammary cell cultures from a lactating gland**

Regulation of the synthesis and secretion of the individual milk proteins LF, \( \alpha_s,1 \)-casein, and \( \alpha \)-lactalbumin in cultures of lactating bovine mammary cells differed drastically from that in developing
Figure 50. Effect of detachment of the substratum on the regulation of \( \alpha_{s1} \)-casein secretion by bovine mammary cells from a developing gland in culture. Bovine mammary cells were cultured on an attached (\( \bullet \)), or a detached collagen gel on day 2 (\( \bigcirc \)), or day 6 (\( \triangle \)). Medium consisted of M199 supplemented with lactogenic hormones Prl, I, and F. Medium was changed every 2 days, and assayed for \( \alpha_{s1} \)-casein concentration by ELISA. A) Mammary cells on 100 ul collagen. B) Mammary cells on 500 ul collagen in an FCS free medium, or C) with 15% FCS supplemented to the medium.
Figure 51. $\alpha_s$-Casein intracellular accumulation in cultures of bovine mammary cells from a developing gland in response to substratum detachment. Cells were collected on day 4, 10, and 14 and assayed for $\alpha_s$-casein concentration by ELISA. A) Mammary cells from a developing gland on 100 ul collagen in an FCS free medium. B) Cells on 500 ul collagen, in an FCS free medium, or C) supplemented with 15% FCS. (O) Cells on an attached substratum, (•) or on a substratum detached on day 2, or (Δ) day 6.
Cells. Detaching the collagen gel did not affect the synthesis and secretion of LF into the medium, with levels remaining unchanged whether the substratum was detached on day 2 or day 6, or kept attached throughout the culture period. No intracellular accumulation of LF was noted under attached or floated substratum conditions, and more than 90% of the LF synthesized was secreted into the medium on days 10 and 14 of the culture (data not shown). The effect of thin or thick substratum (100 ul vs. 500 ul collagen well) on LF secretion was maintained as previously reported (Talhouk et al., 1989a) even after substratum detachment. Cells on 500 ul collagen gel (Fig. 52B) secreted higher levels of LF than cells on 100 ul gels (Fig. 52A), and FCS induced higher LF secretion for cells on 500 ul collagen (Fig. 52B vs. 52C).

\( \alpha_{s,1}-\text{Casein synthesis and secretion, in contrast to LF, was markedly enhanced when the collagen substratum was detached (Fig. 53). Cells cultured in an FCS free medium on a thin layer of collagen secreted lower levels of } \alpha_{s1}-\text{casein into the medium (Fig. 53A) than those same cells cultured on a thick layer of collagen (Fig. 53B), regardless whether the substratum was detached or not. Cells from a lactating gland cultured on 100 ul collagen gel in FCS free medium increased the levels of } \alpha_{s1}-\text{casein secretion into the medium on days 12-14 by 2 fold compared to those on attached gels, whereas detachment on day 6 increased the levels of } \alpha_{s,1}-\text{casein secreted into the medium by 7-8 fold over that for cells on attached gels (Fig. 53A). Detachment of the same} \)
Figure 52. Regulation of LF secretion by bovine mammary cells from a lactating gland in culture in response to substratum detachment. Bovine mammary cells were cultured on an attached (0), or a detached collagen gel on day 2 (●), or day 6 (△). Medium was sampled and assayed as figure 5. A) Mammary cells on 100 ul collagen, in an FCS free medium. B) Mammary cells on 500 ul collagen in an FCS free medium, or C) with 15% FCS supplemented to the medium.
Figure 53. Effect of detachment of the substratum on the regulation of $\alpha_s1$-casein secretion by bovine mammary cells from a lactating gland in culture. Medium was sampled and assayed as in figure 29. Lactating mammary cells on A) 100 ul collagen gel, in FCS free medium. B) 500 ul collagen in an FCS free medium, or C) with 15% FCS supplemented to the medium. Bovine mammary cells were cultured on an attached (O), or a detached collagen gel on day 2 (●), or day 6 (△).
substratum for cells in medium with FCS frequently resulted in the substratum with cells curling and folding onto itself, resulting in a rapid decline in both LF and $\alpha_s,\lambda$-casein secretion into the medium, apparently due to loss of total protein synthetic capacity.

Lactating cells cultured on 500 ul collagen gel exhibited similar induction of $\alpha_s,\lambda$-casein synthesis and secretion to those on 100 ul collagen gels. Detachment of the 500 ul collagen gels in FCS free medium on day 2 increased $\alpha_s,\lambda$-casein secretion by 3-4 fold compared to cells on attached collagen gels, while detaching the collagen substratum on day 6 of culture induced a 10 fold increase (Fig. 53B). Even in the presence of FCS in the medium, detachment induced $\alpha_s,\lambda$-casein synthesis and secretion; however, the degree of induction of casein synthesis and secretion by detachment was less if serum was present in the medium. In FCS supplemented medium, detachment induced a 2-3 fold increase in casein in the medium, irrespective of the day of substratum detachment (Fig. 53C).

$\alpha_s,\lambda$-Casein secretion in cells from either stage of mammary development decreased from the initial stages of the cultures through day 6-8 in culture. However, lactating cells then showed increasing $\alpha_s,\lambda$-casein synthesis and secretion after day 8 (Fig. 53). Detachment of the substratum on day 2 did not prevent the decrease of casein secretion that the cells undergo in the early stages of culture. During
this early period in culture (days 0-6) the mammary cells restructured their acini and spread to form a confluent sheet of cells.

Cells on 500 ul layer of collagen responded to early detachment on day 2 by showing an earlier increase in casein synthesis and secretion manifested during the period between day 6-8 of culture. While detachment of the collagen on day 6 increased \( \alpha_{s,1} \)-casein secretion over days 8-14 (Fig. 53B).

Intracellular accumulation of \( \alpha_{s,1} \)-casein in cultures from lactating mammary cells (in marked contrast to LF), was regulated by detachment of the substratum. Figure 54 shows that intracellular accumulation of \( \alpha_{s,1} \)-casein was only noted in cells whose matrices remained attached. Cells on collagen which were detached on either day 2 or day 6 did not accumulate intracellular \( \alpha_{s,1} \)-casein, and the intracellular \( \alpha_{s,1} \)-casein remained constant throughout the culture period. Cells on attached collagen, although capable of secreting casein into the medium, accumulated comparable intracellular amounts (Fig. 53 vs. Fig. 54). For cells on attached collagen, the sum of total intracellular and secreted \( \alpha_{s,1} \)-casein was not equal to the total amount secreted into the medium by the same cells on detached collagen, suggesting that synthesis of total \( \alpha_{s,1} \)-casein was less for cells on attached collagen than for cells on floating collagen. Furthermore, cells on attached
Figure 54. Intracellular levels of $\alpha_{s,1}$-casein in bovine mammary cultures from a lactating mammary gland. Effect of substratum detachment. Cell sampling and assaying as figure 29. A) Cells on 100 ul collagen, in FCS free medium. B) Cells on 500 ul collagen, in FCS free medium or, C) supplemented with 15% FCS. Cells on attached substratum (0), or on a substratum detached on day 2 (●) or day 6 (△).
collagen apparently were unable to secrete already synthesized \( \alpha_s,1 \)-casein as efficiently as cells on a detached collagen.

Cells from a lactating mammary gland secreted \( \alpha \)-lactalbumin when cultured in FCS free medium on 500 ul of detached collagen gel (Fig. 55). No other substrata supported \( \alpha \)-lactalbumin secretion. Detachment of the 500 ul layer of collagen under FCS free conditions induced \( \alpha \)-lactalbumin secretion in the medium between day 8-14 of the culture. The levels of \( \alpha \)-lactalbumin detected in the medium were only 3-4 ng/ml, and when compared to the relative amounts of \( \alpha_s,1 \)-casein in the medium, they were at least 50 fold lower than their relative concentrations in milk. No attempt was made to determine if lactose was being synthesized under these conditions.

**Prolactin regulation of \( \alpha_s,1 \)-casein and LF in lactating cells on floating collagen gels**

Experiments were performed to determine whether lactating bovine mammary cells were dependent on prolactin in order to secrete casein, and if so, whether prolactin was required throughout the culture, or only for a restricted period, analogous to the bovine mammary gland in vivo for which prolactin is required prepartum, but not postpartum (Akers et al., 1981). We investigated the response of this cell culture system to the lactogenic hormone, prolactin. Cryopreserved cells from a
Figure S3. α-Lactalbumin secretion by mammary cells from a lactating gland on 500 ul collagen in FCS free medium H199 with Prl, I and F. Cells were on substratum which was attached to day 14 (○), or detached on day 2 (●), or day 6 (△).
lactating bovine mammary gland cultured in serum-free medium on 500 ul layer of collagen gel detached on day 6 showed complete dependency on prolactin for casein synthesis and secretion (Fig. 56A), but not for LF synthesis and secretion (Fig. 56B). Such cells grown in medium devoid of prolactin for the entire culture period showed no accumulation of $\alpha_s,\lambda$-casein in the medium, whereas supplementing the culture with prolactin on days zero through 14 induced secretion of $\alpha_s,\lambda$-casein, but as before, it did not begin to increase until days 6-8, by which time a cell sheet had formed. Since those cultures which secreted casein did not begin to do so until days 6-8, we examined whether prolactin could be omitted before or after the onset of casein secretion. Adding prolactin to the medium only on days 8-14 of culture induced $\alpha_s,\lambda$-casein secretion to about 70\% (900 ng/ml) of that from cells that received prolactin throughout the culture up to day 14 (Fig. 56A). On the other hand, supplementing prolactin on day 14-18 in culture to cells grown in a prolactin depleted medium, resulted in a limited response. $\alpha_s,\lambda$-casein concentration in the medium was only 50-100 ng/ml between the period of day 14-18, at which time the experiment was terminated.

Supplementing the culture with prolactin during the first 8 days in culture and then withdrawing it from the medium only partially reduced the levels of $\alpha_s,\lambda$-casein secretion. $\alpha_s,\lambda$-Casein secreted in the medium increased steadily between day 8 and 14 of culture, a period in which the cell culture medium was devoid of prolactin. The $\alpha_s,\lambda$-
Figure 56. Effect of Prol on \( \alpha_{s1} \)-casein (Panel A), and LF (Panel B) secretion by bovine mammary cells on a 500 ul collagen, detached on day 6 of culture. FCS free medium consisted of, M199 with I, and F. Prol is added or depleted from the medium according to the treatment. (O) Prol supplemented to the medium from day 0-14, (•) day 0-8, (▲) and day 8-14. (△) Prol depleted across experimental period (day 0-14).
casein concentration in the medium at day 14, totaling 500 ng/ml, was approximately 30% of the amount of $\alpha_\text{s,1}$-casein secreted by cells cultured with prolactin supplemented to the medium for the entire 14 day experimental period (Fig. 56A). The level of $\alpha_\text{s,1}$-casein secreted on day 14 by cells receiving prolactin throughout the entire culture period showed a high standard deviation in this particular experiment. Typically cells under the same conditions secreted 2-3 ug/ml of $\alpha_\text{s,1}$-casein (see Fig. 53B).

Finally, and in a similar study, depletion of prolactin between days 4-8 of culture yielded the same amount of $\alpha_\text{s,1}$-casein secreted into the medium as when prolactin was supplied at and beyond day 8 (unpublished data).

LF regulation was prolactin independent. LF was continuously secreted into the medium irrespective of the time period that prolactin was supplemented or depleted from the medium. Whereas casein secretion by bovine mammary cells in culture was dependent upon prolactin in the medium, LF secretion increased continuously up to day 14 or until termination of the culture (Fig. 56B). In the absence of prolactin over the entire culture period, LF secretion was consistently decreased by only 30-50% at day 10 and beyond.
DISCUSSION

In this study, we demonstrate that cryopreserved lactating bovine mammary cells responded to detachment of the substratum by increased casein synthesis and secretion. Detachment also induced α-lactalbumin synthesis and secretion, and fat droplet formation and secretion, but did not affect LF regulation in the lactating cell cultures. Emerman and Pitelka (1977) reported similar data on casein secretion in floated cultures of rodent mammary cells. Previously, we compared the performance of bovine mammary cells on different substrata and showed that milk protein synthesis and secretion in bovine mammary cultures was regulated by the substratum (Talhouk et al., 1989a). This has been confirmed by the work of others on rodent mammary cell culture systems (Lee et al., 1985, and Wicha et al., 1982).

Total $^{35}$S-labelled proteins secreted into the medium were not affected by substratum detachment (Fig. 40) contrary to the regulation of the specific milk proteins (LF, $\alpha_{s,1}$-casein and α-lactalbumin). This suggested that the detachment of the substratum induced the regulation of specific milk proteins without affecting the cells total protein synthetic capabilities. This finding was in contrast to Emerman and
Pitelka (1977), who found that total protein synthesis increased 2-3 fold upon detachment of the substratum.

