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.
THE PUTATIVE ACTION OF THE INTERSTITIAL COLLAGENASE GENE AND
THE GENE OF ITS NATURAL INHIBITOR TIMP-1 DURING
THE PROCESS OF CANCER INVASION

DISSERTATION

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

By

Jucheng Chen, BMD, MS

The Ohio State University
1995

Dissertation Committee:
Dr. George E. Milo
Dr. Carl M. Allen
Dr. Charles F. Shuler
Dr. Karl S. Theil

Approved By
Advisor
Department of Pathology

Dr. George E. Milo
To My Parents,

Dr. and Mrs. I-Shih Chen
ACKNOWLEDGEMENTS

I would like to express my deep gratitude to Dr. George E. Milo for his support and guidance, Dr. Bruce C. Casto for his editorial and scientific comments to this dissertation, and Dr. Charles F. Shuler for his advise and insight throughout the research. Gratitude is extended to other members of my dissertation committee, Drs. Carl M. Allen and Karl S. Theil, for their cogent criticisms. Sincere appreciation goes to Dr. David E. Schuller for his providing fresh human tissue samples and Dr. David T. Denhardt, for his gift of the plasmid construct pNMH-aT. My thanks go to Dr. Dawei Li, Dr. Hakjoo Lee, Dr. Daniel Mannix and Inge Noyes for their technical supports and inputs. Special thanks go to Bonnie L. Chavis and Martha K. Leming for their assistance and friendship I have enjoyed during my entire study. I also would like to thank the faculty and staff of the Comprehensive Cancer Center, the Department of Medical Biochemistry and the Section of Oral Pathology at The Ohio State University for making this graduate study an unforgettable experience. Lastly, to my parents, Dr. and Mrs. I-Shih Chen, I thank you for being there.
VITA

1957 . . . . . Born - Keelung, Taiwan, ROC

1983 . . . . . Bachelor of Medicine in Dentistry
(DDS equivalent)
Taipei Medical College
Taipei, Taiwan, ROC

1983 . . . . . ROC Dental Licensure

1983-1984 . . . . Resident, Department of Pathology
Taipei Medical College
Taipei, Taiwan, ROC

1985 . . . . . USA National Dental Board

1987 . . . . . M.S., Experimental Pathology
Saint Louis University
St. Louis, Missouri

1987-1988, 1989-1990 . . . Teaching Associate,
The Ohio State University
College of Dentistry
Columbus, Ohio

1988-1989 . . . . Anatomic Pathology Fellow
The Ohio State University Hospital
Columbus, Ohio

1990 . . . . . Oral Pathology Certificate
The Ohio State University
Columbus, Ohio
1990-1991  .  .  .  .  Research Associate  
The Ohio State University  
Comprehensive Cancer Center  
Columbus, Ohio  

1991  .  .  .  .  Fellow  
American Academy of Oral Pathology  

1991-present  .  .  .  .  Research Associate  
The Ohio State University  
Department of Medical Biochemistry  
Columbus, Ohio  

PUBLICATIONS  


ABSTRACTS  

Chen J, Shuler CF, Milo GE: Altered differentiation in human squamous cell carcinomas in nude mice. J Dental Res 1991; 70: 548  


FIELDS OF STUDY  

Major Field: Pathology  

Studies in Cancer Invasion and Metastasis
TABLE OF CONTENTS

DEDICATION........................................................................................................ii
ACKNOWLEDGEMENTS ..................................................................................iii
VITA..................................................................................................................iv
LIST OF TABLES............................................................................................viii
LIST OF FIGURES..........................................................................................ix
LIST OF ABBREVIATIONS.............................................................................xii
SUMMARY.......................................................................................................xiii

INTRODUCTION..............................................................................................1

CHAPTER
I. THE ROLE OF THE NUDE MOUSE SYSTEM IN TUMOR BIOLOGY
   Introduction...................................................................................................17
   Materials and Methods..................................................................................18
   Results............................................................................................................21
   Discussion.......................................................................................................26
   Conclusion.....................................................................................................31

II. THE PUTATIVE ROLE OF INTERSTITIAL COLLAGENASE GENE AND THE
    GENE OF ITS NATURAL INHIBITOR DURING THE PROCESS OF CANCER
    INVASION
   Introduction...................................................................................................46
   Materials and Methods..................................................................................48
   Results............................................................................................................53
   Discussion.......................................................................................................59
   Conclusion.....................................................................................................69

III. CONTROL OF TIMP-1 EXPRESSION BY AN ANTISENSE RNA
   Introduction...................................................................................................120
   Materials and Methods..................................................................................121
   Results............................................................................................................125
   Discussion.......................................................................................................127
   Conclusion.....................................................................................................130
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nomenclature and natural substrates of the matrix metalloproteinases</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Anatomic Distribution and Xenograft Tumorigenicity of 141 Human Malignant Tumors</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>Tumorigenic Potential of the Overall 141 Primary and Metastatic Lesions</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>Tumorigenic Potential of Pharyngeal/Laryngeal Squamous Cell Carcinomas</td>
<td>35</td>
</tr>
<tr>
<td>5</td>
<td>Tumorigenic Potential of Oral Squamous Cell Carcinomas</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>Histopathologic Comparison Between the Original Tumors and Xenograft</td>
<td>37</td>
</tr>
<tr>
<td>7</td>
<td>Amplimers and Their Product Lengths</td>
<td>70</td>
</tr>
<tr>
<td>8</td>
<td>Expression of TIMP-1 and MMP-1 Genes in Human Patient Tumors</td>
<td>71</td>
</tr>
<tr>
<td>9</td>
<td>Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors</td>
<td>72</td>
</tr>
<tr>
<td>10</td>
<td>Summary of Expression of TIMP-1 and MMP-1 Genes.</td>
<td>73</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>A Nude Mouse with a Xenograft Tumor</td>
<td>38</td>
</tr>
<tr>
<td>2.</td>
<td>Histopathologic Comparison I</td>
<td>40</td>
</tr>
<tr>
<td>3.</td>
<td>Histopathologic Comparison II</td>
<td>42</td>
</tr>
<tr>
<td>4.</td>
<td>Histopathologic Comparison III</td>
<td>44</td>
</tr>
<tr>
<td>5.</td>
<td>A Restriction Map of TIMP-1 mRNA</td>
<td>74</td>
</tr>
<tr>
<td>6.</td>
<td>A Restriction Map of MMP-1 mRNA</td>
<td>76</td>
</tr>
<tr>
<td>7.</td>
<td>A Restriction Map of TIMP-1 genomic DNA</td>
<td>78</td>
</tr>
<tr>
<td>8.</td>
<td>The Expression of TIMP-1 and MMP-1 Genes in Fibroblast Primaries Analyzed by RT-PCR Technique</td>
<td>80</td>
</tr>
<tr>
<td>9.</td>
<td>The Expression of TIMP-1 and MMP-1 Genes in Keratinocyte Primary Analyzed by RT-PCR Technique</td>
<td>82</td>
</tr>
<tr>
<td>10.</td>
<td>The Expression of TIMP-1 and MMP-1 Genes in the Cell Lines 83-01-82SCC and 83-01-82CA-JC1 Analyzed by RT-PCR Technique</td>
<td>84</td>
</tr>
<tr>
<td>11.</td>
<td>The Expression of TIMP-1 and MMP-1 Genes in Five Cell Lines Analyzed by RT-PCR Technique</td>
<td>86</td>
</tr>
<tr>
<td>12.</td>
<td>The Expression of TIMP-1 and MMP-1 Genes in Normal Skin and Uterine Cervix Analyzed by RT-PCR Technique</td>
<td>88</td>
</tr>
<tr>
<td>13.</td>
<td>The Expression of TIMP-1 and MMP-1 Genes in Patient Tumors #1-#4 Analyzed by RT-PCR Technique</td>
<td>90</td>
</tr>
<tr>
<td>14.</td>
<td>The Expression of TIMP-1 and MMP-1 Genes in Patient Tumors #5-#8 Analyzed by RT-PCR Technique</td>
<td>92</td>
</tr>
</tbody>
</table>
15. The Expression of TIMP-1 and MMP-1 Genes in Patient Tumors #9-#11 Analyzed by RT-PCR Technique  .  .  . 94
16. The Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors #1-#3 Analyzed by RT-PCR Technique  .  .  . 96
17. The Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors #4-#6 Analyzed by RT-PCR Technique  .  .  . 98
18. The Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors #7-#9 Analyzed by RT-PCR Technique  .  .  . 100
19. The Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors #10-#12 Analyzed by RT-PCR Technique  .  .  . 102
20. The Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors #13 and #14 Analyzed by RT-PCR Technique  .  .  . 104
21. The Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors #15 and #16 Analyzed by RT-PCR Technique  .  .  . 106
22. The Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors #17 and #18 Analyzed by RT-PCR Technique  .  .  . 108
23. In Situ Hybridization against TIMP-1 mRNA in Normal Laryngeal Mucosa  .  .  .  . 110
24. In Situ Hybridization against TIMP-1 mRNA in Laryngeal Mucosa Exhibiting Pseudoepitheliomatous Hyperplasia .  .  . 112
25. In Situ Hybridization against TIMP-1 mRNA in Laryngeal Mucosa Exhibiting Moderate Dysplasia .  .  . 114
26. In Situ Hybridization against TIMP-1 mRNA in Cancer Cell Nests of the Deep Margin of a Squamous Cell Carcinoma  .  . 116
27. In Situ Hybridization against TIMP-1 mRNA in Other Cancer Cell Nests of the Deep Margin of a Squamous Cell Carcinoma  . 118
28. Diagram of the Expression Vector pNMH-aT  .  .  . 131
29. A Restriction Map of Antisense TIMP-1 .  .  .  . 133
30. The Integration of Plasmid DNA into the Genomic DNA of the Transfectant Analyzed by PCR Technique  .  .  . 135
<p>| 31. | The Expression of TIMP-1, Antisense TIMP-1 and MMP-1 Genes in the Transfectant Lines Analyzed by RT-PCR Technique | 137 |
| 32. | Microphotograph of the Tumor CAT1-JC1 | 152 |
| 33. | Microphotograph of the Tumor CAT1-aTIMP-JC1 | 154 |
| 34. | Postmortem Examination I | 156 |
| 35. | Postmortem Examination II | 158 |
| 36. | Postmortem Examination III | 160 |
| 37. | Postmortem Examination IV | 162 |
| 38. | Postmortem Examination V | 164 |
| 39. | Postmortem Examination VI | 166 |
| 40. | The Expressions of TIMP-1, Antisense TIMP-1 and MMP-1 Genes in the Transfectant Tumor Analyzed by RT-PCR Technique | 168 |</p>
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Matrix Metalloproteinase-1 (Interstitial Collagenase)</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription-Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue Inhibitor of Metalloproteinases-1</td>
</tr>
</tbody>
</table>
SUMMARY

Historically, human tumors have not been invasive in nude mice although invasiveness was demonstrated in the patient tumors. Therefore, the purpose of the research described in this dissertation was to determine those parameters involved in noninvasive property of human tumors in the surrogate host. The nude mouse system provides an opportunity for studies of differential expression of invasiveness between primary and metastatic human tumors. The potential for growth in the nude mouse and the pattern of histodifferentiation was examined in 141 human malignant tumors transplanted to nude mice. Among these 141 transplanted tumors, 44 of 97 primary and 25 of 44 metastatic tumors were found to be tumorigenic in nude mice. Twenty-five of 56 pharyngeal/laryngeal and 24 of 48 oral squamous cell carcinomas also grew in nude mice. The dynamics of transplanted tumor growth in the surrogate host took place independent of the stage of malignancy and site of origin. Xenograft tumors exhibited a higher histologic grade, a decreased cellular pleomorphism, poorer desmoplastic reaction, and a higher mitotic index compared to the original tumors. These data suggest that human cancer cells are plastic with respect to histopathology and expression of tumorigenicity in nude mice.
It has been reported that there is increased MMP-1 enzyme activity in invasive tumors from patients. MMP-1 is negatively regulated by its natural inhibitor TIMP-1 in many physiological conditions by forming a 1:1 stoichiometric complex. Relationship of TIMP-1 and MMP-1 expression to tumor invasion was studied by using RT-PCR and in situ hybridization. Replicating normal cells (keratinocytes and fibroblasts) and six cancer cell lines in vitro exhibited the expression of TIMP-1 and MMP-1. Normal tissues (skin and uterine cervix) in vivo did not express either. Only one of 11 invasive tumors from patients displayed expression of TIMP-1 whereas 8 of 11 invasive tumors demonstrated expression of MMP-1. By contrast, none of 18 xenograft tumors in nude mice exhibited the expression of TIMP-1 or MMP-1. These data strongly support the hypothesis that MMP-1 expression is directly related to tumor cell invasion. In situ localization of TIMP-1 mRNA showed that the TIMP-1 gene was silent in normal mucosal epithelial cells, extremely active in premalignant cells and again silent in the full-blown invasive carcinoma. Therefore, it was concluded that TIMP-1 expression is inversely related to tumor cell invasion. The demonstration of the invasive property of human tumors in patients and the lack of invasiveness in the nude mouse, confirmed that the process of cancer invasion is closely related to uncontrolled collagenolytic activity resulting from upregulated MMP-1 and downregulated TIMP-1, and that the invasive process is controlled by the micro-environment of host tissue.

In order to downregulate the expression of TIMP-1, an expression vector with an antisense TIMP-1 insert, pNMH-aT, was transfected into the squamous cell
carcinoma cell line 83-01-82CA-JC1 by using lipofectin technique. The transfectant line 83-01-82CA-aTIMP-JC1 was cultured in a selective medium for more than 4 months, proving that plasmid DNA was stably integrated into the genomic DNA of 83-01-82CA-JC1. By using RT-PCR technique, the transfectant line 83-01-82CA-aTIMP-JC1 exhibited the expression of TIMP-1, antisense TIMP-1 and MMP-1. The co-expression of TIMP-1 and antisense TIMp-1 indicates that the integrated plasmid pNMH-aT is not 100% inhibitory to the expression of TIMP-1.

The nude mouse system, although not ideal to study the process of human tumor biology, it is the nearest system that mimics the micro-environment of human tissue and one in which we can examine the biologic behavior of cancer cells in vivo. Transfectant line, 83-01-82CA-aTIMP-JC1, was transplanted into the nude mouse for evaluation of invasiveness. The transfectant developed freely movable, encapsulated progressively growing tumors in nude mice in three months; no micro-invasion nor micrometastasis was seen microscopically. Message of antisense TIMP-1, but not TIMP-1 or MMP-1, was detected in the transfectant tumors. The in vivo and in vitro results showed that the transfectant cells were plastic with respect to TIMP-1 and MMP-1 expression. The loss of invasiveness was not due to the failure of antisense TIMP-1 transfection, but due to the downregulated MMP-1 gene of the transfectant line presumably influenced by the micro-environment of mouse tissue.
In closing, human cancer cells are plastic in the nude mouse with respect to tumorigenicity, histopathology and invasiveness. The invasion process by cancer cells is directly related to the expression of MMP-1 and inversely related to the expression of TIMP-1.
INTRODUCTION

Invasion and metastasis are the most life-threatening aspects of the oncogenic process. Approximately 30% of cancer patients at the time of initial diagnosis have clinically detectable metastases. Approximately half of the remaining 70% of cancer patients have clinically nondetectable micro-metastases and will develop detectable metastatic deposits if untreated [Rosenberg 1987]. Invasion and metastasis may represent the biologic endpoint of multistage carcinogenesis [Cotran 1994]. However, it is the endpoint that results in cancer treatment failure and mortality. Oftentimes, cancer patients suffer not from formation of the tumor, but from the destructive nature of invasion and metastasis. Hence, it is clinically significant to understand the process of invasion and metastasis in addition to the processes leading to tumorigenesis.

Tissue Compartments

Before studying the process of cancer invasion, the anatomic relationship between the organs in which cancer arises and surrounding tissues has to be described. In mammals, organs are surrounded by the extracellular matrix consisting of basement membrane and interstitial stroma [Liotta 1983; Yurchenco 1990]. Basement
membrane is a thin and continuous, dense, extracellular matrix marking the barrier between organ parenchyma and interstitial stroma. The basal cells of organ-parenchyma are attached to one side of the basement membrane. The interstitial stroma, containing cells such as fibroblasts and fat cells, is on the other side of the basement membrane. The basement membrane primarily consists of dense type IV collagen, laminin and fibronectin [Timpl 1989], forming a continuous and micro-porous membrane. The pores on this membrane are so small that parenchymal cells cannot passively penetrate through it under normal conditions. In many pathophysiological conditions, such as embryonic morphogenesis, adult tissue remodeling, proliferative disorders, dysplasia, and carcinoma in situ, the cells proliferate on one side of the membrane and do not intermix with the cells on the other side. The only condition that this barrier is disrupted is during the process of active invasion (i.e. the transition from the stage of carcinoma in situ to the stage of invasive carcinoma) by cancer cells. Cancer cells penetrate through the basement membrane and interact with the interstitial stroma and stromal cells.

The interstitial stroma consists of stromal cells distributed in a meshwork of types I and III collagen fibers, glycoproteins (fibronectin), proteoglycans and hyaluronic acid [Tryggvason 1987]. It is reasonable to believe that cancer cells have to break down type I and III collagen in order to advance through the interstitial stroma and to cause the tissue destruction that occurs after the cancer cells have penetrated through the basement membrane. The mechanisms of tumor cell invasion described above are not well understood. The proteolytic breakdown of
extracellular matrix probably plays an important role in the invasive process, and since collagen is the principal structural protein of the extracellular matrix, the process of collagenolysis has been the subject of recent interest. The extracellular matrix consists of the basement membrane and the interstitial stroma [Yurchenco 1990]. There are three major steps in the interaction of cancer cells with the extracellular matrix: attachment to matrix components via cell-surface receptors [Albelda 1993; Casrtronovo 1993; Cioce 1991], proteolysis of the extracellular matrix [Gottesman 1990; Brown 1990; Steeg 1992] and locomotion of tumor cells [Guirguis 1987; Luna 1989; Nabi 1992]. The end result of this interaction is tissue invasion/destruction; a characteristic feature of malignancy.

Matrix Metalloproteinases

A number of proteolytic enzymes responsible for extracellular matrix proteolysis have been found to be correlated with malignant progression, including heparanases, serine- and thiol-dependent proteinase, and metalloproteinases [Goldberg 1991; Mignatti 1993; Nakajima 1990; Schultz 1991; Templeton 1990; Vlodavsky 1992]. All of these enzymes are probably involved in the invasion process in some way. Yet the extracellular matrix, the major target of invasive tumor cells, mainly consists of collagen that is the major substrate of matrix metalloproteinases. This reinforces the importance of matrix metalloproteinases in tumor invasion.
The matrix metalloproteinase family has been sub-grouped into three broad classes based on their substrate preference: collagenase, type IV collagenase, and stromelysin [Woessner 1991]. The chief characteristics of matrix metalloproteinases are: (1) zinc-dependent proteolysis; (2) highly conserved modular structure; (3) multiple substrates of extracellular matrix; (4) inhibition by the Tissue Inhibitor of Metalloproteinases (TIMP); and (5) secretion in zymogen form which can be activated by proteinases or organomercurials [Woessner 1991]. These enzymes share a number of common structural and functional features and have somewhat overlapping substrate specificities [Mignatti 1993]. Their nomenclature and substrate specificity are shown in Table 1.

Simply speaking, type IV collagenases mainly break down the principal structural protein of basement membrane, type IV collagen, whereas interstitial collagenase (MMP-1) primarily degrades the principal structural protein of interstitial stroma, type I-III collagen. Any given invasive tumor, at the time of diagnosis, has broken through the basement membrane and reached the interstitial stroma. The breakdown of the basement membrane by type IV collagenases in cancer invasion has been examined extensively [Stetler-Stevenson 1990b; Tryggvason 1993; Mignatti 1993]. However, little attention has been paid to the degradation of the bulky interstitial stroma behind the thin basement membrane. Once the invasive tumor cells break through the basement membrane, it is reasonable to believe that the matrix metalloproteinases responsible for the degradation of interstitial stroma
must switch to MMP-1 due to the fact that interstitial stroma primarily consists of type I-III collagen.

