MEDICAL INTELLIGENCE UNIT 10

Warren Hoeffler

# Collagenases

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MEDICAL
INTELLIGENCE
UNIT 10

# Collagenases

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R.G. LANDES
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# PREFACE

How we think about a subject has a lot to do with what context we place that subject in. In the case of collagenases, a very important set of enzymes with key roles in development, normal physiology, and pathogenesis, the discussion is usually only an addendum to that on the larger group of enzymes in their category, matrix metalloproteinases. As information about these enzymes has undergone explosive growth in the last few years, any textbook attempting to cover this diverse group of enzymes ironically has diminished room for considering collagenases. The time has come to give this topic its own book, and to consider more broadly the diverse roles these enzymes play in (primarily human) biology.

The authors contributing to this volume have all made major contributions to our understanding of collagenases, and each has recounted some of their own work here. They were encouraged to tell their story from their own personal perspectives, resulting is a more engaging description of the work. In the interests of putting together a more coherent volume devoted to these enzymes the authors were also challenged to consider related work, and how their area of interest fits into a larger picture. The topics for the chapters were chosen to give appropriate emphasis to the major themes associated with collagenases. The opening chapter describes the structural characteristics of collagenases, and is followed by individual chapters devoted to collagenases 1, 2, and 3. Transcriptional regulation of these genes are detailed and put into perspective of the current knowledge base. Since collagenases are uniquely regulated post-translationally, various methods of activation are considered, followed by how these mechanisms come into play in normal physiological functions, and as part of certain pathologies. Interference with the activities of these enzymes for pharmacologic benefit is addressed, and the role of collagenases in wound healing and in cancer receives special attention. Even the ultimate pathology from which we collectively suffer, aging, is also discussed.

In short, the information in this book cuts across a unique sampling of various medical fields, with the common theme that they all share an interest in this very important group of enzymes. Although technical detail is presented, and well documented in the references, more general perspectives are also clearly presented throughout. Since collagens are principle components comprising so much of our bodies, an understanding of the enzymes that orchestrate their constructive modeling, as well as decay, should be an area of importance to anyone interested in the body.

*Warren Hoeffler, Ph.D.*

CHAPTER 1

# Structure of Collagenases and Strategies for Expression and Folding of the Recombinant Proteins

**Robert D. Gray**

## Introduction

The development of recombinant DNA technology and the attendant ability to express virtually any protein in quantities sufficient for biophysical studies is an essential component of modern structural and mechanistic biology. Indeed, most, if not all of the three-dimensional structures of the matrix metalloproteinases (MMPs) currently in the literature were derived from recombinant proteins obtained by expression in *Escherichia coli*. This approach is necessary because culture of mammalian tissues or cells generally produces only relatively small amounts of the enzymes. Expression of heterologous proteins in *E. coli*, however, often is accompanied by problems that must be overcome to ensure that a native structure is being studied. Potential problems include low yields of the target protein, precipitation of the expressed protein within the cell, lack of processing and proteolytic modification such as removal of C and N-terminal residues to produce proteins with heterogeneous termini. The purpose of this article is first to review structural aspects of the collagenases and second, to review systems that have been utilized for heterologous expression of the collagenases and other MMPs. The article is divided into the following parts: (a) a summary of MMP nomenclature and primary structural domains as a reference point for collagenases; (b) aspects of collagenase three-dimensional structure; (c) an overview of protein expression in *E. coli*; (d) an examination of general aspects of protein folding; (e) a discussion of specific recombinant collagenases and other MMPs. All of the examples discussed relate to MMPs expressed in *E. coli*; eukaryotic expression is not covered.

## Matrix Metalloproteinase Structure

### *General Aspects*

The MMPs comprise a group of zinc endopeptidases that degrade proteins of the extracellular matrix. The enzymes share several functional characteristics including the ability to degrade at least one protein of the extracellular matrix, secretion into the extracellular matrix as proenzymes which must be activated to express proteolytic activity, and inhibition

*Collagenases*, edited by Warren Hoeffler. 

***Table 1.1. The matrix metalloproteinase family***

| Group | MMP# | Other Names | EC# |
|---|---|---|---|
| Collagenases | | | |
| Interstitial collagenase | MMP-1 | | 3.4.24.7 |
| PMN collagenase | MMP-8 | | 3.4.24.34 |
| Gelatinases | | | |
| Gelatinase A | MMP-2 | 72 kD gelatinase | 3.4.24.24 |
| Gelatinase B | MMP-9 | 92 kD gelatinase | 3.4.24.35 |
| Stromelysins | | | |
| Stromelysin-1 | MMP-3 | Transin (rat) procollagenase activator | 3.4.24.17 |
| Stromelysin-2 | MMP-10 | Transin-2 (rat) | 3.4.24.22 |
| Stromelysin-3 | MMP-11 | | |
| Others | | | |
| Matrilysin | MMP-7 | punctuated metallopreteinase (PUMP); uterine MP | 3.4.24.23 |
| | MMP-12 | Metalloelastase (mouse) | |
| | MMP-13 | Collagenase-3 | |
| | MMP-14 | Membrane-type | |

Adapted from www.bioscience.org/molglanc/mmp.htm

by specific MMP inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).[1] Historically, three groups of MMPs have been recognized based on substrate specificity: collagenases, which act uniquely on interstitial triple helical collagens (types I, II and III); gelatinases, which rapidly degrade denatured collagens and basement membrane (type IV) collagen; and stromelysins, which cleave the core polypeptide of proteoglycans among other proteins of the extracellular matrix. Two additional groups of MMPs have recently been proposed to accommodate those enzymes which do not fit in the classical scheme.[2] MMP nomenclature is summarized in Table 1.1.

### *Primary Structure*

The MMPs are members of a family of metallopeptidases, the metzincins, so named because of the presence of a catalytically essential zinc ion and a conserved methionine residue within the active site.[3,4] Other members of this family include astacin, meprins, snake venom metalloproteinases (MPs) and the serralysins, which are bacterial metalloproteinases.

Amino acid and DNA[3,4] sequence analysis reveals the modular structure of the MMPs.[2,5] The polypeptide chain of each family member starts with a hydrophobic N-terminal leader sequence that directs the protein to the secretory pathway. The leader sequence, which is removed prior to secretion, is followed by a propeptide that maintains enzymatic latency by coordination of a cysteine thiol to the catalytic zinc. The catalytic domain (CD) follows; it contains binding pockets for the substrate that direct the scissile peptide bond to the active site $Zn^{2+}$ which is ligated to three histidine residues within an HEXGHXXGXXH motif. The conserved Glu is presumed to function as a general base in activating a water molecule that hydrolyzes the substrate peptide bond.

The smallest member of the MMP family, promatrilysin, consists of the propeptide and the CD only. All other MMPs include, as a minimum, a C-terminal domain that exhibits sequence homology to the plasma protein hemopexin. This so-called pexin domain (PD) connects to the CD via a proline-rich hinge region of variable length (5-50 residues). The gelatinases also contain fibronectin-like gelatin-binding modules and gelatinase B contains in addition an α(2)-collagen-like sequence. From the standpoint of folding of the recombinant protein, it should be noted that the three-dimensional structure of the only full-length MMP published, porcine synovial collagenase, reveals that the catalytic and pexin regions appear to be independent structural domains.[5] The crystal structure of the separated CD and PD of human collagenases suggests that these domains are independent folding units as well.

## Collagenase Three-Dimensional Structure

X-ray crystallography and NMR have recently provided detailed models of the collagenases and several related MMPs. Most of these were determined using CDs inhibited by zinc-binding substrate analogues. Initial efforts were focused on fibroblast collagenase,[6-9] neutrophil collagenase[10] and stromelysin-1.[11] Subsequently, structures of full-length porcine fibroblast collagenase,[5] matrilysin[12] and the PDs of collagenase-3[13] and 72 kDa gelatinase[14] were published. The following section summarizes some of the available structural information with an emphasis on comparing human fibroblast and neutrophil collagenases. I have relied on the published structures of Lovejoy et al[6,15] and Spurlino et al[8] for MMP-1 and especially on the extensive work of the group at the Max-Planck-Institute[10,13,16-18] for MMP-8 and MMP-13 for insights into comparisons between MMP-1 and MMP-8.

### *Catalytic Domain Folding Topology*

Collagenases from fibroblasts and neutrophils exhibit similar, but nonidentical folding patterns as illustrated in the topological diagrams in Figure 1.1. The fibroblast CD (residues 100-269) consists of three α-helices (A-C), five β-strands (I-V) and their connecting loops. Strands I, II, III and V run parallel to each other while strand IV is oriented in an antiparallel direction. Helix A is positioned closest to strands I and II, while helix B, which provides the scaffolding for the putative catalytic Glu and two of the three His residues that ligate the catalytic zinc lies next to strand III. Helix C is positioned adjacent to strand IV. The neutrophil enzyme (residues 80-242) is folded in a similar fashion except that helices B and C are arranged in a slightly different topology from that of the fibroblast enzyme: in MMP-1, helix C is positioned more closely to strand III, while in MMP-8, it is closer to strand IV.

Ribbon diagrams depicting the CD of fibroblast and neutrophil collagenases are shown in Figure 1.2. For MMP-1 and MMP-8, the CD are slightly oblate spheroids in overall shape.[7,8,10] The extended substrate binding site consists of a groove that runs across one face of the molecule. From the viewpoint in Figure 1.2, the substrate binding site appears as a cleft in the lower right-hand side of both structures. It is demarcated on the top by strand IV, on its back side by helix B, and at the bottom by the loop connecting helices B and C. The bound inhibitors (Fig. 1.3 for chemical structures) that are positioned to the amino side (Fig. 1.2A and B) or carboxyl side (Fig. 1.2C) of the scissile peptide bond ligate the catalytic zinc of each enzyme through their hydroxamate or thiol functional groups.

The zinc electrophile is situated at the bottom of the catalytic site where it is coordinated to His218, His222 and His228 in MMP-1 and His197, His201 and His207 in MMP-8. In both enzymes, the two His residues in helix B project their Nε2 atoms toward the zinc. Helix B terminates at Gly204 in MMP-8 and at Gly225 in MMP-1; a turn at this position redirects the polypeptide backbone such that His207 or His228 completes the triad of nitrogenous residues that provide nearly perfect tetrahedral coordination of the metal ion.

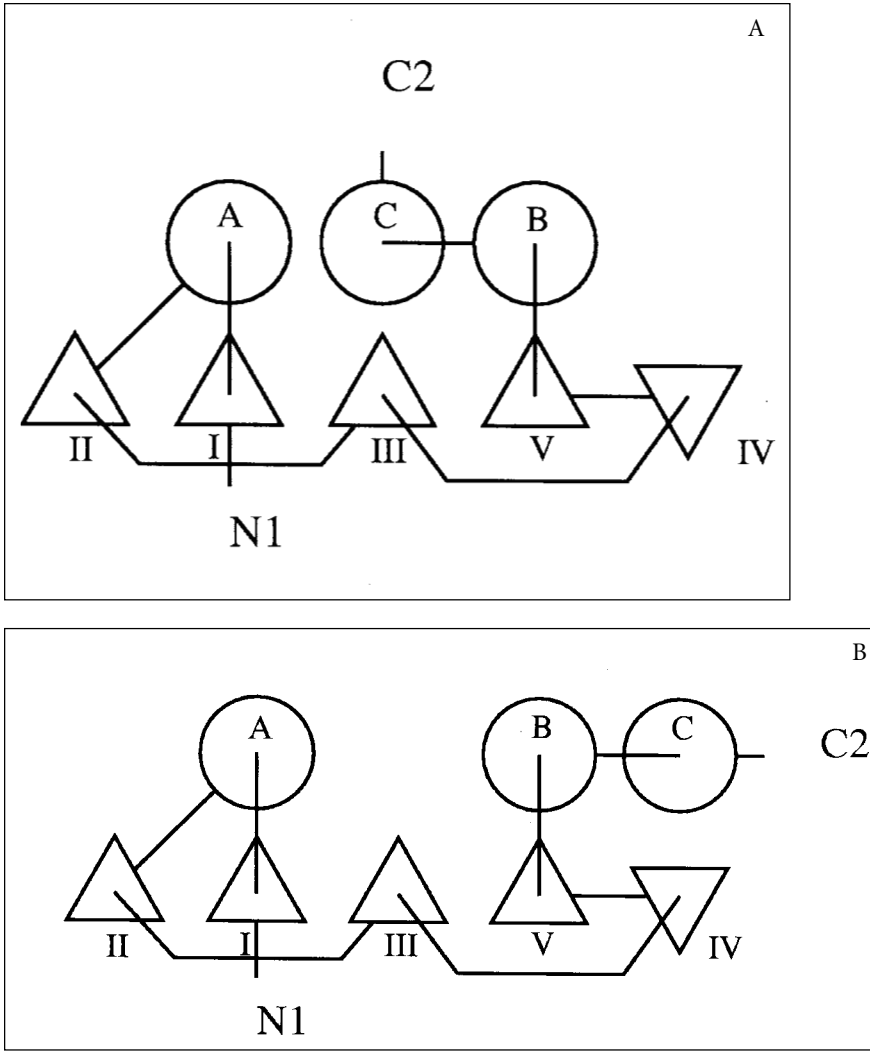


Fig. 1.1. Topology cartoon representing the folding pattern of human collagenase catalytic domain (panel A) and human neutrophil collagenase catalytic domain (panel B). The diagrams were generated with the computer program TOPS65 as modified by Westhead and which is available on the internet at http://tops.ebi.ac.uk/tops/. The triangles represent β-strands and the circles represent helices. Strand direction (N to C) is indicated by the direction of the triangle: upward pointing triangles represent strands pointing toward the viewer and downward pointing triangles indicate strands pointing away from the viewer. Helix direction is indicated by the position of the connecting line: center lines indicate upward pointing helices and edge connectors show downward pointing helices. The diagrams were generated from structural data in the Protein Data Bank (PDB codes 1hfc8 and 1jap10 for MMP-1 and MMP-8, respectively). Helices are labeled A-C and strands are labeled I-V. The diagrams depict the major similarities in secondary structure and folding of the two catalytic domains as well as the different placement of helix C with respect to strands III and IV.

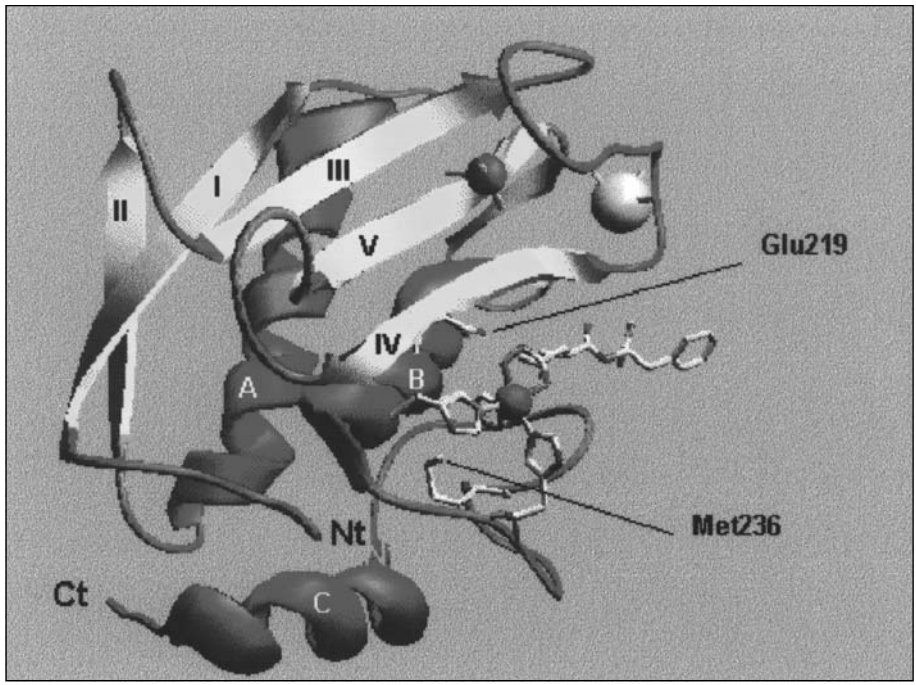


Fig 1.2 A. (above) Ribbon diagrams showing the structure of inhibitor complexes of the catalytic domains of MMP-1 and MMP-8. Panel A shows the complex of MMP-1 with NHOH–Leu–Phe–NMe (Fig. 1.3) and was generated from data of Spurlino et al[8] from Protein Data Base structure 1hfc. The amino and carboxyl termini of the molecule are indicated along with the strand and helix designations. The catalytic zinc (magenta sphere at right center) is shown ligated to three His residues of the protein and the hydroxamate group of the inhibitor. The putative catalytic Glu is shown projecting from helix B above the catalytic zinc, and the side chain of the Met at the bottom of the zinc binding site within the Met turn is also shown. The structural zinc (magenta sphere at upper right) and the calcium (light blue sphere, upper right) are shown in their binding sites where they fasten the large loop structure to the body of the protein. Panel B (see next page) shows a similar view of MMP-8 catalytic domain (data of Grams et al,[19] PDB 1ja0, complexed to HSBzPp–Ala–Gly–$NH_2$. The inhibitor is ligated via its thiol to the catalytic zinc and the P1' benzyl group lies within the S1' binding pocket. The structural zinc and calcium ion is depicted in the top center and top right–hand side of the diagram. The second calcium ion is depicted in the top left–hand side of the diagram. The positions of the catalytic Glu and zinc site Met are also shown. The (carboxyl) prime–side of the substrate binding region is shown by the extended inhibitor. Panel C (see next page) also shows MMP-8 bound to the inhibitor ProLeuGlyNHOH (blue structure) to illustrate the extended substrate binding site on the amino side of the cleavage site. The P3 Pro of the inhibitor resides in a narrow cleft defined by the side chains of His162, Phe164 and Ser151. The data are from Bode et al,[10] PDB 1jap. This figure as well as Figures 1.4 and 1.5 were generated from the referenced structural data using the Swiss Protein Viewer[66] with rendering in conjunction with Quickdraw3D and POV–Ray.

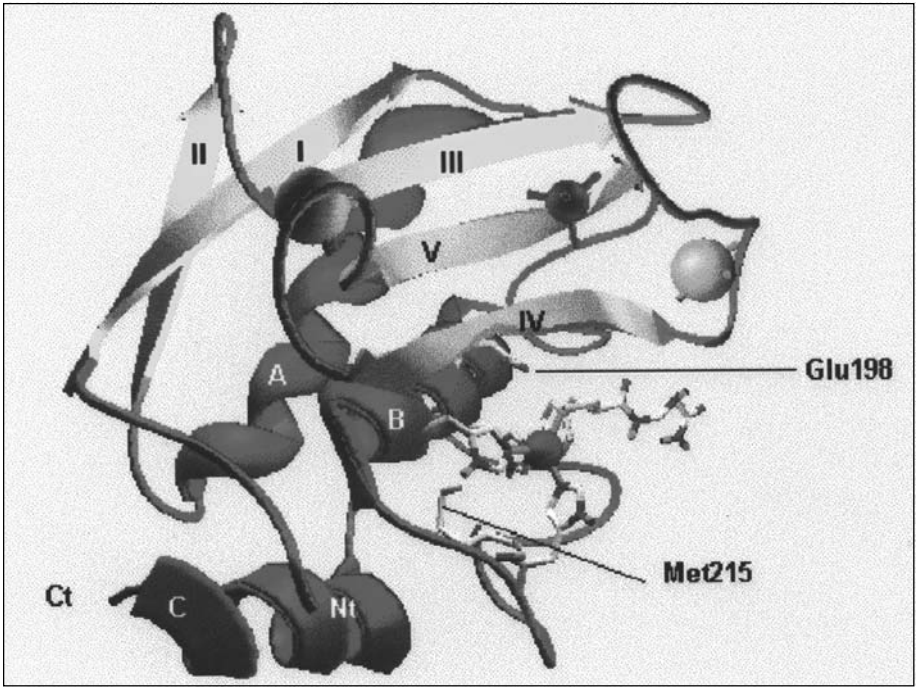


Fig 1.2 B.

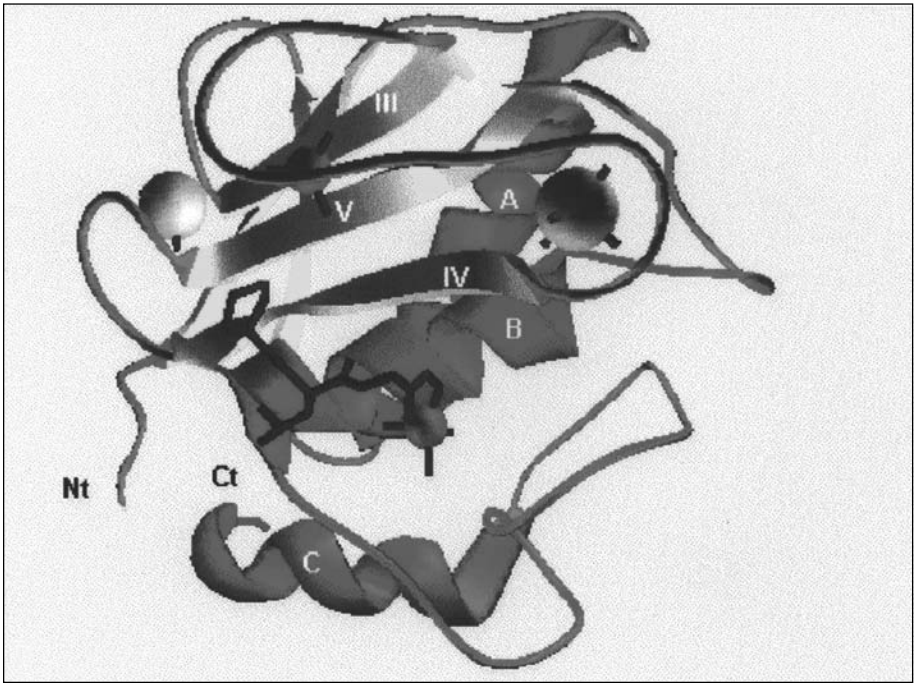


Fig 1.2 C.

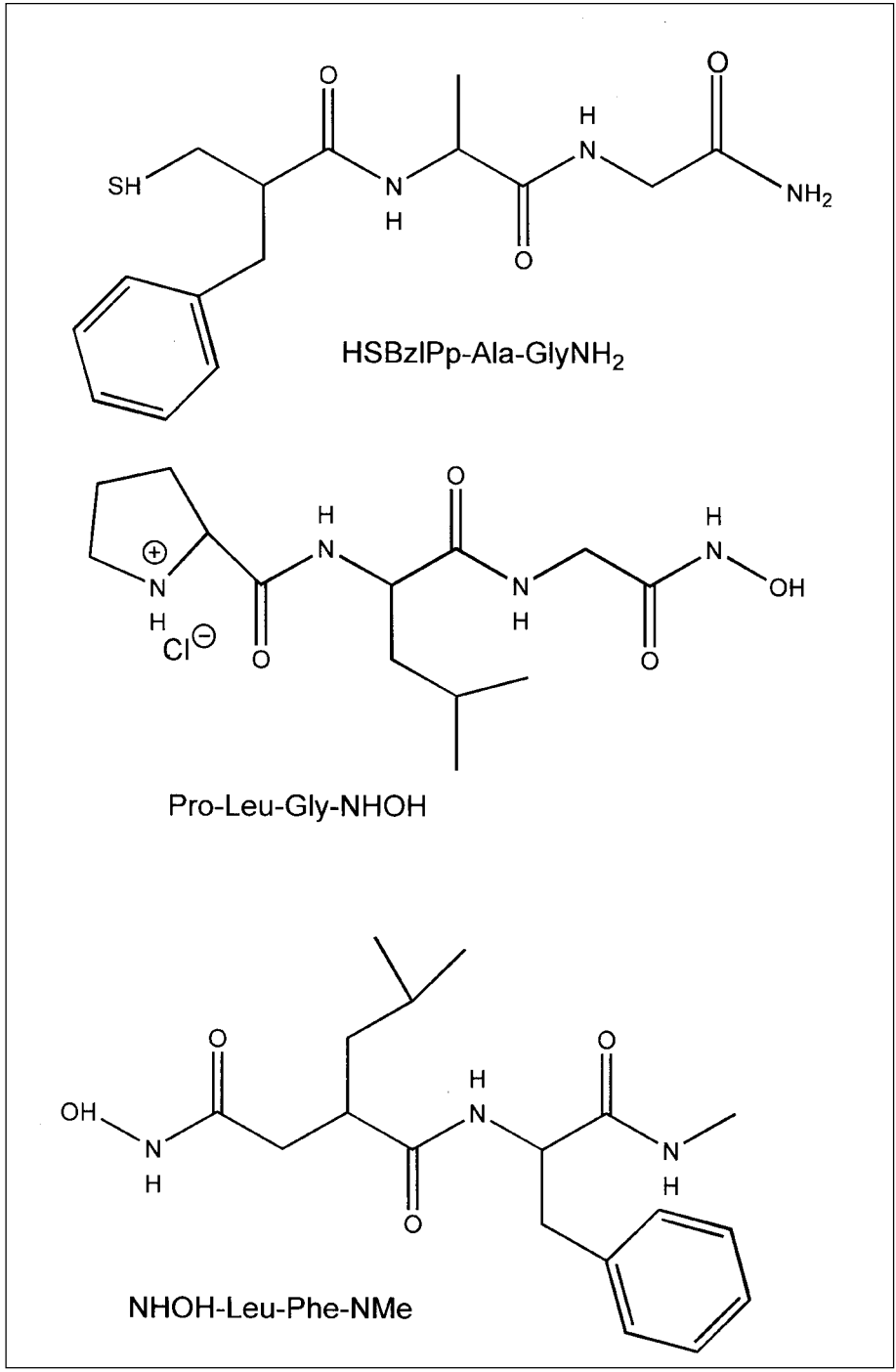


Fig. 1.3. Structure of the three peptide–based metal–coordinating inhibitors in Figure 1.2.

The zinc is positioned below the catalytic Glu, whose carboxyl group points toward it. The crystal structure of the fibroblast enzyme reveals a water molecule in this region that may be H-bonded to Glu219 such that the water's oxygen atom could serve as the nucleophile positioned to attack the scissile carbonyl in the hydrolytic step.[15] The characteristic Met residue at the base of the zinc binding site is found in a 1,4 turn consisting of homologous-Ala-Leu-Met-Tyr- sequences in the two enzymes.

Figure 1.2 also shows the positions of structural zinc and calcium ions. The noncatalytic zinc is bound in the upper right-hand region of the molecule within a tetrahedral site composed of His168, Asp170, His183, His196 (MMP-1) or His147, Asp149, His162, His175 in MMP-8. MMP-1 has one calcium binding site within the S-shaped loop connecting strands III and IV (residues175-181). Binding is mediated by the carboxyls of Asp175, Asp198 and Glu201 along with three peptide carbonyls. MMP-8, on the other hand, has two calcium ions. As with MMP-1, one calcium is bound to the carboxyls of Asp177, Glu180 and Asp 154 and three backbone carbonyls within the loop connecting strands III and IV, and a second binds at the beginning of strand V where Asp173 provides one ligating carboxyl group and the remaining ligands consist of backbone carbonyls and a water molecule. Evidently, the role of the structural zinc and calcium is to fasten the long loop connecting strands III and IV (which comprises the upper lip of the substrate binding groove) to the main body of the molecule. Thus these ions probably function in maintaining the conformational integrity of the substrate binding site.

### *Substrate and Inhibitor Binding Site*

Potential enzyme-substrate binding interactions are suggested in protein structures containing tightly bound inhibitors. These inhibitors (Fig. 1.3) are substrate analogues that incorporate a metal binding moiety such as a carboxyl, thiol or hydroxamate group in place of the scissile peptide bond. The metal binding group may be flanked by peptides designed to interact with the S (amino side) or S' (carboxyl side) subsites of the enzyme. In the structures shown, the inhibitors position themselves in an extended conformation within the substrate binding groove (Fig. 1.2). The metal binding functionality ligates the catalytic zinc at the open coordination position vacated by dissociation of the cysteinyl SH group provided by the propeptide. Inhibitor binding is stabilized by H-bonds with the backbone of the enzyme as well as by extensive interactions with the P1' residue and less extensive contacts with the P3 residue. Interactions with P1, P2' and P3' appear to be less extensive or absent altogether.

These structures reveal that in both the fibroblast and neutrophil enzymes, a primary determinant of substrate specificity lies within the S1' subsite, which consists of a rather expansive hydrophobic pocket that can easily accommodate the side chain of Ile or Leu found at the P1' position of collagen substrates. In MMP-8, the opening of this pocket is ringed by polar groups,[16] which can H-bond to inhibitors. The S1' pocket itself is lined with hydrophobic side chains (Leu 193 and Leu 214) while Arg222, whose guanidinium group is H-bonded to backbone oxygen atoms, defines the bottom of the pocket (Fig. 1.4). Interest-

Fig 1.4. (opposite) Close–up view of the S1' binding pocket of MMP-1 and MMP-8. These diagrams are from the same data as figures 1.2A and 1.2B. In panel A, the structure in gold is the inhibitor NHOH–Leu–Phe–NMe. Panel B shows MMP-8 with the P1' residue (HSBzPp portion only) ligated through its sulfur (yellow sphere) to the catalytic zinc (magenta sphere). The bottom of the S1' site is formed by Arg222 in MMP-8; in MMP-1, the homologous residue is Ser243 (Panel A). In MMP-1, the side chain of Arg214 projects into the S1' site. In both enzymes, strand IV forms the upper edge of the site and helix B forms the back side. Red atoms are oxygen, blue are nitrogen and gray are carbons. Hydrogen atoms are not shown.

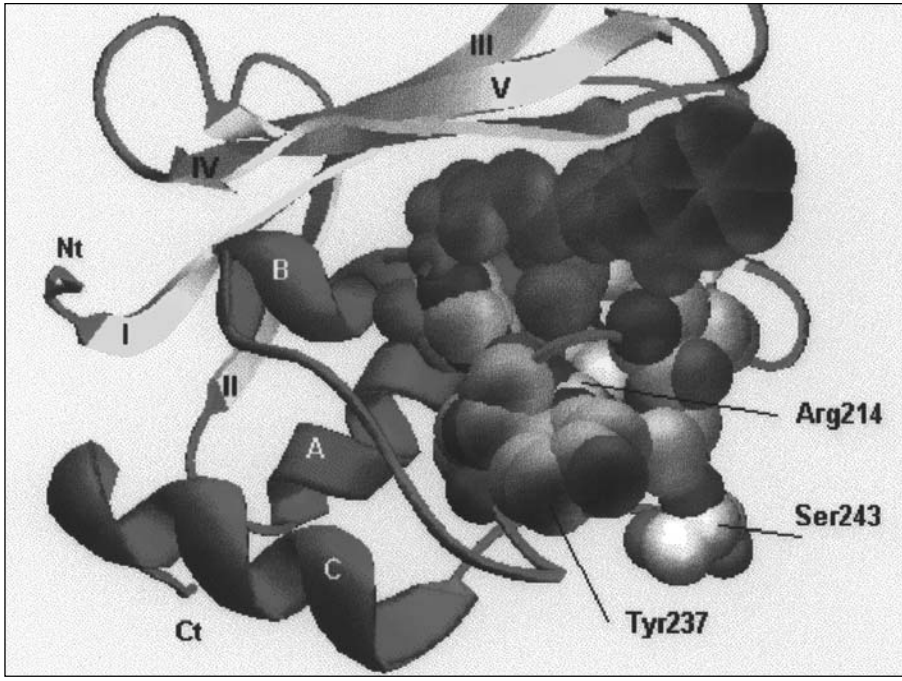


Fig 1.4 A.

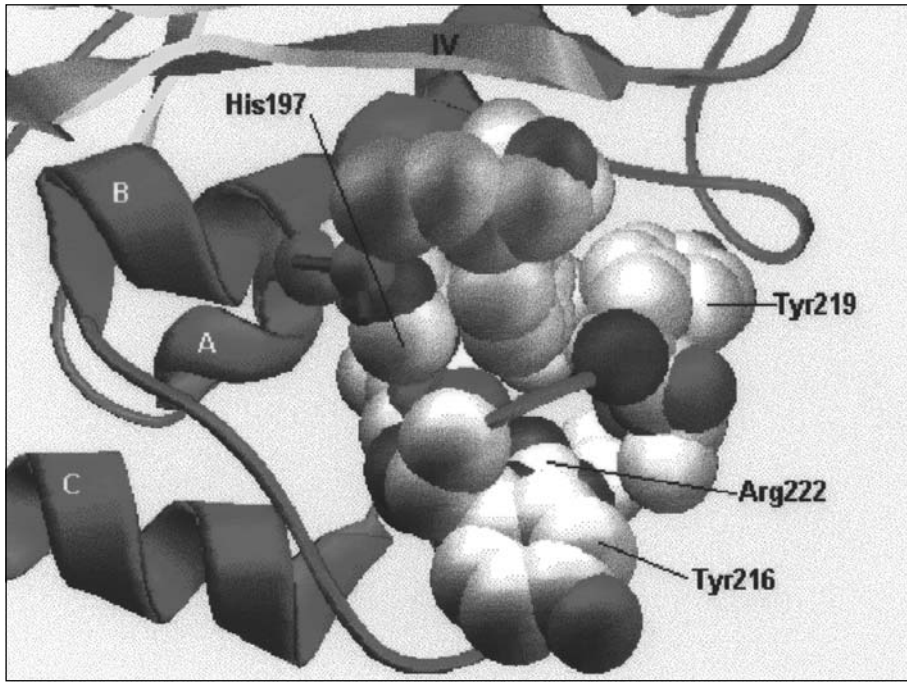


Fig 1.4 B.

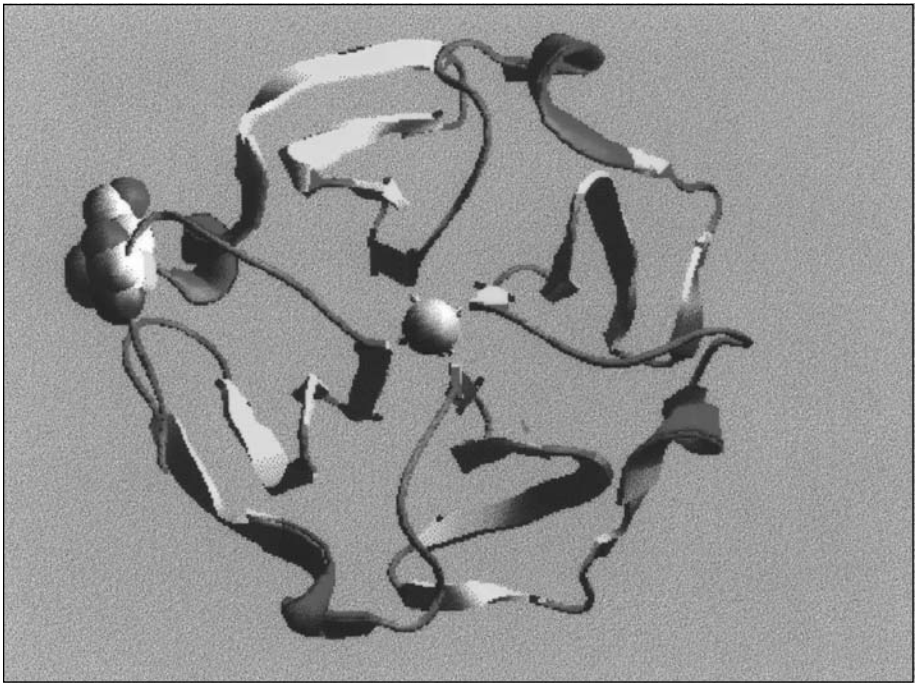

Fig 1.5 A.

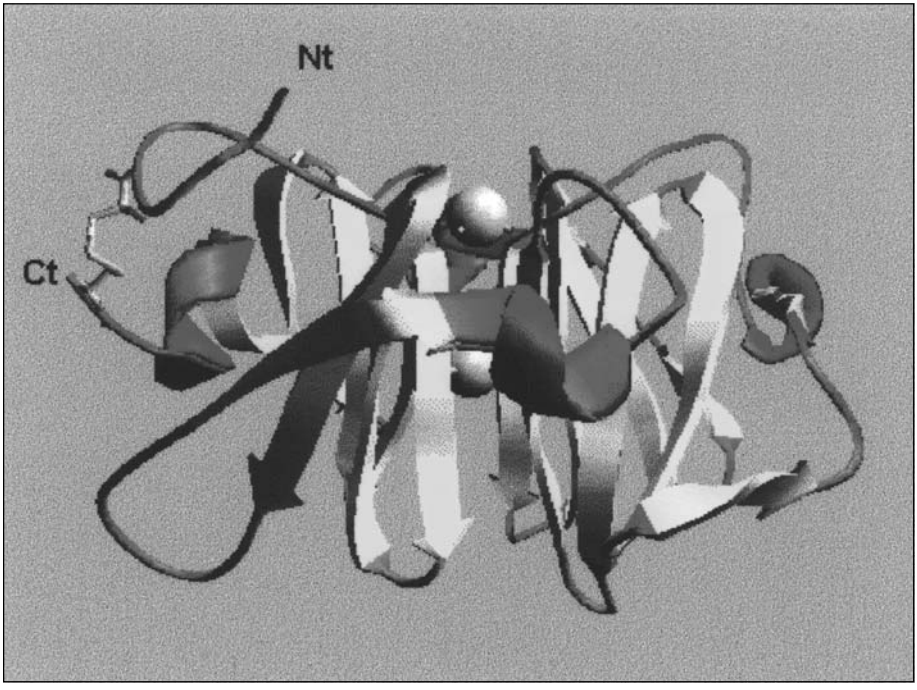


Fig 1.5 B.

ingly, the S1' pocket in MMP-8 contains two water molecules. These features probably account for a preference of the neutrophil enzyme for Tyr at this position.[19]

In MMP-1, the S1' pocket appears to be somewhat smaller than in MMP-8 (Fig. 1.4, panel B). Leu193 and Arg222 of MMP-8 are replaced in MMP-1 by Arg214 and Ser243, respectively; the former projects into the P1' pocket, thereby limiting access to it.

MMP-8 lacks well-defined S1, S2' and S3' sites. The S3 site, which accepts a Pro in substrates and inhibitors, consists of a narrow hydrophobic slot (Fig. 1.2, panel C) and the S2 site is rather shallow.(76)

## The Pexin Domain

The PD is required for triple helicase activity of collagenase.[20,21] Domain swapping experiments show that chimeric enzymes composed of stromelysin CD and collagenase PD or vice versa are unable to cleave triple helical collagen.[22,23] Similarly, a chimeric protein produced by linking the CD of MMP-8 and the PD of stromelysin is not a collagenase.[24]

### *Folding Topology and Structure*

The PD is linked to the CD in collagenases through a 12-residue linker.[10,25] The PD consists of multiple β-strands arranged in subdomains such that the overall structure resembles a four-bladed propeller. Figure 1.5, panel A, is a top view of the PD which clearly shows the remarkable pseudo 4-fold symmetry with one calcium ion positioned on the propeller axis and the other directly below it. At the upper left is illustrated the conserved disulfide bond that tethers the N and C-termini of the PD. Site-directed mutagenesis indicates that the integrity of this disulfide bond is essential for the expression of collagenase activity.[26]

The orientation in panel B reveals both the discoidal shape and the funnel-like topology of the PD. The inner strands are closely packed and completely buried while the outer strands are less tightly packed. Positioned at the top and mid-way down the stem of the funnel are two calcium ions. The disulfide bond is located at the upper left-hand part of the diagram. The PDs of pig collagenase and human 72 kDa gelatinase B are quite similar in overall structure.

The mechanism by which the PD endows collagenolytic activity on CD is unknown. Based on modeling, Gomis-Rüth et al[13] proposed that triple helical collagen binds to one surface of the PD which then folds over the CD and presents the scissile peptide bond to the catalytic zinc. Since the active site groove is too narrow to accommodate all three collagen chains simultaneously, strand separation must precede hydrolysis.[11] Based on comparisons of surface charge distribution in the PDs of MMP-1, MMP-2 and MMP-13 and the above referenced studies with chimeric proteins, these workers suggested that a positively charged region within blade II that is conserved in the PDs of MMP-1 from several species might be

Fig. 1.5. (opposite) Ribbon diagrams of the pexin domain of collagenase-3. The data are from Gomis-Rüth et al[13] (PDB 1pex). The view in panel A is from the top of the pexin domain and shows its four–fold symmetry. One of the chelated calcium ions is represented by the light blue sphere on the symmetry axis; a second calcium and two anions are also located in the central opening but are not shown. The disulfide connecting Cys248 and Cys496 is shown in space–filling form in the upper right hand side of the molecule. Panel B illustrates a side view of the pexin domain to illustrate the funnel–like nature of the structure with a central cavity containing two calcium ions (light blue spheres). The disulfide bond linking the N and C terminal parts of the domain is illustrated in the upper lift. In the full–length molecule, the N-terminal segment proceeds through the proline–rich region to connect to the catalytic domain, as seen in the porcine structure. The figures were generated as described in the legend to Figure 1.2.

important in conferring collagenolytic activity to these enzymes. However, they also pointed out that this patch was not strongly conserved in MMP-8, so the exact role of the PD and its linker in conferring collagenolytic activity remains an enigma.

## Bacterial Expression of Mammalian Proteins

The ability to express native MMPs in heterologous organisms has clearly been essential for the foregoing structural studies. Expression of a foreign protein in bacteria can lead to one of three outcomes.[27] Occasionally protein expression is not discernable. Lack of detectable expression can result from low rates of transcription or translation or to proteolysis of the target protein prior to folding. Alternatively, the protein may be expressed in soluble form, either in the cytoplasm or, if a secretory signal is present, in the periplasm. Most frequently, however, the over-expressed protein localizes in intracellular aggregates referred to as inclusion bodies (IBs). These dense particles contain recombinant protein insoluble in all but the strongest denaturants.

The detailed mechanism of IB formation is unclear, thus making it impossible to predict whether a particular construct will be expressed in soluble or insoluble form. Rudolph[28] suggested that several factors determine whether soluble protein or inclusion bodies are expressed. These include the relative rates of protein synthesis, folding, aggregation, the solubility of the protein, the relative stability of the folded and intermediate states, susceptibility of the partially folded protein to proteolysis and the presence of chaperones.

To address the role of amino acid sequence in IB formation, Kreuger et al[29] studied the distribution of soluble and insoluble forms of the bacterial protein CheB. When over-expressed in *E. coli*, the relative amounts of soluble and insoluble forms of CheB depended on the amino acid sequence of the protein. Wild type CheB was almost exclusively found in IBs. However, some mutants partitioned between soluble and precipitated forms in an unpredictable manner.

