INFORMATION TO USERS

This manuscript has been reproduced 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.

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
Experimental approaches for enhancing wound healing and inhibiting tumor growth

Andreatta-Van Leyen, Sheila, Ph.D.

Case Western Reserve University (Health Sciences), 1994
EXPERIMENTAL APPROACHES FOR ENHANCING WOUND HEALING AND
INHIBITING TUMOR GROWTH

by

SHEILA ANDREATTA-VAN LEYEN

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Thesis Adviser: Dr. Richard L. Eckert

Department of Physiology and Biophysics
CASE WESTERN RESERVE UNIVERSITY
May, 1994
We hereby approve the thesis of

__________________________
Sheila Andreatta-van Leyen

candidate for the PhD

degree. *

(signed)  

__________________________

(chair)

__________________________

__________________________

__________________________

__________________________

(date) 1/21/94

*We also certify that written approval has been obtained for any proprietary material contained therein.
EXPERIMENTAL APPROACHES FOR ENHANCING WOUND HEALING AND INHIBITING TUMOR GROWTH

Abstract

by

SHEILA ANDREATTA-VAN LEYEN

Growth factors are regulatory peptides involved in many processes of cell physiology and pathology, including tissue repair and tumor growth. Part of this work concerned the development of a genetically engineered biological bandage (GEBB) designed to improve wound healing. Human epidermal keratinocytes (SCC-13) were genetically engineered to produce bovine growth hormone (bGH), giving raise to higher producer bGH cell lines (1 ug/ 1x10^6 cells/ 24hs). Selection of biocompatible polymers for bandage composition and appropriate conditions for cell maintenance was also established. The GE BB consists of a hydrophylic and gas permeable top and bottom membranes (Z-bind) united by a gasket. The bottom membrane allows cell
attachment and growth, and diffusion of macromolecules. This system was functional under ambient conditions; when placed onto a full-thickness, surgically generated wound on rats, the cells within the bandage released bGH for three days. The GECC represents a versatile system for delivering a variety of biological agents to wounds.

In the second part of these studies, the role of retinoic acid (RA) and epidermal growth factor (EGF) in the regulation of insulin-like growth factor binding protein-3 (IGFBP-3) was addressed. IGFBP-3 regulates insulin-like growth factors (IGFs) which in turn are important modulators of epithelium proliferation. Treatment of the ectocervical epithelial cell line ECE16-1 with EGF caused a marked reduction in IGFBP-3 levels. In contrast, RA increased mRNA and protein levels in the presence or absence of EGF. This response was concentration-dependent with a half-maximal increase observed at 1 nM RA. RA was able to reverse the EGF suppression in IGFBP-3 when added simultaneously or three days after EGF treatment. Conversely, when ECE16-1 were treated with RA, IGFBP-3 levels increased within 24 h and subsequent addition of EGF was without effect. Thus, the RA-dependent increase in IGFBP-3 is dominant over the EGF
supression. Increased IGFBP-3 levels correlated with suppressed growth. RA also increased IGFBP-3 mRNA in other ectocervical cell lines. These results suggest that RA may act to inhibit ectocervical proliferation by increasing IGFBP-3 levels and/or reducing the extracellular concentration of free IGFs.
DEDICATION

This work was performed with the idea of contributing to the development of mankind. Although it is not often that one reaches its ideals, I would like to dedicate these studies to those who encourage me to accomplish part of them:

My father, Valdir Andreatta; my husband, Alexander van Leyen; and my brother-in-law, Francesco Caterina.

My grandmother, Hilda Hecht; my mother Cyntia Hecht Andreatta; and my sisters, Cathie and Rita.

My friends, in special, to the Corvas.

The scientists, Pedro Guertzenstein, Carlos Ferrario and Toni Scarpa.

All of them have set my life with invaluable examples, support, experiments, and love, without what I could never fulfill my expectations.
ACKNOWLEDGEMENT

To all of those who I once said thank you.

To the members of my thesis committe and to my adviser.

This work was supported by a doctoral Training Grant from Conselho Nacional de Pesquisa e Desenvolvimento (CNPq), Brazil, by grants from the Edison Biotechnology Center and Naval Medical Research and Development Command, and by the American Institute for Cancer Research.
**TABLE OF CONTENTS**

Title i
Abstract ii
Dedication iii
Acknowledgement vi
Table of contents vii
List of figures ix

**CHAPTER I**

Overview 1
1. Wound healing 1
   1.1. Inflammation phase 1
   1.2. Granulation tissue formation 2
   1.3. Matrix formation and remodeling 5

2. Tumors, non-healing wounds 7

**CHAPTER II**

A new approach for wound therapy 12
1. Introduction 12
   1.1. Growth factors and wound healing 13
   1.2. Epidermal growth factor 15
   1.3. Insulin-like growth factor 17
   1.4. Transforming growth factor-beta 19

2. The Genetically Engineered Biological Bandage 20
   2.1. Background 20
   2.2. Concept 28
   2.3. GEBB, a new device to improve wound healing 31
   2.4. Specific Aims 32

**CHAPTER III**

Development and characterization of SCC-13-bGH 34
1. Introduction 34
2. Material and Methods 34
3. Results 38
4. Discussion 48

**CHAPTER IV**

Construction and "in vitro" testing of the biological bandage 53
1. Introduction 53
2. Material and Methods 54
3. Results 56
4. Discussion 69

**CHAPTER V**

Delivery of growth hormone to wounds using a genetically engineered biological bandage 72
1. Introduction 72
CHAPTER VI
A pharmacological approach for the treatment of ectocervical dysplasia
1. Introduction 83
1.1. Growth factors and tumors 83
1.2. Epidermal Growth Factor 85
1.3. Insulin-like growth factor 87
1.4. Insulin-like growth factor binding proteins 88

2. Vitamin A 91
2.1. Background 91
2.2. The ectocervical epithelial cell system 96
2.3. Retinoids, EGF and cervical cancer: a working hypothesis 99
2.4. Specific Aims 99

CHAPTER VII
Regulation of IGFBP-3 level by EGF, IGF-I, and RA 101
1. Introduction 101
2. Material and Methods 102
3. Results 107
4. Discussion 121

CHAPTER VIII
Regulation of ectocervical cell proliferation by EGF, IGF-I, and RA 128
1. Introduction 128
2. Material and Methods 129
3. Results 131
4. Discussion 134

CHAPTER IX
Summary and Conclusions 144

REFERENCES 147
LIST OF FIGURES

Fig. 1A. Bovine growth hormone expression vector 40
Fig. 1B. SCC-13 and SCC13-bGH cell morphology 40

Fig. 2. bGH mRNA expression in SCC13-bGH cells 42

Fig. 3. Synthesis and secretion of bGH by SCC13-bGH cells 45

Fig. 4. Time course of bGH release 47

Fig. 5. SCC13-bGH content of bGH 50

Fig. 6. "In vitro" release of bGH by GEBB 58

Fig. 7. Accumulative profile of bGH release by different bottom membranes 61

Fig. 8. Twenty-four hour profile of bGH release by different bottom membranes 63

Fig. 9. bGH release under ambient conditions 66

Fig. 10. Long term release of bGH under ambient conditions 68

Fig. 11. Wound model and GEBB placement "in vivo" 77

Fig. 12. "In vivo" release of bGH by GEBB 79

Fig. 13. RA regulation of IGFBP-3 mRNA and protein levels in ECE16-1 109

Fig. 14. RA regulation of IGFBP-3 mRNA level in CaSki and ECE16-D1 112

Fig. 15. Concentration-dependent regulation of IGFBP-3 protein and mRNA levels by RA 114

Fig. 16. Concentration dependence of RA regulation of IGFBP-3 mRNA in normal and in cervical cell lines 117

Fig. 17. Time course of IGFBP-3 regulation by EGF and RA 119

Fig. 18. IGFBP-3 ligand blot of conditioned medium of ECE16-1 cells 123

Fig. 19. Effects of IGF-I, EGF and RA on ECE16-1
cell proliferation

Fig. 20. RA dose-response proliferation curves obtained in ECE16-1 under different treatments

Fig. 21. Proliferation data obtained from different ectocervical cell types
CHAPTER I
OVERVIEW

1. WOUND HEALING:

Tissue repair is a dynamic biological process that involves the interaction of multiple cell types. To facilitate understanding of this process, a time-scale compartmentalization of wound healing is presented below. Although somewhat oversimplified, one may propose a model in which three overlapping phases of wound repair exist. These phases include inflammation, granulation tissue formation, and matrix remodeling.

1.1. Inflammation phase: Blood vessel disruption after tissue injury results in extravasation of blood constituents. Platelets aggregate to trigger blood coagulation, while releasing a spectra of biologically active substances. To preserve homeostasis, clot formation occurs within the blood vessel and in the surrounding connective tissue. The first cells to infiltrate an area of inflammation are the neutrophils, shortly followed by monocytes. They are attracted to the injury site by a variety of chemotactic factors that include fibrin degradation products (Stecher and Sorkin, 1972; McKenzie et al., 1975), fibrinopeptides (Kay et al., 1974; Senior et al., 1986), kallikrein (Kaplan et al., 1972; Gallin and Kaplan, 1974), C5a
from complement activation (Snyderman et al., 1972; Fernandez et al., 1978), bacteria-generated formyl methionyl peptides (Freer et al., 1980), leukotriene B4 (Ford-Hutchinson et al., 1980) and platelet-released substances (Deuel et al., 1981). At this stage, a major function of neutrophils is to clear the injury site of contaminating bacteria. When clearance is effective, generation of chemoattractants cease and neutrophils found at the site of injury are phagocytosed by macrophages. Macrophages are critical to the initiation of tissue repair. In the late inflammatory phase, macrophages phagocytose and digest pathogenic organisms, scavenge tissue debris, and release biologically active substances (Humes et al., 1977; Lachman, 1983; Sporn and Roberts, 1986). Growth factors and some chemotactic factors are necessary for the initiation and propagation of the second phase of wound repair, the formation of granulation tissue.

1.2. Granulation tissue formation: Granulation tissue is formed by an accumulation of macrophages, ingrowth of fibroblasts, deposition of loose connective matrix, and angiogenesis. Accumulation of fibroblasts and neomatrix components such as collagen, fibronectin, and hyaluronic acid is referred to as fibroplasia. Growth factors and chemotactic factors released by
platelets and macrophages stimulate fibroplasia and angiogenesis. Fibroblasts respond to these stimuli by proliferation (Kaplan et al., 1979), migration (Seppa et al., 1982; Senior et al., 1983), matrix deposition (Williams et al., 1984), and wound contraction (Ehrlich et al., 1983). The neomatrix provides a substrate for macrophages, endothelial cells and fibroblast migration into the wound. The neovasculature allows nutrients and oxygen to reach the new tissue. When the epithelium is disrupted at the time of injury, re-epithelialization must occur as soon as possible in order to re-establish tissue integrity. This process usually begins within the first 24 hours for a healthy subject, much earlier than the observable formation of granulation tissue. However, re-epithelialization is considered with granulation tissue development, since both represent new tissue generation. When appendages are not present, the movement of epithelial cells is usually from the free edge of the wound across the defect. In the skin (stratified epithelium), cells appear to move one over the other, in the so called leapfrog fashion (Winter, 1962). In the cornea (monolayer) cells appear to move in a single line, with the leading cells remaining always in front (Fujikawa et al., 1984). Alterations in epithelial cell
phenotype are observed during migration, due to conformational changes in intercellular desmosomes, cytoplasmic actin filaments and intracellular tonofilaments (Gabbiani et al., 1978). The driving force for epithelial cell movement is unknown. There is evidence for chemotactic attraction, active contact guidance, and a combination of both. However, migration seems to be independent of cell proliferation (Winter et al., 1972). When re-epithelialization is complete, the cells revert to their normal phenotype and firmly attach to the basement membrane. Fibroblasts also modify their phenotype during migration. The myofibroblast, the genotypically and phenotypically altered fibroblast, gains motility and contractility without loosing the ability to synthesize and secrete structural macromolecules. During fibroblast migration into the defect, a great quantity of loose extracellular matrix is deposited. Fibronectin is one of the most important components of the newly synthetized matrix (Culp et al., 1979), since it binds fibroblasts and extracellular matrix simultaneously. Fibroblasts use fibronectin matrix for movement guidance. The sequence Arg-Gly-Asp-Ser occurs within the cell-binding domain of fibronectin and other extracellular matrix proteins. The tetrapeptide has
been shown to be a critical ligand for the binding of these proteins to cell surface receptors, the integrins (Ruoslahti and Pierschbacher, 1986). Other extracellular matrix components, such as the highly hydrated hyaluronic acid in granulation tissue, provide a matrix that is easily penetrated by ingrowing parenchymal cells. Neovascularization of wounds occurs by capillary buds sprouting from blood vessels adjacent to the defect. Endothelial cell pseudopods project through the fragmented basement membrane towards the stimulus with subsequent migration of the whole cell into the perivascular space. In the parent vessel, cells begin to proliferate and migrate around day two. Interestingly, the cells at the capillary tip never undergo mitosis (Ausprunk et al., 1977).

1.3. **Matrix formation and remodeling:** The third phase of wound repair actually begins simultaneously with the formation of granulation tissue. However, after dissolution of the granulation tissue, the matrix is constantly altered. During this process, rapid elimination of fibronectin is observed and slow accumulation of type I collagen proceeds, followed by accumulation of types III and V collagen fibrils, providing tensile strength for the wound. Ultimately, the composition of the matrix constituents depends not
only on the cells and enzymes present but also on the growth factor profile presented to the cells. As the matrix matures over the next weeks, the fibronectin and hyaluronic acid disappear, collagen bundles grow in size, and proteoglycans are deposited adding resistance-to-deformation to the tissue. During the healing process, the basement membrane does not reform until after migration ceases (Stanley et al., 1981; Clark et al., 1982). As epithelial cells cease to migrate, type IV collagen and then laminin become detectable in the basement membrane zone. The deposition of these two proteins begins at the original wound margin and progresses inward as a zipper mechanism, interlocking the new epidermis to the neodermis.

From the description above, it is clear that the rate and quality of wound healing are dependent on a variety of agents. The metabolic activity of cells in and around the wound site is controlled by nutrients, hormones, growth factors and spatial environment. Increased wound lactate is one of the first local metabolic events to follow tissue injury (Caldwell, 1988). However, evidence has been presented for aerobic glycolysis in the wound site. Blood flow to wounds is usually equal to or greater in the wound when
compared to contralateral non-wounded areas, and so is the oxygen consumption (Wilmore et al., 1977; Tischler et al., 1983; Caldwell et al., 1984). At the wound site, cell growth, proliferation, migration and differentiation are tightly regulated by the presence of several growth factors. Each cell type responds specifically to each growth factor, leading to a fine tune on healing of the tissue.

2. TUMORS, NON-HEALING WOUNDS:

In solid tumors, malignant cells directly climb on or intermingle with stromal elements (Tremblay, 1979). In carcinomas, tumors of epithelial cell origin, a basement membrane is often interposed between the tumor cells and the stroma (Liotta, 1984). The stroma provides the vascular supply for nourishment, gas exchange, and waste disposal. It may also limit the influx of inflammatory cells, providing a barrier to immunologic rejection (Dvorak et al., 1979). Remarkable and interesting similarities exist between the generation of tumor stroma and the healing of wounds.

Tumor stroma is composed of new blood vessels, inflammatory cells, fibroblasts, and myofibroblasts. Tumor connective tissue proteins include fibronectin,
interstitial collagens, elastin, and glycosaminoglycans. Fibrin gel traps plasma proteins and water in the interstitium (Dvorak et al., 1979; Dvorak et al., 1983), forming a provisional matrix later replaced by mature stroma. Tumor fibrin comes from extravasation and extravascular clotting of plasma fibrinogen. Permeability to plasma proteins is highly enhanced in tumor microvasculature. Vasoactive inflammatory mediators do not account for the hyperpermeability of tumor vessels. Instead, the increased permeability is due to secretion of a peptide, vascular permeability factor (VPF), by the tumor cells themselves (Serger et al., 1983). Interestingly, VPF is also detected in keratinocytes and its level is markedly increased in the activated, migrating keratinocytes involved in wound healing (Brown et al., 1992). In tumors, a fibrin-fibronectin gel is first formed as a provisional matrix. This primitive matrix is transformed into mature matrix through a series of events. As in the wound healing process, monocytes enter the growing tumor area and differentiate into macrophages, releasing a series of chemoattractants and growth factors. Fibroblasts and endothelial cells proliferate, migrating into the provisional matrix. The synthesis of the components of
connective-tissue matrix transform the fibrin-fibrinogen stroma into a highly cellular, highly vascularized tissue. This tissue resembles the granulation tissue of the healing wound. Later on, this granulation tissue acquires more collagen and loses its cellularity and vascularity. This process closely parallels the later stages of wound healing.