MMP-1 is a glycosylated 57/52kD matrix metalloproteinase [Nagase 1981 & 1983; Wilhelm 1986; Goldberg 1986]. It is so named not to reflect the localization of the enzyme, but for its major substrate (collagen I-III). The enzyme is characterized by a five-domain modular structure: signal peptide, pro-peptide, catalytic domain, hinge region and hemopexin domain [Woessner 1991]. The hydrophobic signal peptide and pro-peptide are located at the NH$_2$-terminal. The catalytic domain constitutes the catalytic machinery containing a Zn$^{2+}$-binding site. The proline-rich hinge region is the transition to the hemopexin-like COOH-terminal domain that appears to be responsible for substrate specificity [Woessner 1991]. The MMP-1 gene has been cloned and sequenced; it contains ten exons and nine introns [Templeton 1990]. The expression of MMP-1 gene has been reported in many cell types including keratinocytes [Lin 1987; Peterson 1987], endothelial cells [Herron 1986], hepatocytes [Arthur 1990], monocytes/macrophages [Welgus 1985; Campbell 1987], chondrocytes [Lefebvre 1990; Jasser 1994], osteoblasts [Meikle 1991; Quinn 1990] and fibroblasts from skin [Dayer 1985; Reitamo 1994], synovium [Brinckerhoff 1992], gingiva and periodontal ligament [Birkedal-Hausen 1994], cornea [Wilhelm 1986; Tao 1995], lung [Wilhelm 1986], colon [Wilhelm 1986] and uterus [Ito 1988], as well as in many tumor cells [Mignatti 1993].
MMP-1 is not stored as cytoplasmic granules, but is produced by the action of growth factors or cytokines that initiate transcription of the gene; hence, there is a 6-12 hour delay in secretion of the enzyme that accumulates at local sites after initiation due to the complex transcriptional apparatus. This secretion can be sustained for days, and it is estimated that the half-life of human fibroblast MMP-1 mRNA is 53 hours [Brinckerhoff 1986; Overall 1991a]. The initiation, storage and secretion features of MMP-1 are different from those of acute inflammatory proteolytic enzymes that are stored in granules and respond in full force, but do not sustain any destructive activity beyond minutes.

which the basement membrane is intact [Saarialho-Kere 1993]. Furthermore, upon ELISA analysis, no MMP-1 protein is detected in primary cultures of human keratinocytes grown on basement membrane proteins (Matrigel), whereas elevated MMP-1 protein is detected in keratinocytes on type I collagen substrate [Saarialho-Kere 1993]. These observations appear to suggest that, under physiological conditions, the expression of MMP-1 by keratinocytes is upregulated by type I collagen and downregulated by basement membrane proteins.

**Tissue Inhibitor of Metalloproteinases**

Regulation of MMP-1 is stringent and occurs predominantly at the level of gene expression [Matrisian 1990]. A second level of regulation occurs extracellularly, after secretion, by the action of activators of the proenzyme forms as well as natural inhibitor proteins [Dochesty 1990]. The major physiologic inhibitors are \( \alpha_2 \)-macroglobulin and the family of Tissue Inhibitor of Metalloproteinases (TIMP). \( \alpha_2 \)-macroglobulin is restricted in its sites of activity due to its large size (780kDa), hence it is less effective [Curry 1990]. The family of TIMP is specific for matrix metalloproteinases, hence it is more effective [Dochesty 1990]. Thus far, there are at least two members in the family: TIMP-1 and TIMP-2. TIMP proteins form noncovalent complexes with both the activated and some latent forms of matrix metalloproteinases. TIMP proteins inactivate the activity of the mature matrix metalloproteinases and inhibit the activation of some matrix metalloproteinase precursors [DeClerck 1991; Howard 1991].
TIMP-1 is a mannose-rich sialoglycoprotein with a molecular weight of 28.5kD. It can be found in many different cell types from cartilage [Lefebvre 1990], dental pulp [Kishi 1984], liver [Roeb 1993], lung [Welgus 1985; Burnett 1986; Horowitz 1989], ovaries [Curry 1990; Freudenstein 1990], periodontal tissue [Nomura 1993], salivary glands [Drouin 1988], skin [Hembry 1986], synovial tissues [Cawston 1990; MacNaul 1990], tissue fluids [Welgus 1986] and embryonic tissues [Brenner 1989a]. The cDNA encodes a 207-residue polypeptide, consisting of a 23-residue signal sequence and a 184-residue inhibitory domain with a molecular weight of 21kD [Docherty 1985]. Glycosylation increases the mass to 28.5kD. It is most likely that the inhibitory machinery of TIMP-1 protein is encoded by the first 134 residues. This conclusion is justified by the fact that truncation after the 134th residue maintains the inhibitory machinery, and truncation before the 134th residue destroys the inhibitory machinery [Murphy 1991a]. TIMP-1 protein is highly stable due to the six disulfide bonds formed by 12 highly conserved cysteine residues [Williamson 1990]. It is inactivated by serine proteinases such as trypsin, chymotrypsin and elastase, but is resistant to plasmin [Okada 1988]. TIMP-1 protein inhibits MMP-1 protein by forming a 1:1 stoichiometric complex with a high-affinity Kd=10^{-9} to 10^{-11} M [Murphy 1991b]. The inhibitory activity of TIMP-1 will not be destroyed by the TIMP-1/MMP-1 complex conformation. Competent inhibitor can be recovered from the complex [Murphy 1989].

An additional member of the TIMP family is a smaller protein often referred to as TIMP-2. Human TIMP-1 and TIMP-2 show an overall homology of 66% with 41%
identity of residues at the amino acid level, but they are immunologically distinct [Stetler-Stevenson 1989, 1990]. The TIMP-1 protein is more effective against MMP-1 protein, whereas TIMP-2 protein is more effective against the 72-kD and 92-kD type IV collagenases [Howard 1991].


It has been reported that the murine TIMP-1 gene has a 38bp conserved sequence in the first intron which confers TPA-, serum- and transforming growth factor-β inducibility [Campbell 1991]. This conserved sequence contains an AP-1 binding site (5'-843 TGAGTAA 3') [Campbell 1991]. The murine TIMP-1 gene may give rise to mRNA of varying sizes due to multiple transcription start-sites [Campbell 1991]. Shorter TIMP-1 transcripts without AUG start codon are selectively induced by serum stimulation. They are translated 3-fold more effectively than the larger transcripts [Waterhouse 1990]. Multiple transcription start-sites of human TIMP-1 gene have not yet been reported.
MMP-1 and TIMP-1 Interactions in Physiological Processes

Collagen is the principal structural protein of extracellular matrix and the most abundant protein in mammals. Collagen-containing tissues are dynamic structures that constantly undergo extensive remodeling, for example, during the process of embryonic morphogenesis and during wound healing or uterine involution in the adult. The turnover of the extracellular matrix is a fundamental aspect of the development, growth and maintenance of an organism, and thus is a process that must be stringently controlled in order to maintain the correct tissue and cellular organization [Mullins 1983]. The extracellular matrix turnover is tightly controlled by resident connective tissue cells at three levels: (1) the production of proenzymes (e.g. latent matrix metalloproteinases); (2) the activation of proenzymes by proteolytic processing; and (3) the production of specific natural inhibitors such as TIMPs [Murphy 1990]. The latter is demonstrated by the fact that the expression of MMP-1 and TIMP-1 genes is evident in many physiological processes: embryonic growth and differentiation [Brenner 1989a; Brown 1989; Nomura 1989], skeletal growth and remodeling [Dean 1985; Delaisse 1988; Flenniken 1990; Nomura 1989; Seller 1978], ovary development [Nomura 1989], salivary gland development [Hayakawa 1992; Nakanishi 1986], tooth germ development [Nomura 1989], wound healing [Chowcat 1988; Hembry 1986; Saarialho-Kere 1992 & 1993], ovulation [Brännström 1988], parturition [Rajabi 1988 & 1990; Jeffrey 1991] and uterine postpartum involution [Woessner 1988].
The expression of MMP-1 and TIMP-1 genes in many physiologic conditions is highly organized. Studies of TIMP-1 expression have demonstrated a precise spatial and temporal expression relative to matrix metalloproteinases. For example, in studies of lactation and involution of mammary gland, there is a high level of TIMP-1 during lactation. The level of TIMP-1 declines significantly during involution and there is a concomitant increase in extracellular matrix-degrading enzyme activity [Talhouk 1992]. In studies of human chronic ulcers and incision wounds, MMP-1 mRNA was expressed by basal keratinocytes at the advancing edge of ulcers, whereas TIMP-1 mRNA was expressed mostly by nearby stromal cells and less frequently by basal keratinocytes [Saarialho-Kere 1992 & 1993]. In addition, in a study of human hypertrophic scar tissue using indirect immunofluorescence to detect the presence of MMP-1 and TIMP-1, significant extracellular immunoprecipitation of both MMP-1 and TIMP-1 was found in hypertrophic scar areas undergoing active remodeling, whereas inactive areas were mainly negative [Hembry 1986]. In the growth of the young rabbit, TIMP-1 protein is seen in certain regions of the tissue where there are elevated expression of MMP-1 and type IV collagenases [Brown 1989]. Using rabbit colon anastomosis as a wound-healing model, serial studies at specific intervals post-anastomosis revealed that MMP-1 is expressed within 12 hours, whereas TIMP-1 is not expressed until 24 hours [Chowcat 1988]. All of these processes are associated with degradation and restructuring of the extracellular matrix, which are indeed well-controlled collagenolytic activities characterized by balanced MMP-1/TIMP-1 expression both spatially and temporally.
HYPOTHESIS: CANCER INVASION IS DIRECTLY RELATED TO THE EXPRESSION OF MMP-1 AND INVERSELY RELATED TO THE EXPRESSION OF TIMP-1; MESSAGES THAT ARE SUBJECT TO CONTROL BY THE HOST TISSUE MICRO-ENVIRONMENT

The process of invasion is not just a characteristic of malignancy but also is observed in the morphogenesis of embryonic tissues and remodeling of adult tissues. The difference between malignant invasion by cancer cells and benign invasion by embryonic and remodeling tissues is that benign invasion is well-controlled. It has been reported that, during the biological process of morphogenesis and tissue remodeling, elevated levels of TIMP-1 and MMP-1 protein are detected. The activity of MMP-1 protein appears to be negatively regulated by TIMP-1. Findings and observations made by others have shown that no MMP-1 and TIMP-1 mRNAs/proteins are detected in differentiated normal adult tissues. In embryonic tissues, elevated levels of both MMP-1 and TIMP-1 mRNAs/proteins are detected. By contrast, only MMP-1 mRNA/protein, but not TIMP-1 mRNA/protein, appears to be elevated in head and neck cancer tissues. The goal of this research is to approve that invasion by squamous cell carcinoma of the head and neck is directly related to the expression of MMP-1 mRNA and inversely related to the expression of TIMP-1 mRNA and also to demonstrate that the micro-environment of the "cancer" tissue influences the up/down-regulation of these internal enzymatic controls of the invasion process.
EXPERIMENTAL APPROACHES

It has been reported that there is an increased collagenase enzyme activity in invasive tumors from patients [Mignatti 1993]. The experimental approach used in this study examined the roles of interstitial collagenase and its natural inhibitor, TIMP-1, in the invasion of human cancer cells in the nude mouse. This study has been divided into four consecutive experimental periods or chapters as described below.

The experiments in Chapter I were conducted to evaluate (A) tumorigenicities of primary and metastatic patient tumors xenotransplanted into nude mice; and (B) the histopathologic changes of xenograft tumors by comparing the histopathologic features of original patient tumors and their corresponding xenograft tumors.

Invasive tumors from patients that are xeno-transplanted into nude mice will continue to form xenograft tumors, however, these xenograft tumors soon lose the expression of the invasive phenotype, one of the most important features of malignancy. Tumorigenicity and histopathologic studies were conducted to provide information leading to a better understanding of this loss of the invasive phenotype.

Studies in Chapter II were designed to examine the up/down regulation of mRNAs of collagenase and/or TIMP-1 genes in (A) keratinocyte/fibroblast primary cultures
and cancer cell lines *in vitro*; (B) normal and cancerous tissues *in vivo*; and © xenograft tumors (Chapter I) by using the reverse transcription-polymerase chain reaction technique (RT-PCR). In addition, the distribution of TIMP-1 mRNA in normal, hyperplastic, and dysplastic laryngeal mucosa and in invasive squamous cell carcinoma was examined using an *in situ* hybridization technique.

Studies of the role of MMP-1 and its natural inhibitor, TIMP-1, in cancer should shed light on the molecular events that govern tissue destruction of interstitial stroma by cancer cells. However, studies of the roles of MMP-1 and TIMP-1 genes in cancer invasion have historically been incomplete primarily due to a lack of adequate samples for conventional biotechnology analyses. Development of mRNA phenotyping [Brenner 1989] now provides a means to study the roles of MMP-1 and TIMP-1 genes in cancer cell invasion. This method couples reverse transcription (RT) of total cellular RNA and the polymerase chain reaction (PCR) using thermostable DNA polymerase from *Thermus aquaticus* [Rappolee 1988].

In *Chapter III* studies were focused on the regulation of the expression of the TIMP-1 gene and its possible role in tumor invasion by using antisense TIMP-1.

It has been demonstrated that TIMP-1 protein negatively regulates the behavior of collagenase protein by forming a 1:1 stoichiometric complex
[Murphy 1991]. The transcriptional and translational controls of these two genes are independent. Inhibition of TIMP-1 gene expression by using an antisense TIMP-1 [Khokha 1989] may decrease the production of TIMP-1 protein and thereby free more collagenase protein to participate in tissue invasion by cancer cells. With respect to this theory, transfectants of human cancer cells were established using an antisense TIMP-1.

In Chapter IV, using the information obtained from the previous three chapters, the invasiveness of the transfectants with integrated antisense TIMP-1 (Chapter III) was evaluated by xeno-transplantation of the transfectants into nude mice and the degree of invasion assessed by (A) histopathologic examination; and (B) the expression of MMP-1 and TIMP-1 genes in the xenograft tumors derived from the transfectant. The expression of TIMP-1 and MMP-1 genes monitors the activities of these genes in vivo.

Thus far, the nude mouse system is the most reliable system that seems to mimic human physiologic conditions. Such system provides a good environment for the xeno-transplanted transfectant in the study of the invasive phenotype in vivo. Thorough histopathologic examination of the mouse injected with the transfectant can be used to search for evidence of micro-invasion and micro-metastases.
Table 1. Nomenclature and natural substrates of the matrix metalloproteinases

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzyme</th>
<th>MMP#</th>
<th>Natural Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Interstitial collagenase</td>
<td>MMP-1</td>
<td>Collagen I-III, VII, VIII, X; gelatin</td>
</tr>
<tr>
<td></td>
<td>Neutrophil collagenase</td>
<td>MMP-8</td>
<td>Collagen I-III</td>
</tr>
<tr>
<td>II</td>
<td>72-kD gelatinase/ type IV collagenase</td>
<td>MMP-2</td>
<td>Collagen IV, V, VII, X, XI; gelatin; fibronectin; elastin; proteoglycan core protein</td>
</tr>
<tr>
<td></td>
<td>92-kD gelatinase/ type IV collagenase</td>
<td>MMP-9</td>
<td>Collagen types IV, V; gelatin</td>
</tr>
<tr>
<td>III</td>
<td>Stromelysin</td>
<td>MMP-3</td>
<td>Collagen IV, V, IX, X; fibronectin; laminin; proteoglycan core protein; procollagenases</td>
</tr>
<tr>
<td></td>
<td>Stromelysin-2</td>
<td>MMP-10</td>
<td>Same as MMP-3</td>
</tr>
<tr>
<td></td>
<td>Stromelysin-3</td>
<td>MMP-11</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>PUMP-1</td>
<td>MMP-7</td>
<td>Collagen IV; fibronectin; laminin; gelatin; proteoglycan core protein; procollagenases</td>
</tr>
</tbody>
</table>

Ref: Woessner 1991
CHAPTER I
THE EVALUATION OF TUMORIGENICITY AND INVASIVENESS
OF HUMAN TUMORS IN THE NUDE MOUSE

INTRODUCTION

The xenotransplantation of human tumor fragments into the nude mouse is an important system for studying tumor biology. This system provides an in vivo environment [Holmstrup 1985; Ryaggard 1969; Wennerberg 1983] for various types of tumor tissue [Giovanella 1991; Kleine 1986; Mattern 1985; Pratesi 1986; Volm 1987; Zätterström 1992]. Although in vitro cell culture models [Elsdale 1972; Gey 1952; Jones 1973; MacPherson 1964; Paranjpe 1975; Puck 1955] may provide an alternative means for studying the process of carcinogenesis, these in vitro culture models lack the physiologic host tissue micro-environment that is a critical component in the complex nature of tumor pathogenesis. The nude mouse model system provides an environment that somewhat resembles the physiology of the in vivo human situation. Human malignant tumors which are xeno-transplanted into nude mice continue to form xenograft tumors, however, these xenograft tumors frequently lose the expression of the invasive phenotype that is a characteristic of the patient tumor and is one of the most important features of malignancy. The
The purpose of this part of the study is to use the nude mouse tumor biology system to evaluate the tumorigenic potential and histopathology of these xenograft tumors. This may in turn provide information leading to the better understanding of the nature of the invasive and the noninvasive phenotypes.

**MATERIALS AND METHODS**

**Source of Malignant Tissues**

Fresh samples of malignant human tissues were collected through the Tissue Procurement Service of The Ohio State University Comprehensive Cancer Center. Each of the malignant tissues was processed by the Division of Surgical Pathology of University Hospitals for histopathologic diagnosis. Portions of the tissue were aseptically placed in cooled incomplete Minimal Essential Medium (MEM) on wet ice and were used for xeno-transplantation within 24 hours of removal from the patient.

**Immunosuppressive Pretreatment of Nude Mouse**

Male gnotobiotic NCR/SED nude mice (Massachusetts General Hospital, Boston, MA), 3-4 weeks of age, were splenectomized under general anesthesia (70 mg/kg of sodium pentobarbital). Anti-lymphocyte serum (0.1 ml per mouse) was administered subcutaneously twice weekly for four weeks to increase tumor engraftment. It has been demonstrated that tumor engraftment will be higher in gnotobiotic nude mice that receive additional immunosuppressive pretreatment such as splenectomy [Grimwood 1985] and anti-lymphocyte serum injection [Stanbridge...
1969]. The mice were used within two months of the treatments since tumor engraftment rate is dramatically decreased in mice older than three months [unpublished data].

**Tumor Xeno-transplantation Procedures**

Manually-minced tumor fragments less than $1 \text{mm}^3$ (0.2 to 0.3 ml of tumor suspension) were injected subcutaneously into the subscapular area with an 18-gauge needle. The injection site was monitored weekly by palpation and visual inspection. The latent period prior to the observation of a progressively growing tumor xenograft varied with each patient tumor, but was most commonly 3-6 months. Progressively growing tumors were allowed to reach a size of 2 cm in greatest dimension and were then excised. The excised xenograft tumors were subdivided and portions were either fixed in formalin for routine histopathology, subpassaged in additional nude mice to produce second-generation tumors, or snap frozen for the studies described in Chapter II.

**Histologic Analysis of Patient and Xenograft Tumors**

Tumor specimens were routinely sectioned, stained with hematoxylin and eosin, and examined microscopically. The histologic appearance of the tumors was categorized by the following criteria.

1. Histologic grade:

   *Well differentiated: presence of features of epithelial stratification including keratin pearls*
Moderately differentiated: some evidence of epithelial differentiation but lack of keratinization and pleomorphism of replicating cells

Poorly differentiated: lack of any significant features of epithelial terminal differentiation; most cells proliferating

2. Cellular pleomorphism: subjectively graded as 1+, 2+, 3+, and 4+ based on the appearance of the cells and comparison to normal epithelial cells.

   Grade 1+: tumor cells resemble the morphology of the normal tissue
   Grade 2+: tumor cells somewhat resemble the morphology of the normal tissue but lack of uniform size
   Grade 3+: tumor cells are anaplastic and varying in sizes
   Grade 4+: tumor cells are markedly anaplastic and do not resemble cells normally observed in the tissue

3. Desmoplastic reaction: subjectively graded as the amount of fibrovascular tissues around the tumor and recorded as:

   Poor: no response of the surrounding tissue to encapsulate and react to the tumor
   Mild: minimal response of surrounding tissue to encapsulate and react to the tumor
   Moderate: surrounding tissues partially encapsulate the tumor and generate an inflammatory response
   Severe: surrounding tissues completely encapsulate the tumor and the area of fibrovascular tissues in excess of the area of tumor
4. Mitotic Index: determined by counting the number of mitoses in three randomly selected high-power fields in a 5x5 mm area. The number of mitoses was compared between the original patient tumor and the xenograft tumor and recorded as:

- Increased: the xenograft had more mitoses than the original tumor
- Decreased: the xenograft had fewer mitoses than the original tumor
- Unchanged: xenograft and original tumor had the same number

In all cases, these histologic criteria were compared between the original patient and the xenograft tumors in multiple sections.