More recent studies summarized by King et al[30] suggest that a pathway for IB formation in the case of phage P22 tailspike protein involves a particular intermediate that tends to associate in a step-wise fashion to form discrete oligomers. Formation of these multimeric species is sufficient to direct the newly synthesized peptide chains toward the IB pathway rather than the folding pathway. Genetic methods allowed isolation of temperature sensitive mutants and subsequent mapping of the sensitive sites. Interestingly, the mutant proteins did not exhibit lower temperature stability; rather, the folding pathway itself proved the temperature sensitive determinant.

In another set of studies, Wetzel and Chrunyk[31] observed the temperature-dependent of expression of recombinant interleukin-1β (IL-1β) in *E. coli.* They found that the proportion of IL-1β deposited in IBs decreased with deceasing growth temperature. In addition, they observed a strong correlation between the effect of temperature on in vitro aggregation in denaturants and IB formation in vivo, suggesting a common structural intermediate was responsible for protein aggregation in either case. They noted that one particular mutant (Lys97Val) of IL-1b was more prone to IB formation than wild-type IL-1β, even though this mutant was more thermally stable than the wild type protein. They interpreted this to indicate specific amino acids in a particular structural context influence the rate of partition of folding intermediates into the folding and aggregation pathways. Other studies show that IB proteins can have secondary structure, consistent with the hypothesis that IBs consist of partially folded intermediates that self-associate rather than achieving a fully folded and soluble state.[32-34]

## General Aspects of Protein Renaturation

Because recombinant proteins are frequently expressed in an insoluble state that can only be retrieved by dissolving in strong denaturants, understanding protein renaturation is of practical interest in generating a functional recombinant protein. Aspects of the protein folding problem are therefore relevant to the preparation of most recombinant proteins.

### *Mechanism of Protein Folding*

The mechanism of protein folding is defined in terms of the structure of intermediates that exist on the pathway from the unfolded to the folded state.[35] One can define a theoretical folding pathway of a protein in terms of intermediate structural states:

$$U \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow I_3 \leftrightarrow \ldots I_n \leftrightarrow N.$$

U represents unfolded protein, I represents various hypothetical intermediates and N represents native protein. For small single domain proteins, folding is often highly cooperative and the concentration of intermediates therefore immeasurably small.

In practical terms, the yield of native protein in an in vitro folding experiment can be reduced because unproductive side reactions compete with the folding pathway, thereby leading to irreversible loss of protein. For example, Brems[36] suggested that with bovine growth hormone, monomeric intermediate I may associate to form soluble polymeric intermediate(s) $I_{assoc}$ which subsequently aggregate to yield precipitated protein: $I_n \rightarrow I_{assoc} \rightarrow$ aggregates.

Structural properties of the partially folded intermediates $I_n$, such as surface exposured hydrophobic groups normally buried within the interior of the fully folded protein, may render the folding intermediates especially prone to self-association. Folding and aggregation therefore represent mutually exclusive competing processes, both of which minimize thermodynamically unfavorable interactions of exposed hydrophobic groups with aqueous solvent. Clearly, minimizing the rate of intermolecular interactions will favor folding (a first order kinetic process) over aggregation (a higher order kinetic process). Strategies for optimizing the yield of folded protein at the expense of misfolded structures are thus designed to minimize aggregation. Within the cell, this can occur through the action of molecular chaperones, which prevent partially or improperly folded peptides from interacting with each other. In vitro, it can be achieved by dilution or by refolding from intermediate denaturant concentrations which effectively solubilize intermediates with exposed hydrophobic surfaces while they undergo productive folding and incorporation of the offending groups within the protein interior.

In addition to aggregation, recombinant proteins with cysteine residues are also susceptible to formation of incorrectly paired disulfide bonds. Such abnormal structures can be either intramolecular, intermolecular or, when multiple cysteine residues are present, both can form. Refolding strategies must therefore provide for reshuffling mispaired disulfide bonds. Refolding in a redox buffer that contains a mixture of oxidized and reduced thiols such as dithiothreitol (DTT), cysteamine, cysteine, or glutathione (GSH) readily accomplishes this goal. GSH can be especially valuable as a redox buffer because its mixed disulfide with protein thiols provides additional ionic groups to increase the solubility of folding intermediates. In addition, chelating agents such as EDTA prevent redox active metal ions (adventitious iron or copper salts) from participating in -SH autooxidation.[37]

What are the structures of folding intermediates? As pointed out above, the cooperative nature of protein folding generally makes isolation of stable folding intermediates unlikely. However, stable partially folded proteins can be generated and characterized at concentrations of denaturant below those needed for complete unfolding. Such structures may simulate unstable intermediates formed transiently during folding. One structure, termed

the molten globule,[30,38,39] has received considerable attention. This structure is defined as possessing native secondary structure but lacking organized tertiary structure. On exposure to physiological milieu, the unfolded protein assumes a conformation with secondary structure, but without optimal side chain packing.

Physical techniques such as circular dichroism, fluorescence, and molecular sizing reveal characteristic properties of the molten globule state. For example, a low UV circular dichroism spectrum is characteristic of folded protein. In contrast, techniques that assess organization of aromatic side chains (near UV circular dichroism and fluorescence emission) reveal the properties of an unfolded protein. Molecular sieving shows that the molten globule state is more compact than the fully unfolded state but less compact than the folded protein. Hydrophobic surfaces can be detected by binding probes such as anilinonaphthalene sulfonate (ANS) whose fluorescence properties are sensitive to local polarity.

In two recent excellent reviews, Rudolph[40,41] summarized practical aspects of isolation and folding of recombinant proteins from IBs. After disruption of cells, IBs can be separated from other cellular particles by centrifugation at 5000-12000 x g., washed and then solubilized in chaotropes. Guanidinium (Gdn) salts are preferred to urea because urea can lead to irreversible modification of protein amino groups if contaminating cyanate is not eliminated.[42] Detergents have also been used for solubilization and may present the advantage of minimizing the contamination of recombinant proteins with difficult to remove bacterial lipids.[43] Not surprisingly, it has proved impossible to formulate general recipes for refolding all recombinant proteins. However, a number of additives and solution conditions (summarized in ref. 20) have been shown empirically to improved the yield of refolded protein in specific cases.

## Expression and Folding of Recombinant MMPs

Various truncated and full-length forms of MMPs have been successfully expressed in *E. coli* and refolded where necessary to yield active proteinases. The following section reviews specific examples of the purification methods and refolding procedures of these expression systems.

### *Fibroblast Collagenase*

A number of laboratories have reported expressing various forms of fibroblast collagenase, including the full-length enzyme containing the propeptide, the enzyme lacking the propeptide, and the catalytic domain alone. The expression system of Windsor et al[26,44] yielded procollagenase in IBs that were solubilized in 2 M GdnHCl/50 mM Tris-HCl (pH 7.5)/0.2 M NaCl/ 5 mM $CaCl_2$/1 μM $ZnCl_2$. The enzyme was diluted 20-fold into 50 mM Tris- HCl (pH 7.5)/0.2 M NaCl and purified by chromatography on an antibody affinity column. The specific activity of the recombinant enzyme was one-seventh that of the native enzyme, but exhibited the same specific activity as native enzyme when assayed against β-casein. It spontaneously cleaved the propeptide and underwent the autolysis typical of native collagenase.

Lowry et al[45] expressed the catalytic domain of human fibroblast collagenase (residues 101-269). The insoluble enzyme was dissolved in 5 M urea, purified, and renatured by dialysis against 50 mM Tris-HCl (pH 7.5)/0.2 M NaCl.

Hassell et al[46] also expressed fibroblast collagenase catalytic domain using pET11- derived vector. The resulting insoluble protein was separated by centrifugation and dissolved in 6 M urea. After purification by anion exchange and reversed phase chromatography, the protein was dialyzed against 6 M urea/50 mM Tris-HCl (pH 7.5)/ 0.2 M NaCl. It was diluted to 0.1 mg/ml and refolded by dialysis against 50 mM Tris-HCl (pH 7.5)/0.2 M NaCl/1 mM $CaCl_2$/50 μM $ZnCl_2$. 2,4-Methyl pentanediol was also included (0.1%), with the effect of

reducing protein precipitation and enhancing enzyme stability. N-terminal analysis revealed three predominant species: the full-length enzyme, N-1, and N-2 truncated forms.

O'Hare et al[47] generated a mutant rHFC in which autolytic sites Ala258Ile and Pro269Ile were mutated to Ala258Ser and Leu269Ile, respectively. The mutant DNA was expressed as a fusion protein with glutathione-s-transferase (GST). IBs were solubilized in 8 M urea/0.5 M Tris/HCl (pH 7.9)/0.5 M NaCl/10 mM $CaCl_2$/30 mM β-mercaptoethanol/1 mM phenylmethanesulfonyl fluoride (PMSF) followed by folding by dialysis versus 50 mM Tris-HCl (pH 7.9)/0.5 M NaCl. The protein cleaved itself from the GST during folding and the resulting collagenase was purified on a peptide hydroxamic acid (PHA) affinity column.[48] This preparation was stable to autolysis; the specific activity assayed with collagen was reportedly to be the same as native enzyme.

The expression system of Gehring et al[49] is interesting because it yields soluble protein. These researchers generated a fusion protein consisting of the catalytic domain of human fibroblast collagenase C-terminally linked to yeast ubiquitin through a linker doubling as an autocatalytic cleavage site. The soluble protein was purified on Q-Sepharose and allowed to undergo autolytic separation from the ubiquitin.

Using a construct supplied by Dr. G. McGeehan of Glaxo-Wellcome Laboratories, we[50] surveyed conditions for refolding a full-length human fibroblast collagenase construct. Optimal refolding occurred when the purified, denatured protein was diluted from 6 M GdnHCl into a solution containing 2 M GdnHCl, 20% glycerol, 2.5 mM oxidized and reduced glutathione, and 5 mM $CaCl_2$ at 4°C. DTT was not as effective as GSH, and lower yields were observed in the absence of glycerol or at higher temperatures (25°C or 37°C). Removal of the remaining denaturant by a gel filtration spin column afforded a preparation indistinguishable from native collagenase in terms of specific activity measured on peptide and collagen substrates. We speculated that an intermediate denaturant concentration was important for solubilizing intermediates prone to aggregation instead of folding.

### *Porcine Collagenase*

O'Hare et al[47,51] expressed porcine collagenase as an N-terminal fusion protein with *E. coli* β-galactosidase. After solubilization in 8 M urea/0.5 M Tris-HCl (pH 7.9)/0.5 M NaCl/10 mM $CaCl_2$/30 mM β-mercaptoethanol/1 mM PMSF, the protein was refolded by dialysis against 50 mM Tris-HCl (pH 7.9)/0.5 M NaCl. The fusion protein was proteolytically active, resulting in the partial separation of collagenase from β-galactosidase. Complete cleavage was achieved by treatment with Factor Xa and the resulting recombinant collagenase purified by affinity chromatography on PHA Sepharose. Interestingly, the recombinant enzyme exhibited different stability toward autolysis than did native enzyme. In the recombinant enzyme, autolytic cleavage occurred at three sites: Tyr241Gly, Ser244Glu and Ser 251Gly. But native porcine collagenase was cleaved only at Ala239Ile; the corresponding bond was intact in the recombinant enzyme. The authors suggested that the two enzymes were not equivalently folded in the peptide segment (hinge region) harboring the autolytic sites.

## Neutrophil Collagenase

Schnierer et al[52] expressed the catalytic domain of neutrophil procollagenase. The enzyme was produced as IBs solubilized in 6 M urea/100 mM β-mercaptoethanol/20 mM Tris (pH 8.5). Removal of urea by dialysis resulted in activation and autolytic degradation of the recombinant protein. This problem was avoided by refolding the enzyme while bound to Q-Sepharose.

Ho et al[53] also obtained neutrophil collagenase using the pET11a vector. They expressed both the catalytic domain and the catalytic domain containing propeptide. In both cases, IBs containing the protein were separated by centrifugation and solubilized in 6 M urea.

The solubilized material was purified on a MonoQ column and renaturation was accomplished by diluting the soluble protein into buffer containing $Zn^{2+}$ and $Ca^{2+}$. The proteins were further purified by PHA-Sepharose chromatography. Analysis using peptide substrates showed kinetic constants nearly equivalent to the native enzyme.

## Other MMPs

### *Promatrilysin*

At least two laboratories have reported expressing recombinant promatrilysin. In conjunction with a study of zinc binding to MMPs, Soler et al[54] expressed promatrilysin fused to the C-terminus of GST. The chimeric protein was localized in IBs dissolved in 2% palmityl sulfobetaine and purified by affinity chromatography on GSH-agarose. The MMP was separated from GST by thrombolytic cleavage of the fusion protein. Promatrilysin was recovered in homogeneous form by chromatography with MonoS and activated by treatment with organomercurials, resulting in a specific activity equivalent to that observed with protein expressed in a mammalian cell system. The zinc content of both samples was also equivalent (approximately two g-atom per mol of protein).

Itoh et al[55] also recently reported expression and refolding promatrilysin. This group generated a cDNA from a human rectal carcinoma cell line by PCR and inserted it into an expression vector containing T7 promoters, a ribosomal binding site, an initiator codon followed by a hexahistidine coding sequence, and a termination codon. The protein was expressed in *E. coli* BL21(DE3), a strain which carries a copy of T7 RNA polymerase under control of the IPTG-inducible lacUV5 promoter.[56] The (His) 6-tagged protein was found in IBs that were isolated by centrifugation, washed with Triton X-100 (1%) and solubilized in 8 M urea/10 mM Tris-HCl (pH 8.0)/100 mM Na phosphate/100 mM β-mercaptoethanol. It was purified on a nickel chelating resin by washing with 8 M urea. But surprisingly, the enzyme could not be eluted by standard methods with imidazole (0.5 M).

The authors used a novel refolding procedure to maximize the yield of proenzyme. While bound to the resin, they incubated the protein at 4°C for 12 hours in the presence of 6 M urea. The bound enzyme was then resuspended in urea-free buffer and utilized in the solid phase for kinetic studies.

The proenzyme could be eluted from the chelating column at pH 4.5 in 6 M urea/1% Triton X-100/100 mM Na phosphate. The pH of the eluate was increased to 7.5 and the soluble preparation dialyzed against 50 mM Tris-HCl (pH 7.5)/150 mM NaCl/10 mM $CaCl_2$/ 0.05% Brij 35/0.02% $NaN_3$ /1% Triton X-100/6 M urea. The urea concentration was then decreased by dialysis in a step-wise manner to 3, 1.5, 0.5 and 0 M and the Triton concentration was reduced to 0.1%. The resulting preparation contained only about 20% activated enzyme. The authors suggested that refolding the enzyme while bound to the solid matrix was an effective strategy because linkage through the C-terminal $(His)_6$ allowed folding to be initiated at the N- terminus, as would occur naturally on the ribosome. The specific activity measured with a synthetic substrate was only about half that of the native enzyme. This was attributed either to improper folding or to loss of active enzyme by autolysis.

### *Stromelysins*

The catalytic domain of stromelysin was expressed in both soluble and insoluble forms by Ye et al[57] in the vector pGEMEX in *E. coli* strain DH5αF'IQ. When the cells were grown at 37°C, the recombinant protein was insoluble; however, when growth temperature was lowered to 27°C, the recombinant protein was soluble and could be purified by chromatography on phenyl Sepharose using a decreasing gradient of ammonium sulfate (1 to 0 M) and

an increasing gradient of $CaCl_2$ (5 to 20 mM) in 50 mM Tris-HCl (pH 7.6). Further purification was achieved by chromatography on Q-Sepharose.

The insoluble protein obtained by growth at 37°C was dissolved in 8 M GdnHCl and refolded by drop-wise (pulse) dilution into 10 mM Tris-HCl (pH 7.6)/10 mM $CaCl_2$/0.1 mM $ZnCl_2$ containing a mixture of protease inhibitors (leupeptin, aprotinin, and pepstatin). Some of the protein precipitated under these conditions; it could be recovered by centrifugation, resolubilization in 8 M GdnHCl, and a repetition of the refolding procedure. The refolded protein was purified by chromatography on Q-Sepharose.

### *Gelatinase A*

A 19 kDa catalytic domain related to gelatinase A was expressed by Ye et al[58] from a synthetic gene constructed such that the fibronectin-like insert within the catalytic domain of the native enzyme was deleted. The resulting construct was inserted into pGEMEX and was expressed in BL21 (DE3)/pLysS. An insoluble protein was recovered in 6 M urea and purified by anion exchange chromatography under denaturing conditions. The apoenzyme was refolded by dialyzing out the urea and followed by activation through reconstitution with $Zn^{2+}$.

### *Gelatinase B*

Collier et al[59] expressed gelatinase B residues 93-708. IBs were solubilized in 8 M urea/25 mM Tris-HCl (pH 7.5)/5 mM $CaCl_2$/1 mM PMSF/0.2 M NaCl/10 mM EDTA. Soluble protein was obtained after dialysis against buffers containing successively, 6, 4, 2 and 0 M urea. The active recombinant protein was purified by gelatin-agarose chromatography.

Xia et al[60] recently expressed the gelatinase B catalytic domain of rat (107-463). The protein was first solubilized in 6 M urea and purified on a nickel chelating column, and then refolded on the column by washing with a gradient of 4-0.5 M urea in 5 mM $CaCl_2$/20 mM Tris-HCl/0.2 M NaCl. After elution with 80 mM imidazole/5 mM $CaCl_2$/20 mM Tris/0.2 M NaCl/0.5 M urea, the remaining urea was removed by dialysis. The enzyme was reported to be fully active.

Pourmotabbed et al[61] expressed full length neutrophil gelatinase B in *E. coli*. A cDNA encoding gelatinase B, derived from the library of a patient with chronic granulocytic leukemia, was ligated into pET-12C. The resulting construct was expressed as a fusion protein with the bacterial ompT leader sequence to direct the protein to the periplasm. The enzyme was isolated in folded form from the periplasm after osmotic shock or freeze/thaw and subsequently purified on gelatin agarose. The recombinant enzyme exhibited a mass of 72 kDa (expected for the unglycosylated protein) in electrophoresis. Analysis by zymography showed proteolytic activity of apparent masses of 45, 62 and 92 kDa, perhaps reflecting both autolysis and protein aggregation.

### *MT1-MMP*

Sato et al[62,63] expressed two membrane-type matrix metalloproteinase derivatives as fusion proteins with GST. One construct contained the pro, catalytic, and hinge domains; the other contained these domains and the pexin domain. The fusion proteins were proteolytically active; they catalyzed self-cleavage and activated progelatinase A. Lichte et al[64] also reported expression of the catalytic domain of MT-MMP.

## Mechanism of Collagenase Folding

### *Catalytic Domain*

Lowry et al[45] investigated the folding mechanism of the catalytic domain of fibroblast collagenase. Workers in his laboratory utilized intrinsic fluorescence and CD to study the effects of $Ca^{2+}$ and $Zn^{2+}$ on the stability of the truncated recombinant enzyme. The catalytic domain contained three tryptophan residues whose emission maxima shifted from 328-335 nm in the folded protein to ~350 nm in the denatured protein, with an accompanying decrease in emission intensity of roughly 50%. The protein was unexpectedly sensitive to denaturant as indicated by the shift in emission maximum; it was 50% denatured in less than 1 M GdnHCl. $G(H_2O)$ of unfolding was found to be 0.6 kcal/mol for the truncated protein and 2.1 kcal/mol for the full-length protein (catalytic + pexin domains).

### *Full-Length Collagenase*

Zhang and Gray[50] have provided evidence for intermediates in the unfolding pathway of fibroblast collagenase. One intermediate structure observed in 1 M GdnHCl had a slightly larger Stokes radius than the fully folded protein and contained ordered tryptophan residues with a somewhat higher quantum yield than the folded protein. The second intermediate, which was stable between 2 and 4 M GdnHCl, exhibited properties consistent with those of a molten globule state. It contained secondary structure; however, ordered tertiary structure appeared to be lacking, as evidenced by the near UV circular dichroism spectrum. The Stokes radius was intermediate between that of the folded and unfolded states. Binding of the hydrophobic probe ANS was evident in this state, but not in the fully folded or unfolded states. The data are consistent with a folding mechanism that includes at least two intermediates: $U \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow N$.

## Summary

At present, representatives of the major MMPs have been successfully expressed in *E. coli*. Several expression systems have been utilized with or without the propeptide, including MMP catalytic domains. N and C-terminal fusion proteins with glutathione S-transferase, ubiquitin and β-galactosidase have also been also used. In the majority of cases, the expressed protein is found in IBs, even when the expressed MMP was fused to a soluble protein; an exception was the yeast ubiquitin system, which yielded soluble protein. Most workers solubilize the IBs in urea, although guanidine and cationic detergents are also effective. Refolding has been successfully carried out using a variety of techniques, including stepwise lowering of denaturant through dialysis, refolding while bound to an insoluble matrix, and pulse dilution. However, there are relatively few reports of a detailed comparison between the recombinant and native proteins.

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CHAPTER 2

# Chondrocyte Expression of Collagenase 2

**Ada A. Cole and Klaus E. Kuettner**

## Introduction

Collagenase 2, also called neutrophil collagenase or matrix metalloproteinase (MMP)- 8, was originally identified as a select gene product of neutrophils.[1] While this gene was thought to be expressed exclusively by the neutrophil, our studies have shown that it is also expressed by chondrocytes.[2-4] Since two substrates of collagenase 2 are collagen type II and aggrecan,[1,5] the two major components of the cartilage extracellular matrix (ECM), collagenase 2 is a potential candidate for massive matrix destruction that accompanies joint diseases, such as osteoarthritis (OA). The ability of collagenases to disrupt the collagenous framework is thought to be a critical component of a cascade leading to joint dysfunction.[6] Our data[7] have additionally shown an elevation of collagenase 2 gene expression in cartilages from patients with OA. This evidence adds support to the hypothesis that collagenase 2 may be involved in a disease process which includes the destruction of articular cartilage.

## Articular Cartilage

The hyaline cartilage that lines the joint cavity is relatively thin[8](0.6-6 mm). It sits between the synovial cavity on its superficial surface and the subchondral bone on its deep surface. The tissue is avascular and aneural, being composed exclusively of chondrocytes surrounded by an extensive ECM. In the adult the cartilage is relatively acellular with chondrocytes in the deeper zone having a matrix domain of 180,000 $\mu m$,[3] defined as the ratio of matrix mass per unit volume of tissue to the number of cells.[9] Of the total human adult articular cartilage volume, chondrocytes occupy only 2%.[10] The chondrocyte is responsible for the maintenance of this extensive ECM.

Articular cartilage is a stratified tissue from the articular surface to the subchondral bone, with four zones recognized morphologically: zones I through IV, the superficial, middle, deep and calcified zones, respectively.[11] These subdivisions are based on differences in cellularity, ECM composition, and material properties.[12] The superficial zone contains flat, disc-shaped chondrocytes; the ECM has a relatively low proteoglycan (PG) and high collagen content. These specialized cells secrete a novel PG termed superficial zone protein into the synovial fluid,[13] rather than the chondrocytes deeper in the cartilage. In the middle and deep zones, the chondrocytes are more spherical; the ECM has a higher content of PG and is traversed by thick collagen fibrils. In addition to this stratification, the ECM can be subdivided, in both the middle and deep zones according to distance from the chondrocyte

*Collagenases*, edited by Warren Hoeffler. 

plasmalemma, into pericellular, territorial and interterritorial ECM compartments, differing in structure and biochemical composition. The distinct microanatomical and functional units of cartilage, known as chondrons, are found in the middle and deep zones. The chondrons consist of either individual chondrocytes or several cells in a column, with their associated pericellular ECM and surrounding specialized capsular ECM.[14] Articular cartilage is attached to subchondral bone by a zone of calcified cartilage. The interface between the noncalcified and calcified cartilage is demarcated by the tide mark.[15] Arcades of collagen fibers, called cascades, originate as thick bundles in the calcified cartilage (but do not cross the subchondral bone), traverse the deep and middle zones gradually becoming thinner before they become aligned parallel to the articulating surface.[16]

### *Articular Cartilage Collagens*

The articular cartilage serves to spread the biomechanical load over the surface during joint movement. The properties of cartilage are a result of containment of the swelling pressure of highly charged PGs by entrapment in a network of collagen fibrils that are tension resistant. In cartilage, collagen types II, VI, IX, X, XI, XII and XIV are expressed with type II comprising over 90% of the collagen present in the ECM.[17] Type II collagen is the main building block of the fibril with type XI forming a core around which the type II collagen molecules are deposited. Two alternative splice forms of type II collagen are known. Type IIa contains all exons and is present in early embryonic structures such as somites and notochord. Type IIb, from which exon 2 has been removed, is secreted by cartilaginous tissues. The function of type XI appears to be similar to that of type V, which forms a core for type I fibrils in other tissues. Type IX collagen forms bridges between the fibrils or other components of the ECM. Two different forms of type IX are expressed in tissues as a result of variable mRNA splicing. In cartilage, the mRNA contains sequences coding the 250 amino acids of the globular NC4 domain of the N-terminus of the protein. In other tissues this globular domain is absent. The expression of collagen type X is restricted to the hypertrophic zone of growth plates of growing animals and to the calcified layer of articular cartilage. Type X collagen represents approximately 45% of the collagens synthesized by the hypertrophic chondrocyte.[18] The protein sequence of type X collagen has a high homology to type VIII collagen that forms a distinctive cross-linked hexagonal lattice in basal laminae. Type X collagen also appears to be highly cross-linked. This collagen contains two cleavage sites for collagenase within the triple helix.[19] Type VI collagen is found in the pericellular matrix of the chondrocyte;[20,21] the molecule contains several arginine-glycine-aspartic acid (RGD) sequences, suggesting a role in cell attachment.[22]

### *Noncollagenous Components of Cartilage*

In addition to collagens, the ECM of cartilage contains a number of PGs as well as hyaluronan and noncollagenous proteins, including the cartilage oligomeric matrix protein.[11] The PGs include the large aggregating PG, aggrecan, as well as the small PGs, decorin, biglycan and fibromodulin. PGs are composed of a core protein to which at least one sulfated glycosaminoglycan chain is attached. The most space-filling PG in cartilage is aggrecan with a molecular weight of 2,500 kDa. Approximately 100 chondroitin sulfate and 20 keratan sulfate chains are covalently bound to the core protein. The amino-terminal of aggrecan core protein is immobilized in the ECM through its interaction with hyaluronan and link protein. Multiple aggrecan monomers are attached to hyaluronan, forming densely packed aggregates of up to 250 x $10^6$ Da. The molar concentrations of biglycan and decorin are comparable to that of aggrecan in full thickness cartilage. However, the concentration between the cartilage zones is different, with the highest concentration of decorin and biglycan in the superficial zone and lowest in the deep zone.[23,24]

### *Cartilage Changes in Aging*

Articular cartilage thickness, water content and chondrocyte density all decrease with maturation and aging, with the most rapid changes occurring prior to adult life.[10, 25] The composition of the ECM also progressively changes with aging. While some age-related changes result in alterations at the level of synthesis, others are mediated by the action of proteinases on components of the ECM.[26-32] Importantly, some of these age-related changes appear to predispose articular cartilage to OA.[31-33]

### *Cartilage Changes in Osteoarthritis*

The cartilage destruction that accompanies OA results from a metabolic disturbance of cartilage homeostasis with an imbalance of anabolism and catabolism. The distribution of pathological lesions in OA and the rate of their progression are related to the heterogeneity within the tissue, both of the cells and the ECM composition. The earliest sign of OA is a disruption of the stiff protective superficial zone of cartilage. Subsequently, with a loss of the superficial zone, the deeper tissue is exposed to more damaging stresses, and is also less resilient than normal as a result of the diminished content of PG.[34] The chondrocytes in OA cartilage are more sensitive to the anabolic cytokine transforming growth factor-than normal chondrocytes relative to PG synthesis.[35] Although the chondrocytes may be stimulated to increase PG synthesis, PG loss from ECM in early OA probably results from an acceleration of the normal processes of turnover, mediated by the chondrocytic proteinases, termed chondrocytic chondrolysis.[36] Increased hydration of the remaining PGs within a weakened collagen network results in tissue swelling, a lowered osmotic pressure around the chondrocytes, and ultimately a softened, fibrillated tissue incapable of bearing weight and functioning normally during movement. Loss of articular cartilage with joint space narrowing, multiple tide marks and subchondral bone sclerosis are characteristic features of OA.

## MMPs in Cartilage

Collagenases, along with other MMPs, have been implicated in the development of joint diseases, especially arthritis. The degeneration of cartilage is currently thought to involve MMPs since 1) their activity is elevated in OA cartilage (and rheumatoid synovium) and 2) they are secreted by the chondrocytes and act on the ECM at physiological pH.[37] In the disease progression, MMPs work in a cascade of proteolytic activity to release the PGs and degrade the collagens, thereby impairing the function of the cartilage and thus the entire joint.[37] The three collagenases have been identified in normal human adult articular cartilage including collagenases 1-3.[3, 38] The membrane type-MMP (MT1-MMP) has recently been shown to be able to cleave native collagen and is also expressed by chondrocytes.[39,40] All of the collagenases except collagenase 2 are constitutively expressed in normal cartilages.[38]

### *Collagen Degradation*

A direct involvement of collagenases in type II collagen degradation was recently demonstrated in human articular cartilage.[41] Antibodies were developed that recognize the carboxy-terminal and the amino-terminal neoepitopes generated by cleavage of native human type II collagen by the three collagenases 1, 2, and 3. When the antibodies were used to immunoassay cartilage extracts, significantly more neoepitope was present in OA cartilage compared to nonOA cartilages. The neoepitope reflecting collagenase activity within the tissue is detectable particularly in areas depleted of PGs. Collagenases have been detected in the synovial fluid of patients with traumatic arthritis, rheumatoid arthritis as well as OA.[42-45] While in other arthritic diseases the cellular source of the MMPs may be the cells of the

synovial membrane, in OA chondrocytes are the cellular source and are capable of expressing and secreting all three collagenases.[38]

### *Changes in Expression of Collagen in OA*

In normal adult articular cartilage, type II collagen mRNA is not detectable.[46] The collagen network is thought to be very stable with a half-life of approximately 100 years.[47] However, the type II collagen message is up-regulated with early signs of damage and type III collagen synthesis begins, which is atypical for chondrocytes. Accordingly, in this same study there was no detectable type I collagen mRNA within the OA articular cartilage.[48] Aigner and Dudhia[49] hypothesize that the cartilage destruction in OA involves: 1) an increase in collagen type II and aggrecan synthesis; 2) a modulation of the chondrocytic phenotype with the expression of atypical cartilage genes like collagen type III; and 3) finally, a suppression of aggrecan core protein synthesis and collagen types II and III mRNA expression with subsequent quantitative loss of aggrecan molecules from the ECM.

### *Aggrecanase*

Several of the MMPs can cleave the core protein of aggrecan in its interglobular domain, freeing it from its attachment to the hyaluronan-aggregates. The activity of the MMPs on aggrecan is thought to be responsible for the depletion of this molecule in OA cartilage. Stromelysin-1 (MMP-3) was at first thought to be the most likely candidate. The expression of MMP-3 in cartilage has been extensively studied and is thought to play a major role in cartilage destruction.[6] However, the sequence of cleavage sites in its core protein released from cartilage following catabolic stimulation indicated the activity of an as yet unidentified enzyme, called aggrecanase.[44,50-54] Neutrophil collagenase (collagenase 2) became a candidate for aggrecanase when it was shown to be able to also generate specific cleavage products in aggrecan.[55] Our studies[2-4] ask whether collagenase 2 is expressed by human articular chondrocytes. If collagenase 2 is not expressed by the cartilage cells, then collagenase 2 would not be a candidate for aggrecanase or aggrecanase activity.

### *Immunological Markers of Proteolytic Products*

One of the questions in our investigation was to determine whether aggrecanase activity could be detected in human articular cartilages. Previous studies[44,50-54] had concentrated on rat chondrosarcoma or bovine or human articular cartilage in culture. In collaboration with Drs. Clare Hughes and Bruce Caterson, we obtained an antibody (BC-3) that recognizes the N-terminus sequence (ARGSV) on the core protein of aggrecan produced by aggrecanase.[56] Cartilages were obtained from organ donors with no known history of joint disease and from patients (both rheumatoid arthritis and OA) undergoing knee replacement surgery. The PGs were extracted from these cartilages using 4M guanidine HCl in the presence of protease inhibitors. Immunopositive bands for BC-3 were present in extracts from the normal donors cartilages as well as in the extracts from the patients with joint disease. These data showed that the fragments of the aggrecan molecule were retained in the human adult articular cartilages even after the core protein had been cleaved at the aggrecanase site. Following cleavage, the core protein is no longer bound to the hyaluronan aggregates, and the molecule should be free to migrate out of the tissue as it appears to do in culture. However, the unattached molecule is retained in the adult human articular cartilages. Since we were able to identify aggrecanase cleavage products in the cartilage, we then began to investigate these cartilages for the presence of collagenase 2.

### *Normal and Damaged Articular Cartilages*

One major concern in identifying collagenase 2 in chondrocytes was that the cartilage should be absolutely free of contaminating polymorphonuclear leukocytes, especially cartilages obtained from patients undergoing joint replacement surgery. In order to address these concerns, we used for this study cartilages from the knee and ankle (talocrural) joints of human organ donors obtained through collaboration with the Regional Organ Bank of Illinois. Cartilages were collected from 352 donors (age range 16 fetal weeks to 93 years; mean age = 44 years; adults only mean age = 49 years). Although none of the donors had a history of joint disease, not all of the joints, neither knee nor ankle, were entirely normal. Some of the joints had fissures, fibrillations or full thickness defects in the cartilage, and changes in the bony contours of the joints, including osteophytes. We subsequently classified these joints based on a modification of the scale originally published by Collins.[57, 58] The scale has grades 0-4 with 0 having no detectable changes and 4 having full thickness defects in > 30% of the articular surface and osteophytes. Based on this scale we have designated joints with grades 0-1 as normal and grades 2-4 as damaged. Although a recent study[59] concluded that all damage in the knee joints of donors was preclinical OA, we have avoided that classification. There is no possibility of determining whether such cartilage lesions would have become progressively more severe, remained unchanged or undergone repair had the donors continued to live. Thus, we have taken a more conservative approach and have used the term damaged. Among the 352 donors, 37.1% of the knees and 13.7% of the ankles were damaged. While the percentage of damage in the ankle is lower than in the knee, the damage still far exceeds what would be expected of preOA in the ankle, since OA so rarely occurs in the ankle.

### *Chondrocyte Collagenase 2 Protein and mRNA*

For our collagenase 2 investigations the normal cartilages from the donors were originally used so that there was no neutrophil contamination. In collaboration with Dr. Karen Hasty, we began our studies of chondrocyte collagenase 2 using the in situ hybridization technique as described by Sandell et al,[60] and which we later modified.[3, 4] Two cDNA probes were designed specific for neutrophil collagenase complementary to bp 1588-1610 [5'-GGT-AGA-ATG-GAT-ACA-GTG-ATG-GG-3'] and to bp 796-822 [5'-GAG-GGA-GTG-AGT-AGT-TGC-TGG-TTT-CCC-3']. Both of these probes were positive in neutrophil-enriched populations of leukocytes derived from normal human blood obtained by venopuncture, and in rat chondrosarcoma cells and chondrocytes cultured in alginate beads (Fig. 2.1). The hybridization was performed under conditions of highest stringency where only probes with 100% homology should remain bound. Human articular chondrocytes within tissue sections were also positive (Fig. 2.2). The level of expression by the uncultured knee articular chondrocytes was low, but expression was not uniform within the tissue. Chondrocytes in the superficial and middle zones expressed low levels of mRNA, however, no detectable mRNA for collagenase 2 was found in deep zone chondrocytes. In the normal ankles, no mRNA was detectable even in the superficial layers of over 25 different donors. As a positive control to ensure that mRNA was present throughout the knee and ankle cartilage, in situ hybridization to detect MMP-3 (stromelysin-1) was done on adjacent tissue sections. Chondrocytes throughout the knee and ankle cartilages were positive for MMP-3 message.

Polymerase chain reaction (PCR) amplification of mRNA confirmed our in situ hybridization results for collagenase 2: 1) mRNA was detectable in the knee but not in the ankle cartilage; 2) mRNA levels were low even in the knee requiring a second amplification (nested primers) to detect mRNA in the knee. No mRNA was detectable in the ankle cartilage even with this second amplification. The sequence of the PCR product was 100% homologous with bp 439-797 in the neutrophil collagenase gene.

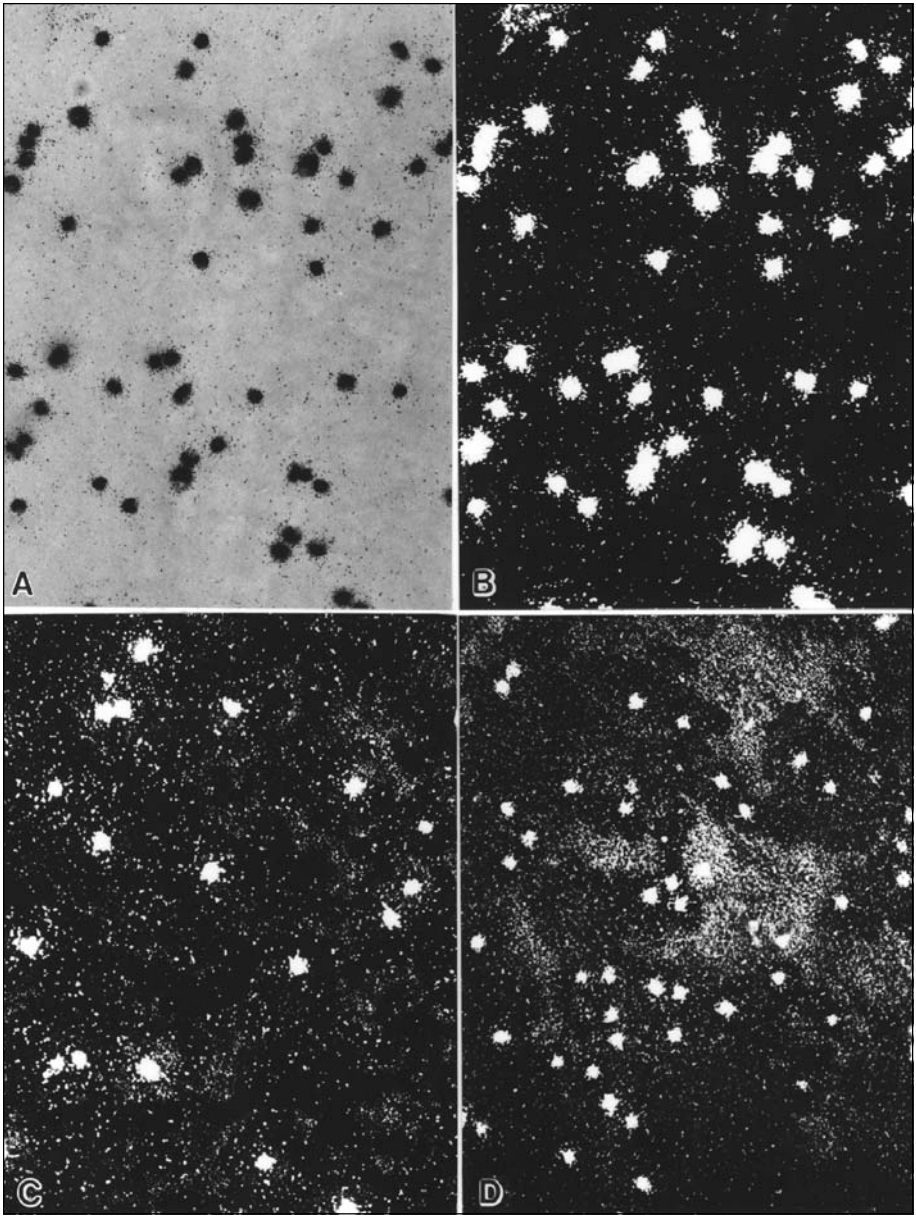


Fig. 2.1. A. Bright field photomicrograph of radiolabeled probe specific for MMP-8 hybridized to mRNA in rat chondrosarcoma cells. B. Darkfield photomicrograph of the same field shown in A. C. MMP-8 mRNA in polymorphonuclear leukocyte–enriched population of cells. D. Human chondrocytes (11 week old) cultured in alginate beads also have mRNA for MMP-8. Original magnification = 37.5.

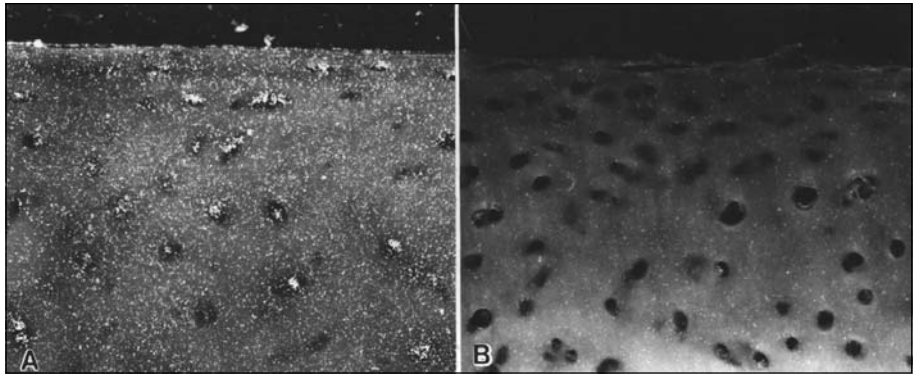


Fig. 2.2. A. Chondrocytes in human adult articular cartilage from a normal knee joint have low levels of MMP-8 mRNA. B. Control section incubated with probe specific for collagen type I. Original magnification = 37.5.

Northern blot analysis also confirmed the presence of mRNA for collagenase 2 in chondrocytes using a collagenase 2 specific probe.[1] A single mRNA of 3.3 kilobases was identified. In order to obtain sufficient quantities of mRNA from the chondrocytes to be detectable by Northern blotting, the cells were isolated by proteolytic digestion of the cartilage and cultured in high-density monolayer as previously described.[12] Only after stimulation with 10 pg/ml IL-1β was mRNA for collagenase 2 detectable. In another study[38] we were able to detect collagenase 2 mRNA by Northern blot analysis in knee cartilages from only two of eight OA patients, while the mRNA was detectable by PCR in all eight. In addition, we were able to increase the expression of collagenase 2 slightly with both IL-1β and tumor necrosis factor α (TNF-α). The results of these studies[38] confirm that collagenase 2 is not constitutively expressed in all chondrocytes and that expression can be up-regulated by the catabolic cytokines.