The structural proteins that accumulate in tumor and wound stroma have been analyzed immunohistochemically (Dvorak et al., 1984). Fibrin is, at first, substantial in both, but then declines more rapidly and completely in normally healing wounds. Fibronectin also increases initially in both tumors and wounds and persists in the tumors. Type I and III collagen progressively increase in both processes. Older tumors contain type I collagen almost exclusively, less mature tumors contain relatively more type III (El-Torky et al., 1985). Type IV collagen is confined to basement membranes such as those that envelop blood vessels and epithelia. A number of benign and malignant epithelial cells synthesize type IV collagen (Barsky et al., 1982; Bano et al., 1984) whereas fibroblasts synthesize collagen types I and III. In the case of healing wounds, epithelial cells are stimulated to divide and to migrate to cover the
defect. At the last stage of wound healing, epithelial cells secrete basement membrane components, such as type IV collagen and laminin (Liotta, 1984; Dvorak et al., 1984). Tumor cells engage in very similar activities, but they are less tightly regulated. Thus, malignant cells also divide, migrate (or invade) and in the case of carcinomas also synthesize type IV collagen and laminin.

A typical example of a carcinoma is uterine cervical cancer. The multilayered cervical epithelium has a cellular organization similar to the epidermis of the skin (Ferenczy and Winkler, 1987). In the cervix, basal cells proliferate moving outward toward the surface. They further differentiate and finally are sloughed off from the surface. In cervical carcinomas, the usual pattern of cell division and differentiation is disrupted (Pattilo et al., 1977; Parmley, 1987, Woodworth et al., 1990). With that, all the layers of the epithelium consist of undifferentiated proliferating cells. The abnormal cells form a patch confined to the epithelial side of the basal lamina. With continuous proliferation, several chromosomal abnormalities may take place and cells will give rise to a truly malignant cervical carcinoma. The malignant cells are then able to break the basal lamina and
invade the connective tissue, generating a "healing-impaired" wound. Therefore, tumors have developed the capacity of subverting the wound healing response of the host as a means to acquire the stroma they need to grow and expand. They mimic wounds by depositing an extravascular fibrin-fibronectin gel. Such gels, in tumors or wounds, signal the host to start the wound healing process. However, in tumors, the wound healing response is not servo-controlled and continues to operate. Therefore, tumors may appear to the host as an endless series of wounds in a continuous healing process that is never complete.
CHAPTER II
A NEW APPROACH FOR WOUND THERAPY

1. INTRODUCTION:
A multitude of growth factors have been structurally and functionally characterized, including the cloning of their respective cDNAs (Heldin and Westermark, 1989). There are also growth inhibitory factors (Sporn and Roberts, 1985). In addition some of these agents exhibit stimulatory or inhibitory activity, depending on the cell type (Sporn and Roberts, 1985). Growth factors are generally named based on the initial source of isolation, sometimes resulting in the designation of a name that does not match what is eventually determined to be the function of the factor. One of the factors discovered in platelets was called platelet-derived growth factor (PDGF) (Kaplan et al., 1979). Subsequently, it was demonstrated that PDGF was also produced by macrophages, endothelial cells and smooth muscle cells (DiCorletto and Bowen-Pope, 1983; Shimokado et al., 1985; Collins et al., 1987; Barret et al., 1988). Transforming growth factors alpha and beta were purified from tumor cells; however, they are also produced by normal cells (Goustit et al., 1986).
Growth factors: i) stimulate fetal and placental growth, ii) regulate growth and differentiation of continuously regenerating tissues, and iii) stimulate tissue repair processes. Plasma contains few growth factors; the presence of them in platelets is thought to facilitate delivery of growth factors to sites of injury where they play a major role in wound healing. Growth factors do not usually act in an endocrine manner; they diffuse in intercellular spaces and act locally.

1.1. Growth factors and wound healing: A role for various growth factors in normal healing has been established in recent years (Nemeth et al., 1988). It has been demonstrated that multiple growth factors are required for maximum stimulation of specific cell types. Exposure to one growth factor can lower the threshold for mitogenicity of a second one (Wharton et al., 1983). The multiplicity of growth factors in various tissues, the varying cell type specificity for them, and the requirement of multiple growth factors for stimulation of specific cell types provide the fine tuning of cellular regulation in different tissues. In wound healing experiments, application of different growth factors leads to improvements in the rate and/or quality of healing (Mustoe et al., 1987; Brown et al.,
1989; Schultz et al., 1991; Langer and Moses, 1992). In these experiments, growth factors are delivered in solution or as a component of a dressing. Synergistic effects of growth factors are also observed in different wound models (Lynch et al., 1989). Although growth factors appear to enhance wound healing, the systems described for their delivery are not adequate.

Growth factors exert their mitogenic effect by interacting with specific receptors on responsive cells (Heldin and Westermark, 1989). The binding of a growth factor to its receptor elicits a cascade of events, including altered protein phosphorylation, increased inositol-lipid breakdown, changes in ion fluxes and changes in gene expression. Many growth factor receptors are protein-tyrosine kinases, indicating that phosphorylation of specific substrates on tyrosine residues is important in growth stimulation (Hunter and Cooper, 1985). In fact, activation of growth factor receptors appears to initiate a cascade of intracellular phosphorylation events.

Three of the well defined growth regulatory factors active on mesenchymal and epithelial cells are described below. Each has been implicated as being of importance in wound healing.
1.2. **Epidermal Growth Factor (EGF):** EGF was isolated from mouse submaxillary glands and from human urine (Cohen, 1962; Gregory, 1975). Human EGF consists of a single chain with a molecular weight of 6000 daltons (Taylor et al., 1972). It contains 53 amino acids, has three disulphide bonds, and has been shown to stimulate the growth of skin and corneal epithelium in a variety of animals (Cohen, 1962; Brown et al., 1986). EGF receptors have been found in a diversity of cell types and the receptor protein was purified from A431 cells (Cohen et al., 1982). *In vivo* studies have shown an increased rate of healing, increased proliferation, and increased skin thickness in different wound models treated topically with EGF (Brown et al., 1989; Schultz et al., 1991). Sustained release of EGF by polyvinyl alcohol sponge implanted subcutaneously, accelerates wound repair (Buckley et al., 1985). The increased rate of repair was achieved by a marked increase in the extent and organization of the granulation tissue, a doubling in the DNA content, and a 33% increase in protein content and wet weight, as compared to placebo controls. Studies were also undertaken to evaluate the effect of EGF on the healing of uniform partial-thickness wounds. Topical application of EGF on paired donor sites significantly
accelerated epidermal regeneration as compared to control (Brown et al., 1986; Brown et al., 1989). The ability to accelerate the rate of regeneration at donor site allows earlier reharvest of this region. This is essential in burn patients, because it permits earlier coverage of burn wounds with autologous skin grafts. Another potential beneficial use of EGF is for treatment of impaired healing and chronic ulcers. In summary, wound healing is enhanced in animals and patients treated with EGF. These findings suggest a potential application of EGF for improvement of wound healing.

The cellular receptor for EGF is the best understood of the growth factor receptors. It is an integral 170 kDa membrane protein composed of an extracellular binding domain, a transmembrane region, and an intracellular domain (Cohen et al., 1982; Hunter and Cooper, 1985). The latter exhibits a tyrosine kinase activity for ATP phosphorylation substrates. The addition of EGF to cells increases the level of phosphotyrosine in proteins as well as the rate of cellular metabolism. EGF induces expression of the c-fos and c-myc genes (Muller et al., 1984), markers of cell proliferation.
1.3. **Insulin-like growth factors (IGF-I and IGF-II):** IGF-I and IGF-II are growth hormone-dependent peptides, ancestrally related to proinsulin (Blundell and Humbel, 1980). Both peptides have remarkable growth-promoting and insulin-like effects "in vivo" and "in vitro". IGF-I, purified from human serum, is a single chain of 70 amino acids with three internal disulfide bonds. The human IGF-II gene is closely linked to the human insulin gene (Bell et al., 1985). The IGFs are processed from larger precursors. IGF-I (70 amino acids) is derived from a 130-residue precursor, whereas the IGF-II (67 amino acids) is derived from a 180-residue precursor (Bell et al., 1984). Chemical cross-linking of radiolabelled IGFs to cells reveal the presence of two distinct receptors. The IGF-I receptor shows homology to the insulin receptor. It is composed of a heterotetrameric 450-kDa complex, consisting of two transmembrane B subunits each bonded to one A subunit. The A subunits provide the growth factor binding domain and the B subunits possess ATPase and tyrosine kinase activities (Pilch and Czech, 1979; Van Obberghen et al., 1983). The IGF-II receptor is a single chain polypeptide, exhibiting a 250 kDa component. It consists of a large extracellular domain (93% of the receptor), a
transmembrane region and a small cytoplasmic domain. The IGF-II receptor has no intrinsic tyrosine kinase activity but contains several potential serine, threonine and tyrosine phosphorylation sites.

IGF-I has been shown to act in several phases of wound healing (Spencer et al., 1988; Steenfos and Jansson, 1990; Steenfos and Jansson, 1991). At the onset of tissue injury, the lysis of platelet alpha-granules release IGF-I and insulin-like growth factor binding protein-3 (IGFBP-3) in addition to other growth factors with which IGF-I may synergize (Spencer et al., 1993). During the next stages of healing, fibroblasts secrete IGF-I that can act in an autocrine mode, stimulating proliferation and collagen synthesis. IGF-I may also play a paracrine role in angiogenesis and re-epithelialization since both basal epithelial and endothelial cells have IGF-I receptors (Bar et al., 1981; Misra et al., 1986). In this context, IGF-I-deficient rats show a significant reduction in healing (Steenfos et al., 1989). In these animals, lower levels of DNA, hydroxyproline, and total protein are extracted from the ingrown tissue collected from subcutaneously implanted wound chambers. The actual amounts of protein correlates to the level of IGF-I present in the wound fluid.
1.4. **Transforming growth factor-beta (TGF-beta):**

TGF-beta was first described as a factor stimulating the growth of AKR-2B (Moses et al., 1981) and NRK cells (Roberts et al., 1981) in soft agar. TGF-beta has been purified from different sources (Assoian et al., 1983; Frolik et al., 1983; Roberts et al., 1983; Massague, 1984) and the human gene has been cloned (Derynk et al., 1985). TGF-beta is the prototype of a large family of structurally related factors that regulate various cellular functions. There are three isoforms of TGF-beta which are highly conserved among species. TGF-beta₂ consists of a disulfide-linked homodimer (Derynk et al., 1985), each subunit is synthesized as a biologically inactive 350 amino acid precursor (Sporn et al., 1987; Bennett and Schultz, 1993). The subunits are cleaved at the carboxyl end to produce a 112 amino acid active protein. In several cell types, the amino terminal segment of the protein remains associated with the carboxyl segment to form a latent complex (Sporn et al., 1987). Activation of the latent complex is achieved through proteolysis by plasmin or exposure to acidic pH (Sporn et al., 1987). Although TGF-beta is mitogenic for fibroblasts (Massague, 1984; Moses et al., 1985; Roberts et al., 1985; Shipley et al., 1985), it acts as an inhibitor of cell growth in a variety of
epithelial cells (Moses et al., 1985; Roberts et al., 1985). Up to five different classes of TGF-beta receptor have been reported in the literature (O'Grady et al., 1992). Although combinatorial interactions between ligand and receptor are highly variable, a common feature among them is the intracellular protein kinase domain with predicted serine-threonine specificity.

The alpha granules of human platelets contain significant amounts of TGF-beta (Assoian et al., 1983), and wound fibroblasts and macrophages also produce it (Lawrence et al., 1984; Rappolee et al., 1988). In addition, TGF-beta induces monocyte chemotaxis and subsequent release of mediators that stimulate fibroblast growth (Wahl et al., 1987). Although TGF-beta inhibits keratinocyte proliferation, it has been shown to improve healing in several wound models. It does so by increasing connective tissue volume (Lynch et al., 1989), granulation tissue formation and tensile strength of dermal wounds (Sporn et al., 1983; Mustoe et al., 1987). It also attracts macrophages to the site of injury (Sporn et al., 1986; Wahl et al., 1987).

2. THE GENETICALLY ENGINEERED BIOLOGICAL BANDAGE:

2.1. Background:
In the past century, the dogma was to allow wounds to heal without any intervention. A major advance was made in the mid 20th century when Dillman and Penn (1956) demonstrated that a moist environment encourages the epithelialization of partial-thickness wounds. Dillman and Penn used inexpensive fine mesh gauze as wound dressings. Later on, these mesh dressings were replaced by a superior plastic-based product (Band Aids). Several forms of these dressings are presently available in the market. They are classified either as occlusive (gas impermeable) or semi-occlusive (gas-permeable), both types have been shown to increase the rate of epithelialization and to decrease pain (Madden et al., 1989). These bandages are called "passive" because they are used to cover and protect the wound, but do not actively promote wound healing.

The search for more effective ways of wound treatment led to the employment of autographs, allographs and xenographs to the the wound bed. A more recent approach for the treatment of acute, chronic and burn wounds is the treatment of the injured site with sheets of keratinocytes and/or skin sheets. Evidence that application of the sheets of keratinocytes to wound surfaces promote healing was first established by Green and associates (1979). Subsequently, several
laboratories have shown that keratinocyte sheets alleviate pain and promote healing (Leigh et al., 1987; Gallico et al., 1984; Gallico et al., 1989; Phillips et al., 1989; Fratianne et al., 1993) of wounds of different etiologies. However, there are several potential problems with this approach. These include the need to screen keratinocytes for the presence of virus, the weeks required to obtain a confluent monolayer of keratinocytes for grafting and the possibility of activating the patient's immune system. Furthermore, wounds covered with keratinocytes are fragile and subject to sheer stress (Fratianne et al., 1993).

Another way of improving wound healing is through the application of skin sheets onto the wound (Phillips and Dover, 1991). This is called split thickness skin grafting. This technique consists of the removal of "skin sheets" (down to the midermis) from intact segments of the patient's skin (donor site) and subsequent grafting them onto the injured site (Wheeland, 1989). However, availability of donor sites depends on a series of variables, including the presence of an area for harvesting and the general health status of the patient. To cover extensive wounds, the donor site needs to be harvested several
times. Therefore, increasing the rate of reepithelialization of the donor site area and promoting healing of chronic wounds are major objectives in this field of research.

Recombinant growth factors have been shown to improve the rate of epithelialization, when added to wounds (Buckley et al., 1985; Lynch et al., 1989). Thus, by delivering the appropriate growth factor at the right time and level to the injured area, one should expect an improvement in the rate of healing at that site. In this context, recombinant or purified growth factors have been added to cutaneous wounds as a liquid or dispersed in collagen/gel suspensions (Mustoe et al., 1987; Brown et al., 1989; Langer and Moses, 1991; Schultz et al., 1991), with some notable results. EGF, TGF-alpha, TGF-beta, IGF-I, PDGF and bFGF have been tested in several wound model systems. Overall, there is good evidence for improvement of the repair process; however, the rate of wound healing is far from optimal. Problems that must be overcome to improve the rate of healing include the following. First, the rate of peptide delivery is not constant in the presently available delivery systems. Most of the kinetic work carried out in these systems show a burst of growth factor delivery in the first few hours, followed by a
decrease in the rate of delivery (Siegel et al., 1989; Brunstedt and Anderson, 1992; Gombotz et al., 1992). Second, growth factor delivery is dependent on the properties of the vehicle (Siegel et al., 1989; Brunstedt and Anderson, 1992). If the vehicle is undergoing destruction or does not contact the wound, delivery of growth factors will be uneven. Third, the tridimensional structure and integrity of the growth factor can be compromised during preparation of the delivery system. The short half-life of some growth factors and changes in their bioactivity due to conformational change or cleavage of the molecule, may reduce biological activity. In addition, the presence of proteases in the wound fluid may contribute to a decrease in growth factor levels in the wound environment (Varghese et al., 1986; Emonard and Grimaud, 1990; Woessner, 1991). Thus, efforts to improve wound healing include i) keeping the wound moist, ii) applying keratinocytes and/or skin sheets to the site of injury, and iii) applying growth factors to the wound.

Based on the above observations, we have developed a controlled release system aimed to increase the rate and quality of healing. Before a description of our prototype model, a brief review of various modes of
drug delivery presently available in the market is presented.

Reservoir devices are composed of a core (active agent) enveloped by an inert diffusion barrier. The active agent is present in a highly saturated state and is able to diffuse through the surrounding membrane in response to a gradient. Two well known systems are included in this category. The first and simplest, is the osmotic pump (Zentner et al., 1985; Gallo et al., 1989). Here, the core consists of a solid, water-soluble drug, enclosed in a water-permeable, drug-impermeable membrane containing a small aperture. When in contact with aqueous solutions such as body fluids, water is transported into the reservoir, generating a hydrostatic pressure. This leads to release of the drug solubilized in water, through the opening. The second system in this category is microencapsulation. The active agent is microencapsulated by polymeric materials, ranging from 5 to 500 um in diameter. Here, the drug release is achieved by a combination of erosion of the polymeric shell and the diffusion through the polymer.