Statistical Analysis

The data were analyzed by $\chi^2$ analysis since the type of data are either qualitative ordinal or nominal [Neter 1982].

RESULTS

Tumorigenic Potential of Primary and Metastatic Patient Tumors in Nude Mice

In order to evaluate the relationship between the stage of human tumor progression (primary vs. metastatic) and the tumorigenic potential of patient tumors xenografted into the nude mouse host, 141 human malignant tumors (97 primary and 44 metastatic lesions) of various tissue origin (Table 2) were xenotransplanted into nude mice. Not all of the original patient tumors produced progressively growing tumors in the nude mouse host (Table 3), however sixty-nine of the 141 original
patient tumors did form tumors. The overall frequency of tumorigenicity was 48.9% (Table 3).

The stage of tumor progression in the patient (primary vs. metastatic) was then analyzed statistically to determine the correlation with tumorigenic potential of each tumor in the model system. The tumorigenic group of 69 transplanted tumors was composed of 44 primary and 25 metastatic tumors (Table 3) that developed into xenograft tumors larger than 2.0 cm (Figure 1). The patient tumors that did not successfully form xenograft tumors (nontumorigenic group), were obtained from 72 tumors that consisted of 53 primary and 19 metastatic tumors (Table 3). In the pool of these 141 human malignant tumors, the tumorigenic potentials of primary and metastatic patient tumors in the nude mouse system appeared to be statistically similar (p>0.05).

In this study, the 141 human malignant tumors were further subgrouped according to their anatomic locations. There were 56 pharyngeal/laryngeal squamous cell carcinomas (36 primary and 20 metastatic lesions) of which 13 of the 36 (36.1%) primary tumors and 12 of the 20 (60%) metastatic tumors from patients developed xenograft tumors (Table 4). The tumorigenic potentials of primary and metastatic pharyngeal/laryngeal patient tumors in the nude mouse system appeared to be statistically similar (p>0.05).
In addition, there were 48 oral squamous cell carcinomas (37 primary and 11 metastatic) of which 19 of the 37 (51.4%) primary tumors and 5 of 11 (45.5%) metastatic tumors from patients developed xenograft tumors (Table 5). The tumorigenic potentials of primary and metastatic patient oral squamous cell carcinomas in the nude mouse system appeared to be statistically similar (p>0.05). Statistical analyses on 141 tumors, 56 pharyngeal/laryngeal tumors and 48 oral tumors, indicated that the tumorigenic potential of primary and metastatic patient tumors in the nude mouse host all appeared to be statistically similar (p>0.05). These findings suggested that the tumorigenic potential of human malignancies in this surrogate host, when injected subcutaneously, is independent of the stage of malignancy regardless of the site of tumor origin.

Histopathologic Comparison of Patient and Xenograft Tumors

The histodifferentiation of the original patient tumors that were used to initiate the xenograft were compared to the progressively growing tumors that ultimately developed in the nude mouse (Table 6). The histopathologic sections of 34 original patient tumors were available for comparison. There were both similarities and differences between the patient tumors and their corresponding xenograft tumors.

The major similarity found was the histomorphology. These 34 patient tumors were classified as squamous cell carcinoma (Figure 2 A to D; Figure 3 A and F; Figure 4 A and B) and the xenograft tumors continued to express the squamous cell
carcinoma phenotype (Figure 2 E to H; Figure 3 B to E; Figure 3 G to I; Figure 4 C and D).

The major difference found was the pattern of invasion. The patient tumors characteristically exhibited multiple infiltrating processes into the adjacent normal tissue causing massive tissue destruction (Figure 2 A to D; Figure 3 A and F; Figure 4 A and B), whereas the xenograft tumors became nodular with a thin fibrous capsule (Figure 2 F and G; Figure 3 B to E; Figure 4 C and D). There was no evidence of invasion observed in the xenograft tumors. This nodular pattern was characteristic of the xenograft tumors, which rarely invaded or metastasized. Besides the difference in the invasive phenotype, there were differences that were somewhat subjective, but reproducible. These included histologic grading, cellular pleomorphism, desmoplastic reaction, and mitotic index.

Histologic grading was recorded as well-differentiated (presence of features of epithelial stratification including keratin pearls), moderately differentiated (some evidence of epithelial differentiation but lack of keratinization and pleomorphism of replicating cells) or poorly differentiated (lack of any significant features of epithelial terminal differentiation; most cells proliferating). Among 34 tumor pairs (i.e. patient tumor matched with its corresponding xenograft tumor), there were 21 tumor pairs that remained at the same histologic grading and 13 tumor pairs that exhibited a higher grade in the xenograft tumors (Table 5, Histologic Grade). The range of higher grade varied among the tumors. In one pair, the change was minimal (Figure
2 A vs. E); in a second case, the change was moderate (Figure 2 B vs. F); and in another pair, the change was considerable (Figure 2 C vs. G). Interestingly, with two tumors that were serially passaged 4 and 3 times respectively and formed two tumor lines (Figure 3 A to E; and Figure 3 F to G), a good opportunity was provided to examine the change of histologic grading in a long term xeno-transplantation study. In one tumor line, each subsequent subpassage exhibited higher grading than either the original tumor or the previous subpassage (Figure 3 A to E). The second tumor line retained the same histologic grading throughout passage (Figure 3 F to G). Finally, in a study in which primary and metastatic tumors from the same patient were available from separate occasions (Figure 4 A and B), both eventually developed progressively growing tumors in the nude mouse system (Figure 4 C and D). Comparisons between these two passaged tumors revealed that both xenograft tumors exhibited a nodular pattern with a thin capsule (Figure 4 C and D).

Cellular pleomorphism was subjectively graded as 1+, 2+, 3+, and 4+ based on the appearance of the cells and comparison to normal epithelial cells. Grade 1+ represented tumor cells that resemble the morphology of the normal tissue and grade 4+ represented the other end of the spectrum in which tumor cells are markedly anaplastic and do not resemble cells normally observed in the tissue. Among the 34 tumor pairs, nineteen xenograft tumors had a decreased range of cellular pleomorphism than that observed in the patient tumors; twelve tumor pairs retained a similar range of cellular pleomorphism (Table 6, Cellular Pleomorphism).
Desmoplastic reaction was rated poor (no tendency of the surrounding tissue to encapsulate and react to the tumor), mild (minimal tendency of the surrounding tissue to encapsulate and react to the tumor), or moderate (surrounding tissues partially encapsulate the tumor and generate an inflammatory response). Among the 34 tumor pairs, thirty-two xenograft tumors exhibited a decreased desmoplastic reaction in contrast to that observed in the patient tumors. Only two tumor pairs retained the same degree of desmoplastic reaction (Table 6, Desmoplastic Reaction).

Mitotic index was recorded as increased (the xenograft had more mitoses than the original tumor), decreased (the xenograft had fewer mitoses than the original tumor), or unchanged (xenograft and original tumor had the same number). Among the 34 tumor pairs, twenty-four xenograft tumors exhibited increased mitotic indices when compared to those observed in the patient tumors, whereas seven xenograft tumors exhibited decreased mitotic indices (Figure 6, Xenograft Mitotic Index).

**DISCUSSION**

The historical methods for analyzing tumors, such as various *in vitro* cell culture models [Elsdale 1972; Gey 1952; Jones 1973; Leighton 1967; MacPherson 1964; Paranjpe 1975; Puck 1955] were instigated to provide alternative means for studying the process of carcinogenesis. A large body of data has been generated on the behavior of human tumor cells *in vitro* that has been extrapolated to the
aggressive behavior of tumor cells in vivo. However, in vitro culture models lack the physiologic host tissue micro-environment that may be critical to the expression of tumor pathogenesis in vivo. A xenotransplantation model in animals (hamsters or nude mice) provides a means for prospective studies on tumor behavior in an in vivo environment. Among these animal model systems, the nude mouse system provides an in vivo environment [Wennerberg 1983; Ryaggard 1969; Holmstrup 1985] that is suitable for various types of tumor tissue [Giovanella 1991; Kleine 1986; Matterm 1985; Pratesi 1986; Volm 1987; Zätterström 1992]. The xenotransplantation of human tumor fragments into the nude mouse model has become an important model in tumor biology. The nude mouse model system provides a simulated environment that somewhat reflects the physiology of the in vivo human situation. Ideally in such a model, the xenogeneic tumors should grow in a pattern similar to that of the original tumor. Changes in biologic behavior of the xenograft tumor in this model could be due to physiologic differences from the host tissue micro-environment of the original tumor. The absence of a host immune response and the failure of xenograft tumors to invade are two of the major differences that exist between the nude mouse model and the human tumor in situ. Although these and other differences do exist, the nude mouse model still provides the best opportunity to examine tumorigenic potential, tumor growth, differentiation, and stromal response in an in vivo environment that simulates the original tumor site, as opposed to growing tumor cells in monolayer culture or in different cell culture matrices.
In multistage carcinogenesis, the events of tumor initiation and growth precede the event of metastasis. However, the relative tumorigenic potential of the carcinogenically-advanced tumors (i.e. metastatic lesions) to that of less advanced tumors (i.e. primary lesions) remains unknown. Primary and metastatic human tumor fragments were xeno-transplanted into nude mice to compare their tumorigenic potential. Of a total of 141 human malignant tumors, 69 eventually developed progressive growing tumors in the nude mouse system. The overall tumorigenic potential was 48.9% (Table 3). Among these 141 human malignant tumors, there were 56 pharyngeal/laryngeal and 48 oral squamous cell carcinomas. The tumorigenic potentials of primary and metastatic human tumors were compared from these 141 tumors. Statistical analyses showed that the tumorigenic potentials of the primary and metastatic human tumors in the nude mouse system were similar regardless of the site of tumor origin (p>0.05). These observations suggest that the tumorigenic potential is independent of stage of malignancy when tested in the nude mouse model. Metastatic tumors are derived from cells in the primary tumor that exhibit aggressive behavior, however the tumorigenic potential of the secondary tumor may not necessarily be higher than that of the primary tumors. Tumor formation results from oncogenic changes leading to imbalanced growth regulation and uncontrolled proliferation. However, uncontrolled growth itself does not result in invasion and metastasis. Additional genetic changes appear to be required for the acquisition of a life-threatening invasive and metastatic phenotype by the malignant cells. Tumorigenic potential and invasion/metastasis have both overlapping and separate features and may represent different biologic endpoints.
The most striking difference between the patient tumors and the xenograft tumors noted in this model was the loss of the invasive phenotype by the xenograft tumors. Other differences included a higher histologic grade, decreased cellular pleomorphism, decreased desmoplastic reaction, and an increased mitotic index in the xenograft tumors over those observed in the patient tumors. The loss of the invasive phenotype appeared to be correlated with the features of a higher histologic grade and decreased cellular pleomorphism. In this study, a higher histologic grade was defined as the presence of features of epithelial stratification with less numbers of pleomorphic replicating cells. The decreased cellular pleomorphism was defined as the resemblance of tumor cells to the morphology of normal cells. The combination of a higher histologic grade and decreased cellular pleomorphism was interpreted as a highly differentiated pattern. The tumor cells with a highly differentiated pattern underwent terminal differentiation and behaved less aggressively (i.e. invasion). Although the reason for a higher degree of differentiation is not clear, the microscopic findings partially explain the non-invasiveness of the xenograft tumors.

The loss of the invasive phenotype also appeared to be correlated with features of decreased desmoplastic reaction. Microscopic examination also revealed that the xenograft tumors exhibited a nodular growth pattern with a thin fibrous capsule. Inside these nodules, the fibrovascular component was decreased (i.e. decreased desmoplastic reaction) around the tumor cells. The thin fibrous capsule further walled off the tumor mass and decreased the contact surface area between the
tumor mass and surrounding normal tissues. Although the reason for the encapsulated nodular growth pattern with a decreased amount of fibrovascular component is not clear, the microscopic findings partially explain the non-invasiveness of the xenograft tumors.

The increased mitotic index among the xenotransplanted tumors was also an interesting finding. The human cancerous tissue fragments were packed into a small subcutaneous space in the nude mouse. The critical mass of cells in this confined space resulted in formation of tumors that was condensed into a short period of time. It is reasonable to expect that under these optimal conditions for growth and in the absence of immune competence that the mitotic index could be increased.

Although the reasons for all these changes (loss of the invasive phenotype, higher histologic grading, decreased cellular pleomorphism, decreased desmoplastic reaction and increased mitotic index) are unclear, the sources of these changes may partially come from the mouse tissue micro-environment. It is clear that the mouse and human physiologic micro-environments are not identical. For example, the mouse tissue micro-environment may contain/lack signal(s) that contribute to the phenotype of human cancers features present in their original micro-environment. Cancer cells of xenograft tumors might respond to these signals and exhibit phenotypes that are different from what they were in the human tissue micro-environment (i.e. noninvasiveness). However, the mouse model does provide an
environment in which the human tumors retain their capacity for progressive growth and similar morphologic characteristics.

CONCLUSION

The xenotransplantation of human tumor fragments into the nude mouse is an important model in the field of tumor biology. The nude mouse model system provides an environment that simulates the physiology of the in vivo human situation. The absence of a host immune response and the failure of xenograft tumors to invade are differences that exist between the nude mouse and human tissue micro-environments. Although these differences do exist, the nude mouse model still provides an excellent opportunity to examine tumorigenic potential, tumor growth, differentiation, and stromal response in an in vivo environment that more closely approximates the original tumor site in comparison to an in vitro model.

The tumorigenic potentials of human primary and metastatic tumors in nude mice showed no significant differences statistically (p>0.05). These observations suggest that tumorigenicity is independent of the stage of malignancy when tested subcutaneously in nude mice. Metastatic tumors are derived from the primary tumors, however the tumorigenic potential of the metastatic tumors is not necessarily higher than that of the primary tumors. Although the growth of tumor results from oncogenic changes leading to imbalanced growth regulation and uncontrolled proliferation, uncontrolled growth itself does not result in invasion and
metastasis. Additional genetic changes seem to be required to develop invasive and metastatic tumors that are life-threatening. Tumorigenesis and invasion/metastasis have features that are both overlapping and separate depending upon the host tissue micro-environment and internal molecular signals that direct the cells into a more aggressive phenotype.

Human tumors will grow as xenografts in the nude mouse system, however, only a fraction of such tumors were shown to exhibit such a capability in this study. The xenograft tumors continue to exhibit the features of their original histologic type. The most striking difference between the patient tumors and the xenograft tumors is the loss of the invasive phenotype by the xenograft tumors. Other differences include a higher histologic grading, decreased cellular pleomorphism, decreased desmoplastic reaction, and an increased mitotic index observed in the xenograft tumors over that observed in the patient tumors. The morphologic features of a higher histologic grade, decreased cellular pleomorphism and decreased desmoplastic reaction were consistent with the loss of the invasive phenotype. The mouse tissue micro-environment is not identical to the human tissue micro-environment and may contain/lack signal(s) that contribute to the phenotypic expression of human cancers in their original environment. Cancer cells of xenograft tumors apparently respond to these signals and exhibit the phenotypes described above.
Table 2. Anatomic Distribution and Xenograft Tumorigenicity of 141 Human Malignant Tumors

<table>
<thead>
<tr>
<th>Primary Sites</th>
<th>No. Cases</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Age Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Male</td>
</tr>
<tr>
<td>Larynx</td>
<td>32&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Pharynx</td>
<td>24&lt;sup&gt;f&lt;/sup&gt;</td>
<td>8</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Paranasal Sinuses</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Nasal Septum</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Oral</td>
<td>48</td>
<td>19</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>Salivary Glands</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lungs</td>
<td>8</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Esophagus</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Stomach</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Uterine Cervix</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vulva</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Skin&lt;sup&gt;g&lt;/sup&gt;</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>141</td>
<td>44</td>
<td>53</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>a</sup>primary lesions  
<sup>b</sup>Metastatic lesions  
<sup>c</sup>Tumorigenic  
<sup>d</sup>Non-tumorigenic  
<sup>e</sup>Three pairs of primary and metastatic lesonal materials were available.  
<sup>f</sup>Portions of a primary lesion from one patient were available twice.  
<sup>g</sup>Skin of the head and neck region.
Table 3. Tumorigenicity of All Primary and Metastatic Lesions

<table>
<thead>
<tr>
<th></th>
<th>Primary</th>
<th>Metastatic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumorigenic</td>
<td>44</td>
<td>25</td>
<td>69</td>
</tr>
<tr>
<td>Nontumorigenic</td>
<td>53</td>
<td>19</td>
<td>72</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>44</td>
<td>141</td>
</tr>
</tbody>
</table>

\[ x^2 = 1.590 \]
\[ p = 0.207 \]
Table 4. Tumorigenicity of Pharyngeal/Laryngeal Squamous Cell Carcinomas

<table>
<thead>
<tr>
<th></th>
<th>Primary</th>
<th>Metastatic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumorigenic</td>
<td>13</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Nontumorigenic</td>
<td>23</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>20</td>
<td>56</td>
</tr>
</tbody>
</table>

\[ x^2 = 2.969 \]
\[ p = 0.085 \]
Table 5. Tumorigenicity of Oral Squamous Cell Carcinomas*

<table>
<thead>
<tr>
<th></th>
<th>Primary</th>
<th>Metastatic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumorigenic</td>
<td>19</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>Nontumorigenic</td>
<td>18</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>11</td>
<td>48</td>
</tr>
</tbody>
</table>

$x^2=0.118$

$p =0.731$

*The oral squamous cell carcinomas include squamous cell carcinoma of tongue, floor of mouth, gingiva, buccal mucosa, lips, soft palate, palatal tonsil and an unspecified site.
Table 6. Histopathologic Comparison between the Patient Tumors and Xenografts

<table>
<thead>
<tr>
<th>Histologic Grade</th>
<th>Cellular Pleomorphism</th>
<th>Desmoplastic Reaction</th>
<th>Xenograft Mitotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>No.</td>
<td>No.</td>
</tr>
<tr>
<td><strong>Original Cases</strong></td>
<td><strong>Xenograft Cases</strong></td>
<td><strong>Original Cases</strong></td>
<td><strong>Xenograft Cases</strong></td>
</tr>
<tr>
<td>Well</td>
<td>1</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Mod/Well</td>
<td>1</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>Mod</td>
<td>1</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>Mod</td>
<td>4</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>Poor/Mod</td>
<td>1</td>
<td>2+</td>
<td>4+</td>
</tr>
<tr>
<td>Poor/Mod</td>
<td>4</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>Poor/Mod</td>
<td>10</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>Poor</td>
<td>1</td>
<td>4+</td>
<td>2+</td>
</tr>
<tr>
<td>Poor</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. A nude mouse with a xenograft tumor. A male nude mouse (3-4 weeks old) was xenotransplanted with a minced human malignant tumor fragments (0.2mg in a volume of 0.3ml) using an 18-gauge needle (Procedure described in the Materials and Methods of Chapter I). This mouse developed a xenograft tumor larger than 2 cm in greatest dimension in 3 months.
Figure 2. Histopathologic comparison I. Human squamous cell carcinomas of the head and neck were xenotransplanted into nude mice and developed xenograft tumors (Procedure described in the Materials and Methods of Chapter I). The original patient tumor and the xenograft tumors were compared histopathologically. Microphotographs represent four tumor pairs. Left panels (A to D) represent the original tumors and right panels (E to H) represent their corresponding xenograft tumors. (H&E stains, original magnification=160X)
Figure 3. Histopathologic comparison II. Two human squamous cell carcinomas of the head and neck were serially passaged into nude mice four and three times respectively and produced two separate tumor lines (Procedure described in the Materials and Methods of Chapter I). The subsequent xenograft in one tumor line was compared with its previous xenograft and the original tumor. Panels A to E represent microphotographs of one tumor line that was subpassaged four times in the nude mouse system. Panels F to I represent the other tumor line that was subpassaged three times. (H&E stains, original magnification=160X)
Figure 4. Histopathologic comparison III. Both the primary and the metastatic human squamous cell carcinomas from the same patient were available for xenotransplantation study and both lesions gave rise to xenograft tumors (Procedure described in the Materials and Methods in Chapter I). The original primary and metastatic tumors and their corresponding xenograft tumors were compared histopathologically. Panel A is the original primary tumor, panel B is the original metastatic tumor, panel C is the xenograft tumor derived from the primary tumor and panel D is the xenograft tumor derived from the metastatic tumor. (H&E stains, original magnification=160X)
CHAPTER II

THE PUTATIVE ROLES OF INTERSTITIAL COLLAGENASE AND ITS NATURAL INHIBITOR IN CANCER INVASION

INTRODUCTION

The studies in Chapter I demonstrated the plastic nature of cancerous tissues once they are relocated from the native human environment to the nude mouse environment. The changes observed in the new phenotypes of these plastic cancer cells include, but are not limited to, loss of invasion/metastasis, altered histodifferentiation, increased mitotic index and loss of fibrovascular stroma. These alterations suggested that phenotype may be altered by the host environment. The loss of the invasive phenotype in a surrogate host environment provides a good opportunity to determine those factors that account for the altered expression of the invasive phenotype.