Collagenase 2 protein was detectable both by immunohistochemistry and immunoblotting with an antiserum prepared against a synthetic peptide of collagenase 2 and is monospecific for this enzyme.[1] The protein was detected throughout the zones of the normal cartilage, including the deep zone where no mRNA was detectable. The collagenase 2 protein was distributed throughout the matrix with more intense staining in the pericellular and territorial matrices. By Western blotting, collagenase 2 with a molecular mass of 55 kDa is similar to that synthesized by transfected COS cells, but not to that of the neutrophil. The neutrophil collagenase 2 has a molecular mass of 75 kDa and is heavily glycosylated. Collagenase 2 has 6 glycosylation sites in the molecule. The sugar residues may play a function in sequestering the proteinase in the specific granule until the neutrophil is activated, releasing the contents of the granules. By contrast, the chondrocyte, immobilized in its ECM, releases the collagenase 2 into the ECM. The underglycosylated protein may provide specificity for its function in cartilage.

### *Expression of Collagenase 2 in Immature and Rapidly Growing Cartilage*

In young cartilages (less than 4 years old) the epiphyseal articular cartilage (Fig. 2.3) does not have well defined zones, nor do the chondrocytes appear to have territorial and interterritorial matrix. In addition, the cells do not have the typical shape of those in zones of adult articular cartilage. Cells throughout this immature human cartilage have higher

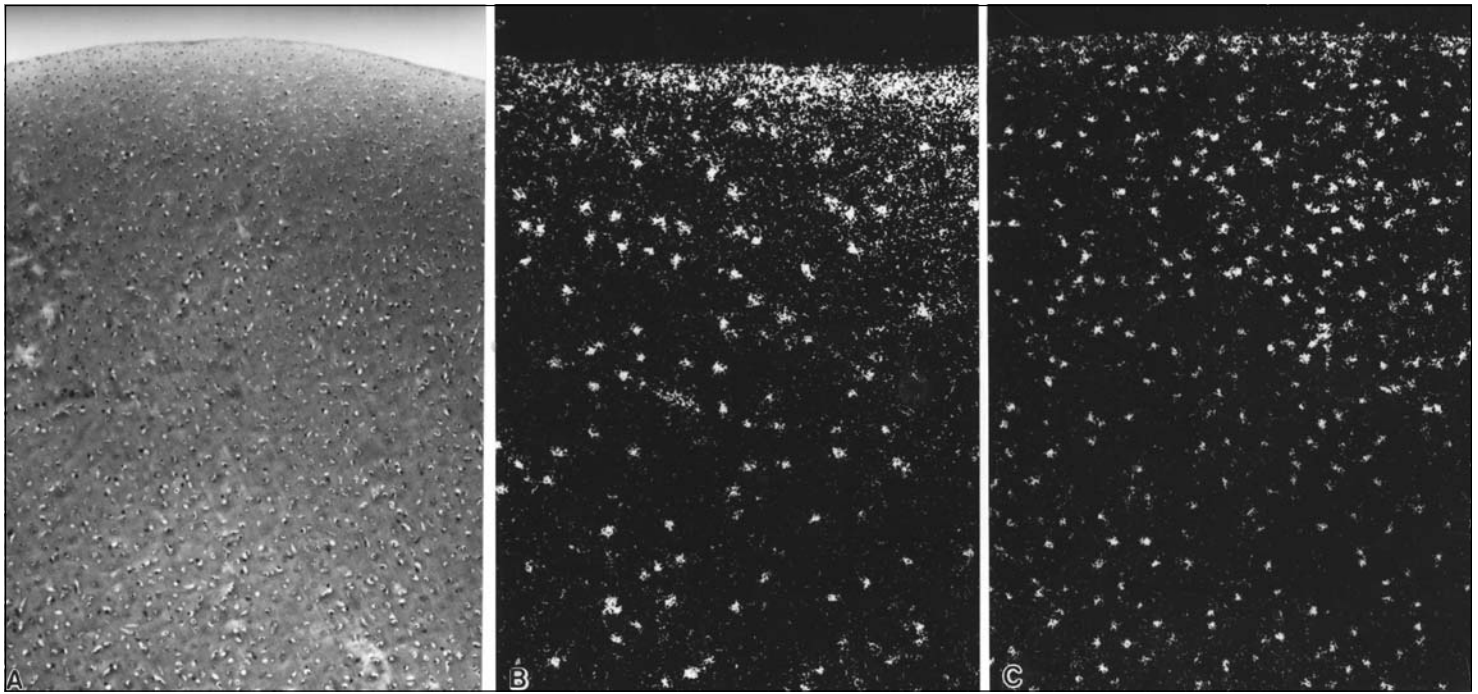


Fig. 2.3. A. Section of 11 week old epiphyseal cartilage stained with Safranin O to show PG localization and morphology. B. and C. Adjacent sections from the same cartilage are in situ hybridizations done with probes specific for MMP-8 (B) and MMP-3 (C). Original magnification A = 15; original magnification B&C = 37.5.

levels of expression of collagenase 2 than adult chondrocytes. This observation was also confirmed by PCR amplification showing decreased mRNA in normal adult cartilage (68 years) compared to that in cartilages from 1 day, 3.5 months, 4 years and 22 years.

### *Expression of Collagenase 2 in Damaged and Osteoarthritic Cartilage*

In damaged cartilages the expression of collagenase 2 is up-regulated. Especially when the superficial zone is disrupted, expression is higher in the superficial and middle zones, while mRNA is now evident in deep zone chondrocytes. With OA cartilages, the superficial and middle zones are often absent and expression in the remaining zone(s) is very high (Fig. 2.4). We conclude that expression of collagenase 2 is elevated in young, rapidly growing cartilages. It then becomes down-regulated in the adult cartilages, but ends progressively up-regulated as damage to the cartilage increases.

### *Activation of Collagenase 2*

MMPs are synthesized in a latent form with a reduction in molecular mass of approximately 10 kDa upon activation. Latent chondrocyte collagenase 2 is approximately 55 kDa. Western blots of cartilage extracts also contain immunoreactive bands at 46, 42, 36 and 32 kDa (Fig. 2.5). Smith et al[61] reported that recombinant collagenase 2 (55 kDa) is converted to active enzyme with a molecular weight of 45 or 46 kDa. In the chondrocyte, the 46 and 42 species appear to be the active enzyme, while the 36 and 32 may represent fragments of the protein as was shown for the recombinant molecule.[61]

### *Modulation of Expression by Cytokines*

The synthesis of many of the MMPs is up-regulated by the catabolic cytokine IL-1. While the involvement of IL-1 in rheumatoid arthritis is well accepted, its role in OA is still questionable. Nevertheless, many studies have pointed toward some role for IL-1 in the cartilage degeneration of OA. For example, IL-1 has been identified in synovial fluid of joints of patients with OA,[62] and during inflammatory episodes IL-1 may disturb tissue homeostasis by suppressing the synthesis and repair mechanisms of cartilage.[63] The direct effects of IL-1 on articular cartilage have been investigated both by injection into the joints of experimental animals (e.g. rabbits), and by many studies on cultured articular cartilage and chondrocytes from several different species, including human. A comparison of genes up-regulated by IL-1 stimulation and analyzed by differential display reverse transcriptase-PCR revealed a total of 123 recognized differentially displayed cDNA fragments. The genes for which sequences are known include fibronectin, osteopontin, calnexin and TNF-stimulated gene 6.[64] In most animal cartilages (including bovine and porcine) IL-1 both inhibits the anabolism of PGs and stimulates catabolism.[65, 66] In human cartilage, however, the catabolic response, measured by PG loss from the cartilage, is either lacking, or only elicited by extremely high concentrations of IL-1.[65-67] Even so, low concentrations of IL-1 acting on human cartilage explants over long periods of time may result in ECM damage, because of suppressed synthesis, even in the absence of enhanced ECM catabolism.[68] Our preliminary studies using in situ hybridization and immunostaining of human cartilage cultured with IL-1 have shown that low doses of IL-1 (0.5 pg/ml) result in up-regulation of collagenase 2 and generation of neoepitopes for aggrecan core protein in the territorial ECM suggesting that IL-1 does elicit a select catabolic response, although at a reduced level.

## Conclusions

Our studies of collagenase 2 in cartilage have shown that chondrocytes do indeed express collagenase 2, along with collagenases 1 and 3. The data additionally shows that

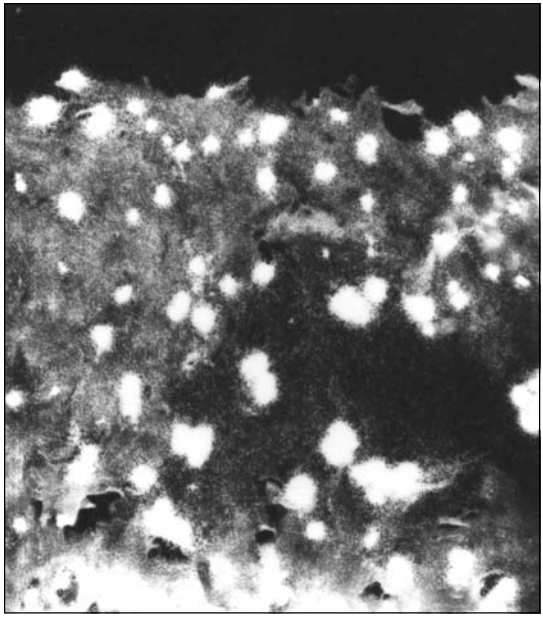

Fig. 2.4. Cartilage from OA patient's knee joint. The superficial and middle zones are absent from this sample. Chondrocytes in the remaining deep zone have high levels of collagenase 2 mRNA. Original magnification = 15.

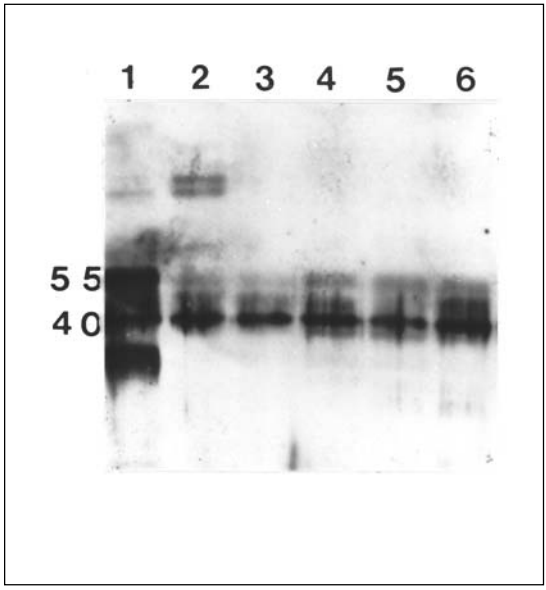


Fig. 2.5. Immunoblot of cartilage extracts from 5 OA patients with collagenase 2-specific antibody. Lane 1. Recombinant collagenase 2 with immunoreactive bands at 55, 42, 40, 36 and 32 kDa. The latent proteinase is 55 kDa with activated form at 42 and 40 kDa.

collagenase 2 is developmentally regulated being more highly expressed in young, rapidly growing cartilages and down-regulated in normal adult cartilage. The chondrocytes in normal cartilage retain the capacity to up-regulate expression of the proteinase upon stimulation with catabolic cytokines, both IL-1 and TNF-α. There is also an in vivo up-regulation of collagenase 2 when the cartilage has sustained damage in vivo and further in the diseased cartilage from OA patients during joint replacement surgery. Although a recent study with a truncated recombinant collagenase 2 and a specific inhibitor of collagenase 2 activity reported that collagenase 2 was not identical to aggrecanase,[69] we suggest a further role for this MMP in OA since its mRNA is elevated and the activated form of this proteinase was detectable in the damaged and OA cartilages.

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CHAPTER 3

# Collagenase-3
## Identification, Characterization, and Physiological and Pathological Relevance

**Carlos López-Otín**

### Identification and Structural Characterization of Human Collagenase-3

The identification of human collagenase-3 resulted from studies intended to identify the presence of new proteolytic enzymes in breast carcinomas.[1] One of the distinctive features of malignant tumors is their ability to invade normal tissues and spread to distant sites, in the process giving rise to metastasis.[2] These events involve degradation of the different macromolecular components of the extracellular matrix, and require the combined action of several proteolytic systems. Among various enzymes implicated in tumor invasion and metastasis, a number of previous studies focused on matrix metalloproteinases because of their unique ability to collectively degrade all connective tissue protein components at neutral pH.[3-5]

These considerations, led us to speculate that samples of human tumor specimens were an appropriate starting material to identify putative novel members of the MMP family potentially involved in the spread of cancer. To test this idea, we designed a PCR-based homology cloning strategy, using RNA isolated from breast carcinomas and degenerate oligonucleotides encoding structural motifs conserved in MMPs. When we initiated this work, a total of nine human MMPs had been isolated and characterized at the amino acid sequence level (interstitial collagenase, neutrophil collagenase, gelatinases A and B, stromelysins-1, -2, -3, matrilysin, and macrophage metalloelastase).[3,4] A comparison of their amino acid sequences revealed two sequences conserved in all of them, the activation locus (PRCGVPD) and the Zn-binding site (VAAHEXGH). After synthesizing two degenerate oligonucleotides encoding these conserved motifs and performing RT-PCR of total RNA isolated from a mammary carcinoma, a band of the expected size was obtained and cloned. Analysis of the nucleotide sequence of different clones revealed that some of them had a sequence similar to, but distinct from all previously characterized human MMPs. Screening of a breast cancer cDNA library prepared from the same tumor employed for the RT-PCR experiment and using the PCR-generated fragment as probe, we identified a positive clone with an insert of 2.7 kb. The isolated cDNA encoded a polypeptide of 471 amino acids,

*Collagenases*, edited by Warren Hoeffler. 

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        1*            **       *  *                  ** **   *       **              * ****** *  *****   *
human   M-HPGVLAAF LFLSWTHCRA LPLP-SGGDE DDLSEEDLQF AERYLRSYYH P-TNLAGILK ENAASSMTER LREMQSFFGL EVTGKLDDNT
mouse   M-HSAILATF FLLSWTPCWS LPLPYGDDDD DDLSEEDLVF AEHYLKSYYH P-ATLAGILK KSTVTSTVDR LREMQSFFGL EVTGKLDDPT
rat     M-HSAILATF FLLSWTHCWS LPLPYGDDDD DDLSEEDLEF AEHYLKSYYH P-VTLAGILK KSTVTSTVDR LREMQSFFGL DVTGKLDDPT
Xenopus MAPSSLSVFV LSLSFTYC—  LSAPVSQDED SELTPGALQL AEHYLNRLYS SSSNPAGMLR MKDVNSVETK LKEMQSFFGL EVTGKLNEDT
newt    MMPSVLSAAI FFLSLAFG—  LPVPVPHERD SDVTEQELRL AEKYLKTFYV A-SDHAGIMT KKGGNALASK LREMQSFFDL EVTGKLDEDT

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human   LDVMKKPRCG VPDVGEYNVF PRTLKWSKMN LTYRIVNYTP DMTHSEVEKA FKKAFKVWSD VTPLNFTRLH DGIADIMISF GIKEHGDFYP
mouse   LDIMRKPRCG VPDVGEYNVF PRTLKWSQTN LTYRIVNYTP DMSHSEVEKA FRKAFKVWSD VTPLNFTRIY DGTADIMISF GTKEHGDFYP
rat     LDIMRKPRCG VPDVGVYNVF PRTLKWSQTN LTYRIVNYTP DISHSEVEKA FRKAFKVWSD VTPLNFTRIH DGTADIMISF GTKEHGDFYP
Xenopus LDIMKQPRCG VPDVGQYNFF PRKLKWPRNN LTYRIVNYTP DLSTSDVDRA IKKALKVWSD VTPLNFTRLR TGTADIMVAF GKKEHGDYYP
newt    LEVMKQPRCG VPDVGEYNVF PRSLKWPRFN LTYRIENYTP DMTHAEVDRA IKKAFRVWSE VTPLNFTRLR SGTADIMISF GTKEHGDFYP

        **** ***** *****   ** * ******       *****  ***** **  ****** ***  ** * * **   *   **** ****** ***
human   FDGPSGLLAH AFPPGPNYGG DAHFDDDETW TSSSKGYNLF LVAAHEFGHS LGLDHSKDPG ALMFPIYTYT GKSHFMLPDD DVQGIQSLYG
mouse   FDGPSGLLAH AFPPGPNYGG DAHFDDDETW TSSSKGYNLF IVAAHELGHS LGLDHSKDPG ALMFPIYTYT GKSHFMLPDD DVQGIQFLYG
rat     FDGPSGLLAH AFPPGPNLGG DAHFDDDETW TSSSKGYNLF IVAAHELGHS LGLDHSKDPG ALMFPIYTYT GKSHFMLPDD DVQGIQSLYG
Xenopus FDGPDGLLAH AFPPGEKIGG DTHFDDDEMF STDNKGYNLF VVAAHEFGHA LGLDHSRDPG SLMFPVYTYT ETSRFVLPDD DVQGIQALYG
newt    FDGPNGLLAH AFPPGQRIGG DTHFDDDETF TSGSNGYNLF IVAAHEFGHA LGLDHSRDPG SLMYPVYSYT EPSRFLLPDD DVQGIQSLYG

         *  ** *** **** ****  *  ****  * **  ****** *** ***     ** *   ** ****   *** ** *  **   **** *****
human   PGDEDPNPKH PKTPDKCDPS LSLDAITSLR GETMIFKDRF FWRLHPQQVD AELFLTKSFW PELPNRIDAA YEHPSHDLIF IFRGRKFWAL
mouse   PGDEDPNPKH PKTPEKCDPA LSLDAITSLR GETMIFKDRF FWRLHPQQVE AELFLTKSFW PELPNHVDAA YEHPSRDLMF IFRGRKFWAL
rat     PGDEDPNPKH PKTPEKCDPA LSLDAITSLR GETMIFKDRF FWRLHPQQVE PELFLTKSFW PELPNHVDAA YEHPSRDLMF IFRGRKFWAL
Xenopus SGNRDPHPKH PKTPEKCDPD LTIDAITELR GEKMIFKDRF FWRVHPQMTD AELVLIKSFW PELPNKIDAA YEHPAKDLIY IFRGKKFWAL
newt    PGNRDPNPKH PKTPEKCDPE LSLDAITEMR GEKLIFKDRF FWRQHPQMTD VELVLIRNFW PELPSKIDAA YEYPEKDLIY IFRGRKFWAL
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          ****    **  *   ** **       ***      * ** *      *  *        *  *   *   **** *  *** *   ******* *
human     NGYDILEGYP KKISELGLPK EVKKISAAVH FEDTGKTLLF SGNQVWRYDD TNHIMDKDYP RLIEEDFPGI GDKVDAVYEK NGYIYFFNGP
mouse     NGYDILEGYP RKISDLGFPK EVKRLSAAVH FENTGKTLFF SENHVWSYDD VNQTMDKDYP RLIEEEFPGI GNKVDAVYEK NGYIYFFNGP
rat       NGYDIMEGYP RKISDLGFPK EVKRLSAAVH FEDTGKTLFF SGNHVWSYDD ANQTMDKDYP RLIEEEFPGI GDKVDAVYEK NGYIYFFNGP
Xenopus   NGYDFVEDYP KKLHELGFPK TLKAIDAAVY NKAIGKTLFF AEDSYWSFDE EARTMDKGFP RLISEDFPGI GEKVDAAYQR NGYIYFFNGA
newt      NGYDILADYP KKIQELGFPK SLRTIDAAVY NRAMGKTLFF TGEKYWSFDE EKQTVEKGYP RFIADDFPGI GETVDAAYQR NGYIYFFSGS

           ***** **     *    *   * *471
human     IQFEYSIWSN RIVRVMPANS ILWC
mouse     IQFEYSIWSN RIVRVMPTNS ILWC
rat       IQFEYSIWSN RIVRVMPTNS LLWC
Xenopus   LQFEYSIWSK RITRILKTNF VLMC
newt      LQFEYSTWSN KVIRVLKTNS ILWC
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Fig. 3.1. Comparison of the amino acid sequences of collagenase–3 (MMP–13) from different species. The amino acid sequences of human, mouse, rat, Xenopus, and newt collagenase–3 were extracted from the SwissProt data base and the multiple alignment was performed with the PILEUP program of the University of Wisconsin Genetics Computer Group package. Identical residues in all five sequences are indicated by asterisks.

having all the structural features characteristic of MMPs, including the predomain with a hydrophobic leader sequence, the pro-domain having the PRCGVPD activation locus, the Zn-binding site of the catalytic domain, and a C-terminal extension with sequence similarity to hemopexin (Fig. 3.1). This novel human MMP contained in its amino acid sequence several residues specific to the collagenase subfamily of MMPs (Tyr-214, Asp-235, and Gly-237), but lacked the 9-residue insertion present in the stromelysins and in the fibronectin domain characteristic of the gelatinases. According to these structural characteristics, this human MMP was designated collagenase-3 (MMP-13), since it represented the third member of the subfamily composed at that time of fibroblast and neutrophil collagenases.[1]

Pairwise comparisons for amino acid sequence similarities between collagenase-3 and other human MMPs confirmed that the highest percentage of identities (about 50%) was found with the human collagenases. However, and somewhat surprisingly, when the sequence comparison was extended to all sequences present in the databank, a higher degree of identities was found with rat and mouse interstitial collagenases.[6,7] Since their description, it had been assumed that these enzymes were the murine counterparts of human interstitial collagenase (MMP-1). However, the finding that they were more closely related to human collagenase-3 indicated that these murine enzymes represented counterparts of MMP-13 instead of MMP-1. To date, there is no evidence for a homologous MMP-1 in either rat or mouse, and the possibility exists that rodents do not possess this gene.

To avoid future confusion with the nomenclature of the collagenase subfamily of MMPs, I would like to propose the use of collagenase-1 to designate interstitial or fibroblast collagenase (MMP-1), and collagenase-2 for neutrophil collagenase (MMP-8). In fact, the novel human collagenase is also produced by fibroblast and inflammatory cells,[8-10] whereas neutrophil collagenase is also synthesized by articular chondrocytes,[11] also a source of collagenase-3.[12-15] Consequently, cellular origin and tissue distribution are not distinctive characteristics of these enzymes. Furthermore, this nomenclature system would facilitate the description of as yet uncharacterized human collagenases, as may be the case of the putative human homologue of the recently described collagenase-4 (MMP-18) identified in *Xenopus laevis*.[16]

The collagenase-3 family has recently grown with the finding of homologue enzymes in two additional species: *Xenopus laevis* and *Cynopus pyrrhogaster* (newt). The Xenopus collagenase-3 was identified as a thyroid hormone-induced gene during tail resorption,[17] whereas the newt homologue was cloned from early bud-stage regenerating limbs.[18] A comparison of the amino acid sequences of the five collagenase-3 sequences reported so far is shown in Figure 3.1. The percentage identities with the human enzyme ranges from 86% with the murine enzymes to about 60% with Xenopus collagenase-3. A more detailed analysis of these sequences reveals an uneven distribution of conserved sequences between them, especially between the amphibian enzymes and the human and murine sequences. The catalytic domains are highly conserved and contain all the essential residues for catalytic properties as well as the distinctive residues of the collagenase subfamily. In contrast, the signal sequence, the prodomain and hemopexin domains show a higher divergence between species. The availability of all five sequences will be very useful for future studies directed at identifying specific residues responsible for the distinctive enzymatic properties of collagenase-3.

In research that will aid in the structural analysis of collagenase-3, recent studies have solved the crystal structure of the C-terminal hemopexin-like domain of the human enzyme by molecular replacement.[19] This structure reveals a disk-like shape with the polypeptide chain folded into a β-propeller structure of pseudo 4-fold symmetry. The four propeller blades are arranged around a funnel-shaped tunnel that harbors two calcium and two chloride ions. This structure thus has a high degree of homology to the equivalent domain

of porcine collagenase-1.[20] The elucidation of the complete three-dimensional structure of collagenase-3 has been hampered by the high instability of the enzyme. In fact, collagenase-3 possesses a potent autoproteolytic activity, which leads to an extensive autolysis of the molecule, with the exception of the C-terminal domain, which remained intact.[21] Nevertheless, the complete crystal structure of this enzyme must be elucidated to gain a better understanding of its functional relevance in both normal and pathological conditions.

### *Biochemical Characterization of Human Collagenase-3*

The inclusion of human collagenase-3 in the collagenase subclass of MMPs was initially based on structural comparisons, since its deduced amino acid sequence contained a number of features characteristic of these enzymes. Subsequent studies performed with recombinant protein produced in different eukaryotic expression systems provided definitive support for the proposal that this novel human enzyme was a bona fide collagenase. In this regard, the first studies were performed with a recombinant protein produced in a vaccinia virus expression system.[1] This protein was active against type I collagen as well as against synthetic peptides used for assaying vertebrate collagenases. In addition, its proteolytic activity was fully abolished by EDTA, a typical inhibitor of metalloproteinases.

A more detailed analysis of collagenase-3 substrate specificity was performed by Knäuper et al[22] using a recombinant enzyme produced in mouse myeloma cells. As anticipated from its amino acid sequence, human collagenase-3 was able to hydrolyze the fibrillar collagens types I, II, and III, generating fragments approximately three-quarters and one-quarter the size of the original molecules. Collagenase-3 was most efficient at cleaving type II collagen, while in contrast, collagenase-1 preferentially cleaves type III collagen and collagenase-2 is more active against type I collagen. Similar results were obtained by Mitchell et al[12] with recombinant collagenase-3 produced in a baculovirus expression system. The distinct substrate preferences of these enzymes for fibrillar collagen suggests that they may have evolved as specialized enzymes to degrade tissues with different collagen composition. Since type II collagen is the main fibrous constituent of cartilage, the preferential hydrolyzing activity of collagenase-3 on this collagen was the first indication that this enzyme could be very important in the turnover of articular cartilage.[12,22] Consistent with this, several groups have provided evidence that collagenase-3 is produced by human chondrocytes and appears to play an essential role in mediating the cartilage destruction associated with arthritic processes.[9,10,12-15]

In addition to its proteolytic activity on fibrillar collagens, collagenase-3 may also act as a potent gelatinase with the ability to degrade the initial cleavage products of collagenolysis into small fragments suitable for further metabolism.[22] The high gelatinolytic activity of collagenase-3 may be due to the fact that this enzyme has similarities within the active site cleft of gelatinases. All of these enzymes contain a Leu residue preceding the first His residue of the zinc binding motif, thus enlarging the $S'_1$-pocket, and facilitating the hydrolysis of a broader range of substrates. Furthermore, collagenase-3 and the gelatinases contain an acidic residue immediately preceding the third His residue of the catalytic site. This acidic residue is substituted by Ser or Ala in collagenase-1 and -2, respectively. Knäuper et al[22] have proposed that the presence of a negatively charged residue at this position has implications on the polarization of the zinc-bound water molecule within the gelatinases and collagenase-3, likely increasing their nucleophilic nature and enhancing the proteolytic efficiency of these MMPs. Consistent with this, further analysis of the substrate specificity of collagenase-3 has revealed an ability to degrade a wide range of extracellular matrix proteins including the large tenascin C isoform, fibronectin, and type IV, IX, X, and XIV collagens.[21] In addition, it also has efficient N-telopeptidase activity against fibrillar collagens, a feature distinguishing collagenase-3 from the two other human collagenases, which are devoid of detectable

telopeptidase activity. The murine homologues of collagenase-3 also display a potent N-telopeptidase activity as demonstrated by using transgenic animals having a mutant type I collagen that prevents cleavage into the characteristic 3/4 and 1/4 fragments.[23,24] Interestingly, Krane et al[24] (see chapter 10) have proposed that in rodents, this telopeptidase activity might be sufficient for resorption of type I collagen during embryonic and early adult life, while triple helicase activity should be necessary in processes involving intense tissue resorption, such as those taking place in the postpartum uterus. Human collagenase-3 can also cleave cartilage aggrecan at three sites within the interglobular G1-G2 domain, providing additional support to the proposed involvement of this enzyme in cartilage destruction during arthritic processes.[25]

According to these studies, human collagenase-3 is a potent enzyme with a wide spectrum of enzymatic activity. Nevertheless, like other MMPs, collagenase-3 is synthesized in a proenzyme form which must be activated prior to proteolytic enzyme action. Preliminary studies of cellular mechanisms mediating procollagenase-3 activation revealed that it can be directly activated by stromelysin-1, which cleaves the $Glu^{84}$-$Tyr^{85}$ peptide bond, generating the final active enzyme of 48 kDa.[22] However, further studies have revealed that other activation mechanisms can occur and may be of physiological and pathophysiological significance. In fact, Knäuper et al[26] have shown that procollagenase-3 can be efficiently activated by MT1-MMP, a member of the membrane-type subfamily of MMPs,[27] through a stepwise proteolytic removal of the propeptide domain. The initial cleavage occurs at the $Gly^{35}$-$Ile^{36}$ peptide bond, and is followed by a secondary cleavage which generates $Tyr^{85}$ N-terminal active collagenase-3. It is of great interest that this activation mechanism is specific for collagenase-3 within the collagenase subfamily, since the two other human collagenases, MMP-1 and MMP-8, are resistant to proteolysis by MT1-MMP and remain inactive.[26] Furthermore, these studies have shown that gelatinase A also activates procollagenase-3, even at a much faster rate than MT1-MMP. In the presence of progelatinase A, which is itself activated by MT1-MMP,[27] the activation of procollagenase-3 by MTI-MMP is potentiated even further. Similarly, progelatinase A can considerably increase the activation rate of procollagenase-3 by fibroblast-derived plasma membranes expressing MT1-MMP, thus confirming the results obtained by using purified MT1-MMP.[26] On the basis of these results, it has been proposed that MT1-MMP, gelatinase A, and collagenase-3 are members of an activation cascade which could lead to the generation of potent extracellular collagenolytic activity (Fig. 3.2). This activation cascade may be of importance in vivo since these three proteins have all been implicated independently in connective tissue remodeling under both normal and pathological conditions. Consistent with this, recent studies have shown that in human fetal osteoblasts, as well as in invasive laryngeal carcinomas, collagenase-3 is coexpressed with MT1-MMP and gelatinase A.[15,28] These three MMPs can act in a coordinate manner in these pathological conditions, contributing to the collagenolysis required for bone formation, or to the dissolution of the extracellular matrix surrounding laryngeal carcinomas, thereby facilitating tumor growth and invasion.

A final mechanism described for procollagenase-3 activation takes place through the action of plasmin, which cleaves the $Lys^{38}$-$Glu^{39}$ and $Arg^{76}$-$Cys^{77}$ peptide bonds in the propeptide domain.[26] Autoproteolysis then results in the release of the rest of the propeptide domain, generating the $Tyr^{85}$ N-terminal fully active form of collagenase-3. However, this plasmin-mediated activation pathway is accompanied by loss of the C-terminal hemopexin domain of collagenase-3, which leads to a significant decrease in its collagenolytic activity. Therefore, activation by plasmin fails to produce enzyme with a high specific collagenolytic activity, suggesting that it may only play a minor role in the cellular processing of procollagenase-3.

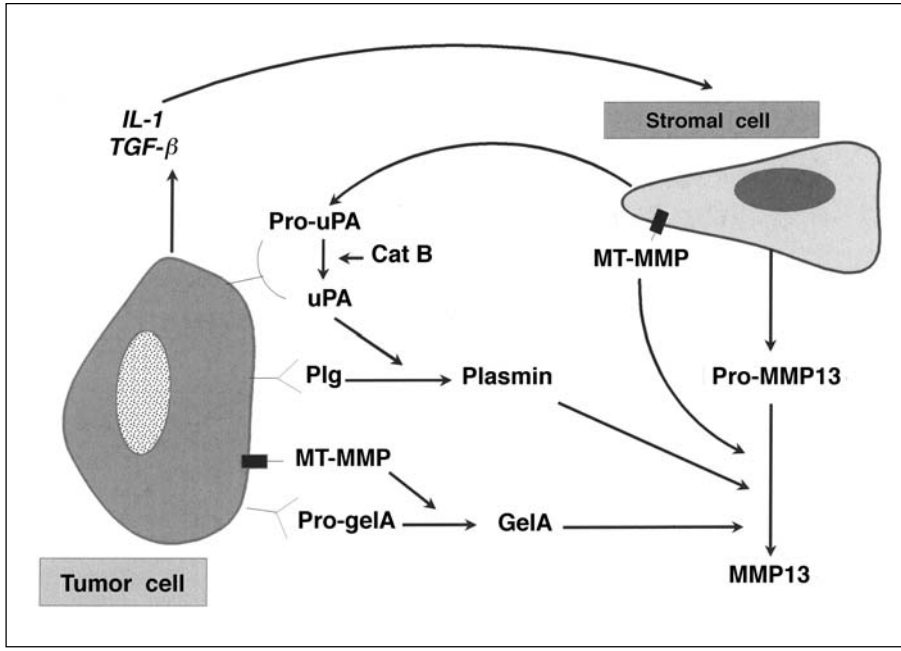


Fig. 3.2. Mechanisms controlling expression and activation of human collagenase-3 in breast carcinomas. Human collagenase-3 is predominantly expressed in stromal cells stimulated by soluble factors (IL-1, TGF-β) released from epithelial tumor cells. After synthesis, procollagenase-3 is activated through the action of other proteases, including MT1-MMP, gelatinase A or plasmin.

The sensitivity of human collagenase-3 to natural and synthetic inhibitors has been extensively analyzed by Knäuper et al.[22] The inhibition profile of the active enzyme by human TIMP-1, -2, and -3, revealed that all react in 1:1 stoichiometry by forming noncovalent, tight-binding complexes, in agreement with earlier published data on other MMPs. Nevertheless, kinetic analysis of collagenase-3/TIMP interaction demonstrated that TIMP-3 reacts 1.2 times faster than TIMP-1 and 5.5 times faster than TIMP-2. The inhibition of active collagenase-3 by hydroxamic acid-based inhibitors like CT1399 and CT1847 has also been examined.[22] According to the data obtained, both are efficient inhibitors and react with 1:1 stoichiometry via a simple bimolecular collision. Comparative analysis of the efficacy of both synthetic inhibitors revealed that CT1399 reacts 12.1 faster than CT1847. The $K_i$ values of these inhibitors for collagenase-3 and gelatinases were very similar, and significantly differ from those for other MMPs like collagenase-1. These results confirm the existence of structural similarity in the active site between collagenase-3 and gelatinases, and suggest that inhibitors directed against these latter enzymes could be also successful in blocking the activity of collagenase-3. In this regard, Billinghurst et al[29] have reported the use of a synthetic inhibitor (RS 102,481), which preferentially acts against human collagenase-3. The availability of the complete three-dimensional structure of this protease will be essential in the design of specific inhibitors that control its undesired activity in pathological conditions.

## Structure and Regulation of the Human Collagenase-3 Gene

The gene encoding human collagenase-3 has been recently characterized using genomic clones obtained from a DNA library prepared in EMBL3 phage.[30] The gene spans more than

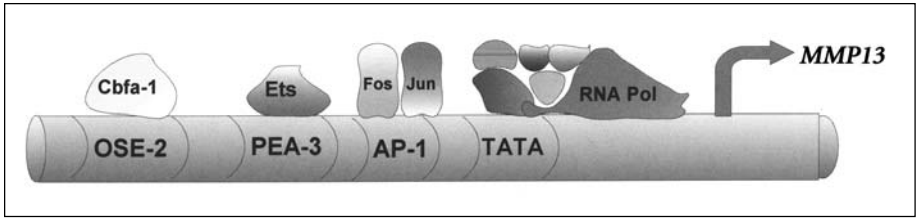


Fig. 3.3. Structure of the promoter region of the human collagenase-3 gene. Sequences relevant for the transcriptional activity of the gene, including TATA box, and AP-1, PEA3, and OSE-2 motifs are indicated.

12.5 kb, and is composed of 10 exons and 9 introns. Its overall organization in terms of size of exons and distribution of intron-exon junctions is similar to other human MMP genes like collagenase-1[31] and macrophage metalloelastase,[32] but more distantly related to genes coding for gelatinases[33,34] and stromelysin-3.[35] In addition, the collagenase-3 gene has the unique features, of an extremely short first intron (92 bp) and an unusually large last exon (1371 bp), the largest among all the equivalent exons from MMP genes.[30] The increased length of this exon derives mainly from the presence of a large 3'-untranslated region that could be important in posttranscriptional regulatory events. In fact, this region contains three potential polyadenylation sites that are used to generate at least three different collagenase-3 mRNAs, differing in size and relative abundance.[1] The human collagenase-3 gene is located on chromosome 11q22.3,[36,37] clustered to at least seven other members of the MMP gene family: collagenases-1 (MMP-1), and -2 (MMP-8); stromelysins-1 (MMP-3) and -2 (MMP-10), matrilysin (MMP-7), macrophage metalloelastase (MMP-12), and enamelysin (MMP-20).[38] Fine physical mapping of this region of the human genome using pulsed-field gel electrophoresis has shown that MMP-13 is localized to the telomeric side of the MMP cluster, the relative order of the loci being centromere/MMP-8/MMP-10/MMP-1/MMP-3/MMP-12/MMP-7/MMP-20/MMP-13 /telomere.[35,38] On this basis, MMP-13 would be located close to the ataxia telangiectasia gene, a human disease characterized by its association to an increased likelihood of developing diverse human carcinomas.[39]

The promoter region of the human collagenase-3 gene has been analyzed both structurally and functionally,[30,40] looking for regulatory motifs that could affect its transcription (Fig. 3.3). Like most MMP genes, the human collagenase-3 gene contains at equivalent positions a TATA box sequence (TATAAA), an AP-1 motif (TGACTCA), and a PEA3 site (TAGGAAGT). The presence of adjacent AP-1 and PEA3 motifs is of special interest in relation to the high-level expression of collagenase-3 in human carcinomas, since a combination of these two elements appears to confer responsiveness to growth factors, oncogene products and tumor promoters.[41] Functional characterization of these elements has revealed that the AP-1 site is indeed functional, recognized by members of the Fos and Jun family of transcription factors, and responsible, at least in part, for the observed inducibility of this gene by TPA, TGF-β and IL-1β in human fibroblasts.[8,30,42] The involvement of AP-1 binding factors in mediating collagenase-3 expression in murine fibroblasts has been confirmed by using cells derived from *c-fos* deficient mice generated through gene knockout techniques. In fact, collagenase-3 cannot be induced by growth factors or tumor promoters in 3T3-type cell lines derived from *c-fos*$^{-/-}$ mice embryonic fibroblasts.[43] However, unlike collagenase-1, in which a cooperative effect between AP-1 and PEA3 is required, the PEA3 site does not appear to play a significant role in the transcriptional regulation of the human collagenase-3 gene.[30] Nevertheless, the presence of a functional AP-1 site that may contribute to the

induction of this gene by oncogenic proteins, tumor promoters, or inflammatory mediators is a clear indication at the molecular level that collagenase-3 expression may be linked to oncogenic transformation or destructive joint diseases like rheumatoid arthritis. A final point of interest is that the promoter of the human collagenase-3 gene contains an OSE-2 element (CAAACCACA), proposed to be essential for the osteoblastic-specific expression of the mouse osteocalcin gene.[44] The presence of this sequence in the human collagenase-3 gene, as well as in their murine homologues,[45,46] could provide an explanation for recent results indicating that this gene is expressed in osteoblasts during fetal ossification as well as in postnatal bone remodeling processes.[15,47]

In summary, the mechanisms controlling expression of human collagenase-3 present both similarities and differences with those operating in other MMP genes. Of special relevance are the differences between collagenase-3 and collagenase-1, which belong to the same subfamily of MMPs and display similar substrate specificity on fibrillar collagens. These differences could provide a molecular basis for the different expression pattern of both enzymes, collagenase-1 being widely distributed and collagenase-3 highly restricted.

## Physiological Significance of Human Collagenase-3

Originally identified in breast carcinomas, a survey of a wide variety of normal human tissues failed to reveal any significant expression of human collagenase-3 any of the tissues examined.[1] Subsequently, Mitchell et al[12] detected the expression of collagenase-3 in human osteoarthritic cartilage and confirmed its absence in a number of normal adult tissues including brain, heart, kidney, liver, lung, skeletal muscle, small intestine, spleen, testis and thymus. Although these initial studies suggested that the expression of collagenase-3 was linked to pathological conditions like cancer and arthritis, it was unlikely that this enzyme could be unique to these processes. One explanation of the apparent absence of collagenase-3 expression in normal tissues is that this potent proteolytic enzyme is only produced in a specific temporal manner by tissues undergoing remodeling or breakdown events, such as those occurring during embryonic development or in the course of reproductive processes like uterine-postpartum involution, ovulation, or mammary gland involution. Studies performed by different groups have confirmed the expression of collagenase-3 in some of these processes, suggesting potential physiological roles for this enzyme.

The first studies assigning potential roles for human collagenase-3 during fetal development have been reported by Stahle-Bäckdahl et al,[15] and Johansson et al.[47] Their results demonstrate that, starting at week 10 and continuing through the gestation, strong expression of collagenase-3 mRNA is found in the primary ossification centers in the shaft of the long bones. This collagenase-3 specific signal is confined to hypertrophic chondrocytes and to osteoblastic cells associated with periostal blood vessels. In contrast, no signal can be detected in osteoblasts nor in any other fetal tissue during the entire gestation period. These findings were also confirmed at the protein level by using a specific collagenase-3 antiserum.[15] The expression of collagenase-3 is not limited to the process of endochondral ossification, and also appears in the calvarial bone of the skull. This bone develops through intramembranous ossification, a process involving the formation of mineralized bone directly from primitive connective tissue. Similar results have been obtained by Gack et al[48] and Mattot et al[49] in studies of murine collagenase-3 expression during fetal development. Murine collagenase-3 is exclusively expressed by osteoblasts and hypertrophic chondrocytes in the forming bone, in complete agreement with a series of previous studies demonstrating that cultured rat osteoblasts[6,7] produce an interstitial collagenase, which must be now considered as the murine homologue of human collagenase-3.[1] The mechanisms regulating expression of this osteoblastic collagenase have been closely scrutinized (for a review see ref. 50). These analyses have allowed the finding of factors such as PTH, aFGF, bFGF,

PDGF-BB, prostaglandins, retinoic acid, cortisol, and interleukin-6, which induce rat collagenase-3 expression in osteoblast or osteosarcoma cells.[51-58] Similarly, a series of factors like IGF-I, IGF-II, BMP-2, and TGF-β have been characterized as negative regulators of rat collagenase-3 expression in these cells.[59-61] The identification of these factors, with their ability to positively or negatively modulate rat collagenase-3 expression in cultured osteoblastic cells, will prove helpful in future studies of the mechanisms controlling human collagenase-3 expression during fetal ossification.