Matrix devices include several systems in which the active agent is dissolved in the polymer (Brunstedt and Anderson, 1992). The active agent is
preferentially released from the surface layers followed by release from deeper regions of the polymer matrix. When the active agent is dispersed within a polymer matrix, solute transport may occur by either a partition mechanism involving diffusion along and between the polymer segments or a pore mechanism in which transport occurs through microchannels (Haleblian et al., 1971; Bawa et al., 1985; Siegel et al., 1989; Golomb et al., 1990).

In carrier systems, the active agent is bound to a soluble polymer. The agent may be converted to a polymerizable moiety and then incorporated into the main chain of a polymer (Veronese et al., 1989; Ouchi, 1989). The active agent may also be incorporated into an existing polymer by its reaction with groups located on the side chains or end groups of the polymer carrier. A longer pharmacological action was demonstrated for polymer-bound drugs. This may be due to restricted transport of the polymer-bound drug through barriers between body compartments, by slower degradation, or by reduced excretion.

It is clear that with controlled release polymeric devices, one must not only be concerned with the effects of the active agent, but also with the effects of the polymer on the host. Its toxicity,
biocompatibility, and immunogenicity are critical since the device interfaces directly with biological environments. The mechanism of release of active agents through a given polymer is by diffusion of the agent in response to a decreasing concentration gradient away from the releasing device. Usually it is assumed that the agent is released into an infinite sink where its concentration is zero (Langer and Folkman, 1976; Ouchi, 1989). However, these systems are mostly suitable for drug delivery into body cavities. They are usually implanted subcutaneously or taken orally. Currently, for external delivery few materials are available, including collagen, polyhydroxyethylmethacrylate, and ethylene-vinyl acetate copolymer (Polverini et al., 1977; Gospodarowicz et al., 1979; Glaser and D'Amore, 1980; Silberstein and Daniel, 1987; Folkman et al., 1989).

Because these systems are far from ideal, we have designed a biological bandage for release of active agents into external wounds. The "core" of our bandage contains biologically active cells, designed to function as a growth factor factory. The cells are engineered to secrete specific proteins or growth factors into a matrix (hydrogel), strategically placed underneath the bandage. When this system is placed in
contact with the wound bed, the proteins diffuse out of the hydrogel into the wound site. Since these cells are confined inside a polymeric envelope, they will not come into direct contact with the wound. The bandage is kept in place by means of a commercially available wound dressing.

2.2. Concept:

The biological bandage is a new approach for wound therapy. It combines the use of living cells and the delivery of a specific growth factor (or any other protein) to the injured site. It is analogous to a closed envelope inside which cells have been seeded and kept alive. The bandage consists of: 1) a top membrane; 2) a bottom membrane; 3) a separator gasket to maintain the positions of both membranes; and 4) the genetically engineered cells. The top membrane of the envelope is a protective cover for the upper surface of the bandage and is composed of a gas permeable, biocompatible polymer. The bottom membrane is highly permeable, thin and pliable, with a pore size that allows free diffusion of macromolecules (< 50 kD). However, the cells are retained inside the envelope for the patient's safety. This polymer must also allow cell attachment and growth. The membranes are sealed
to the separator gasket by means of a biocompatible adhesive. This separator should also be biocompatible and flexible, so as to conform to the contours of the wound. The living cells within the bandage are either of epidermal or mesenchymal origin. They can either be normal cells, an established cell line, or a genetically engineered cell line. The biological bandage is analogous to the keratinocyte sheets, but the cells do not make direct contact with the wound. This has the advantages of decreasing rejection and preventing the transfected cells to take residency at the wound. The genetically engineered biological bandage (GEBB) contains cells that were engineered to produce and release specific biological reagents (growth factors or other proteins). The growth factors are selected based on their potential to promote wound healing. The GEBB is designed to accelerate healing of donor sites, skin grafts, and chronic wounds. The overall goals of this approach are to significantly accelerate the rate of wound healing and to facilitate closure of healing-impaired wounds. For cell line derivation, the growth factor gene is transfected into keratinocytes and/or fibroblasts on DNA vectors, using a variety of gene transfer techniques. Included in the vectors is a gene allowing selection of cells. Stable
cell lines are derived by selection with a specific antibiotic.

A unique feature of this approach is the possibility of using genetically engineered cells to produce growth factor(s). Moreover, the cell lines are readily available since they are kept in continuous culture. The biological bandage is a versatile system of delivering active substances to wounds. Although several methodologies for improving wound healing are presently utilized, all of them have potential problems. The GEBB represents a new concept in wound therapy. This technology does not presently exist. This system provides the following advantages:

1) The bandage can be applied to and removed from the wound as desired. No residual material is left behind.

2) The growth factor-producing cells cannot escape from the bandage into the wound, significantly increasing patient's safety.

3) The bandage provides uniform and continuous delivery of growth factor over the entire application area.

4) With this system, the probability of the growth factor being biologically active increases,
since it is released from the producer cells immediately after synthesis.

5) Different bandages each producing different growth factors could be applied sequentially to optimize healing.

6) Composite bandages could be produced that would produce multiple growth factors simultaneously.

7) Growth factor delivery rate can be set by changing the ratio of factor producing/factor nonproducing cells within the bandage.

8) GEBB can be engineered to release a wide variety of agents other than growth factors.

9) High compatibility with existing wound healing dressings allows the GEBB to be used in conjunction with these dressings.

10) The GEBB size can be varied to cover wounds of various sizes.

2.3. GEBB, a new device to improve wound healing:

The idea driving our experiments is the creation of a system for proper growth factor delivery to wounds, since such a system does not presently exist. To demonstrate that growth factor-producing cells can be genetically engineered, confined inside a polymeric envelope, maintained biologically active at ambient
conditions and readily available for placement at the injured site, the following objectives were outlined.

2.4. **Specific Aims:**

1. Production of a cell line expressing a marker gene. The cell line will be selected and secretion of the marker gene will be monitored by specific antibodies.

2. Testing of cells for appropriate attachment and growth on the bandage.

3. Verifying conditions for survival of cells in the bandage under ambient conditions.

4. Testing of the ability to continue secretion of the marker protein to the outside compartment by cells sealed into the bandage.

5. Testing to assure complete retention of the engineered cells inside the polymeric envelope.

6. Evaluating cell viability inside the bandage.
7. Demonstrating delivery of the marker protein to wound bed in experimental animals.
CHAPTER III

DEVELOPMENT AND CHARACTERIZATION OF THE SCC13-bGH

1. INTRODUCTION:

The production of a cell line expressing high levels of a specific protein is the first aim towards the development of the genetically engineered biological bandage (GEBB). The keratinocyte line SCC-13 (Rheinwald and Beckett, 1981) was chosen for several reasons. Although derived from a human squamous cell carcinoma, SCC-13 is a line that forms unstratified colonies and does not require EGF for sustained growth in culture. These cells grow poorly in soft agar and form small encapsulated cysts in nude mice. We transfected SCC-13 cells with a vector expressing the bovine growth hormone gene (bGH). The bGH gene (Woychick et al., 1982) is well characterized and its product has been used as a marker protein in other systems. We utilized bGH in our studies, since specific antibodies make it possible to distinguish bGH from rat and human growth hormone in tissues and fluids.

2. MATERIALS AND METHODS:
Cell culture: The cell lines were maintained in growth medium consisting of DMEM:F-12 (3:1) supplemented with 18 mM adenine, 100 uM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 4 ng/ml hydrocortisone, 5 ng/ml insulin, 50 ug/ml gentamicin, and 8% fetal calf serum. For passage, cells were harvested with Hank's Balanced Salt Solution (HBSS) containing 0.025% trypsin and 1 mM EDTA (Trypsin-EDTA) and replated in growth medium. Bandage test medium was identical in composition, except that it lacked fetal bovine serum and was supplemented with 50 mM HEPES buffer.

Generation of SCC13-bGH cells: SCC13-bGH was derived from SCC-13 cells by transfection with the pCMV-bGH-NEO vector. SCC-13 cells were plated at a density of 4000 cells/cm². After attachment, cells were incubated overnight with 10 ug of pCMV-bGH-NEO in 3.0 ml of growth medium containing 10 ug/ml polybrene. Polybrene is a polycation that has been successfully employed in transfection of low molecular weight DNA (Charney et al., 1986). Cells were then treated with 30% dimethylsulfoxide for 3 minutes at room temperature, washed with HBSS, and fed with fresh medium. After 48 hours, the cells were treated with 225 ug/ml geneticin (G418) for 10 days. The surviving
cells were pooled and designated SCC13-bGH. In parallel dishes, after antibiotic selection, cell clones were isolated and kept in culture for expansion. The clones were designated SCC13-Wn bGH (W stands for wild type bGH).

Nucleic acid extraction: Total RNA was isolated from 9 confluent dishes (175 cm²) of cultured SCC-13 or SCC13-bGH cells, using the guanidine/CsCl gradient method (Boedtke, 1971). For extraction of messenger RNA (mRNA), total RNA was chromatographed on an oligo(dT) cellulose column (Aviv and Leder, 1972). The purified poly(A)+ RNA was resuspended in TE (10 mM Tris-HCl/ 1 mM EDTA, pH 7.4) and quantitated by spectrophotometry. Equal amounts of poly(A)+ RNA were diluted in RNA sample buffer (Lehrach et al., 1977) and electrophoresed on a 1% formaldehyde agarose gel at 50 mv for 16 h. The mRNA was then transferred by cappillary elution to a Byodine A membrane and hybridized with ³²P-dCTP labeled plasmids encoding bGH (pCMV-bGH-NEO) or actin (pA-1). The plasmids were labelled by random priming (Feinberg and Vogelstein, 1983). Church hybridization mix [250 mM NaPi (pH 7.2), 1 mM EDTA, 1% BSA (w/v), 1% salmon sperm (w/v, 10 mg/ml), 7% SDS (w/v)] was used to hybridize the probes overnight at 60° C. The blots were then washed five
times, ten minutes each time in Church wash II [20 mM NaPi (pH 7.2), 1 mM EDTA, 1% SDS], at 60°C to reduce non-specific binding and exposed on X-ray film.

**Immunological detection of bGH:** To monitor the level of bGH released into the culture medium, 1 - 10 ml of medium was concentrated 10 fold by filtration using a Centricon-10 concentrator (Amicon). After addition of one volume of 2X sample buffer (Laemmli, 1970), an aliquot was electrophoresed on gels containing 10 or 12% acrylamide. As a standard, a known quantity of authentic recombinant bGH was electrophoresed in a parallel lane. The proteins were then transferred to nitrocellulose, blocked in a 2% nonfat dry milk/TBS (10 mM Tris-HCl, 200 mM NaCl, pH 7.4) solution for four hours, and incubated overnight with a polyclonal rabbit anti-bGH antibody (diluted 1:3000, in blocking buffer), kindly provided by Dr. Fritz Rottman (CWRU, Dept. of Mol. Biol. and Microbiol.) The blot was then washed three times with TBS, and ¹²⁵I-protein A (diluted 1:1000, in blocking buffer), was then added to the blot for one hour. Finally, the blot was washed three times with 1% gelatin/0.2% triton/TBS, dried and exposed to a X-ray film. Specific binding of the antibody was visualized after 3 days. To assay the level of intracellular bGH,
cells were harvested with Trypsin-EDTA, washed with HBSS, and resuspended at 5000 cells/ul in 1X SDS sample buffer. Extracts (50,000 cells/lane) were fractionated by gel electrophoresis and bGH was detected as described above.

3. RESULTS:
A partial map of the plasmid vector used to transfer the bGH gene to SCC-13 cells is depicted in Fig. 1A. pCMV-bGH-NEO contains the neomycin phosphotransferase gene which encodes resistance to G418 (NEO-R) under the control of the SV40 promoter and terminator. The wild type bGH gene, delineated by the arrow, is 1.8 kb in length and consists of 5 exons (solid boxes) and 4 introns (Woychick et al., 1982). It is transcribed under the control of the cytomegalovirus (CMV) promoter and terminated by the native bGH polyadenylation signal. It produces a 0.95 kb mRNA product. SCC-13 cells were transfected with pCMV-bGH-NEO using the polybrene method and selected with G418 to yield the SCC13-bGH cell lines. Figure 1B shows the change in morphology acquired by SCC13-bGH. Compared to the parent cells, these cells are smaller and pack together, forming a tighter monolayer. Figure 2 shows the high level of bGH mRNA transcribed by SCC13-bGH.
Fig. 1A. Bovine growth hormone expression vector. The bovine growth hormone gene (Woychick et al., 1982) was driven by the cytomegalovirus promoter/enhancer (CMV) elements. The five exons of the bGH gene are indicated by the solid boxes and the introns are indicated by solid lines. The arrow indicates the extent of the bGH transcript. The plasmid also contains a selection cassette encoding the aminoglycoside phosphotransferase gene (neo-\textsuperscript{r}) that confers resistance to the drug G418. NEO-\textsuperscript{R} is transcribed by the SV40 promoter/enhancer and an SV40 stop signal.

Fig. 1B: Transfection and expression of the bGH gene to SCC-13 alters cell morphology. SCC-13 (top) or SCC13-bGH (bottom) cells (5 x 10\textsuperscript{5} cells) were plated in 50 cm\textsuperscript{2} dishes and grown for 4 days. The cells were then photographed at 100X magnification using a Nikon microscope with phase contrast optics. SCC13-bGH form smaller, more compact colonies and cells are readily released from the surface of the dish. This result suggests that production of bGH by SCC-13 changes morphology of the cells. The compaction of the colonies suggests that bGH may be accelerating the rate of cell division.
pCMV-bGH-NEO
Fig. 2  Production of bovine growth hormone mRNA by SCC13-bGH cells. Confluent cultures of SCC13-bGH and SCC-13 cells were harvested and poly(A)$^+$ RNA was prepared as previously described. Equal quantities of RNA (5 ug/lane) was electrophoresed on an RNA denaturing gel and transferred to Biodyne A membrane. The blots were then hybridized with $^{32}$P-dCTP-labeled plasmids encoding actin (A) or bovine growth hormone (bGH), washed and exposed on x-ray film. The arrow indicates migration of authentic bovine growth hormone messenger RNA. As both cell types produce similar quantities of actin protein and mRNA, the intensity of the actin hybridization signal indicates that approximately equal amounts of mRNA were layered in each lane.
To determine whether the SCC13-bGH cells produce bGH, an equal quantity of poly (A)$^+$ RNA (5 ug) isolated from SCC-13 (Fig. 2, lanes 1) or SCC13-bGH cells (Fig. 2, lanes 2) was electrophoresed on a RNA-denaturing agarose gel, transferred to Biodyne A membrane and hybridized with $^{32}$P-labeled plasmids encoding bovine growth hormone (bGH) or actin (A). As indicated by the arrow, SCC13-bGH cells produce large amounts of the 0.95 kb bGH mRNA, while no bGH mRNA is produced by the parent SCC-13 cells. The actin hybridization signal serves as an internal control to evaluate whether equal amounts of mRNA have been layered in each lane. The signal intensity indicates that similar amounts of mRNA are present in each lane. Figure 3 shows the high level synthesis and release of bGH from different clones of SCC13-W$_n$ bGH cells. With the exception of the SCC13-W$_7$bGH clone, all other clones secreted at least 1 ug of bGH per 24 hours per $1 \times 10^6$ cells. To quantitate the amount of bGH produced by SCC13-bGH cells, the cells were extensively washed with HBSS to remove any previously synthesized bGH and then shifted to 10 ml of serum-free medium. Figure 4 is the result of a particular experiment in which conditioned medium was collected at different time points. At 0, 1, 2, 5, 8, and 24 hours after the shift, culture medium was
Fig 3. High level synthesis and secretion of bGH in different SCC13-bGH clones. Clonal cell lines were derived by bGH transfection onto SCC-13. Cells were plated (4000 cells/cm²) and incubated overnight with 10 ug of the CMV-bGH-NEO vector in growth medium containing 10 ug/ml polybrene. Cells were then treated with 30% DMSO, washed and fed with fresh medium. Selection with geneticin (225 ug/ml) started 48 hours later, for 10 days. Individual colonies were designated SCC13-W₇-bGH. Each clone was plated into 50 cm² dish and allowed to reach confluency. They were then washed 3 times with HBSS to remove serum and fed with 10 ml of serum-free medium. Twenty four hours conditioned medium was collected from each clone and concentrated 10 fold. Samples were prepared in sample buffer and a 10 ul of the concentrate was electrophoresed on a 10% acrylamide gel. The proteins were transfer blotted to nitrocellulose and incubated with a bGH specific antibody/¹²⁵I-protein A and exposed to x-ray film. From a total of eighteen clones, only two were bGH negative. SCC13-W₇-bGH was the only one expressing low levels of bGH. The numbers in the figure correspond to the clone number. The standard lane (std) contains 1 ug of rbGH.
Fig. 4  Time course of release of bovine growth hormone from SCC13-bGH cells. SCC13-bGH cells (2.5 x 10^7) were plated into each of six 50 cm² dishes, allowed to attach overnight, washed three times to remove serum and fed with serum-free medium. At 0, 1, 2, 5, 8 and 24 h the medium (10 ml) was harvested from a single dish and concentrated 10 fold by filtration. A sample (10 ul) of the concentrate was electrophoresed on a 10% acrylamide gel, transfer blotted and incubated with a bGH-specific antibody/¹²⁵I-protein A and exposed on x-ray film. The standard lane (std) contains 500 ng of authentic recombinant bGH. In these experiments, a calculated net secretion of approximately 0.8 - 3.0 ug of bGH/1x10⁶ cells/24 hours was obtained.
collected from separate, parallel dishes and measured for bGH production. bGH is detected in the medium within 1 h and the levels steadily increase thereafter. SCC13-bGH cells produce 0.8 - 3.0 pg of bGH/cell/24 h. This represents a net production of approximately 0.8 - 3.0 ug of bGH/1 x 10^6 cells/24 h. Parallel studies conducted using cell extracts from SCC13-bGH indicate that each cell contains approximately 0.2 pg bGH (shown in Fig 5).