There is limited understanding of the proteolytic mechanisms involved in the tissue invasion/destruction of interstitial stroma by cancer (see pages 1-13, Introduction). It has been reported that there is an increased interstitial collagenase (MMP-1) enzyme activity in invasive tumors from patients [Abramson 1975; Tarin 1982;
Muller 1991]. It has also been reported that MMP-1 protein is negatively regulated by its natural inhibitor: Tissue Inhibitor of Metalloproteinases-1 protein (TIMP-1) under physiological conditions [Murphy 1991b]. Studies of the role of MMP-1 and its natural inhibitor TIMP-1 in cancer should shed light on the molecular events that govern tissue destruction of interstitial stroma by cancer cells. It has been reported that MMP-1 and TIMP-1 mRNA levels reflect the subsequent accumulation of each protein in the conditioned medium [MacNaul 1990]. In our study, the messages of MMP-1 and TIMP-1 genes were examined as an indicator of MMP-1 and TIMP-1 protein activities. However, studies of the roles of MMP-1 and TIMP-1 mRNA in cancer invasion are incomplete primarily due to the lack of adequate sample quantity for conventional biotechnology analysis. In this respect, mRNA phenotyping [Brenner 1989a] provides a method for studies to determine the association between MMP-1 and TIMP-1 mRNA and the invasive phenotype. This method couples reverse transcription (RT) of total cellular RNA with the polymerase chain reaction (PCR) using the thermostable DNA polymerase from *Thermus aquaticus* [Rappolee 1988].

In order to study the role of MMP-1 and TIMP-1 in cancer invasion, the expression of MMP-1 and/or TIMP-1 genes in the following cells/tissues were examined: (A) keratinocyte/fibroblast primary cultures and cancer cell lines *in vitro*; (B) normal and cancerous tissues *in vivo*; and (C) xenograft tumors (Chapter I) by using reverse transcription-polymerase chain reaction technique (RT-PCR). In addition, the distribution of TIMP-1 message in normal, hyperplastic, and dysplastic laryngeal
mucosa as well as invasive squamous cell carcinoma was evaluated using an *in situ* hybridization technique.

**MATERIALS AND METHODS**

**Primary Cultures**

Fresh human neonatal foreskins were collected through the Tissue Procurement Service of The Ohio State University Comprehensive Cancer Center. The neonatal foreskins were aseptically placed in cooled incomplete Minimal Essential Medium (MEM) on wet ice and used for primary cultures within 48 hours.

A. Keratinocyte Primary Culture

The trimmed neonatal foreskins (5x5 mm²) were trypsinized in 0.1% trypsin/0.02% EDTA (pH 3.5) at 4°C for 18 hours. This trypsinization causes a suprabasal separation. The suprabasal layer was detached and the underlying keratinocytes on basal layer were scraped into complete MEM with a curved ophthalmic forceps. The cells were then seeded into a T-25 flask and incubated at 37°C, 4% CO₂ without disturbance for the first 24 hours.

B. Fibroblast Primary Culture

Each neonatal foreskin was digested in 0.25% collagenase/MEM (20% fetal bovine serum) at 37°C for 6 hours. The digested foreskin was then transferred to fresh MEM with 10% fetal bovine serum, seeded into a T-75 flask and incubated at 37°C, 4% CO₂ without disturbance for the first 24 hours.
Cancer Cell Lines

There were six cancer cell lines used in this study. These cell lines were originally isolated from human cancerous tissues of the head and neck region in Dr. George E. Milo's laboratory and routinely maintained in long-term monolayer culture using MEM with 10% fetal bovine serum (FBS). These six lines included: human squamous cell carcinoma cell line, 83-01-82SCC (tongue); human squamous cell carcinoma cell line, 83-01-82CA-JC1 (tongue); human adenocarcinoma cell line, 83-01-175 (parotid); human oat cell carcinoma, 83-05-45 (tonsil); human basal cell carcinoma cell line, 83-02-08 (skin); and a human myxoid chondrosarcoma cell line, 82-5-74 (heel), also named Sarc 2. The cell line 83-01-82SCC is nontumorigenic when tested in the nude mouse system and can be converted to a transient tumorigenic phenotype by treating with methylmethane sulfonate to become a daughter line 83-01-82CA-JC1.

Source of Malignant and Normal Tissues

Fresh malignant and normal human tissues used in this study were collected through the NIH-supported Tissue Procurement Service of The Ohio State University Comprehensive Cancer Center. Portions of the human tissue were snap-frozen in liquid nitrogen by the Tissue Procurement Service, transported on dry ice to Dr. Milo's laboratory, and stored in liquid nitrogen until use.
Xenograft Tumors

The source of xenograft tumor for this study was described in the Materials and Methods section of Chapter I. Briefly, portions of manually minced fresh human malignant tissue (1mm³ fragments in a volume of 0.2ml) were transplanted into pretreated nude mice to produce xenograft tumors. Portions of the xenograft tumors were snap frozen and stored in liquid nitrogen until use.

Total Cellular RNA Extraction and Reverse Transcription (RT)

This technique incorporates a micro-procedure for isolating total cellular RNA and reverse transcription of the total cellular RNA. Thus, only the mRNA in the total cellular RNA is reverse transcribed to produce cDNA [Brenner 1989c]. Briefly, total cellular RNA was extracted from cultured cells or minced tissue by boiling the cells or tissue for 5 minutes and IMMEDIATELY transferring 64μl of the supernatant to a 31μl reverse transcription reaction mix. The reverse transcription was carried out with 5μl Mo-MLV reverse transcriptase (200U/μl) and 1.25μg oligo(dT) priming at 42°C for 45 minutes in the presence of 50mM KCl, 1.5mM MgCl₂, 1mM dNTP mix and the RNase inhibitor, RNasin 100U. Immediately after the reverse transcription (RT), the RT product was stored at -20°C until use.

Polymerase Chain Reaction (PCR)

The RT product was then amplified using PCR in the presence of the thermostable Taq DNA polymerase. The technique was performed as previously described [Rappolee 1989]. Briefly, one μl of RT product was mixed with one unit of Taq DNA
polymerase (BRL) and 5 pmoles of amplimer pair specific for the mRNA of interest (see the next section: Amplimers and Their Product Lengths) in a buffer containing 20mM Tris-HCl (pH 8.3), 1.5mM MgCl₂, 50mM KCl and 0.2mM dNTP mix, in a 25-μl volume. The mixture was overlaid with one drop of mineral oil to prevent evaporation and amplified by a thermocycler (Perkin-Elmer Cetus) in repeated three-temperature cycle (94°C for 1 minute, 66°C for 2 minutes and 72°C for 3 minutes) for 35 cycles. The PCR product was then stored at 4°C until use.

Amplimers and Their Product Lengths

The amplimer pairs used in this study included the amplimers for MMP-1 and TIMP-1. In addition, an amplimer pair for β-actin was used here as a control [Nomura 1993]. Amplimers were designed according to the methods outlined by Rappolee et al. [1989]. The structures of the amplimers used in this study were designed from known sequences published in the Genbank Data Base. The amplimers used in this study are listed in Table 7.

Restriction Enzyme Digestions

After the PCR, the overlying mineral oil was removed from the PCR products with chloroform, and the PCR products were then digested with the restriction enzymes described below, 1μl at 37°C for 1 hour, for confirmation of the amplified products. The enzymes used for TIMP-1 product were Hinfl and Hha I; Hha I and Sca I for MMP-1 product; and Hinfl and Apa I for genomic TIMP-1 product. The digested PCR products were then separated on a 3% Wide Range Agarose gel (Sigma) and
stained with ethidium bromide (1μg/10ml) for photography under UV illumination (wavelength=312nm).

**In Situ Hybridization**

The probe labeling system and *In Situ* hybridization/detection system were purchased from BRL and the manufacturer's protocol was followed [Zheng 1993]. Briefly, the probe (1μg/50μl) was biotinylated by using biotin-14-dATP with DNA polymerase I. Frozen sections were fixed in 4% paraformaldehyde/5mM MgCl₂ for 15 minutes, pre-hybridized at 42°C for 2 hours and hybridized at 42°C overnight. The signals were detected by alkaline phosphatase (4μg/ml) and visualized by freshly made dye solution NBT (nitroblue tetrazolium, 0.66mg/ml)/BCIP (5-bromo-4-chloro-3-indolylphosphate, 0.36 mg/ml).

**RESULTS**

**PCR Products and Restriction Enzyme Digestions**

Throughout the entire RT-PCR studies presented in this chapter, there were two major electrophoresis bands observed: a 386bp band detected by using the amplimer pair for TIMP-1 mRNA and a 611bp band detected by using the amplimer pair for MMP-1 mRNA. In order to further confirm the nature of these products, restriction enzyme digestions were performed.
There was a 386bp band detected by using the amplimer pair for TIMP-1 mRNA (Figure 5: Lane 2). The 386bp product was digestible by *Hinf* I and yielded 192bp and 194bp fragments (Figure 5: Lane 3). This 386bp product was also digestible by *Hha* I and yielded 133bp and 253bp fragments (Figure 5: Lane 4). The size of this 386bp product and its *Hinf* I and *Hha* I restriction enzyme digestion patterns were consistent with the amplified TIMP-1 mRNA.

There was a 611bp band detected by using the amplimer pair for MMP-1 mRNA (Figure 6: Lane 2). The 611bp product was digestible by *Hha* I and yielded 145bp and 466bp fragments (Figure 6: Lane 3). In addition, this 611bp product was digestible by *Sea* I and yielded 276bp and 335bp fragments (Figure 6: Lane 4). The size of this 611bp product and its *Hha* I and *Sea* I restriction enzyme digestion patterns were consistent with the amplified MMP-1 mRNA.

There was an extra 1626bp band observed in the RT-PCR products of some samples by using the amplimer pair for TIMP-1 mRNA (Figure 7). This 1626bp PCR product was digestible by *Hinf* I and yielded 585bp and 1041bp fragments (Figure 7: Lane 1). This 1626bp product was further digested by *Apa* I and yielded 386bp and 1239bp fragments (Figure 7: Lane 2). The size of this extra 1626bp product and its *Hinf* I and *Apa* I restriction enzyme digestion patterns were consistent with the amplified genomic DNA of TIMP-1. Amplification of this large fragment provided
a positive control for the PCR protocol, but did not interfere with amplification of the RT-PCR products.

**Expression of TIMP-1 and MMP-1 Genes in Fibroblasts and Keratinocytes In Vitro**

Using RT-PCR with the amplimer pairs for TIMP-1 and MMP-1 mRNA respectively, the expression of MMP-1 and TIMP-1 genes in the human neonatal foreskin fibroblasts (harvested 24 hours, 48 hours and 72 hours post-seeding) and the human neonatal foreskin keratinocytes (harvested 24 hours post-seeding) were evaluated.

The RT-PCR products of fibroblast primaries on gel electrophoresis (Figure 8) showed that the fibroblasts harvested at 24 hours post-seeding expressed both TIMP-1 (Figure 8: Lane 2) and MMP-1 (Figure 8: Lane 3). The fibroblasts harvested at 48 hours post-seeding also expressed both TIMP-1 (Figure 8: Lane 5) and MMP-1 (Figure 8: Lane 6). The fibroblasts harvested at 72 hours post-seeding again expressed both TIMP-1 (Figure 8: Lane 8) and MMP-1 (Figure 8: Lane 9). In summary, human fibroblasts in monolayer culture exhibited the expression of TIMP-1 and MMP-1 genes. The expression levels of TIMP-1 gene remained the same throughout the three time points (Figure 8: Lanes 2 vs. 5 vs. 8). The expression levels of MMP-1 gene were about the same at 24 and 48 hours post-seeding (Figure 8: Lanes 3 vs. 6), however, the level decreased at 72 hours post-seeding (Figure 8: Lane 9).
The RT-PCR products of keratinocytes on gel electrophoresis (Figure 9) showed that the keratinocytes harvested at 24 hours post-seeding expressed both TIMP-1 (Figure 9: Lane 2) and MMP-1 (Figure 9: Lane 3). In summary, human normal keratinocytes in monolayer culture exhibit the expression of TIMP-1 and MMP-1 genes at various times, presumably due to cell replication at the periphery of cell clones.

**Expression of TIMP-1 and MMP-1 Genes in Six Cancer Cell Lines In Vitro**

Total cellular RNAs of six cancer cell lines described below were extracted for RT-PCR with the amplimer pairs for TIMP-1 and MMP-1, respectively. An amplimer pair for β-actin was used as a control [Nomura 1993]. The six cell lines were: human squamous cell carcinoma cell line, 83-01-82SCC (tongue); human squamous cell carcinoma cell line, 83-01-82CA-JC1 (tongue); human parotid adenocarcinoma cell line, 83-01-175; human tonsil oat cell carcinoma cell line, 83-05-45; human basal cell carcinoma cell line, 83-02-08; and the human chondrosarcoma cell line, Sarc 2. The RT-PCR products of these six lines on gel electrophoresis showed that TIMP-1 and MMP-1 messages were detected in the samples of the cell lines 83-01-82SCC (Figure 10: Lanes 2 and 3; also see Figure 11: Lane 2 and 3), 83-01-82CA-JC1 (Figure 10: Lanes 5 and 6), 83-01-175 (Figure 11: Lane 5 and 6), 83-02-08 (Figure 11: Lane 8 and 9), 83-05-45 (Figure 11: Lane 11 and 12) and Sarc 2 (Figure 11: Lane 14 and 15). In brief, all six cancer cell lines studied in monolayer culture exhibited the expression of TIMP-1 and MMP-1 genes.
Expression of TIMP-1 and MMP-1 Genes in Normal Skin and Cervix Tissues In Vivo

Total cellular RNAs of the human normal tissues sampled from skin and uterine cervix were extracted for RT-PCR with the amplimer pairs for TIMP-1 and MMP-1, respectively. An amplimer pair for β-actin was used as a control [Nomura 1993]. The RT-PCR products of normal skin and uterine cervix on gel electrophoresis showed that neither TIMP-1 nor MMP-1 was expressed by normal skin (Figure 12: Lane 2 and 3) or uterine cervix (Figure 12: Lane 5 and 6). The genomic TIMP-1 of the skin sample was amplified (Figure 12: Lane 2). To recapitulate, human skin and uterine cervix tissues in vivo did not exhibit the expression of TIMP-1 and MMP-1 genes.

Expression of TIMP-1 and MMP-1 Genes in Human Patient Tumors

Total cellular RNAs of human patient tumors sampled from 11 patients were extracted for RT-PCR with the amplimer pairs for TIMP-1 and MMP-1, respectively. An amplimer pair for β-actin was again used as a control [Nomura 1993]. The expression of TIMP-1 and MMP-1 genes in human patient tumors is presented in Table 8 and the RT-PCR products of 11 patient tumors on gel electrophoresis are shown in Figures 13, 14 and 15. Patient tumor # 1 exhibited the expression of TIMP-1, but not MMP-1. By contrast, patient tumors #’s 2, 3, 4, 5, 6, 8, 9 and 11 demonstrated the presence of MMP-1 mRNA, but not TIMP-1 mRNA. Patient tumors #’s 7 and 10 did not manifest the presence of either. In addition, patients #’s 2 and 4 exhibited the contamination of total cellular RNA by small amount of genomic DNA. In summation, only 1 of 11 human cancerous tissues exhibited the
expression of TIMP-1 gene and 8 of 11 cancerous tissues exhibited the expression of MMP-1 gene.

**Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors**

The xenograft tumors were generated as previously described in the Materials and Methods section of Chapter I. Total cellular RNAs of 18 xenograft tumors were extracted for RT-PCR with the amplimer pairs for TIMP-1 and MMP-1, respectively. An amplimer pair for β-actin was used here as a control [Nomura 1993]. The expression of TIMP-1 and MMP-1 genes in xenograft tumors is presented in Table 9 and the RT-PCR products of these 18 xenograft tumors on gel electrophoresis are displayed in Figures 16 through 22. All 18 xenograft tumors did not demonstrate the presence of either TIMP-1 mRNA or MMP-1 mRNA. Xenograft tumors #’s 1, 2, 4, 5, 6, 7, 8, 9, 13, 14, 16, 17 and 18 manifested the contamination of total cellular RNAs by a small amount of genomic DNA. Succinctly, none of the 18 xenograft tumors exhibited the expression of TIMP-1 or MMP-1 genes. Thirteen of the 18 xenograft tumors exhibited contamination by genomic DNA.

In closing, the expression of TIMP-1 and MMP-1 in the cells and tissues examined in this study is summarized in Table 10. In brief, two normal cell types (keratinocytes and fibroblasts) as well as six cancer cell lines in vitro exhibit the expression of TIMP-1 and MMP-1. Two normal tissue types (skin and uterine cervix) did not display the expression of TIMP-1 and MMP-1. Only one of 11 patient tumors demonstrated the expression of TIMP-1 and 8 of 11 patient tumors exhibited the
expression of MMP-1, whereas none of 18 xenograft tumors demonstrated the expression of TIMP-1 and MMP-1 genes.

**In Situ Localization of TIMP-1 mRNA**

The distribution of TIMP-1 mRNA was determined in normal laryngeal mucosa, laryngeal mucosa exhibiting pseudoepitheliomatous hyperplasia, laryngeal mucosa exhibiting moderate dysplasia, and in the deep margin of a laryngeal squamous cell carcinoma by using an *in situ* hybridization technique. The *in situ* hybridization results showed there was no NBT/BCIP dye deposited on the normal mucosa, which is indicative of the presence of TIMP-1 mRNA (Figure 23). An increased amount of the NBT/BCIP indicator dye was seen in the laryngeal mucosa exhibiting pseudoepitheliomatous hyperplasia (Figure 24). Heavy deposits of the indicator dye were observed in the basal and suprabasal cells (Figure 24). In addition, an increased amount of the NBT/BCIP indicator dye was observed in the laryngeal mucosa exhibiting moderate dysplasia (Figure 25). The indicator dye was deposited in the lower half of the mucosal epithelium, especially in the basal and suprabasal cells (Figure 25). However, in the deep margin of the laryngeal squamous cell carcinoma, no NBT/BCIP indicator dye was seen (Figures 26 and 27). In summation, the TIMP-1 mRNA was expressed by hyperplastic and dysplastic squamous epithelial cells (Figures 24 and 25), but not by normal squamous epithelial cells (Figure 23) and invasive squamous carcinoma cells (Figures 26 and 27).
DISCUSSION

Our understanding of the molecular events that govern the process of cancer invasion has historically been incomplete due to the difficulty in obtaining sufficient amounts of clinical cancerous materials for conventional analyses such as Northern blot analysis. The technical advance of reverse transcription-polymerase chain reaction (RT-PCR) has allowed us to overcome such limitations. RT-PCR is a rapid and highly sensitive method for the detection of mRNA in a very small quantity of sample [Rappolee 1988a]. It is sensitive down to 10 copies of mRNA transcript and can detect mRNA in a single cell [Rappolee 1988a, 1988b, 1989]. In the present study, approximately 0.2g of cancerous tissue from each case was used for RT-PCR analysis. This small quantity provided a tremendous amount of information which was previously not demonstrable.