The restricted expression of both human and murine collagenase-3 to forming bone is in marked contrast to that of other MMPs produced in a variety of tissues during prenatal development. On this basis it has been proposed that collagenase-3 serves a distinct and specific role during bone morphogenesis.[15,47] A plausible function in this context is to digest matrix components of the bone anlage to initiate the formation of mature bone. Consistent with this, collagenase-3 is a potent proteolytic enzyme capable of degrading different extracellular matrix components, such as types I, II, and X collagens during bone development. An observation of great interest was that no expression of collagenase-1 was detected in either cartilage or calvaria,[15,47] strongly suggesting collagenase-3 as the predominant collagenase expressed during both endochondral and intramembranous ossification.

In addition to this putative role of collagenase-3 in human fetal bone development, a number of studies have examined the participation of this enzyme in other tissue remodeling processes. Nevertheless, care must be taken in extrapolating results obtained in rodents to the situation present in humans, especially in light of data suggesting that murine collagenase-3 may also have to function as a murine collagenase-1, whose existence has yet to be demonstrated. One of the studies looking for potential collagenase-3 functions in murine tissues concluded that it may be involved in the ovarian function during the reproductive cycle.[62] In fact, in Northern blot analysis of rat tissues, Balbin et al[62] have demonstrated that collagenase-3 is expressed at high levels in the ovary and thus has a potential role in ovulation. The dynamics of collagenase-3 expression in rat ovary, with increasing levels at proestrus and estrus as the time of ovulation approaches and declining thereafter, is consistent with the participation of this enzyme in the ovulatory process. One likely function in ovulation could be the involvement of collagenase-3 in the rupture of the follicular wall, which precedes the release of the mature oocyte. In fact, there are numerous pieces of evidence indicating that a cascade of proteolytic enzymes, including plasminogen activators, plasmin, and MMPs, act in concert to degrade the ovarian follicular wall.[63] However, collagenase-3 may also be involved in other connective tissue remodeling processes occurring within the ovary during the reproductive cycle, such as atresia, cumulus cell expansion, or the massive angiogenesis taking place during early corpus luteum formation. A role for collagenase-3 in human ovarian function still requires evidence that the findings in rodents can be extend to human tissues. Although further studies are required in this regard, promising preliminary studies (Balbín et al, unpublished results) seem to indicate that the human ovary is also a site of collagenase-3 expression, but only in premenopausal women and at specific days of the menstrual cycle.

In addition to its potential role in ovulation, rat collagenase-3 is also expressed in postpartum uterus[64] and during wound healing,[65] suggesting additional roles for the enzyme in these processes. The finding of murine collagenase-3 expression during uterine postpartum involution is consistent with previous studies showing that rat uterine smooth muscle cells in culture produce an interstitial collagenase,[66,67] which in light of current data would correspond to collagenase-3.[1] Analysis of mechanisms regulating the expression of this uterine collagenase has also identified serotonin as a potential in vitro inducer of this enzyme.[68,69] However, levels of collagenase-3 during postpartum involution of the uterus are at least 100-fold lower than those found for other MMPs like matrilysin, indicating that the

participation of collagenase-3 in the process should be secondary to other abundantly expressed MMPs.[64] In contrast, studies of MMP expression during rat skin wound healing have revealed that collagenase-3 is one of the enzymes highly expressed throughout this remodeling process.[65] A more detailed in situ RNA hybridization analysis on skin wound sections after cutaneous incision revealed that collagenase-3 was intensely expressed in the epithelial basal cell layer and the hair follicle sheath, but not the dermis. This data suggests that collagenase-3, in coordination with other MMPs, contributes to the restoration of connective tissue during rat skin wound healing. The extension of these observations to human tissues would lend new insights and perspectives into the physiological significance of collagenase-3.

### *Pathological Significance of Human Collagenase-3*

To date, human collagenase-3 expression has been associated with two pressing pathological conditions: cancer and arthritic diseases. In fact, collagenase-3 was originally identified as an enzyme produced by mammary carcinomas,[1] but further studies have extended its participation to other tumor processes distinct from breast cancer. In addition, the relevance of collagenase-3 as a degradative enzyme in joint diseases has been the focus of notable interest, with a number of papers examining distinct aspects of this connection.[9,10,12-15,70]

The first studies linking collagenase-3 to tumor processes were performed by Freije et al,[1] who detected the expression of collagenase-3 mRNA in some breast carcinomas but not in benign lesions or unaffected normal mammary gland. This expression analysis also revealed three different mRNA species in breast carcinomas which result from the alternative use of different polyadenylation sites in the 3'-flanking region of the collagenase-3 gene. In situ RNA hybridization analysis of collagenase-3 expression in breast carcinomas has shown that collagenase-3 transcripts are detectable in fibroblastic cells surrounding epithelial cells, but not in the carcinoma cells themselves or normal breast glandular epithelium.[8] In addition, the fibroblastic cells most positive for collagenase-3 expression were those immediately adjacent to islands of cells present at the invasive edge of the tumor. This pattern of expression resembles that previously noted for other MMPs produced by breast carcinomas, including stromelysin-3, MT1-MMP and gelatinase A, which are also predominantly expressed by fibroblast cells within the tumor stroma adjacent to breast cancer cells.[71-73] The stromal expression of collagenase-3 is consistent with coculture experiments using human fibroblasts and MCF-7 breast cancer cells, demonstrating that conditioned medium from these latter cells stimulates the fibroblastic expression of collagenase-3 mRNA. By contrast, no stimulatory effect is observed when medium from fibroblasts is added to epithelial breast cancer cells.[8] These results suggest that transcription of the collagenase-3 gene in stromal cells of breast carcinomas is activated by diffusible factors released from epithelial tumor cells. To date, the precise nature of these soluble factors remains unknown, although a survey of cytokines and growth factors in human fibroblasts, has identified IL-1 as a potential candidate to induce collagenase-3 expression in breast carcinomas.[8] The expression of collagenase-3 in breast carcinomas has been confirmed by Heppner et al,[74] although in this work the enzyme was reportedly produced preferentially by tumor cells. These variations in intracellular location have been reported for other MMPs, and stromal expression may play a role in earlier events in tumor progression, whereas epithelial cell expression may be more relevant in late stage carcinomas.[75] Although both epithelial and stromal expression of collagenase-3 may occur, the presence of this enzyme in breast carcinomas mainly results from stromal expression rather than from synthesis by the tumor cell themselves. This is supported by the finding of collagenase-3 mRNA in different fibroblastic cell lines but not in any of the analyzed breast cancer cells, and by the results of coculture experiments. Conse-

quently, collagenase-3 should be included among the molecular factors detected during the stromal reaction to invasive breast cancer, whose concerted action may be essential for tumor growth and progression.[5] At present, this theory is without direct evidence, although preliminary clinical studies of collagenase-3 expression in these tumors suggest that production of high levels of this enzyme by breast carcinomas is a factor in poor prognosis in breast cancer patients (F. Vizoso, unpublished observations). Further support was provided by Lochter et al[76] who have recently provided evidence that collagen invasion by TCL1 mouse mammary carcinoma cells producing collagenase-3 is reduced by half in the presence of collagenase-3 antisense oligonucleotides, but in contrast, invasion of SCg6 cells not expressing collagenase-3 remain unaffected. Treatments that increase collagenase-3 expression such as culture of TCL1 cells in the presence of Matrigel promote collagen invasion, whereas further up-regulation of other MMPs does not cause any additional effect.[76] Taken together, these results support the proposal that collagenase-3 expression is one of the proteolytic enzymes relevant to mammary tumor cell progression in the invasive phenotype. Further studies with animal models, including transgenic mice overexpressing collagenase-3 in their mammary glands, will be required to elucidate the precise role of this enzyme in breast cancer.

In addition to these studies implicating collagenase-3 expression in breast carcinomas, more recent studies have revealed that this protein is also produced by other malignant tumors, including laryngeal carcinomas, chondrosarcomas, and ovarian carcinomas.[28,77,78] Northern blot analysis of collagenase-3 mRNA in a series of 35 matched squamous cell carcinomas of the larynx and the corresponding non-neoplastic adjacent tissues showed significant levels of expression in 20 of the 35 carcinomas (57%), but not in any of the normal mucosas.[28] Western blot analysis confirmed the presence of collagenase-3 protein in those carcinomas with high levels of mRNA expression, whereas no protein was detected in the carcinomas with negative collagenase-3 mRNA expression, or in any of the normal tissues. Immunohistochemical analysis of these carcinomas localized collagenase-3 predominantly in squamous cancer cells. This observation complements recent findings that this enzyme is produced by transformed human epidermal keratinocytes in culture, but not by cultured primary epidermal cells.[79] Interestingly, collagenase-3 overexpression in laryngeal carcinomas correlated significantly with advanced local invasion of the tumors, suggesting that this protein may contribute to the progression of a significant subset of squamous cell carcinomas of the larynx.[28] On the other hand, and since collagenase-3 has been found in normal chondrocytes and ovarian cells, recent studies have evaluated the possibility that tumors involving these cells also could be a source of collagenase-3 expression. In fact, most chondrosarcomas analyzed produce significant levels of collagenase-3, whereas the protein is either present at low levels or undetectable in benign chondromas.[77] The high levels of collagenase-3 present in human chondrosarcomas is of interest in light of previous observations demonstrating that *c-fos*-transgenic mice develop osteosarcomas and chondrosarcomas which overexpress collagenase-3.[78] Similarly, ovarian carcinomas produce collagenase-3, and furthermore mucinous carcinomas produce even higher levels than other types of ovarian tumors (M.Balbín, unpublished results). Since mucinous carcinomas have a relatively bad prognosis when compared with other ovarian carcinomas,[80] these results are consistent with those derived from analysis of other collagenase-3-producing tumors, in which the presence of high levels of this protease seems to be associated with tumor progression and poor clinical outcome.

Besides the evidence linking collagenase-3 to tumor processes, data has been also accumulating that implies a possible role for this enzyme in destructive joint diseases. To this end, Mitchell et al[12] demonstrated that collagenase-3 was expressed by chondrocytes in human osteoarthritic cartilage but not in normal articular cartilage. Relative mRNA levels

varied significantly between different samples, in some cases showing high levels of collagenase-3 but not collagenase-1, and vice versa. Shortly after, other groups confirmed and extended these findings, showing that collagenase-3 is not specific to osteoarthritic lesions but also can be found in rheumatoid arthritis.[9,10,15] The enzyme mainly localizes to chondrocytes from arthritic cartilage, but its presence has been noted in fibroblasts and macrophage-like cells in both the synovial lining and stroma. Furthermore, a series of proinflammatory cytokines, such as IL-1α, IL-1β, and TNF-α, have been identified as potential mediators of the increased synthesis of collagenase-3 in osteoarthritic cartilage.[12-14] The finding of collagenase-3 in both osteoarthritis and rheumatoid arthritis raised new insights into the potential pathogenetic role of collagenases in the arthritides. According to previous data, collagenase-1 produced by fibroblasts and macrophage-like cells at the advancing edge of the pannus was believed to be the major enzyme responsible for the degradation of the collagenous structures of the rheumatoid joint. In addition, collagenase-2 released from synovial fluid neutrophils could also contribute to degradation of type II collagen in the inflamed joint. However, none of these enzymes is particularly effective against the major collagen of the articular cartilage. Therefore, the finding of a third type of collagenase very efficient in the cleavage of type II collagen and expressed in the distinct cell types involved in the degradation of articular cartilage strongly suggests that it represents a significant mediator of tissue destruction in the arthritides. The relative contribution of the three human collagenases to these destructive joint events is presently unknown and will likely depend on a number of factors, including their relative expression levels, degree and mechanism of activation, sensitivity to endogenous inhibitors, and relative activity against other cartilage macromolecules distinct from type II collagen. Nevertheless, Billinghurst et al,[29] through the use of both antibodies reactive to neoepitopes generated by MMP cleavage of type II collagen and a synthetic preferential inhibitor of collagenase-3, have provided evidence that this enzyme plays the major role in cleavage of type II collagen in osteoarthritic cartilage. Therefore, collagenase-3 must be included among the molecular targets for therapeutic intervention designed to restrict the cartilage damage in destructive joint diseases.

## Conclusions and Perspectives

In the short time since its discovery, human collagenase-3 has gained recognition because of its association with cancer and rheumatic diseases, two pathological conditions of unquestionable clinical and social relevance. In addition, the enzyme appears involved in the normal physiological processes of fetal ossification, ovulation, and wound-healing, raising the possibility of future studies examining its precise involvement in these processes. Interestingly, collagenase-3 expression in normal tissues is restricted both spatially and temporally, and seems subject to stringent regulatory mechanisms that are most likely lost in pathological conditions. At present, very little information is available on these regulatory mechanisms, and more extensive studies will be required to address this question. Of special interest are comparative studies with other MMPs, such as collagenase-1, that may have overlapping substrate specificity but distinct tissue distribution. Similarly, elucidation of the complete three-dimensional structure of collagenase-3 and its comparison with that of other MMPs is also necessary for a better understanding of its enzymatic properties, including substrate specificity and activation mechanisms. These structural studies, together with those based on the use of combinatorial chemistry, will also help in the design of specific inhibitors which can control the unwanted activity of collagenase-3 in cancer and arthritic processes. Finally, the generation of both collagenase-3 overexpressing and collagenase-3 deficient mice will also illuminate the functional relevance of this enzyme in both normal and pathological conditions.

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CHAPTER 4

# Transcriptional Regulation of the Collagenase-1 (Matrix Metalloproteinase-1; MMP-1) Gene

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## Introduction

We first learned of the "collagenolytic principle" in 1962 when Gross and Lapiere[1] studied explants of tail fin tissue of the tadpole to document the resorption that results from metamorphosis as the tadpole matures into a frog. They performed a simple experiment by adding small amounts of tissue explant from the tadpole to slides coated with radiolabeled type I collagen. After incubation in 5% $CO_2$ at 27° or 37°C for 48 hours, the results were striking. Around the tail fin explant, there was a halo, or clearing, in the collagen due to collagenolytic activity. This was particularly unique and exciting because the protein was degraded under normal physiological conditions and at neutral pH. Later, the protein was purified and was found to be secreted as a latent enzyme that required activation.[2-4] Not until the mid-1980s, however, were investigators able to research this collagenolytic principle more thoroughly at the molecular level. Before this time, the tools were not in place to isolate the gene/cDNA or to investigate the mechanisms regulating its expression. With the advent of molecular biology and approximately 20 years later, what was originally termed the "collagenolytic principle" is now called "interstitial collagenase" or "collagenase-1," and comprises one member of the collagenases, a sub-group of enzymes that belong to the family of Matrix Metalloproteinases (MMPs).

In normal physiology, the major role of interstitial collagenase (MMP-1) is modeling the extracellular matrix (ECM), a critical aspect of organogenesis and skeletal formation from development throughout adult life.[5-6] MMP-1 also plays a pivotal role in normal remodeling processes (i.e. embryonic development, postpartum involution of the uterus, bone and growth plate remodeling, ovulation, and wound healing).[5,7,8] Transcription of the MMP-1 gene is a tightly controlled process in normal cells. In general, basal transcription is low, but is upregulated by a variety of factors that will be addressed later in this chapter. It is not surprising, therefore, that the regulation of MMP-1 gene expression occurs at several levels: transcriptional, post-transcriptional, and post-translational, as well as the interaction of the secreted enzyme with tissue inhibitors of metalloproteinases (TIMPs).[9-15] The corollary to the multi-leveled regulation of MMP-1, however, is that there is opportunity for abnormal expression, which may contribute to the pathogenesis of diseases, such as joint destruction (rheumatoid and osteoarthritis),[16-17] atherosclerosis,[18-19] angiogenesis,[20-21]

*Collagenases*, edited by Warren Hoeffler. 

***Table 4.1. Relative substrate specificities and $K_M$s for the collagenases***

| Enzyme | Substrate | Molecular Mass (latent, unglycosylated) | ~$K_M$ (μM) |
|---|---|---|---|
| MMP-1<br>interstitial collagenase<br>collagenase-1 | collagen types III>I>II, VIII, X | ~57 kDa | 0.8 - 1.7[a] |
| MMP-8<br>neutrophil collagenase<br>collagenase-2 | collagen types I>III>II, proteoglycan | ~53 kDa | 1 - 2.5[a] |
| MMP-13<br>collagenase-3 | collagen types II>III, I, IV | ~54 kDa | ~2[b] |
| MT1-MMP<br>membrane type 1 MMP | collagen types I>III>II, proteoglycan, fibronectin, gelatin | ~56 kDa | ~3[c] |

Relative substrate specificities and KM's for the Collagenases. Representative substrates are shown in the order of enzyme specificity. The KM's are listed for the most preferred substrate.
[a]Birkedal-Hansen et al.[30a]
[b]Mitchell et al.[30b]
[c]Ohuchi et al.[30]

periodontitis,[22] corneal ulcerations,[23-24] and tumor invasion and metastasis.[25-27] Clearly, this enzyme is critical in many biological processes, and much attention has been focused on the mechanisms controlling its induction and repression.

## MMP-1 Biology and Biochemistry

Interstitial collagenase is one of four collagenases able to degrade the interstitial collagens types I, II, and III at neutral pH (Table 4.1). MMP-8 (neutrophil collagenase),[28] MMP-13 (collagenase-3),[29] and MT1-MMP (membrane-type 1- MMP), a membrane bound form of the MMPs,[30] are also members of the collagenase subfamily able to degrade collagen (Table 4.1), and their roles are described in chapter 1. The interstitial collagens (types I, II and III) are the most abundant proteins in the body, comprising ~30% of the total protein[31-32] and, therefore, it is important to understand their role in the ECM and how the balance between these collagens and levels of MMP-1 influence matrix metabolism. Collagen is produced as early as the cleavage of the fertilized ovum. It provides tissues with structural support and a means of cell-cell communication, thus facilitating development, cell migration, remodeling, attachment, differentiation, and repair.[33]

All three of the major types of collagen are degraded by MMP-1. Type I collagen is the most abundant collagen and consists of two α1(I) chains and one α2(I) chain. It is the major collagen type in bone, skin, and tendon, and it is found at high levels in scar tissue and in most chronic inflammatory conditions.[33] Type II collagen is comprised of three α1(II) chains and is the major collagen in articular cartilage.[34] Type III collagen contains three α1(III) chains and is most abundant in embryonic tissues. It is also present in the gas-

trointestinal tract, blood vessels, and uterus, and is the first type of collagen deposited in wound healing.[33] The collagens are made up of triple helical domains with an amino acid triplet repeat of Gly-X-Y, where X and Y can be any amino acid, but most often are hydoxylated proline and lysine residues.[32-33] Although there are other collagen types (VIII and X) that are degraded by interstitial collagenase,[35] collagens type I, II, and III are the predominant substrates. Furthermore, the substrate preferences of MMP-1 for the interstitial collagens differ from those of the other collagenases. For example, MMP-1 cleaves Type II collagen relatively inefficiently. However, it is more active against collagen Types III and I, respectively,[36] whereas MMP-13 degrades type II collagen most efficiently.[37] This enzyme appears to have a dominant role in the pathology of osteoarthritis, where it is produced by the chondrocytes and where it degrades articular cartilage.[38] Although the rates of substrate cleavage are different with each collagen type, most vertebrate collagenases cleave the triple helix at a single peptide bond (Gly—Ile at position 775/776) at the same locus in the three constituent chains, releasing two large fragments consisting of 1/4 and 3/4 lengths of the collagen molecule.[39] Once cleavage has taken place, the molecules unwind at body temperature[40] and are then degraded by gelatinases and nonspecific extracellular proteases, or by lysosomal enzymes after phagocytosis of the fragments.[41]

## MMP-1 Structure, Organization, and Location

The induction of MMP-1 gene expression relies on transcription and de novo protein synthesis.[42-43] The enzyme is synthesized as a single polypeptide, preprocollagenase. It secreted from the cell in a latent form, procollagenase, and the major species is unglycosylated and has an approximate molecular weight of 57 kDa, with a minor glycosylated species is ~61 kDa. MMP-1 is secreted within 40 minutes after synthesis, and levels of MMP-1 mRNA are generally paralleled by levels of the secreted protein.[44] It is important to note, however, that protein production is not necessarily indicative of active enzyme. In order for collagen degradation to occur, the secreted latent collagenase must first be activated. This occurs by proteolytic cleavage of the proenzyme. Serine proteinases such as plasmin and kallikrien, or a related MMP, stromelysin-1, comprise the majority of the physiological activators of MMP-1.[45-46] Upon activation, the enzyme is truncated at the N-terminus to ~41 kDa[47] and is then able to degrade collagen.

In 1986, Fini et al[48] cloned and characterized the rabbit synovial cell collagenase cDNA (rabbit MMP-1), while Goldberg et al[49] cloned and sequenced the human homologue from skin fibroblasts. The structure of the human gene was determined to have 10 exons, spanning ~8.2 kbp, and to be similar to the rabbit gene.[50] MMP-1 is cytogenetically located on human chromosome 11q22.2-22.3[51] and, interestingly, this gene is tightly linked to a cluster of other MMP genes, including stromelysin. Physical mapping of this region by pulsed-field gel electrophoresis has shown that the order of these gene loci are centromere—Stromelysin-2—Collagenase-1—Stromelysin-1—Collagenase-3—telomere.[52] The human macrophage elastase gene has also been linked to this cluster, and its relative location is within 62 kb of stromelysin-1.[53] The facts that the MMP-1 gene is conserved through many species and that it is located in a cluster of other MMPs suggest that it diverged from a single gene, which has been duplicated and rearranged throughout the course of evolution to become not only MMP-1, but also other members of the MMP family.[50] The conservation of the cDNAs and of the protein sequences, along with comparable conservation of mechanisms of induction and suppression of MMP-1 among species, suggest that the regulatory promoter sequences also share similar features. Indeed, Figure 4.1, which compares the MMP-1 promoter regions from the rabbit and human genes, documents this similarity.

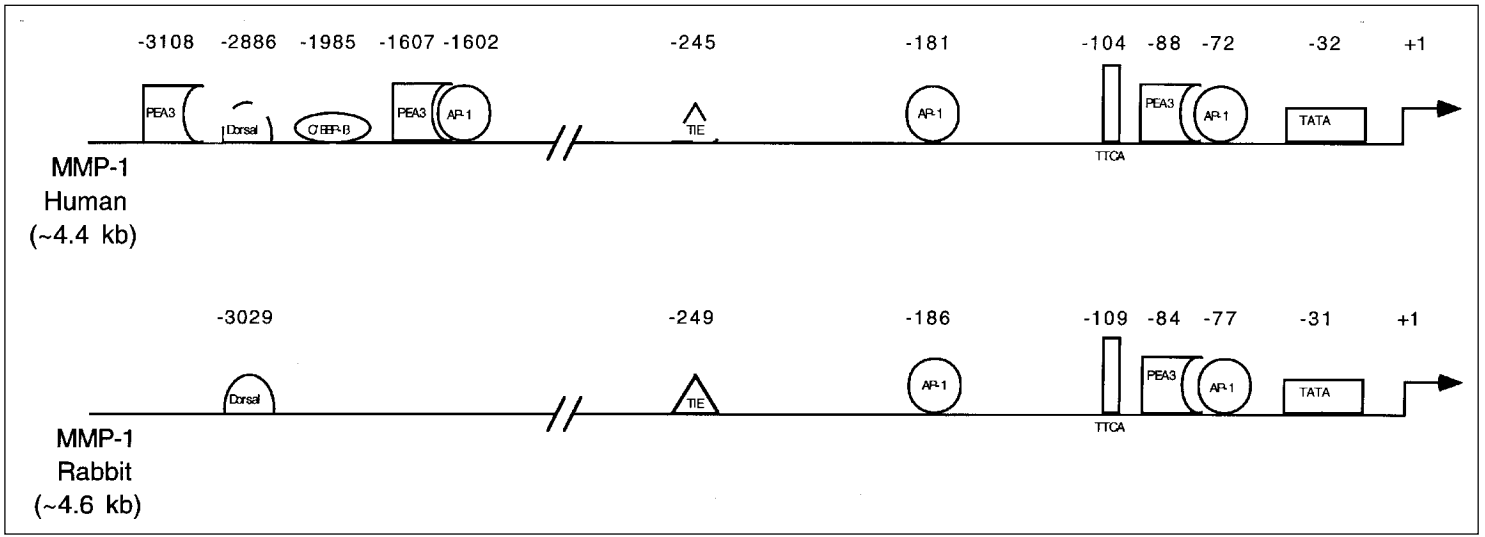


Fig. 4.1. Comparison of the cis-acting elements involved in MMP-1 transcriptional regulation. The symbols represent different elements. Positions (bp) are indicated above each symbol. The transcriptional start site is designated with an arrow at +1. Sites that have not yet been shown to be functional are outlined with a dashed line. See text for details on the sites and their functional relevance to transcription. (AP-1, Activator Protein-1; PEA3, Polyomavirus Enhancer A-binding protein 3; TIE, TGF-β Inhibitory Element; C/EBP-β, CCAAT/Enhancer-Binding Protein-β)

## Transcription of MMP-1: Basal/Constitutive and TPA-induced Expression

Most of the literature accumulated thus far regarding the transcription of MMP-1 describes the rabbit and human promoters. Both of these promoter sequences (~4.6 kbp, rabbit; ~4.4 kbp, human) have been studied for some time,[42-54,58,13,15] and their characterization is still ongoing. Since the rabbit and human genes share a high degree of homology both in the cDNA and promoter, investigators have used a rabbit model as a tool to study diseases, such as rheumatoid arthritis, in which there is overexpression of MMP-1. This rabbit model of cultured monolayers of synovial fibroblasts has been crucial in creating a strong foundation of basic mechanisms controlling MMP-1 gene expression, which can be used to formulate and test hypotheses that may be translated into the clinic. However, the promoter regions, although homologous, are not identical. Most striking is the strong similarity in the proximal promoter regions, while sequences in the distal regions are more divergent (Fig. 4.1). Furthermore, the intracellular trans-activating factors in the rabbit cells may be somewhat different from the trans-activating factors in the human cells, since each species and cell-type is unique. Thus, although animal and in vitro models are useful, the results obtained from them must be carefully interpreted. Nowhere is this paradigm more apparent than in animal models that use rats and mice to study MMP-1 gene expression. Within the past few years, it has become clear that these animals do not express the MMP-1 gene. Rather, their collagenase activity is due to collagenase-3 (MMP-13), the product of an entirely different gene, with its own distinct regulatory mechanisms.[59]

One well-accepted method for studying mechanisms controlling gene transcription is the assay in which the promoter DNA is chimerically linked to a reporter gene in a plasmid. Such assays often use either the chloramphenicol acetyltransferase (CAT) gene or the firefly luciferase (LUC) gene as a reporter of promoter expression, and are useful in delineating and defining important regulatory regions in the promoter DNA. Generally, these assays will localize a regulatory element so that other methods, such as the electrophoretic-mobility shift assay (EMSA), DNA footprinting techniques, or mass spectroscopy can then be applied to verify the identity of a specific element, its precise location, and even the proteins that bind to it.

Angel et al[11] performed the initial studies of the human collagenase promoter utilizing these techniques. By differential screening of cDNA libraries prepared from untreated cells and from cells treated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA, a strong, nonphysiologic tumor promoting agent and a protein kinase C agonist), they found that a short 5'-flanking fragment containing only 517 bp of the promoter DNA was sufficient to strongly enhance MMP-1 transcription. Deletional analyses of the promoter, along with CAT assays led to the discovery of an AP-1 enhancer element at -72 to -65 bp.[11] In addition to the AP-1 site, these studies also identified the TATA box located at -32 bp. The core AP-1 sequence (also known as the TPA responsive element, TRE) is 5'-TGAGTCA-3', and the proteins that bind to this site are members of the Fos and Jun family of transcription factors.[60-63] The interaction of these proteins with the DNA is a highly complex process that is, ultimately, the target of a cascade of intra- and extracellular signals. The Jun family is comprised of c-Jun, Jun B, and Jun D proteins, all of which are capable of binding to the AP-1 site either as homodimers or as Jun-Fos heterodimers. The known Fos proteins are c-Fos, Fos B, Fra-1, and Fra-2. Depending on the makeup of the Jun/Jun homodimers or the Jun/Fos heterodimers can actually dictate the level of expression and result in either induction or repression. The AP-1 site plays an important role in the basal/constitutive transcription of MMP-1[55,63,64,154] and in induction by TPA and, perhaps by other factors, including interleukin-1$\alpha$ and $\beta$ (IL-1$\alpha$, $\beta$),[65] tumor necrosis factor $\alpha$ (TNF-$\alpha$),[66] UV irradiation,[66-68] and urate crystals.[69] Initially, the AP-1 containing constructs were studied in conjunction

with heterologous promoters and/or were linked to the reporter in mulitmeric repeats. Often, these constructs were transfected into transformed cell lines that do not express the MMP-1 gene endogeneously, and thus the results obtained may not have been completely representative of mechanisms controlling expression of the endogenous gene. Nonetheless, these studies were important because they identified prominent elements in the proximal promoter that seem to contribute substantially to MMP-1 transcription in most cell types. However, more recent studies in cells that express the MMP-1 gene have revealed that, although the proximal enhancers are necessary for transcription, they are not sufficient. It was, therefore, essential to study larger fragments of the promoter in its native configuration and to use cells that express MMP-1 endogenously.

As noted above, analysis of the nucleic acid sequence of the rabbit and human promoter reveals a high degree of conservation within 1 kb (Fig. 4.1).[42,70] On the basis of this information, along with earlier experimental data that demonstrated the similarity in the behavior of rabbit and human synovial cells,[2,71] the rabbit gene has been a model for studying mechanisms controlling the expression of the MMP-1 gene. These studies used fragments of the native rabbit MMP-1 promoter transfected into monolayers of rabbit synovial fibroblasts. With a 127 bp promoter fragment, it was found that, although the AP-1 site at -77 bp is a key player, it alone, could not support induction by TPA. Auble and Brinckerhoff[54] showed that the presence of at least two additional sites were required for TPA-inducibility. A polyoma enhancer adenovirus 3 (PEA3) element functionally cooperated with the AP-1 site since mutations at this site abolished transcriptional activity. Another element was a 5'-TTCA-3' motif located at -109 bp, which when mutated, displayed reduced activity.[54] These data suggest transcriptional cooperativity among the AP-1, PEA3, and TTCA sequences, which are all necessary to confer full TPA-induced expression of MMP-1 (see below). Taken together, they also show that when studied in the context of the native promoter and in the synovial cells that normally express MMP-1, the AP-1 site, alone, is not as potent as are tandems of AP-1 sites transfected into transformed cells that do not express the MMP-1 gene. Perhaps the milieu of transcription factors found in the transformed cells differs from that seen in normal cells in that there are higher levels of Jun and Fos proteins, and these proteins interact with the AP-1 multimers and drive transcription.

There is also mutational evidence of the AP-1 site at -77 bp that suggests a role for additional functional elements. A T → G transversion introduced at -77 bp of the rabbit promoter abolished binding of Fos and Jun proteins[72] and displayed dramatically reduced levels of transcription, supporting a role for this site in constitutive expression, as reported in the stromelysin-1 gene.[73] However, mutant constructs of the MMP-1 promoter that contain at least -321 bp responded to TPA similarly to the native gene, indicating that additional sequences within -321 bp of the promoter also mediate the TPA response. Deletional studies with a -321 bp fragment of the rabbit promoter suggested the presence of yet another enhancer sequence.[54,55] Using mutational analysis, White and Brinckerhoff[13] searched for additional elements that could affect transcription. They found another AP-1 site, located at -186 bp. This site contains the sequence 5'-TTAATCA-3', corresponding to a two bp difference to the site at -77 bp, and an upstream AP-1 site, at -186 bp. When this site was mutated at position -186 bp, basal transcription was not greatly affected, although the TPA-induced activity was reduced by 50%. These data show that there are two AP-1 sites located in the first -186 bp of the 5'-flanking DNA, and that they regulate the promoter differentially. There may be altered affinities of the -186 bp site for the Fos and Jun protein members for this site compared to the -77 bp site. Indeed, the -186 bp site binds Jun D and c-Fos proteins, while the -77 bp site also binds Jun D and c-Fos, but also binds Fra-2.[13] Interestingly, this suggests that heterodimer formation influences the activity of the promoter,[74-75] and that the nucleotide sequence of the site, and perhaps adjacent sequences as well, may

confer specific transcriptional activities. Thus, subtle changes in the identity and affinity of the transcription factors that bind to elements appear to influence transcriptional activity.

## Transcriptional Regulation by Cytokines and Growth Factors

As mentioned previously, inappropriate expression of MMP-1 has been associated with several diseases. One example is the large overexpression of MMP-1 in rheumatoid arthritis (RA), with the role of this enzyme in RA being studied since the late 1960s. This disease is characterized by inflammation and erosion of the joint,[16] as the thin layer of normal synovial fibroblasts proliferates into a huge mass of invading cells. The accompanying deformity arises from the degradation of the collagens within the cartilage, tendon, and bone and from extensive synovial cell proliferation, which has been likened to that of tumor growth.[76] The conclusive experiments that implicated MMP-1 in RA stemmed from the pioneering efforts of Gross and Lapiere.[1] Subsequently, Evanson and colleagues showed that tissue of rheumatoid synovium could degrade and release $^{14}$C-labeled collagen fragments when the tissue was cultured on collagen gels.[16,77,78] Since then, the pathology of RA has provided a system in which to investigate the molecular events involved in upregulating MMP-1 gene expression, and the degradation of the ECM.

Several cytokines, [IL-1α and β; TNF-α] and growth factors [(epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)], are important mediators in inducing MMP-1 expression.[79-81] Each of these cytokines and growth factors has been implicated in the pathophysiology of RA, and each can upregulate MMP-1 gene expression. Both IL-1 and TNF- α are major products secreted from monocytes/ macrophages and are involved in numerous physiological functions of cells. Cytokines contribute to inflammation and autoimmunity, while growth factors are important in events such as cell proliferation, tissue remodeling, and wound repair.[82-83] However, because IL-1 has been detected in the synovial fluid of patients with RA, many studies have focused on it.[15,58,84-88]

There are two distinct, but structurally related members of IL-1, known as IL-1α and IL-1β.[89] Both forms exhibit similar biological properties and bind to the same receptor.[90] A third member, an endogenous receptor antagonist (IL-1Ra), binds to the receptor but does not elicit a biological response.[91] Three forms of the IL-1Ra exist and include two intracellular forms and one secreted form.[92] Several lines of evidence indicate that an imbalance between IL-1 and IL-1Ra may be important in monitoring the state of arthritic disease, and suggest that restoring this balance to its normal state may be an appropriate goal of therapeutic treatments.[38,93-97] Therefore, elucidating the mechanisms by which IL-1 increases collagenase production has been an important area of research.

Initial studies on the transcriptional regulation of MMP-1 by IL-1 revealed that the induction of MMP-1 was similar to that by TPA.[98] There was a prolonged activation of c-jun gene expression, and this enhanced the binding of c-jun to one or both of the AP-1 sites, resulting in increased transactivation of MMP-1. Additionally, a -72 bp construct of the human promoter, which contained the AP-1 site, responded to IL-1.[65] However, as more promoter DNA was added, MMP-1 induction by IL-1 was reduced,[65] leading the authors to postulate the existence of a repressor element upstream. Other evidence argues against a strict role for AP-1 in IL-1 induction, and it stems from an experiment that used a mutant form of the IL-1β protein. This mutant contains a Gly to Arg substitution at position 127 of the IL-1β protein and binds to the cellular receptors with high affinity, but fails to evoke significant proliferation of T-helper cells. Since this mutant IL-1β induces c-jun and c-fos genes, but not transcription of the MMP-1 gene[99] the authors suggested that Fos and Jun expression does not necessarily correlate with increased transcription of genes containing AP-1 elements, and that IL-1β-induced MMP-1 gene expression requires at least two events.[99] Thus, IL-1 and TPA appear to transactivate MMP-1 via different mechanisms.

Additional data support this concept. In both rabbit and human fibroblast cells, maximal induction of MMP-1 transcription is achieved with promoter sequences containing greater than 3000 bp of DNA.[47,15] Using deletional and mutational analysis, Vincenti et al[100] have delineated an IL-1β-responsive element between -3030 and -3000 bp in the upstream region of the rabbit MMP-1 promoter, which results in maximal activation of the IL-1β - induced transcript.[103] When IL-1β inducibility is compared between a 4.6 kb construct containing either a wild-type or mutant AP-1 site at -77 bp, the inducibility decreased 10-fold in the mutant, suggesting that cooperativity between these two sites may be required to provide transcriptional activation of the gene. Interestingly, the upstream region between -3000 and -3030 bp contains a Dorsal binding motif (5'-ATGGAAAAACAC-3'), which in Drosophila, binds to proteins of the Nuclear Factor κB (NFκB) family.[101,102] Indeed, induction of MMP-1 expression in the rabbit fibroblasts by IL-1β involves binding of the NFκB-related protein to an element located between -3000 bp and -3030 bp of the rabbit MMP-1 promoter.[103] Although the function of this site has yet to be determined in the human promoter, the fact that it is conserved both in sequence and relative location suggests that it may have significance in this promoter as well.

In normal rabbit synovial fibroblasts and in human dermal fibroblasts, IL-1β treatment induces a 10- to 20-fold increase in MMP-1 mRNA. While this increase depends on an increase in transcription, it also results from enhanced mRNA stability. The 3'-untranslated region of the MMP-1 mRNA contains three 5'-AUUUA-3' motifs that are conserved in sequence and location in both the rabbit and human genes.[58] The presence of these sequences enhances the turnover of MMP-1 mRNA, and the addition of IL-1β antagonizes this decay, thereby stabilizing the MMP-1 message.[15,58] Thus, along with an increase in transcription, an increase in the stability of the mRNA represents another mechanism by which IL-1 increases MMP-1 gene expression. This point is noteworthy in considering the design of therapies that antagonize IL-1 induction of the MMP-1 gene. Indeed, a recent report describes a reduction in MMP-1 mRNA by adenosine receptor agonists.[104] The authors show that these agents down regulate MMP-1 production by destabilizing mRNA. This concept suggests that along with antagonizing transcription, destabilizing MMP-1 mRNA may prove to be a new target for therapies needed to combat the overexpression of MMP-1.[104]

To date, little is known about the effects of other cytokines such as TNF-α and growth factor induction of MMP-1. However, one report demonstrates that TNF-α and interferon-β (IFN-β) increase the steady-state levels of MMP-1. Interestingly, these authors suggest that both TNF-α and IFN-β increase c-fos and c-jun mRNA levels. Consequently, the induction of MMP-1 may rely on the AP-1 proteins.[105] Alternatively, these cytokines may mimic the induction of MMP-1 by IL-1β, and two reports show that they are important in regulating MMP-1 in an autocrine fashion.[106-107] These authors suggest that the activation of a cytokine feedback loop may explain the act of remodeling by resident cells. EGF appears to act by increasing MMP-1 mRNA stability with a slight increase in transcription, similar to what has been shown with IL-1β induction.[108] Thus taken together, the evidence indicates an undisputed role for the proximal AP-1 sites. However, increased accumulation of new data on transcriptional regulation of MMP-1 implicate an essential role for distal elements in the promoter.

## Role of PEA3 Sites and AP-1 Sites: The Concept of Cooperativity

Analysis of the nucleic acid sequences of the human MMP-1 promoter reveals several additional AP-1 sites (bp location: -436, -562, -891, -1062, -1602, -1955, and -3471), PEA3 sites (bp location: -1390, -2101, -3108, and -3837), as well as a host of other putative cis-acting elements.[15] Table 4.2 lists these elements in the human MMP-1 promoter and compares them with elements in the rabbit promoter. Although functional significance of many

***Table 4.2. Comparison of cis-acting sequences in the human and rabbit MMP-1 promoter***

| Consensus Element | Sequence/Location (bp) (Human) | (Rabbit) | Regulation: Agent |
|---|---|---|---|
| AP-1 | **TGAGTCA/-72**<br>**TTAATCA/-181**<br>TCAGTCA/-436<br>TGAGTAA/-562<br>TGAGTAA/-891<br>AGAGTCA/-1062 | **TGAGTCA/-77**<br>TTAATCA/-101<br>**TTAATCA/-186**<br>TGAGTTA/-1724 | Positive:<br>TPA, IL-1,<br>TNF<br><br>Negative:<br>dexamethasone, |
| | TGACTTA/-1602<br>TGAGTTA/-1955<br>TGAGTGA/-3471 | | retinoic acid |
| Pea3 | **AGGAT/-88**<br>AGGAA/-1390<br>AG*GAT/-1608<br>AGGAT/-2101<br>**AGGAA/-3108**<br>AGGAA/-3837 | **AGGAT/-92**<br>AGGAA/-864<br>AGGAA/-1076<br>AGGAA/-1314<br>AGGAA/2030 | Positive:<br>TPA<br><br><br>Negative:<br>retinoic acid |
| TIE | GAATTGGAGA/-245 | **GAAATGGAGA/-249** | Negative: TGF-β |
| CCAAT (C/EBP-β) | **CCAAT/-1985**<br>CCAAT/-2326 | CCAAT/-1754<br>CCAAT/-2188<br>CCAAT/-2520<br>CCAAT/-3363 | Positive<br>monocyte<br>differentiation |
| CACCC Box | CACCC/-385<br>CACCC/-1821<br>CACCC/-1937 | CACCC/-698<br>CACCC/-998<br>CACCC/-1422<br>CACCC/-2638 | |
| CREB | TGACG/-1540<br>TGACG/-3187 | TGACG/-3955 | |
| Dorsal | ATGGAAAAA/-2886 | **ATGGAAAAA/-3029** | Positive: IL-1 |
| SP-1 | GGCGG/-2208 | | |
| OCTA Motif | ATGCAAAT/-4078 | | |
| AP-2 | TGGGGA/-960<br>TGGGGGA/-2695 | TGGGGGA/-2821 | |

Sequences known to participate in transcription are shown in bold. All other sequences are putative sites. The (*) in the PEA3 site at -1608 bp of the human promoter designates an allelic variation. The effects on MMP-1 gene regulation through the known sites are shown in italics along with representative stimuli. (CREB, cyclic AMP Response Element Binding Protein;[128] OCTA, octamer sequence) See text for additional details and references.

of these sites is not yet known, it is possible that they may be involved in transcription in response to various stimuli and/or they serve to regulate MMP-1 transcription in a cell-type specific manner.[63]

Conversely, it is also possible that an allelic variation(s) in the human MMP-1 promoter may influence the transcriptional response to IL-1β, and perhaps to other inducers.[15,56] The variation is located at position -1607 bp of the human MMP-1 promoter, where the sequence contains either one or two Gs (5'-AAGAT-3' or 5'-AAGGAT-3'). Interestingly, if two Gs are present, this site constitutes a consensus PEA3 element, and its presence influences the basal level of MMP-1 transcription.[57] This is intriguing because an AP-1 consensus element is located 1 bp 3' of the variation,[15] and PEA3/AP-1 site cooperativity has been documented previously.[64] DNA elements containing single PEA3 sites are not sufficient for Ets (a known transcription factor that binds to PEA3) induction, and all genes activated by an Ets protein require a nearby AP-1 site.[65-110] Supporting this concept is the fact that in the MMP-1 promoter the AP-1 site at -77 bp (rabbit)/-72 bp (human) interacts with the PEA3 site at -94 bp (rabbit)/-88 bp (human).[13-54] Thus, the interaction/cooperation between AP-1/PEA3 sites may be evident again in the upstream region of the MMP-1 promoter with a (potential) PEA3 site at -1607 bp and the AP-1 site at -1602 bp.