The expression of milk proteins, LF, $\alpha_s,1$-casein, and $\alpha$-lactalbumin was regulated according to the stage of the cell and the detachment of the substratum. In contrast, no major changes were evident in the secreted protein patterns on SDS-PAGE (Fig. 41). The above findings on lactating and developing bovine mammary cells in culture, were similar to previous results by Blum et al. (1987), Bissell and Hall (1987), and Li et al. (1985) on rodent mammary cultures from a mid-pregnant gland. The authors reported that non-milk proteins were secreted in vitro by cultures of mammary cells, and the substratum specifically regulated milk protein expression without affecting the total protein output by the cultured mammary cells.

The concept of coordinated regulation of milk protein in vivo is attractive; however, it does not translate to in vitro conditions. In fact Harmon et al. (1976) showed that upon bovine mammary gland injury the cells induced LF secretion without affecting the levels of other milk proteins in the mammary secretions. This argued against the concept of coordinated regulation of milk protein synthesis under in vivo conditions as well. Schanbacher and Smith (1975) and Hoppe (1976) described changes in milk and mammary proteins and related that to the stage of development of the mammary gland. LF was reported as a marker
for involuted or dedifferentiated bovine mammary glands, whereas high levels of micellar casein, α-lactalbumin, and other proteins reflected a differentiated, lactational status of the mammary gland. However, during mammary infection, high levels of both LF and micellar casein could be found in the bovine mammary secretions.

The regulation of milk proteins in vitro does not appear to be coordinately regulated. Durban (1987) reported that mouse α-caseins are less responsive than β-casein to hormonal and extracellular matrix regulation. Similar results by Lee et al. (1985) further suggested independent regulation of α-caseins and β-caseins in vitro. MacKenzie et al. (1985) demonstrated α-lactalbumin secretion by bovine mammary cells in culture; however, no evidence for casein secretion was shown, even though caseins and α-lactalbumin are both co-localized in casein containing secretory vesicles (Shappell et al., 1986). We were able to specifically induce αS,1-casein synthesis by 3-10 fold in cultures of cryopreserved lactating bovine mammary cells by detaching the collagen substratum upon which the cells were overlaid (Fig. 53). This induction of αS,1-casein did not alter LF levels in the medium (Fig. 52) or LF intracellular pools. Whether this increase in αS,1-casein expression in the medium was due to changes in cell shape which was induced by contraction of the released collagen, (Haeuptle et al., 1983; Emerman and Pitelka, 1977; Neville, 1987), or whether it is an indirect effect of a change in cell shape, whereby the cells were then able to
synthesize their own matrix components necessary to initiate casein synthesis and secretion (Blum et al., 1987) is still unresolved. Our observations suggested that both factors, contractility of the collagen and synthesis of extracellular matrix components by the cells, contributed to augmentation of casein levels secreted into the medium for the following reasons: 1) Bovine mammary cells on a 500 ul collagen layer, secreted higher levels of αs,1-casein than cells plated on a 100 ul layer of collagen. Although this could be attributed to better access of nutrients to the basal side of cells that are on the 500 ul collagen, our preliminary data do not support this. Culturing the same cells on a very thin film of collagen (as described by the manufacturer, Millipore Corporation, Bedford, MA) on solid non-contractile membrane filters (well inserts) such that nutrients have free access to cell basal membrane, resulted in low levels (~200 ng/ml) of αs,1-casein output into the medium (unpublished observation). This suggested that contractility of the substratum plays a role in inducing αs,1-casein synthesis and secretion by bovine mammary cells in culture. 2) On the other hand, cells on detached collagen gels were more difficult to free from their substratum by collagenase treatment than cells on attached collagen, even though the collagen gel was completely digested. In cultures of bovine mammary cells on an attached gel, a suspension of cell clumps was obtained, and the cell clumps were easily dispersed by using a pasteur pipet. In contrast, cells on detached and contracted collagen floated freely in the digesta as a well complexed sheet of cells. This sheet of
cells required extensive vortexing before the cells were dispersed into suspension. This suggested that cells on detached and contracted collagen gel synthesized a basement membrane different than that of cells on attached collagen.

Detaching the collagen gel that the mammary epithelia cells were plated on was not by itself sufficient to induce $\alpha_{s,1}$-casein synthesis, even though the cells contracted the collagen within hours after detachment. This was clearly evident when lactating bovine mammary cells were detached on day 2 of culture, while casein synthesis and secretion was not noted prior to day 6 or 8 of culture (Fig. 53). Thus, it appears that the cells underwent a transition phase in the early period of the culture that allowed them to acquire a casein synthetic capability. Perhaps the mammary cells in culture replicate during this early culture period to reach a certain critical cell density beyond which they are able to acquire casein synthesis (Talhouk et al., 1989b) and cellular polarization in culture. Rapraeger et al. (1986) and Harzlinger and Ojakian (1984) demonstrated that mouse mammary epithelial cells and MDCK cells respectively required a minimal cell density that allowed them to form confluent sheets, with tight junctions between neighboring cells. This caused basal membrane proteins to localize and orient properly to achieve epithelial cell polarization. This concept (Rapraeger et al. 1986 and Harzlinger and Ojakian, 1984) agrees with the observation that $\alpha_{s,1}$-casein synthesis and secretion in our culture was
initiated only at, or after, the mammary cells achieved confluency on the proper extracellular matrix. Detachment of the collagen on day 2 in an FCS free culture increased α_s,1-casein synthesis by 3-4 fold over that of cells on an attached collagen; but, the total α_s,1-casein secreted was less than that secreted by cells on collagen that was detached on day 6. In both cases, when the collagen substratum was detached the cells achieved confluency, and no apparent differences were noted at the light microscopy level.

Detaching the collagen not only increased α_s,1-casein secretion but also increased the efficiency of α_s,1-casein secretion for cells derived from a lactating bovine mammary gland. Transmission electron microscopy showed micellar casein in secretory vesicles of cells on detached collagen, but none in cells on attached gels (Talhouk et al., 1989a). However, α_s,1-casein was detected by ELISA assays in significant amounts both intracellularly and extracellularly in cells on attached gels. This observation suggested that synthesis and secretion of α_s,1-casein by lactating bovine mammary cells on attached collagen was partly impaired, and that the caseins were probably non-micellar in nature, similar to casein found in secretions of mid-pregnant cows (unpublished observation). Rocha et al. (1985) have shown that intracellular pools of casein from cells on attached collagen co-migrated on electrophoretic gels with the caseins secreted by cells on floated collagen or from a lactating gland only when the latter two samples were enzymatically
dephosphorylated. The authors suggested that casein phosphorylation, an essential step that leads to micellar formation, was required for efficient secretion of caseins. In addition, Lee et al. (1985) showed inefficient casein secretion and intracellular degradation by mammary cells on plastic. The above observations (Rocha et al., 1985, and Lee et al., 1985) would explain the absence of micellar casein or secretory vesicles in bovine mammary cells on attached matrices, and their intracellular accumulation, and inefficient secretion when compared to cultures of bovine mammary cells on floated collagen gels.

Thus, we suggest that the change in cell shape induced upon floating and the apparent synthesis of extracellular matrix components induced organized restructuring of the cytoskeletal matrix leading to more efficient transcription and secretion of milk proteins, namely, αs,1-casein (Bissell et al, 1982; Bissell and Hall, 1987).

Detaching the substratum further induced fat droplet formation and secretion, as well as synthesis and secretion of α-lactalbumin in an FCS-free culture of bovine mammary cells derived from a lactating gland. Again, the process of fat droplet formation was not a direct effect of detaching the substratum, since fat droplets were noted only in cells at day 12 (Fig. 47 and 48), but not day 10 (results not shown). Hence, the process of fat synthesis and formation of fat droplets commenced after induction of casein synthesis and secretion into the medium, and the two
events do not appear to be coordinately regulated: Expression of α-lactalbumin in cultures of bovine mammary cells was a result of detaching the collagen. This has been previously reported by MacKenzie et al. (1985); however, no evidence for micellar casein in secretory vesicles was demonstrated. This was not the case in our culture since α-lactalbumin and micellar caseins were only detected as a result of detaching the substratum; nevertheless, comparison of our results to that of MacKenzie et al. (1985) suggested that the two events, α-lactalbumin and casein micelle synthesis and secretion are not coordinately regulated, at least in vitro. Similar observation has been suggested by Blum et al. (1987) for cultures of rat mammary system, while Lao et al. (1985) suggested that α-lactalbumin was not regulated by the substratum in cultures of mouse mammary epithelial cells.

The complexity of interactions needed to induce casein synthesis and secretion was demonstrated by our inability to enhance αs,1-casein secretion into the medium by cells from a developing mammary gland. Intracellular accumulation of αs,1-casein was detected in cultures on attached collagen gels, and only when supplemented with FCS. Levels of secreted αs,1-casein in these cultures declined across the culture period (Fig. 50). Interestingly, Durban (1987) and Durban et al. (1985) showed that cultures of mammary cells embedded in, or grown on detached collagen from immature virgin mice failed to express and accumulate intracellular casein unless properly primed with lactogenic hormones,
and only in the presence of high levels of serum in the medium. In addition, this was not noted except after 2-3 weeks of adaptation in culture. The authors further demonstrated that supplementing the medium with estrogen and progesterone alleviated the need for high levels of serum in the medium, and suggested that unprimed virgin mammary cells may not be synthesizing a putative mammary growth factor responsible to set the stage for mammary differentiation events. Whether a similar event is occurring in our cultures is not known, although it has to be noted that our tissue was obtained from a estrogen/progesterone primed developing mammary gland, and not from virgin nulligravida heifer. Further studies are needed to clarify the inability of cells from a developing bovine mammary gland to efficiently synthesize and secrete caseins.

In contrast to cells from lactating bovine mammary gland, detaching the collagen did not alleviate the partial block on secretion noted to occur in attached FCS supplemented cultures from a developing mammary gland. Instead, intracellular accumulation of $\alpha_s,1$-casein was not evident, and no increase in the extracellular or secreted levels of $\alpha_s,1$-casein were detected in the detached cultures (Fig. 510). It was not determined whether the loss of $\alpha_s,1$-casein attained upon detachment of the matrix was manifested at the pre- or post-transcriptional level.
FCS was required in cultures of mammary cells from a developing gland to initiate and maintain \( \alpha_s,1 \)-casein synthesis (Fig. 51). In cultures of mammary cells from a lactating gland, FCS had an opposite effect (Fig. 53B vs. 53C). Detached cultures supplemented with FCS secreted 25 to 50% of \( \alpha_s,1 \)-casein compared to cells in an FCS-depleted medium. In addition, preliminary studies have shown that when the medium is supplemented with epidermal growth factor, \( \alpha_s,1 \)-casein secreted in the medium by cells from a lactating mammary gland is down regulated (unpublished data). Similarly, Taketani and Oka (1983) reported that epidermal growth factor decreased casein m-RNA levels and casein production in organ cultures of mouse mammary cells, and that this effect was less pronounced from mice at less advanced stages of pregnancy, suggesting different effects of the same growth factor, depending on the stage of mammary development. It is important to note that FCS in cultures of bovine mammary cells from a lactating gland does not inhibit \( \alpha_s,1 \)-casein synthesis and secretion, but reduces it. Previously, we showed that FCS was needed to maintain \( \alpha_s,1 \)-casein secretion in low density cultures of bovine mammary cells from a lactating gland, on attached collagen. This effect was abrogated with higher plating density (Talhouk et al., 1989b). In this study we used a plating density of 5-6x10^5 cells/0.5 ml/1.0 cm well, necessary to obtain \( \alpha_s,1 \)-casein synthesis in FCS free medium. We further suggest that FCS has a negative effect on optimal \( \alpha_s,1 \)-casein synthesis and secretion in cultures from a lactating gland on detached collagen.
The effect of detachment on the regulation of LF was very different from that noted for casein in both types of mammary cells (from either developing and/or lactating gland). In other studies the regulation of transferrin in cultures of rodent mammary cell suggested that it was not regulated by lactogenic hormones to the same extent that caseins were, and that transferrin was secreted via different routes than was casein (Parry et al., 1987; Lee et al., 1987; Lee et al., 1984). On the other hand, synthesis and secretion of transferrin in mouse mammary cells in vitro, and LF in bovine mammary cell cultures were highly affected by the extracellular matrix (Chen and Bissell, 1987; Talhouk et al., 1986). In this report, we demonstrated that detachment of the collagen on which lactating mammary cells were plated did not affect the levels of secreted LF (Fig. 52) or that accumulated in the cells. Furthermore, LF synthesis and secretion was not affected by prolactin (Fig. 56). In contrast to lactating cells, developing cells secreted less LF into the medium upon detachment of the collagen substratum (Fig. 49).