Using the RT-PCR technique, in conjunction with histopathologic examination, the molecular events (i.e. MMP-1 mRNA) associated with the process of cancer invasion and massive tissue destruction were examined. The histopathologic comparisons between patient tumors and xenograft tumors in Chapter I revealed that patient tumors exhibited invasiveness and massive tissue destruction in the human host, whereas xenograft tumors in the nude mouse exhibited encapsulated non-invasive nodules without tissue destruction. The current RT-PCR study demonstrates the presence of MMP-1 mRNA in 8 of 11 patient tumors (Table 8) and the absence of MMP-1 mRNA in all 18 xenograft tumors (Table 9). The results of the current RT-PCR studies are correlated with the histopathologic observations in
Chapter I concerning the non-invasive nature of xenograft tumors and the invasiveness of patient tumors. The presence of MMP-1 mRNA in patient tumors was associated with tumor invasion and massive tissue destruction; and the absence of MMP-1 mRNA in xenograft tumors with non-invasiveness. Such correlations suggest that MMP-1 mRNA may be involved in the process of cancer invasion. To confirm the supposition, there was no MMP-1 mRNA detected in normal skin and uterine cervix (Figure 12). This is further evidence suggestive of a role of MMP-1 mRNA in the process of cancer invasion. It has been reported that there is an extensive collagen destruction in the vicinity of invading tumor cells of many human and animal malignancies [Tarin 1967 & 1969]. Interstitial collagenase (MMP-1 protein) is the only proteinase known to date that is capable of extracellular collagen degradation at physiological pH [Brickerhoff 1992]. It is quite likely that MMP-1 protein is the proteinase responsible for the destructive changes in tissues that are observed morphologically adjacent to tumors. There is cumulative evidence correlating the expression of type IV collagenases with the progression of invasive tumors [Liotta 1986; Stetler-Stevenson 1990; Tryggvason 1993]. However, the study on the participation of MMP-1 protein/mRNA in cancer invasion and massive tissue destruction has been sporadically reported [Abramson 1975; Tarin 1982; Muller 1991]. In a separate study, a series of 14 human squamous cell carcinomas of the oral cavity and larynx produced a collagen-dissolving enzyme in vitro as demonstrated by the breakdown of $^{14}$C-labeled collagen [Abramson 1975]. Oral and laryngeal squamous cell carcinomas showed a higher collagenolytic activity than uninvolved mucosa from the same patients [Abramson 1975]. Northern
blot analysis on total RNAs prepared from 107 human head and neck squamous cell carcinomas and 26 lung carcinomas demonstrated the frequent and concomitant over-expression of the MMP-1 gene in the cancerous tissues as compared to their corresponding normal tissue samples [Muller 1991]. Similar findings have also been observed in laboratory animals. Naturally-occurring murine mammary tumor cells were reported to secrete a mammalian collagenase active against type I collagen [Tarin 1982]. The tumors of high pulmonary-colonization potential secreted significantly more of this enzyme than those with low pulmonary-colonization potential, or non-neoplastic proliferating (e.g. lactating) mammary tissue [Tarin 1982]. The findings in this study together with the observations by others [Abramson 1975; Tarin 1982; Muller 1991] suggest that MMP-1 protein/mRNA is involved in the process of cancer invasion.

In addition to the process of invasion, it is reasonable to believe that cancer cells also must proliferate in order to build up the critical tumor mass to become clinically detectable. In the current study, the relationship between cell proliferation and the expression of MMP-1 gene was evaluated in monolayer cells in vitro by using the RT-PCR technique. Although the RT-PCR protocol in this study is not designed for quantitative purposes, the expression levels of MMP-1 gene by normal fibroblasts harvested 24 and 48 hours post-seeding were roughly the same, however the expression level of MMP-1 gene by normal fibroblasts at confluent dense culture (i.e. 72 hours post-seeding) decreased dramatically (Figure 8). It is well known that normal cells exhibit contact inhibition in monolayer culture at confluence, but in
contrast, cancer cells in \textit{in vitro} culture lose the feature of contact inhibition and continue to proliferate at confluence. In this context, all six cancer cell lines that were harvested at confluent dense population exhibited the expression of MMP-1 gene (Figures 10 and 11). These findings suggest that expression of MMP-1 gene is closely associated with cell proliferation. In addition, both embryonic tissues and adult remodeling tissues that undergo extreme cell proliferation, also exhibit the expression of MMP-1 gene [Brenner 1989a; Brännström 1988; Brown 1989; Chowcat 1988; Dean 1985; Delaisse 1988; Flenniken 1990; Hayakawa 1992; Hembry 1986; Jeffrey 1991; Koob 1974; Nakanishi 1986; Nomura 1989; Rajabi 1988 & 1990; Ryan 1971; Saarialho-Kere 1992 & 1993; Seller 1978; Woessner 1988]. The observations in the present studies also support the concept that the expression of MMP-1 gene is associated with cell proliferation.

By definition, all human malignant tumors are invasive while in their native environment (e.g. human environment), however once these human malignant tumors are xenotransplanted into nude mice, xenograft tumors become non-invasive and form encapsulated nodules. If we accept the concept that MMP-1 protein/mRNA is involved in the process of cancer invasion, then the absence of MMP-1 mRNA in xenograft tumors may be the molecular evidence for the loss of the invasive phenotype by the xenograft tumors.

MMP-1 gene expression is inducible by the stimulating signals of interleukin-1\(\alpha\), \(\beta\) [Lyons 1991; MacNaul 1990], tumor necrosis factor-\(\alpha\) [Brenner 1989b, Dayer 1985,
MacNaul 1990], platelet-derived growth factor [Bauer 1985], transforming growth factor-α [Lyons 1991], epidermal growth factor [Chua 1985] and basic fibroblast growth factor [Edwards 1987]. Repression of MMP-1 has been related to the inhibitory signals of transforming growth factor-β [Edwards 1987], glucocorticoids [Brinckerhoff 1986], progesterone [Newsome 1977], prostaglandin E₂ [Lyons 1991] and interferon-γ [Andrews 1990; Wahl 1990]. These signals exist in the normal environment of the cells. The nude mouse environment is not identical to the human environment, therefore it is reasonable to believe that the physiologic conditions and the quantities of these inducing or inhibitory signals (growth factors, cytokines and/or hormones regulating the expression of MMP-1 gene in the nude mouse environment) are different from those of the human environment. The human cancer cells that are relocated into nude mice apparently do not respond to these signals in the same way as they do in their native environment. The absence of MMP-1 mRNA in xenograft tumors may be due to inadequate stimulating signals or excessive inhibitory signals from the nude mouse environment.

Using RT-PCR in conjunction with the in situ hybridization technique, the molecular events (e.g. TIMP-1 mRNA) that are associated with cancer invasion were further explored. Histopathologic examination of patient tumors in Chapter I revealed that there was massive tissue invasion by patient tumors in the natural host. The process of invasion, however, is not unique to cancer. It can be observed in many physiologic conditions such as embryonic morphogenesis and adult tissue remodelling [Brown 1989; Nomura 1989; Saarialho-Kere 1992 & 1993]. There is
a great body of evidence showing the expression of TIMP-1 genes in embryonic
growth and differentiation [Brenner 1989a; Brown 1989; Nomura 1989], skeletal
growth and remodelling [Dean 1985; Delaisse 1988; Flenniken 1990; Nomura 1989;
Seller 1978], salivary gland development [Hayakawa 1992; Nakanishi 1986], tooth
germ development [Nomura 1989], ovary development [Nomura 1989], wound
healing [Chowcat 1988; Hembry 1986; Saarialho-Kere 1992 & 1993], ovulation
[Brännström 1988], parturition [Rajabi 1988 & 1990; Jeffrey 1991] and uterine post-
partum involution [Koob 1974; Ryan 1971; Woessner 1988]. However, in the
present study, only one of 11 patient tumors exhibited the expression of TIMP-1
gene upon RT-PCR examination (Table 8). This indicates that there was decreased
expression of TIMP-1 gene in patient tumors that were competent for cancer
invasion.

In this study, the role of TIMP-1 gene in cancer invasion was further studied by
using in situ hybridization techniques. The distribution of TIMP-1 mRNA in normal
laryngeal mucosa, laryngeal mucosa exhibiting pseudoepitheliomatous hyperplasia,
laryngeal mucosa exhibiting moderate dysplasia, and the deep margin of a
laryngeal squamous cell carcinoma were examined. The results showed there was
no TIMP-1 mRNA found in the normal mucosal epithelial cells of the larynx (Figure
23) but an increased amount of the TIMP-1 mRNA was exhibited in the laryngeal
mucosal cells exhibiting moderate dysplasia (Figure 25). No TIMP-1 mRNA was
demonstrated in the cancer cell nests of the deep margins of laryngeal squamous
cell carcinoma (Figures 26 and 27). To recapitulate in a sequential manner, the
TIMP-1 gene was silent in normal tissue, extremely active in the premalignant lesion (i.e. dysplasia) and back to silent again in the full-blown carcinoma (i.e. deep margin of a squamous cell carcinoma). These observations suggest that the TIMP-1 gene expression appears to be inversely related to the advancing stage of carcinogenesis. An inverse relationship between TIMP-1 protein levels and invasive potential has been observed in human intracranial tumors [Halaka 1983] and in many different cell lines of the mouse [Hichs 1984; Ponton 1991]. Invasive human meningiomas tended to produce less TIMP-1 protein than noninvasive meningiomas [Halaka 1983]. Similar relationships were observed in many murine models. TIMP-1 protein secreted by highly invasive murine fibrosarcoma cells was decreased 10- to 20-fold when compared with normal fibroblasts or poorly invasive fibrosarcoma cells [Hichs 1984]. An 2.5-fold decrease of TIMP-1 mRNA was observed in metastatic SP1 murine mammary adenocarcinoma cells as compared to their non-metastatic equivalent [Ponton 1991]. The findings in this study and the observations by others [Halaka 1983; Hichs 1984; Ponton 1991] insinuate that TIMP-1 gene expression is indeed inversely related to the advancing stage of carcinogenesis.

The role of the TIMP-1 gene in xenograft tumors was also studied by using RT-PCR technique. The studies in Chapter I revealed that patient tumors were invasive, whereas xenograft tumors in the nude mouse lost the invasive phenotype. Although the RT-PCR studies showed that only one of 11 patient tumors and none of 18 xenograft tumors exhibited the expression of TIMP-1 gene, the data did not provide
additional insight into the nature of the loss of the invasive phenotype by xenograft tumors. RT-PCR is a highly sensitive technique that can detect as few as 10 copies of mRNA transcripts [Rappolee 1988a, 1988b, 1989]. Needless to say, any trace amount of DNA/RNA contaminant may become visible upon RT-PCR analysis. One potential contaminant may come from the genomic DNA. During the process of total RNA preparation, genomic DNA cannot be completely removed. Such genomic DNA contaminants may then be amplified upon RT-PCR [Rappolee 1988a, 1988b, 1989]. The easiest method to avoid amplification of such unavoidable genomic DNA contaminants is to design the amplimer pair in such way that the amplimers cover the junctions between exons so that the intron-containing genomic DNA would not be amplified [Rappolee 1988a, 1988b, 1989].

In the current study, the amplimer pair for TIMP-1 was originally designed by Nomura et al. [1993]. Using this amplimer pair for the analysis of 18 xenograft tumors, 13 of 18 PCR products on gel electrophoresis exhibited an extra 1626bp band. This 1626bp PCR product was digestible by \textit{Hin}f I and \textit{Apa} I restriction enzymes and consistent with the amplified genomic DNA of TIMP-1 by the adumbrated size and restriction enzyme digestion pattern (Figure 7). It appears that both the 5'-amplimer and the 3'-amplimer do not cover the exon junctions, so that a genomic DNA fragment of 1626bp in length was amplified. Interestingly, this 1626bp PCR product was observed mostly in the samples of tumor tissue. It was only observed occasionally in the samples of monolayer cultured cells. Thus, it appeared that there was a higher chance of genomic DNA contamination in total
RNAs prepared from tumor tissue. During the process of total RNA preparation, the tumor tissues were homogenized manually, whereas the monolayer cells were collected in cell suspension. Apparently the homogenization procedure damaged the nuclear membrane and released genomic DNA fragments into the crude cell extract. In many occasions (Figure 10: Lane 1; Figure 11: Lanes 2, 5, 14; and Figure 13: Lane 1), both 1626bp and 386bp RT-PCR products were present in the same samples suggesting that this 1626bp fragment did not interfere with amplification of the 386bp fragment. Although this 1626bp extra PCR product demonstrated the contamination of genomic DNA, it serves as a quality control mechanism for the RT-PCR procedure for TIMP-1. The presence of this 1626bp product indicates that the RT-PCR for TIMP-1 was working and that the absence of a 386bp (e.g. the PCR fragment of TIMP-1 mRNA) in these 13 xenograft tumors was not due to a poor RT-PCR technique; they were truly negative.

The interaction between TIMP-1 and MMP-1 proteins has been demonstrated biochemically [Murphy 1991b]. TIMP-1 protein inactivates MMP-1 protein by forming a 1:1 stoichiometric complex with a high-affinity $K_d=10^{-9}$ to $10^{-11}$ M [Murphy 1991b]. Biologically, the expression of TIMP-1 and MMP-1 genes is in a highly organized relationship. Immunolocalization studies of TIMP-1 and MMP-1 gene expression have demonstrated that in the developing growth of the young rabbit, TIMP-1 protein was seen in certain regions of the tissue where there was elevated expression of MMP-1 protein [Brown 1989]. A rabbit wound-healing study showed that MMP-1 was expressed within 12 hours, whereas TIMP-1 was not expressed
until 24 hours [Chowcat 1988]. In addition, in a study of human hypertrophic scar tissue by indirect immunofluorescence for the presence of MMP-1 and TIMP-1 proteins, a significant extracellular immunoprecipitation of both MMP-1 and TIMP-1 was found in the hypertrophic scar areas undergoing active remodelling, whereas inactive areas were mainly negative [Hembry 1986]. The degradation and restructuring of the extracellular matrix thus are normally well-controlled activities characterized by balanced MMP-1/TIMP-1 expression both spatially and temporally. By contrast, invasive human meningiomas tended to produce less TIMP-1 protein than noninvasive meningiomas, whereas cultures of dura from an invasive meningioma and cultures of bone involved by a meningioma produced MMP-1 protein [Halaka 1983]. In the present study, 8 of 11 patient tumors exhibited the expression of MMP-1 gene, whereas only one of 11 patient tumors exhibited the expression of TIMP-1 gene. TIMP-1 mRNA was also localized in premalignant dysplastic cells, but not in the full-blown invasive cancer cells. The data clearly demonstrate that the process of cancer invasion is closely related to uncontrolled collagenolytic activity resulting from upregulated MMP-1 gene and downregulated TIMP-1 gene.
CONCLUSION

During the biological process of embryonic morphogenesis and adult tissue remodelling, the expression of MMP-1 and TIMP-1 genes is highly organized. The proliferating cells in embryonic and adult remodelling tissues express the MMP-1 gene for synthesis, assembly, and turnover of the extracellular matrix macromolecules. This synthesis/assembly/turnover process by MMP-1 is negatively regulated by TIMP-1. This well-balanced TIMP-1/MMP-1 interaction is a fundamental feature of the normal development, growth and maintenance of an organ. An aberration of this TIMP-1/MMP-1 balance appears to be associated with the stage of cancer invasion. An unbalanced TIMP-1/MMP-1 ratio favors a surplus MMP-1, resulting in tissue invasion and massive tissue destruction. This unbalanced TIMP-1/MMP-1 may be due to downregulated TIMP-1 and/or upregulated MMP-1. In either case, one would expect increased collagenolytic activity, destruction of interstitial stroma, and invasion of tumor cells into the surrounding tissues.
Table 7. Amplimers and Their Product Lengths

<table>
<thead>
<tr>
<th>Amplimer</th>
<th>Sequence</th>
<th>Tm</th>
<th>GC%</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1 [Templeton 1990]</td>
<td>F 5'-cat cca agc cat ata tgg acg ttc c-3'</td>
<td>66.4</td>
<td>48%</td>
<td>611</td>
</tr>
<tr>
<td></td>
<td>R 5'-tct gga gag tca aaa ttc tct tcg t-3'</td>
<td>61.9</td>
<td>40%</td>
<td>(42%)</td>
</tr>
<tr>
<td>TIMP-1 [Dochesty 1985]</td>
<td>F 5'-agt caa cca gac cac ctt ata cca-3'</td>
<td>61.0</td>
<td>46%</td>
<td>386</td>
</tr>
<tr>
<td></td>
<td>R 5'-ttt cag agc ctt gga gga gct ggt c-3'</td>
<td>69.9</td>
<td>56%</td>
<td>(54%)</td>
</tr>
<tr>
<td>pNMH-aT [Edwards 1986]</td>
<td>F 5'-act ccg tag ctc cag ctt ca-3'</td>
<td>60.2</td>
<td>55%</td>
<td>534</td>
</tr>
<tr>
<td></td>
<td>R 5'-att caa ggc tgt ggg aaa tg-3'</td>
<td>59.9</td>
<td>60%</td>
<td>(51%)</td>
</tr>
<tr>
<td>β-Actin [Ponte 1984]</td>
<td>F 5'-gcg aga aga tga ccc aga tca tgt t-3'</td>
<td>66.8</td>
<td>48%</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>R 5'-gct tct cct taa tgt cac gca cga t-3'</td>
<td>66.7</td>
<td>48%</td>
<td>(60%)</td>
</tr>
</tbody>
</table>

F= 5'-amplimer, R= 3'-amplimer, Tm= melting temperature, GC%= GC content, Length= PCR product length
Table 8. Expression of TIMP-1 and MMP-1 Genes in Human Patient Tumors

<table>
<thead>
<tr>
<th>Case No.</th>
<th>TIMP-1 mRNA</th>
<th>MMP-1 mRNA</th>
<th>Reference</th>
<th>Figure</th>
<th>Lanes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td></td>
<td>13</td>
<td>1 and 2</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td></td>
<td>13</td>
<td>4 and 5</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td></td>
<td>13</td>
<td>7 and 8</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td></td>
<td>13</td>
<td>10 and 11</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td></td>
<td>14</td>
<td>1 and 2</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>+</td>
<td></td>
<td>14</td>
<td>4 and 5</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td></td>
<td>14</td>
<td>7 and 8</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td></td>
<td>14</td>
<td>10 and 11</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>+</td>
<td></td>
<td>15</td>
<td>1 and 2</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td></td>
<td>15</td>
<td>4 and 5</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>+</td>
<td></td>
<td>15</td>
<td>7 and 8</td>
</tr>
</tbody>
</table>
Table 9. Expression of TIMP-1 and MMP-1 Genes in Xenograft Tumors

<table>
<thead>
<tr>
<th>Case No.</th>
<th>TIMP-1 mRNA</th>
<th>MMP-1 mRNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Figure</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>-</td>
<td>22</td>
</tr>
</tbody>
</table>
Table 10. Summary of Expression of TIMP-1 and MMP-1 Genes