4. DISCUSSION:
Two major goals in wound healing therapy are to normalize abnormal healing processes (i.e., keloids, chronic wounds) and to accelerate normal wound closure. Multiple studies suggest that the rate of wound healing can be improved using allografts of cultured cells applied directly to the wound surface or by direct application of growth factors to the wound (Thornton et al., 1982; Leigh et al., 1987; Mustoe et al., 1987; Schultz et al., 1987; Brown et al., 1989; Phillips et al., 1989; Cony et al., 1990; Regauer and Compton, 1990; Kane et al., 1991; Langer and Moses, 1991). Growth factors have been shown to modify and/or accelerate the rate of wound healing (Thornton et al., 1982; Mustoe et al., 1987; Schultz et al., 1987; Brown
Fig. 5  bGH is specifically secreted from transfected cells. To estimate the net production of bGH by SCC13-bGH cells, cell extracts from either SCC-13 or SCC13-bGH were prepared in 1 X SDS buffer. Conditioned medium from SCC13-bGH cells was collected and concentrated as before. Recombinant bGH was reconstituted and samples were prepared for electrophoresis. SDS-PAGE and immunoblot were performed as described in Figure 3. Intracellular bGH was quantitated according to the standards. Lane 1: 50 ng of rbGH; lane 2: 100 ng of rbGH; lane 3: 250 ng of rbGH; lane 4: 500 ng of rbGH; lane 5: 10 ul sample from SCC13-bGH conditioned medium; lane 6: SCC-13 cell extract (50,000 cells); lane 7: expected molecular weight from protein standards; lane 8: SCC13-bGH cell extract (50,000 cells). Aproximately 0.2 pg of intracellular bGH/cell was detected in these experiments.
et al., 1989). In these experiments the growth factor is delivered in solution or as part of a dressing. There are several limitations with this approach, including uncertainties regarding the stability of the growth factor and/or the dressing, the ability to provide uniform delivery of the growth factor over the wound surface and the high cost of producing purified growth factors.

An alternate possibility is the use of living sheets of keratinocytes to promote wound healing (O'Connor et al., 1981; Gallico et al., 1984; Madden et al., 1986; Leigh et al., 1987; Morgan et al., 1987). Recent reports indicate that autografted and allografted sheets of diploid human epidermal keratinocytes can alleviate pain and promote healing of chronic ulcers (Carter et al., 1987; Gallico et al., 1989; Premachandra et al., 1990; Fratianne et al., 1993). Growth factors released by the cells of the epidermal sheet are thought to play a major role in promoting healing. However, this method requires that the patient come in direct contact with the applied cell sheet which limits the types of cells that can be used. It also implies that genetically engineered cells could not be safely used in the grafting system, since the vectors utilized for transfection inevitably
contain virus fragments. The development of a biological bandage takes into consideration all of these problems. It allows the delivery of growth factors and/or proteins to wounds using living cells that have been programmed to overproduce such molecules and are confined inside a polymeric barrier. The ability to confine cells within the GEPP envelop opens up the possibility that immortalized cells can be employed in this system. This would make it possible to utilize fully characterized permanently available cell lines.

We have utilized human epidermal keratinocytes as the recipient cells for our test gene. The immortalized SCC-13 cells were transfected with a plasmid encoding bGH under the control of the CMV promoter. This gave rise to the high producer bGH cell lines. bGH was selected as our test molecule because of the availability of an antibody specific for bovine growth hormone which does not cross-react with rat or human GH. This is important, since in animal tests we must be able to distinguish the gene product released from our bandage from the endogenous experimental animal or human gene product.

In the following chapter, we describe the design and development of such a system.
CHAPTER IV

CONSTRUCTION AND "IN VITRO" TESTING OF THE GENETICALLY
ENGINEERED BIOLOGICAL BANDAGE

1. INTRODUCTION:

Several methods of wound healing treatment have been recently proposed; however the efficiency of these systems is questionable. Most of them are simply bandage types of coverings that serve to protect the wound but are not effective at promoting wound healing and closure. Although there is good evidence that application of growth factors to the injured area can cause enhanced rates of wound healing (Mustoe et al., 1987; Schultz et al., 1987; Brown et al., 1989), in most of these studies the rate of improvement is not dramatic. Application of sheets of keratinocytes to wound surfaces have also been implicated in promoting healing (O'Connor et al., 1981; Gallico et al., 1984; Madden et al., 1986; Leigh et al., 1987; Morgan et al., 1987). In the present study, we propose to develop a biological bandage that will allow the safe delivery of peptides to the wound site. In our view this system has many advantages including prompt availability of cells, immediate synthesis and secretion of the
peptide, and uniform delivery at a known rate all over the wound bed.

2. MATERIALS AND METHODS:

Membranes and adhesives Celgard and 3501 (isostatic polypropylene membranes), Z-bind (0.2 or 0.45 um pore size, low protein binding polysulfone), Millicell-CM (extracellular matrix-coated teflon membrane), and Anocel (glass matrix) membranes were obtained from Hoechst-Celanese (Germany), Gelman Sciences (Ann Arbor, MI), Millipore (Massachusetts) and Anotec (United Kingdom), respectively. Silastic type A silicone medical adhesive (silicon adhesive) was obtained from Dow Corning (Midland, MI). Hydrocolloid adhesive and closed cell polyurethane foam were obtained from Variseal Inc. (Pittsburgh, OH). Hydrogel biocompatible adhesive wound dressing was obtained from Nepera, Inc. (Harriman, NY).

Construction of the bandage: The bandage consists of two membranes attached with silicon adhesive to each face of a ring-shaped separator gasket made from medical grade silicon rubber. The circular central well has a 2 cm diameter and the height and width of the gasket wall are 0.6 and 0.5 cm, respectively. A circular sheet (d = 2.5 cm), cut from Z-bind or another
membrane, is fixed to the bottom of the separator gasket using silicon adhesive. Similarly, the upper membrane (z-bind) is fixed in place. After overnight attachment, the bandages are sealed in a plastic bag and sterilized by exposure to cobalt-60 irradiation (5000 Rads/min, 30 minutes). SCC13-bGH cells (1 x 10^6) are harvested with trypsin-EDTA, resuspended in fresh growth medium and injected through a 18 gauge needle into the chamber formed by the gasket and the membranes. The cells are allowed to attach for at least 10 h and then the growth medium is replaced with test medium (for medium components refer to the previous chapter).

Growth factor release from bandage "in vitro": For "in vitro" tests of bGH release, bandages were assembled using either Millicell-CM, Anocel or z-bind as the bottom membrane. In most cases pore sizes of 0.20 and 0.45 um were tested. SCC13-bGH cells were seeded onto the inner surface of the bottom membrane and the bandage was placed atop glass rods in a culture dish (9.6 cm^2) containing 3 ml of normal culture medium. The bandages were incubated overnight at 37° C in a standard CO_2 incubator and then shifted to serum-free medium and placed at 33° C or at room temperature in ambient atmospheric conditions. At various times
after the temperature shift, the interior medium and the medium surrounding the bandage were removed, the system was washed with fresh test medium and fresh medium was added for an additional 24 h. The 24 h conditioned internal and surrounding medium was collected and concentrated to 0.2 ml by centricron-10 filtration. A 10 ul sample was then mixed with 10 ul of 2 x SDS sample buffer (Laemmli, 1970) and boiled for 5 minutes. The level of bGH present was then assayed by electrophoresing the sample on a 10% acrylamide gel, transferring to nitrocellulose and incubating with the bGH-specific antibody and 125I-protein A, as described in the previous chapter.

3. RESULTS

Growth hormone release from the bandage: Figure 6A shows the "in vitro" system used to test the various bandage membranes. SCC13-bGH cells were seeded in the inner side of the bottom membrane and allowed to attach overnight atop glass supports in tissue culture dishes kept in a CO2 incubator. Bandages were then shifted to serum-free medium and bGH accumulation in the inside and outside compartments (Figure 6B) was monitored after 6 days. In this experiment, Millicel (0.4 um
Fig. 6  Growth hormone release from the bandage "in vitro". A schematic of the "in vitro" bandage test system is displayed in panel A. The bandage consists of top (outer) and bottom (inner) membranes and a separator. The interior of the bandage contains serum-free cell culture medium and approximately 1 x 10^6 SCC13-bGH cells. Bandages were assembled and placed on glass rods in a culture dish. Panel B shows the results of bGH accumulation tests. The bandage containing serum-free culture medium was incubated at 37° C in 5% CO₂ for 6 days. The medium from the culture dish (3 ml) was harvested and concentrated to 0.2 ml by Centricon-10 filtration. A 10 ul sample was electrophoresed on a 10% acrylamide gel, transferred to nitrocellulose and detected with a bGH-specific primary antibody and ^125^I-protein A. Medium from inside the bandage was similarly concentrated and assayed. bGH was detected in both compartments, indicating a free diffusion of the molecule through the bottom membrane of the bandage.
pore size) was used as the bottom membrane of the bandage. Figure 7 shows the profile of bGH release by different bottom membranes. Bandages containing SCC13-bGH cells were assembled as before. After shifting the medium, they were reshelved into the incubator for 24 hours, when the outside medium was collected and replaced. Forty eight hours later, the conditioned medium from both inside and outside compartments was collected. Altogether, the cells were kept inside the bandage for 3 days. From the three materials tested in this experiment, only the Millicell-CM membrane allowed diffusion of bGH to the outside compartment. Two other membranes, anocel and z-bind, were also tested and compared to the Millicell-CM. As shown in Figure 8, z-bind, Anocel and Millicell-CM membranes all demonstrate significant and comparable release of bGH into the external medium. From the above experiments, the release rate of bGH into the outside medium could be calculated. When 1 x 10^6 cells were present in the bandage in a 0.5 ml volume, the level of bGH inside approaches 3 ug/ml within 24 h and the concentration in the external medium exceeds 1 ug/ml (2-3 ml volume). Thus, under these conditions, the rate of bGH production inside the bandage is adequate to produce a
Fig. 7  Different bottom membranes were examined for bGH release at the outside compartment. The polymers tested and shown in this figure were: Celgard (CG), Millicel-CM (CM) and 3501 (3501). SCC13-bGH cells were seeded into the bandage and serum-free medium was added after cell attachment. Bandages were kept in the incubator (37° C / 5% CO2) for 24 h, the outside medium was harvested and replaced. Forty eighth hours later, the conditioned medium from the outside (3501, CG, CM) and from the inside (,3501', ,CG', ,CM') the bandages was harvested, concentrated to 0.2 ml and 10 ul samples were eletrophoresed in 10% acrylamide, transferred and bGH detected as previously described. Only the bandage utilizing Millicell-CM as the bottom membrane allowed diffusion of bGH to the outside compartment. std= 1 ug of recombinant bGH.
Fig. 8 Anocel and z-bind membranes are comparable to the Millicell-CM. One million cells (SCC13-bGH) were seeded into bandages containing culture medium and assembled with different bottom membranes. To compare bGH release, bandages were assembled in duplicate and procedures similar to those described in figure 6 were undertaken. The bottom membrane in the test bandages consisted of Z-bind (Z-bind), Millicell-CM (CM) or Anocel (An). In the Z-bind test, twenty four hour conditioned medium from inside (in) and outside (out) the bandage was tested for bGH, as described in the previous figure. In addition, Z-bind membrane having pore sizes of 0.25 and 0.45 microns were tested in parallel. Only the outside medium was assayed in the Millicell-CM and Anocel tests. Each test was run in duplicate. The std lane contains 250 ng of authentic recombinant bovine growth hormone.
net concentration gradient in favor of bGH release into the surrounding medium.

We next determined whether bGH production would be maintained after prolonged incubation of SCC13-bGH cells under atmospheric conditions. To keep the pH around physiological levels, HEPES buffer was added to the culture medium. Cells kept in medium containing 10 to 60 mM HEPES survived for at least three days outside the incubator. The cells were biologically functional, as observed by the bGH detected in the outside medium (Fig 9). Under these conditions, the best viable cell counts and holding of the pH was observed at 50 mM (not shown). To verify whether bandages would be reliable for extended periods, duplicate bandages containing bGH cells were incubated for 2, 3 or 6 days in serum-free medium/50mM HEPES. At those specific time points, they were washed with HBSS and shifted to fresh serum-free medium. After 24 h, the conditioned medium was collected and analysed for the presence of bGH. Fig. 10 shows that in these conditions, high level of bGH was found up to 3 days; however, after six days of serum-free maintenance, the bGH level had decreased. Parallel studies showed that the level of bGH per cell is maintained at 0.8 - 3.0 pg bGH/cell/24 h at each
Fig. 9 SCC13-bGH cells are biologically active under atmospheric conditions. One million cells were seeded into six experimental bandages assembled as described in Figure 6. After 24 h incubation, the culture medium (inside and outside) was shifted to serum-free medium containing HEPES in concentrations varying from 10-60 mM (lanes 1-6, respectively). The bandages were moved to a 33°C incubator for three days. After this period, the outside conditioned medium was harvested from each bandage, concentrated, and tested for the presence of bGH as previously outlined. Lane 7: molecular weight markers; std = 250 ng of recombinant bGH.
Fig. 10  Bandage function under ambient conditions. Bandages containing SCC13-bGH cells and normal growth medium were assembled as shown in Fig. 6 with a Millicell-CM filter as the bottom membrane and placed in a culture dish containing 1.5 ml of normal growth medium. After cell attachment, serum-free medium was added to the bandage and to the outside medium. The culture dish was then transferred at time zero to ambient atmospheric conditions at 33\(^\circ\) C. Duplicate dishes were incubated in serum-free medium/50 mM HEPES for 2, 3 and 6 days, respectively, without medium change. Fresh serum-free medium was then added to the outside compartment and after 24 h the outside medium was harvested. In each case the medium was concentrated 10 fold and a 10 ul aliquot was electrophoresed in 10% acrylamide gel and immunobblotted for detection of bGH. In addition, the cells were harvested from each bandage, counted and viability was determined using trypan blue exclusion. Authentic bGH (250 ng) was electrophoresed as an internal standard (std).
time point. The decline in bGH production is due to a reduction in the number of viable cells at six days. This decrease in bGH production and cell viability was not observed when fresh medium was added to the internal bandage compartment on alternate days.

4. DISCUSSION:

The design and the development of a biological bandage which may improve wound healing is described. This system consists of a bottom and top membrane, a separator gasket and a genetically engineered growth hormone-producing cells. The membrane of choice for construction of the bandage is a low protein binding polysulfone named z-bind. This membrane was selected over four other surfaces that are commonly used for cell growth, molecular transport and differentiation studies. Our results show that under our experimental conditions, the cells attach efficiently and are well maintained on z-bind. The z-bind membrane permits efficient release of bGH from the bandage interior. In addition, due to its small pore size (0.25 um), only proteins smaller than 50 kDa are expected to have access to the wound bed.

Although we demonstrated that bGH is found in the outside compartment it is important to emphasize that
other proteins secreted by these cells also have the ability to diffuse to the wound. Thus, the end result would be an orchestration of several bioactive substances acting either in a synergistic fashion with the overexpressed growth factor or in antagonism with it. Conversely, proteins present in the wound exudate may get access to the inner compartment of the bandage. In this regard, one should be concerned with the protease levels in the wound fluid. In a normal wound healing process, we do not expect that to interfere with the genetically engineered cells since they do not usually interfere with the cells present in the normal healing process. However, in chronic wounds they may play a negative role by lysing the cells inside the GEPP. Thus, an exchange of the bioactive molecules between both compartments is predict, the overall result being reflected by the cooperativism (positive or negative) of these substances.

Z-bind is also preferred for use in the bandage because of its high tensile strength, durability and ability to support cell growth. These are important features for a membrane that must maintain separation of the engineered cells from the patient's wound. Parallel tests show that the cells do not escape from the z-bind membranes and hydrostatic pressure tests
show that the junctions between the membranes and the separator gasket do not leak. In addition, our results indicate that the cells remain physiologically active in a completely defined medium for extended periods of time. At present, we do not know what will be the buffering capacity of the bandage medium when in contact with the wound exudate. However, we anticipate a pH change towards the acidic side, due to cellular metabolism. This should not really affect the genetically engineered cells since the extracellular pH of the epidermis, and hence, the keratinocyte environment is acidic (Kaufman and Berger, 1988).