We thus suggest that LF in cultures of bovine mammary cells is independently regulated from $\alpha_{s,1}$-casein, and is probably secreted via different intracellular routes. This is based on the following facts:

1) No intracellular accumulation of LF was noted irrespective of the
amounts secreted in the medium when cells were cultured on attached or detached collagen gels. 2) If both LF and α₅,₁-casein were secreted via the same secretory vesicles, we would expect their intracellular levels to increase or decrease in parallel when both proteins were being secreted into the medium. This was not the case. 3) Furthermore, in the early period of cultures from a lactating mammary gland, a decrease in α₅,₁-casein secreted into the medium was noted, followed by an increase at the time the cells achieve confluency. In contrast, LF levels were constantly on the increase during the culture period. This implied that different factors affect the regulation of both proteins, since under the same conditions α₅,₁-casein decreases and then increases, while LF only increased.

The dedifferentiation noted at the early stages of the culture from a lactating bovine mammary gland, followed by differentiation on day 6-8, and only in the presence of prolactin in the medium, reflected either of two situations. 1) The lack of prolactin receptors at the early stages of culture (day 0-6) that were lost due to the tissue dissociation procedure. Or, 2) the cells undergo reorganizational changes to achieve confluency and hence polarization (Rapraeger et al., 1985) before they re-differentiate and secrete caseins. The former option is highly unlikely since mammary epithelial cells have been shown to possess prolactin receptors after dispersion of the cells with collagenase (Suard et al., 1979; Sahai et al., 1978). Thus, we propose
that the culture underwent a critical change from a dedifferentiated state to a casein secretory state that was emphasized by detachment of the collagen gel. This important window of events occurred between day 4-8 of culture. Thus sensitivity and response of this FCS free system to prolactin was examined around the day 4-8 interval.

There is no evidence that prolactin increases milk yield; however, Goodman et al. (1983) and Gertler et al. (1982) demonstrated that prolactin increased the synthesis of α-lactalbumin and casein in explant cultures of bovine mammary tissue. Reports in the literature show marked variation on the effect of prolactin among different species. It is generally accepted that in ruminants, prolactin is needed for the initiation of lactation, but not for its maintenance (Schams et al., 1972; Akers et al., 1981; Smith et al. 1974). In rodents, this does not seem to be the case (Tucker et al., 1979). The above studies on prolactin were either done in whole animals or organ cultures, and while this has its advantages, nonetheless, several factors as hormones, growth factors, and others could interact with the treatment and affect the results reported. Studies on mammary cell cultures of rodents (Wiens et al., 1987) and rabbits (Suard et al., 1983) showed the effect of prolactin on the induction and maintenance of casein synthesis and secretion. The authors further demonstrated the influence of the extracellular matrix, and cell shape on the responsiveness of mammary cells to the lactogenic hormone, prolactin. Chen and Bissell (1987), on
the other hand, showed that transferrin synthesis and secretion was partially affected by prolactin but not to the extent that the caseins were. In this study, the mammary cells cultured in an FCS free medium (except for the first two days in culture, whereby FCS was used to facilitate cell attachment), were shown to be completely dependent on prolactin for the initiation of casein production. Our data further supported the earlier reports (Smith et al., 1974) that ruminants were dependent on prolactin for the initiation of casein secretion, but not for its maintenance. Withdrawal of prolactin at day 8-14 in culture only partially reduced the levels of αs,1-casein secreted into the medium. αs,1-Casein continued to be detected in the medium in increasing amounts up to at least day 14 in culture. It could be argued that the very high concentrations of prolactin used in the medium (8 ug/ml) up to day 8 were not completely washed out of the medium, and could still be acting at low levels (physiological or sub-physiological) to maintain casein synthesis and secretion. The presence of prolactin as a carry over beyond day 8 in a prolactin depleted medium was not examined; however, cells on day 8 and prior to medium depletion were washed 3 times with M199 and medium was changed at 2 days intervals beyond that for a period of 6 days. αs,1-Casein in the medium after this 6 day period was still secreted in increasing amounts (Fig. 56A). The extensive washes in addition to the medium changes, and the question of stability of prolactin for 6 days in culture at 37°C, would suggest the wash out of all active prolactin from the cell environment.
Cells maintained their ability to respond to prolactin even after 14 days in culture in a prolactin depleted medium. However, their ability to redifferentiate into casein secretory cells decreased with time in culture. A 30\% decrease of $\alpha_{s,1}$-casein secretion was observed on day 14 upon stimulation with prolactin on days 8-14, when compared to $\alpha_{s,1}$-casein levels in cell cultures receiving prolactin on days 0-14. A later study showed that 100\% of $\alpha_{s,1}$-casein output was acquired by supplementing prolactin on day 6-14 of culture. Interestingly, supplementing prolactin between day 0-4 and beyond day 8 resulted in similar casein output as that of prolactin supplemented beyond day 8 (data not shown). This suggest that the cells in culture undergo a refractory stage in the first 4 days, during which they are non-responsive to prolactin. However, at a certain window of events between day 4-8, the cells become responsive to prolactin. At a later stage, beyond day 8 the cells gradually lose their ability to redifferentiate in response to lactogenic hormones. It would be interesting to determine the effect of a spike of prolactin only on day 6 when the cells are responsive to prolactin. Perhaps this would mimic the effect noted when prolactin was supplemented only up to day 8 prior to withdrawal. Further studies are needed to clarify the ruminant mammary cell responsiveness to prolactin.

We have previously demonstrated (Talhouk et al., 1989c) that cells from a lactating gland secreted a growth promoting activity in culture. This growth activity peaked at a stage in culture coincident with
confluent sheet formation, and thus with the period of optimal prolactin sensitivity, and initiation of casein synthesis. Perhaps this peak of growth factor activity is required to set the stage for differentiation events as suggested by Durban (1987) and Durban et al. (1985).

The loss of cells' ability to respond to prolactin as the culture progressed did not reflect the loss of the protein synthetic and secretory ability of the cells, since, in contrast to $\alpha_{s,1}$-casein, LF continued to be secreted throughout the entire experimental period, and it was not affected by the presence or absence of prolactin in the medium. Chen and Bissell (1987) reported a partial decrease of mRNA levels for transferrin in the absence of prolactin, when compared to the decrease noted for casein mRNA. Similarly, data showed approximately a 40% decrease in LF secretion on day 14 of culture, in the absence of prolactin from the medium. LF was not affected by other permutations of prolactin supplementation to the medium (Fig. 56B). Thus, LF synthesis and secretion was governed by a different set of regulatory events unlike those of caseins, since neither lactogenic hormone (prolactin) nor detachment of the collagen gel affected it in the same pattern as caseins.

In conclusion, we have demonstrated that cryopreserved cells from a lactating bovine mammary gland, cultured on a 500 ul layer of collagen
detached on day 6 in culture, secreted $\alpha_s,\lambda$-casein, $\alpha$-lactalbumin and LF. Fat droplet formation was also noted. Detachment of the substratum increased $\alpha_s,\lambda$-casein concentration in the medium beyond day 6-8 by increasing the efficiency of synthesis and secretion of the casein. In addition, detachment induced $\alpha$-lactalbumin secretion and fat synthesis, while detachment did not affect LF synthesis and secretion. Mammary cells on detached 500 ul collagen in an FCS-free environment were responsive to prolactin. A period of optimal prolactin sensitivity was suggested to occur between day 4-8. This period coincided with the formation of a confluent sheet of cells. Furthermore, prolactin was found to be necessary for initiating but not maintaining $\alpha_s,\lambda$-casein secretion. Cells from a developing gland responded differently to substratum detachment. LF was specifically down regulated, while none of the other milk proteins were induced upon substratum detachment. Finally we suggest that the milk proteins are independently regulated in vitro, and that LF and $\alpha_s,\lambda$-casein are secreted via independent routes.
REFERENCES


264


PARTIAL CHARACTERIZATION AND PURIFICATION OF GROWTH FACTORS
IN PRIMIGRAVIDA BOVINE MAMMARY SECRETIONS FROM A
DEVELOPING MAMMARY GLAND

INTRODUCTION

Milk is known to contain a variety of polypeptide growth factors (GF) which vary with the stage of lactation and species, and are presumed to modulate growth of either mammary or neonatal systems or both (Shing and Klagsbrun 1984 and Dembinski and Shiu 1987). The presence of GF in milk was first suggested by the observation that milk and/or colostrum supported the growth of cells in serum free culture. Sereni and Bergen (1981) used bovine milk as a serum replacement for the growth and maintenance of several cell lines. Klagsbrun and Neuman (1979) and Read et al. (1984) reported that BALB/c 3T3 cells, as well as other cell lines, were maintained in serum free bovine colostrum supplemented medium. Further analysis of the mitogenic activities appearing in colostrum and milk demonstrated the presence of a basic polypeptide GF in goat colostrum with a mol. wt. of approx. 35,000,
which was chemically and functionally similar to human platelet derived growth factor (h-PDGF) (Brown and Blakeley, 1984), and the presence of three GF in human milk, two of which appeared to be epidermal growth factor (EGF) or EGF-like (Carpenter, 1980; Shing and Klagsbrun, 1984), and insulin like growth factor (IGF)-1 (Baxter et al., 1984). In addition, Kidwell and Salomon (1987) reported at least 14 GF in human milk and suggested that some of them, including EGF, transforming growth factor (TGF) alpha and TGF-beta, and human mammary derived growth factor-1 (HMDGF1), were synthesized in the mammary gland and may play a role in mammary development.

Studies defining and characterizing growth promoting activities in bovine mammary secretions have been limited. Brown and Blakeley (1983a,b) showed that bovine, sheep, and goat colostrum inhibited $^{125}\text{I}$-EGF binding to 3T3 cells and suggested that bovine colostrum contained a growth factor (GF) similar to the hPDGF-like found in goat colostrum (Brown and Blakeley, 1984). Campbell and Baumrucker (1988) recently demonstrated the presence of insulin-like GF (IGF)-1 and IGF-1 binding proteins in bovine milk, while Francis et al. (1986) and Francis et al. (1988) purified IGF-1 and IGF-2 from bovine colostrum and determined their amino acid sequences. Spitzer and Grosse (1987) identified EGF receptors on bovine mammary plasma membranes at different stages of mammary development and lactation, and reported highest concentrations of receptors on the plasma membrane of pregnant animals.
No studies are available that describe the changes, if any, in GF occurring at different stages of gestation, parturition, and lactation. In contrast, the changes in whey proteins during mammary development, morphogenesis, and basement membrane synthesis have been described (Schanbacher and Smith, 1975; Hoppe, 1976), with suggestions from these studies of local growth regulation within the developing bovine mammary gland. Kidwell et al. (1984) described a collagen stimulating activity extracted from hormonally-induced, developing bovine mammary tissue. Aside from this study, no one has investigated the presence of polypeptide GF in mammary secretions during active mammary development in mid to late pregnancy, and hence, we describe here efforts to identify and characterize GF activity in mammary secretions during bovine mammary development. We found at least 2 major GF activities in secretions whose activity varied dramatically during the different stages of the mammary development from post-pubertal mammary secretion, through gestation and lactation, with a peak at the 7th month of gestation. The partial characterization of the GF occurring in secretions at the 7th month of gestation showed a GF activity of 30,000 mol. wt. which is EGF-like on the basis of binding activity, while another GF activity eluted under gel filtration at 50,000 and/or 150,000 mol. wt. In addition we have shown that bovine mammary cells in culture secreted a growth factor into the medium distinct from mEGF, and regulated by the extracellular matrix on which the cells were grown.
MATERIALS AND METHODS

Materials

Tissue culture media were from M.A. Bioproducts (Walkersville, MD). Cell culture plates were purchased from Bellco (Vineland, N.J.). The AKR-2B cell line was a generous gift from H. L. Moses (Vanderbilt University, TN). Receptor grade mouse epidermal growth factor (mEGF) and laminin were from Collaborative Research, (Bedford, MA). 125I-mEGF receptor grade (107 uCi/ug) and methyl-\textsuperscript{3H} thymidine (32 Ci/mmole) were obtained from ICN Biochemicals (Irvine, CA) and New England Nuclear (Boston, MA), respectively. Cortisol was purchased from Steraloids, (Wilton, NH) and collagenase Type III was obtained from Worthington Biochemicals (Freehold, NJ); all other hormones, enzymes, and liquid chromatography gels (DEAE-Sepharose CL-6B, CM-Sepharose CL-6b, and Sephacryl S200 superfine) were from Sigma Chemical Company (St. Louis, MO). For growth of bovine mammary gland cells on collagen gels, type I collagen was prepared from rat tail tendons as a modification of the procedure described by Bornstein (1958).
Collection of mammary secretions

Mammary secretions were collected by manual expression from either primiparous adult dairy cattle at different stages of mammary development, or from nulligravida dairy heifers at selected stages during hormonally induced bovine mammary development (Smith and Schanbacher, 1973). Thus, samples were examined from varying stages of mammary development ranging from non-pregnant postpubertal, mid to late pregnancy, colostrum formation, and lactation. Mature milk was obtained from the refrigerated bulk tank at the OARDC dairy research facility. All milk and secretion samples were transported to the laboratory on ice. Cell debris and fat were removed by centrifugation at 30,000xg, 30 min., 4°C. The supernatant fat layer and the debris pellet were discarded; the recovered supernatant (skimmed secretion or milk) was decaseinated by acidification to pH 4.5 with acetic acid and centrifuged at 30,000xg, 30 min., 4°C to remove precipitated casein. The supernatant whey preparation was then dialyzed (mol. wt. size exclusion at 14,000) overnight against 0.01N NaAc pH 5.6, or pH adjusted to 5.6 with NaOH, and then either loaded onto an ion exchange chromatography column, or stored at -20°C until further use. The defatted, decaseinated, and dialyzed mammary secretions is referred to as "decaseinated secretions" hereafter.
For the determination of GF activity in cultured bovine mammary cells, medium from culture was removed by aspiration and stored at -20°C until assayed.