## Inhibition of MMP-1 Transcription

Because of the therapeutic implications, inhibiting MMP-1 has been a major focus of research. Reducing MMP-1 levels could prevent the irreversible degradation of cartilage, tendon, and bone in arthritis and impede the process of tumor migration and invasion in cancer. Considerable attention has, therefore, been directed at the inhibition of collagenase activity, with either naturally occurring protein inhibitors (the TIMPs) or with synthetic compounds. Alternatively, inhibition of MMP-1 can occur by inhibiting the synthesis of this enzyme. The focus of this section is a discussion of mechanisms that inhibit the synthesis of MMP-1 at the transcriptional level.

Transforming growth factor β (TGF- β), and the vitamin A derivatives, all-trans-retinoic acid and the synthetic retinoids, suppress the transcription of MMP-1.[111-112] TGF- β mediates its effect through a TGF- β inhibitory element (TIE 5'-GAGTTGGTGA-3'), which is located at -709 bp of the rat stromelysin-1 promoter.[113] Interestingly, these authors show that c-Fos binding is necessary for the inhibitory effects on transcription. The presence of this site has been described in the rabbit[114] and human[113] MMP-1 promoters located at -249 bp and at -245 bp, respectively. Due to the similarity in both sequences (rabbit: 5'-GAAATGGAGA-3'; human: 5'-GAATTGGAGA-3', a 2 to 3 bp difference from the originally described TIE in the stromelysin-1 gene) and the conserved location of this element in the two MMP-1 promoters, it is possible that it is functional. Indeed, in the rabbit promoter, this element also binds c-Fos. Moreover, mutations in this element result in a significant increase in both basal and TPA-induced transcription in fibroblasts, suggesting that the TIE may have a novel role as a constitutive repressor of the MMP-1 gene in these cells.[114]

Considerable attention has been focused on the mechanism(s) by which retinoids inhibit MMP-1 transcription.[112] Retinoids mediate their effects through one family of the nuclear hormone receptors: the retinoic acid and retinoid X receptors (RARs and RXRs, respectively).[115-116] Both RARs and RXRs have an α, β, and γ subtype and their functional activity depends on their ability to form RXR/RXR homodimers, or RAR/RXR heterodimers. These dimers can then either bind to the DNA directly, via retinoic acid response elements (RAREs), or indirectly to other sites in the DNA. The formation and precise effect of either the RXR homodimer or the RXR/RAR heterodimer is most likely dependent on the ligand availability and receptor concentration within the cell type.[117]

The MMP-1 promoter does not contain an RARE, and it has been demonstrated that in fibroblasts, RARs/RXRs suppress transcription of MMP-1 by interacting with the proximal AP-1 site via protein-protein interactions.[118-121] Other studies suggest that alternative mechanisms contribute to suppression of MMP-1 by retinoids. These include 1) a conformational change in the DNA resulting from the association of the RARs/RXRs with AP-1 proteins bound to the DNA, with a subsequent inhibition of transcription; 2) the interaction of RARs/RXRs with corepressors, which downregulate transcription;[122] 3) the interference of RARs/RXRs with the transcriptional machinery, since the -77 bp AP-1 site is in close proximity to the preinitiation complex;[123] 4) the sequestration of c-Jun by the RARs/RXRs prevents them from binding to the promoter and trans-activating the gene;[118-122,124,125] or 5) PEA3/protein interactions (see below). It is possible that multiple mechanisms may be operational simultaneously, a concept that emphasizes both the redundancy in the pathways regulating transcription and the importance of precise control of MMP-1 gene expression.

Recently, novel mechanisms of MMP-1 repression have been reported. Schneikert et al[126] have shown that the androgen receptor directly interacts with a PEA3/Ets-related protein, ERM, resulting in the inhibition of MMP-1. In addition, Benbow et al[127] have demonstrated that a PEA3 site at -3108 bp constitutively binds RAR/RXR proteins, probably via protein-protein interactions. Despite this constitutive binding, however, repression of MMP-1 transcription occurs only in the presence of ligand. Possibly, the addition of retinoic acid triggers a conformational change in the RAR/RXR proteins,[121,127,128] or a post-translational event, such as phosphorylation, that culminates in the repression of transcription.[129] There is also evidence that the repression by the nuclear hormone receptors occurs in a ligand-independent manner, in which the receptors become translocated to the nucleus by non-hormonal stimuli, such as heat shock. Interestingly, this suggests that the receptors can repress transcription even under conditions where hormone is absent.[112]

It is already well accepted that retinoids have potent antiproliferative effects,[117] and much effort has been directed towards developing their therapeutic potential in cancer. Since these compounds also suppress MMP-1 synthesis, it is equally possible that they may have an additional role in suppressing the process of tumor invasion and metastasis, by suppressing the transcription of MMP-1. Since tumor cells can contribute to their own invasiveness[130] and some tumor cells constitutively express MMP-1,[15,64] the concept that decreasing MMP-1 may decrease the invasive potential of certain tumor cells is intriguing. Understanding the molecular mechanisms behind this suppression may be a goal of future studies and, eventually, form the basis for therapeutic intervention.

## Summary and Conclusion

Cell-type and species-specific mechanisms regulate transcription of MMP-1. This regulation depends not only on the array of cis-acting sequences and their juxtaposition in the promoters of MMP-1 from different species, but also on the assortment of transcription factors found in these species and cell types. This concept is becoming increasingly apparent as MMP-1 gene expression is studied in a variety of cell types. The previous contributions made by investigators studying the crucial role of the proximal promoter with its adjacent AP-1 and PEA3 sites have been essential in developing our understanding of how this gene is regulated. The efforts in the future will be focused on determining how this gene, with such a critical role in extracellular matrix metabolism, is carefully regulated in different cell types under different physiological and pathological conditions.

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CHAPTER 5

# Interpreting Transcriptional Control Elements

**Warren Hoeffler**

## The Promise of Transcription 'Factorology'

In this book devoted to collagenases, regulation of collagenase genes necessarily has an important place. In the previous chapter a complete review was given of work that has been done on understanding the transcriptional control of the collagenase I promoter by a leader in the field. Complementary to the previous chapter, here some of this information is put into the perspective of what is known about transcriptional control in general. Although this discussion is general, and therefore applicable to consideration of a variety of gene specific gene regulation systems, the discussion and conclusions provide focus on the particulars of collagenase gene regulation. I discuss transcriptional regulation with a bias, based on my own experience in this field. I do not attempt to recount or reference the great many important contributions in the transcription field, since this is outside the scope of this volume that focuses specifically on collagenases.

One of the reasons for my own involvement in the collagenase field has been from the standpoint of trying to decipher transcriptional control signals. Unfortunately, the promise of understanding how genes are regulated by identification and characterization of the transcription factors involved is still largely an unfulfilled dream. Not to say that there has not been great progress in certain areas. Over the last ten years the technology that was initially developed in laboratories investigating mechanisms of transcription has been harnessed to study potential roles of transcription factors in controlling gene specific transcription, but problems remain in the interpretation of these data.

The collagenase promoter was one of the first promoters studied in detail, because it was a gene that was greatly increased in abundance by the treatment of cells with phorbol esters. So the collagenase promoter was initially studied because it happened to be responsive to a class of tumor promoting chemicals, rather than any inherent interest in the physiological processes controlled by collagenase. From these studies a region in the collagenase promoter was identified that when deleted nearly obliterated the phorbol ester response.[1,2] These studies were conducted by linking a reporter gene to a processive series of collagenase promoter mutants. The constructs were transfected into cells and reporter gene activity compared for untreated and phorbol ester treated cells. A phorbol ester responsive element was narrowed down as a discrete element within the promoter. This experimental design, sometimes called 'promoter bashing', has been an important method in the effort to localize specific regions within the DNA of a gene's promoter region that can control transcription.

*Collagenases*, edited by Warren Hoeffler. 

The 'pros' of this methods include the fact that it is reasonably straightforward. Methodology for the creation of recombinant DNA molecules has been sufficiently advanced to make the creation of deletion mutants a simple exercise. Transfection of the DNA into living cells gives a reliable in vivo assay for the contribution of some individual promoter elements to the overall transcription of many promoters. Among the 'cons' of the method is that promoters that are contained in extrachromosomal plasmids may not be regulated in the same ways as endogenous promoters contained in the chromosomal environment. Therefore, some promoter elements may underrepresent their contribution to the control of overall transcription levels in transfection assays. Also, since typically a great deal of plasmid DNA enters only a subset of the transfected cells, the pool of transcription factors in these cells may be altered by the quantity of the transfected DNA, resulting in transcription levels unrepresentative of what they would be for the endogenous promoters, that are often present endogenously in only single copies. For example, certain aspects of transcription factor cooperativity, so important for regulation, may not be detected in these assays as a result of the unnatural ratios of transfected DNA to cellular transcription factors.

Another important tool in transcription studies is the biochemical extraction and purification of transcription factors from eukaryotic cell nuclei. The enzymes that catalyze transcription, RNA polymerases, had been purified from a variety of eukaryotic cell types.[3,4] The presence of additional 'transcription factors' needed to control the process of transcription had been postulated from analogy with bacterial systems that required auxiliary factors. The finding that gene specific transcription could proceed in a nuclear extract[5,6] meant that the general factors needed to catalyze transcription starting at the correct initiation point for many genes could be solubilized and retained their activity in nonliving reconstituted models, i.e., in vitro. These discoveries made possible the biochemical fractionation of extracts to yield preparations of individual components of the transcription machinery.[7,8]

## Functional Assays for Transcriptional Activation Largely Unavailable

Given the excitement of being able to pick apart the eukaryotic transcription machinery by biochemical purifications of soluble systems, a certain problem was quickly overlooked, or at least not widely discussed. Significantly, only a tiny part of the promoter was needed for correct initiation of transcription, the region within about 30 bp of the transcriptional initiation site, termed the TATA box. Further upstream regions, later shown to contain many of the binding sites for enhancing binding proteins (EBPs) that control transcriptional levels, were nonfunctional in the in vitro systems. For investigators interested in what is termed the general transcription machinery, the components of all transcription complexes needed to correctly initiate transcription, these models offered a powerful tool for characterization of all components needed for the start of transcription.

Soon other researchers with an interest in specific gene systems began asking about the specific controlling elements regulating the genes found to be important in their fields of study. Certain investigators saw the potential of in vitro transcription systems as a tool for purification and characterization of the set of transcription factors active in regulating their gene of interest. The power of in vitro transcription reactions is that they are functional assays. If active transcription factors can be separated from each other biochemically, then they could presumably be added back to the in vitro reactions to determine how each factor functionally alters the amount of transcript made. The assay would provide direct proof of how a factor functions. Other assays that do not measure the amount of transcript made in response to added factor are not functional assays, and so cannot speak directly to the effect of the added factor on transcription levels.

Some of the researchers starting out did not fully realize that the in vitro systems had not shown any effects mediated by upstream DNA elements, or they hoped to be among the first to find such effects. They made nuclear extracts from HeLa cells and analyzed series of deletion mutant constructs for their gene of interest in hopes of seeing differences in levels of in vitro transcription products dependent upon upstream transcription factor (enhancer) binding sites. Unfortunately, these efforts were largely unfruitful, since effects of upstream elements were generally not observed. Sporatic claims of upstream effects from promoter enhance elements were often not followed up, perhaps indicating irreproducibility. Extracts were also prepared from other cell types, but these extracts were inactive or dramatically less active than the Hela cell extracts for in vitro transcription reactions, dampening hopes that the in vitro transcription systems could be prepared from a variety of cell types known to display interesting gene regulatory phenomena. Enthusiasm for the promise of in vitro transcription reactions as providing a means for dissecting transcriptional gene regulation died out, and awaits a resurgence once key obstacles to achieving regulatable gene transcription in vitro can be surmounted.

Some laboratories over the last few years have reported altered gene expression levels dependent upon upstream elements. For example, Bert O'Malley's lab[9,10] and more recently Roeder's laboratory[11] have reported stimulation of transcription mediated by upstream factors. O'Malley's extracts and reaction conditions are similar to those used by others, but certain unique features may help his group see these effects. Interestingly they claim that superhelicity of the plasmid template added to the reactions is crucial to seeing these effects, so that linear DNA templates used in the classic in vitro transcription reactions do not allow the upstream enhancer effects to be seen. One limitation of the methodology seems to be that it is not easily adapted by other laboratories, since others have not reported the same results, again potentially indicating problems with reproducibility.

## DNA Binding Assays Are Helpful, but Inconclusive

The whole approach of learning about gene regulation at promoters through biochemical methods might have fallen into complete disfavor, due to critical limitations of the in vitro transcription systems. Fortunately, a technique was developed to allow researchers to quickly and easily visualize the interaction of DNA binding proteins, obtained from transcription style extracts, with specific recognition sequences on the DNA.[12,13] The gel shift assay, or electrophoretic mobility shift assay, is easy to perform, requiring the incubation of a DNA fragment containing a consensus DNA binding site for a transcription factor with a cell extract containing the factors, and resolving the transcription factor—DNA complex on a nondenaturing polyacrylamide gel. Much of the data obtained for the collagenase promoter, as well as many other gene specific promoters, has been obtained using gel shift experiments. They are a simple method for visualizing many of the variety of transcription factors that bind to gene promoter regions, and so are appropriate for the identification of candidate factors that may be involved with transcriptional control.

The transcription factors are often provided by a crude extract prepared from the cells of interest. Preparation of extracts is viewed as a sampling of the factors that are controlling the transcription of the cells at the time that the extract was prepared. Thus, the population of various proteins that bind to a promoter region as visualized using gel shifts are thought to accurately represent the proteins that were controlling the promoter at the time of extract preparation. The identities of the DNA binding proteins are often inferred from the consensus binding sites that they bind to. Often, short double stranded oligonucleotides containing consensus binding sites for known transcription factors, whose sequences are present in the gene promoter being studied, are used to assay for their presence in the extract. Thus, factor binding to the promoter is often assumed based on gel shift results. Additional proof

of the identity of a particular factor binding to DNA can be obtained by additionally incubating an antibody preparation that will bind to a specific transcription factor. The protein-DNA complex visualized as the gel shift band becomes 'supershifted' when an antibody also binds the factor part of the complex.

Among the limitations of gel shifts is the lack of information they provide about which of these factors help to catalyze transcription, since the method is not a functional assay. Stated plainly, DNA binding is not the same as transcriptional activation. For example, the binding of the yeast transcription factor GCN4 to its binding site does not correlate with activation of genes.[14] This said, it has often been noted that increased DNA-binding is observed for certain key transcription factors in extracts prepared from cells that are transcribing a particular gene in greater amounts. In the case of collagenase, for example, treating cells with phorbol ester has been noted to increase the DNA binding activity of the AP-1 transcription factor. Many other factors in a variety of gene systems follow this pattern. How should we interpret these sort of results?

## Molecular Mechanisms of Transcriptional Activation

The fundamental problem is that the molecular mechanism of transcriptional regulation has not been established. It could be argued that the transcription field has gotten ahead of itself. The tools have been developed to identify many different transcription factors that regulate genes, but we do not understanding the molecular basis of how any of the factors help catalyze transcription. The presumption has been that the mechanism is complicated because of the large number of proteins that have been found to be involved with transcription. As time has gone by increasing complexity is ascribed to transcriptional mechanisms because a larger number of the factors have been identified. There is little doubt that a variety of proteins are required to get the job done, however, my personal bias is that there are simple principles at work that we have yet to decipher. Some of the best talent in the transcription field has been focusing on the role of so called 'general transcription factors' (GTFs), i.e., those factors required by all promoters to achieve the transcription of the gene.[7,8] Most of these workers are concentrating on the initiation of transcription because of the belief that a detailed understanding of how the general factors work together to initiate transcription will elucidate how EBPs work to alter transcription rates. The presumption by these workers is that these proteins interact with the components of the general transcription complex directly to effect changes in transcription rates. They argue that understanding the roles of the variety of proteins involved in the initiation complex is a prerequisite for understanding how enhancers work to activate transcription. Perhaps the mechanism for how enhancers work will simply follow as a consequence of this complete picture of general transcription factor function.

Data along this line has been presented. For example, one paper makes the claim that it provides "a general model for the mechanism of action of upstream regulatory factors."[15] The paper states that TFIID is a direct target for the enhancer binding protein ATF, and that their interaction results in an increase of transcription by facilitating assembly of a preinitiation complex. The data was largely obtained by DNA footprinting analysis. Chromatographic fractions containing the protein factors ATF (an enhancer factor) and TFIID (a GTF) are incubated with a DNA template that contained part of a gene promoter. After these factors have had a chance to bind to their specific recognition sequences on the DNA, the DNA is digested with an enzyme that will degrade DNA, called DNase. The DNA that has a factor bound to it will be protected from the digestion, whereas the surrounding DNA will be cleaved. The amount of DNase added is carefully titrated so that, on average, there is only one fairly random site of digestion for each DNA molecule. Afterwards, when the digested DNA is resolved on a DNA sequencing gel, a fairly uniform ladder of DNA fragments

appears, but wherever a factor was specifically bound to its consensus DNA recognition site there is a clear region in the ladder. The region of clearing in the otherwise fairly uniform DNA ladder is termed a 'footprint'. Both ATF and TFIID were shown to produce footprints over their recognition sequences. But the crucial experiment was obtained by mixing the two factors together and looking to see if the footprint obtained for the two together looked different than just the sum of the two individual footprints. In fact, the footprint was extended over the initiation site of transcription in the presence of the two factors! Thus, the investigators concluded that TFIID must be altered in its conformation by the presence of ATF to allow it to protect a larger portion of the promoter from DNase digestion. These data were taken as proof that enhancer binding proteins work by direction interaction with GTFs and facilitate the assembly of preinitiation complexes at the promoter.

Numerous reviews are available describing what is presently known about transcription initiation complexes.[7,8] I describe only a few of these ideas here. Biochemical fractionation of extracts and reconstitution in vitro transcription reactions has lead to the identification of the following general transcription factors (GTFs) required for initiation: TFIIA, -B, -D, -E, -F, -H. Rather than focusing on the limitations of the in vitro transcription system discussed previously, i.e., the lack of regulation from enhancer binding proteins that typically regulate transcription in vivo, workers studying GTFs were satisfied to make use of "an intrinsic ability to effect low levels of accurate transcription", that makes possible the study of transcription initiation mechanisms.[16]

The most widely accepted model for events at the initiation of transcription come from models surmised from the study of prokaryotic promoters.[17] Eukaryotic promoters are thought to operate in an analogous fashion to prokaryotic promoters. The steps in prokaryotic transcription, as outlined by the experts in the field, are 1) the formation of the preinitiation complex (PIC) by assembly of the general factors and RNA polymerase that are necessary for transcription, 2) activation of the PIC by DNA melting to separate the two DNA strands, and 3) initiation of transcription. Additional steps are required to complete transcription, these are 4) promoter clearance 5) elongation of the transcript, and 6) termination. The prokaryotic system has served as a model for an understanding of eukaryotic transcription mechanisms. The stated reason for a detailed isolation and characterization of all of the eukaryotic general transcription factors is that this information is necessary in order to understand promoter activation by enhancer elements.[7] Although the information that has been collected is quite interesting, and is certainly needed for a complete understanding of transcription, this effort has not yet yielded the expected answers to the question of how enhancers work. It may have been disappointing to find that all of the general transcription factors isolated and characterized work together as the functional equivalent of a σ factor in prokaryotes.[7]

The σ factors can be considered components of RNA polymerase in bacteria, and are required for the polymerase to initiate transcription. Several unique σ factors have been identified, and they have been shown to specify the transcription of different groups of promoters. How the σ factor works with other transcriptional regulators is currently being investigated. Studies of four different bacterial activators have shown that they all facilitate transcription at a step following the recruitment of polymerase to the promoter (nitrogen regulatory protein C-NtrC, N4 SSB, cyclic AMP-dependent activation protein-CAP, and phage lcI protein).[18] A key step in the regulation of bacterial promoters is when the activator joins the RNA polymerase with σ factor on the DNA and the promoter is converted from a closed state to an open state, i.e., PIC activation by DNA melting.[19]

How good of an analogy for eukaryotic transcriptional control is the prokaryotic model? If all of the GTFs function as a σ factor, is there evidence for variation in the GTFs that could result in some promoter specificity? One of the GTFs called TFIID recognizes common

start site signals for transcription, the TATA box and the initiator. TFIID is composed of TATA-binding protein (TBP), and TBP associated factors (TAFs). Multiple TAFs have been found to be associated with TBP, and there is now evidence that specific eukaryotic activators interact with subsets of these TAFs.[20] Although work on prokaryotes had indicated that the crucial step for controlling transcription rate is the conversion of the promoter complex from closed to open,[17] workers on eukaryotes suggest that the earlier first step of factor recruitment for PIC formation may be the controlling step.[20] Clearly those proposing analogous models of transcriptional regulation for prokaryotes and eukaryotes must still conduct additional studies to determine the extent to which the analogy holds.

## TFIIIC as a Prototypical Transcriptional Activator

Despite all that is now known about the GTFs that comprise the general transcription machinery, some knowledge of the enhancer binding proteins themselves is certainly required for understanding how they catalyze transcription. Although some well known laboratories have emphasized dissection of the functions of the general transcription machinery as a prerequisite for understanding gene specific transcription, at least part of the secret to how enhancer binding proteins work must be inherent in the properties of these factors themselves. In this, and the following section, I review my own work in the area of enhancer proteins. The significance of the findings to gene specific transcriptional regulation in general, and ultimately to how collagenase genes may be regulated is discussed.

In this section, I diverge to discuss class III gene regulation, before subsequent sections which describe class II genes, the class to which collagenase genes belong. RNA polymerase III (class III) gene transcription is more simply regulated with fewer components, as compared to RNA polymerase II transcription, which is responsible for the transcription of mRNAs. Most pol III genes, that code for small structural RNAs, such as the tRNA genes, require only two transcription factors, TFIIIB and TFIIIC, as well as RNA polymerase III.[21,22] TFIIIB contains the same factor, called TBP, that is contained in TFIID used for class II transcription,[23-25] so class II and class III genes share this factor, and may also share other peptides contained in the GTFs.

The key to understanding gene specific gene regulation seems to be in understanding how enhancer binding proteins work. So why diverge to discuss class III genes, which are generally not thought to be regulated through enhancers? I argue here that TFIIIC actually does function as an enhancer binding protein, and can provide a clear example of how such proteins work to catalyze transcription of a gene. To discuss the more current literature on TFIIIC we need to invoke the somewhat confusing nomenclature that appears in the literature. TFIIIC is comprised of several polypeptide chains, and some of these are present only some of the time. My argument is that at least one of these peptides from TFIIIC can be considered to function as an enhancer.

TFIIIC was biochemically fractionated into two components, termed TFIIIC1 and TFIIIC2, that are both required for activity.[26-28] In my own work, we showed that TFIIIC2 appears in two distinct forms in the cell, upper and lower band forms.[29] We showed that when class III gene transcription was increased, such as during adenovirus infection or during stimulation with serum, more of the upper band form was observed in DNA binding experiments (gel shifts) than the lower band form. It was latter shown that these two forms may also differ by a single subunit of 110 kD, termed TFIIICβ, that has now been cloned.[30] The inactive lower form (TFIIIC2b?) is presumably converted to the active upper form (TFIIIC2a?) by the addition of TFIIICβ.[30] Our paper was also the first to show that eukaryotic transcription factors are modulated in their transcription activity by phosphorylation, since dephosphorylation of the upper band form complex yielded the lower band form.[29] Our paper was referenced in an important review on the topic of transcription

factor modulation at that time.[31] Our results appeared in a good journal because of their editorial policy to cover all three gene systems, since lessons learned in the other simpler transcription systems are likely to teach us important principles about class II gene regulation as well. Unfortunately, because TFIIIC has not been recognized as a prototypical enhancer binding protein, the lesson that enhancer binding proteins are modulated in their activity by phosphorylation in response to signal transduction pathways may have not been fully appreciated from our early work. Important principles are illustrated in the figures of our papers, so I would like to review a few of these figures here.

Two of the figures are reproduced here.[29] Figure 5.1 shows the phenomenon we observed, that two distinct species of TFIIIC—DNA (containing the DNA consensus sequence for TFIIIC binding) complexes are observed depending upon the how the cells used for extraction preparation were treated. In slow growing cells only a lower band complex was observed (lane 1). When these cells were infected with a virus that we were studying (adenovirus), an upper band complex could also be observed (lane 2). In faster growing cells (stimulated by higher serum concentrations) both complexes were seen, but in these cells that were also infected with the virus only the upper band species was observed. Therefore, the presence of the upper band complex of TFIIIC increased in cells more actively transcribing pol III transcripts, faster growing cells or cells during virus infection.

The identity of the upper band complex as the transcriptionally active form of TFIIIC is shown in Figure 5.2. When extracts were fractionated on a phosphocellulose column and eluted with a salt gradient a clean separation of the two forms of TFIIIC could be achieved. Reconstituted in vitro transcription reactions conducted with these chromatographic fractions showed that the TFIIIC fractions that contained the upper band complex were transcriptionally active, whereas the TFIIIC that formed the lower band complex were not transcriptionally active (Fig. 5.2B). This makes a very important point, an enhancer binding protein can bind to its recognition site on DNA, but the complex may not have transcriptional activity. Over the last ten years many experiments using gel shift analysis have been conducted by researchers. Often the presence of a DNA-protein complex for an enhancer binding protein has been taken as evidence that a particular enhancer binding protein has activity in a certain cell extract. This methodology has been used as an important tool in discerning which enhancer binding proteins are controlling a gene of interest in a particular cell type and in response to certain stimuli. Yet Figure 5.2 clearly shows that specific binding to the DNA consensus sequence is not sufficient to ensure transcriptional activity; so binding of an inactive form of the enhancer binding protein does not enhance transcription of the gene. Some of the data on the collagenase gene has been obtained using gel shifts, and conclusions have been drawn about the activation of the gene based on the presence of DNA binding proteins present in particular cell extracts. The presumption has been that data showing unique proteins binding upstream of the collagenase gene must indicate that these proteins are indeed activating the gene. Yet, it was already clear from our experiments on TFIIIC that DNA binding does not indicate whether a gene is being activated.

The importance of utilizing a functional assay to evaluate transcription factor activity needs to be emphasized. The best proof that certain transcription factors are acting to enhance transcription is obtained by showing that when they are added back to in vitro transcription systems that they act to increase the amount of transcription. As already discussed, the pol II in vitro systems have not worked to show transcriptional activation by enhancer binding proteins, until perhaps recently in purified systems. In contrast, the pol III in vitro system allows dramatic response to its enhancer binding protein, TFIIIC. Therefore, the type of study we did early on is only now becoming possible for pol II genes with the advent of more purified systems where effects of enhancers are seen. In the years ahead many of the crucial experiments will be done that indicate the mechanism of how enhancer binding

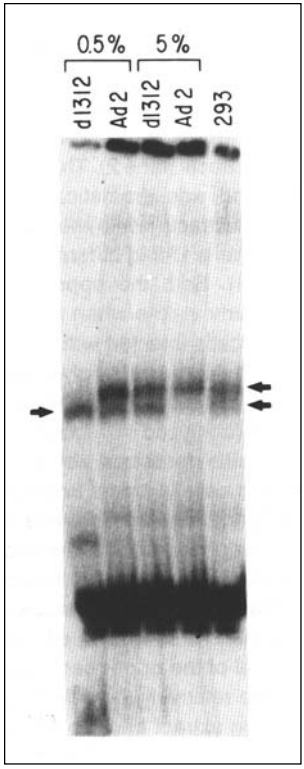


Fig. 5.1. Two Distinct Complexes for TFIIIC Binding its DNA Consensus Sequence. A $^{32}$P end–labeled fragment of the VA gene was incubated in a binding reaction with nuclear extracts prepared from cells maintained in either 0.5% or 5.0% serum and infected with either a control virus (dl312) that does not produce the viral oncogene capable of altering transcriptional activity, or the wild–type virus (Ad2). 293 cell nuclear extracts are also analyzed, since they are a cell line transformed by the viral oncogene. The specific TFIIIC–VA gene complexes are indicated by the arrows. More of the upper band complex is observed in cells more actively expressing RNA polymerase III gene transcripts, such as cells stimulated by higher serum concentrations or by cells infected by virus. (reprinted by permission of Cell Press).

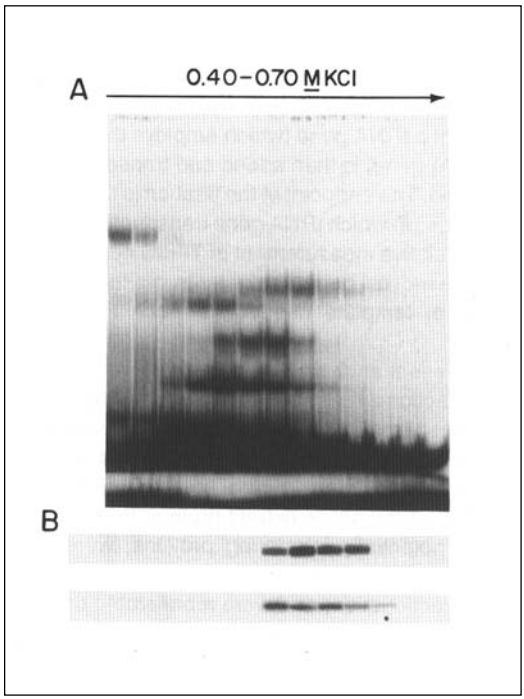


Fig. 5.2. The Upper Band Form of TFIIIC and Enhanced TFIIIC Transcriptional Activity Coelute on Phosphocellulase. Nuclear extracts that yield the two TFIIIC–DNA complexes were chromatographed on a phosphocellulose column. Fractions that eluted in a gradient between 0.40 and 0.70 M KCl were analyzed (A) by gel shift assay using a VA gene probe and (B) for TFIIIC activity in a reconstituted in vitro transcription reaction of the VA gene. (Figs. 5.1 and 5.2 appeared in the article "Activation of transcription factor TFIIIC by the Adenovirus E1A protein", Warren K. Hoeffler, Robert Kovelman, and Robert G. Roeder, 1988, Cell, 53, 907–920. and are reprinted by permission of Cell Press).

proteins work to catalyze transcription. I argue that we can anticipate the results by applying lessons learned in the pol III system.

The more recent identification of multiple subunits comprising TFIIIC leads to the question of whether a single subunit is responsible for the proposed enhancer function. If only one subunit is actually the functional equivalent of the enhancer, then the other subunits could still play the role of a GTF. In this regard TFIIIC1 (and perhaps TFIIICβ) may be a good candidate to be the enhancer, since its presence is required to reconstitute the high levels of expression we have come to expect from pol III genes. The categorization of transcription factors as being GTFs is based upon their need to achieve transcription in soluble systems. The basal level of transcription seen in vitro for pol II genes is very low and occurs in the absence of enhancer binding proteins. Reconstituted reactions, using some recombinant transcription factors and some purified fractions respond similarly to reactions using cruder cell extracts. I believe that the pol III system would also have a low level of expression without its enhancer binding protein during transcription in vitro. Thus, I postulate that TFIIIB (together with TFIIIC2?) and pol III would support transcription of class III tRNA genes in vitro. In support of this notion, earlier attempts to purify TFIIIB away from TFIIIC have always been frustrated. Notably, the level of transcription is roughly 100-fold greater in the presence of TFIIIC, but the residual transcription observed using even the cleanest TFIIIB and pol III preparations is still at the approximate level of transcription of class II genes in the in vitro systems. I argue that the high levels of in vitro transcription seen for pol III genes is the result of a functioning enhancer. This is why Pol III transcription in vitro is as much as 100-fold greater than pol II transcription, quantitated by the amount of gene specific transcript produced. Furthermore, early experiments indicated that pol III genes reinitiate transcription at their promoters, whereas pol II genes do not. I argue that the very phenomenon of reinitiation at the promoter is a characteristic of enhancer driven transcription. This important idea can serve to help ultimately dissect the mechanism of enhancer driven transcription. In current discussions on enhancer mechanisms reinitiation at the promoter has yet to be even considered a key property.

I recall from my own studies that very little DNA template is required for pol III transcription in the crude in vitro systems, but there is a need for nonspecific DNA to titrate out nonspecific DNA binding proteins. Less than one-tenth of the DNA is needed as a template. In comparison, much less of the template DNA can be substituted with nonspecific DNA for pol II gene transcription. This is further evidence of the high efficiency of pol III transcription as compared to pol II transcription, all due to the importance of reinitiation of transcription on active transcription templates. Calculating the number of transcripts produced in vitro from pol II genes as compared to pol III genes, the number becomes many times greater than 100-fold. I argue that this huge difference in transcription efficiency is due to the driving of pol III transcription by its enhancer, the somewhat mislabeled TFIIIC. I believe the evidence is good that TFIIIC contains an enhancer protein that actively functions in vitro.

Early on I believed in the applicability of pol III results to the pol II gene regulation system. Perhaps a common theme in many enhancer binding proteins is the presence of two populations of enhancer binding proteins, one inactive for transcription and the other active. These forms might be interconvertable by phosphorylation, as shown first in the pol III system.[29] During the next phase of my career I sought to find out if these principles would indeed hold up for pol II enhancer binding proteins.

## Enhancer Binding Protein AP-1 (aka Jun/Fos)

Therefore, I switched systems to the best characterized pol II enhancer binding protein for eukaryotes, AP-1 aka Jun/Fos. The Jun/Fos binding site in the collagenase I gene is thought

to be the primary regulator of collagenase. For me at the time, one advantage of studying Jun was that the gene sequence was already known, therefore the amino acid sites of phosphorylation could be mapped. I embarked on this endeavor at Genentech in Arthur D. Levinson's laboratory with the assistance of William Kohr from the protein chemistry department. We found one main site of phosphorylation in c-Jun at Ser 73, our data also showed a second more minor site that we did not characterize (latter shown to be at Ser 63). I presented our data at the Keystone conference in January 1991. The abstract is insightful considering the date of presentation, and is shown below:

MAPPING A PHORBOL ESTER RESPONSIVE PHOSPHORYLATION SITE ON C-JUN: IMPLICATIONS FOR V-JUN ONCOGENICITY.

*Warren K. Hoeffler, and Arthur D. Levinson, Department of Cell Genetics, Genetech, Inc., South San Francisco, CA*

C-Jun is a cellular transcription factor known to mediate the regulation of gene expression in response to phorbol esters. Its viral homologue, v-jun, bears close structural similarity except for the deletion of 27 amino acids. To assess the function of the deleted region, human 293 cells were transfected transiently with expression vectors encoding c-jun, v-jun, or delta c-jun ( a deletion mutant of the 27 amino acid region) with or without c-fos. The deletion resulted in more efficient heterodimer formation of jun with the fos protein. Additionally, the following responses of the heterodimer to phorbol ester treatment were impaired: increased binding to the consensus binding site on DNA, subsequent increased transcription from a promoter containing these sites, and increased phosphorylation of Jun. A phorbol ester inducible phosphorylation site on c-Jun was mapped to Serine 73, adjacent to the region deleted in v-Jun. We propose that the 27 amino acid region modulates the response of c-Jun to phorbol ester ( and presumably other signals) by regulating phosphorylation of Serine 73, and the deletion of this region, as occurs in v-Jun, impairs its ability to respond to the signaling pathway.

J. Cellular Biochemistry, Keystone Symposia on Molecular and Cellular Biology, January 18-25, 1991. Reprinted by permission.

I personally presented our poster to a group of scientists that subsequently beat us to the publication of these results,[32] although our group was clearly first in identifying the phosphorylation of Ser73 as an important regulatory site in controlling c-Jun transcriptional activity. Figure 5.3 shows our mapping of the phosphorylations sites by sequential Edman degradations of a peptide fragment derived from in vivo labeled c-Jun. The main peak is at Serine 73, but the minor peak at Serine 63 is also visible. I was not able to publish our results even though they were submitted prior to the articles that were published, even after multiple submissions. I realize most of us have had this experience.

A few years later, at Stanford, we were able to publish some of the data on Jun that had not yet been published by others. The question addressed was whether the substitution of either an acidic or neutral amino acid for Serine 73 would alter the transcriptional activity of Jun. In some enzyme systems an acidic residue, such as aspartic acid, can be substituted for a phosphorylated residue resulting in an increase in activity, presumably due to the presence of a net negative charge at the residue. Likewise, substitution of an uncharged amino acid, such as alanine, can respond as the functional equivalent of an unphosphorylated residue. I tested whether any alteration in activity could be observed with these amino acid substitutions. The following figures reproduced from our paper show a trend toward increased Jun transcriptional activity in the construct mimicking the phosphorylated serine and decreased activity in the construct mimicking the unphosphorylated serine. Shown in Figure 5.4 is the transfection of expression vectors for either the wild-type c-Jun or the amino acid substitutions at 73, along with a Jun/Fos responsive reporter gene.

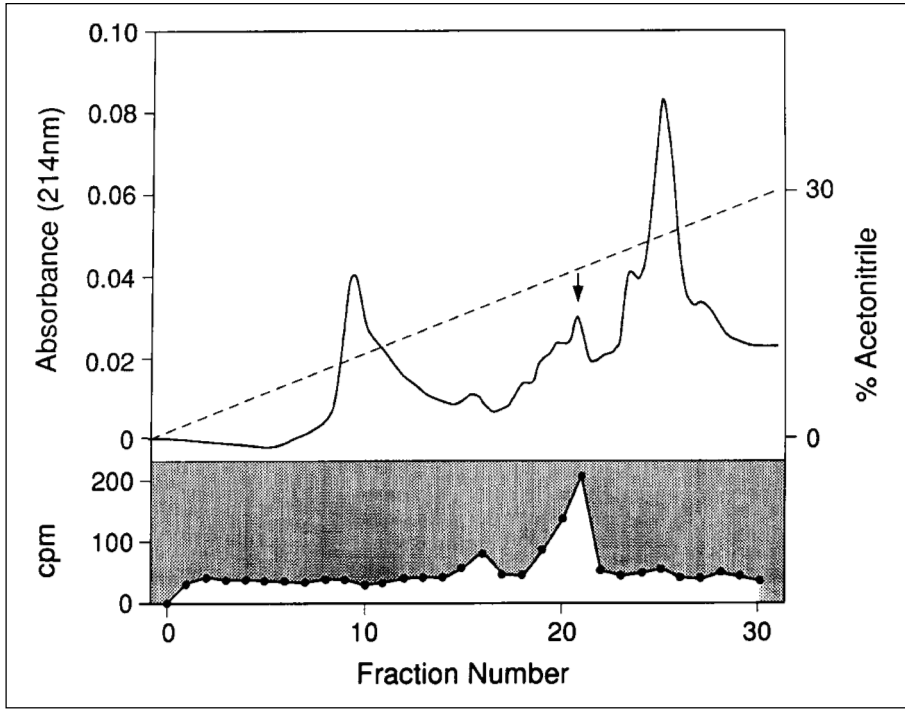


Fig. 5.3. In Vivo $^{32}$P–Labeling of C–Jun and Resolution of Two Proteolytic Fragments Containing Phosphorylated Residues. Cells were labeled in vivo with $^{32}$P inorganic phosphate in the presence of 50 mM TPA for 2 hr. Labeled C–Jun was immunoprecipitated using Jun antisera, and digested with the endopeptidase lysine C. Proteolytic fragments were resolved on a hydrophobic HPLC column. The top part of the figure shows an U.V. absorbance tracing of the material eluted with a linear gradient of acetonitrile. Individual fractions were collected, counted in a scintillation counter, and are plotted on the second curve shown below. The arrow marks the position of the major peak of radioactivity eluted from the column that was further characterized and shown to be due to phosphorylation at Serine 73. A second smaller peak was also seen, was not characterized, and is presumably due to phosphorylation at Serine 63.

It would be more ideal to study the effects of amino acid substitutions in the transcriptional activation domain in a system where no background binding of endogenous transcription factor interferes with assessing the performance of the mutants. Therefore I fused the N-terminal transcription activation domains of the mutants to the DNA-binding domain of the bovine papilloma virus E2 protein. There is no endogenous DNA-binding protein in the cells tested that specifically binds the E2 protein, therefore all of the DNA-binding and transcriptional activities observed come from the mutants tested. Figure 5.5A diagrams the placement of the alanine (A) and aspartic acid (D) substitution for Serine 73 (S) within the transcriptional activation domain A1 of c-Jun. Gel shift extracts prepared from cells transfected with these constructs are shown in Figure 5.5B. This experiment shows a potentially significant effect. The complex is seen as a doublet, with both upper and lower band species reminiscent of the complexes seen for TFIIIC earlier in my studies.