A prerequisite for the successful clinical use of the biological bandage is the complete definition of its constituents. This has been achieved throughout the several tests described in this section. Our next objective is to verify whether the biological bandage is also functional in "in vivo" experiments.
CHAPTER V

DELIVERY OF GROWTH HORMONE TO WOUNDS USING A GENETICALLY ENGINEERED BIOLOGICAL BANDAGE

1. INTRODUCTION:

The healing of surface wounds is a complex process that involves multiple cell types and the rate and quality of wound healing can be modulated by a variety of agents. Growth factors, delivered in solution or as components of a dressing, have been shown to accelerate wound healing (Thornton et al., 1982; Mustoe et al., 1987; Schultz et al., 1987; Brown et al., 1989). The interest in growth factors as stimulants of wound healing is based on observations that various growth factors differentially regulate the growth and/or differentiation of epithelial and mesenchymal cell populations (Lynch et al., 1989; Aaronson et al., 1990). This includes stimulation of production of extracellular matrix proteins, collagenase and other proteins involved in tissue remodeling. Although growth factors appear to enhance wound healing, efficient delivery systems have not been developed. In the present chapter we describe a prototype system in which cells that have been engineered to produce bovine growth hormone, remain viable for an extended period of
time when encased in this bandage and release bGH onto the wound surface. We discuss the advantages of this system and conclude that delivery of specific proteins from a living bandage is feasible and may be useful in the treatment of chronic and acute wounds.

2. MATERIALS AND METHODS:

Construction of the bandage: The bandages were prepared as described in the preceding chapter. In these experiments, the bottom membrane of the bandages consisted of z-bind (.45 um pore size). After cell attachment and changing to test medium, a hydrogel dressing (preequilibrated in test medium) was affixed to the bottom of the bandage before use.

Animal wound models: Full thickness or partial thickness wounds were surgically prepared on the dorsal surface of Sprague-Dawley rats (average body weight = 250 g). The animals were anesthetized with nembutal (40 mg/kg, i.p.), shaved and cleaned with a sterile alcohol swab. The dermis, epidermis and panniculus carnosus were removed with sterilized scissors to create a circular, full thickness wound having a 2.4 cm diameter. To create partial thickness wounds, the epidermis and mid-dermis were removed by means of a dermatome (set at 0.5 um depth). Bandages were
assembled and seeded with either 1 X 10^6 SCC-13 or SCC13-bGH cells. In some experiments, bandages containing cell cultured medium alone were also utilized. The wound chamber gasket, preformed from closed cell polyurethane foam and designed to conform to the animals body surface, was affixed to the skin using hydrocolloid adhesive. Under these conditions the gasket remains in place for a minimum of 8 days. The gasket prevents the animal from destroying the bandage and also prevents wound contraction. The top of the wound chamber is closed using a transparent sheet of polyurethane film or transpore (3M) and the whole gasket is wrapped with a bandaging tape (Vetrap, 3M).

In these experiments, bandages were left in place for 1, 2, and 3 days. Upon removal of the bandages, 1/4 of each hydrogel dressing (100 - 250 mg wet weight) was harvested, weighed and boiled for 3 min in the presence of 1 vol of 2 x SDS sample buffer (weight:volume). The hydrogel and associated sample buffer was electrophoresed on a 12% acrylamide gel, transferred to nitrocellulose and incubated with the anti-bGH antibody and ^125_I-protein A as previously outlined. Cell viability and histological sections from the wounded area were analysed at the specific
time points. NIH guidelines for the care and use of laboratory animals have been observed.

3. RESULTS:

Delivery of bGH to wounds "in vivo": To determine whether bGH could be delivered to wounds on animals using the bandage system, we assembled several bGH-producing bandages consisting of the bandage and an adhesive hydrogel dressing as shown in Fig. 11A. Each bandage was placed into a wound chamber affixed to the back of a rat (Fig. 11B). A single bandage was removed for detection of bGH in the hydrogel dressing at 1, 2, and 3 days after bandage placement (Fig. 12). A fraction (1/4th) of the hydrogel dressing was combined with 1 volume of 2 x SDS sample buffer and electrophoresed on a 12% acrylamide gel prior to immunodetection. The bandage continues to release high levels of bGH for at least three days on the animal. The dressings contained approximately 2, 1.5 and 0.5 ug of bGH on days 1, 2, and 3, respectively. In parallel experiments, cell viability studies indicate that the highest decline in cell number occurs at day 3, after which cell number remains almost constant until day 7. Due to loosening of the protective gasket after a
Fig. 11 Description of wound model. The animals are shaved and full thickness wounds are prepared by excising, using scissors, a circular area of skin having a diameter of 2.4 cm. Panel A shows a schematic of the bandage. The wound chamber gasket is applied directly to the back of each rat, using adhesive, to form a circular wound chamber having a diameter of approximately 2.5 cm. The wound chamber gasket provides a protected environment for the bandage and also prevents contraction of the wound edges. An absorptive dressing is then placed adjacent to the wound followed by the bandage. The top of the wound chamber is sealed with a transparent plastic sheet (wound chamber cover). Panel B shows a schematic of the bandage after placement onto the backs of rats.
Fig. 12 The bandage assembled as shown in Fig. 11A and B, was placed onto the backs of rats and after 1, 2 or 3 days, the bandage was removed and the adhesive dressing was assayed for the presence of bGH. One quarter of the dressing was combined with 1 volume of 2 x sample buffer (w/v), boiled and electrophoresed on a 12% acrylamide gel. The bGH was detected by immunoblotting. The quantity of bGH/lane was estimated by laser densitometry and compared to a lane containing 1.0 ug of recombinant bGH (std).
longer exposition to the animal, we could not extend our experimental periods. To assure that the bandage did not induce a host inflammatory response, we histologically compared wounds that received medium-containing bandages to wounds that received cell-containing bandages. There was no difference in the extent of inflammation of these two forms of treatment up to day seven (result not shown).

4. DISCUSSION:

We demonstrated the feasibility of creating a biological bandage which is functional under ambient conditions. When placed onto a surgically generated full thickness or partial thickness wound on rats, this bandage releases bGH into the wound for at least three days. This system is a safe and reliable way of providing real-time delivery of any desired biomolecule into the wounded area. The use of living bandages for peptide delivery to wounds would be much less expensive than producing purified proteins from recombinant or natural sources. Moreover, growth factor addition in dressings or as a liquid solution provides short or intermittent delivery of the factor. In contrast to the potential loss of activity of growth factors linked to dressings, in our system the peptide
is released immediately after synthesis from the producer cells. This should make it more likely for the protein to be biologically active. Furthermore, the delivery of the peptide is expected to be continuous and uniform over the application area. Unlike other forms of biological therapy, this bandage can be applied and removed as desired, facilitating treatment. The chance of generating an immune response (cell rejection) will be smaller, since the cells do not make straight contact to the host wound. This fact also prevent the genetically enginnered cells to take residency into the host. The use of different bandages each producing a different growth factor could be applied to wounds sequentially to optimize wound healing. In some wounds it may be desirable to place an insulin-like growth factor-producing bandage on the wound to enhance fibroblast proliferation and collagen deposition, while later on, it may be important to enhance re-epithelialization with an epidermal growth factor-producing bandage. Since it is likely that wound closure involves a complex interplay of growth factors released simultaneously, composite bandages could be produced that would produce multiple growth factors simultaneously. Finally, the bandage could also be used to deliver a variety of other biologically
important molecules. These include, for example, metalloproteinases or other enzymes or substrates that would be important in wound remodeling.

Several potential problems with delivery using viable cells should also be noted. First, viability of the cells must be maintained while the bandage is on the patient. In the present animal wound studies, viability was maintained for three days; however, it would be desirable in future studies to extend this period. Second, the biological bandage can utilize both normal and immortalized cells to produce the growth factor. In the case of immortalized cells, safeguards must be built into the bandage design to guarantee that the cells cannot accidentally escape into and populate the wound. This can be accomplished by designing safety barriers into the bandage and by treating the cells to prevent cell division. Cobalt-60 irradiation of cells has been employed to produce functional cells that cannot divide but remain metabolically active (Rheinwald and Green, 1975). However, in spite of these potential difficulties, the biological bandage represents a versatile system that could be utilized to deliver a wide variety of biological agents to wounds.
CHAPTER VI

A PHARMACOLOGICAL APPROACH FOR THE TREATMENT OF
ECTOCERVICAL DYSPLASIA

1. INTRODUCTION:

The major thesis governing the experiments outlined in this section is that growth factors and/or vitamins can be utilized to manipulate the milieu in the vicinity of a tumor so as to slow and perhaps reverse the expansion of the tumor mass or as a method of preventing the initial appearance of neoplasia. I will begin by providing background information on several of the agents that are important stimulators of tumor growth or are potential therapeutic agents for treating cervical neoplasia and cancer.

1.1. Growth factors and tumors: The loss of requirement for specific growth factors is a common finding in many cancer cells (Goustin et al., 1986). Such loss can be mediated by activation of autologous growth factor synthesis (autocrine), synthesis of an altered growth factor receptor, or activation of a post-receptor pathway that bypasses the normal growth factor receptor requirement (Reddy and Pardee, 1980; Kaplan et al., 1982; Erikson et al., 1983; Bowen-Pope et al., 1984; Hunter, 1984; Goustin et al., 1985;
Raines et al., 1985; Sporn et al., 1985; Reiss et al., 1991). A prerequisite for autocrine growth stimulation is that the cell synthesizes both the growth factor and its receptor. The abnormal expression of a growth factor receptor or abnormal production of a growth factor in a cell can lead to autocrine stimulation (Lang and Burgess, 1990). Truncated receptors that are constitutively activated, is another way of achieving autocrine stimulation (Raines et al., 1985). The autocrine model can explain the growth of transformed cells in soft agar and the serum factor independence of transformed cells. Paracrine stimulation is also involved in tumor development in some cancers (Yee et al., 1989; Barreca et al., 1992). In this model, growth factor produced by cancer cells stimulates proliferation of stromal cells. Alternatively, stromal cells may produce growth factors that stimulate cancer cells. Such a situation in which tumor components cross-feed each other with factors, could explain why it is sometimes difficult to grow certain malignant cells in culture.

In addition, growth factor regulation of target genes can be altered in cancer. An important example is the enhanced sensitivity of the keratin genes to regulation by retinoids in HPV16-immortalized cervical
epithelial cells and HPV16-immortalized keratinocytes (Agarwal et al., 1991; Pirisi et al., 1992).

1.2. Epidermal Growth Factor: Several lines of evidence indicates that the EGF-stimulated cell regulatory system may play a role in tumorogenesis. EGF directly elicits transformation-associated phenotype in certain cell types (Stoscheck and King Jr., 1986). Many of these effects are reversible upon removal of EGF, but in a small number of transformed cells they are not reversible. The addition of EGF to normal cells induces a partial loss of density-dependent inhibiton of growth and reduces dependence on serum for growth (Carpenter and Cohen, 1976; Westermark, 1976; Kirkland et al., 1979). EGF also elicits certain responses which are associated with cancer. These include a decrease in fibronectin secretion and an increase in secretion of plasminogen activator (Chen et al., 1977; Lee and Weinstein, 1978; Eaton and Baker, 1983). Growth of cells in soft agar is potentiated by EGF or EGF-like molecules (Roberts et al., 1982; Anzano et al., 1983; Carpenter et al., 1983). This potentiation is even greater for transformed cells and tumor cells.

Although only one cell line has been reported to spontaneously synthesize EGF (Sato et al., 1985),
detectable TGF-alpha or EGF-like species are present in several tumors. EGF and TGF-alpha both recognize the same cellular receptor and they are equally effective in most systems (Dailey et al., 1978; Carpenter et al., 1983). It has been proposed that TGF-alpha is an embryonic form of the adult EGF. Because tumors may ectopically reactivate embryonic genes, TGF-alpha and EGF-like species are molecules often found in tumor cells (Gousten et al., 1986; Stoscheck and King Jr., 1986; Heldin and Westermark, 1989; Reiss et al., 1991). EGF is also known to modulate the activity of growth factors, proteins and hormones (Corps and Brown, 1988; Corps and Brown, 1991; Irwin et al., 1991; Hembree et al., 1992). This modulatory effect may play a role in autocrine and paracrine loops present in some types of tumors (Tremblay, 1979; Gousten et al., 1986; Heldin and Westermark, 1989; Barreca et al., 1992).

EGF can also induce proliferation in other ways. For example, the presence of an intact EGF receptor is not always necessary for tumor development. The oncogene v-erb B codes for a protein homologous to a portion of the EGF receptor (Downward et al., 1984). v-erb B product has a deletion on the EGF-binding domain. Consequently, the truncated receptor is autophosphorylated and constitutively activated. This
leads to a constant mitogenic signalling activation regardless of the presence of the ligand.

In addition, EGF regulation of gene expression can markedly influence the response of the tumor cell to other growth regulators (Corps and Brown, 1988; Corps and Brown, 1991; Hembree et al., 1992). This is an important area of interest for the experiments outlined in this thesis, since EGF may influence the IGF signalling system. The IGF signalling system is described in the next section.

1.3. **Insulin-like Growth Factor:** The insulin-like growth factors are an important family of growth regulatory proteins (Goustin et al., 1986, Heldin and Westmark, 1989, Yee et al., 1989, Lang and Burgess, 1990). Although hepatic production provides the major source for circulating IGFs, it is well established that IGFs also act in an autocrine and/or paracrine fashion. Locally produced IGF-I may contribute to the malignant transformation of cells (Huff et al., 1986; Minuto et al., 1988; Yamada and Serrero, 1988). CALU-6, a human lung carcinoma cell line has been found to produce IGF-I. The growth of this cell line was inhibited by monoclonal antibodies against IGF-I (Minuto et al., 1988). Autocrine stimulation of cell growth by IGF-I has also been suggested for breast
carcinoma and human osteosarcoma cell lines (Blatt et al., 1984; Huff et al., 1986). In human breast cancers, IGF-I mRNA was found to be expressed in the stroma but not in normal or malignant epithelial cells (Yee et al., 1989). Taken together, these findings suggest that IGF-I may function as a paracrine stimulator of epithelial cell growth as well as an autocrine stimulator of mesenchymal cells.

1.4. IGF-binding proteins (IGFBPs): IGFs associate with distinct insulin-like growth factor binding proteins (IGFBP) present in serum and other biological fluids and appear to be circulating regulators of IGF-I activity (Hintz, 1984; Martin and Baxter, 1986). IGFBPs are thought to maintain IGFs in an inactive state, protecting them from breakdown, prolonging their half-life, and partitioning IGFs into different compartments (Hardouin et al., 1987; Martin and Baxter, 1988; Sara and Hall, 1990). Most actions of IGFBPs on IGFs appear to be inhibitory; however, under certain conditions, stimulatory actions have also been observed (Elgin et al., 1987; De Mellow and Baxter, 1988; Conover, 1992; Cohen et al., 1993). To date, six different binding proteins have been described: IGFBP-1 through IGFBP-6. Their primary
structure, molecular weight, and human gene localization have been determined as outlined below.

-IGFBP-1: cDNA clones isolated from human hepatoma cell line, placenta and decidual cells (Brinkman et al., 1988; Julkunen et al., 1988; Lee et al., 1988). Molecular weight: 30 kD, gene localized to chromosome 7.

-IGFBP-2: cDNA clones isolated from BRL-3A cell line, rat liver and human fetal library (Binkert et al., 1989; Brown et al., 1989; Margot et al., 1989). Molecular weight: 34 kD, gene localized to chromosome 2.


-IGFBP-4: cDNA clones isolated from rat liver and human placenta library (Shimasaki et al., 1990). Molecular weight: 27 kD, gene localized to chromosome 17.


-IGFBP-6: cDNA clones isolated from rat ovary and human placenta library (Shimasaki et al., 1991).
Molecular weight: 22 kD, gene localized to chromosome 12.

In body fluids, the majority of IGF-I and IGF-II is bound to a large 150 kD complex, although a smaller 40 kD complex also exists. The large complex is a ternary, growth hormone-dependent complex of one molecule of IGF (-I or -II), IGFBP-3, and "acid-labile" subunit (ALS) (Hintz, 1984; Martin and Baxter, 1986; Baxter and Martin, 1989; Baxter et al., 1989). IGFBP-3 is the only IGFBP that associates with ALS and only after IGF binding. At low pH, ALS is inactivated and IGF dissociates from IGFBP-3 (Hintz, 1984; Baxter et al., 1989). In serum, the mean concentration of IGFBP-3 is correlated with the sum of IGF-I and IGF-II levels, suggesting that all IGFBP-3 in the large complex is saturated with IGFs (Gargosky et al., 1992). It has been demonstrated that purified mouse IGFBP-3 can inhibit cell growth (Delbe et al., 1990), an effect independent of IGF-I receptor activation (Liu et al., 1992). Cell growth inhibitors such as TGF-beta and trans-retinoic acid have recently been reported as IGFBP-3 inducers, and their inhibitory effects on cell growth seems to be partly mediated through IGFBP-3 action (Fontana et al., 1991; Adamo et al., 1992).
2. VITAMIN A:

Vitamin A has been reported to inhibit the growth of cervical tumors in patients (Lippman et al., 1992). However, the mechanism responsible for this regulation is not known. As will become clear in the following report, retinoic acid upregulates IGFBP-3 levels in cultured cervical cells and may inhibit the activity of IGF-I via this mechanism. The following provides an introduction to vitamin A and retinoids.