**Cell cultures**

Mitogenic activity was measured by stimulation of [³H]-thymidine incorporation in target cell lines. For routine mitogenic activity assays, AKR-2B mouse embryo fibroblast cells were routinely used. Human A431 epidermoid cells (American Type Culture Collection, Rockville MD), and BALB/c 3T3 cells from the NIH (Bethesda, MD) were also used for selected receptor or mitogenesis studies. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS) and gentamycin (50 µg/ml) in a humidified incubator (95% air:5% CO₂) at 37°C.

Primary cultures of bovine mammary cells were prepared (Talhouk et al., 1986; Talhouk et al., 1989a, manuscript in preparation) by modifications of the procedures of Larson (1976) and Mackenzie et al. (1982). Briefly, acini, acinar fragments, and cells were prepared from lactating bovine mammary tissue by enzymatic dissociation. The cells were plated in M199 with insulin (5 µg/ml), cortisol (5 µg/ml), prolactin (8 µg/ml), and gentamycin (50 µg/ml) and supplemented with 15% FCS and laminin (1-2 µg/cm² of growth area). FCS and Laminin were only
supplemented upon plating to facilitate cell attachment. The resulting bovine mammary cells and acini were plated on 100 ul type I collagen gel or a 100 ul of type I collagen mixed with extracellular matrix (10 parts collagen: 1 part extracellular matrix) prepared as described by Wicha et al. (1982) at approximately 5 x 10^5 cells per 0.5 ml medium per 1.0 cm well of 24 well plates.

**Mitogen assay**

The \(^{3}H\)-thymidine assay used to determine the mitogenic activity was similar to that used by Brown and Blakeley (1984), Klagsbrun (1978), and Simmen et al. (1988). AKR-2B, A431, and 3T3 cells were sub-cultured into 24 well plates by seeding at 1 x 10^5 cells/1.0 cm well in 1.0 ml DMEM supplemented with 10% FCS, with growth to confluent monolayers (usually by 72 hrs.). Upon confluency, cells were changed to serum deficient medium (DMEM with 2% FCS) and incubated for 48 hrs to stop proliferation by serum depletion and density dependent arrest before application of mitogen preparation. For assessing GF activity, skimmed secretion, decaseinated secretion, or fractions from column chromatography were added to arrested cells after dilution to the concentration desired. The final volume of the test solution was adjusted to 100ul/well with sterile HBSS. After 20 hrs. at 37°C, \(^{3}H\)-thymidine (1 uCi/well, diluted to 50 uls with HBSS) was added to each well, and incubation continued another 4 hrs at 37°C. After \(^{3}H\)-
thymidine labelling period, the cells were washed once with 1 ml of phosphate buffered saline (PBS, 15 mM HPO$_4$, 0.85% NaCl; pH 7.2), 2x with 1 ml methanol/well (5 min.) each, then 4x 1 ml water/well. Cellular proteins were precipitated in situ by two 5 min washes with cold 5% TCA (1 ml each), then solubilized with 300 ul of 0.3N NaOH, and transferred to liquid scintillation vials for liquid scintillation counting (Ecolume, ICN, Irvine, CA).

**Liquid chromatography**

Anion (DEAE-Sepharose CL-6B) or cation (CM-Sepharose CL-6B) exchange chromatography was performed on columns equilibrated at 4°C in 0.01M NaAc, pH 5.6. The decaseinated secretions were loaded onto the column. Seventy-five mls. of 0.01M NaAc pH 5.6 were then passed through the column before a 800 mls NaCl gradient (0-2M) was started to elute bound proteins. Fractions were collected, and the protein elution profile determined at 280nm, while growth promoting activity was determined by addition of aliquots to AKR-2B cells for mitogenesis assays as described.

To further purify and determine the apparent molecular weight of the mitogenic activity, peaks of growth promoting activity were pooled and concentrated by precipitation with 80% saturated ammonium sulfate. The precipitate was dissolved in minimal volume of PBS, pH 7.2, dialyzed
overnight against PBS, pH 7.2, and loaded onto a Sephacryl S200 column (2.6 cm x 85 cm) equilibrated with PBS at 4°C, pH 7.2. Column fractions were analyzed for total proteins by absorbance at 280 nm and for growth promoting activity by stimulation of [3H]-thymidine incorporation in AKR-2B cells.

**Enzyme Linked Immunoassay**

Specific milk proteins were determined in medium of bovine mammary cell cultures and as internal markers in column fractions with biotin-avidin-horseradish peroxidase enzyme linked immunoassay (ELISA) using biotinylated antibody mono-specific for bovine immunoglobulins, bovine lactoferrin, bovine serum albumin, β-lactoglobulin, and α-lactalbumin (Talhouk et al., 1989a; Guesdon et al., 1979; Kendall et al., 1983; Bayer et al., 1979).

**Radio-receptor competitive binding assay**

The radio-receptor competitive binding assay for 125I-mEGF is a modification of Carpenter et al. (1975) and Brown and Blakeley (1983b). Confluent serum arrested AKR-2B cells, as previously described, were washed twice with 1 ml of binding buffer (0.1% BSA, and 5 mM MgCl₂ in PBS; PH 7.4). Skimmed secretions, or fall-through fractions off a DEAE-Sepharose column were diluted to the desired concentration in binding
buffer. The treatment in a total of 0.5 ml in binding buffer containing 1ng/ml of $^{125}$I-mEGF (200,000 cpm/ng; specific activity = 107-144 uCi/ug) was added per well and incubated at 37°C and 4°C for 90 minutes. Binding at 37°C vs. 4°C allowed the distinction of true competitive binding from indirect modulation of receptor levels by the competing test ligand. The cells were then washed twice with 0.5 ml of cold binding buffer to remove unbound ligand, solubilized in 0.5 ml of 0.3N NaOH for 10-20 minutes, at 37°C, and the solubilized well contents were transferred to polystyrene tubes for gamma counting (Isodyne, model 1185) to determine the $^{125}$I-mEGF bound.

To confirm binding, a standard competitive binding assay was performed on AKR-2B cells with mEGF, at concentration ranging from 0-20 ng/ml, with nonspecific binding determined in the presence of 2 ug of unlabelled mEGF/ml. All binding assay samples were run in triplicates.
RESULTS

Skimmed secretions from a primigravida heifer at 7 months gestation were tested for their ability to stimulate $[^3\text{H}]-\text{thymidine}$ incorporation into different cell lines. The cell lines used (mouse 3T3, and AKR-2B, as well as human A431) showed varied responses to the addition of the mammary secretions. The majority of BALB/c 3T3 cell monolayers detached upon the addition of the skimmed mammary secretions even at very low concentrations, while A431 cells showed low levels of $[^3\text{H}]-\text{thymidine}$ incorporation upon stimulation. The AKR-2B cells responded with high levels of $[^3\text{H}]-\text{thymidine}$ incorporation, and henceforth this cell line was used to monitor mitogenic GF activity from mammary gland secretions at different stages of pregnancy, and during purification and characterization.

**Growth factor activity at different stages of mammary development**

Skimmed mammary secretions from a 7 months pregnant heifer induced $[^3\text{H}]-\text{thymidine}$ incorporation into AKR-2B cells. The mitogenic activity was retained in the whey fraction of the developing secretion. The mitogenic activity of a 1% solution of skimmed and decaseinated mammary
secretions from a 7 months primigravida dairy heifer was typically 3-4 times that for 10% FCS (Fig. 57), and induced peak DNA synthesis in AKR-2B cells within 12-20 hours after stimulation (Fig. 57A). \(^{3}\text{H}\)-Thymidine incorporation dose response profiles for FCS, mEGF, and decaseinated mammary secretions from 7 months gestation, a colostrum-forming gland and a lactating gland (milk) are shown in Fig. 57B, 57C and 57D. Maximum activity was found in secretions from a developing mammary gland at 7 months gestation, at a concentration of 0.5 - 1.5%, and then declined at higher concentrations. This decline in \(^{3}\text{H}\)-thymidine incorporation activity was not due to cell death, since the cells failed to take up trypan blue, and they retained their ability to resume cell proliferation after exposure to 10% FCS (results not shown).

The mitogenic activity in decaseinated mammary secretion from a 7 months primigravida dairy heifer was at least 3-4 fold that of colostrum. The dose response profile for colostrum showed activity that plateaued (at about 5%) in a pattern similar to that noted for FCS and mEGF. No evidence of inhibition of GF activity at higher concentrations was noted. No significant stimulation of \(^{3}\text{H}\)-thymidine incorporation was induced by bovine milk.

The presence of such a potent mitogenic activity in decaseinated and/or skimmed secretion from a 7 months primigravida heifers that was several fold that found in colostrum suggested that this activity could be modulated at the different stages of pregnancy and mammary
Figure 57. Effect of mammary secretions, FCS, and mEGF on $[^3\text{H}]$-thymidine incorporation into AKR-2B cells. A) Rate of $[^3\text{H}]$-thymidine incorporation, or DNA synthesis at different time intervals after stimulation of cells with 1% skimmed mammary secretions. B) Dose response curves for increasing concentrations of decaseinated mammary secretions from (O) primigravida heifer at 7 month gestation, (△) colostrum forming stage and (□) lactating state. C) Saturation profiles of (O) FCS and D) of (O) mEGF. All samples were assayed in triplicates.
Figure 57.
development. This theory was examined by testing skimmed mammary secretions obtained from individual heifers at different stages of pregnancy (pre-pregnant up to and through lactation), as well as secretions during hormone induced mammary development. The mitogenic activity (Fig. 58) peaked during the latter part of pregnancy (approx. at 7 months, or day 11-14 in a hormonally induced developing gland) and then declined until the colostrum forming stage, after which the activity decreased to virtually undetectable levels in milk. Interestingly, a mitogenic activity was noted in mammary secretions of non-pregnant heifers (Fig. 58B, day zero of hormone induced development) that was several fold that found in colostrum (Fig. 57B) or FCS (Fig. 57C) at 1% concentration. This activity in the mammary secretions of non-pregnant heifers was lower than that found in the 7th month of gestation (Fig. 58A), a period of extensive alveolar development and extracellular matrix remodeling (Cowie 1971).