<table>
<thead>
<tr>
<th></th>
<th>TIMP-1</th>
<th>MMP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Cells</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Cancer Cells</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Normal Tissues</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Human Tumors</td>
<td>1/11</td>
<td>8/11</td>
</tr>
<tr>
<td>Xenograft Tumors</td>
<td>0/18</td>
<td>0/18</td>
</tr>
</tbody>
</table>
Figure 5. A restriction map of TIMP-1 mRNA. Upper panel represents a diagrammatic restriction map of the TIMP-1 mRNA fragment showing two restriction points that were examined in this study. Lower panel represents a gel electrophoresis of TIMP-1 mRNA RT-PCR product and its restriction digested products. The RT-PCR product, using an amplimer pair specific for TIMP-1 mRNA, was digested with the restriction enzymes indicated in the upper panel and its digested fragments separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lane 2 represents undigested RT-PCR product. Lane 3 represents HinfI digestion. Lane 4 represents HhaI digestion.
Figure 6. A restriction map of MMP-1 mRNA. Upper panel represents a diagrammatic restriction map of the MMP-1 mRNA fragment showing two restriction points that were examined in this study. Lower panel represents a gel electrophoresis of MMP-1 mRNA PCR product and its restriction digested products. The RT-PCR product, using an amplimer pair specific for MMP-1 mRNA, was digested with the restriction enzymes indicated in the upper panel and its digested fragments separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lane 2 represents undigested RT-PCR product. Lane 3 represents Hha I digestion. Lane 4 represents Sca I digestion.
Figure 6
Figure 7. A Restriction map of TIMP-1 genomic DNA. Upper panel represents a diagrammatic restriction map of the TIMP-1 genomic DNA fragment showing two restriction points that were examined in this study. Lower panel represents a gel electrophoresis of restriction digested TIMP-1 genomic DNA PCR product. The PCR product, using an amplimer pair for TIMP-1, was digested with the restriction enzymes indicated in the upper panel and its digested fragments separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represents Hinf I digestion. Lane 2 represents Apa I digestion. Lane 3 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 7
Figure 8. The expression of TIMP-1 and MMP-1 genes in primary fibroblast cultures analyzed by RT-PCR technique. Total cellular RNA of normal fibroblast primary cultures harvested at 24 hours (Lanes 2-4), 48 hours (Lanes 5-7) and 72 hours (Lanes 8-10) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lanes 2, 5 and 8 represent the TIMP-1 PCR product. Lanes 3, 6 and 9 represent the MMP-1 PCR product. Lanes 4, 7 and 10 represent the β-actin PCR product.
Figure 9. The expression of TIMP-1 and MMP-1 genes in keratinocyte primary cultures analyzed by RT-PCR technique. Total cellular RNA of normal keratinocyte primary cultures harvested at 24hr was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lane 2 represents the TIMP-1 PCR product. Lane 3 represents the MMP-1 PCR product. Lane 4 represents the β-actin PCR product.
Figure 10. The expression of TIMP-1 and MMP-1 genes in the cell lines 83-01-82SCC and 83-01-82CA-JC1 analyzed by RT-PCR technique. Total cellular RNA of the cell lines 83-01-82SCC (Lanes 2-4) and 83-01-82CA-JC1 (Lanes 5-7) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lanes 2 and 5 represent the TIMP-1 PCR product. Lanes 3 and 6 represent the MMP-1 PCR product. Lanes 4 and 7 represent the β-actin PCR product.
Figure 11. The expression of TIMP-1 and MMP-1 genes in the cell lines 83-01-82SCC, 83-01-175, 83-02-08, 83-05-45 and Sarc 2 analyzed by RT-PCR technique. Total cellular RNA of the cell lines 83-01-82SCC (Lanes 2-4), 83-01-175 (Lanes 5-7), 83-02-08 (Lanes 8-10), 83-05-45 (Lanes 11-13) and Sarc 2 (Lanes 14-16) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lanes 2, 5, 8, 11 and 14 represent the TIMP-1 PCR product. Lanes 3, 6, 9, 12 and 15 represents the MMP-1 PCR product. Lanes 4, 7, 10, 13 and 16 represent the β-actin PCR product.
Figure 12. The expression of TIMP-1 and MMP-1 genes in normal skin and uterine cervix analyzed by RT-PCR technique. Total cellular RNA of normal skin (Lanes 2-4) and uterine cervix (Lanes 5-7) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lane 1 represent a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lanes 2 and 5 represent the TIMP-1 PCR product. Lanes 3 and 6 represent the MMP-1 PCR product. Lanes 4 and 7 represent the β-actin PCR product.
Figure 13. The expression of TIMP-1 and MMP-1 genes in patient tumors #1-#4 analyzed by RT-PCR technique. Total cellular RNA of patient tumors #1 (Lanes 1-3), #2 (Lanes 4-6), #3 (Lanes 7-9) and #4 (Lanes 10-12) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1, 4, 7 and 10 represent the TIMP-1 PCR product. Lanes 2, 5, 8 and 11 represent the MMP-1 PCR product. Lanes 3, 6, 9 and 12 represent the β-actin PCR product. Lane 13 represent a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 13
Figure 14. The expression of TIMP-1 and MMP-1 genes in patient tumors #5-#8 analyzed by RT-PCR technique. Total cellular RNA of patient tumors #5 (Lanes 1-3), #6 (Lanes 4-6), #7 (Lanes 7-9) and #8 (Lanes 10-12) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1, 4, 7 and 10 represent the TIMP-1 PCR product. Lanes 2, 5, 8 and 11 represent the MMP-1 PCR product. Lanes 3, 6, 9 and 12 represent the β-actin PCR product. Lane 13 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 14
Figure 15. The expression of TIMP-1 and MMP-1 genes in patient tumors #9-#11 analyzed by RT-PCR technique. Total cellular RNA of patient tumors #9 (Lanes 1-3), #10 (Lanes 4-6), and #11 (Lanes 7-9) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1, 4 and 7 represent the TIMP-1 PCR product. Lanes 2, 5 and 8 represent the MMP-1 PCR product. Lanes 3, 6 and 9 represent the β-actin PCR product. Lane 10 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 16. The expression of TIMP-1 and MMP-1 genes in xenograft tumors #1-#3 analyzed by RT-PCR technique. Total cellular RNA of xenograft tumors #1 (Lanes 1-3), #2 (Lanes 4-7) and #3 (Lanes 7-9) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1, 4 and 7 represent the TIMP-1 PCR product. Lanes 2, 5 and 8 represent the MMP-1 PCR product. Lanes 3, 6 and 9 represent the β-actin PCR product. Lane 10 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 17. The expression of TIMP-1 and MMP-1 genes in xenograft tumors #4-#6 analyzed by RT-PCR technique. Total cellular RNA of xenograft tumors #4 (Lanes 1-3), #5 (Lanes 4-7) and #6 (Lanes 7-9) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1, 4 and 7 represent the TIMP-1 PCR product. Lanes 2, 5 and 8 represent the MMP-1 PCR product. Lanes 3, 6 and 9 represent the β-actin PCR product. Lane 10 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 18. The expression of TIMP-1 and MMP-1 genes in xenograft tumors #7-#9 analyzed by RT-PCR technique. Total cellular RNA of xenograft tumors #7 (Lanes 1-3), #8 (Lanes 4-7) and #9 (Lanes 7-9) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1, 4 and 7 represent the TIMP-1 PCR product. Lanes 2, 5 and 8 represent the MMP-1 PCR product. Lanes 3, 6 and 9 represent the β-actin PCR product. Lane 10 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 19. The expression of TIMP-1 and MMP-1 genes in xenograft tumors #10-
#12 analyzed by RT-PCR technique. Total cellular RNA of xenograft tumors #10
(Lanes 1-3), #11 (Lanes 4-7) and #12 (Lanes 7-9) was analyzed using RT-PCR
priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An
amplimer pair for β-actin was used as a control. These RT-PCR products were
separated on a 3% Wide Range Agarose gel that was then stained with ethidium
bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure
described in the Materials and Methods in Chapter II). Lane 1 represents a DNA
size marker, the size of the standard fragments in the region of interest are shown
to the left. Lanes 2, 5 and 8 represent the TIMP-1 PCR product. Lanes 3, 6 and
9 represent the MMP-1 PCR product. Lanes 4, 7 and 10 represent the β-actin PCR
product.
Figure 20. The expression of TIMP-1 and MMP-1 genes in xenograft tumors #13 and #14 analyzed by RT-PCR technique. Total cellular RNA of xenograft tumors #13 (Lanes 1-3) and #14 (Lanes 4-7) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1 and 4 represent the TIMP-1 PCR product. Lanes 2 and 5 represent the MMP-1 PCR product. Lanes 3 and 6 represent the β-actin PCR product. Lane 7 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 21. The expression of TIMP-1 and MMP-1 genes in xenograft tumors #15 and #16 analyzed by RT-PCR technique. Total cellular RNA of xenograft tumors #15 (Lanes 1-3) and #16 (Lanes 4-7) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1 and 4 represent the TIMP-1 PCR product. Lanes 2 and 5 represent the MMP-1 PCR product. Lanes 3 and 6 represent the β-actin PCR product. Lane 7 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 22. The expression of TIMP-1 and MMP-1 genes in xenograft tumors #17 and #18 analyzed by RT-PCR technique. Total cellular RNA of xenograft tumors #14 (Lanes 1-3) and #15 (Lanes 4-7) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter II). Lanes 1 and 4 represent the TIMP-1 PCR product. Lanes 2 and 5 represent the MMP-1 PCR product. Lanes 3 and 6 represent the β-actin PCR product. Lane 7 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 23. *In situ* hybridization against TIMP-1 mRNA in normal laryngeal mucosa.

*In situ* hybridization was performed on a frozen section of normal laryngeal mucosa using a biotinylated TIMP-1 cDNA probe. The hybridized signal was then visualized with indicator dye NBT/BCIP (Procedure described in the Materials and Methods in Chapter II). This figure shows no deposit of fine NBT/BCIP granules in the mucosal epithelial cells. (Original magnification= 160X)
Figure 23
Figure 24. *In situ* hybridization against TIMP-1 mRNA in laryngeal mucosa exhibiting pseudoepitheliomatous hyperplasia. *In situ* hybridization was performed on a frozen section of laryngeal mucosa exhibiting pseudoepitheliomatous hyperplasia using a biotinylated TIMP-1 cDNA probe. The hybridized signal was then visualized with indicator dye NBT/BCIP (Procedure described in the Materials and Methods in Chapter II). This figure shows deposits of the fine NBT/BCIP granules in the entire thickness of the mucosal epithelium. Heavy deposits were observed in the basal and suprabasal cells. (Original magnification= 160X)
Figure 24
Figure 25. *In situ* hybridization against TIMP-1 mRNA in laryngeal mucosa exhibiting moderate dysplasia. *In situ* hybridization was performed on a frozen section of laryngeal mucosa exhibiting moderate dysplasia using a biotinylated TIMP-1 cDNA probe. The hybridized signal was then visualized with indicator dye NBT/BCIP (Procedure described in the Materials and Methods in Chapter II). This figure shows deposits of the fine NBT/BCIP granules in the lower half of the mucosal epithelium. Heavy deposits were observed in the basal and suprabasal layers. (Original magnification= 160X)
Figure 25
Figure 26. *In situ* hybridization against TIMP-1 mRNA in the cancer cell nests of the deep margin of a squamous cell carcinoma. *In situ* hybridization was performed on a frozen section of the deep margin of a squamous cell carcinoma using a biotinylated TIMP-1 cDNA probe. The hybridized signal was then visualized with indicator dye NBT/BCIP (Procedure described in the Materials and Methods in Chapter II). This figure shows no deposition of NBT/BCIP in the cancer cell nests. (Original magnification= 160X)
Figure 27. *In situ* hybridization against TIMP-1 mRNA in other cancer cell nests of the deep margin of a squamous cell carcinoma. *In situ* hybridization was performed on a frozen section of the deep margin of a squamous cell carcinoma using a biotinylated TIMP-1 cDNA probe. The hybridized signal was then visualized with indicator dye NBT/BCIP (Procedure described in the Materials and Methods in Chapter II). This figure shows minimal deposition of fine NBT/BCIP granules in the cancer cell nests. (Original magnification= 160X)
INTRODUCTION

The studies presented in Chapter II revealed that the expression of MMP-1 mRNA was predominantly associated with an invasive phenotype, and the production of TIMP-1 mRNA was associated with hyperplastic and premalignant tissues. These findings suggested that the invasive phenotype may be controlled by the relationship that is established between the expression of MMP-1 and TIMP-1 genes. The transcriptional and translational controls of the expression of these two genes have been purported to be independent of each other, however, the protein products of these two genes were shown to be antagonistic. TIMP-1 protein inhibits the function of MMP-1 protein by forming a 1:1 stoichiometric irreversible complex [Murphy 1991b]. If MMP-1 protein/mRNA is involved in the active process of cancer invasion, then to decrease the formation of TIMP-1 protein by decreasing the expression of the TIMP-1 gene may produce an invasive phenotype.

In a recent report, a murine TIMP-1 antisense construct, pNMH-aT, was demonstrated to cause nontumorigenic/non-invasive Swiss 3T3 cells to form
progressively growing tumors and metastases in nude mice [Khokha 1989]. It is known that the protein product of the TIMP-1 gene negatively regulates the behavior of the collagenase protein [Murphy 1991b]. In the reports described above, it appears that the inhibition of TIMP-1 gene expression by antisense TIMP-1 decreased the production of TIMP-1 protein, thereby indirectly liberating extracellular collagenase protein. This liberation and cellular release of mature collagenase increased the invasiveness of progressively growing cancer cells. Thus, the antisense construct pNMH-aT provides a good model to study the role of TIMP-1 and MMP-1 in cancer invasion.

In the experiments described below, the construct, pNMH-aT, was transfected into a squamous cell carcinoma cell line 83-01-82CA-JC1 to study the putative role of antisense TIMP-1 mRNA in the downregulation of the TIMP-1 gene. The cell line 83-01-82CA-JC1 is tumorigenic but non-invasive when tested in the nude mouse system.

MATERIALS AND METHODS

Plasmid Amplification

The plasmid construct pNMH-aT was a gift from Dr. David T. Denhardt [1992]. This construct is a pBR322-based expression vector containing a neomycin resistant gene and a murine MT-1 promoter (metallothionein-1 promoter) driven antisense TIMP-1 cDNA (Figure 28). This antisense TIMP-1 insert was genetically removed to become pNMH (This procedure was carried out by Dr. Dawei Li), as a negative
control in this study. The plasmids were then amplified in *E.Coli* strain HB101 following the procedure previously described [Sambrook 1989a]. Briefly, the transformed HB101 containing plasmid pNMH-aT or pNMH was cultured in terrific broth in the presence of ampicillin (100μg/ml) at 37°C overnight. The collection of transformed bacteria was lysed by lysozyme (1mg/ml) in 10mM Tris (pH 8.0) and the plasmid was then precipitated in an equal volume of ice-cold isopropanol. The pellet was stored in sterile 0.1X TE until used in the transfection protocol.

**Transfection (Lipofectin Method)**

The transfection protocol was carried out using lipofectin reagent (BRL) as described in the manufacturer's protocol [Felgner 1987]. Briefly, the monolayer cells, 83-01-82CA-JC1, at 30-50% confluence were overlaid with 2 ml serum-free MEM (containing 15 μg plasmid DNA and lipofectin 35 μl) and cultured at 37°C, 4% CO₂ overnight. After 16-24 hours, the experimental medium was replaced with fresh MEM (10% fetal bovine serum) and the cells were grown under the same conditions for two more days. Neomycin (G418 sulfate) (400μg/ml) selection against neomycin-resistant transfectants was begun on day four.

**Genomic DNA Isolation (Phenol Extraction Method)**

After the transfection protocol, the genomic DNA of the transfectants was isolated in order to determine whether integrated plasmid DNA was present. Genomic DNAs were isolated as previously described [Sambrook 1989b]. Briefly, the cell membranes of harvested cells were disrupted with 1 mM Nonidet P-40 and the
naked nuclei collected for proteinase K digestion (0.2 mg/ml in 0.5% SDS) at 37°C for three hours. Genomic DNA was then extracted from the digested nuclei using a phenol/chloroform/isoamyl alcohol mixture and precipitated by using 100% ethanol.

**Total Cellular RNA Extraction and Reverse Transcription (RT)**

Parallel to the examination of integrated plasmid DNA, the expression level of the integrated plasmid DNA was also examined. This technique incorporates a micro-procedure for isolating total cellular RNA and reverse transcription of the total cellular RNA. Thus, only the mRNA in the total cellular RNA is reverse transcribed to produce cDNA [Brenner 1989c]. Briefly, total cellular RNA was extracted from cultured cells by boiling the cells for 5 minutes and IMMEDIATELY transferring 64μl of the supernatant to a 31μl reverse transcription reaction mix. The reverse transcription was carried out with 5μl Mo-MLV reverse transcriptase (200U/μl) and 1.25μg oligo(dT) priming at 42°C for 45 minutes in the presence of 50mM KCl, 1.5mM MgCl₂, 1mM dNTP mix and the RNase inhibitor, RNasin 100U. Immediately after the reverse transcription (RT), the RT product was stored at -20°C until use.

**Polymerase Chain Reaction (PCR)**

The RT product was then amplified using PCR in the presence of the thermostable *Taq* DNA polymerase. The technique was performed as previously described [Rappolee 1989]. Briefly, one μl of RT product was mixed with one unit of *Taq* DNA polymerase (BRL) and 5 pmoles of amplimer pairs specific for the mRNA of interest.
(see the next section: Amplimers and Their Product Lengths) in a buffer containing 20mM Tris-HCl (pH 8.3), 1.5mM MgCl₂, 50mM KCl and 0.2mM dNTP mix, in a 25-μl volume. The mixture was overlaid with one drop of mineral oil to prevent evaporation and amplified by a thermocycler (Perkin-Elmer Cetus) in repeated three-temperature cycle (94°C for 1 minute, 60°C for 2 minutes and 72°C for 3 minutes) for 35 cycles. The PCR product was then stored at 4°C until use.

**Amplimers and Their Product Lengths**

The amplimer pairs used in this study included the amplimers for MMP-1, TIMP-1 and antisense TIMP-1. In addition, an amplimer pair for β-actin was used here as a control [Nomura 1993]. Amplimers were designed according to the methods outlined by Rappolee et al. [1989]. The structures of the amplimers used in this study were designed from known sequences published in the Genbank Data Base. The amplimers used in this study are shown in Table 7 (p. 70).

**Restriction Enzyme Digestion**

After PCR, the overlying mineral oil was removed from the PCR products with chloroform and the PCR product was digested with the restriction enzymes Ava II and Pst I, 1μl at 37°C for 1 hour, for confirmation. The digested PCR products were then separated on 3% Wide Range Agarose gel (Sigma) and stained with ethidium bromide (1μg/10ml) for photography under UV illumination (wavelength=312nm).
RESULTS

PCR Product and Restriction Enzyme Digestion

There was one major gel electrophoresis band observed in the PCR product amplified from the genomic DNA and mRNA of the transfectant line CA-aTIMP-JC1, i.e. a 535bp band was demonstrated by using the amplimer pair for antisense TIMP-1 (Figure 29: Lane 2 of the lower panel). The 535bp PCR product was then digested with Ava II and Pst I restriction enzymes for further confirmation. It was digestible with Ava II and yielded 225bp and 310bp fragments (Figure 29: Lane 3 of the lower panel). It was also digested with Pst I to yield 155bp and 380bp fragments (Figure 29: Lane 4 of the lower panel). The size of this 535bp product and its restriction enzyme digestion patterns were consistent with the amplified antisense TIMP-1.

Transfection

The parental line 83-01-82CA-JC1 was transfected with the plasmid pNMH-aT to produce a transfectant line CA-aTIMP-JC1. In addition, the parental line 83-01-82CA-JC1 in a separate batch was also transfected with the negative-control plasmid pNMH to produce a control line CA-pNMH-JC1. The transfectant line and control line were cultured in neomycin medium (400µg/ml) for more than four months. The genomic DNAs of the parental line 83-01-82CA-JC1, transfectant lines CA-aTIMP-JC1, and control line CA-pNMH-JC1 were then extracted for PCR with the amplimer pair specific for antisense TIMP-1 gene sequence. Electrophoresis
of the PCR products showed no electrophoresis band observed in the samples of 83-01-82CA-JC1 (Figure 30: Lane 2). The antisense TIMP-1 gene sequence was amplified from the sample of CA-aTIMP-JC1 (Figure 30: Lane 3) and no electrophoresis band observed in the samples of CA-pNMH-JC1 (Figure 30: Lane 4). This suggested that the antisense TIMP-1 sequence was only present in the genomic DNA of transfectant line CA-aTIMP-JC1 and not in the genomic DNAs of the control line CA-pNMH-JC1 and the parental line 83-01-82CA-JC1. The antisense TIMP-1 gene sequence appeared to be permanently integrated into the genomic DNA of the transfectant line CA-aTIMP-JC1.

Expression of TIMP-1, MMP-1 and Antisense TIMP-1 in the Transfectants

Since the antisense TIMP-1 gene sequence was confirmed to be integrated into the genomic DNA of the transfectant line CA-aTIMP-JC1, expression of TIMP-1, MMP-1 and antisense TIMP-1 genes was then examined. Total cellular RNAs of the parental line 83-01-82CA-JC1, the transfectant line CA-aTIMP-JC1 and the control line CA-pNMH-JC1 were extracted for RT-PCR with the amplimer pairs for TIMP-1, MMP-1 and antisense TIMP-1, respectively. An amplimer pair for β-actin was used as a control. Electrophoresis of the RT-PCR products showed that the parental line 83-01-82CA-JC1 exhibited the expression of TIMP-1 (Figure 31: Lane 1), no expression of antisense TIMP-1 (Figure 31: Lane 2) and the expression of MMP-1 (Figure 31: Lane 3). The transfectant line CA-aTIMP-JC1 exhibited the expression of TIMP-1 (Figure 31: Lane 5), antisense TIMP-1 (Figure 31: Lane 6) and MMP-1 (Figure 31: Lane 7). The control line CA-pNMH-JC1 exhibited the expression of
TIMP-1 (Figure 31: Lane 9), no expression of antisense TIMP-1 (Figure 31: Lane 10) and the expression of MMP-1 (Figure 31: Lane 11). To summarize, all three lines exhibited the expression of TIMP-1 and MMP-1 genes, but only the transfectant line CA-aTIMP-JC1 exhibited the expression of antisense TIMP-1 gene.