2.1. Background: Vitamin A has been recognized as an important factor in the visual system, in regulating cell growth and differentiation, in maintenance of reproductive capacity and in glycoprotein production (Olson, 1968; Beitch, 1970; Fisher et al., 1970; Barnett and Szabo, 1973; De Luca and Yuspa, 1974; Mayer et al., 1978; Gilfix and Eckert, 1985; De Luca, 1991). Vitamin A was first discovered in eggs, milk, butter and fish liver oils and was later found in plants in the form of provitamin beta-carotene (Olson, 1968; Fisher et al., 1970; Lotan, 1980). Beta-carotene is converted to vitamin A in mammals. The parent substance is called trans-vitamin A alcohol or trans-retinol. Its naturally occurring oxidation products are trans-retinal and trans-retinoic acid. The family of vitamin A-like compounds are called retinoids. Both
natural and synthetic retinoids have a basic structural formula composed of a cyclic end group, a polyene chain and a polar end group (Lotan, 1980). Synthetic vitamin A analogs may share with trans-retinol or trans-retinoic acid all or only some of their biological activities.

A relationship between retinoid effects on cell differentiation and its inhibitory effects on the development of epithelial cancer was suggested by several authors (Sporn et al., 1976; Sporn, 1977; Mayer et al., 1978). They observed an inverse correlation between vitamin A intake and the incidence of malignant lesions in epithelial tissues. Some of the actions of retinoids at the cellular level have been studied "in vivo" and "in vitro". Histological and biochemical changes are produced by vitamin A excess or deficiency (Rothberg, 1967; Sporn, 1977). In experimental animals, vitamin A deficiency causes squamous metaplasia in the epithelium of the eye, respiratory tract, genito-urinary tract and others (Beitch, 1970; Hayes et al., 1970; Wong and Buck, 1971; Harris et al., 1972). In the absence of vitamin A, mucous-secreting epithelia are replaced by keratinizing squamous epithelia and epidermal keratinocytes become hyperkeratotic. Similarly, removal of vitamin A from
the growth medium caused squamous metaplasia in tracheal and prostate gland organ cultures and in cervical epithelial cells (Lotan, 1980; Agarwal et al., 1991). These phenomena are completely reversed by reintroduction of vitamin A. Biochemical analysis of epithelial tissues of vitamin A-deficient animals reveals changes in the biosynthesis of glycoconjugates, proteins and nucleic acids. A reduction in DNA synthesis is found in vitamin-A deficient rats when compared to normal controls (Lotan, 1980).

Other studies demonstrate that an excess of vitamin A suppresses keratinization and replaces squamous cells by mucous-producing cells (Wilkoff et al., 1976; Peck et al., 1977). While mucous metaplasia is not found in adult mammalian epidermal cells "in vitro" morphogenetic and biochemical changes are observed. These included formation of microvilli (Barnett and Szabo, 1973) and production of new glycopeptides (De Luca and Yuspa, 1974). In cultures of newborn mouse epidermis, retinoids supressed DNA synthesis and cell proliferation. However, the lifespan of individual cells is increased due to the reduced rate of cell death associated with inhibition of keratinization (Yuspa et al., 1977).
Several retinoids have been found to possess anti-carcinogenic and anti-tumor activities (Sporn et al., 1976; Sporn, 1977; Mayer et al., 1978; De Luca, 1991). The susceptibility of many epithelial tissues to carcinogenesis is enhanced by vitamin A-deficiency (Sporn, 1977). Moreover, retinoids can inhibit the process of carcinogenesis (Tannock et al., 1972; Sporn et al. 1976; Yuspa et al., 1977; Lippman et al., 1992). Retinoids are able to prevent the development of chemical-derived tumors including cancers of the skin, respiratory tract, urinary tract, and mammary gland (Sporn et al., 1976; Sporn, 1977; Mayer et al., 1978). Other studies show that retinoids can inhibit the growth of papillomas (McMichael, 1965) and sarcomas (Cohen and Carbone, 1972; Tannock et al., 1972). Whether this effect is due to an enhanced host immune defense reaction is not clear. However, the inhibitory effects of retinoids are also observed in cell culture systems. Kochhar et al. (1968) reported the first case of growth inhibition by retinol on an established cell line, the 3T6 fibroblasts. The inhibition was dose- and time-dependent and did not result from cytotoxic effects. Extensive reports from our and other laboratories indicate that retinoids can suppress the
growth of transformed cervical cells (Lippman et al., 1992; Agarwal et al., 1993; Sizemore and Rorke, 1993).

Retinoic acid (RA) is the major mediator of the non-vision-related functions associated with retinoids (Ross, 1993). Retinoic acid is formed intracellularly through the oxidative metabolism of diet-derived retinol. RA is the ligand for a family of nuclear retinoic acid receptors (RAR) which have strong homology to members of the steroid/thyroid hormone/vitamin D receptor family. Two subfamilies of RAR exist, the RAR (De Luca, 1991) and the RXR (Mangelsdorf et al., 1991). Each subfamily consists of three receptors (alpha, beta, and gamma). Based on homology, the amino acid sequences of RAR and RXR can be divided into six regions (Leid et al., 1993). The amino terminal regions A and B contain a ligand-independent transactivation function(s). Region C is the DNA-binding domain, responsible for specific binding to DNA-response elements. The ligand binding domain, region E, is functionally complex because it also contains a ligand-dependent transactivation function and a dimerization interface. Regions D and F have unknown functions. RAR isoforms arise from the different use of two promoters and alternative splicing (Leid et al., 1993). Although little is known about
the formation of RXR isoforms, preliminary evidence suggests a difference in the N-terminal A/B region. RAR and RXR differ in affinity for various retinoids. The RAR forms bind to retinoic acid with high affinity, but have a low affinity for 9-cis-retinoic acid. The RXR receptor forms bind to 9-cis-retinoic acid with high affinity (Heyman et al., 1992; Mangelsdorf et al., 1992). RXR have been shown to form heterodimers with and function as coregulators with RAR and thyroid hormone receptors (Zhang et al., 1992). These heterodimers interact with different subsets of triiodothyronine/retinoic acid response elements. Thus, isomerization of RAR and RXR produces additional levels of gene regulation and help to fine tune signal transduction.

2.2. The ectocervical epithelial cell system:

The ectocervix is a stratifying squamous non-keratinizing epithelium. Continuous proliferation in the basal layer replenishes the superficial epithelial layers (Singer, 1975; Ferenczy and Winkler, 1987; Parmley, 1987). Cultured ectocervical epithelial cells (ECE cells) are responsive to a variety of hormones, growth factors, and retinoids (Gorodeski et al., 1989; Gorodeski et al., 1990; Agarwal et al., 1991; Lippman, 1992). Several immortalized ECE lines were derived in
our laboratory by transfection of normal ECE cells with the human papillomavirus 16 (HPV16) genome (Agarwal et al., 1991). These include ECE16-1, ECE16-D1 and ECE16-D2 (Agarwal et al., 1991, Choo and Eckert, unpublished). HPV is associated with the development of cervical dysplasia and carcinoma (Durst et al., 1983; Boshart et al., 1984; Lorincz et al., 1986; Woodworth et al., 1990; Sizemore and Rorke, 1993). Almost 90% of anogenital cancers are positive for one or more HPV subtypes (Durst et al., 1983; Boshart et al., 1984; Lorincz et al., 1986). Under normal growth conditions, the HPV16-immortalized ECE16-1 cells are less differentiated than the normal cervical cells (Agarwal et al., 1991). However, growth of ECE16-1 cells in retinoid-free medium results in a partial restoration of normal phenotype. In addition, retinoids regulate the expression of biochemical markers of differentiation (Agarwal et al., 1993), transglutaminase activity (Sizemore et al., 1993), EGF receptor density (Sizemore and Rorke, 1993) and TGF-beta production (Wakefield et al., 1990). The retinoids are also known to play an important role in regulating ECE16-1 cell growth (Agarwal et al., 1993). A dose-dependent inhibition of cellular growth is
obtained when these cells are challenged with different forms of retinoids (Agarwal et al., 1993).

Results from our laboratory indicate that EGF stimulates ECE16-1 cell growth via two distinct mechanisms (Hembree et al., 1992). First, EGF directly stimulates growth via the EGF receptor signalling pathway. Second, EGF promotes cell growth through an indirect mechanism, by reducing the level of ECE16-1 cell expression and synthesis of IGFBP-3. As noted above, IGFBPs are produced by a great variety of cell types and their effects can be complex. They can stimulate or inhibit cell proliferation by potentiating or inhibiting IGF-I action (De Mellow and Baxter, 1988; Blum et al., 1989; Conover, 1992). They appear to inhibit IGF-I stimulation of cell proliferation by binding to IGF-I and removing it from the active pool (Gopinath et al., 1989; Bicsak et al., 1990; Conover et al., 1990; Cohick and Clemons, 1991). In ECE16-1, EGF suppression of IGFBP-3 is detected at the message and protein levels (see Chapter VII). Moreover, the IGF-I growth response of these cells is increased in the presence of submaximal doses of EGF (Hembree et al., 1992). Taken together, these findings suggest a complex interaction of the EGF and IGF-I signalling systems in cervical cells.
2.3. **Retinoids, epidermal growth factor and cervical cancer: a working hypothesis.**

The hypothesis that forms the basis for the experiments outlined below is that retinoids may inhibit the growth of cervical tumor cells, at least in part, by increasing the levels of IGFBP-3 which in turn binds to IGF-I and inhibits the mitogenic effects of IGF-I. Removal of IGF-I stimulation of growth would then decrease the rate of tumor cell proliferation and tumor growth. To evaluate this hypothesis, we carried out the specific aims as outlined below.

2.4. **Specific Aims:**

1) To evaluate retinoic acid regulation on IGFBP-3 levels in ectocervical cell lines.

2) To disclose the mechanism responsible for the regulation.

3) To correlate the growth inhibitory effect of retinoic acid with release of IGFBP-3 in the culture medium.

4) To determine whether retinoic acid antagonizes the stimulation of cell growth by IGF-I and if so, to analyze the IGFBP-3 component of the response.
5) To evaluate whether retinoic acid influences the effects of EGF and/or IGF-I on IGFBP-3 levels.
CHAPTER VII
REGULATION OF IBBFP-3 LEVELS BY EGF, IGF-I AND RETINOIC ACID

1. INTRODUCTION:

Vitamin A and its analogs (retinoids) are important physiological regulators of differentiation in stratifying epithelia (Gorodeski et al., 1989, Agarwal et al., 1991, De Luca, 1991, Agarwal et al., 1993). They regulate cell morphology and the expression of biochemical markers of differentiation. Retinoids have also been demonstrated to have substantial therapeutic potential in the treatment of ectocervical cancer (Lippman et al, 1992, Agarwal et al., 1993). Cervical epithelium is a major target of human papillomavirus and the oncogenic HPV types are present in over 90% of all high grade cervical carcinomas (Durst et al., 1983; Boshart et al., 1984; Lorincz et al., 1986). Growth factors such as IGF-I and EGF have been shown to be important mitogens in a variety of systems and to contribute to the process of malignant transformation (Goustin et al., 1986; Heldin and Westermark, 1989; Lang and Burgess, 1990). The role of these growth factors in the process of ectocervical malignancy is poorly understood, although
the level of the EGF receptor in cervical tumor cells is known to be important (Sizemore and Rorke, 1993). The effects of IGF-I are tightly regulated by IGFBPs, a family of secreted proteins that are produced in a cell type specific manner (Hardowin et al., 1987; Hossenlopp et al., 1987; Sara and Hall, 1990). IGFBP-3 is an important member of the IGFBP family that has been reported to inhibit the effects of IGF-I in many systems (Bicsak et al., 1990; Conover and Powell, 1991). The mechanism of inhibition appears to involve IGFBP-3 binding to IGF-I, thus reducing the free extracellular IGF-I concentration (Gopinath et al., 1989; Conover et al., 1990; Bicsak et al. 1990; Cohick and Clemmons, 1991). Because of the importance of EGF, IGF-I and retinoids in regulating ectocervical cell proliferation and differentiation (Agarwal et al., 1991; Hembree et al., 1992; Agarwal et al., 1993; Sizemore and Rorke, 1993), we have examined the effects of retinoids on the expression of IGFBP-3 in three immortal but non-tumorigenic ectocervical epithelial cell lines, ECE16-1, ECE16-D1 and ECE16-D2 and in the fully transformed CaSki cell line.

2. MATERIALS AND METHODS:
Materials: Dulbecco's Modified Eagle's Medium (DMEM), F12 medium, nonessential amino acids, L-glutamine, trypsin and antibiotics were purchased from Gibco (Grand Island, NY). Fetal calf serum, insulin, bovine serum albumin (BSA), hydrocortisone, T₃, transferrin and adenine were obtained from Sigma Chemicals (St. Louis, MO). Cholera toxin was purchased from ICN Biomedicals (Costa Mesa, CA). Human recombinant EGF was from Upstate Biotechnology Inc. (Lake Placid, NY) and recombinant IGF-I was obtained from Collaborative Biomedical Products (Bedford, MA). ¹²⁵I-IGF-I and ³²P-dCTP were purchased from Amersham (Arlington Hts, IL). All-trans-retinoic acid was purchased from Sigma Chemical (St. Louis, MO). Pre-stained molecular weight standards were purchased from BioRad Laboratories (Richmond, CA). Human IGFBP-3 cDNA, spanning nucleotides -50 to +1264 base pairs relative to the translation start codon (Wood et al, 1988), was kindly provided by Dr. D.R. Powell (Baylor College of Medicine, Houston, TX). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) partial cDNA (Tso et al, 1985) was kindly provided by Dr. R. Wu (Cornell University, Ithaca, NY). Proteins were transferred to nitrocellulose membranes (.45 um, Schleicher and Schuell, Keene, NH) and RNA was
transferred to Biodyne A membranes (ICN Biomedicals, Costa Mesa, CA). Densitometry of autoradiograms was performed using a SciScan 5000 optical scanner (United States Biochemicals, Cleveland, OH).

Cell culture: The ectocervical cells and the ectocervical cell lines were maintained in culture medium containing DMEM:F12 (3:1) supplemented with 5% fetal calf serum (FCS), 5 ng/ml insulin, 0.1 nM cholera toxin, 5 ug/ml transferrin, 2 nM T3, 10 ng/ml EGF, 4 nM hydrocortisone, 0.18 mM adenine, 100 uM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin and 50 ng/ml gentamycin. For experiments, cells were plated in culture medium at a known density (5,000 - 20,000 cells/cm²) onto either 9.6 cm² dishes (biochemical and proliferative studies) or 176.6 cm² dishes (RNA studies). After 24 h, cells were shifted to defined medium (DM). DM is identical to the complete medium, except it lacks FCS, insulin, cholera toxin, and EGF, and is supplemented with bovine serum albumin (BSA, 1 mg/ml) and ascorbic acid (50 ug/ml). After a 24 h equilibration in DM, DM + growth factor was added at 24 h intervals for 3 days. Unless otherwise noted, conditioned medium was collected from the last incubation period, centrifuged to remove cell debris and stored at -20°C for IGFBP-3 analysis.
Cells were then harvested with HBSS containing 1 mM EDTA and 0.025% trypsin (w/v), resuspended in an isotonic solution containing 3.7% formaldehyde and kept on ice until counting with a Coulter counter.

**Ligand blot analysis:** The level of IGFBP-3 in conditioned medium was detected as described by Hossenlopp et al (1987). Briefly, aliquots of conditioned medium, corresponding to an equivalent number of cells (usually 3000 - 5000 cells), were prepared in reducing agent-free Laemmli sample buffer (Laemmli, 1970). Fifty to one hundred microliter samples were electrophoresed on a 10% acrylamide gel at 25 mA/gel and the separated proteins were then transblotted to nitrocellulose. After transfer, the membranes were sequentially treated with phosphate buffered saline (PBS) containing 3% NP40 (30 min), PBS containing 1% BSA (60 min) and PBS containing 0.1% Tween 20 (10 min). 1.5 X 10^6 cpm of ^125^I-IGF-I (specific activity = 2,000 Ci/mmol) was added to 10 ml of PBS containing 3% NP40 and 1% BSA and the mixture was incubated with the membrane for 20 h at 4° C. The membrane was then washed with PBS containing 0.1% Tween 20 (2 x 15 min) and PBS alone (3 x 15 min), dried and exposed to x-ray film for 7-10 days at -80° C with intensifying screen.
Time-course experiments: Cells were plated at 5,000 cells/cm², in triplicate 9.6 cm² wells in serum-containing medium, allowed to attach for 24 h and then shifted to DM. Cells were then treated at 24 h interval and conditioned medium was collected within the same time frame. The experiment consisted of four groups of cells, submitted to one of the following treatments: 1) cells treated with EGF for 5 days; 2) cells treated with EGF for 3 days and then shifted to EGF + retinoic acid for another 2 days; 3) cells treated with retinoic acid for 5 days; 4) cells treated with retinoic acid for 2 days and then shifted to RA + EGF for another 3 days. The EGF concentration was 20 ng/ml and the retinoic acid concentration was 1 μM. The conditioned medium from the last 24 h was assayed for IGFBP-3 by ligand blot, as previously described.