Fractionation of growth factors in mammary secretions

Previous reports of multiple GF activity in bovine colostrum, together with the differences in the dose response profiles for colostrum and secretions from a developing gland, and particularly the very high GF activity of secretions from a developing gland (at 0.5-1.5%) which exceeded the maximum plateau of colostrum, and the inhibition of GF activity at higher concentrations, suggested multiple
Figure 58. The mitogenic activity of skimmed mammary secretions assayed at 1% concentration taken at different stages of bovine mammary gland development. The mitogenic activity is determined as the ability of the secretion to stimulate $^{3}$H-thymidine incorporation into AKR-2B cells, as described in the materials and methods section. A) Samples from different primigravida heifers, at intervals during gestation, B) Mammary secretions from 4 different nulligravida heifers, induced into mammary development by estrogen/progesterone injection according to Smith and Schanbacher (1973).
Figure 58.
and complex interactions for the GF's of developing secretions. To determine whether one or more growth promoting activities exist in secretions at 7 months gestation, we attempted to fractionate the mammary secretions with ion exchange and gel filtration chromatography. A whey preparation was dialyzed overnight against 0.01M NaAc pH 5.6 (or pH adjusted to 5.6 with 1N NaOH), and subjected to cation exchange CM-Sepharose or anion exchange DEAE-Sepharose chromatography, both equilibrated and developed in 0.01N NaAc pH 5.6, with a 0-2M NaCl gradient. All quoted fractions (100ul) were assayed for mitogenic activity. At least 2 different growth promoting activities were observed (Fig. 59A, 59B); designated here as bMDGF-1 and bMDGF-2 (Bovine mammary derived growth factor 1 and 2). The former was in the fall-through of the DEAE-sepharose, and from this has a pI of > 8.6, since it remained in the fall-through portion when run on a DEAE-Sepharose, pH 8.6, but was retained on CM-sepharose and was eluted at about 0.65M NaCl. A second growth promoting activity, bMDGF-2, was retained on DEAE-Sepharose and eluted at 0.3M NaCl, but was not retained on CM-Sepharose at pH 5.6. Typically bMDGF-1 stimulated \(^{3}\text{H}\)-thymidine uptake by AKR-2B cells 3-4 fold greater than that stimulated by bMDGF-2 under the same conditions. However, exceptions were noted, and in certain samples the two activities were almost equal, but never did we observe greater stimulation by bMDGF-2 than bMDGF-1.
Figure 59. Ion-exchange chromatography of decaseinated mammary secretions from a 7 months primigravida heifer were loaded onto: A) a CM-Sepharose column or, B) a DEAE-sepharose column, prequilibrated in 0.01N NaAc, pH 5.6, 0-2M NaCl gradient. Purification was monitored by ability of 100 ul aliquots of fractions eluted from the columns to stimulate [\(^3\)H]-thymidine incorporation into AKR-2B cells. Fractions were monitored for protein content by absorbance at 280nm (O) and assayed for mitogenic activity (*), in triplicate.
Figure 59.
To determine the apparent mol. wt. of non-denatured and non-reduced bMDGF-1 and bMDGF-2 and to further purify them, the ion exchange fractions containing bMDGF-1 and bMDGF-2 were pooled separately, each was concentrated by precipitation with 80% saturated ammonium sulfate, redissolved in a minimal volume of PBS and dialyzed overnight against PBS (pH 7.2). The dialyzed and concentrated bMDGF1 and bMDGF2 were fractionated on Sephacryl-S200 gel filtration column. bMDGF-1 activity eluted at a peak that corresponded to approx. 30000 mol. wt., while the apparent mol. wt. noted for bMDGF-2 was found in peaks with growth promoting activity at 50,000 mol. wt. and 150,000 mol. wt. (Fig. 60A, 60B), values too high to fall within the mol. wt. range of most known polypeptide GF. This finding suggested that bMDGF-2 was complexed with binding proteins of different mol. wt., or that bMDGF-2 was two different GF complexed to binding proteins.

Attempts to fractionate skimmed or decaseinated mammary secretions by loading on a Sephacryl-S200 gel filtration column always showed a broad peak of activity ranging from 30,000-150,000 mol wt. No growth activity lower than 30,000 mol wt. was noted. This procedure was cumbersome and often resulted in compressing the Sephacryl-S200 column and blocking the flow due to the thick consistency of the developing mammary secretions (data not shown).
Figure 60. Gel-filtration chromatography of pooled fractions containing growth promoting activity (bMDGF-1 and bMDGF-2) eluting from CM-Sepharose. Fractions containing bMDGF-1 and bMDGF-2 were pooled separately, concentrated and resuspended in minimal volume of PBS, pH 7.2 as described in the materials and methods section, and loaded on a gel filtration column (S-200 Sephacryl) pre-equilibrated in PBS, pH 7.2, for determination of their apparent mol. wt.. A) Apparent mol. wt. of bMDGF-1, and B) bMDGF-2 as monitored by the ability of fractions to stimulate \(^{3}\text{H}\)-thymidine incorporation into AKR-2B cells. Fractions were monitored by absorbance at 280nm (O), and their ability to stimulate \(^{3}\text{H}\)-thymidine incorporation (♦), each value is a mean of 3 samples.
Figure 60.
Competitive displacement of $^{125}$I-mEGF

Skimmed mammary secretions from a 7 month primigravida heifer, partially purified bMDGF-1 eluted from DEAE-Sepharose, and mEGF were tested for their ability to compete with $^{125}$I-mEGF for binding to AKR-2B cells at both 4°C and 37°C (Fig. 61A, 61B and 61C). Both preparations of mammary secretions competed with $^{125}$I-mEGF in a dose dependent manner at 4°C and 37°C. The $^{125}$I-mEGF displacement at both temperatures was not total, and only represented 60% inhibition. On the other hand, purified receptor grade mEGF showed 80-90% inhibition at both temperatures. These findings suggested that the mitogenic activity in the processed whey fraction and bMDGF-1 bound to mEGF receptor directly and did not inhibit binding of the $^{125}$I-mEGF via receptor transmodulation (Sporn and Roberts 1988), and that at least bMDGF-1 is an EGF-like growth promoter. The ability of bMDGF-2 to compete with $^{125}$I-mEGF binding to AKR-2B cells was not tested.

Physical and chemical characterization

Table 2 summarizes the effects of trypsin-chymotrypsin and heat treatment, pH and storage on the stability of the mitogenic activity of bMDGF-1. bMDGF-1 was subjected to proteolysis with both trypsin-chymotrypsin (0.5 mg/ml) for 4 hr. at 37°C, followed by the addition of soybean trypsin inhibitor (STI, 2mg/ml) to inactivate the proteases and
Figure 61. Inhibition of $^{125}$I-mEGF binding to AKR-2B cells by: A) Skimmed mammary secretions from a 7 month primigravida heifer, B) partially purified bMDGF-1 (from DEAE ion exchange chromatography Fig. 59B), and C) Receptor grade mEGF, at 37°C (□) and 4°C (■). Each point represents the mean value of 3 samples, expressed as % of $^{125}$I-mEGF bound to untreated cells.
Figure 61.
Table 2. Sensitivity of growth promoting activities in decaseinated mammary secretions from a primigravida heifer at 7 month gestation, partially purified bMDGF-1 (from DEAE-Sepharose, Fig. 59B), and GF detected in mammary cell culture medium, to heat and trypsin/chymotrypsin treatment.

<table>
<thead>
<tr>
<th>Growth Promoter</th>
<th>Control</th>
<th>Heat 100°C (5 min)</th>
<th>Protease (37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS</td>
<td>39094 +-6078 (100%)</td>
<td>11813 +-980 (30%)</td>
<td>-</td>
</tr>
<tr>
<td>bMDGF-1</td>
<td>73346 +-8458 (100%)</td>
<td>59861 +-1011 (82%)</td>
<td>447 +-371 (0%)</td>
</tr>
<tr>
<td>GF (in medium)</td>
<td>38896 +-2962 (100%)</td>
<td>31193 +-1915 (80%)</td>
<td>985 +-75 (2.5%)</td>
</tr>
</tbody>
</table>

* Results are expressed as the ratio of [3H]-thymidine incorporation induced in AKR-2B cells by the treated growth promoting activity to that of its control (untreated). Each sample was tested in triplicates.
thereby allow assay for the mitogenic activity. The control consisted of
the same concentration of bMDGF-1 and proteolytic enzymes incubated
under the same conditions as above except that STI, used to inactivate
the proteases, was added at the beginning of the incubation period.
Separate assays showed that the proteolysis was completely inhibited by
concentrations of 2mg/ml of STI added to the trypsin-chymotrypsin
mixture. bMDGFl was completely inactivated by the proteolytic enzymes.

Sensitivity to heat was tested by subjecting bMDGF-1 to 100°C for 5
min., with the control maintained on ice for that same period of time.
After heat treatment, only 18% inactivation of the GF ability to
stimulate $[^3H]$-thymidine incorporation was noted, showing high stability
to 100°C heat treatment. The ability of bMDGF-1 and bMDGF-2 to stimulate
$[^3H]$-thymidine uptake in AKR-2B cells persisted after subjection to pH
ranging from 4.5 to 8.6.

Handling mammary secretion from a 7 month primigravida dairy heifer
was difficult, due to their dense, gluey consistency, as well as the
instability of the mitogenic activity in the decaseinated secretions
prior to fractionation on column chromatography. Storage as decaseinated
secretions for more than 2 weeks at -20°C caused significant loss of
bMDGF-1 and bMDGF-2 activity. Growth promoting activity from
decaseinated mammary secretions of a 7 month primigravida heifer was
also lost after repeated freezing and thawing of the samples.
However, partially purified forms of bMDGF-1 and bMDGF-2, eluted from both ion exchange columns and size exclusion columns were stable for at least 3 months at -20°C, and after multiple freezings and thawings.

Growth factor profiles in bovine colostrum and milk

Reports on the presence of several GF in bovine milk and colostrum (Campbell and Baumrucker, 1988; Brown and Blakeley, 1983), and the differences in the dose response profiles noted for colostrum, milk and decaseinated secretions from a developing gland (Fig. 57B) warranted a comparison of the elution profiles of the growth promoting activities in the mammary secretions from each of the above stages. Figure 62 shows the A$_{280}$ and $[^3H]$-thymidine incorporation patterns of a decaseinated preparation from colostrum, and bovine milk after elution from a CM-Sepharose, pH 5.6, with 0-2M NaCl gradient. Equal amounts of protein from decaseinated secretions (Fig. 59A) from the colostrum forming stage to that from a 7 month primigravida heifer were loaded onto the CM-Sepharose column, while only 6% of the total protein content of decaseinated mammary secretion during lactation (milk) was used. The peaks of the GF in colostrum and milk eluted at similar NaCl concentrations as the GF in the mammary secretions of a 7 month primigravida dairy heifer.
Figures 62. CM-Sepharose cation exchange chromatography of decaseinated mammary secretions: from A) Colostrum of a primigravida heifer obtained at parturition, and B) Milk obtained from the bulk tank container. The decaseinated mammary secretions were loaded onto a CM-sepharose column, pre-equilibrated in 0.01N NaAc, pH 5.6. The fractions were monitored for total protein absorbance at 280nm (O), and their ability to stimulate [3H]-thymidine incorporation into AKR-2B cells (♦), as described in the legend for figure 59.
Figure 62.
Growth factor in bovine mammary cell culture conditioned medium

The ability of developing mammary secretions to stimulate $[^3]H$-thymidine incorporation in cultures of bovine mammary cells (unpublished data), and the observation that this activity peaked at periods of maximal mammary gland growth raised the question of the origin and site of synthesis of these GF. Mitogenic activity observed in serum free medium from bovine mammary cell cultures (Fig. 63A) increased slowly up to day 6 of culture then sharply increased to peak at day 8, followed by return to basal levels at day 10 and beyond. This sharp peak of GF activity, although variable in different cultures, was usually observed at day 8-10. This correlated with the formation of a cell monolayer and the initiation of casein synthesis and secretion (Talhouk et al., 1989b). Furthermore, this spike of GF activity did not correlate with any changes in the profile for lactoferrin secretion by those same mammary cells (Fig. 63B). In addition, the extracellular matrix, a strong modulator of milk protein synthesis and secretion of bovine mammary cells in culture (Talhouk et al., 1986), also appeared to regulate the growth promoting activity secreted by the cells into the culture medium.