DISCUSSION

The phenomenon that eukaryotic cells can take up foreign DNA and localize the foreign DNA in their nuclei under appropriate conditions has been exploited to study the molecular biology of various genes [Kawai 1984; Farber 1975; McCutchan 1968; Graham 1973; Loyter 1982; Cudd 1984; Cepko 1984; Graessmann 1986; Neumann 1982; Schaffner 1980; Cooper 1982]. Several methods have been developed to deliver the foreign DNA into the nucleus. These methods include the use of polycations [Kawai 1984; Farber 1975; McCutchan 1968], calcium phosphate [Graham 1973; Loyter 1982], liposome fusion [Cudd 1984], retrovirus [Cepko 1984], micro-injection [Graessmann 1986], electroporation [Neumann 1982] and protoplast fusion [Schaffner 1980]. The disadvantages of these methods include, but not limited to, the problems related to cytotoxicity, poor reproducibility, inconvenience or insufficient delivery. A recent report showed that the lipofectin method is a highly efficient lipid-mediated DNA-transfection procedure [Felgner 1987]. Lipofectin reagent is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphotidylethanolamine (DOPE). In the DOTMA-DNA complex, the cationic DOTMA neutralizes the negatively charged DNA and provides the complex with a
net positive charge that can associate with the negatively charged surface of the cell [Felgner 1987]. This new reagent appears to overcome the above mentioned problems such as cytotoxicity, poor reproducibility, inconvenience or insufficient delivery associated with other methods. In the present study, the lipofectin reagent was used as a vehicle to deliver the plasmid pNMH-aT into the parental line 83-01-82CA-JC1. The procedures were simple and only required a standard cell culture facility and supplies. Besides these technical advantages, the lipofectin reagent also overcomes a chronic problem. In the past in Dr. Milo's laboratory, the cell line 83-01-82 and normal keratinocytes in calcium phosphate-mediated transfection studies underwent terminal differentiation when treated with calcium phosphate [unpublished observation]. The phenomenon of terminal differentiation was not present in the transfectant line CA-aTIMP-JC1, therefore the lipofectin reagent appeared to overcome the above described problem. By using this lipofectin reagent, the parental line 83-01-82CA-JC1 was smoothly transfected with the plasmid pNMH-aT.

Conventionally, the demonstration of the integration of foreign DNA into genomic DNA is analyzed by the Southern blot technique [Khokha 1989]. The obvious drawbacks of Southern blot analysis include the amount of time need for the procedure and radiation biohazard. In the present study, rather than using Southern blot analysis, conventional PCR with a pair of amplimers specific for the antisense TIMP-1 expression cassette was used to amplify the genomic DNA of the transfectants. In case of negative integration, there would not be a PCR product.
The PCR product obtained by using this amplimer pair indicates that there was integration, since the antisense TIMP-1 gene sequence was only detected in the genomic DNA of the transfectant line CA-aTIMP-JC1 by using an amplimer pair specific for the antisense TIMP-1 gene sequence (Figure 30). In addition, the transfectant line CA-aTIMP-JC1 was cultured in neomycin medium (400μg/ml) for more than four months. In short, the expression vector pNMH-aT appeared to be permanently integrated into the genomic DNA of the transfectant line CA-aTIMP-JC1. Furthermore, the presence of antisense TIMP-1 mRNA in the transfectant line CA-aTIMP-JC1 (Figure 31) indicated that this expression vector was not only integrated into the genomic DNA but was also carrying out its full function to express the antisense TIMP-1 gene.

Naturally occurring antisense RNA in gene regulation has been reported in many physiological conditions [Kelly 1991; Munroe 1991; Okano 1991]. In terms of practical application, the antisense RNA has been used in the agricultural industry. For example, an antisense RNA to the polygalacturonase mRNA is genetically engineered to prolong the post-harvest storage of tomatoes [Sheehy 1988]. The mechanism of action of antisense RNA is not fully understood, however the mechanism appears to be inhibition at the level of splicing, inhibition at the level of transport, or inhibition at the level of translation [Denhardt 1992]. The antisense TIMP-1 gene in this study was designed to inhibit the expression of TIMP-1 [Khokha 1989] and has been reported to downregulate TIMP-1 expression by 30-60% at the level of splicing [Denhardt 1992]. In the present study, both TIMP and antisense
TIMP-1 mRNAs were detected in the transfectant line CA-aTIMP-JC1. The co-expression of TIMP-1 and antisense TIMP-1 genes is consistent with the observations of Khokha et al. [1989]. However, current RT-PCR techniques in this study were designed for a qualitative assay and not for a quantitative assay. It is unknown if the expression of TIMP-1 gene in this study is downregulated or not. Use of quantitative RT-PCR in the future may provide an answer to this question.

**CONCLUSION**

Transfection by using lipofectin reagent is a reproducible method for delivery of the plasmid pNMH-aT into the squamous cell carcinoma cell line 83-01-82CA-JC1. The expression vector carrying the antisense TIMP-1 is not only permanently integrated, but is also constitutively expressed in the transfectant CA-aTIMP-JC1 in neomycin medium for more than four months. The results suggest that the expression vector pNMH-aT is compatible with the cell line 83-01-82CA-JC1. This vector may be used in future studies of the expression regulation of TIMP-1 gene.

The action of antisense RNA on its target gene is not 100% inhibitory, a well-known phenomenon in this field. Co-expression of TIMP-1 and antisense TIMP-1 genes *in vitro* is consistent with the observations of others [Khokha 1989]. The degree of inhibition of TIMP-1 gene expression by this antisense TIMP-1 gene may be determined later by using quantitative RT-PCR.
Figure 28. Diagram of the expression vector pNMH-aT [Khokha 1987].
Diagram of the expression vector pNMH. A full-length I6c8 cDNA clone was inserted in reverse orientation into the Bam HI site between the mouse metallothionein I promoter and the 3' terminal portion of the human growth hormone gene (exon 5 and 3' nontranslated region). Neo gene is flanked on its 5' end by SV40 DNA containing the SV40 origin of replication and early promoter, small splice site and polyadenylation signals are in the 3' segments. pBR322 contains the pBR322 origin of DNA replication and BamHI gene (AmpR).
Figure 29. A restriction map of antisense TIMP-1. Upper panel represents a diagrammatic restriction map of the antisense TIMP-1 fragment showing two restriction points that were examined in this study. Lower panel represents a gel electrophoresis of antisense TIMP-1 PCR product and its restriction-digested products. The RT-PCR product, using an amplimer pair for antisense TIMP-1, was digested with the restriction enzymes indicated in the upper panel, and its digested fragments were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter III). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lane 2 represents undigested PCR product. Lane 3 represents Ava II digestion. Lane 4 represents Pst I digestion.
Figure 29
Figure 30. The integration of plasmid DNA into the genomic DNA of the transfectant analyzed by PCR technique. Genomic DNA of the parental line CA-JC1, the transfectant line CA-aTIMP-JC1, and the control line CA-pNMH-JC1 was amplified using an amplimer pair for antisense TIMP-1 sequence. The PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1µg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter III). Lane 1 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the left. Lane 2 represents the parental line CA-JC1. Lane 3 represents the transfectant line CA-aTIMP-JC1. Lane 4 represents the control line CA-pNMH-JC1.
Figure 31. The expression of TIMP-1, antisense TIMP-1 and MMP-1 genes in the transfectant lines analyzed by RT-PCR technique. Total cellular RNA of the parental line CA-JC1 (Lanes 1-4), the transfectant line CA-aTIMP-JC1 (Lanes 5-8) and the control line CA-pNMH-JC1 (Lanes 9-12) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1 and MMP-1 mRNA respectively. An amplimer pair for β-actin was used as a control. These RT-PCR products were separated on a 3% Wide Range Agarose gel that was then stained with ethidium bromide (1μg/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter III). Lanes 1, 5 and 8 represent the TIMP-1 PCR product. Lanes 2, 6 and 10 represent the antisense TIMP-1 PCR product. Lanes 3, 7 and 11 represent the MMP-1 PCR product. Lanes 4, 8 and 12 represent the β-actin PCR product. Lane 13 represents a DNA size marker, the size of the standard fragments in the region of interest are shown to the right.
Figure 31
CHAPTER IV
EVALUATION OF THE TRANSFECTANT FOR AN INVASIVE PHENOTYPE

INTRODUCTION
The studies in Chapter III demonstrated that the transfectant line, CA-aTIMP-JC1, exhibited the expression of TIMP-1, MMP-1 and antisense TIMP-1 genes in vitro. This antisense TIMP-1 gene was designed to downregulate the expression of TIMP-1 [Denhardt 1992] and eventually enhance the invasive phenotype of the transfectant [Khokha 1989]. By using RT-PCR technique in Chapter III, it was unknown if the expression of TIMP-1 gene was downregulated or not. Despite the limitation of current RT-PCR technique, it would be interested to evaluate the invasive phenotype of the transfectant in a bioassay system. It has been reported that this antisense TIMP-1 construct converts the non-invasive Swiss 3T3 cells into invasive cells when xenotransplanted into the nude mouse [Khokha 1989]. The observation suggested that the nude mouse system may be a suitable bioassay system for studying the invasion process. Although the results in Chapter II showed that the invasive phenotype was not exhibited by xenograft tumors derived from human tumors, the invasiveness of human monolayer cells was never evaluated in
the nude mouse system. The cell population of human tumor is far more heterogeneous than that of monolayer cells. The biologic behavior of these two populations (e.g. human tumor tissue and monolayer cells) in the nude mouse tissue micro-environment may be different. The nude mouse system may not be ideal for studying the process of human tumor biology, however, it somewhat resembles the human tissue micro-environment and providing a setting in which we can examine the biologic behavior of cancer cells \textit{in vivo}. The transfectant line, CA-aTIMP-JC1, was hence xenotransplanted into nude mice and the features of the invasive phenotype examined in this surrogate host.

In the current studies, evidence of micro-invasion in the surrogate host was searched for microscopically and the molecular events (i.e. TIMP-1 and MMP-1 genes) possibly governing the process of cancer invasion were examined by using RT-PCR.

\textbf{MATERIALS AND METHODS}

\textbf{Immunosuppressive Pretreatment of Nude Mouse}

Nude mice were used as a surrogate host in this study since the nude mouse is the nearest system that mimic the human environment. Male gnotobiotic NCR/SED nude mice (Massachusetts General Hospital, Boston, MA), 3-4 weeks of age, were splenectomized under general anesthesia (70 mg/kg of sodium pentobarbital). Anti-lymphocyte serum (0.1 ml per mouse) was administered subcutaneously twice weekly for four weeks to increase tumor engraftment. It has been demonstrated
that the tumor take will be higher in gnotobiotic nude mice that receive additional immunosuppressive pretreatments such as splenectomy [Grimwood 1985] and anti-lymphocyte serum injections [Stanbridge 1969]. The mice were used within two months of the treatments since tumor incidence rate is dramatically decreased in mice older than three months [unpublished data].

Cell Suspension Xenotransplantation
The pretreated nude mice then received xenotransplantation of the transfectant line which was cultured in vitro. Monolayer cells were harvested using a trypsinization method (trypsin 0.625% in versene solution), and the trypsin enzymic activity was neutralized in MEM with 5% fetal bovine serum. The cell suspension (3X10⁷ in a 0.2-ml volume) was aspirated into a tuberculin syringe without a needle and the cell suspension injected with a 22-gauge needle into the subcutaneous flank region of the pretreated nude mouse. The injection site was monitored weekly by palpation and visual inspection. The latent period prior to the observation of a progressively growing tumor xenograft was approximately 3 months. Progressively growing tumors were allowed to reach a size of 2 cm in greatest dimension and were then excised. The excised xenograft tumors were subdivided and portions were either fixed in formalin for routine histopathology or snap frozen for the study described below.
Total Cellular RNA Extraction and Reverse Transcription (RT)

Once the nude mouse developed a 2cm tumor, the tumor was removed for the study of the expression level of TIMP-1, antisense TIMP-1 and MMP-1 genes. This technique incorporates a micro-procedure for isolating total cellular RNA and reverse transcription of the total cellular RNA. Thus, only the mRNA in the total cellular RNA is reverse transcribed to produce cDNA [Brenner 1989c]. Briefly, total cellular RNA was extracted from minced tissue by boiling the tissue for 5 minutes and IMMEDIATELY transferring 64μl of the supernatant to a 31μl reverse transcription reaction mix. RT was carried out with 5μl Mo-MLV reverse transcriptase (200U/μl) and 1.25μg oligo(dT) priming at 42°C for 45 minutes in the presence of 50mM KCl, 1.5mM MgCl₂, 1mM dNTP mix and the RNase inhibitor, RNasin 100U. Immediately after RT, the product was stored at -20°C until use.

Polymerase Chain Reaction (PCR)

The RT product was amplified using PCR. The technique was performed as previously described [Rappolee 1989]. Briefly, one μl of RT product was mixed with one unit of Taq DNA polymerase (BRL) and 5 pmoles of amplimer pairs specific for the mRNA of interest (see the next section: Amplimers and Their Product Lengths) in a buffer containing 20mM Tris-HCl (pH 8.3), 1.5mM MgCl₂, 50mM KCl and 0.2mM dNTP mix, in a 25-μl volume. The mixture was overlaid with one drop of mineral oil to prevent evaporation and amplified by a thermocycler (Perkin-Elmer Cetus) in repeated three-temperature cycle (94°C for 1 minute, 60°C for 2 minutes
and 72°C for 3 minutes) for 35 cycles. The PCR product was then stored at 4°C until use.

**Amplimers and Their Product Lengths**

The amplimer pairs used in this study included the amplimers for MMP-1, TIMP-1 and antisense TIMP-1. In addition, an amplimer pair for β-actin was used as a control [Nomura 1993]. Amplimers were designed according to the methods outlined by Rappolee et al. [1989]. The structures of the amplimers used in this study were designed from known sequences published in the Genbank Data Base. The amplimers used in this study are presented in Table 7 (p. 70).

**RESULTS**

**Invasiveness of the Xenograft Transfectant Tumors**

In order to evaluate the molecular events surrounding the invasiveness of the transfectant line CA-aTIMP-JC1 *in vivo*, the transfectant line CA-aTIMP-JC1 (3x10⁷ cells in a 0.2-ml volume) was xeno-transplanted into three nude mice from which developed two xenograft, transfectant-induced tumors in three months. The xenograft transfectant tumors were designated CAT1-aTIMP-JC1. The parental line 83-01-82CA-JC1 (3x10⁷ cells in a 0.2-ml volume) was also xenotransplanted into three other nude mice as negative controls. Two of these mice developed xenograft tumors in three months, which were designated CAT1-JC1. Grossly, these xenograft tumors were freely movable subcutaneous nodules, there was no adhesion between these nodules and the adjacent mouse tissues. Upon
microscopic examination, both the tumor CAT1-JC1 (Figure 32) and the transfectant tumor CAT1-aTIMP-JC1 (Figure 33) exhibited the features of squamous cell carcinoma with a thin fibrous capsule (Figure 33). No invasion was seen microscopically (Figure 33). Postmortem examination of the mice carrying the transfectant tumor CAT1-aTIMP-JC1 was then performed to identify possible micro-metastatic deposits which may have been overlooked by visual inspection. Multiple histology sections revealed no evidence of metastatic deposits in the lungs (Figure 34), the trachea (Figure 35), the liver (Figure 36), the kidneys (Figure 37), the gastro-intestinal tract (Figure 38) or the heart (Figure 39).

Expression of TIMP-1, MMP-1 and Antisense TIMP-1 Genes in the Transfectant Tumors

Although the transfectant tumors CAT1-aTIMP-JC1 were found to be non-invasive in the surrogate host microscopically, the expression of TIMP-1, MMP-1 and antisense TIMP-1 genes in the transfectant tumors was examined to study the possible roles of these genes in the non-invasiveness of the transfectant tumor CAT1-aTIMP-JC1. Total cellular RNA of the tumors CAT1-JC1 and CAT1-aTIMP-JC1 was extracted for examination of the presence of TIMP-1, MMP-1 and antisense TIMP-1 messages by using RT-PCR technique. Electrophoresis of the RT-PCR products showed that the parental tumor CAT1-JC1 did not exhibit the expression of TIMP-1 (Figure 40: Lane 2), antisense TIMP-1 (Figure 40: Lane 3) or MMP-1 (Figure 40: Lane 4). The transfectant tumor CAT1-aTIMP-JC1 did not exhibit the expression of TIMP-1 (Figure 40: Lane 6), exhibited the expression of
antisense TIMP-1 (Figure 40: Lane 7) and did not exhibit the expression of MMP-1 (Figure 40: Lane 8). In summation, the transfectant tumor CA-aTIMP-JC1 expressed antisense TIMP-1 only and did not express TIMP-1 or MMP-1 genes.

DISCUSSION

In the present study, both the transfectant line CA-aTIMP-JC1 and the parental line 83-01-82CA-JC1 were xeno-transplanted into nude mice for the observation of an invasive phenotype. Two out of three nude mice that received the transfectant line CA-aTIMP-JC1 developed xenograft tumors. At the same time, two out of three nude mice that received the parental line 83-01-82CA-JC1 also developed xenograft tumors. The tumorigenic potential of these two lines appeared to the same. Histopathologic comparison between these two xenograft tumor series exhibited the features of squamous cell carcinoma with a similar histodifferentiation pattern (Figures 32 vs. 33). The similar tumorigenic potential and histodifferentiation pattern suggests that the transfectant line CA-aTIMP-JC1 and the parental line 83-01-82CA-JC1 responded to the nude mouse tissue micro-environment in the same way regardless of the slightly different genetic make-up between them. Apparently, the extra DNA in the transfectant line CA-aTIMP-JC1 did not alter some properties such as the tumorigenic potential and histodifferentiation pattern.

Further histopathologic examination of the mice carrying the transfectant tumors CAT1-aTIMP-JC1 revealed no micro-invasion by the tumors nor was there any evidence of micro-metastasis in the lungs (Figure 34), the trachea (Figure 35), the
liver (Figure 36), the kidneys (Figure 37), the gastro-intestinal tract (Figure 38) or the heart (Figure 39). The results in this study with human cells were different from the observations of Khokha et al. [1989] using mouse cells. Khokha et al. reported that the plasmid pNMH-aT converted the non-invasive Swiss 3T3 cells into invasive cells when xenotransplanted into the nude mouse [Khokha 1989]. The major difference between the current study and the study by Khokha et al. is that the transfectant in the current study was tested in a xenogeneic host (i.e. human cells in nude mouse) and the transfectant of Khokha et al. was tested in a syngeneic host (i.e. mouse cells in nude mouse). The invasive phenotype of mouse cells was expressed in the mouse tissue micro-environment, whereas the invasive phenotype of human cells was suppressed in the nude mouse tissue micro-environment. This suggests that the host tissue micro-environment may influence the phenotype of cancer cells. To further study the non-invasive nature of the transfectant tumor CAT1-aTIMP-JC1, the expression of TIMP-1, MMP-1 and antisense TIMP-1 genes in the transfectant tumors was examined. The results of this study demonstrated the expression of antisense TIMP-1 gene by the transfectant tumor CAT1-aTIMP-JC1 (Figure 40), however, neither TIMP-1 mRNA nor MMP-1 mRNA were present in the transfectant tumor CAT1-aTIMP-JC1 or the tumor CAT1-JC1 (Figure 40). The studies in Chapter III revealed that the transfectant CA-aTIMP-JC1 expressed both TIMP-1 and MMP-1 in vitro, but interestingly, both TIMP-1 and MMP-1 genes of the transfectant CA-aTIMP-JC1 in the nude mouse tissue micro-environment were turned off. This again suggests that the host environment may influence the phenotype of cancer cells. The findings presented in this Chapter are consistent
with the observations presented in Chapters I and II. The studies in Chapter I demonstrated the plastic nature of cancerous tissues once they are relocated from the native human tissue micro-environment to the nude mouse tissue micro-environment. The changes observed in the new phenotypes of these plastic cancer cells include, but are not limited to, loss of invasion/metastasis, altered histodifferentiation, increased mitotic index and loss of fibrovascular stroma. The studies in Chapter II demonstrated that MMP-1 gene was down-regulated once patient tumors were relocated to the nude mouse. In the present study, the MMP-1 and TIMP-1 genes were down-regulated once the transfectant was relocated from \textit{in vitro} to \textit{in vivo} conditions. Taken together, the properties of cancer cells are plastic and the plasticity is strongly influenced by the host tissue micro-environment.