Could the presence of two distinct complexes be indicative of transcriptionally active and inactive complexes, as seen earlier for TFIIIC? If amino acid substitutions at Serine 73

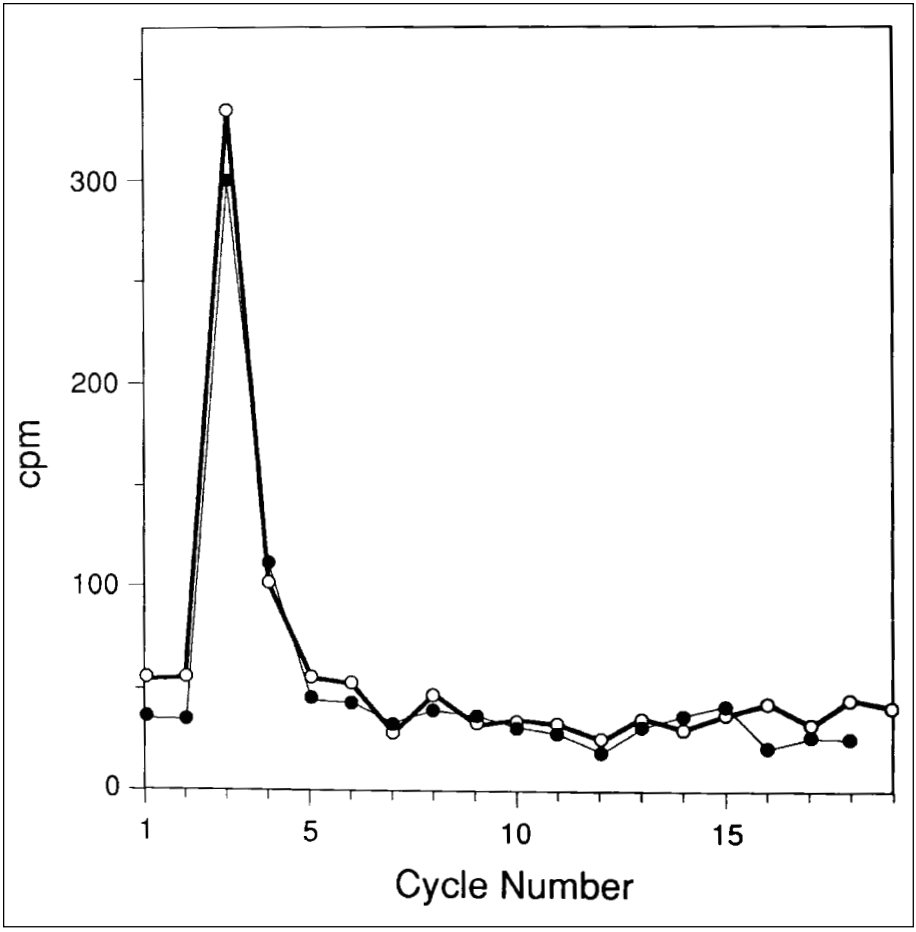


Fig. 5.4. Phosphorylation at Serine 73 Identified by Amino Acid Sequence Analysis. The three fractions comprising the peak of activity were pooled and used for amino acid sequence analysis. Protein in the peak fractions were coupled to a solid support and Edman degradation was conducted from the N–terminal end. Each cycle of degradation was collected in a single fraction, and counted in a scintillation counter. Two repetitions of the procedure are plotted on the same axis. A phosphorylated residue eluted on the third cycle. Of the possible proteolytic fragments generated, Serine 73 is the only residue at the third position from the N–terminal end that is a target for phosphorylation, contained within the following peptide: LASPELERLIIQSSNGHITTTPTPTQFLCPK. (Figs. 5.3 and 5.4 from Warren K. Hoeffler, William Kohr, and Arthur D. Levinson, unpublished data)

can mimic dephosphorylation or phosphorylation that control, in part, the transcriptional activity of Jun, then these substitutions may alter the proportion of upper to lower band species. The substitution of an alanine at 73 did not alter the proportion of the doublet bands, but the substitution of an aspartic acid residue results in predominantly the upper band complex (Fig. 5.5B, marked with the arrow). The presence of an additional Serine at 63 that is also phosphorylated in response to signal transduction may complicate the results. An alanine mutant at 73 in Jun may still be modulated by phosphorylation at Serine

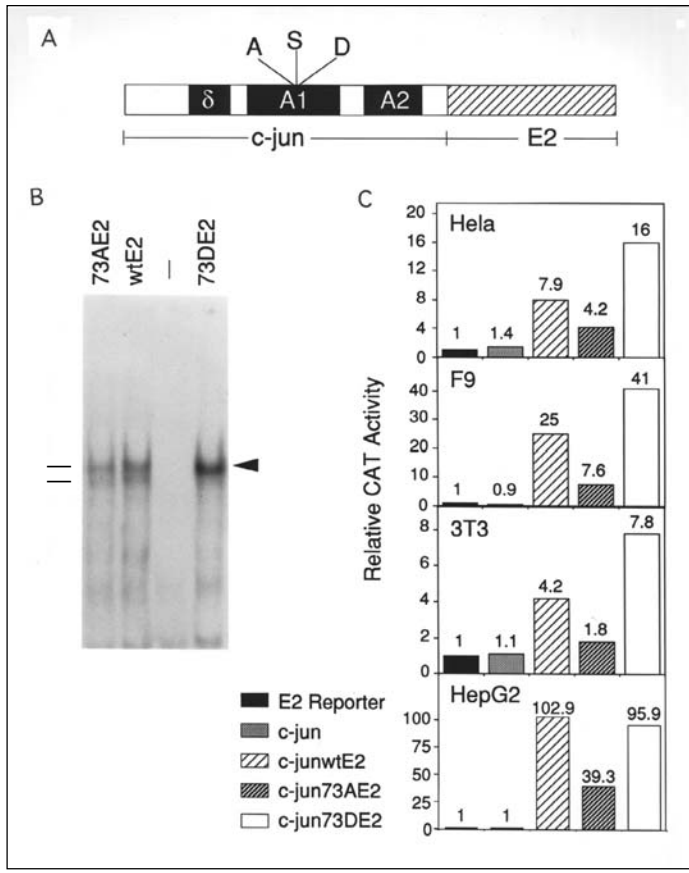


Fig. 5.5. An Upper Band Form of cJun/BPV–E2 fusion protein correlates with transcriptional activity. (a) Diagram of c–Jun/BPV–E2 fusion proteins. The N–terminal of c–Jun containing both activation domains A1 and A2 and either the wild–type serine at position 73 or alanine or aspartic acid substitutions, were fused to the DNA–binding region of the BPV–E2 protein. (b) Upper band complex of c–Jun/BPVE2 fusion protein bound to its DNA consensus site is increased when the c–Jun portion contains a single amino acid substitution of aspartic acid at position 73. Gel shift assays were conducted using labeled oligonucleotide containing the DNA binding site homology for the BPV–E2 protein, and were incubated in whole cell extracts prepared from cells either not transfected (lane–) or transiently expressing fusion proteins. The available upper band DNA–binding activity for the fusion protein either containing c–Jun (lane wtE2) or expressing the amino acid substitutions (lanes 73AE2 and 73DE2) was the greatest for the aspartic acid substitution (lane73DE2). (c) Relative transcriptional activity of c–Jun/BPV–E2 fusion proteins. Hela, F9, 3T3, and HepG2 cells were transiently transfected with 2 μg CAT (chloramphenicol acetyltransferase) reporter gene construct alone, or with 2 μg of c–Jun or Jun/BPV–E2 expression vectors. E2CAT, which contains an E2 DNA–binding site was cotransfected to measure the transcriptional activation by each of the c–Jun constructs. CAT expression is shown as counts per minute of benzene–extractable [$^3$H]–mono–acetylated chloramphenicol per μg extract protein. The data are the average of three transfections each done in duplicate.

63, causing no substantial change in the proportion of upper and lower bands in this mutant. The transcriptional activation by the upper and lower bands cannot be easily assayed, since in vitro transcription systems that respond to the binding of enhancer proteins reproducibly are not commonly available. In the case of TFIIIC, transcriptional activity corresponded with only the upper band complex, whereas fractions containing the lower band complex had no activity. What is clear for Jun is that there is a trend toward lower transcrip-

tional activity for Jun with an alanine at 73, and a trend toward increased activity with an aspartic acid at 73 in reporter gene assays (Fig. 5.5C).

My interpretation of these results, and other so far unpublished experiments of mine, is that the presence of upper and lower band complexes is a common property of many enhancer binding proteins. The modulation of their transcriptional activities is often mediated through phosphorylations within their transcriptional activation domains. Besides the documentation of transcriptional activation differences mediated through phosphorylations at specific sites in c-Jun, there are also now other well documented examples for other transcription factors.

## Other Influences on Transcriptional Control

More recent work from a variety of laboratories has continued to build upon the hypothesis that EBPs operate to increase transcription by direct interaction with GTFs, by showing which specific GTF is the primary target of this interaction. The conclusions of many of these papers is that the EBPs target the TFIIB assembly step.[33-36] Yet, if the EBP did facilitate TFIIB assembly into the PIC then it would be expected that increased concentrations of TFIIB would decrease the effects of EBPs, but it does not.[35] The problem with these experiments is that they are frequently conducted with crude cell extracts that are depleted in one or more specific individual factors, rather than being conducted with purified fractions of each of the individual factors. Currently, a completely reconstituted in vitro transcription system from purified factors is rarely achieved, and is not therefore available for the multiple experimental perturbations required to test the dominant paradigm of how EBPs work. A role for TAFs in these experiments was hypothesized because of the observation that higher levels of activation were achieved when the preincubation experiments were conducted in the crude extracts as compared to when more purified preparation of the GTFs were used.[36] RNA polymerase has been shown to be the next component to join the preinitiation complex, right after TFIID and TFIIB.[37-39]

Although the traditional view of the GTFs is that they sequentially join in the assembly of a complex at the gene promoter, new data suggests that RNA polymerase is precomplexed with the GTFs before being added to the promoter.[40,43] If this model holds up, then the GTFs should be seen analogously to the σ factors of bacterial polymerases. The mechanism of how EBPs work to enhance transcription would then most likely require the recruitment of the complete complex to the promoter, rather than just effecting the recruitment of a single component.

To see the effects of enhancer binding proteins on the in vitro transcription of a gene it may be generally necessary to provide a closed circular plasmid. Interestingly, the de facto standard for template conformation used in experiments designed to see the effects of enhancer binding proteins has been closed circular DNA templates, particularly supercoiled preparations. Detection of the transcripts generated in these assays is usually conducted by primer extension analysis.[44,45] However, certain researchers have also used linearized promoter fragments, and could still see increases in transcription dependent upon enhancer binding proteins.[11]

Many groups have noted that the multimerization of enhancer binding sites results in further increases in the amount of transcription, indicating additive effects. Cooperativity has been proposed in the assembly of multiple enhancer binding proteins that together work to increase transcription.[37] Multiple factors work together in a complex, and cooperativity in the assembly of these complexes can lead to greater than additive effects when bringing together multiple enhancer binding proteins.

Other recent findings in the transcription field are also likely to contribute to our understanding of transcription regulatory mechanisms in the future. One of these is the in-

creasing number of instances where gene transcription and DNA replication share common components. An early example was the finding that the transcription factor CTF (CCAAT-binding transcription factor) is the same protein as NF-1, a cellular factor that is required for DNA replication of adenovirus.[46] The recent observation that one of the GTFs is both a transcription factor and a DNA replication factor, TFIIH, is also of interest. Although the function of TFIIH is not yet fully understood, it binds to damaged regions in the DNA to facilitate their repair.[47]

Another level of control of gene expression is due to effects of the packaging of genes into regions of chromosomal structure. Classically the regular arrangement of nucleosomes on DNA, and its remodeling in regions of transcriptional activity, has been detected as sites of DNase I hypersensitivity.[48,49] The regular arrangement of DNase hypersensitive sites (DHS) along chromosomal DNA reflects the 200-215 bp repeat length of nucleosomes. Notably, in vivo the promoter regions of genes being actively transcribed contain DHS, indicative of an absence of nucleosomes in these regions. The packaging of DNA with nucleosomes precludes the binding of key factors required for active gene transcription, and overcoming this inhibition is another level for the control of gene transcription.[50]

## Summary

Much of the phenomena of gene regulation is controlled at the transcriptional level. Thus, the synthesis of mRNA from the DNA template is the primary control point determining which regions of the DNA will be expressed as proteins in any given cell. RNA polymerase, the enzyme that catalyzes this synthesis requires a variety of protein cofactors in order to accomplish this task. One set of these factors interact with RNA polymerase largely right at the initiation site of transcription, the so called 'general transcription factors' or GTFs. Participation of the GTFs is required for transcription of all genes. Yet since the set of GTFs utilized is the same, or similar, for most genes it is unlikely that these factors provide the major control point for gene expression.

A larger and more diverse set of proteins is most likely responsible for providing the control of transcription, and can be referred to collectively as enhancer binding proteins. These proteins bind to a region in the DNA just 5', or upstream, of the coding region, that is called the promoter. The DNA binding locations of the enhancer binding proteins (EBPs) can also be far away for the promoter, with thousands of bases of DNA on either side of the promoter site being possible. For the collagenase I gene, several EBPs have been identified, including Jun/Fos, PEA3, CdxA, and C/EBP. By preparing extracts that contain solubilized transcription factors from cells that express collagenases we can assay for the presence of EBPs that may be controlling collagenase expression in vivo. Yet current methodology does not allow us to determine which of the EBPs are activating transcription of a gene of interest, such as the collagenase I gene. Ideally, we would like to utilize a functional assay to evaluate the role of each of the EBPs in controlling collagenase gene expression. Unfortunately, the available functional assay, the in vitro transcription reaction does not generally show any effect of EBPs on transcription, and the extracts used in these assays are typically inactive when prepared from the variety of cell types that normally express collagenase. DNA binding assays often indicate increased binding of EBPs to their DNA consensus sites in extracts prepared from cell types that express collagenase, but we cannot interpret the meaning of these results. The binding of EBPs to DNA does not guarantee their role in transcriptional activation, and we currently do not have an appreciation of how multiple upstream EBP sites work together to control transcription.

However, I believe that in the near future we will be able to decipher how the availability of certain EBPs work together to regulate the transcription of genes. There is good reason for optimism. Work on the GTFs has advanced to the point that most of these factors

have been cloned, leading to the possibility of the reconstitution of in vitro transcription reactions using clean components. Since many of the EBPs have also been cloned, adding them to the reactions should allow the study of the mechanism of transcriptional activation in vitro. Likewise the more complex interaction of multiple EBPs working together to activate transcription should also be dissectable in the new reactions. No doubt, a breakthrough is needed in our understanding of transcriptional activators. Once the basic mechanism of transcriptional activation is understood investigators will finally have the powerful tool that seemed so close at hand earlier on. We will understand exactly which EBPs are turning collagenase, and other genes, on and off in response to physiological conditions.

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CHAPTER 6

# Activation and Induction of Collagenases

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## Introduction

Matrix metalloproteinases (MMPs) play an essential role in extracellular matrix (ECM) remodeling that occurs under a variety of physiological and pathological conditions such as embryonal development, inflammation and tumorigenesis. So far 15 distinct members of the MMP family have been identified by cDNA cloning and sequencing. The MMP family can be subdivided into four subclasses based on their substrate specificity: (i) the collagenases: interstitial collagenase (MMP-1) (see chapter 1), neutrophil collagenase (MMP-8) (see chapter 2), and collagenase 3 (MMP-13) (see chapter 3), with a substrate preference for native fibrillar collagen; (ii) the gelatinases: 72 kDa (MMP-2) and 92 kDa (MMP-9) gelatinases, with a preference for type IV collagen and denatured collagen (gelatin); (iii) the stromelysins: stromelysin-1 (MMP-3), -2 (MMP-10), and matrilysin (MMP-7), recognizing a wide variety of ECM substrates, such as proteoglycans, laminin or fibronectin; and (iv) metalloelastase (MMP-12), with a preference for elastin.[1,2] In addition to the above, a group of integral plasma membrane MMPs, termed membrane-type MMPs (MT-MMPs), has recently been identified. The MT-MMP subgroup so far includes MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), and MT4-MMP (MMP-17). MT1-MMP is believed to be an important activator of 72 kDa gelatinase.[3] The MT-MMPs do not fall conveniently into the above categories, but stromelysin-3 and MT-MMPs contain a furin-type protease recognition sequence, RXXR, and are activated prior to secretion or expression at the plasma membrane, respectively.[4,5] Thus, stromelysin-3 and MT-MMPs can be subclassified as furin-activated, RXXR-containing MMPs.[2] An additional member of the MMP family (MMP-18), of unknown substrate specificity, has been recently identified.[6]

All currently known members of the MMP gene family share the characteristic that they are synthesized and secreted as inactive precursors, except for the furin-activated MT-MMPs and stromelysin-3, which are activated intracellularly as mentioned above. Hence, extracellular activation of the proenzymes is one of the major regulatory mechanisms responsible for activity of most MMPs, including interstitial collagenases, which is the focus of this review. This extracellular activation involves several MMPs and serine proteases in the form of a cascade, and is further influenced by the presence of specific tissue inhibitors of metalloproteinases (TIMPs). In addition to activation, production of MMPs is highly regulated at the transcriptional level as a result of the action of several different growth factors, cytokines, and other mediators, and increased production usually leads to enhanced

*Collagenases*, edited by Warren Hoeffler. 

extracellular MMP activity. Regulation of MMP production, as well as the synthesis of MMP-activating proteases and TIMPs, usually requires paracrine or direct cell-cell interactions. EMMPRIN is one of the key factors mediating such cell-cell interactions, especially between cancer and stromal cells during tumorigenesis.[7] The first part of this chapter deals with mechanisms by which collagenases, especially interstitial collagenase, are activated. The second part deals with stimulation of collagenase production via cell-cell interactions, with special reference to EMMPRIN.

## Procollagenase Activation

### *An Overview*

The existence of a latent form of tadpole collagenase, presumably a zymogen, was first proposed by Harper, Bloch, and Gross.[8] Evidence for the presence of a zymogen activator in the same culure media was provided shortly thereafter.[9] Studies on the collagenase precursors rapidly progressed after the discovery that trypsin[10] and organomercurial compounds[11,12] activate latent collagenase in vitro. Purification of latent human interstitial collagenase,[13] analysis of molecular changes during activation,[14] and the cloning and sequencing of the collagenase gene[15] proved the existence of a procollagenase zymogen. In particular, the deduced primary structure of collagenase resolved many of the questions pertaining to acceptance of latent collagenase as a zymogen. Subsequently, intramolecular cleavage sites during activation of procollagenase, the nature of the activation process, and the possible structure surrounding the putative active site which is conserved among all MMPs were clarified and the "cysteine switch" mechanism was proposed for activation of interstitial procollagenase and other MMPs.[16,17] These issues will be discussed in more detail in the following sections.

### *Domain Structure of Procollagenase and the Structural Basis for Latency*

Human interstitial collagenase is synthesized as a preproenzyme of 469 amino acids, of which the first 19 are the signal peptide[15](see chapter 1). After removal of the signal peptide, collagenase is converted to the proenzyme with a molecular mass of approximately 52 kDa. Analysis of the primary structure of cloned MMPs demonstrates that these proteins contain several distinct domains that are conserved in various different combinations among the members of the MMP family.[18] Interstitial procollagenase consists of three such domains (Fig. 6.1). The N-terminal domain is the 'pro' domain of approximately 80 amino acid residues, which is autolytically cleaved subsequent to activation, leading to a reduction in molecular mass of approximately 10 kDa. This domain contains a highly conserved sequence surrounding Cys[73]. The central portion of the enzyme, with an approximate molecular mass of 19 kDa, is the catalytic domain and contains conserved histidine residues that complex the active-site zinc atom. In addition to the catalytic zinc, there is a second zinc ion and a calcium ion which play a major role in stabilizing the tertiary structure of collagenase, which is comprised of a five-stranded β-sheet, two bridging loops, and two α-helices.[19] The C-terminal domain is referred to as the hemopexin domain because of its sequence similarity to this heme-binding protein, and is linked via a short hinge sequence motif to the catalytic domain. The hemopexin domain is involved in substrate specificity and is thus critical for collagenolytic (triple helicase) activity.[20,21]

As mentioned above, the amino acid sequence surrounding the conserved Cys of the 'pro' domain (PRCGVPDV) and that surrounding the zinc-binding histidines (HEXGHXXGXXHS) are highly conserved in all of the MMPs (Fig. 6.1).[18] X-ray crystallographic analysis of the catalytic domain has revealed that the conserved histidine-containing region constitutes the active site, and is arranged in a similar way to the active sites of bacterial thermolysin and crayfish astacin despite a lack of primary sequence homology.[19,22]

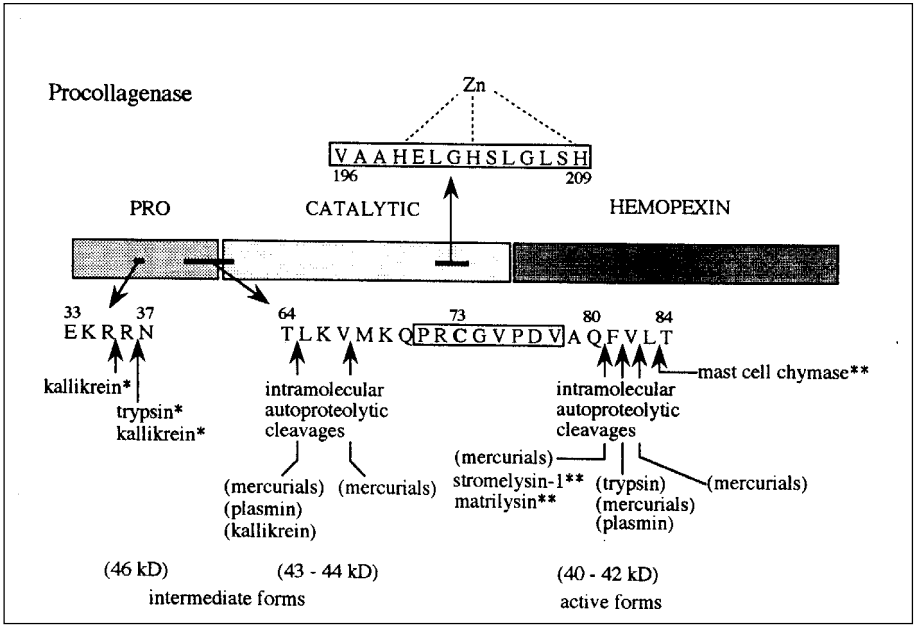


Fig. 6.1. Domain structure of interstitial procollagenase and intramolecular cleavage sites during activation. Amino acid sequences that are highly conserved among the members of the MMP family are designated by boxes. Pro, catalytic and hemopexin are the names of domains. *Trypsin and kallikrein directly cleave procollagenase within the 'pro'domain. **Stromelysin-1, matrilysin and mast cell chymase are reported to cleave procollagenase directly, between $Gln^{80}$ and $Phe^{81}$, between $Gln^{80}$ and $Phe^{81}$, and between $Leu^{83}$ and $Thr^{84}$, respectively.

It has been speculated that the 'pro' domain of the proenzyme may be folded such that the sulfhydryl group of the conserved $Cys^{73}$ residue forms a complex with the active-site zinc molecule.[17] This folded 'pro' domain would cover the active site and render the zymogen latent.[23]

### *Procollagenase Activators*

A variety of agents or stimuli are capable of activating interstitial procollagenase. These include: (i) proteases; (ii) organomercurials; (iii) conformational perturbants, such as sodium dodecyl sulfate (SDS)[24] and chaotropic ions (SCN-);[25] (iv) disulfide compounds, such as oxidized glutathione; sulfhydryl alkylating agents such as N-ethylmaleimide; and oxidants such as NaOCl,[16] and (v) endogenous nonenzymatic activators.[26] Activation occurs on modification, exposure, or proteolytic release of the $Cys^{73}$ residue from the active-site zinc atom, with the concomitant exposure of the active site (Fig. 6.2). Of the known activators, the best characterized are trypsin and the organomercurials.

### *Activation of Procollagenase by Organomercurials*

The most commonly used organomercurials include 4-aminophenylmercuric acetate (APMA),[12] sodium o-[(3-hydroxymercuri-2-methoxypropyl)carbamoyl] phenoxyacetate (mersalyl),[27] phenylmercuric chloride (PMC), p-hydroxymercuribenzoate (pHMB),[28] and p-chloromercuribenzoate (PCMB).[16] These organomercurials efficiently activate not only

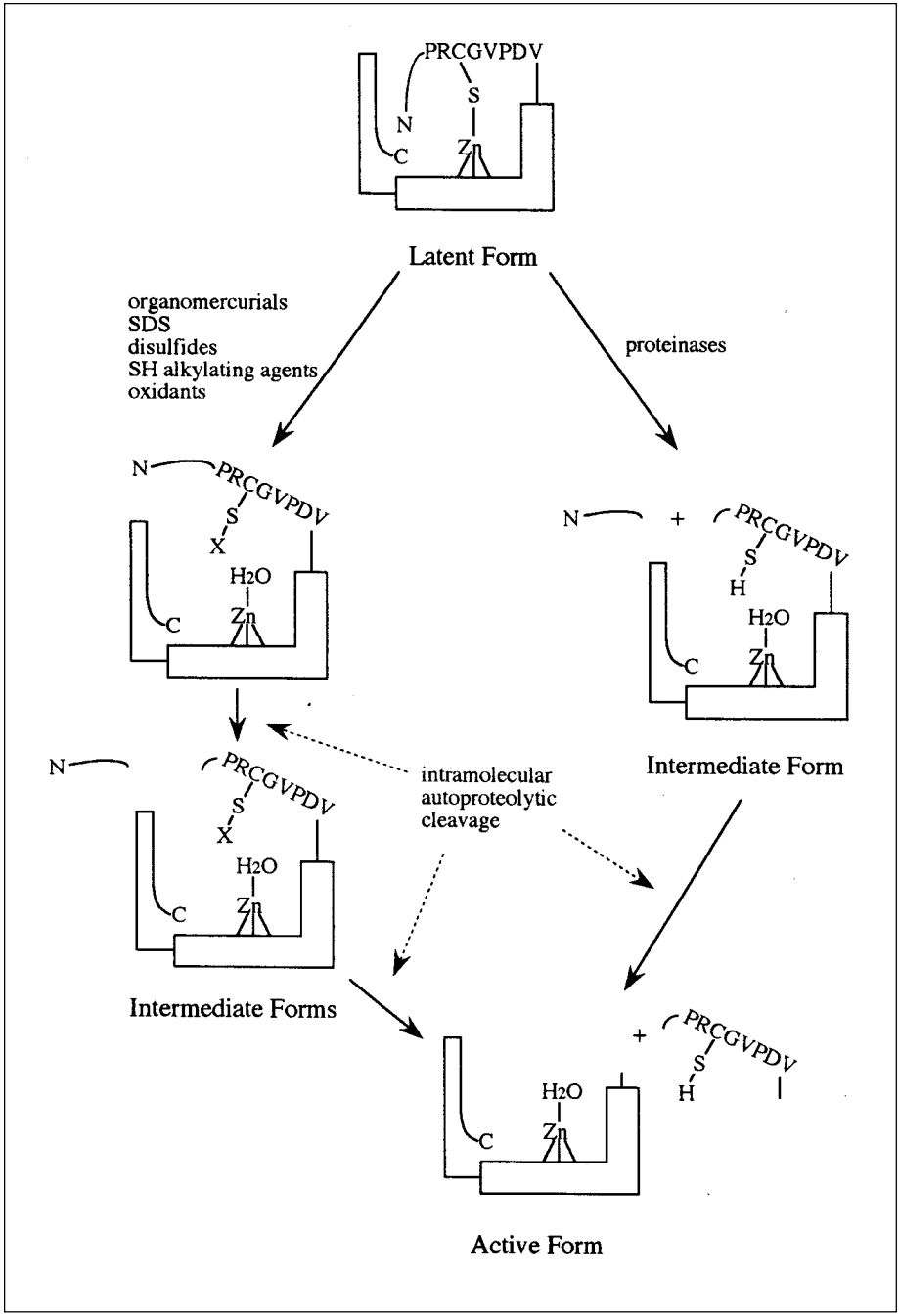


Fig. 6.2. Schematic representation of activation of procollagenase.

interstitial collagenase but also other members of the MMP family, including stromelysins, gelatinases and matrilysin.[29]

Activation of interstitial procollagenase (52 kDa) with organomercurials rapidly produces a 43-44 kDa intermediate form which is then converted to a product with a molecular mass of 42 kDa, which is the stable active form (Fig. 6.1).[14,28,30,31] The intermediate form is produced by cleavage between $Thr^{64}$ and $Leu^{65}$ or between $Val^{67}$ and $Met^{68}$ and the final 42 kDa activation product results from cleavage between $Gln^{80}$ and Phe,[81] between $Phe^{81}$ and Val,[82] or between $Val^{82}$ and $Leu^{83}$.[28,30] An important observation regarding this organomercurial-induced activation is that a very significant amount of collagenolytic activity is generated by several organomercurials prior to any discernible loss of molecular weight.[14,28] In addition, the subsequent cleavages appear to be autocatalytic in nature because of the following observations: (i) the continuing presence of organomercurials is critical for the entire activation process;[14] (ii) the reaction is independent of procollagenase concentration or the presence of substrates;[14,30] (iii) the addition of trypsin-activated collagenase does not enhance the activation process and the newly activated collagenase is unable to perpetuate the activating process.[14]

The organomercurial-induced activating process of procollagenase is inhibited by tissue inhibitor of metalloproteinases-2 (TIMP-2).[31] During the organomercurial-induced activation of 92 kDa gelatinase, TIMP-1 complexed with progelatinase inhibits the conversion of the intermediate form (83 kDa) to the active form of 67 kDa, but formation of the intermediate form from the proenzyme is not blocked.[32] These results suggest that formation of the intermediate form from the proenzyme and the subsequent conversion of this intermediate form to an active enzyme are somehow different from each other although both processes are supposed to be due to intramolecular proteolytic cleavages.

### *Activation of Procollagenase by Trypsin*

Trypsin-induced activation of procollagenase involves an initial cleavage between $Arg^{36}$ and Asn,[37] immediately after the triplet of basic amino acids, $K^{34}RR$ (Fig. 6.1), generating a major intermediate form of 46 kDa.[28] This intermediate form is then converted to a stable 42 kDa active enzyme by cleavage between $Phe^{81}$ and $Val^{82}$ While formation of the 46 kDa intermediate form is not affected by metalloproteinase inhibitors, EDTA or o-phenanthroline, subsequent conversion to the 42 kDa active form is completely inhibited by these reagents.[28] Moreover, the conversion of the intermediate form to the 42 kDa species does not require trypsin activity, cannot be blocked by serine protease inhibitors, and is independent of the initial procollagenase concentration.[14,28] These lines of evidence suggest that the proteolytic cleavage responsible for production of the 42 kDa stable active enzyme species is an autocatalytic reaction requiring initial activation by trypsin.

Procollagenase is activated in the similar manner also by other serine proteases such as plasmin and plasma kallikrein.[30,33] Plasma kallikrein causes initial cleavage in or after the same triplet of basic amino acids as does trypsin, i.e. between $Arg^{35}$ and $Arg^{36}$ and between $Arg^{36}$ and Asn,[37] the latter is identical to the trypsin cleavage site.[30] The aminoterminal sequence of plasmin-activated 42 kDa collagenase is identical to that of the trypsin-activated enzyme.[33]

Trypsin can also efficiently activate stromelysin, but gelatinases are poor substrates for trypsin-mediated activation.[29] While stromelysin has a triplet of basic amino acids ($R^{36}RK$) in the 'pro' domain, like interstitial collagenase, gelatinases lack such a triplet.

### *Activation Mechanisms*

As described above, the activation process for interstitial collagenase consists of a two-step reaction. The first step involves activation of procollagenase either by proteolytic cleavage

(trypsin) or conformational change (organomercurials). The second step is an intramolecular autoproteolytic reaction leading to formation of the stable active enzyme.[28]

In procollagenase the sulfhydryl group of $Cys^{73}$ is coordinated with the catalytic zinc atom in a manner that occludes the active site and renders the enzyme latent. Organomercurials convert the $Cys^{73}$ sulfhydryl group to a species that cannot serve as a ligand for the zinc atom, thus causing release of $Cys^{73}$ from the zinc atom with the concomitant formation of a catalytically competent active site (Fig. 6.2).[16] In this catalytically active form, the fourth coordination site of the zinc atom is occupied by water and the substrate binding site becomes accessible. This rearrangement precedes the intramolecular autoproteolytic reaction. The organomercurial-induced active forms of interstitial collagenase,[30] 72 kDa gelatinase,[34] stromelysin,[35] and matrilysin,[36] lack the N-terminal propeptides containing the conserved PRCGVPDV sequence, due to autoproteolytic cleavage of peptide bonds at positions two to four amino acids downstream from this sequence. However, this removal of the conserved Cys residue itself is not critical for its permanent dissociation from the zinc atom since the organomercurial-induced active form of 92 kDa gelatinase retains the conserved Cys.[32]

Cleavage of interstitial collagenase within the 'pro' domain by trypsin leads to removal of the polypeptide. This cleavage also modifies the network of secondary structures in the conserved region surrounding $Cys^{73}$ and causes its dissociation from the active-site zinc atom (Fig. 6.2).[29] Alteration of noncysteine residues in this conserved region in stromelysin leads to spontaneous activation of the zymogen,[37] confirming that the secondary structure in this region is essential to maintain MMPs as inactive zymogens. Taken together, dissociation of $Cys^{73}$ from the zinc atom by any means exposes the active site, acting as a "switch" that leads to activation (the "cysteine switch" model).

Although both organomercurial- and trypsin-induced activation cause formation of intermediates, the intermediates are different from each other in terms of collagenase activity. A significant amount of collagenolytic activity is generated prior to the formation of intermediates in the case of organomercurials while the 46 kDa intermediate produced by trypsin lacks collagenolytic activity. In the latter case, the activity is generated when the 46 kDa intermediate is further converted to the 42 kDa active enzyme.[14,28] Since the 46 kDa to 42 kDa conversion is inhibited by TIMP, TIMP may play an important role in the case of protease-induced activation of procollagenase.

### *Possible Biological Collagenase Activators*

Under normal as well as pathological conditions in vivo, collagenase activation appears to be mediated by proteolysis of the 'pro' domain, although some nonproteolytic activation routes, such as protein factor-mediated modulation of the secondary structure surrounding the cysteine switch[26] and oxidative activation by neutrophils,[38] are also possible. Proteinases that have been reported to activate interstitial collagenase include trypsin,[10] plasmin,[30,31,33,39,40] kallikrein,[30,40] elastase,[40] cathepsin B,[40] mast cell chymase,[41] stromelysin,[30,33,42] matrilysin,[36] stromelysin-2 (MMP-10),[43] stromelysin-3 (MMP-11),[44] and 22/25 kDa interstitial collagenase fragments[20] (Fig. 6.3). These proteases are classified as either serine proteases (trypsin, plasmin, kallikrein, mast cell chymase and elastase) or matrix metalloproteinases (interstitial collagenase fragments, stromelysin-1, -2, and -3, and matrilysin), except for cathepsin B.

### *Stromelysins*

Of the above proteases, stromelysin appears to be the most potent and physiologically available collagenase activator, but its action on interstitial collagenase requires cooperation from other proteases. Stromelysin is able to "superactivate" interstitial collagenase, generat-

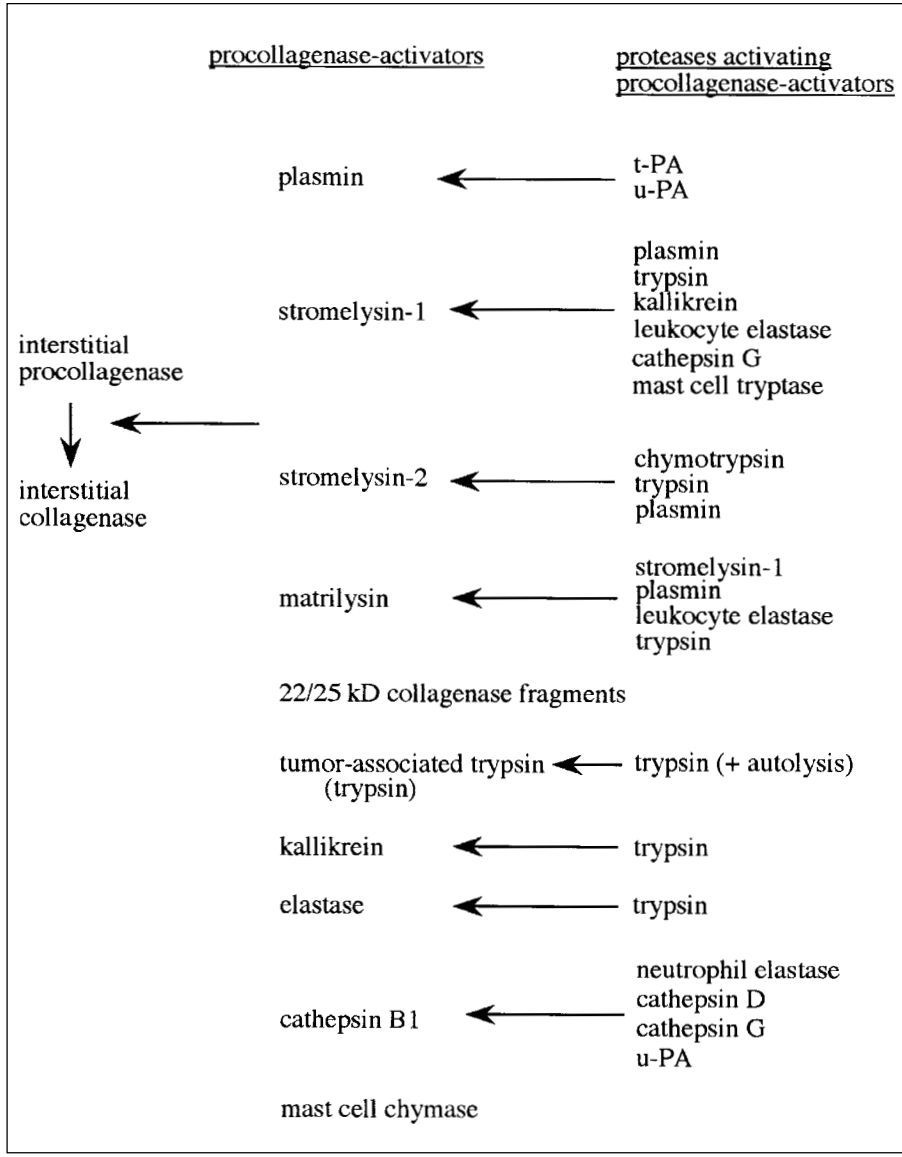


Fig. 6.3. Interstitial procollagenase activators. Procollagenase-activators can directly activate procollagenase to varying extents, and proteases that activate procollagenase-activators influence procollagenase activation indirectly. These activations occur via cell-cell interactions in vivo.

ing 7- to 12-fold higher specific activity than that resulting from organomercurials or limited proteolysis by plasmin or trypsin.[30,33,42] Stromelysin-2 is as potent as stromelysin-1 in superactivating procollagenase.[43] However, superactivation by stromelysin requires the presence of trypsin, plasmin, and kallikrein. Stromelysin alone causes slow activation[30] or is unable to activate interstitial collagenase efficiently.[33] Since two other collagenases,

collagenase-3 and neutrophil collagenase, are efficiently activated by stromelysin alone by a two-step or a single-step mechanism respectively,[45,46] the peptide bonds cleaved within procollagenase-3 and neutrophil collagenase seem to be more readily accessible to stromelysin than interstitial collagenase. The latter is resistant until proteolysis upstream within the propeptide has been affected by combined trypsin/plasmin-stromelysin treatment, leading to superactivation.[46] Such a cascade mechanism may play an important role in collagenase activation by stromelysin in vivo since plasmin, stromelysin, and collagenase are usually coordinately produced by cells.[42,47] On the other hand, requiring this cascade activation mechanism for collagenase may also provide more opportunities for endogenous inhibitors such as TIMPs or α2-antiplasmin to regulate extracellular matrix degradation in a precise manner.

Contradictory results have been published concerning the cleavage site of procollagenase by stromelysin. He et al[33] have claimed that truncation of the C-terminal domain of procollagenase by stromelysin is necessary for full activation of interstitial collagenase, while Suzuki et al[30] have reported that N-terminal truncation between $Gln^{80}$ and $Phe^{81}$ by stromelysin leads to full activation.

Since stromelysin can activate not only collagenases (MMP-1, MMP-8 and MMP-13) but also matrilysin[36] and 92 kDa gelatinase,[32] and since very high levels of stromelysin are detected under certain pathological conditions, stromelysin may be a major physiological activator of MMPs in vivo. On the other hand, stromelysin is generated by proteolytic activation of prostromelysin with trypsin, plasmin, kallikrein, leukocyte elastase, cathepsin G and mast cell tryptase[35] (Fig. 6.3). Close association of stromelysin with other MMPs and neutral proteases such as plasmin at the cell surface or on the substrate itself would be key events for their sequential activation.

### *Other Proteinases*

Plasmin is a more potent activator of interstitial procollagenase than trypsin.[39] However, the extent of procollagenase activation by plasmin alone is about 15-20% of the maximal activation, e.g. by a combination of plasmin with stromelysin,[30,33] indicating that plasmin can participate in a cascade-type activation. The involvement of plasmin in a multi-enzyme activation process, especially via cell-cell interaction, has been demonstrated by He et al.[33] When dermal fibroblasts and epidermal keratinocytes are cocultured, activation of interstitial procollagenase and prostromelysin occurs in the presence of plasminogen. This activation is mediated by keratinocyte-derived urokinase plasminogen activator (uPA), which converts plasminogen into plasmin. Since the uPA pathway of plasminogen activation is implicated in tissue degradation and cell migration under a variety of normal and pathological conditions, plasmin may be frequently involved in interstitial collagenase activation in vivo. A high local level of uPA can be obtained by concentration of the enzyme on its specific membrane receptor.[48] However, procollagenase-3 in or around cultured human fibroblasts is activated by MT1-MMP and 72 kDa gelatinase but not by plasmin, although plasmin itself can activate purified procollagenase-3 in a test tube,[49] suggesting that the activation process may be specific to each cell type.

Matrilysin activates interstitial procollagenase to the level obtained by organomercurial-activation.[36] Activation occurs by the cleavage between $Gln^{80}$ and $Phe$,[81] which is identical to the cleavage site for stromelysin reported by Suzuki et al.[30] Matrilysin also activates 92 kDa gelatinase and uPA,[36,50] and can itself be activated by stromelysin, trypsin, plasmin, and leukocyte elastase, the first two of which cause full activation.[36] Since matrilysin mRNA and protein are detected in carcinoma cells and matrilysin protein localizes to secretory and ductal epithelium in various glands of normal human tissue,[50] matrilysin may play an im-

portant role in collagenase activation during remodeling of extracellular matrix by cells of epithelial origin.

Trypsinogen/trypsin production is not solely confined to the pancreas. Koivunen et al[51] purified two trypsinogen isoenzymes from the cyst fluid of human ovarian carcinoma and named them tumor-associated trypsinogen-1 (TAT-1) and TAT-2. TAT-1 and TAT-2 have structures very similar to human pancreatic trypsinogen 1 and 2 respectively, but there are some differences in substrate specificity and susceptibility to proteinase inhibitors. TAT-1 and TAT-2 can efficiently activate uPA. More recently, trypsin-related proteins have been purified from the conditioned medium of a human gastric carcinoma cell line and identified as one- and two-chain forms of trypsinogen 1 and their activated forms.[52] Since these enzymes are very similar or identical to trypsinogen and trypsin, they could possibly activate collagenases, stromelysins, matrilysin, 92 kDa gelatinase, and uPA around cancer cells, although this has not yet been demonstrated.