The above mentioned GF activity found in the extracellular medium of bovine mammary cell cultures was subjected to protease and heat treatment as described earlier. It was found to have about the same
Figure 63. Secretion of growth promoting activity and lactoferrin into the medium by cultured bovine mammary cells cultured on different substrata. A) Evidence of a growth promoting activity secreted by bovine mammary cells in culture, and its regulation by the substratum, as determined by its ability to stimulate $[^3H]$-thymidine incorporation into AKR-2B cells (each point represent the mean of 3 values). B) Profiles of lactoferrin secreted into the medium, and its regulation by the substratum, as determined by ELISA (each point is the mean of 2 values). (□) Cells grown on type I collagen and, (■) cells grown on a type I collagen : ECM (extracellular matrix, mixed in the ratio of 10:1 respectively) as described by Wicha et al (1982).
Figure 63.
resistance to heat as the partially purified bMDGF1 from mammary secretions, and also to be sensitive to trypsin-chymotrypsin (Table 2). However, unlike the activity in mammary secretions, that from the culture medium did not compete for $^{125}$I-mEGF binding to AKR-2B cells at 4°C or at 37°C (data not shown). No further characterization was possible for this growth factor(s) in mammary cell culture medium due to insufficient amounts available.
DISCUSSION

In this report we demonstrated the presence of growth promoting activity in mammary secretions from a nulligravida and from a mid-late developing mammary gland of primigravida dairy cattle. The GF activity was regulated by the stage of gestation and peaked at the 7th month. At that stage of gestation the growth activity was several fold that in colostrum. Two major GF were identified, an EGF-like bMDGF1, 30,000 mol wt heat resistant basic (pI > 8.6) polypeptide, and bMDGF2 which was found in 50,000 and/or 150,000 apparent mol wt. complexes. The ability of milk and colostrum of several species to maintain growth of cell lines in serum free cultures has been demonstrated by many workers (Klagsbrun, 1978; Klagsbrun and Newman, 1979; Sereni and Basegna, 1981). Carpenter (1980) demonstrated that EGF is the major GF in human milk responsible for routine maintenance of human fibroblast cultures. Kidwell and Salomon (1987) found human milk to contain an array of GF activities described as EGF, PDGF, IGF-1, TGF-alpha and TGF-beta, MDGF1 and MDGF2, and milk growth factors I-III. On the other hand rodent milk contained EGF (Beardmore and Richards, 1983; Byyny et al., 1974). Ruminant colostrum was also shown to contain several growth promoting activities. Brown and Blakeley (1984) demonstrated the presence of a
PDGF-like growth factor (colostrum basic growth factor; CBGF), approx. mol. wt. 35000 in the colostrum of cows, sheep and goats, and showed it to inhibit EGF binding to 3T3 cells in a temperature dependent manner, suggesting it was different from EGF. In addition, Read et al. (1983) and Francis et al. (1986) purified and determined the partial amino acid sequence of a GF in bovine colostrum with a mol wt of 7,800 and a pi between 8 and 9. The first 30 N-terminal amino acids showed complete homology to IGF-1. In contrast to other species, bovine milk contained little if any growth promoting activity (Klagsbrun and Newman, 1979; Brown and Blakeley, 1983a). Recent evidence showed that bovine milk contained IGF-1 and was mostly bound to a 45,000 mol wt binding protein from which it is readily dissociated under acid/ethanol treatment or acid chromatography. IGF-1 levels decreased substantially (5-10 fold) by 56 days of lactation (Campbell and Baumrucker, 1988). In contrast to the information about GF in colostrum and milk, no previous reports described GF's in mammary secretions from the different stages of mammary development during gestation. Brown and Blakeley (1983) found pre-colostrum obtained from goats immediately before parturition to also contain a PDGF-like activity, similar to that of colostrum.

While most mammary gland development and growth occurs throughout gestation (Anderson, 1985), the aggressive mammary gland growth occurs during the latter part of gestation, which is mostly characterized by extensive lobulo-alveolar development (Cowie 1971) under hormonal
control (Anderson, 1985), with evidence from in vitro studies for the involvement of GF's as well (reviewed by Dembinski and Shiu, 1987). Interestingly, the ability to stimulate [³H]-thymidine incorporation into AKR-2B cells by mammary secretions from first mammary development in dairy cattle peaked during the latter part of gestation (7-8 months), a period characterized in the mammary gland by extensive lobulo-alveolar development (Cowie, 1971). This suggested the involvement of GF's such as those described here in the development of the bovine mammary gland, particularly in that latter part of gestation. The existence of GF activity in mammary secretions from a nulligravida post-pubertal heifer, prior to inducing mammary development (i.e., day zero, Fig. 58B) that was several fold that detected in FCS at equal concentration (1%) inferred that a resident GF in mammary secretion prior to pregnancy could be involved in the limited pre- and post-pubertal mammary development. We did not determine whether the growth promoting activities found before hormone injections (day zero, Fig. 58B) in nulligravida heifers were the same as those detected at day 11-14 of hormonally induced developing mammary gland, or at 7-8 month of gestation.

The profile of the growth promoting activity in mammary secretions of hormone-treated heifers (Fig. 58B) followed the growth and development pattern of the mammary gland as monitored by regular daily observation. Growth in the size of the gland commenced at 4-7 days after
hormone injection and achieved its maximum size at day 11-14. The growth of the gland and increase in size was limited after this, and the mammary secretions attained a milky appearance only beyond day 17. The GF activity peaked at day 11, a period (day 11-14) which was characterized by aggressive growth of the mammary gland. GF activity decreased beyond day 11 gradually into the colostrum forming stage (day 17-23) and then dropped to minimal levels in milk (after day 23). Interestingly, secretions from one heifer, retained growth promoting activity beyond the first milking day (day 23, Fig. 58B). Mammary secretions from that particular heifer, unlike the colostric secretions from the other heifers, were opaque and viscous. These secretions were characteristic of a non-lactating gland and retained their growth promoting activity until the secretions attained their typical colostric appearance (day 35). This further suggested that growth promoting activity was involved in the development of the mammary gland.

Earlier reports suggested that the abrupt decrease in the mitogenic activity between that found in colostrum and that in milk was due to a reduction in total milk protein concentration, because of the dilution effect as the volume of milk produced increased (Corps et al., 1987; Read et al., 1984). However, the authors noted that the protein composition of mammary secretion changed according to the stage of gestation. Schanbacher and Smith (1975) demonstrated that the change in total protein content was not simply a dilution effect due to an
increase in the volume of mammary secretion, but instead was itself a parameter of mammary development. In our studies, the growth activity in mammary secretions across gestation peaked at 7 months of pregnancy or Day 11-14 in a hormonally induced mammary development. The volume of mammary secretions increased in the period from pre-gestation up to the 7th month of gestation. This increase in both GF activity and volume of secretion argued against a dilution effect for the GF in the secretions and suggested that specific GF circulating in the plasma during gestation were targeted to the mammary gland, or that the gland at this stage of pregnancy synthesized and secreted its own GF to promote its own development and extracellular matrix remodeling (Vonderhaar et al., 1983; Mohanam et al., 1987).

The developing secretions and colostrum showed marked differences in their dose response curves (Fig. 57C) as evidenced by their $[^3H]$-thymidine incorporation into AKR-2B cells (Fig. 57C). Partial separation of the different entities in colostrum led to higher levels of $[^3H]$-thymidine incorporation (150,000 cpm) than that achieved by colostrum (approx. 50,000 cpm). This suggested separation of an inhibitory activity from the growth promoting activities detected in colostrum. On the other hand, the decrease in $[^3H]$-thymidine incorporation observed in skimmed mammary secretions from a 7 month primigravida heifer at concentrations more than 0.5-1.5% in the assay suggested the presence of an inhibitor at that stage of growth as well. Bohmer et al. (1984)
purified a 12-14 Kd protein from lactating bovine mammary gland that inhibited the growth of Ehrlich ascites mammary carcinoma cells. Later that same group of investigators reported a TGF activity in acid/ethanol extracts of lactating bovine mammary gland, and in Ehrlich ascites mammary carcinoma cells (Eckert et al., 1985). This activity co-eluted with an activity that competed for EGF receptor. The authors concluded that TGF's and the growth inhibitor (Bohmer et al., 1984), co-existed in the mammary gland, and possibly modulated the growth and development of the gland via an autocrine fashion. Knubbe et al. (1987) showed that TGF-beta produced by MCF7 cells was under hormonal regulation, and acted as a negative growth regulator for human breast cell lines. Whether either of the growth promoting activities we have shown acted as a positive and/or a negative growth regulator on AKR-2B cells and thereby might account for the inhibition, was not determined.

In this study we have demonstrated the presence of at least 2 growth promoting activities in mammary secretions from a 7 month primigravida heifer. One activity was an EGF-like bMDGF-1 basic polypeptide with a mol. wt. of approximately 30,000. Similar to EGF, bMDGF-1 stimulated \(^{3}\text{H}\)-thymidine incorporation into AKR-2B cells; however, maximal EGF stimulation plateaued at around 80,000 cpm while DNA synthesis, and \(^{3}\text{H}\)-thymidine incorporation induced by bMDGF-1 attained levels as high as 300,000 cpm's (Fig. 57). In addition, bMDGF-1 competed with labelled \(^{125}\text{I}\)-mEGF for binding to AKR-2B cells in a dose dependent manner at both
4°C and 37°C, suggesting that the displacement was a direct binding of the bMDGF-1 to the EGF receptor and not a result of receptor "transmodulation" (receptor-cross-talk; Sporn and Roberts, 1988) as can be caused by PDGF-like GF in bovine colostrum (Brown and Blakeley 1983b). The ability of partially purified preparations of bMDGF-1 to stimulate \[^{3}\text{H}\]-thymidine incorporation into AKR-2B cells at levels at least 3 times that of purified preparation of EGF, in addition to bMDGF1 displacement of \(^{125}\text{I}-\text{mEGF}\) binding on AKR-2B cells suggested that: (1) bMDGF-1 is an EGF-like factor capable of stimulating higher levels of \[^{3}\text{H}\]-thymidine incorporation than EGF, or (2) an EGF-like factor that competed for EGF binding on AKR-2B cells co-eluted with bMDGF-1 and both factors interacted to produce a synergistic effect.

The activity described above (bMDGF1) was partially purified by procedures similar to those described by Shing et al. (1987) for the separation of a basic GF from bovine colostrum. The authors reported elution of the colostric GF activity from Bio-Rex 70 cation exchange chromatography column at about 0.6M NaCl at pH 7.0 with an activity peak with a mol. wt. of about 30,000 by size exclusion chromatography. Based on the above criteria for the purification of a colostric GF, and the pattern of elution of GF activity as detected in colostrum in our laboratory (Fig. 62A), it appeared that both factors bMDGF-1 and CBGF (described by Brown and Blakeley 1983a,b) were the same. However, while Brown and Blakeley (1983b) demonstrated a temperature dependent
Inhibition of CBGF by $^{125}$I-mEGF to 3T3 cells, bMDGF-1 inhibited $^{125}$I-m-EGF at both temperatures 37°C and 4°C (Fig. 61B). Thus, in contrast to Brown et al. (1983), bMDGF-1 appears to be an EGF-like activity. This discrepancy in the results could be attributed to the use of different cell lines in the respective assays namely 3T3's and AKR-2B cells.

Being different from CBGF (PDGF-like), the other GF described in the literature within similar mol. wt. ranges are HMGF-II (mol. wt. 32,000-60,000) (Shing and Klagsbrun, 1984) and TGF-beta; mol. wt. 25,000 (Sporn et al., 1986). HMGF-II was described by the authors as a non-EGF like activity, and hence different from bMDGF-1 (EGF-like). On the other hand, TGF-beta, differed from bMDGF-1 in several aspects. Although they both stimulate DNA synthesis in AKR-2B cell lines, Shipley et al. (1985) reported that this was only induced after a long pre-replication period of >24 hrs., while most other GF required only 12-14 hrs. period. In contrast to TGF-beta, decaseinated mammary secretions induced peak DNA synthesis in AKR-2B cells within the first 20 hrs. (Fig. 57B). In addition, TGF-beta was highly specific and only interacted with its specific receptors, with no demonstration of cross reactivity between TGF-beta receptors and other growth factors, or of TGF-beta reacting with other GF receptors (Frolik et al., 1984). The above suggested that bMDGF-1 is a unique growth promoting activity belonging to the EGF-type growth factors.
bMDGF-2, a growth promoting activity with an apparent mol. wt. of approximately 50,000 and/or 150,000 was detected in the fall-through from the CM-Sepharose column (Fig. 59A). A similar activity is detected in the colostrum and bovine milk separation profile (Fig. 62A, 62B). The high mol. wt. of bMDGF-2 suggested that IGF-1 or IGF-2 bound to their carrier proteins, were possible candidates for bMDGF-2. Insulin like growth factors (approximately 8000 mol. wt.), circulate in the plasma bound to carrier proteins with mol. wt. of approx. 50,000 and/or 150,000; however, they only exert insulin-like effects when dissociated from their carriers either at a low pH environment, or via an "active mechanism" at the cellular level (Froesch et al., 1985). Thus, the high mol. wt. forms of bMDGF-2 (50,000 and 150,000) could be carrier proteins bound to an IGF.

Francis et al. (1988) and Campbell and Baumrucker (1988) have demonstrated an IGF-like activity in bovine milk and colostrum. On the other hand, purified preparation of porcine IGF-1 failed to stimulate $[^3H]$-thymidine incorporation into AKR-2B cells (F. Simmen, personal communication). At present, no other data is available to us that would allow a speculation of bMDGF-2 identity.

Brown and Blakeley (1983) reported a minor GF activity in goat's colostrum (different from CBGF) which did not compete with $^{125}$I-mEGF binding to its receptors. Our competition curves showed only a 60%
displacement of $^{125\text{I}}$-mEGF by the skimmed secretion from a 7 month primigravida heifer (Fig. 61A). This displacement was not affected when only bMDGF-1 was assayed for its ability to compete with $^{125\text{m}}$-EGF binding (Fig. 61B). This suggested that the other growth promoting activity (bMDGF-2) found in the skimmed mammary secretions but not in the partially purified preparations of bMDGF-1, did not contribute to $^{125\text{I}}$-mEGF displacement. Another GF with characteristics similar to bMDGF-2 was described by Bano et al (1985). MDGF1 a 62,000 mol wt under denaturing conditions was isolated from human milk. MDGF1 did not compete with mEGF and was sensitive to protease treatment.