Nucleic acid analysis: Total RNA was prepared from 50% confluent dishes (176.6 cm²) of cultured cells using the guanidine/CsCl gradient method (Boedtke, 1971) and poly (A)⁺ RNA was prepared by chromatography on oligo (dT) cellulose (Aviv and Leder, 1972). The purified poly (A)⁺ RNA was resuspended in TE (10 mM Tris-HCl / 1 mM EDTA, pH 7.4) and quantitated by spectrophotometry. Equal amounts of poly (A)⁺ RNA were diluted in RNA sample buffer (Boedtke, 1971) and
electrophoresed on a 1% formaldehyde agarose gel at 50 V for 16 h. The RNA was then transferred to Biodyne A membrane and hybridized with IGFBP-3 or GAPDH probes using the conditions previously described (Gilfix and Eckert, 1985). The probes were generated by using a random primed DNA kit (Boehringer Mannheim) in which $^{32}$P-dCTP is randomly incorporated to the denatured plasmid (Feinberg and Vogelstein, 1983). The blots were then washed (Gilfix and Eckert, 1985) and exposed on x-ray film.

3. RESULTS:

Fig. 13 shows the effects of epidermal growth factor and retinoic acid on IGFBP-3 protein and mRNA levels. As depicted by the ligand blot, IGFBP-3 is the predominant IGF binding protein produced and secreted by the ECE16-1 cervical cell line. Growth of cells in defined medium (DM) lacking EGF (DM, -) and retinoic acid results in a basal level of IGFBP-3 production (Fig. 13C). Addition of 20 ng/ml EGF reduces IGFBP-3 levels (EGF, -). In contrast, addition of 1 μM retinoic acid to cells growing in DM (DM, +) or DM containing 20 ng/ml EGF (EGF, +) results in a 2.5 fold increase in IGFBP-3 protein level. Fig. 13A shows that the change in IGFBP-3 protein level correlates with a
Fig. 13  Retinoic acid regulation of IGFBP-3 RNA and protein levels. In panel A, ECE16-1 cells were treated for 3 days with DM or DM supplemented with 20 ng/ml EGF in the presence (+) or absence (-) of 1 μM retinoic acid. Poly(A)^+ RNA was isolated, electrophoresed on an agarose gel, transferred to Biodyne A membrane and hybridized with ^32P-labeled cDNAs encoding IGFBP-3 (BP3) or GAPDH. On average, a 2.7 fold increase in IGFBP-3 RNA level was observed after RA treatment, independent of the EGF presence. Panel B, shows a sample of RNA prepared from ECE16-1 cells following treatment with 1 μM RA for 3 days. Parallel blots (2 μg RNA/lane) were hybridized with the radiolabeled IGFBP-3 (lane 2) or GAPDH (lane 1) cDNAs. The migration of the bands corresponded to 2.4 kb (BP3) and 1.45 Kb (GAPDH), values that coincides with those previously reported (Tso et al., 1985; Wood et al., 1988). Panel C is a ligand blot of cell culture medium taken from cells after 3 days of treatment with DM or DM supplemented with 20 ng/ml EGF in the presence (+) or absence (-) of 1 μM RA. Medium was harvested that had been conditioned for 24 h during the 3rd day of treatment. IGFBP-3 levels increase 2.5-fold after treatment with RA.
corresponding change in the level of mRNA encoding IGFBP-3 (i.e., EGF suppresses and RA increases mRNA levels). RA produces on average (n=4) a 2.7 fold increase in IGFBP-3 mRNA in the presence or absence of EGF. Fig. 13B shows the migration of IGFBP-3 mRNA in comparison to the mRNA encoding GAPDH.

To determine whether the retinoic acid dependent increase in IGFBP-3 is a general effect on ectocervical cells, additional cell lines were tested. The CaSki cell line, originally derived from a human cervical carcinoma (Pattillo et al., 1977), is a fully malignant cell line and ECE16-D1 was derived in our laboratory by transfection of the dimerized HPV16 genome (Choo and Eckert, unpublished). Like ECE16-1, this line is immortal but does not form tumors in nude mice (Choo and Eckert, unpublished). As shown in Fig. 14A and 14B, EGF reduced and RA increased IGFBP-3 mRNA levels in CaSki and ECE16-D1 cells, respectively. We next examined the concentration dependence of the RA increase in IGFBP-3. Fig. 15A shows a ligand blot performed using 24 h conditioned medium of cells growing for 3 days in the presence of 0, 0.1, 1.0, 10, 100 and 1000 nM RA. Retinoic acid treatment resulted in a concentration-dependent increase in the level of IGFBP-3 which was half-maximal at around 1 nM and
Fig. 14  Retinoic acid regulation of IGFBP-3 mRNA level in CaSkI and ECE16-D1 cells. Cells were plated in growth medium and allowed to attach overnight. They were then shifted to DM and allowed to equilibrate for 24 h. After equilibration, the cells were treated for 3 days with DM or DM supplemented with 20 ng/ml EGF in the presence (+) or absence (-) of 1 uM RA (fresh medium and supplements daily). After 3 days, poly(A)^+ RNA was isolated from CaSkI (A) and ECE16-D1 (B) cells, electrophoresed on an agarose gel, transferred to Biodyne A membrane and hybridized with a ^32P-labeled cDNA encoding IGFBP-3. The IGFBP-3 band is indicated by the arrows.
Fig. 15  Concentration-dependent regulation of IGFBP-3 protein and RNA levels by RA. ECE16-1 cells were treated for three days in DM medium containing 20 ng/ml EGF and 0 to 1000 nM RA. Conditioned medium (from the last 24 h of treatment) was removed and assayed for IGFBP-3 by ligand blot (panel A). The migration of IGFBP-3 is indicated (BP3), as is the migration of the 32 and 49 kDa molecular weight markers. In a parallel study (panel B), poly(A)^+ RNA was isolated from cells growing in EGF supplemented DM in the presence of 0, 10, 100 and 1000 nM RA. The RNA was electrophoresed, transferred to membrane and simultaneously hybridized with ^32P-labeled cDNAs encoding IGFBP3 (BP3) and GAPDH.
maximal at concentrations ≥ 10 nM. Figure 15B shows that the IGFBP-3 mRNA level in ECE16-1 is also maximally increased by ≥ 10 nM RA.

We also tested the regulation of IGFBP-3 in normal ectocervical epithelial cells and in ECE16-D2 (Choo and Eckert, unpublished). Quantitatively, the ECE16-1 cell line is more responsive with respect to RA stimulation of IGFBP-3 mRNA levels than ECE16-D1 or ECE16-D2 cells (shown in Fig. 16). The normal ectocervical epithelial cells are the least responsive. Qualitatively, the IGFBP-3 response to RA in all cell lines is consistent with that observed in ECE16-1 cells. Interestingly, although RA treatment increases IGFBP-3 mRNA levels in ECE16-D1, ECE16-D2, and CaSki and in normal ectocervical epithelial cells, the level of IGFBP-3 protein is only increased in ECE16-1 and ECE16-D2 cells.

We next compared the kinetics of change in IGFBP-3 levels in response to EGF and RA, in ECE16-1 cells. After two days of treatment with RA, ECE16-1 cells were shifted to medium containing RA or medium containing RA + EGF for an additional 3 days (Fig. 17A). IGFBP-3 levels were measured every 24 h during the treatment period. Retinoic acid alone increased IGFBP-3 in 24
Fig. 16 Concentration-dependence of RA regulation of IGFBP-3 RNA in normal ectocervical cells and cervical cell lines. Normal cervical epithelial cells and the HPV16-immortalized ECE16-1 cells, ECE16-D1 and ECE16-D2 cells were plated in 175 cm² dishes and allowed to attach overnight. They were shifted to EGF supplemented DM (10 ng/ml) for 24 h and then treated for 6 d in the presence of 0, 10, 100 or 1000 nM RA. Poly(A)⁺ RNA was prepared, electrophoresed on agarose gels and hybridized with ³²P-labeled cDNAs encoding GAPDH (indicated by arrowhead) and IGFBP-3 (indicated by arrow).
Fig. 17  Time course of IGFBP-3 regulation by EGF and retinoic acid. ECE16-1 cells were seeded at 5,000 cells/cm² in 9.6 cm² wells and allowed to attach in growth medium overnight. They were then washed and shifted to DM. After 24 h, the conditioned medium was harvested (control, C) and the cells were shifted to DM containing 1 μM RA (panel A). RA treatment was continued on days 1 and 2. At the end of day 2, half of the cultures were shifted to medium containing 1 μM RA + 20 ng/ml EGF (RA, EGF) and the other half was maintained in 1 μM RA (RA) and treatment was continued on days 3, 4 and 5. Appropriately supplemented fresh medium was added daily. The 24 h conditioned medium was collected daily and an equivalent quantity (based on cell number) was assayed by ligand blot using ¹²⁵I-IGF-I as the ligand (Hossenlopp et al., 1986). The experiment was similar in panel B, except that the cells were treated with 20 ng/ml EGF for days 1 (not shown), 2 and 3. At the end of day 3, the cells were shifted to medium containing 20 ng/ml EGF + 1 μM RA or maintained in medium containing 20 ng/ml EGF. The 24 h conditioned medium was harvested and assayed exactly as described in panel A. The numbers at the top of each lane refer to treatment duration (in days).
hours and the levels remained constant with continued treatment. Concomitant addition of EGF to cells treated with retinoic acid for two days did not change the pattern of IGFBP-3 secretion. In a parallel experiment, cells were treated for three days with EGF and then shifted to medium containing EGF or EGF + RA. At day 2 of EGF treatment, IGFBP-3 secretion is reduced, on average, to 50% and maximum inhibition is observed at day 3. However, if retinoic acid is added in conjunction to EGF, this effect is reversed within 24 h (Fig. 17B).

IGF-I, one of the ligands for IGFBP-3, also regulates IGFBP-3 levels in some systems (De Mellow and Baxter, 1988; Blum et al., 1989; Conover et al., 1989; Conover et al., 1992). To evaluate the effects of IGF-I on the level of IGFBP-3 and the interaction of IGF-I, EGF and RA in regulating IGFBP-3 levels, we treated ECE16-1 cells with combinations of these agents for three days and measured the levels of IGFBP-3 present in the medium during the last 24 h of treatment (Fig. 18). ECE16-1 cells were grown in DM (DM) or DM supplemented with 20 ng/ml EGF (EGF), 5 or 50 ng/ml IGF-I (IGF 5, 50) and EGF + 5 or 50 ng/ml IGF-I (EGF/IGF 5 or EGF/IGF 50). Parallel dishes in each group were treated with (+) or without (-) RA. At both
5 and 50 ng/ml concentrations, IGF-I promoted increased IGFBP-3 levels. However, reduced IGFBP-3 levels were observed when EGF and IGF-I were added simultaneously. This response demonstrates that the EGF is dominant over IGF-I in the regulation of IGFBP-3. However, increased IGFBP-3 levels were observed in the presence of RA, regardless of the presence of EGF or IGF-I.

4. DISCUSSION:
Retinoic acid interacts with the retinoic acid receptors, which are members of the steroid hormone receptor superfamily (Giguere et al., 1987; Petkovich et al., 1987; Mangelsdorf et al., 1991). These are soluble receptors that are activated by interaction with ligand and then bind to DNA to regulate gene expression (Umesono et al., 1988; Yang et al., 1991). Retinoic acid alters gene expression in cervical cells, including regulating transglutaminase activity (Sizemore et al., 1993), cytokeratin gene expression (Agarwal et al., 1993) the level of the EGF receptor (Sizemore and Rorke, 1993) and TGF-beta1 production (Wakefield et al., 1990). These results suggest that RA can effect cell proliferation and differentiation via two mechanisms, direct effects on gene expression
Fig. 18  IGFBP-3 ligand blot of conditioned medium of ECE16-1 cells after 3 days of treatment with various agents. ECE16-1 cells were grown for 3 d in DM or DM supplemented with 20 ng/ml EGF, 5 ng/ml IGF-I, 50 ng/ml IGF-I, 20 ng/ml EGF + 5 ng/ml IGF-I or 20 ng/ml EGF + 50 ng/ml IGF-I either in the absence (-) or presence (+) of 1 uM retinoic acid. Fresh RA and growth factor were added daily. At the end of the 3 d treatment, conditioned medium from the last 24 h of treatment was harvested and assayed for IGFBP-3 by ligand blot (Hossenlopp et al., 1986). CS indicates the migration of IGFBPs present in human cord serum. The migration of IGFBP3 (BP3) and the 32 and 49 kDa molecular weight markers are indicated. The arrowhead indicates the migration of an additional lower molecular weight IGFBP that is sometimes detected in these cells.
and modulation of the production of other growth factors that in turn affect cell function.

The present studies were predicated on the idea that the tumor suppressive effects of RA (Lippman et al., 1992) and the growth suppressive effects "in vitro" (Pirisi et al., 1992; Agarwal et al., 1993) could be mediated, in part, by inhibition of the IGF-I stimulatory cascade.

Our results show that treatment of several ectocervical epithelial cell lines with all-trans-retinoic acid upregulates IGFBP-3 mRNA levels. This effect is also observed in normal ectocervical cells. In the case of the ECE16-1 and ECE16-D2 cells, the increase in IGFBP-3 mRNA was correlated with increased IGFBP-3 secreted into the culture medium. Further studies will be necessary to determine whether the regulation of IGFBP-3 mRNA levels is at the level of new RNA synthesis and/or RNA degradation. Thus, the amount of IGFBP-3 present in the medium and able to bind IGF-I is directly controlled by the level of IGFBP-3 mRNA produced by these cells. At the present time we lack information regarding the IGF-I-induced increase in IGFBP-3 levels. Although in these experiments we did not evaluate what is (are) the mechanism(s) involved in this response, the possibility
of transcriptional and translational regulation of IGFBP-3 by IGF-I, as well as the release of a membrane-bound form of IGFBP-3 by IGF-I exists.

Recently, it has been reported that cervical cell lines immortalized by human papillomavirus are more retinoid responsive, with respect to regulation of cytokeratin gene expression and suppression of growth compared to normal cervical cells (Agarwal et al., 1993). This response has also been described in HPV-immortalized epidermal keratinocytes (Pirisi et al., 1992). Other changes are also apparent. For example, EGF receptor levels respond differently to retinoic acid in transformed cervical cells compared to normal cells (Sizemore and Rorke, 1993). In the present study, all of the immortalized cell lines (ECE16-1, ECE16-D1, ECE16-D2) and the tumorigenic cervical line (CaSki) express higher levels of IGFBP-3 mRNA in response to RA. Moreover, lower concentrations of RA are required to increase IGFBP-3 mRNA levels in these lines. This suggests that enhanced retinoid sensitivity may be a general property of cervical dysplasia and cancer.

In contrast, although retinoic acid markedly upregulates IGFBP-3 mRNA levels in CaSki and ECE16-D1 cells, we failed to observe any increase in IGFBP-3
protein levels. It has recently been reported that RA inhibits the proliferation of human breast carcinoma cells (Fontana et al., 1991; Adamo et al., 1992). Unlike the cervical cells, MCF-7 breast carcinoma cells synthesize and secrete IGFBP-2 and IGFBP-4, but not IGFBP-3. During RA inhibition of MCF-7 growth, there is a general increase in IGFBP levels, as well as the appearance of IGFBP-3. When MCF-7 cells are co-incubated with IGF-I and RA, the increase in IGFBP-3 is much larger than the increase in IGFBP-3 mRNA. Their results show that changes in IGFBP-3 RNA levels are not always correlated with changes in protein level (Fontana et al., 1991). Therefore, as previously described in MCF-7 cells (Adamo et al., 1992), regulation of IGFBP-3 translation, secretion or degradation may be important in the CaSki and ECE16-D1. Any change in these regulatory pathways may contribute to the observed decreased levels of IGFBP-3 in these cells. Thus, although retinoic acid increased IGFBP-3 mRNA and protein levels in ECE16-1 cells and ECE16-D2, our results suggest that increased mRNA levels may not always correlate with increased IGFBP-3 protein in all cervical carcinomas.