The influence by the host tissue micro-environment indicates that carcinogenesis involves not only the inherent genetic changes of the cancer cells but also epigenetic regulation. It is reasonable to believe that the absence of MMP-1 and TIMP-1 message of the transfectant \textit{in vivo} was partially due to the inhibitory signals or the lack of stimulating signals from the mouse tissue micro-environment. These signals are beyond the control of the inherent genetic regulation of the cancer cells (i.e. epigenetic regulation). Apparently, in the present study, epigenetic regulation dominates and inhibits the invasive phenotype.

In this study, the invasive phenotype was not observed in the tumors established by the transfectant carrying antisense TIMP-1. However, an inverse relationship
between TIMP-1 protein levels and invasive potential has been observed by others in intracranial human tumors [Halaka 1983] and in many different mouse cell lines tested in in vitro invasion assay [Hichs 1984; Ponton 1991]. Experimentally, an increased level of TIMP-1 protein/mRNA does decrease the aggressiveness of many cancer cell lines. In a study with recombinant TIMP-1 protein [Schultz 1988], the recombinant TIMP-1 protein was found to inhibit the invasion of B16-F10 murine melanoma cells through the human amniotic membrane. C57BL/6 mice treated with intraperitoneal injections of recombinant TIMP-1 protein every 12 hours for 6.5 days showed a significant inhibition of metastatic B16-F10 murine melanoma cells in a pulmonary colonization assay. The size of metastatic colonies in the lungs was not influenced by the recombinant TIMP-1 protein suggesting that TIMP-1 protein is likely involved in metastasis but not tumorigenesis in this model system [Schultz 1988]. In another study, an expression vector was used to overproduce TIMP-1 mRNA/protein that also decreased the aggressiveness of cancer cells. Upregulation of murine TIMP-1, by an expression vector to overproduce TIMP-1, in a metastatic B16-F10 murine melanoma cell line led to (i) a suppression of invasive ability in a Matrigel transwell invasion assay [Khokha 1992a], (ii) a decreased growth ability in vitro [Khokha 1992b], (iii) a suppressed experimental metastatic ability in the chicken embryo assay [Khokha 1992b], and (iv) a decreased tumorigenicity and experimental metastasis in both nude (BALB/c) and syngeneic (C56BL/6) mice [Khokha 1994]. The latter two studies clearly demonstrate that increased TIMP-1 mRNA/protein will inhibit the aggressiveness of cancer cells. On the other hand, decreased TIMP-1 mRNA (by using antisense TIMP-1) promotes
the aggressiveness of Swiss 3T3 cells [Khokha 1989]. In short, the level of TIMP-1 mRNA/protein is inversely related to the invasiveness of cancer cells. This appears to certify TIMP-1 as a suppressor [Khokha 1989; Liotta 1991]. A mutation in the tumor suppressor p53 gene has been found at the codon 126 in the parental line 83-01-82CA-JC1 [unpublished data] of the transfectant CA-aTIMP-JC1. Also, this parental line has been found to exhibit a mutation at the codon 12 of H-ras [unpublished data]. If we accept the concept that TIMP-1 is a suppressor gene, it is evident that the downregulation of the suppressor TIMP-1, in addition to the inherent mutations of p53 and H-ras in the current system, are apparently insufficient to confer an invasive phenotype. It is suspected that further genetic changes and proper host tissue micro-environment are required.

Although the present study did not demonstrate an increased invasiveness as was expected, it does not deny the roles of TIMP-1 and MMP-1 genes in the process of cancer invasion. Lack of MMP-1 mRNA in the non-invasive transfectant tumors indirectly suggests that MMP-1 mRNA might play an active role in the process of cancer invasion. Both TIMP-1 and antisense TIMP-1 mRNAs were present in the transfectant line in vitro, whereas only the antisense TIMP-1 mRNA was present in the transfectant tumor in vivo. Antisense TIMP-1 mRNA is designed to inhibit the expression of TIMP-1 gene. The absence of TIMP-1 mRNA in the transfectant tumors may be due to inhibitory signals from the nude mouse tissue micro-environment and/or due to the inhibitory effect of antisense TIMP-1 mRNA. The presence of antisense TIMP-1 mRNA again suggests that the antisense TIMP-1
construct was permanently integrated into the genomic DNA and continued to express the antisense TIMP-1 gene both in vitro and in vivo. The antisense TIMP-1 sequence of the construct pNMH-aT is driven by murine metallothionein-I promoter (MT-I promoter). Placing such a construct with a murine promoter into the nude mouse tissue micro-environment may allow the continuous expression of antisense TIMP-1 gene in the nude mouse system. TIMP-1 and MMP-1 genes of the transfectant are driven by their own native promoters and transcription regulatory elements. Theoretically, replacing these native promoters with murine promoters may maintain the expression of these genes in the nude mouse tissue micro-environment so that the role of TIMP-1 and MMP-1 during cancer invasion can be further studied.
CONCLUSION

The transfectant line CA-aTIMP-JC1 will form progressive growing tumors in the nude mouse system. These tumors retain a tumorigenic potential and continue to express the characteristic features of squamous cell carcinoma. Apparently the extra new DNA in the transfectant line, CA-aTIMP-JC1, did not alter some features such as tumorigenic potential and histodifferentiation pattern. Like other xenografts derived from human tumors, the transfectant tumors lost the invasive phenotype and formed encapsulated nodules. The transfectant tumors did not exhibit the expression of TIMP-1 and MMP-1 genes. The loss of the invasive phenotype was not due to the failure of antisense TIMP-1 transfection, but was due to the downregulated MMP-1 gene of the transfectant line, presumably influenced by the nude mouse tissue micro-environment. If we accept the concept that MMP-1 protein/mRNA is involved in the process of cancer invasion, then lack of MMP-1 mRNA may be the molecular evidence for the non-invasiveness of the transfectant in nude mice. The non-invasiveness of the transfectant line in the nude mouse system does not deny the role of TIMP-1 in cancer invasion, nor the value of the nude mouse system. Any approach(es) in the future that can maintain/stimulate the expression of both TIMP-1 and MMP-1 genes of the transfectant may shed light on the roles of TIMP-1 and MMP-1 genes/proteins in the process of cancer invasion.
Figure 32. Microphotograph of the tumor CAT1-JC1. A male nude mouse (3-4 weeks old) was xeno-transplanted with the tumorigenic cell line 83-01-82CA-JC1 (ca. $3 \times 10^7$ cells in a volume of 0.2ml). A tumor, CAT1-JC1, developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Microphotograph of this lesion demonstrates the features of squamous cell carcinoma. (H&E stains, original magnification=160X)
Figure 33. Microphotograph of the tumor CAT1-aTIMP-JC1. A male nude mouse (3-4 weeks old) was xeno-transplanted with the transfectant line CA-aTIMP-JC1 (ca. 3X10^7 cells in a volume of 0.2ml). A tumor, CAT1-aTIMP-JC1, developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Microphotograph of this lesion demonstrates the features of squamous cell carcinoma growing in a nodular pattern with a thin fibrous capsule. No microscopic invasion is seen. (H&E stains, original magnification=160X)
Figure 34. Postmortem examination I. A male nude mouse (3-4 weeks old) was xeno-transplanted with the transfectant line CA-aTIMP-JC1 (ca. $3 \times 10^7$ cells in a volume of 0.2ml). A tumor CAT1-aTIMP-JC1 developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Postmortem examination of the mouse carrying the tumor CAT1-aTIMP-1 revealed a picture of nearly normal lung microscopically. No micro-metastatic deposit was seen. (H&E stains, original magnification=160X)
Figure 35. Postmortem examination II. A male nude mouse (3-4 weeks old) was xeno-transplanted with the transfectant line CA-aTIMP-JC1 (ca. $3 \times 10^7$ cells in a volume of 0.2ml). A tumor CAT1-aTIMP-JC1 developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Postmortem examination of the mouse carrying the tumor CAT1-aTIMP-1 revealed a picture of nearly normal trachea microscopically. No micro-metastatic deposit was seen. (H&E stains, original magnification=160X)
Figure 36. Postmortem examination III. A male nude mouse (3-4 weeks old) was xeno-transplanted with the transfectant line CA-aTIMP-JC1 (ca. 3X10^7 cells in a volume of 0.2ml). A tumor CAT1-aTIMP-JC1 developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Postmortem examination of the mouse carrying the tumor CAT1-aTIMP-1 revealed a picture of nearly normal liver microscopically. No micro-metastatic deposit was seen. (H&E stains, original magnification=160X)
Figure 37. Postmortem examination IV. A male nude mouse (3-4 weeks old) was xeno-transplanted with the transfectant line CA-aTIMP-JC1 (ca. 3X10^7 cells in a volume of 0.2ml). A tumor CAT1-aTIMP-JC1 developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Postmortem examination of the mouse carrying the tumor CAT1-aTIMP-1 revealed a picture of nearly normal kidney microscopically. No micro-metastatic deposit was seen. (H&E stains, original magnification=160X)
Figure 38. Postmortem examination V. A male nude mouse (3-4 weeks old) was xeno-transplanted with the transfectant line CA-aTIMP-JC1 (ca. 3X10⁷ cells in a volume of 0.2ml). A tumor CAT1-aTIMP-JC1 developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Postmortem examination of the mouse carrying the tumor CAT1-aTIMP-1 revealed a picture of nearly normal gastro-intestinal tract microscopically. No micrometastatic deposit was seen. (H&E stains, original magnification=160X)
Figure 39. Postmortem examination VI. A male nude mouse (3-4 weeks old) was xeno-transplanted with the transfectant line CA-aTIMP-JC1 (ca. $3 \times 10^7$ cells in a volume of 0.2ml). A tumor CAT1-aTIMP-JC1 developed from these cells in three months (Procedure described in Materials and Methods of Chapter IV). Postmortem examination of the mouse carrying the tumor CAT1-aTIMP-1 revealed a picture of nearly normal cardiac valve microscopically. No micro-metastatic deposit was seen. (H&E stains, original magnification=160X.)
Figure 40. The expression of TIMP-1, antisense TIMP-1 and MMP-1 genes in the transfectant tumor analyzed by RT-PCR technique. Total cellular RNA of the parental tumors CAT1-JC1 (Lane 2-5) and the transfectant tumor CAT1-\textit{aTIMP-JC1} (Lanes 6-9) was analyzed using RT-PCR priming with amplimer pairs for TIMP-1, antisense TIMP-1, and MMP-1 mRNA respectively. An amplimer pair for \( \beta \)-actin was used as a control. These RT-PCR products were separated on a 3\% Wide Range Agarose gel that was then stained with ethidium bromide (1\( \mu \)g/10ml). These bands were visualized under UV=312nm. (Procedure described in the Materials and Methods in Chapter IV). Lane 1 represents a DNA size marker; the size of the standard fragments in the region of interest are shown to the left. Lanes 2 and 6 represent TIMP-1 PCR product. Lanes 3 and 7 represent antisense TIMP-1 PCR product. Lanes 4 and 8 represent MMP-1 PCR product. Lanes 5 and 9 represent \( \beta \)-actin PCR product.
CONCLUSIONS

The xenotransplantation of human tumor fragments into a surrogate host system such as the nude mouse can be an important model in tumor biology. The nude mouse model provides an environment that somewhat mimics the physiology of the \textit{in vivo} human situation. Although differences do exist between the human and the mouse, the nude mouse model provides an opportunity to examine such characteristics as tumorigenic potential, tumor growth pattern and differentiation, and stromal response in an \textit{in vivo} environment that more closely approximates the original tumor site.

Human tumors will grow as xenografts in the nude mouse system, however, only a subset of these tumors exhibit such an ability. This subset of tumors provides an opportunity for studying such aspects of tumor biology as factors contributing to tumorigenic potential and the loss of the invasive phenotype. Tumorigenic potential in the nude mouse system is independent of the stage of malignant progression of the original human lesion (primary vs. metastatic) regardless of the site of tumor origin. Metastatic tumors are secondary tumors derived from aggressive cells in the
primary tumors, however the tumorigenic potential of the secondary tumor may not be higher than that of the primary tumors.

Human cancer cells are plastic. Biologically, tumors in a surrogate host often lose their invasive phenotype, an important feature in the original patient tumors. Morphologically, tumors in a surrogate host exhibit a higher histologic grading, decreased cellular pleomorphism, decreased desmoplastic reaction and an increased mitotic index when compared to their original human tumors. These morphologic features are consistent with the biologic feature of loss of the invasive phenotype. Using techniques of molecular biology, tumors in the surrogate host do not demonstrate the expression of Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) and interstitial collagenase (MMP-1) genes. The downregulated MMP-1 gene in the surrogate host environment may be molecular evidence for the loss of the invasive phenotype in xenograft tumors.

During the biological process of embryonic morphogenesis and adult tissue remodelling, the expression of MMP-1 and TIMP-1 genes is highly organized. This TIMP-1/MMP-1 balance appears to be abrogated in the process of cancer invasion. TIMP-1 gene expression is inversely related and MMP-1 gene expression directly related to the advancing stage of carcinogenesis. While TIMP-1 gene appears to be unexpressed in normal mucosal epithelium, it becomes active in hyperplastic and dysplastic mucosal epithelium and silent again in invasive squamous cell carcinoma. MMP-1 gene expression is directly related to the invasive phenotype, is silent in
normal skin and uterine cervix, active in invasive patient tumors, and silent again in non-invasive tumors in the surrogate host. This unbalanced TIMP-1/MMP-1 expression may be the result of downregulated TIMP-1 and/or upregulated MMP-1.

Transfection by using lipofectin reagent is a suitable method for delivery of foreign DNA into squamous cell carcinoma cells which in the past often underwent terminal differentiation when foreign DNA was delivered using the calcium phosphate method. The expression vector carrying the antisense TIMP-1 is not only permanently integrated, but also constitutively expressed in the transfectant line CA-aTIMP-JC1, suggesting that the expression vector pNMH-aT is compatible with the cell line 83-01-82CA-JC1. This vector may be used for future studies of the expression/regulation of the TIMP-1 gene. The interaction of antisense RNA with its target gene is not 100% inhibitory. Co-expression of TIMP-1 and antisense TIMP-1 genes in vitro is consistent with the observations of others [Khokha 1989]. The degree of inhibition of TIMP-1 gene expression by this antisense TIMP-1 gene may be determined in future studies by using quantitative RT-PCR.

The transfectant line CA-aTIMP-JC1 will form progressive growing tumors in the nude mouse system with similar tumorigenic potential and features of human squamous cell carcinoma. The extra DNA in the transfectant line CA-aTIMP-JC1 did not alter some attributes, such as tumorigenic potential and histodifferentiation pattern. The transfectant CA-aTIMP-JC1 appears to be plastic, in that both TIMP-1 and MMP-1 genes of the transfectant are active in vitro but inactive in vivo. Like
other xenografts derived from human tumors, the transfectant tumors had an inactive MMP-1 gene, lost the invasive phenotype, and formed encapsulated nodules. This again indirectly suggests that the MMP-1 gene is related to the invasive phenotype. The loss of the invasive phenotype was not due to the failure of antisense TIMP-1 transfection, but was most likely due to the downregulated MMP-1 gene of transfectant tumor influenced by the nude mouse tissue micro-environment.

Additional studies need to be performed in order to understand the roles of TIMP-1 and MMP-1 genes in the process of cancer invasion. Such studies may include:

1. Study of TIMP-1 and MMP-1 mRNA phenotyping in collaboration with oncologic surgeons. TIMP-1 and MMP-1 mRNA distribution may vary from area to area in the same tumor due to the heterogeneity of cancer cells. The tumors used in this study were random samples supplied by the Tissue Procurement Service. Careful mapping of the tumor samples by surgeons may provide further information on the roles of TIMP-1 and MMP-1 genes in cancer invasion.

2. Develop a convenient and reliable quantitative RT-PCR system, so that the levels of TIMP-1, MMP-1 and the decreased level of TIMP-1 by antisense TIMP-1 can be confirmed and determined. Current RT-PCR is a qualitative assay, not a quantitative assay.
3. Construct an expression vector to constitutively overproduce MMP-1 both *in vitro* and *in vivo*, so that the active role of the MMP-1 gene in cancer invasion can be determined.

4. Develop an *in vitro* invasion assay system. Currently the most popular *in vitro* system (e.g. Matrigel invasion chamber) is for evaluation of type IV collagenase, not for MMP-1.
BIOBIBLIOGRAPHY


Albelda SM: Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab Invest 1993; 68:4-17


Brinckerhoff CE, Plucinska IM, Sheldon LA, O’Connor GT: Half-life of synovial cell collagenase mRNA is modulated by phorbol myristate acetate but not by all-trans-retinoid acid or dexamethasone. *Biochemistry* 1986; 25: 6378-6384


Clark SD, Kobyashi DK, Welgus HG: Regulation of the expression of tissue inhibitor of metalloproteinases and collagenase by retinoids and glucocorticoids in human fibroblasts. *J Clin Invest* 1987; 80: 1280-1288


Farber FE, Melnick JL, Butel JS: Optimal conditions for uptake of exogenous DNA by Chinese hamster lung cells deficient in hypoxanthine-guanine phosphoribosyltransferase. *Biochim Biophys Acta* 1975; 390: 298-311


Golde DW, Bersch N, Quan SG, Lusis AJ: Production of erythroid-potentiating activity by a human T-lymphoblast cell line. *Proc Natl Acad Sci USA* 1980; 77: 590-595


Hicks NJ, Ward RV, Reynolds JJ: A fibrosarcoma model derived from mouse embryo cells: growth properties and secretion of collagenase and metalloproteinase inhibitor (TIMP) by tumour cell lines. Int J Cancer 1984; 33: 835-844


Ito A, Goshowaki H, Sato T, Mori Y, Yamashita K, Hayakawa K, Nagase H: Human recombinant interleukin-1α-mediated stimulation of procollagenase production and


Khokha R: Suppression of the tumorigenic and metastatic abilities of murine B16-F10 melanoma cells in vivo by overexpression of the tissue inhibitor of metalloproteinases-1. JNCI 1994; 86: 299-304


Koob TJ, Jeffrey JJ: Hormonal regulation of collagen degradation in the uterus. Inhibition of collagenase expression by progesterone and cyclic AMP. *Biochim Biophys Acta* 1974; 354: 61-69


McCutchan JH, Pagano JS: Enchancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *JNCI* 1968; 41: 351-357


Moscatelli D, Jaffe E, Rifkin DB: Tetradecanoyl phorbol acetate stimulates latent collagenase production by cultured human endothelial cells. *Cell* 1980; 20: 343-351


Nicolau C, Tosi PF, Arvinte T, Mouneimne Y, Cudd A, Sneed L, Madoulet C, Schulz B, Barhoumi R: CD4 inserted in red blood cell membranes or reconstituted in liposome bilayers as a potential therapeutic agent against AIDS. *Prog Clin Biol Res* 1990; 343:147-177


Ponton A, Coulombe B, Skup D: Decreased expression of tissue inhibitor of metalloproteinases in metastatic tumor cells leading to increased levels of collagenase activity. *Cancer Res* 1991; 51: 2138-2143


Puck TT, Marcus PI: A rapid method for viable cell titration and clone production with HeLa cell in tissue culture. The use of x-irradiated cells to supply conditioning factors. *Biochemistry* 1955; 41: 432-437


Recklies AD, Poole AR, Mort JS: A cysteine proteinase secreted from human breast tumors is immunologically related to cathepsin B. Biochem J 1982; 207: 633-636


Schaffner W: Direct transfer of cloned genes from bacteria to mammalian cells. *Proc Natl Acad Sci USA* 1980; 77: 2163-2167


Sheehy RE, Kramer M, Hiatt WR: *Proc Natl Acad Sci USA* 1988; 85: 8805-8809


Stuart GW, Searle PF, Chen HY, Brinster RL, Palmiter RD: A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc Natl Acad Sci USA* 1984; 81: 7318-7322