Fragments of 22 and 25 kDa of interstitial collagenase, possibly produced by autocatalytic cleavages, can superactivate procollagenase in the presence of trypsin in a way similar to stromelysin.[20] The 25 kDa fragment is a glycosylated form of the 22 kDa fragment, and the N-terminal sequence of the 22 kDa fragment is identical to that of active collagenase. These fragments have proteolytic activity against casein and gelatin but not against collagen. Moreover, the activity of these fragments is not inhibited by TIMP because they lack the C-terminal domain. Similarly, generation of a 45 kDa fragment of 72 kDa gelatinase has been reported.[53] This 45 kDa fragment lacks both the N-terminal pro-domain and the C-terminal domain, and thus is less susceptible to TIMP-2 inhibition. However, it remains catalytically competent and capable of activating 92 kDa progelatinase, and although the physiological significance of these fragments of collagenase and gelatinase remains obscure, they may easily penetrate the extracellular matrix and gain access to substrate because of their smaller sizes. Alternatively, they may be involved in activation of collagenase and gelatinase or degradation of matrix proteins at sites of matrix remodeling even if excess inhibitor is present.[20]

Elastase and cathepsin B are also capable of activating collagenase, although they are less effective than trypsin.[40] Procathepsin B released from human carcinoma cells is efficiently activated by neutrophil elastase, cathepsin D, cathepsin G and uPA.[54,55]

Heparin proteoglycan-bound chymase exocytosed from human mast cell granules may also be an efficient activator of collagenase in vivo. This is supported by the observations that procollagenase and collagenase bind to extracellular mast cell granules in human skin, and chymase is relatively resistant to the physiologic serine protease inhibitors present in plasma and interstitial fluids.[41]

Some serine proteases can affect the collagenase activation process through their activity against TIMP. The inhibitory activity of TIMP against stromelysin is destroyed by degradation into small fragments by human neutrophil elastase, trypsin, or alpha-chymotrypsin, but not by cathepsin G, pancreatic elastase, or plasmin.[56] Elastase released from neutrophils infiltrating into inflammed tissues may decrease TIMP activity in vivo and thus help activation of collagenases.

## Collagenase Induction by Cell-Cell Interactions

In addition to extracellular regulation by proteolytic activation of the proenzyme and by the inhibitory effects of TIMPs, collagenase is also regulated at the level of synthesis as a result of the action of various cytokines, growth factors, and other soluble and cell surface mediators. These mediators usually act by paracrine or direct cell-cell interactions with target cell receptors, leading to various signal transduction events and transcriptional changes via positive or negative regulatory elements of the collagenase gene. They may have an

additional effect on regulation of collagenase activity by modulating expression of procollagenase-activating enzymes and their inhibitors. Since most of these factors act in a paracrine or direct cell-cell fashion, regulation of collagenase production cannot be fully understood without consideration of cell-cell interactions in vivo.

### *Epithelial Cell-Fibroblast Interactions*

The possibility that interactions between two different cell types might significantly modulate the production or activation of interstitial collagenase was first suggested by Grillo and Gross.[57] Using a wound healing system in guinea pig skin they demonstrated that the wound edge, containing both epithelium and mesenchyme (granulation tissue), had a high level of collagenolytic activity, whereas the separated epithelium and mesenchyme alone showed low activity. When sheets of epithelium isolated from the wound edge were recombined with the mesenchyme from the same region, the enzyme activity was restored to the same level as that of whole tissue.

The importance of epithelial-stromal interaction in stimulation of collagenase has also been demonstrated using cellular components of normal rabbit cornea and skin. Cultured corneal epithelial cells, in the presence of cytochalasin B, were shown to secrete soluble factors, of molecular weights 19, 54 and 90 kDa, which stimulated collagenase production by corneal stromal cells.[58] When fetal and adult skin epidermal cells were cultured with dermal fibroblasts, a modest increase in level of collagenase also occurred in the absence of cytochalasin B.[59] Subsequently, a soluble factor of 20 kDa secreted by the epidermal cells was shown to stimulate collagenase production, probably through enhanced transcription of collagenase mRNA.[60] Recently, a similar collagenase-stimulating cytokine of 20 kDa has been isolated from human gingival epithelial cell-conditioned medium and shown to induce high levels of collagenase activity in periodontal ligament fibroblast or gingival fibroblast cultures.[61] The collagenase stimulatory activity of the 20 kDa cytokine was markedly inhibited in the presence of anti-human interleukin (IL)-1$\alpha$ neutralizing antibody, indicating that the factor is identical to, or antigenically similar to, IL-1 $\alpha$.

### *Tumor Cell-Fibroblast Interactions*

Degradation of extracellular matrix components by MMPs is a crucial step in tumor cell invasion and metastasis[62-64](see chapter 11). The role of tumor cell-fibroblast interactions in regulation of MMP levels in neoplasms has been demonstrated by several investigators, including ourselves.[65-72] Recently several important findings have been reported that are consistent with a major role for these interactions in tumorigenesis in vivo.

First, in situ hybridization studies have clearly demonstrated that, in vivo, some tumor-associated MMPs are mainly synthesized in peritumoral fibroblasts, rather than in tumor cells themselves. For example, interstitial collagenase mRNA has been detected in stromal cells of gastrointestinal, breast, and head and neck cancers;[73,74] stromelysin-1 has been demonstrated in the stroma of colon and basal cell carcinomas;[75,76] and stromal elements surrounding tumors are responsive for the production of 72 kDa gelatinase in colon, breast and skin cancers.[77-81] MT-MMP is also expressed in the stroma of breast, colon, and head and neck cancers.[74] On the other hand, tumor cell-specific expression of 92 kDa gelatinase and matrilysin is stimulated by stromal fibroblasts.[70-72,82,83] Even the expression of an inhibitor of MMPs, TIMP-1, is regulated by tumor cell-fibroblast interactions.[70,79,84]

Second, recent detection of a receptor for the 72 kDa gelatinase/TIMP-2 complex on the tumor cell surface suggests the possibility of tumor cell binding and utilization of stromal MMPs.[85] Also, stromal-derived uPA activity can be localized to the tumor cell surface by binding to the uPA receptor (uPAR).[86]

Third, cell/tissue type specificity of MMP gene expression may play a role in the above interactions. For example, interstitial collagenase and stomelysin-1 and -3 are detected in some normal mesenchymal tissues and in the stromal compartments of tumors, whereas matrilysin is almost exclusively produced by epithelia and is not associated with stromal elements of tumors. Based on such observations, MacDougall and Matrisian[18] have suggested that carcinoma cells may not express stromelysin-1, stromelysin-3, and interstitial collagenase because they are somehow restricted in their MMP gene expression due to their epithelial lineage. Thus, these MMPs are produced by stromal fibroblasts under the influence of cancer cells. Moreover, since MT-MMP, which is a physiological activator of 72 kDa gelatinase is produced by stromal cells in several tumor types,[74] not only production but also extracellular activation of MMPs may be aided by stromal cells in tumors. These various lines of evidence strongly suggest that it is important for our understanding of the regulation of MMPs to elucidate further the molecular events involved in interactions between tumor cells and stromal fibroblasts.

### *Mechanisms of Stromal Interstitial Collagenase Induction*

A variety of agents are capable of inducing interstitial collagenase synthesis. These include: (i) cytokines and growth factors such as tumor necrosis factor (TNF)-α, TNF-β, IL-1, leukoregulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF);[1,87-92] (ii) ECM components such as collagen, heparin and the RGD-containing cell-binding region of fibronectin;[93-95] (iii) extracellular, regulatory macromolecules such as SPARC (osteonectin/BM40);[96] and (iv) plasma membrane proteins such as integrins.[94,97] The transmembrane neural cell-adhesion molecule (NCAM) down-regulates secretion of interstitial collagenase.[98]

With respect to tumor cell-stimulated stromal collagenase production, both a tumor cell-derived cytokine(s) and an integral plasma membrane protein on tumor cells appear to play fundamental roles. Collagenase production in cultures of fibroblast-like cells derived from rabbit V2 carcinoma is enhanced by coculture with epithelial-like cells from the same tumor or by addition of medium conditioned by these epithelial-like cells, indicating the presence of a soluble stimulator.[68] Goslen et al[69] isolated an IL-1-like cytokine of 19 kDa from tissue extracts of basal cell carcinoma, which induced collagenase synthesis in skin fibroblasts. On the other hand, Biswas and her colleagues have demonstrated that carcinoma cells stimulate fibroblasts to produce interstitial collagenase via an integral plasma membrane protein expressed on the carcinoma cells.[7,65-67,99-104] This protein was first called "tumor cell-derived collagenase stimulatory factor (TCSF)", and was later renamed "EMMPRIN" to indicate its role in extracellular matrix metalloproteinase induction via normal, as well as pathological, cellular interactions. In fact, EMMPRIN stimulates expression, not only of interstitial collagenase, but also of fibroblast-derived stromelysin-1 and 72 kDa gelatinase.[102,104] A similar membrane factor produced by MCF-7 breast cancer cells, which stimulates fibroblastic interstitial collagenase, stromelysin-1 and 72 kDa gelatinase, has also been reported.[105] Recently, it was shown that human breast and lung tumor cells, but not nearby normal cells, express EMMPRIN mRNA in vivo, whereas stromal fibroblasts adjacent to the tumor cells, but not the tumor cells themselves, express 72 kDa gelatinase mRNA.[106] In this same study it was shown that the level of EMMPRIN mRNA increased from near zero in normal tissue to high levels in all stages of breast and lung carcinomas. An earlier study demonstrated elevated levels of EMMPRIN in the tumor cells and in urine of patients with transitional cell carcinoma of the bladder.[107] In the light of these results, further elaborated in the next section, and since cell-cell contact has often been implicated in the stimulation of production of MMPs,[58,70-72,83,99] EMMPRIN or EMMPRIN-type stimu-

latory protein appears to play a central role in regulation of MMP production by tumor/epithelial cell-fibroblast interactions.

## EMMPRIN

### *Identification and Purification*

In earlier work, Biswas et al[108] obtained evidence that stromal collagenase can be induced by tumor cells in vivo using a heterologous host-tumor system where rabbit V2-carcinoma was implanted into the nude mouse. Immunological identification of the source of enzyme revealed that a significant proportion of collagenase was derived from host cells. Subsequently, coculturing normal fibroblasts with tumor cells was shown to result in increased collagenase production as compared to cultures of either cell type alone.[65,67] The collagenase stimulatory activity was at first shown to derive from the tumor cells since addition of tumor cell-conditioned medium to the fibroblasts stimulated collagenase production, but not vice versa. Under some conditions, however, the stimulatory factor(s) is not secreted but appears to remain associated with the tumor cell surface since cell contact is required to elicit stimulation of fibroblast collagenase production.[99]

In subsequent work, the properties of EMMPRIN as a membrane-intercalated protein were demonstrated,[100] and Biswas and Toole[62] proposed that EMMPRIN is attached to the plasma membrane via a transmembrane domain and interacts with a receptor on fibroblasts via its extracellular domain. EMMPRIN was extracted from the membranes of LX-1 human lung carcinoma cells, and a monoclonal antibody (E11F4) that neutralizes the collagenase stimulatory activity of LX-1 cells or LX-1 membrane extracts was obtained.[101] EMMPRIN purified using an E11F4-conjugated immunoaffinity column contains a doublet of 58 kDa and 54 kDa proteins; the former is present in higher concentration than the latter, and the collagenase stimulatory activity resides in the 58 kDa protein.[101,103] Moreover, addition of this purified 58 kDa protein to cultured fibroblasts has revealed that EMMPRIN stimulates expression of stromelysin-1 and 72 kDa gelatinase as well as interstitial collagenase.[102,104] Interestingly, EMMPRIN stimulates stromelysin-1 more effectively than interstitial collagenase; the extent of stimulation of mRNA expression is about 6-fold for stromelysin-1 while 2- to 4-fold for interstitial collagenase. This would be advantageous for the activation of interstitial procollagenase by stromelysin; possibly, the fibroblast-derived stromelysin may also activate other cancer cell-derived proMMPs such as 92 kDa progelatinase or matrilysin.

### *Molecular Cloning of EMMPRIN*

cDNA encoding EMMPRIN has been successfully isolated by RT-PCR using oligonucleotide primers derived from N-terminal and internal peptide sequences of the EMMPRIN protein.[7] The cDNA encodes a 269-amino acid residue polypeptide that contains a putative signal peptide of 21 amino acid residues. The 248 codons after the signal sequence encode a 185-amino acid extracellular domain consisting of two regions characteristic of the immunoglobulin superfamily, followed by 24-amino acid residues comprising the transmembrane domain and a 39-amino acid cytoplasmic domain (Fig. 6.4). The 248-amino acid residues of the mature protein correspond to an approximate molecular weight of 27 kDa. Because native EMMPRIN purified from LX-1 cells has a larger molecular weight, 58 kDa, the difference is most likely due to post-translational glycosylation of the protein. This post-translational processing is critical for EMMPRIN activity because recombinant EMMPRIN (r-EMMPRIN) produced by bacteria, which has a molecular mass of ~29 kDa and is not post-translationally processed, is functionally inactive, while r-EMMPRIN isolated from CHO cells transfected with EMMPRIN cDNA successfully stimu-

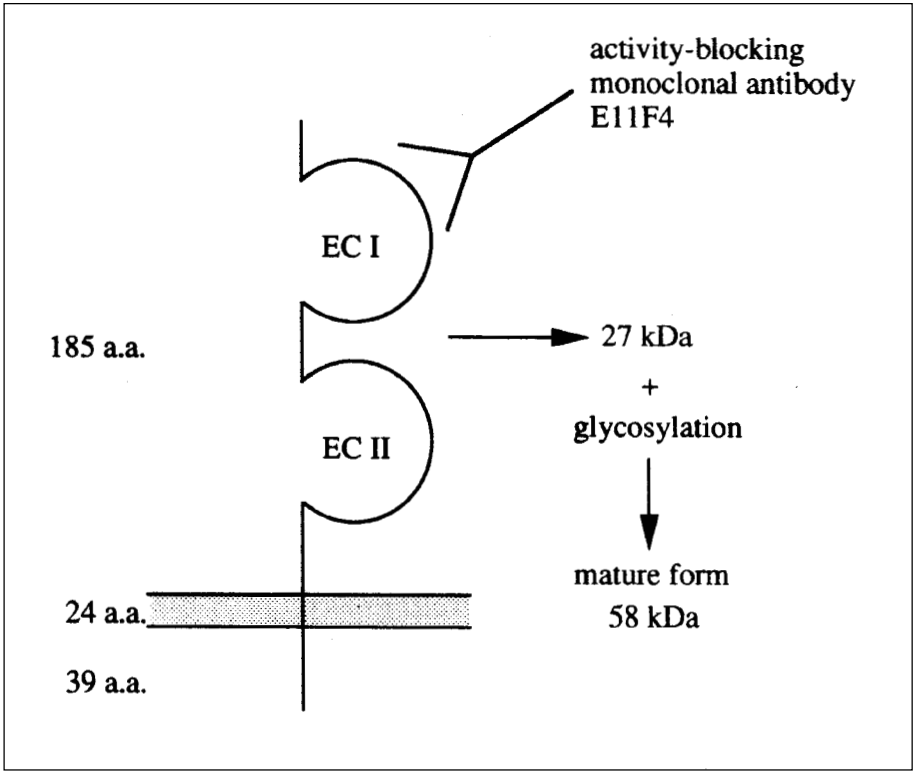


Fig. 6.4. A molecular model for the EMMPRIN molecule. The MMP-stimulatory active site of EMMPRIN, which is blocked by monoclonal antibody E11F4, is likely to be localized to sequences contained in ECI. ECI, the extracellular immunoglobulin domain I; ECII, the extracellular immunoglobulin domain II.

lates production of interstitial collagenase, 72 kDa gelatinase and stromelysin-1.[109] The CHO cell-derived r-EMMPRIN is post-translationally modified to a molecular mass of ~58 kDa.

The location of the epitope for activity-blocking monoclonal antibody E11F4 has been determined by using modified EMMPRIN expression plasmids in which the following regions were deleted: (a) extracellular immunoglobulin domain I; (b) extracellular immunoglobulin domain II; (c) transmembrane domain; and (d) cytoplasmic domain. The mutated protein lacking extracellular immunoglobulin domain I lost reactivity with E11F4, indicating that the antibody epitope exists in this domain (Fig. 6.4). Since E11F4 is an activity-blocking antibody, the functional site for the metalloproteinase stimulatory activity of EMMPRIN may be localized at or near this epitope in the immunoglobulin domain I region.

### *Plasma Membrane Localization and Shedding of EMMPRIN*

Immunoaffinity purified EMMPRIN stimulates fibroblast collagenase activity by 6- to 8-fold and expression of collagenase mRNA by 2- to 4-fold, while carcinoma (LX-1) cells, which have intact EMMPRIN molecules on their surface, can stimulate collagenase activity by 15- to 20-fold and the mRNA level by 8- to 10-fold. This indicates that integration into

the plasma membrane may enhance EMMPRIN activity. Purified EMMPRIN may acquire an altered or partially denatured configuration, and interaction with other membrane proteins might aid EMMPRIN in its function.

LX-1 carcinoma cell-conditioned medium contains several protein species of 58 kDa and smaller molecular weight which are crossreactive with the EMMPRIN antibody.[101,103] The 58 kDa protein, which is the same size as the intact cell surface EMMPRIN, might be released as a component of shed membrane vesicles, whereas the smaller species might be derived from membrane bound EMMPRIN by proteolytic cleavage. In support of the latter, we have obtained data indicating that elastase treatment of the 58 kDa membrane form generates a 35 kDa species identical in molecular weight to a species consistently present in conditioned medium (unpublished data). Although the released forms are less active compared with intact membrane EMMPRIN, they might have some role in vivo since they would have better accessibility to nearby cells. In addition, since culture of tumor cells on matrix deposited by fibroblasts increases the level of EMMPRIN in conditioned media,[99] determination of the matrix component(s) that stimulates EMMPRIN production or release may contribute to our understanding of EMMPRIN-based tumor cell-fibroblast interactions in vivo.

### *EMMPRIN Stimulation of Fibroblasts*

Identification of the molecule on the surface of fibroblasts that interacts with tumor cell-derived EMMPRIN to cause increased fibroblast MMP production is crucial to understanding the mechanism of MMP induction via cell-cell interactions. Although neural cell-adhesion molecule (NCAM), which is also a member of the immunoglobulin superfamily, operates via both homophilic (NCAM to NCAM) and heterophilic binding mechanisms (e.g., NCAM to heparin/heparan sulfate proteoglycan and various collagens),[98] EMMPRIN seems to act on fibroblasts via heterophilic binding mechanisms since EMMPRIN expression has not been detected on the fibroblast cell surface.[101,110] Recently we have used EMMPRIN-affinity chromatography to isolate the putative fibroblast receptor for EMMPRIN and are in the process of characterizing this protein (Guo and Toole, unpublished data).

The mechanism whereby EMMPRIN upregulates transcription of the interstitial collagenase gene in fibroblasts is another interesting question yet to be solved. The interstitial collagenase promotor region contains a TATA box, a TPA responsive element (TRE), and a binding motif for the transcription factor PEA3[1,111] (see chapter 4). The TRE alone or in combintion with the PEA3 site, and possibly other upstream elements, controls the transcription of this gene. These regulatory sequences are recognized by transcription factors which are composed of proto-oncogene products. The PEA3 binding site interacts with the c-Ets protein, whereas the TRE element binds AP-1, a protein complex of homodimers and heterodimers of the fos and jun family. Thus, agents that modulate the expression of these proto-oncogenes also influence the transcription of the collagenase gene.[1] Recently it has been shown that c-Ets-1 is expressed in mesenchymal cells adjacent to epithelial structures during morphogenetic processes and in actively remodeling tissues,[112] and also in the stromal fibroblasts adjacent to lung, breast, colon, pancreas, and thyroid carcinomas.[113,114] Expression of c-Ets is induced in human fibroblasts by coculture with human squamous carcinoma cells of the skin, and this induction is dependent on a combination of cell-cell contact and a tumor cell-derived soluble factor.[83] These lines of evidence suggest that c-Ets-1 is a potential candidate for a mediator involved in EMMPRIN-induced collagenase expression. Since c-Ets-2 activates the stromelysin-1 promoter more effectively than collagenase,[115] which is compatible with the action of EMMPRIN, participation of c-Ets-2 is another possibility. However, EMMPRIN can also upregulate 72 kDa gelatinase, albeit to a lesser extent than

stromelysin-1 and collagenase. Since the 72 kDa gelatinase gene lacks a TATA box, a TRE sequence and a PEA3-binding site,[1] its regulatory mechanisms may not be as simple.

### *Protein Homologues of EMMPRIN and Their Possible Functions*

Analysis of its cDNA-derived amino acid sequence revealed that EMMPRIN is a member of the immunoglobulin superfamily,[7] and that it is identical to human basigin[116] and the human leukocyte activation-associated M6 antigen.[117] These proteins are also the species homologue of the rat OX-47 antigen[118] and spermatozoon membrane protein, CE9;[119] mouse gp42[120] and basigin;[121] and a molecule associated with blood-brain barrier endothelium and the retina of the chicken, known as HT7, 5A11 and neurothelin.[122-124] Comparison of human EMMPRIN with the other species homologues reveals that the putative transmembrane region is almost identical in all cases, and that the cytoplasmic domain is highly homologous (~70%).[116] Within the putative transmembrane region of the chick and human homologues,[116,122] three leucines are repeated every seventh amino acid residue. This sequence is characteristic of leucine zippers found in DNA binding proteins but has also been identified in several other types of proteins, including other members of the immunoglobulin superfamily.[122] This suggests the possibility of protein-protein interactions within the membrane; however, mouse basigin has only two of these leucines.[121] The conserved presence of a glutamic acid residue in the transmembrane region of chick, mouse and human homologues[116] also suggests that the protein might functionally interact with other membrane proteins[122,125] and thereby exhibit various functions.

It seems likely that formation of a complex between the molecules above and a cell-surface protein on adjacent cells is important for their function. For example, the 5A11 antigen may mediate heterotypic cell-cell recognition events in the developing neural retina.[123] Addition of anti-5A11 antibody to rotation cultures of trypsin-dissociated embryonic retina cells results in reduced reaggregation of cells when compared to control antibody. Addition of the antibody to monolayer cultures of embryonic retina cells also reduces neuron-dependent glial cell maturation. The M6 antigen might be involved in cell-substratum interactions since anti-M6 antibody inhibits neurite extension in cultured neurons which express the M6 antigen on their cell surface.[126] Another structurally-related molecule, embigin, which is preferentially expressed in early embryonic cells of the mouse, is also involved in cell-substratum adhesion.[127] Transfectant fibroblasts expressing embigin have enhanced cell-substratum adhesion activity which is $Ca^{2+}$-dependent and is inhibited by an arginine-glycine-aspartic acid (RGD) peptide that competitively inhibits integrin binding and by anti-integrin antiserum. These experiments indicate that embigin plays a role in promotion of integrin-mediated cell-substratum adhesion.[127] Taken together, these observations indicate that EMMPRIN homologues are involved in cell-cell recognition and cell-substratum interactions, supporting the role of EMMPRIN in tumor/epithelial cell-fibroblast interactions.

Significant levels of EMMPRIN and its homologues are present in a wide variety of normal embryonic and adult tissues (T. Nakamura and C. Biswas, unpublished results).[107,121,128,129] The function of EMMPRIN and related molecules in many of these locations is not at all clear. However, it will be of interest to determine whether the EMMPRIN homologues and related molecules also act via cell-cell interactions to stimulate MMP production. For example, the presence of M6 antigen, which is identical to EMMPRIN, on granulocytes in patients with rheumatoid arthritis[117] may indicate a role for EMMPRIN in stromal MMP production and in the consequent matrix degradation that occurs in the arthritic joint. EMMPRIN may also cause an increase in MMP production by dermal fibroblasts during wound healing or embryonic development since EMMPRIN is expressed in

the epidermis.[110] Thus EMMPRIN and related molecules may be important mediators of matrix remodelling in normal and pathological tissues.

## Conclusions and Perspectives

As summarized in the first half of this review, procollagenase activation is a cascade process. Furthermore, as was shown in the latter half of this chapter, cancer/epithelial cell-fibroblast interactions play an important role in regulating collagenase activity at the level of synthesis. Similar interactions also have a significant role at the level of procollagenase activation. The following is a possible scenario for the cascade of events involved in regulation of collagenase activity (also see Fig. 6.5).

In colon and breast cancers, stromal fibroblasts are stimulated by carcinoma cells to produce pro-uPA, which then binds to the uPA receptor (uPAR) expressed on the carcinoma cell surface at the tumoral-stromal interface.[86,130-132] Upon binding to its receptor, pro-uPA is effectively converted to active uPA, possibly by receptor bound plasmin, and this uPA in turn efficiently activates plasminogen, which is also bound to its receptor in close proximity on the carcinoma cell surface, to plasmin. The generated plasmin possibly takes part in activation of prostromelysin and procollagenase, which are synthesized by stromal cells via induction by EMMPRIN expressed on carcinoma cells. Stromelysin may then superactivate procollagenase in the presence of plasmin. Carcinoma cell-derived TAT, cathepsin B or elastase, and plasma kallikrein or leukocyte elastase may also contribute to activation of procollagenase. Since EMMPRIN is a powerful inducer of stromelysin, EMMPRIN may give rise to efficient activation of other proMMPs, including procollagenase, promatrilysin, and 92 kDa gelatinase. In addition, close cell proximity or direct cell contact would be advantageous for one protease to activate another because of accessibility and protection from protease inhibitors present in tissue fluid at the inflammed site. In this light, EMMPRIN may play a central role in direct cell contact-dependent regulation of procollagenase activity. Furthermore, since production of TIMP-1 is probably not stimulated by EMMPRIN,[109] an imbalance of active versus inactive MMP production may also result from EMMPRIN action on stromal fibroblasts.

The role of stromal collagenase or other MMP activity induced by cancer/epithelial cell-fibroblast interactions has yet to be evaluated directly. One approach is the use of 'knockout' animals, which could lead to stromal cells free of some of the MMPs. A preliminary report was recently published on a knockout of mouse basigin, which is a homologue of EMMPRIN.[133] One of the major defects in the null mice appears to be inefficient embryo implantation, which coincides well with the involvement of stromal MMPs in implantation.[111,134] The influence of stromal MMPs on tumor growth, invasion, and metastasis could be examined in such a system. Introduction of EMMPRIN-overexpressing or EMMPRIN-deficient carcinoma or epithelial cells into in vitro or in vivo systems would be another useful approach to assess the role of EMMPRIN as well as stromal MMPs. Identification and cloning of the EMMPRIN receptor on fibroblasts might lead to development of therapeutic agents such as EMMPRIN analogues that can bind to the receptor but cannot stimulate the intracellular signal pathway thereafter.

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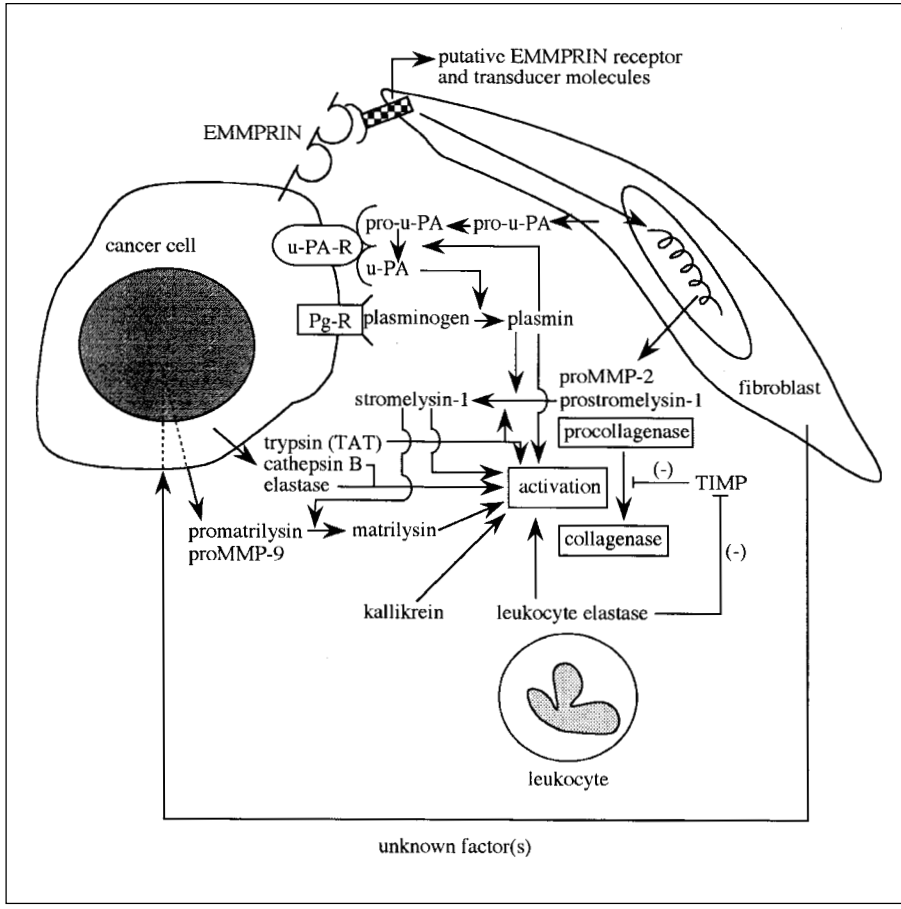


Fig. 6.5. Induction and activation of interstitial procollagenase via cancer cell-fibroblast interaction, including EMMPRIN. uPA, urokinase type plasminogen activator; uPA-R, uPA receptor; Pg-R, plasminogen receptor; TAT, tumor-associated trypsin.

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CHAPTER 7

# Role of Reactive Oxygen Species in the Induction of Collagenases, and Other MMPs—Pathogenic Implications for Photoaging and Tumor Progression

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## Introduction

There is a strong association between exposure to ultraviolet irradiation (UV) induced reactive oxygen species (ROS), human skin cancer, and premature aging of the skin.[1-3] Reactive oxygen species (ROS) are part of normal regulatory circuits and the redox state is tightly controlled by antioxidants. Cells and tissues are equipped with a complex enzymatic and nonenzymatic antioxidant defense system.

UV generation of ROS results in a loss of cellular antioxidant homeostasis, thus tilting the balance towards a prooxidant state. The increase in UV irradiation on earth due to the stratospheric ozone depletion will lead to a substantially higher risk of photooxidative damage to the skin, resulting in a dramatic increase in skin tumor incidence.[4]

Increased ROS level and loss of cellular redox homeostasis can contribute to photoaging and photocarcinogenesis. ROS and free radicals have the capacity to cause permanent structural damage in different cellular target structures, such as DNA, lipid membranes, RNA, and proteins. Furthermore, ROS-induced mutations or activation of cellular signal transduction pathways results in the overexpression of cellular protooncogenes. These cellular protooncogenes include proteins that function as transcription factors such as c-fos, c-myc, and AP-1. The DNA binding activity of AP-1 is redox controlled.[5] Along these lines, it is interesting that the conversion of benign papillomas to malignant squamous cell carcinomas involves the constitutive activation of the AP-1 transcription factor complex.[6] AP-1 transactivates different promoters, including the promoters of several matrix-metalloproteinases. Matrix-degrading metalloproteinases have been causally linked to tumor cell invasion and metastasis. Collectively, the modulation of cytoplasmic and nuclear signal transduction pathways by ROS and changes in the cellular redox status most likely contribute to photocarcinogenesis and photoaging of the skin.

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In this review we will concentrate on the role of ROS in the cellular UV-response leading to MMP induction.

### *Generation of Reactive Oxygen Species*

It is well accepted that low levels of reactive oxygen species are continuously produced in vivo and are involved in signal transduction pathways, cell activation, differentiation and growth control.[7,8] However, there is accumulating indirect evidence for the damaging effect of higher concentrations of reactive oxygen species generated in vivo upon UVA and UVB-irradiation of the skin.[2,9-11] Beside direct absorption of UVB-photons by DNA and subsequent structural changes, generation of reactive oxygen species following irradiation with UVA and UVB requires the absorption of photons by endogenous photosensitizer molecules. Photosensitized oxidations occur as type I or type II reactions.[12] Mechanistically, the absorption of UV-photons by a sensitizer results in its electronically excited state. Endogenous photosensitizers include riboflavin, porphyrins, quinones, and bilirubin in mammalian cells.[13] The excited sensitizer subsequently reacts with another substrate (type I reaction) or with oxygen (type II reaction). The resulting products of type I reactions are radicals or radical ions. Type II reactions produce reactive oxygen species including the superoxide anion ($O_2^{-\cdot}$) and singlet oxygen ($^1O_2$ ). $O_2^{-\cdot}$ may also be formed from other sources, e.g. when chelated $Fe^{3+}$ is reduced and undergoes autoxidation.[14] Superoxide dismutases convert $O_2^{-\cdot}$ to $H_2O_2$. $H_2O_2$ is able to easily cross cellular membranes. However, neither of these species react directly with DNA.[15] Therefore, $H_2O_2$ and $O_2^{-\cdot}$ are thought to participate in the generation of more dangerous species such as the hydroxyl radical ($OH^{\cdot}$). This can happen in vitro and in vivo by two related though different mechanisms. $O_2^{-\cdot}$ can reduce Fe(III) or Cu(II) and the subsequent Fe(II), or Cu(I) can reduce $H_2O_2$, finally resulting in the generation of the hydroxyl radical ($OH^{\cdot}$) via the Fenton reaction.[16] In vivo, $O_2^{-\cdot}$ enhances a release of Fe(II) from [4 Fe-4S] clusters of dehydratases, and the released Fe(II) subsequently reduces $H_2O_2$ to $OH^-$ and $OH^{\cdot}$.[17] In addition, $O_2^{-\cdot}$ is able to release Fe(II) from ferritin.[18, 19] This release could be mediated by a UVB-generated superoxide anion.[20]

Using electron spin resonance techniques, $OH^{\cdot}$ has been detected in the skin upon UV-irradiation.[21] Like singlet oxygen, $OH^{\cdot}$ is able to initiate the lipid peroxidation chain reaction, resulting in the oxidative deterioration of polyunsaturated fatty acids. The deterioration produces lipid radicals, hydroperoxyl radicals and lipid hydroperoxide. The lipid radical perpetuates the chain reaction. The end products of this lipid peroxidation process include aldehydes, carbonyles and various other degradation products that can diffuse and influence the cellular homeostasis.[22]

Singlet oxygen is a particularly damaging molecule with a short half life of μ seconds in water. It can react with a variety of biological molecules such as DNA, proteins, and lipids.[23,24] UVA-irradiation generates singlet oxygen by energy transfer from a photosensitizer molecule to ground-state oxygen. In addition to endogenous photosensitizers, the skin has access to an increasing number of exogenous photosensitizers in cosmetics, medications, drugs, plants, and industrial emissions. Singlet oxygen and other reactive oxygen species have been implicated in the pathogenesis of photodermatologic disorders which include drug-induced phototoxicity, porphyrias, photoaging, and photocarcinogenesis.[25-28] ROS, which have increased upon UV-irradiation, cause extensive damage to DNA, as demonstrated by tumor suppressor genes and oncogenes, in addition to proteins and lipids with genotoxic and mutagenic effects. These ROS induced alterations are variations of a common theme involved in aging, cancer, and other degenerative diseases. In this review we will concentrate on ROS induced up-regulation of matrix-degrading metalloproteinases related to tumor progression.

## The Role of UV-induced Reactive Oxygen Species (ROS) and Collagenases in Photoaging and Photocarcinogenesis

Substantial effort from a variety of laboratories, including our own laboratory, has been made to accurately define the involvement of ROS in photocarcinogenesis and photoaging. Both pathological processes share common features, however, reveal unique molecular characteristics which finally determine the fate of the cell and its host. For example, ROS cause permanent genetic changes which involve protooncogenes and tumor suppressor genes. In addition, ROS activate cytoplasmic signal transduction pathways that are related to growth differentiation, senescence, and tissue degradation, all of which determine the malignant invasive and metastatic phenotype.[1, 29]

### *Photocarcinogenesis*

There is a strong association between exposure to reactive oxygen species generating sunlight and human nonmelanoma skin cancer. The relation between UV induction and melanoma is less clear and still controversially discussed in the scientific community. However, recent epidemiological studies and results from animal studies[30-36] support the concept that recreational UV-exposure and sunburns with subsequent influx of ROS- generating inflammatory cells into the skin may play an important role in the etiology of cutaneous malignant melanoma. In a recent paper, the long-term combined application of the photosensitizing agent 8-methoxypsoralen and UVA irradiation, widely used for the treatment of psoriasis and other dermatological diseases, resulted in an increased incidence in melanoma development.[37]

Most likely similar to nonmelanoma skin cancer, melanoma undergoes a multistage development towards the fully malignant phenotype. Operationally, this process can be subdivided into three stages, termed initiation, promotion, and progression. Initiation is thought to involve permanent genetic alterations in protooncogenes and tumor suppressor genes that make epidermal cells resistant to signals for terminal differentiation. Tumor progression appears to involve clonal expansion of initiated cells in response to tumor promoters, finally giving rise to benign papillomas. The tumor promoting agents were shown to transiently induce the expression of genes related to hyperproliferation and tissue invasion, like the matrix-metalloproteinases and distinct members of the serine protease family. Whereas benign papillomas constitutively express genes associated with hyperproliferation but not with tissue invasion, the latter property is a characteristic for the conversion of papillomas to squamous cell carcinomas during tumor progression. It was shown that these protease genes are transcriptionally regulated by the transcription factor complex AP-1, which transactivates the promoter of different matrix-degrading metalloproteinases. Constitutive AP-1 activity was observed in malignant squamous cell carcinomas, but not in benign papilloma producing cell lines.[6]

ROS generated by UVA/UVB irradiation have been shown to be involved in all three stages of (photo) carcinogenesis.[1, 38] Currently both UVA and UVB are considered to be complete carcinogens. For many of the tumors studied, progression from benign hyperproliferation through dysplasia to invasion and metastasis is accompanied by changes in cell-matrix interactions (i.e. localized proteolysis of the basement membrane allowing migration of the tumor cells into the connective tissue of the dermis or of distant organs due to MMP activity). The family of matrix-metalloproteinases (MMPs) is growing and comprises at least 19 members.[39, 40] While MMP-1 (collagenase) cleaves collagen type I, MMP-2 is able to degrade basement membrane compounds, including collagen type IV and type VII. MMP-3 reveals the broadest substrate specificity for proteins such as collagen type IV, proteoglycans, fibronectin, and laminin.[41-44] As to their proteolytic activity, UV-induced MMPs may contribute to the dissolution of the basement membrane and der-

mal structural proteins. This process directly leads to tumor invasion and metastasis at distant body sites.[45,46] Furthermore, MMP-1 appears also to be involved in the initiation of carcinogenesis.[47] Beside UV generation of ROS, there is now evidence that tumor cells may constitutionally overproduce reactive oxygen species.[48] An overproduction of a reactive oxygen species may stimulate the synthesis and activation of matrix-metalloproteinases in the peritumoral connective tissue, enhancing the invasion and spreading of tumor cells. In fact, there is evidence from in situ hybridization experiments that specific mRNAs of MMPs are synthesized in fibroblasts adjacent to basal cell carcinomas (Mauch and Krieg, unpublished observation). The activity of all MMPs is inhibited by a special class of tissue inhibitors of metalloproteinases (TIMPs). Like MMPs, TIMPs are synthesized by ordinarily resident fibroblasts. Interestingly, ROS are able to inactivate TIMPs by a direct oxidative attack, thus contributing to tumor progression and photoaging.[49]

### *Photoaging*

Several intrinsic and extrinsic factors contribute to the complex phenomenon of aging. Chronological aging affects the skin in a manner similar to other organs.[50] Superimposed on this innate process, photoaging is related to severe UV-induced damage of the dermal connective tissue. There is increasing evidence that chronological aging and photoaging have different biological, biochemical and molecular mechanisms. [27] These differences are reflected in the histological picture, the content of various extracellular matrix proteins, and the formation of cross-links within the collagen molecule[51,52] and the capacity of fibroblasts to organize extracellular matrix molecules.[53] Photoaging in humans becomes apparent as a final stage after several decades of chronic sun exposure. Clinically, it is characterized by wrinkle formation, loss of recoil capacity, increased fragility of the skin with blister formation and impaired wound healing. At the histological level, a loss of mature collagen, a distinct basophilic appearance of collagen ("basophilic degeneration"), a greatly increased deposition of glycosaminoglycans, and an increase in fragmented elastic fibers[54, 55] are constant features in cutaneous photodamage.[56] Biochemically, quantitative and qualitative alterations of dermal extracellular matrix proteins such as elastin, [57,50] glycosaminoglycans,[58,59] and interstitial collagens[60,61] are involved. Collagen type I belongs to a family of closely related but genetically distinct proteins,[62] providing the dermis with tensile strength and stability. It has also been found to be diminished in photoaged skin.[28] Because the enzymatic capacity for extracellular matrix synthesis and its degradation resides in the dermal fibroblast, much effort has been devoted to study these fibroblast controlled processes. In addition to the UV effects on post-translational modification of the newly synthesized collagen molecule, [63,28] we and others have shown that various MMPs are responsible for the breakdown of dermal interstitial collagen and other connective tissue components. Distinct MMPs were induced in a dose-dependent manner in vitro and in vivo by UVA- and UVB-irradiation.[64-71]

## The Regulation of Matrix-Degrading Metalloproteases by Ultraviolet Irradiation (UV) Induced Reactive Oxygen Species (ROS)

### *MMP Induction by Distinct Reactive Oxygen Species*

Reactive oxygen species may prove particularly relevant to future developments of protective agents for the skin. Thus, there have been efforts to better define the involvement of distinct oxygen species in the up-regulation of matrix-metalloproteinases which are responsible for the connective tissue degradation in photoaging and photocarcinogenesis. For this purpose, fibroblast monolayer cultures were subjected to various reactive oxygen species generating systems or to UV-irradiation at different wavelengths and spectra. Exposure of

fibroblasts to ROS generating systems may accurately model the situation that occurs when tumor cells or inflammatory cells release high concentrations of ROS. The tumor cells may stimulate synthesis of collagenase in the peritumoral fibroblasts or in fibroblasts adjacent to inflammatory cells. Using inhibitors for the activity of ROS detoxifying enzymes and iron chelators to block the Fenton reaction, distinct ROS have been increased peri- or intracellularly.