It is important to note that the RA-dependent increase in IGFBP-3 which we observe is likely to be
physiologically significant, as the level of IGFBP-3 mRNA in ECE16-1 cells and in the other cervical cell lines is increased at RA concentrations near the $K_d$ of RA for the retinoic acid receptors (i.e., 1 - 10 nM) (Cavey et al., 1990; Yang et al., 1991). This suggests that regulation of IGFBP-3 levels by RA is likely to be substantial "in vivo". Our studies also demonstrate that IGF-I increases and EGF decreases IGFBP-3 levels. When added simultaneously, the EGF effect predomnates and IGFBP-3 levels fall. Retinoic acid is dominant over all of these agents. Time course studies indicate that RA is able to reverse the suppression even after the IGFBP-3 levels are down regulated by EGF pretreatment.

These results suggest that in a scenario where uncontrolled cell proliferation occurs due to surplus of IGF-I and/or EGF, retinoic acid may, at least in part, inhibit cell proliferation via upregulation of IGFBP-3 levels.
CHAPTER VIII

REGULATION OF ECTOCERVICAL EPITHELIAL CELL PROLIFERATION BY EGF, IGF-I, AND RETINOIC ACID

1. INTRODUCTION:

Numerous studies have shown that transformed and tumor cell lines secrete growth factors that function to enhance growth via autocrine or paracrine mechanisms (Bowen-Pope et al., 1984; Sporn et al., 1985; Yee et al., 1989; Reiss et al., 1991; Barreca et al., 1992). At least two recognized mitogens from the EGF family are produced by epithelial cells: transforming growth factor alpha and amphiregulin (Li et al., 1992; Reiss et al., 1991). TGF-alpha binds to the EGF receptor, thus activating the cellular growth signaling system. Overexpression of TGF-alpha occurs in squamous epithelium-derived carcinoma, suggesting that high availability of this growth factor may induce tumorigenicity. IGFs are typically produced by mesenchymal cells and are not normally expressed in epithelia (Han et al., 1987; Barreca et al., 1992; Park et al., 1992). However, epithelial cells are readily accessible to IGF-I produced by the neighboring stromal cells.
Type I IGF receptors are known to exist in epithelial cells such as breast epithelium (De Leon et al., 1988) and in ECE16-1 (Hembree, unpublished). IGFBPs regulate IGF activity and have been reported to be produced in kidney epithelial cells (Cohick and Clemmons, 1991) and breast epithelium (De Leon et al., 1988). It has been demonstrated that IGFBPs modify breast tumor cell growth response to IGFs (Fontana et al., 1991). More recently, Adamo et al. (1992) reported retinoic acid modulation of IGFBPs secreted by MCF-7, with subsequent inhibition in cell growth.

In this section we examine the interactions of IGF-I, EGF and RA in the regulation of ECE16-1 proliferation. We also examine the effects of RA on growth of other ectocervix-derived cell lines.

2. MATERIALS AND METHODS:

Materials: Dulbecco's Modified Eagle's Medium (DMEM), F12 medium, nonessential amino acids, L-glutamine, trypsin and antibiotics were purchased from Gibco (Grand Island, NY). Fetal calf serum, insulin, bovine serum albumin (BSA), hydrocortisone, T3, transferrin and adenine were obtained from Sigma Chemicals (St. Louis, MO). Cholera toxin was purchased from ICN Biomedicals (Costa Mesa, CA). Human
recombinant EGF was from Upstate Biotechnology Inc. (Lake Placid, NY) and recombinant IGF-I was obtained from Collaborative Biomedical Products (Bedford, MA).

Cell culture: The ectocervical cell lines ECE16-1, ECE16-D1, ECE16-D2 and CasKi were maintained in culture medium containing DMEM:F12 (3:1) supplemented with 5% fetal calf serum (FCS), 5 ng/ml insulin, 0.1 nM cholera toxin, 5 ug/ml transferrin, 2 nM T₃, 10 ng/ml EGF, 4 nM hydrocortisone, 0.18 mM adenine, 100 uM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin and 50 ng/ml gentamycin.

Cell proliferation assay: Cells were plated in culture medium at a known density (5,000 - 10,000 cells/cm²) onto 9.6 cm² dishes (6 well cluster), in duplicate. After 24 h, cells were shifted to defined medium (DM). DM is identical to the complete medium, except it lacks FCS, insulin, cholera toxin, and EGF, and is supplemented with BSA (1 mg/ml) and ascorbic acid (50 ug/ml). After a 24 h equilibration in DM, DM + growth factor was added at 24 h intervals for 3 days. Cells were then harvested with HBSS containing 1 mM EDTA and 0.025% trypsin (w/v), resuspended in an isotonic solution containing 3.7% formaldehyde and kept
on ice until counting with a Coulter counter. The data are presented as the mean +/- SE of three experiments.

3. RESULTS:

To study the growth response of ECE16-1 cells to RA treatment, we measured the combined effects of EGF and IGF-I on cell proliferation either in the presence or absence of RA (Fig. 19). IGF-I at 5 ng/ml increased cell number 1.5 fold. EGF at 3 ng/ml increased cell number 2.6-fold. Coincubation with 5 ng/ml IGF-I and 3 ng/ml EGF produced a synergistic increase (4.2-fold increase in cell number). Addition of 1 uM RA in combination with IGF-I and EGF reduced the growth response to the EGF level of treatment. As shown by the insert, under the conditions of this growth experiment, the level of IGFBP-3 in the culture medium was regulated in a manner consistent with the experiments described in the previous chapter. These results suggest that the RA may be specifically countering the IGF-I augmentation of EGF stimulated growth. This could be mediated by a RA-dependent increase in the level of IGFBP-3, thus reducing the level of free IGF-I in the extracellular space. To determine if this effect is specific for IGF-I-stimulated growth, we compared the ability of RA to
Fig. 19 Effects of IGF-I, EGF and RA on ECE16-1 cell proliferation. ECE16-1 cells were plated (5,000 - 10,000 cells/cm²) in growth medium and allowed to attach overnight. At day 0 (8422+/−992 cells/cm²) They were then shifted to DM or DM supplemented with 5 ng/ml IGF-I, 3 ng/ml EGF, 5 ng/ml IGF-I + 3 ng/ml EGF or 5 ng/ml IGF-I + 3 ng/ml EGF + 1 uM RA and treated for three days. The number of cells in the DM group (6,521+/−135 cells/cm²) at the end of 3 days (no treatment) is equal to 100%. At 5 ng/ml IGF-I, the increase in cell number is not statistically significant. A concentration of 3 ng/ml EGF induced a 2.5-fold increase in proliferation (p < 0.05, n = 3, compared to the DM group). Simultaneous treatment with IGF-I + EGF results in an enhanced growth response (p < 0.01, n = 3, compared to the DM effect). However, retinoic acid given in conjunction with EGF and IGF-I, reduced cell growth. The insert is a ligand blot from a representative experiment showing the levels of secreted IGFBP-3 detected in the conditioned medium harvested from the last 24 h of treatment.
modulate growth and IGFBP-3 levels in the presence of other growth factors. Fig. 20 shows that RA inhibits growth of ECE16-1 cells, regardless of the combination of growth factors present, or even in the absence of any growth factor (DM). Moreover, ligand blots of conditioned medium obtained from each treatment group shows a consistent concentration-dependent increase in IGFBP-3 levels in RA treated cells. In the previous chapter, we demonstrated that cell line sensitivity to retinoic acid differs, as evaluated by IGFBP-3 mRNA. Because of this difference in sensitivity, we next examined the effects of RA on the proliferation of other ectocervical cell lines.

We treated ECE16-1, ECE16-D1, ECE16-D2 and CaSki cells with 1 μM RA for three days either in the presence or absence of EGF (20 ng/ml). The proliferative studies shown in Fig. 21 indicate that RA does not inhibit the growth of CaSki or ECE16-D1 cells. Conversely, ECE16-D2 proliferative response to RA is similar to the ECE16-1, i.e., they are both growth inhibited by RA.

4. DISCUSSION:

Retinoic acid has been shown to have potential as a therapeutic agent for the treatment of ectocervical
Fig. 20  RA effects on ECE16-1 proliferation curves obtained under different treatments. ECE16-1 cells, 10,000 cells/cm², were seeded in 9.6 cm² dishes in triplicate and allowed to attach overnight. They were then washed, shifted to DM and equilibrated for 24 h. Parallel wells were then treated with DM, 5 ng/ml IGF-I, 20 ng/ml EGF or 5 ng/ml IGF-I + 20 ng/ml EGF in the presence of 0, 0.1, 1, 10, 100 and 1000 nM RA. After 3 days of treatment the cells were harvested and counted and equal quantities of conditioned medium (based on cell number) from the last 24 h of treatment were assayed for IGFBP-3 levels (inserts) by ligand blot.
Figure 21. Proliferation data obtained from different ectocervical cell types (n=3). Cells were plated in duplicate wells (5,000 - 10,000 cells/cm²) in growth medium and allowed to attach overnight. They were shifted to DM for 24 h and then treated with EGF (open bars) or EGF + RA (dotted bars) for a period of three days. The EGF concentration utilized in these experiments was 20 ng/ml and the RA concentration was 1 μM. Each group had its own control (DM) and the number of cells obtained after 3 days in DM is equal to 100%. Qualitatively, ECE16-1 (16-1) and ECE16-D2 (D2) show a similar pattern of response; i.e., they are both growth inhibited by RA. CaSki (CASKI) and ECE16-D1 (D1) cells were not affected by any of the treatments. The insert is a ligand blot from a representative experiment showing the levels of secreted IGFBP-3 detected in the conditioned medium harvested from the respective cells during the last 24 h of treatment.
carcinoma (Lippman et al., 1992; Agarwal et al., 1993). Moreover, in human cervical epithelial cell culture models, retinoids have been shown to regulate cell proliferation and differentiation (Gorodeski et al., 1989; Agarwal et al., 1991; De Luca, 1991; Agarwal et al., 1993). However, the molecular basis underlying these responses is not well understood.

IGFs are typically produced by mesenchymal cell types (Han et al., 1987; Barreca et al., 1992;) and a variety of epithelial cell types are the targets of IGF action. Thus, IGF could be an important mitogenic agent in the development of cervical tumors. Regulation of the free IGF concentration in the extracellular environment is achieved by IGF binding to members of a family of binding proteins, the insulin-like growth factor binding proteins (Martin and Baxter, 1988; Baxter and Martin, 1989; Schmid et al., 1989; Clemmons et al., 1990; Ernst and Rodan, 1990; Martin and Baxter, 1990). An important member of this family is IGFBP-3, which binds IGFs and in many systems attenuates the mitogenic effects of IGF-I. This effect appears to be mediated, at least in part, by the ability of IGFBP-3 to bind IGF-I and therefore reduce the concentration of free IGF-I in the extracellular environment (Bicsak et al., 1990; Conover et al.,
Overexpression of IGFBP-3 in Balb C-3T3 cells has been shown to slow cell growth and reduce responsiveness to IGF-I (Cohen et al., 1993). Because of this effect, IGFBP-3 has been referred to as a tumor suppressor gene product (Cohen et al., 1993).

We have recently noted that HPV-immortalized cervical cell lines are more retinoid responsive, with respect to regulation of cytokeratin gene expression, compared to normal cells (Agarwal et al., 1991), an effect that has also been described in immortalized epidermal keratinocytes (Pirisi et al., 1992). EGF receptor levels also respond differently to retinoic acid in transformed cervical cells compared to normal cells (Sizemore and Rorke, 1993). In the present study, all of the immortalized cell lines (ECE16-1, ECE16-D1, ECE16-D2) and the tumorigenic cervical line (CaSki) express higher levels of IGFBP-3 mRNA in response to RA. Moreover, lower concentrations of RA are required to increase IGFBP-3 mRNA levels in these lines. This further supports the suggestion (Agarwal et al., 1991; Lippman et al., 1992) that increased sensitivity to retinoids may be a general property of dysplastic cervical cells.

In the previous chapter we showed that addition of IGF-I to the culture medium leads to an increase in the
extracellular pool of IGFBP-3. Paradoxically, under this condition we did not observed a growth inhibitory effect. The reasons for that could be several fold. First, if the IGF-I concentration is greater than the IGFBP-3 concentration at any given time (in other words, IGFBP-3 is saturated by the exogenous IGF-I), then IGF-I should bind to its receptor inducing a mitogenic response. Second, the possibility exists for an endogenous production of IGFs (I or II) by these cells. Thus, IGF-I and/or IGF-II competes for IGFBP-3 (IGFBP-3 is saturated by endogenous IGFs) and the exogenously added IGF-I binds to its receptor. Third, we do not know the time course of the IGF-I-induced IGFBP-3 increase, thus the mitogenic activity of IGF-I may be dominant over the inhibitory effects of IGFBP-3 on cell proliferation. Fourth, IGF-I may induce release of membrane bound IGFBP-3 which can also form IGF-I-IGFBP-3 complex with stabilization of the IGF-I receptor (Cohen et al., 1993) and further induction of cell proliferation. More indepth studies are necessary to conclude this initial observation.

Our initial growth experiments with ECE16-1 cells indicated that an increase in IGFBP-3 levels in response to RA is correlated with a decrease in IGF-I-dependent cell growth. Further investigation showed
that growth under a variety of other conditions was also suppressed by RA. This suppression correlates with an increase in IGFBP-3 levels. Although our results indicate a strong correlation between IGFBP-3 secretion and growth inhibition, we cannot at present determine whether this effect is mediated by effects of IGFBP-3 on the level of free IGF-I or is due to a more generalized effect of IGFBP-3 on the cells. In general, at receptor saturating levels of the growth factors, we would expect cells cotreated with EGF and IGF-I to be the most resistant to growth suppression by RA. This is because these cells would have the benefit of optimal stimulation by the EGF and IGF-I signalling pathways (i.e., the EGF-mediated suppression of IGFBP-3 would increase the free IGF-I concentration and facilitate the effects of IGF-I). A definitive test of this hypothesis will require additional experiments. In addition, it is also likely that RA operates via multiple pathways and may be having dual effects - a direct growth suppressing effect via the retinoid receptor signalling pathway and a secondary (indirect) growth suppressing effect via effects on the IGF-I signalling system.

Thus, our results indicate that the regulation of IGFBP-3 levels in cervical carcinoma cells is complex
and involves multiple levels of regulation. However, the results are consistent with the idea that RA may inhibit the growth of some cervical tumor cells via interference with IGF-I signalling.
Growth factor administration to wounds through indirect methods (autografts, allografts, xenografts) or by direct topical application in dressings, has been shown to modify the rate of healing. However, several limitations with these methodologies exist, including uncertainties regarding the stability of the growth factor and/or the dressing, the ability to provide uniform delivery of the growth factor over the wound surface and the high cost of producing purified growth factors.

To overcome these limitations, we have developed a genetically engineered biological bandage prototype. This bandage is designed to improve wound healing and may defeat the potential problems presently found in this area.

We demonstrated that SCC-13 cells can be easily engineered to produce a specific protein (bGH). In addition, "in vitro" tests of these cells showed that they are able to grow confined inside the bandage and can be kept biologically functional (secreting bGH) for a period of six days. The "in vivo" test of this system revealed that the bandage is functional up to
three days, i.e., the cells inside the bandage continue to produce bGH for this period of time. It would be desirable in future studies to extend the longevity of the cells. Preliminary data indicate that this can be accomplished by refeeding the cells on alternate days.

We conclude that the genetically engineered biological bandage is safe and reliable, since cells do not escape from it and since desired levels of secreted proteins can be achieved by manipulation of cell number inside the bandage. This system provides a new approach for the potential delivery of biomolecules into wounded areas.

Retinoid effects on cell differentiation and its inhibition of epithelial cancer cell growth have been observed by several authors. The underlying mechanisms of action for tumor growth suppression by retinoids have not yet been established. Growth factors such as EGF and IGF-I have also been implied in cancer etiology.

These studies were predicted on the idea that the growth suppressive effect of RA on ectocervical epithelial cells could be mediated by inhibition of the IGF-I stimulatory cascade, namely, regulation of IGFBP-3 protein levels.
We demonstrate that treatment of different ectocervical epithelial cell lines with all-trans-retinoic acid upregulates IGFBP-3 mRNA levels. In the case of lines ECE16-1 and ECE16-D2, the increase in IGFBP-3 mRNA was correlated with increased IGFBP-3 secretion and inhibition of cell proliferation.

We also demonstrate that IGF-I increases and EGF decreases IGFBP-3 levels and EGF effects predominate over IGF-I. However, RA is dominant over all these agents and is also able to reverse the suppression of IGFBP-3 after its down regulation by EGF.

We conclude that in a scenario where uncontrolled cell proliferation occurs due to surplus of IGF-I and/or EGF, RA may inhibit cell proliferation via upregulation of IGFBP-3 levels.
REFERENCES


Cavey, M.T., B. Martin, I. Carlavan and G. Shroot. 1990. In vitro binding of retinoids to the


Van Obberghen, E., B. Rossi, A. Kowalski, H. Gazzano and G. Ponzio. 1983. Receptor-mediated phosphorylation of the hepatic insulin receptor:
evidence that the M, 95,000 receptor subunit is its own kinase. Proc. Natl. Acad. Sci. USA 80:945-949.


Yamada, Y. and G. Serrero. 1988. Autocrine growth induced by the insulin-related factor in the