The authors are unaware of other reports describing GF in secretions from a developing mammary gland and demonstrate their temporal pattern across mammary development. Schanbacher and Smith (1975) reported that expression of milk proteins was regulated by the stage of mammary development. Spitzer and Grosse (1987) have shown that EGF receptors in the bovine mammary gland were modulated according to the stage of pregnancy, and that the level of EGF receptors was enhanced in "fast proliferating normal mammary tissue". In addition, Smith et al. (1986) reported insulin receptors on the mammary gland with different affinities, and suggested the presence of IGF receptors. Indeed Baumrucker et al. (1986 a,b) later showed IGF-1 receptors on bovine mammary cells. Our results defined GF activities in developing mammary
gland and inferred that their occurrence and identity in mammary secretion was regulated according to the stage of pregnancy.

A mammary derived growth factor was purified from human milk (MDGF1, 62,000 mol. wt., pI of 4.8) and was shown to bind to normal mouse mammary cells (Bano et al., 1985). The authors suggested that MDGF1 was involved in the regulation of basement membrane synthesis during mammary development. Similarly, several reports demonstrated that EGF was involved in the regulation of mammary gland development both in vivo and in vitro. EGF induced cell proliferation in rodent mammary epithelial cells and inhibited their differentiation (Taketani and Oka, 1983). Others have shown EGF to affect morphology of mammary processes (Vonderhaar et al., 1983; Kidwell et al., 1980; Vonderhaar, 1985). We have demonstrated that decaseinated secretions at 7-10% concentration (vol/vol) from a 7 month primigravida heifer induce [³H]-thymidine incorporation in day 12 cultures of bovine mammary cells (unpublished data). This implied that the GF activity appearing in this latter part of pregnancy during extensive lobulo-alveolar growth (Cowie, 1971) was involved in the regulation of the mammary development.

The site of synthesis of GF affecting mammary development is not clear. However it was evident that at least some GF were synthesized in the mammary gland. Kidwell and Salomon (1987) and Rall et al. (1985) identified m-RNA in normal mammary tissue for TGF-beta, TGF-alpha and
EGF, while Eckert et al. (1985) detected a TGF activity that co-eluted with EGF-receptor competing activity in acid/ethanol extracts of bovine mammary gland. Similarly, we have shown that bovine mammary cells in culture, secreted a growth promoting activity in the medium (Fig. 63A), suggesting autocrine stimulation by the secreted GF to the mammary cells in culture as demonstrated by Sirbasku (1981, 1983) and Knubbe et al. (1987).

This GF activity secreted by the mammary cells was modulated (Fig. 63) by the type of substratum provided for the mammary cells in culture, and the stage of the culture (i.e., experimental day). A similar finding was reported by Dano et al. (1985); they demonstrated that MDGF1 stimulated synthesis of extracellular matrix components, and that this stimulation was attenuated when those cells were provided with their own matrix components. Later, Mohanan et al. (1988) noted that the substratum modulated the expression of EGF (TGF-alpha) surface receptors on rodent mammary cells in culture.

The effect of the stage of the cells in culture on the secretion of the GF into the culture medium was clearly evident by the sharp peak noted on day 8 of culture regardless of the substratum provided (Fig. 63A). Interestingly, our laboratory has demonstrated that for bovine mammary cells cultured on top of a collagen substratum casein synthesis and secretion was initiated only after 8 days in culture and increased
thereafter. The period of initiation of casein secretion (day 8) was coincident with the formation of a confluent sheet of cells (Talhouk et al., 1989a,b), as well as this sharp peak of growth activity. Whether this GF peak in culture was responsible for inducing casein synthesis and secretion was not determined. However, Durban (1987) speculated that mammary cells from a virgin gland did not differentiate and secrete casein in culture, because of their inability to synthesize a GF that was necessary to initiate casein synthesis. On the other hand, lactoferrin secretion (Fig. 63B) was not affected by the growth activity secretion into the medium, and was secreted continuously and in increasing concentrations from day 2 of culture up to day 14, with the effect of substratum clearly evident on its pattern of secretion as previously demonstrated (Talhouk et al., 1986). These differences in the lactoferrin secretion pattern and that of the GF factor showed that the peak of DNA synthesis induced in AKR-2B cells upon addition of conditioned medium from day 8 bovine mammary cell cultures, was not due to lactoferrin acting as an autocrine growth modulator by supplying iron (Laskey et al., 1988) to the cultured cells. Parry et al. (1987) speculated that transferrin, an iron binding protein was involved in the development of the mammary gland by acting as an autocrine growth modulator.

The identity of this GF in the culture medium, was not thoroughly investigated due to limitations on its availability. However, and in
marked contrast to bMDGF-1, preliminary data (not shown) on competition with \(^{125}\)mEGF binding to AKR-2B cells showed that the GF synthesized in culture did not displace binding of \(^{125}\)mEGF to its receptors. In addition, this GF was resistant to heat inactivation but was completely inactivated by protease treatment and not by pepstatin, suggesting that it is a protein, but not a cathepsin-D like that is able to induce mitogenesis in mammary cell lines (Capony et al., 1987).

In conclusion, we have shown that mammary secretions from a nulligravida and a primigravida heifer contained growth promoting activities that were modulated according to the stage of gestation. The GF activity peaked during the 7\(^{th}\) month of gestation and declined after that to the colostrum forming stage and into lactation. At least 2 GF's existed in the mammary secretions at the 7\(^{th}\) month of gestation, bMDGF-1 an EGF-like polypeptide with a mol. wt. of 30,000, and bMDGF-2 with an apparent mol. wt. of 50,000 and/or 150,000. In addition, mammary cells in culture secrete a non-EGF like GF, and its secretion was regulated by the substratum the cells were grown on.
REFERENCES


318


CHAPTER VI

CONCLUSION

In this study we developed serum free bovine mammary cell culture systems derived from lactating and from developing mammary glands, capable of long term synthesis and secretion of milk proteins. The procedure allowed for the dissociation of 100-200 gms of mammary tissue into acini and acinar fragments and their cryopreservation in liquid nitrogen, thereby representing a 5-10 fold increase in the scale of cell preparation previously described even from cattle. Furthermore, such large scale preparation and cryopreservation of cells as described here substantially reduces the need for fresh mammary tissue and animals. The cryopreserved bovine mammary cells did not lose their ability to synthesize and secrete milk proteins (Chapter II).

Cellular organization and the regulation of milk protein synthesis and secretion in culture were markedly affected by the substratum provided for the cells to grow on, the developmental stage of the mammary gland from which the cells were obtained, and the presence or absence of serum in the culture medium.
Cellular organization in vitro

Effect of the substratum: The substratum altered the cell organization in culture (Chapter III and IV), with two distinctive patterns of growth evident. Lactating and developing cells grown on collagen or plastic restructured their acini and spread into a two dimensional cell sheet which covered the entire growth area. In contrast, lactating or developing cells on Matrigel or embedded within collagen maintained their 3 dimensional acinar structure.

Effect of developmental stage of the gland: Light microscopy showed no difference between the increase in cell population and cellular organization of lactating and developing bovine mammary cells (Chapter III) when cultured on similar substratum. However, analysis at the transmission electron microscopy level showed that developing cells on a 500 ul layer of collagen formed a cell sheet of 3-4 cells thick and lacked specific cellular organization. Lactating cells spread into a sheet of 2-3 cell layers thick with the top layer consisting of epithelial secretory cells, and underlaid by another type of cells, probably myoepithelial.

Lactating or developing cells on Matrigel or embedded within collagen showed no differences at the transmission electron microscopy levels.
Effect of fetal calf serum: Fetal calf serum had no effect on cell organization in culture (Chapter III), however cells plated at low density (2x10^5 cells/well; Chapter II) in the presence of 15% FCS in their medium throughout the culture period spread and achieved confluence by day 6, whereas cells cultured without FCS did not form confluent sheets until later in the culture (Chapter II).

Regulation of milk protein synthesis and secretion

Effect of substratum and developmental stage of the gland: Both lactating and developing cells synthesized and secreted \( \alpha_s,1 \)-casein. Secretion of \( \alpha_s,1 \)-casein by developing cells gradually declined across the culture period and was not affected by substratum type or detachment (Chapter III). In contrast, \( \alpha_s,1 \)-casein secretion by lactating cells was dependent on a critical cell density (Chapter II), and was regulated by the cell substratum. Collagen proved to be the only substratum used in this study which supported the long term synthesis and secretion of \( \alpha_s,1 \)-casein by lactating cells (Chapter III). Detachment of the collagen substratum further induced additional synthesis and efficiency of \( \alpha_s,1 \)-casein secretion by lactating cells, and initiated synthesis and secretion of \( \alpha \)-lactalbumin and fat (Chapter IV).

Lactating cells in FCS free culture on detached collagen were responsive to, and dependent on prolactin for \( \alpha_s,1 \)-casein synthesis and
secretion. Prolactin was absolutely required for the initiation but not the maintenance of casein synthesis and secretion (Chapter IV). LF was only slightly affected, if at all, by the prolactin depletion from the medium (Chapter IV).

LF was secreted in similar patterns by lactating and developing cells when plated on the same substratum. The developing cells secreted higher LF than did lactating cells, and FCS induced higher LF secretion on a per cell basis (Chapter III). In addition, synthesis of LF was regulated by the substratum, but differently from casein. LF was synthesized and secreted into the medium regardless of the substratum and in increasing concentrations with time in culture; however, the ultimate concentration secreted was regulated by the substratum (Chapter III). Synthesis and secretion of LF by lactating cells was not regulated by collagen detachment, but was down regulated by developing cells in culture upon collagen detachment (Chapter IV).

LF did not accumulate in cells or acini except for cells on Matrigel, for which case we proposed that LF was secreted by cells on Matrigel but remained trapped within the intercellular spaces of the acinar structure (Chapter III).
Growth factors and bovine mammary development

Bovine mammary cells cultured from a lactating gland secreted a polypeptide growth factor (GF) that was modulated by the cell substratum (Chapter V). The GF was secreted at a stage in the culture that coincided with the attainment of cellular confluence, the reemergence of prolactin responsiveness, and the induction of casein synthesis and secretion. Whether the secretion of a GF by lactating mammary cells in culture resulted from the cells achieving confluence, and whether the GF acted in an autocrine fashion and initiated casein synthesis and secretion was not determined. However, the recent recognition of the role of GF in mammary development and differentiation (Vonderhaar et al., 1983; Spitzer and Grosse, 1987), prompted us to investigate GF's in mammary secretions at different stages of mammary development and differentiation, from a virgin nulligravida heifer, up to and through gestation and into early lactation. Results (Chapter V) revealed the presence of a growth promoting activity that peaked at the 7th month of gestation, and then declined as the gland entered colostrum formation, with further decline into lactation. This activity noted in mammary secretions at the 7th month of gestation was fractionated into two GF activities, one being EGF-like and termed bMDGF1 (apparent mol wt approximately 30,000), and the other termed bMDGF2 and distributed at apparent mol wts of 50,000 and/or 150,000.
Suggested mechanisms and concepts of regulation of milk protein synthesis and secretion by bovine mammary cells in culture.

The effect of the extracellular matrix on the proper growth and development of the mammary gland was first recognized by Daniel and DoOmo (1965). Later, Emerman and Pitelka (1977) utilized a floated collagen gel substratum to achieve the first long term culture of rodent mammary cells that were capable of regulating and secreting one of the main milk proteins, casein. The substratum affected the multicellular organization and the regulation of milk protein synthesis and secretion of bovine mammary cells in culture, similar to that for rodent mammary cell cultures (Neville, 1987; Blum et al., 1987). As previously demonstrated in separate studies, and in accordance with our direct comparisons, bovine cells cultured on (MacKenzie et al., 1982 and MacKenzie et al., 1985) or embedded within (McGrath, 1987) collagen assumed different cellular organization depending on the substratum provided for growth. Bovine mammary cells on collagen substratum restructured their acini into a two dimensional sheet of cells, whereas bovine mammary cells embedded within collagen grew in a three dimensional ductular structure. No reports are available that describe the performance or growth of bovine mammary cells on Matrigel, however, our studies showed that cells plated on Matrigel maintained their three dimensional morphology.
Bissell et al. (1982) proposed the "Dynamic Reciprocity" model for the regulation of milk protein synthesis and secretion by the extracellular matrix. Briefly, the authors proposed that proper interaction of the mammary cell with the extracellular matrix components leads to an organized cytoskeletal-nuclear, and a cytoskeletal-polysome matrix. The extent of intracellular organization affects the ability of the cells to differentiate and secrete milk proteins. One would expect that if the proposed model of "Dynamic Reciprocity" was valid, then the different types of interactive and cellular reorganization of the bovine cells on the extracellular substratum provided would affect their ultrastructural morphology, and subsequently, their ability to secrete milk proteins. Our studies support this model in that bovine mammary cell organization in culture was highly affected by the substratum, and the ultrastructural organization was required (Chapter IV) for the efficient synthesis and secretion of fat and milk proteins that are indicative of a differentiated mammary cell (casein and α-lactalbumin). On the other hand, providing the cells with the extracellular matrix components (Matrigel) normally found in vivo in the mammary gland was not by itself sufficient to induce cell polarization and differentiation (Chapter III and IV). It appears that the remodeling of the bovine acinar structures in culture into a confluent sheet with one side attached to the substratum (basal) and the other unattached and directly in contact with the medium (apical), as induced by the substratum plastic or collagen was essential but not
sufficient to induce casein synthesis and secretion (Chapter III). Instead, the cell required the proper interaction with the extracellular matrix to differentiate and polarize in culture (Chapter IV). It is worthwhile mentioning that while "Dynamic Reciprocity" appears true in our system for casein synthesis and secretion and for the differentiation of the bovine mammary cell in culture, it does not apply for LF synthesis and secretion, since LF was secreted by the mammary cells regardless of the cell substratum, ultrastructural organization, and stage of cell or whether the cell was polarized or not (Chapter III and IV).

Lactoferrin, an iron binding protein secreted by bovine mammary epithelial cells (Groves, 1960; Mason, 1970), is similar to transferrin (Goodman et al., 1988), an iron binding protein that is secreted by rodent mammary epithelial cells (Chen and Bissell, 1987; Kraehenbuhl et al., 1977). In rodent mammary cell cultures, transferrin synthesis and secretion was reported to be regulated by the extracellular matrix, and in contrast to casein, was always synthesized and secreted into the medium regardless of the cell substrata. Only the final concentration of transferrin in the medium was regulated by the substrata. In addition, prolactin partially affected the regulation of transferrin (Chen and Bissell, 1987; Kraehenbuhl et al., 1977). Transferrin (Parry et al., 1987) was also secreted via the basolateral and apical membrane of COMMA-D1 mammary cells. This was in contrast to caseins, which were
only secreted through the apical membrane. The authors (Parry et al., 1987) suggested a different route of secretion for the two milk proteins, transferrin and casein. No reports are available that describe intracellular routing and mechanisms of secretion of LF by bovine mammary cells.

The regulation of transferrin in rodent mammary culture appears to be similar to LF regulation by bovine mammary cells in culture, as outlined below. Different from the patterns noted for the regulation of $\alpha_s$-$\lambda$-casein synthesis and secretion, LF in bovine mammary cell culture was secreted in increasing concentration with time in culture, on all the substrata provided for growth. The concentrations of LF secreted into the medium were regulated by the substratum. LF synthesis and secretion was independent of the presence or absence of prolactin (Chapter III and IV). Furthermore, no intracellular accumulation of LF was detected in cultures of bovine mammary cells except for those cultured on matrigel.

From the ultrastructure of the cells on Matrigel (Chapter III), we suggested that the intracellular/acinar LF noted in these cells on Matrigel may have been secreted by the cells, but was then trapped within the intracellular spaces within the acinar structures. The virtual absence of intracellular LF in cells that spread and formed sheet structures, and in cells embedded within collagen, in addition to
the lower amounts of intracellular/acinar LF levels in cells on Matrigel-37 that partially restructured their acini compared to the cells that did not (on Matrigel-4), led to the conclusion that LF was probably secreted from the cells on Matrigel, but remained trapped within the intercellular spaces of the acinar structure.

The secretion of both LF and αs,1-casein secretion was regulated by cell density in culture. However, while LF secretion increased linearly as the cell plating density was increased, αs,1-casein was only secreted by cells plated at densities above a threshold critical cell density, and below which no casein was secreted into the medium.

αs,1-Casein synthesis and secretion by bovine mammary cells was dependent on the stage of the mammary gland that the cells were derived from, and the type of cellular organization in culture, extracellular substratum, and prolactin. In addition to the critical cell density necessary for induction of casein but not LF synthesis (Chapter II) by bovine mammary cells in culture, three other findings further suggested that the synthesis and secretion of αs,1-casein was under separate patterns of regulation than was LF. First, the mammary cells maintained their ability to secrete LF during the early period of the culture (day 2-6) while they gradually lost their ability to synthesize and secrete casein. The cells regained their casein synthesis capability after 6-8 days in culture, and only under proper hormonal and substratum
conditions (Chapter III and IV), and then only for lactating cells. Second, $\alpha_s,1$-casein but not LF accumulated intracellularly as well as in the medium, and this was regulated by the type of extracellular substrata (Chapter III). Third, detachment of the collagen gel for mammary cells from a lactating bovine mammary gland enhanced $\alpha_s,1$-casein synthesis and secretion, similar to the induction of casein upon detachment of mouse mammary cell cultures (Neville, 1987), whereas detachment of the collagen substratum in bovine mammary cell cultures did not affect the synthesis and secretion of LF (Chapter IV).

The regulation of casein synthesis and secretion in cultures of bovine mammary cells was similar in certain aspects to that reported for rodent mammary cells in culture, yet, the two systems differed considerably for certain experimental conditions, as outlined below.

McGrath (1987) and Shamay and Gertler (1986) reported that bovine cells embedded in collagen grew vigorously, maintained a three dimensional structure in culture and formed a lumen. Neither group (McGrath, 1987; Shamay and Gertler, 1986) investigated milk protein synthesis and secretion in their embedded cultures. In contrast to their studies, we demonstrated that bovine mammary cells embedded within collagen attained the same three-dimensional branching structure, and, although actively secreting LF, they rarely formed a lumen when embedded in collagen (Chapter III). No evidence of secretory vesicles,
casein micelles or fat droplets was noted in such embedded cells. Hamamoto et al. (1988) showed lumen formation in cultures of mouse mammary epithelial cells embedded in collagen with vectorial secretion of casein secretory vesicles and fat into the lumen.

Lactating and/or developing bovine mammary cells cultured on Matrigel also grew with a three-dimensional pattern appearing as acinar-like and distinctly non-ductular, similar to structures described for developing rodent mammary cells cultured on Matrigel (Blum et al., 1987; Barcellos-Hoff et al., 1987). However, unlike mouse mammary epithelial cells on Matrigel which formed an acinar lumen (Bissolli and Hall, 1987), the bovine mammary cells did not form a lumen and showed no evidence of intracellular secretory vesicles or casein micelles. The bovine mammary cells grew in rounded clumps, and were highly disorganized with no particular orientation or evidence of polarization.

No α₅₁-casein was secreted into the medium by bovine mammary cells on Matrigel, even though low intracellular/acinar α₅₁-casein levels were evident (up to 150-200 ng/ml on day 14) in cells from a lactating gland on Matrigel-4. The cells under these conditions maintained an acinar-like structure with no sheet-like structures of cells migrating away from it.
Blum et al. (1987) and Li et al. (1987) demonstrated the superiority of the Matrigel over a detached collagen substratum for rodent mammary cultures as measured by casein mRNA levels, synthesis, and secretion. In marked contrast, bovine mammary cells showed polarized morphology, casein synthesis and secretion, casein micelle formation, α-lactalbumin synthesis and secretion, and fat synthesis only for cells from a lactating gland grown on floated collagen gel. The αs,1-casein output by lactating bovine mammary cells on a floated collagen gel (secreted) was 15-20 fold that of the combined secreted and intracellular/acinar αs,1-casein output by cells on Matrigel. Indeed, bovine mammary cells on detached collagen gel out-performed similar cells cultured on all other matrices (Chapter III vs IV) that we examined (including Matrigel), and were similar to mouse mammary cells as described by Emorman and Pitolka (1977).

A correlation was noted between ultrastructural organization and morphology of bovine mammary cells in culture and their ability to synthesize and secrete αs,1-casein, α-lactalbumin, and fat. Optimal induction of αs,1-casein, α-lactalbumin, and fat synthesis and secretion was only demonstrated with cells from a lactating bovine mammary gland, and only when the cells were cultured on detached collagen substratum that allowed the spreading of the cells and restructuring of the acini into sheet-like structures. This pattern of growth allowed polarization of the cells with the basal membrane
oriented towards the collagen layer and separated from it by what appears ultrastructurally as myoepithelial cell. It appears that this reorganization of the acinar structure on top of a proper substratum may allow the cells to synthesize their own basement membrane components and polarize during the early periods of culture (Chapter IV). After this reorganization of the acinar structure on the proper substratum, the lactating mammary cells in culture redifferentiated and secreted casein, α-lactalbumin, and fat vectorially into an open and unrestricted environment.

The sensitivity of cells from a lactating gland grown on a detached collagen for prolactin (Chapter IV) supported the concept that prolactin in ruminants is needed to initiate but not maintain lactation (Smith et al., 1974). Furthermore, our results suggested that initiation of αs,1-casein synthesis and secretion by the cells on day 8 was not dependent on prolactin during the early periods of the culture (day 0-6), but became absolutely dependent on prolactin between day 6-8 (Chapter IV), an abrupt window of acquired hormone sensitivity.

This period (day 6-8) coincided with two events occurring in the culture: 1) formation of a confluent sheet of cells, and 2) the secretion of a GF by the cells in culture. This GF possibly acts in an autocrine fashion. It would be interesting to determine whether either or both of these events were responsible for the responsiveness of the
cells to prolactin, and eventual αs,1-casein synthesis and secretion, or was otherwise related to induction of differentiated functions (or termination of undifferentiated states).

Whether this GF peak in culture is paralleled under in vivo conditions is not known. However, our studies described here were the first to show the presence of GF activity in mammary secretions taken during the latter half of gestation from primigravida heifers. The GF activity in mammary secretions was modulated during gestation and peaked during the 7th month.

We suggested that the partially purified growth promoting activities bMDGF1 (EGF-like) and bMDGF2 play a role in mammary development and differentiation. GF are postulated to play a role in mammary development (Kidwell and Salomon, 1987; Bano et al., 1985). In fact, Dembinski and Shiu (1987) demonstrated EGF receptors on the plasma membrane of bovine mammary cells.

The availability of the bovine culture system, and further purification of bMDGF1 and bMDGF2 would allow us to examine the role of GF in the development and differentiation of the mammary gland in a focused and detailed study.
In conclusion, the studies reported in this dissertation provided a basic description of performance of bovine mammary cells in culture, and established a bovine mammary cell culture that can be specifically induced to synthesize and secrete milk fat and the milk proteins, LF, casein, α-lactalbumin, and β-lactoglobulin. The different culture permutations tested caused differences in cell growth, morphology, and milk protein synthesis and secretion, and provided insights into the mechanism relating ultrastructure and cell morphology to function and differentiation of the bovine mammary cells in culture. The preliminary characterization of the GF in the mammary secretions of a developing mammary gland and their implication in mammary development and differentiation in vivo and in vitro (Kidwell et al., 1984, Vonderhaar et al., 1983; Dembinski and Shiu, 1987), increased our awareness of the biological phenomena involved in the development of the gland.

However, the results of this study have left us with even more unanswered questions. To list a few: Is LF actually secreted through a different intracellular route than caseins? What is the role of LF, and why is it secreted in the culture under all conditions tested, regardless whether other milk proteins were secreted or not, or whether the cell was polarized, lactating, or developing? Could LF be acting as an autocrine growth regulatory factor? What is the role of the GF secreted by the mammary cells in culture? Do bMDGF1 and bMDGF2 affect proliferation and differentiation of bovine mammary epithelial cells in
culture? Why is it that in culture we were only able to induce cells from a lactating but not developing mammary gland to synthesize and secrete casein?

Many interesting questions remain unanswered, but the development of an FCS-free culture of bovine mammary cells that is responsive to hormones and extracellular substrata, and able to maintain long term synthesis and regulation of the milk proteins, is the ideal tool to address such questions in the future.
REFERENCES


**BIBLIOGRAPHY**


345
Baumrucker, G. R. 1986a. Insulin-like growth factor-1 (IGF-1) and insulin stimulates DNA synthesis in bovine mammary tissue explants obtained from pregnant cows. J. Dairy Sci. 69:(Suppl. 1):120 (Abstr.).

Baumrucker, G. R. 1986b. Insulin-like growth factor (IGF-1) and insulin stimulates lactating bovine mammary tissue DNA synthesis and milk production in vitro. J. Dairy Sci. 69:(Suppl. 1):120 (Abstr.).


Hoeffer's Instruction Manual for mighty small lab gel electrophoresis unit (SE 200), pp. 1-27.