Exposure of cultured human fibroblasts to singlet oxygen, generated in a dark reaction by thermodecomposition of the endoperoxide of the sodium salt of 3,3´-(1,4-naphthylidene) dipropionate ($NDPO_2$), induced collagenase (MMP-1) mRNA steady state levels in a dose-dependent manner.[72] The increase in collagenase expression after singlet oxygen exposure generated with 3 mM $NDPO_2$ was equivalent to that observed with UVA at a dose of 200-300 kJ/m$^2$ and developed in a similar time course. In contrast, mRNA levels of TIMP-1, the tissue inhibitor of metalloproteinases-1, remained unchanged. Indirect evidence for the role of singlet oxygen in the UVA-induction of collagenase (MMP-1) comes from studies using enhancers or quenchers of singlet oxygen. Accordingly, incubation in deuterium oxide, an enhancer of the life span of singlet oxygen led to an additional increase in steady state levels of collagenase mRNA, after exposure to $NDPO_2$ or to UVA-irradiation. In contrast, sodium azide, a potent quencher of singlet oxygen and other reactive oxygen species, almost totally abrogated the induction of collagenase after exposure of fibroblasts to $NDPO_2$ or to UVA-irradiation. Similar results were obtained on the protein level.[73]

The importance of singlet oxygen in photoaging becomes particularly clear in patients suffering from porphyria cutanea tarda. Those patients show substantially accelerated photoaging and a more frequent and severe blister formation compared to UV-exposed individuals. Biochemically, an increase in photosensitizing porphyrins mainly of uroporphyrin in the skin has been reported.[74] Photoexcitation of uroporphyrin I enhances the formation of singlet oxygen. In order to simulate this metabolic disorder, fibroblast monolayer cultures have been subjected to a combined treatment with uroporphyrin and subsequent irradiation (340-450 nm, UVA). This combined treatment resulted in a much stronger induction of MMPs compared to irradiation alone.[67] These results are in line with the clinical experience of a substantial improvement of photoaging after a therapeutical decrease of abnormally elevated porphyrins by means of chloroquin. This improvement further underlines the central role of singlet oxygen in dermal photoaging and its particular relevance to future development of UV-protective agents for the skin. Because even high doses of singlet oxygen quenchers could not completely inhibit the UVA-induced collagenase, it was concluded that other reactive oxygen species, besides singlet oxygen, are involved. In order to define distinct ROS other than singlet oxygen (which are possibly involved in the up-regulation of MMPs), we have used paraquat, a redox cycling agent known for its capacity to increase the intracellular concentration of the superoxide anion. A time-dependent increase in steady state mRNA levels for interstitial collagenase (MMP-1) with a maximal increase of 8-fold was observed at 72 h, following exposure of fibroblasts to paraquat at nontoxic concentrations.[75] The isolated and combined inhibition of glutathione peroxidase and catalase, in addition to the Fenton reaction by buthionine sulfoximine (BSO), aminotriazole (ATZ) and iron chelators like desferrioxamine (DFO) or N-2-hydroxybenzyl N´-benzylethylenediaminediacetic acid (HBED), respectively enhances MMP-1 mRNA levels up to 6-fold after paraquat treatment or UVA-irradiation compared to the simply paraquat treated or UVA-irradiated controls. Both isolated and combined treatment of cells only marginally increased TIMP-1 mRNA levels. While inhibition of the Cu, Zn superoxide dismutase by diethyldithiocarbamate (DDC) diminished the steady state MMP-1 mRNA level after paraquat treatment, MMP-1 mRNA levels were increased after UVA-irradiation.[75] These results indicate that following paraquat treatment, $H_2O_2$ may be responsible for the

induction of MMP-1. Following UVA-irradiation in addition to $^1O_2$, both $H_2O_2$ and $O_2^{-\cdot}$ appear to play a role in the induction of MMP-1 synthesis.[67, 73] By contrast, following UVB-irradiation, in particular iron chelators are able to inhibit the up-regulation of MMP-1 mRNA levels by 60%. This points to the importance of the iron driven Fenton reaction with subsequent generation of $OH^{\cdot}$ and lipid peroxides. Interestingly, inhibition of the Fenton reaction and lipid peroxidation by iron chelators, the vitamin E derivative Trolox, and scavenging $OH^{\cdot}$ by DMSO or mannitol resulted in a significant reduction of lipid peroxidation and MMP-1 mRNA levels after UVB irradiation.[94] These results imply potential antioxidant approaches for controlling tumor progression and aging. In fact, due to a pathologically leaky vasculature in tumor angiogenesis, erythrocyte extravasation with hemosiderin formation and release of "free" iron into the connective tissue is a common feature in tumor angiogenesis. Upon $H_2O_2$ release by inflammatory or tumor cells it is most likely that the Fenton reaction may be initiated.

The involvement of transitional metals in accelerating photoaging is further supported by in vivo studies.[76] Chronic exposure of hairless mice with sub-erythemal doses of UVB results in an increased level of nonheme iron in the skin. A similar increase in nonheme iron was observed in sun-exposed human skin compared to nonexposed body sites. Topical application of certain iron chelators to hairless mice skin substantially delayed the onset of UVB-induced histological alterations, such as hyperplasia and fragmentation of elastin fibers, and basophilic degeneration of collagen.[76] These in vitro and in vivo results, and the delay in photoaging by antioxidant strategies, provide strong evidence for an important role of UV-generated reactive oxygen species in the up-regulation and activation of matrix metalloproteinases. In addition, reactive oxygen species either generated by UV-irradiation or by inflammatory cells present in photodamaged skin are able to directly attack and destroy extracellular matrix proteins.[77, 78]

For gelatinase A (MMP-2) expression, a modulation by ROS has been demonstrated pointing to a more general induction of matrix-metalloproteinases by ROS. Kawaguchi et al[79] demonstrated that ROS generated by the xanthin/xanthinoxidase system resulted in a two-fold induction of the 72 kDa gelatinase A (MMP-2). This observation implies a more general mechanism of induction, because MMP-2 expression is controlled by promoter sequences distinct from MMP-1 and MMP-3.

Taken together, recent work has identified distinct ROS involved in connective tissue damage. However, there are strong indications that beside ROS, photoactivated reactions following UVB irradiation may cause depletion of antioxidants, such as ascorbate. The oxidation of ascorbate can be prevented by the introduction of complementary redox recycling systems.[80] Further studies are required to outline preventive strategies which may enhance the rational development of protective agents for cutaneous photodamage.

### *ROS Dependent Modulation of Transcriptional Pathways*

There is good experimental evidence that different UV-modalities with different emitted wavelength spectra, and differences in photosensitizing molecules, mediate the up-regulation of matrix-degrading metalloproteinases via different reactive oxygen species. However, it is not yet clear whether reactive oxygen species exert their actions via different signal transduction mechanisms. Due to their short half lives ($10^{-5}$ to $10^{-8}$ sec), reactive oxygen species would be ideal signaling molecules. In this capacity, they are involved in several physiological and pathological states. ROS are able to modulate the expression of different genes through specific mechanisms.[7] The redox regulation of transcription factors/activators has received much attention recently.[5] There is evidence from in vitro studies for p53, AP-1 and NF-kappa B that reducing environments actually increase, while oxidizing conditions inhibit sequence-specific DNA binding and/or transactivation activities in a transcrip-

tional activator-dependent and cell type-dependent manner. The analysis of events downstream from the initiation of cancer-related mutations demonstrated recently[81] may give promising opportunities for pharmacological intervention. Unfortunately, a complete view of signaling events leading to the up-regulation of matrix-degrading enzymes is not yet available. Hence, it is not yet resolved whether membrane associated changes or DNA damage can be considered as the first event in initiating UV and ROS induced signaling. However, there are some indications from UV studies that activation of AP-1, which transactivates the MMP-1 promoter (see chapter 4), is mediated by membrane associated Src-Tyrosine kinases and Ha-Ras GTP binding proteins.[82] We also were able to show that irradiation of fibroblasts with visible light (wavelength > 450 nm) in the presence of rose bengal, attached to silica beads, results in an activation of MMP-1 mRNA levels. This result suggests that extracellularly generated singlet oxygen most likely is responsible.[83]

It has been demonstrated that the UVB mediated MMP-1 induction is based on activation of stress-activated protein kinases (SAPK), such as Jun N-terminal kinases (JNKs), which rapidly phosphorylate c-Jun, thus activating the AP-1 (heterodimeric c-jun/c-fos) transcription factor. Furthermore, an increase in c-jun synthesis increases the likelihood that the c-jun activator, rather than the inhibitor Jun D, will be present in the AP-1 complexes, resulting in greater transactivation through the AP-1 site of the MMP-1 promoter.[65] We were recently able to corroborate and extend the data showing that the iron driven Fenton reaction by generation of $OH^{\cdot}$ and lipid peroxides dramatically enhances the UVB dependent JNK2 activity, and c-jun transcription as well.[94] By contrast, no induction of the mitogen-activated protein kinase (MAPK), ERK-1 and ERK-2, was reported following UV treatment, [65] though in UV-independent settings ERKs are involved in the induction of c-fos expression and AP-1 activity.[84] It is possible that the MAPK-dependent signaling pathway is active in ROS mediated up-regulation of growth factors, though at this moment direct data are not available.

### *Role of ROS in Autocrine Cytokine Loops*

There is circumstantial evidence that the UVA and UVC induced up-regulation of matrix-metalloproteinases is mediated by signaling peptides, which comprise growth factors, cytokines, and interleukines. Using antisense strategies, function-blocking antibodies, and bioassays, we have provided evidence for an UVA-induced cytokine network consisting of interleukin-1α, interleukin-1β, and interleukin-6 which induced interstitial collagenase (MMP-1) via interrelated autocrine loops. While an early peak of IL-1 bioactivity at one hour post irradiation is responsible for the induction of IL-6 and together with IL-6 leads to an increase in MMP-1, the latter controls synthesis and release of IL-1 at a post-transcriptional level, and thus perpetuates the UV-response.[85,86] Similarly, Kraemer and coworkers showed that UVC-irradiation of HeLa cells induced the synthesis and release of IL-1 α and basic fibroblast growth factor (bFGF),[87] which together stimulate the synthesis of collagenase (MMP-1). Obviously, the UV-induction of collagenase (MMP-1) can be mediated by different cytokines, depending on whether the light is UVA, UVB, or UVC, and possibly on the cell type.

We now have indications that UVA-generation of singlet oxygen and $H_2O_2$ precedes the synthesis and release of IL-1α, IL-1β and IL-6. Accordingly, UV-generated ROS are rather initial intermediates in the complex signaling cascade. They finally lead to the up-regulation of matrix-metalloproteinases, and possibly connective tissue degradation. In this context, it may be of interest that $H_2O_2$ was shown to induce the activation of nuclear factor kappa B (NFkB), which is responsible for the up-regulation of IL-6 gene expression.[88] Also, Meier and coworkers[89] showed that IL-1 stimulates the release of ROS by human fibroblasts,

thus perpetuating the response. Hence, ROS may represent ideal targets for pharmacological intervention.

### *Redox Regulation of Transcription Factors*

Another line of investigation concerns the redox regulation of transcription factors, like AP-1, which transactivates the interstitial collagenase (MMP-1) gene, resulting in its enhanced expression. Most of this work, however, has been performed with epithelial cell lines. It is unclear whether redox regulation of AP-1 also plays a role in human dermal fibroblasts, and how the cytokine network would fit into this complex signaling cascade.

In HeLa cells, redox regulation of AP-1 DNA binding activity is mediated by a conserved cysteine residue, which is localized in the DNA binding domain of the AP-1 protein.[90] Substitution of Cys 154 in Fos and Cys 272 in Jun with a serine residue leads to enhanced DNA binding activity and a loss of redox control.[91] In the prooxidant state, the critical cysteine residue is converted to a state that does not effectively bind to DNA. The cysteine residue is in close contact with DNA, and treatment with reducing agents restores its DNA binding activity. There is evidence that AP-1 binding is regulated by a tightly controlled redox cascade involving the redox factor-1 (Ref-1) and thioredoxin.[90,91] Confocal immunocytology has identified Ref-1 to be localized in the nucleus. Immunodepletion analysis with function blocking antibodies against Ref-1, furthermore, suggests that Ref-1 is a major AP-1 redox activity in HeLa cell nuclear extracts. Interestingly, beside its redox controlling properties, Ref-1 possesses an apurinic/apyrimidinic (AP) endonuclease DNA repair activity, suggesting a link between regulation of transcription factors, oxidative signaling and repair processes of oxidative DNA damage in human cells.[93] It remains to be established whether Ref-1 plays a similarly essential role in fibroblasts, and whether it is induced upon UV-irradiation.

## Conclusions

Reactive oxygen species play a major role in the UV-dependent changes of matrix-metalloproteinase gene expression. The expected increase in the UV-irradiation on earth due to the depletion of the stratospheric ozone layer[4] will therefore contribute to the ROS load of the skin and will have consequences for matrix-metalloproteinase expression in different pathological states. Hence, a decrease in the ROS load by efficient sunscreens and protective agents may represent a promising strategy to prevent or at least minimize ROS induced cutaneous damage. The rational design of antioxidants for topical and systemic application depends on our understanding of the molecular mechanism and the identification of distinct oxygen species in the induction of matrix-metalloproteinase expression contributing to photocarcinogenesis and photoaging.

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CHAPTER 8

# Integrins as Regulators of Collagenase Expression

**Terhi Lehtinen and Jyrki Heino**

## Introduction

Cell adhesion has a central role in tissue integrity and organ formation. The integrin family is an important group of proteins mediating the attachment of cells to extracellular matrix (ECM) and facilitating cell movement on different connective tissue components. Integrins are comprised of two subunits $\alpha$ and $\beta$, which together form the functional receptor spanning the plasma membrane. Although most integrins are receptors for extracellular matrix molecules, some of them, especially leukocyte specific $\beta 2$ integrins and integrins containing the $\alpha 4$ subunit, adhere to other cell surface receptors like members of the immunoglobulin superfamily. To date, out of the 22 known integrin heterodimers, seventeen are known receptors for ECM proteins. Several integrins show overlapping ligand binding specificities, and it is not uncommon for one cell to express different integrins sharing the same ligand. Integrin receptors are also characterized by their ability to bind to more than one ligand molecule. Integrin expression is highly variable during development and many cytokines and growth factors have dramatic effects on integrin expression. Malignant transformation is also a potent regulator of integrin expression, both upregulating some integrin subunits and downregulating those integrins associated with a well differentiated cell phenotype.

Integrins do not merely anchor cells to their surroundings but also act as signal transducing proteins. The cell regulates integrin function by incompletely known mechanisms probably involving the interaction of integrin intracellular domains with other proteins, producing an altered conformation of the integrin extracellular domains. This type of regulation is known as "inside-out signaling". Once initiated, integrin ligation and clustering activate signal transduction pathways while integrins themselves do not have any intrinsic enzymatic activity signaling through them involves protein phosphorylation. Tyrosine phosphorylation of p125 focal adhesion associated kinase (FAK) is considered one of the key factors in integrin mediated signaling. Also closely associated with integrin derived signaling are accumulation of several signal transduction adapters, activation of Src, and the activity of other tyrosine kinases and protein kinase C (PKC). This type of signal also leads to alterations in intracellular $Ca^{2+}$ concentration and $H^+$ influx. Downstream events are associated with the ERK and JNK MAP-kinase pathway activation which eventually induced changes in gene expression. These pathways may cross, and induce changes in gene expression. Binding the same ligand by two different integrins may lead to distinct signals that might have even opposite effects on gene expression. Integrins may also function in an

*Collagenases*, edited by Warren Hoeffler. 

additive or synergistic fashion with growth factors such as platelet-derived growth factor (PDGF).

Integrin binding to extracellular matrix molecules is also a potent regulator of matrix metalloproteinase (MMP) genes. At least five integrins are known to generate signals altering the expression of MMPs and include receptors for collagens, laminins, fibronectin, vitronectin, and tenascin. Understanding the exact mechanisms by which integrins transduce signals may in the future reveal molecular mechanisms behind cancer cell invasion or other diseases involving altered MMP expression. Our own research focused on the action of collagen receptor integrins on osteogenic cells grown inside a three-dimensional collagen lattice. In this chapter we discuss the regulation of MMP expression by cell adhesion in general and investigate their possible importance in physiological and pathological processes.

## Extracellular Matrix Receptor Integrins

### *Structure and Function of Integrins*

Proper adhesion of cells to their surroundings is essential for most biological events during the lifespan of an individual. The members of integrin family mediate the interaction of cells with extracellular matrix and also participate in cell-cell binding (see references 1 & 2). Beginning with sperm-egg fusion,[3] integrin expression and functionality are important in phenomena like cell differentiation, tissue and organ formation, and angiogenesis. Integrins participate in wound healing and inflammation and in tumor cells some integrins are associated with a malignant cell phenotype and with metastasis formation. Further proof that integrins have a key role in tumorgenesis comes from studies where reconstituted expression of some integrins may return these cells to a differentiated phenotype.[4,5] Concomitant with contributing to cell adhesion, movement, division and differentiation, integrins are potent regulators of gene expression.[6–8] Integrins also mediate the effect of extracellular matrix as a cell surviving factor,[9,10] and are currently under intense scrutiny because they are putative targets for the therapy of many diseases.

Integrins can be defined in groups by the common β1, β2 or αV subunit.[1,2] To date 22 different integrin α/β heterodimers are known of which 17 are receptors for extracellular matrix molecules. Integrins are comprised of two noncovalently bound subunits, both having a single transmembrane segment and short cytoplasmic tail. There are eight β subunits (β1-β8), of which at least three (β1, β3 and β4) are expressed in alternatively spliced forms. Of the 16 known α subunits, at least four (α3, α6, α7 and αIIb) can be differentially spliced. The size of the β subunit varies from 90-110 kDa with the exception of the considerably larger 210 kDa β4 subunit. The sequence identity between them is 40-50%. The α subunits are slightly larger in size (150-200 kDa) and there is less similarity in their amino acid composition. The extracellular domains of both α and β subunits are thought to be involved in the ligand binding function. Some α subunits, including α1, α2, αD, αE, αL, αM and αX, contain an inserted-domain (I-domain), a domain of about 200 amino acids binding independently to ligand.[11] It is possible that a similar domain is also included in β subunits.[12] The I-domain in α subunits contains a $Mg^{2+}$ cation, which is essential for ligand binding.[13,14] $Ca^{2+}$ inhibits the function of I-domain integrins and ligand recognition by all integrins requires the presence of divalent cations.[14,15]

Integrins may adopt different conformations having different ligand binding affinities. This is especially important in phenomena where strong affinity to ligand would not be preferred until the cell has reached the correct site. In leukocytes the activation of β2 integrins is required before the receptor acquires the conformational enabling it to bind the ligand

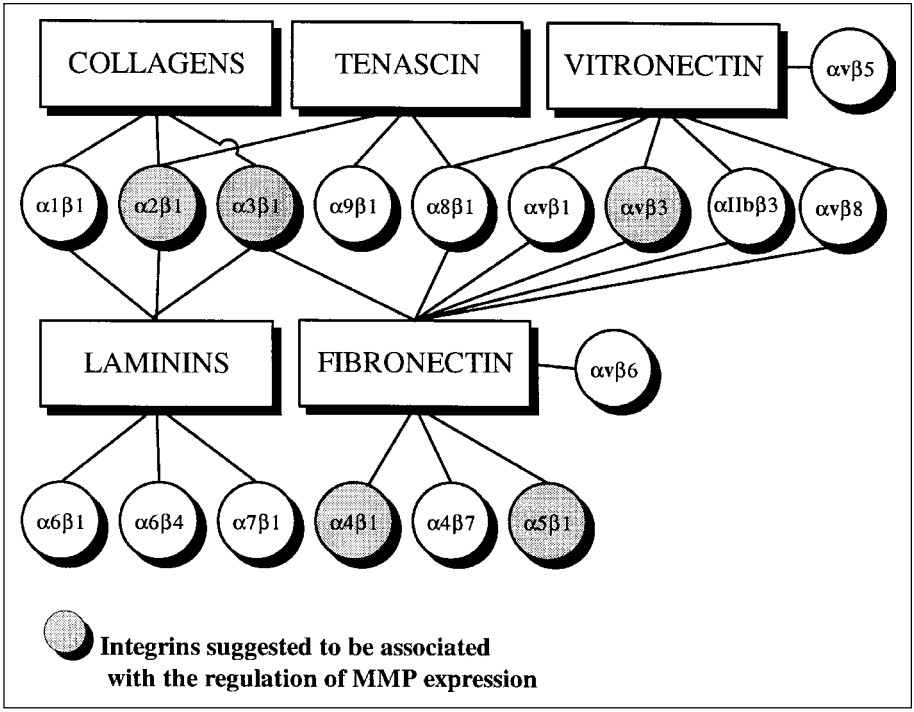


Fig.8.1. Extracellular matrix receptor integrins. 17 integrins mediating cell attachment to extracellular matrix molecules are shown. Each integrin is connected only to its most important ligand molecules.

molecule. This is called "inside-out signaling", and is mediated through cytoplasmic signals to which membrane proximal sequences of both integrin subunits are needed.[16]

### *Integrin Ligands*

Integrins serve as receptors for various matrix molecules, cell surface counter receptors and plasma proteins.[1,2] The ten members of the β1-integrin subfamily (α1-α9β1 and αVβ1), together with the αV-integrins (αVβ3, αVβ5, αVβ6, αVβ8) are the most important adhesion receptors mediating binding to ECM molecules (Fig. 8.1). Integrins α1β1, α2β1 and α3β1 can bind collagens, although the role of α3β1 integrin as a collagen receptor seems to be limited to only a few cell lines. The α1β1 and α2β1 binding sites are distinct, but both are in the triplehelical area of the collagen molecule.[17,18] Native collagen conformation is required as a binding site for both of these collagen receptors, whereas denatured collagen molecules can be recognized by fibronectin and vitronectin integrins binding to the arginine-glycine-aspartic acid (RGD) motif.[17] Six integrins can bind to laminins, namely the α1β1, α2β1, α3β1, α6β1, α7β1 and α6β4 heterodimers. Fibronectin is recognized by α3β1, α4β1, α5β1, α8β1, αVβ3 and α4β7 integrins; integrins α2β1, α8β1, α9β1 and αVβ3 bind tenascin; and α3β1 and αVβ3 bind entactin/nidogen. Vitronectin receptors include α8β1, αVβ1, αVβ3, αVβ5, αVβ8 and αIIbβ3 integrins. Another function of integrins is to mediate the attachment of cells to other cell's surface receptors, examples of which are intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), and E-cadherin. Fibrinogen,

components of the complement system and von Willebrand factor are other examples of proteins recognized by integrins.

Several pathogens are also known to exploit cell surface integrins. For example, viruses such as echovirus-1,[19] coxsackie virus A9,[20] and adenoviruses[21] need cell surface integrins for adhering to the cell surface or internalization. There is some evidence for intercellular adhesion of integrin-integrin type as well. α3 and α4 subunits are thought to be involved in a homotypic intercellular adhesion and α2-α3 binding has also been proposed.[22-24]

In general one integrin may recognize several ligands, and in fact, only few are known to bind to just one matrix molecule. The ligand binding specificity of an integrin receptor is often cell line specific[25] and it is not uncommon for a single cell to express several integrin receptors binding to the same ligand. It appears that different integrin-type receptors on a same cell have distinct roles. For example, two fibronectin receptors may recognize distinct domains inside the same fibronectin molecule. Furthermore, the binding of the same ligand molecule with two distinct integrins may lead to different and even opposite cellular responses.[8,26]

### *Integrin Expression*

With the exception of red blood cells, all major cell types express integrins.[1,2] Some integrins are cell type specific, e.g. αIIbβ3 integrin for platelets, but most integrins are expressed in a variety of tissues. The cellular integrin pattern is not stable, but regulated at different stages of differention as well as at the stimulation of growth factor and cytokine action. Peptide factors known to modify integrin gene expression include transforming growth factors-β (TGF-β),[27,28] interferon-γ (IFN-γ),[29] epidermal growth factor (EGF),[30] bone morphogenetic protein-2 (BMP-2),[31] platelet derived growth factor (PDGF),[32] interleukin-1 (IL-1),[33] and tumor necrosis factor α (TNF-α).[29,33]

While the expression of some integrins has been linked to malignant cell phenotype, most are considered markers of more differentiated cells.[4,5] Integrin α2β1 has been described as the melanoma progression antigen[34] and it is upregulated by malignant transformation of osteogenic cells.[35] In vitro studies, α2β1 enhances the invasion of cells through type I collagen gels and basement membranes.[36,37] Overexpression of α5β1 integrin on hamster ovary cells reduces their tumorigenity.[38] While in highly invasive colon carcinoma cells the integrin is expressed in elevated levels compared to less malignant ones, suggesting that in these cells α5β1 integrin contributes to malignant progression.[39] Integrin αVβ3 is needed for angiogenesis and is often upregulated in cells with invasive and metastatic capacity.[40] Antagonists for αVβ3 integrin successfully disrupted human tumors transplanted onto chick chorioallantoid membrane.[41] Using differential display to evaluate mRNA, it has been shown that α6 can function as a putative tumor suppressor gene in breast cancer cells.[42]

## Osteogenic Cells Inside Collagen Lattices as a Model for Cell–Matrix Interaction

### *Integrin Expression on Osteogenic Cells*

We had several reasons to select osteogenic cell lines, instead of fibroblasts, for use in our experiments. Most importantly, several cell lines with different cell surface collagen receptor integrin patterns are readily available (all the cell lines mentioned here are available from American Type Culture Collection). For example, MG-63 cells express predominantly α2β1 integrin, whereas α1β1 integrin expression level is very low or entirely missing[28,33,43] HOS and Saos-2 cells predominantly express α1β1 integrin and the expression level of α2β1 integrin is low or can not be detected.[35,36,43] However, the HOS cell line has turned out to be problematic because after intensive subculturing, α2β1 integrin levels rise.

Some laboratories seem to have HOS cell lines expressing α2β1 integrin exclusively. By using flow cytometry, Northern blot hybridizations, or immunoprecipitations, we have never been able to detect α2 integrin in Saos-2, even in cells that have been subcultured for several passages.

Originally, MG-63, HOS and Saos-2 cells were derived from osteogenic tumors, more precisely, from osteosarcomas. However, they do not behave like malignant cells and grow in soft agar or generate tumors in nude mice. In cell culture they differentiate under appropriate conditions, but by treating HOS cells with a chemical mutagen, like N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or exposing them to murine Kirsten sarcoma virus, tumorigenic derivatives of HOS cells are generated (in this case, named HOS-MNNG, and KHOS-NP). These cell lines show, in addition to α1β1 integrin, strongly enhanced of α2β1 integrin[35] expression. Two nontumorigenic revertants of KHOS-NP, KHOS-240 and KHOS-312, have moderate expression of both α1β1 and α2β1 integrins.[35,36] The link between α2β1 integrin expression and malignant cell phenotype in osteogenic cell lines also raises the question regarding the putative contribution done by α2β1 integrin to the cancer cell behavior. The fact that α2β1 integrin is associated with melanoma progression and invasion makes the function of this integrin even more interesting.[34,37]

In addition to collagen receptor integrins, all the osteogenic cell lines mentioned above express α3β1 and α5β1 integrins. We have recently analyzed αV integrin expression on Saos-2 cells and they seem to have three αV containing heterodimers: αVβ3, αVβ5, and αVβ6 (Koistinen and Heino, unpublished results).

Distinguishing between the effects of different β1 integrins can be done by blocking their function with specific antibodies. The problem with anti-α chain antibodies is that they rarely are efficient enough. Antibodies against β1 subunit are more potent, but depending on the expression pattern on the cell surface, they usually inhibit several integrin heterodimers. A more efficient method is to alter the cell surface integrin expression by cDNA transfections. We have described a model of integrin heterodimer formation and their maturation as glycoproteins.[44,45] According to our observations in a wide variety of cell lines, precursor β1 integrin is produced in large excess over the precursor α subunits. The excess pool of precursor β1 integrin accumulates in the endoplasmic reticulum, where β1 subunit awaits an α subunit to form the α/β complex which allows transport to the Golgi and finally to the cell surface.[44] Due to the large intracellular pool of precursor β1 subunit, the overexpression of integrin α subunits alone is often enough for efficient surface expression. However, in cell types that lack an adequate precursor β pool, transfection may alter the expression of other integrins.

To this end, we have successfully transfected α2 integrin subunit to α2 integrin negative HOS and Saos-2 cell lines. A commonly used cytomegalovirus promoter was too weak to drive the expression of integrin subunits, so we instead used a construct carrying a spleen focus forming virus LTR promoter.[46] The stable expression of an integrin was achieved by having the neomycin analogue, G418, resistance gene in the same expression construct. The increased expression of α2β1 integrin was assessed by measuring the plasmid derived mRNA with Northern blot analysis and the protein levels with immunoprecipitation. Flow cytometry was used to measure the cell surface expression.

We have also used the antisense approach to downregulate the integrin expression.[46] This was accomplished by using the same construct carrying the α2 integrin sequence in an antisense orientation. We found this approach efficient since the surface expression of α2β1 integrin on antisense transfected cells was reduced to 10-50% when compared to the original cells.[46] The antisense approach has also successfully reduced the amount of β1 integrin synthesis.[47] Our repeated attempts to manipulate the expression of other integrin subunits

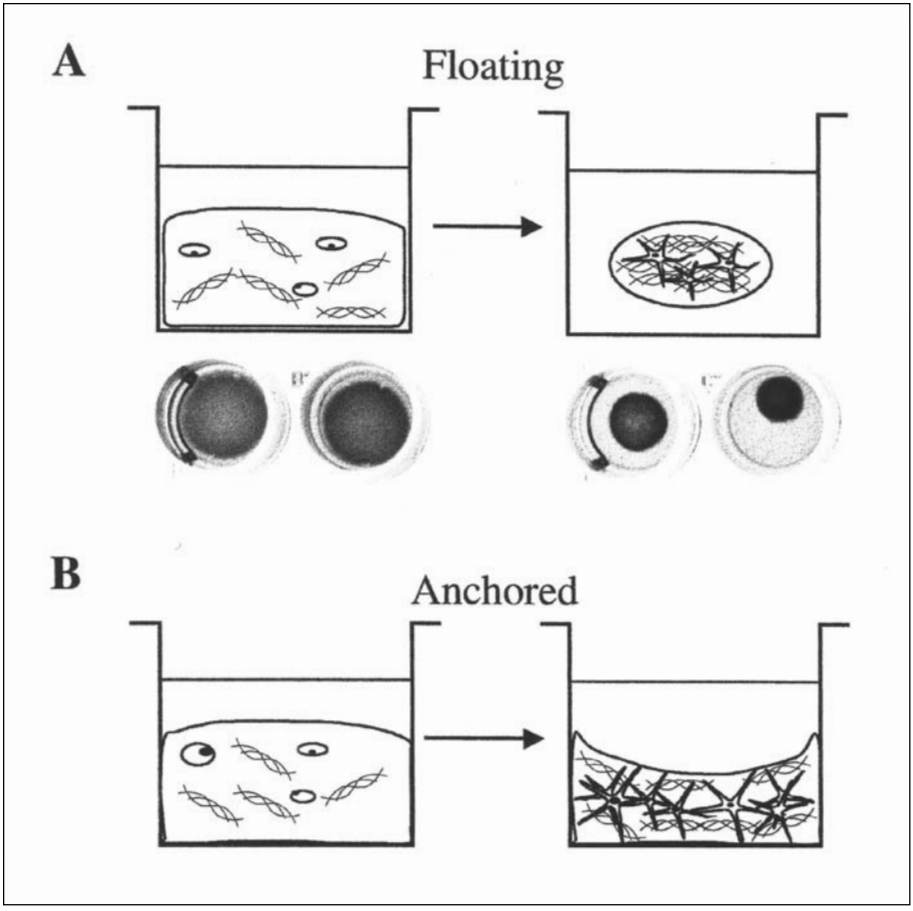


Fig.8.2. A schematic picture of in vitro models for collagen fiber reorganization. A. In a floating collagen gel model, the gel is detached from the sides and the bottom of the cell culture well after the gelation is complete. The cells begin to rearrange the collagen fibers until a dense collagen lattice is formed. B. In an anchored collagen gel, the end result is a stressed tissue like structure containing cells with prominent actin stress fibers. Unlike floating gels, the cells plated here continue to synthesize DNA and increase in cell number.

by the same system have however failed. In general, only a few reports have indicated successful use of antisense strategies in the reduction of integrin expression.

### *Collagen Gel as a Model for Tissue Organization*

Three-dimensional collagen gel is commonly used as a differentation substrate for various cell lines because it supports the formation of differentiated structures like the glands in colon carcinoma cells[48] or duct like structures in mammary tumor cells.[49] Culturing skin fibroblasts inside a collagenous environment has also been recognized as a valuable tool for studying collagen fiber reorganization during wound healing.[50]

Collagen gels are usually prepared from acidic solutions rich in type I collagen. When neutralized, the solution gelates during incubation at 37°C. Prior to gelation, the cells are

mixed within the solution and trapped into this three-dimensional collagen environment. The two types of collagen gels typically used in in vitro studies are floating, where the gel is detached from sides of the cell culture wells, and anchored, where the gel adheres to the sides (Fig. 8.2).

Skin fibroblasts inside a floating collagen gel can be used to mimic the formation of dermis.[50] When embedded inside the three-dimensional collagenous matrix, originally loose network of collagen fibers becomes a dense tissue like structure.[51] This ability to reorganize collagen fibers can be measured by assessing gel reductions in the area. The force needed to retract the gel is derived from the spreading and elongation of cells inside the gel.[52,53] Adherence of cells attempting to migrate on the proximal collagen fibers on them leads to rearrangement of the collagen network, producing a tightly packaged collagen gel. Called collagen gel contraction this process requires an intact cytoskeleton and the presence of serum. This model has been used to mimic wound healing and has many quantifiable effects on cell behavior.[50] Actin stress fibers are needed for a proper wound contraction, and in this model fibroblasts also start to resemble myofibroblasts with their large bundles of actin stress fibers.[54] There is a decline in cellular DNA synthesis, indicating that the cells do not proliferate under these conditions.[55] The simultaneous decrease in collagen mRNA is accompanied by the increased expression of MMP-1.[56] Growth factors responsible for stimulating the wound healing process, of which TGF-β and PDGF are examples, have been used to enhance the ability of fibroblasts to contract collagen gels. [57, 58]

Another model for cell collagen interaction is to embed the cells in an anchored collagen gel,[50] which morphologically resembles granulation tissue rather than dermis. Inside these gels fibroblasts continue to synthesize DNA and increase in cell number.[55]

In our own studies we have shown that osteogenic cell lines in general behave like fibroblasts when inside this collagen lattice. With some exceptions, they promote the contraction of gels, a process which TGF-β enhances.[46] Osteogenic cell number, do not increase inside floating gels,[59] and thus these phenomena are not restricted to fibroblasts.

Type I collagen is the most abundant matrix molecule in bone and therefore, culturing osteogenic cells inside three-dimensional collagenous environment mimics their normal growth conditions. Reorganization of the collagenous matrix around cells may also use similar mechanisms to bone fracture healing. This model enables researchers to test the effects of growth factors such as TGF-β in the modulation of osteoblast metabolism and behavior. Experimental approaches like this facilitates the study of individual collagen receptors that is not possible with fibroblasts. Given the fundamentally similar behavior of fibroblasts and osteogenic cells inside collagen gels, we suggest that observations conducted with our research model may be relevant with other mesenchymal cells as well.

### *The Functions of Collagen Receptor Integrins*

The adhesion of osteogenic cell lines to collagen is mediated by α1β1 and α2β1 integrins. Integrin α3β1 might also to be a receptor for collagens, but, at least in Saos-2 cells, anti-α3 integrin antibody blocks adhesion to laminin-5 only.[31] Overexpression of α2 integrin in osteogenic cells increases cell adhesion to type I collagen but not to type IV collagen or laminin-1.[46]

We measured the migration of HOS cells on collagen using an assay in which cell culture plates were coated with type I collagen. In a metal cylinder, the cells adhere to an area of 6 $mm^2$ in the culture well. After removing the cylinder and nonadherent cells, the migration rate is easily assessed by staining the cells at different time points or under different conditions.[36] The migration rate of chemically transformed MNNG-HOS cells was significantly faster than the migration rate of HOS cells.[36] Furthermore, overexpression of α2 integrin in

HOS cells increased their ability to move on collagen, indicating the importance of α2β1 heterodimer in cell migration.[36]

In addition to cell adhesion and migration α2β1 integrin has been connected to reorganization of pericellular collagen fibers.[60] This reorganization can be viewed as a contraction of floating collagen lattices.[60] Our experiments have shown that HOS and Saos-2 cells are unable to contract collagen gels, whereas osteogenic cells expressing α2 integrin can.[46] Treatment of MG-63 cells with TGF-β increases contraction and TGF-β enhanced α2β1 integrin expression is alone sufficient to explain the phenomenon.[46]

In osteogenic cells, both α1β1 and α2β1 integrin mediate cell adhesion to collagens, whereas α2β1 integrin is essential for cell migration on collagen and collagen gel contraction.[36,46] In these phenomena, α1β1 integrin can not replace α2β1 but, reports by others indicate that in other cell types, including smooth muscle cells, α1β1 integrin might share some of these functions with α2β1.[61]

### *Regulation of MMP and Other Genes in Osteogenic Cells by Integrins*

Integrin-mediated signals activate regulatory pathways modulating gene expression.[62,63] We have recently used RT-PCR based RNA fingerprinting techniques[64] to compare RNAs expressed in α2 integrin negative (vector transfected) and positive (α2 cDNA transfected) Saos-2 cells both in monolayer and inside three-dimensional collagen gels. The numbers of differentially expressed mRNAs clearly indicate that in Saos-2 cells, α2β1 integrin-type I collagen interaction more potently modulates gene expression than does α1β1 integrin-collagen interaction, or general change from monolayer to three-dimensional growth conditions together (Lehtinen, Koivisto, Ralph, Heino, manuscript in preparation).

When fibroblasts are embedded inside collagen gels, the expression of MMP-1 is increased and the expression of type I collagen is reduced.[56] The fact that different osteogenic cells show distinct collagen receptor patterns made it possible to analyze whether these patterns could be correlated with the expression of either MMP-1 or collagen. The cellular effects of different osteogenic cells inside collagenous environment differed greatly. MG-63 cells inside collagen induced MMP-1 expression, but did not reduce α1(I) collagen mRNA levels.[8] HOS cells and KHOS-240 cells decreased collagen mRNA levels, but only the latter induced MMP-1.[8] Thus, in osteogenic cells the presence of α1β1 integrin correlated with the reduction of collagen expression, and the presence of α2β1 with the increase in MMP-1 expression. Experiments with sense and antisense α2 integrin cDNA transfected MG-63 cells confirmed a correlation between cell surface expression levels of α2β1 integrin and the cellular MMP-1 mRNA levels.[8] Further evidence for distinct of α1β1 and α2β1 signaling pathways came from experiments indicating that the induced expression of MMP-1 can be inhibited by tyrosine kinase inhibitors, which constrastingly do not effect type I collagen expression.[59]

In our experiments, we noticed the importance of three-dimensional structure in mediating MMP-1 expression since cells plated on cell culture wells coated with collagen express minute amounts of MMP-1. A similar phenomenon has been demonstrated using fibroblasts.[65] It appears, therefore, that three-dimensional collagenous structure is a more suitable environment than monolayer cultures for studying cell-collagen interaction, at least when investigating the wound or tissue healing processes which also have cells in contact with three-dimensional extracellular collagen.

Inside a collagenous environment, the two collagen binding integrins clearly have separate roles, the one upregulating MMP-1 expression and the other downregulating type I collagen expression. Both processes contribute to the same end: preventing the accumulation of collagen in unproper amounts. However, it is unknown whether the two effects act simultaneously or whether they can compensate for each other. In bone fracture healing,

the two integrins might be needed at different stages. One hypothesis is that α2β1 integrin mediates the degradation and reorganization of newly found matrix, and at the end of the healing process, α1β1 integrin replaces α2β1 integrin and downregulates collagen synthesis and thereby inhibits the formation of excess collagen. Bone matrix constantly remodels under normal conditions, and this activity may also be partially regulated by α2β1 integrin derived signals.

## Integrins as Regulators of MMP Expression

### *Integrins Associated With Altered MMP Expression*

A combination of approaches have attempted to identify integrins role in MMP regulation. Culturing cells on or inside different substrata[8,65–68] can alter MMP expression, the integrin ligation with specific antibodies has triggered the expression of certain MMPs[40,69-72] and in situ localization has shown coordinate regulation of integrins and MMPs.[73,74] Altered MMP expression has been connected to several integrin type receptors (Table 8.1). Studies thus far have revealed five different integrins purportedly involved in the regulating MMP expression. These integrins include receptors for collagens, fibronectin, and vitronectin. However, the ligand responsible for MMP induction is not necessarily known since some results have been obtained by antibodies binding the integrin receptor only.[40,69–72] The inductions seen in MMP expression may not always be due to binding to a single ligand but could require a combination of ligand molecules.

### *Collagen Receptor Integrins and MMP Expression*

During wound healing, keratinocytes express MMP-1 when in contact with type I collagen.[73] This expression is absent in cells contacting intact basement membrane, similar to their in vivo characteristics, cultured keratinocytes on type I collagen coated culture plates also produce MMP-1.[75] In conditions that require degradation of type I collagen, the collagen receptor mediated signals through cell membrane seems to trigger the expression of MMP-1. MMP-1 action denatures collagen and uncovers the binding sites for integrins recognizing the RGD sequence.

There are two integrins responsible for cell collagen interaction, α1β1 and α2β1. As described above, we have used several different osteogenic cell lines in the collagen gel model to show that α2β1 integrin is responsible for signals regulating MMP-1 expression and that in these cells, α1β1 integrin can not replace α2β1.[8] Others have offered similar results, demonstrating with collagen-exposed human skin fibroblasts that functional antibodies against α2 integrin in combination with anti-β1 integrin antibodies can block the induction of MMP-1.[65]

### *Fibronectin Receptor Integrins and MMP Expression*

Two fibronectin binding integrins, α4β1 and α5β1 heterodimers, are known to alter the expression of MMPs.[6,26] They recognize different binding motifs in fibronectin sequences and have opposite effects on MMP expression. Integrin α5β1, the classical fibronectin (FN) receptor binding the RGD motif, has been implicated in the growth regulation of tumor cells. In rabbit synovial fibroblasts plated on intact FN, the expression of MMP-1, MMP-3 and MMP-9 is not up-regulated. However, the induction has been seen with cells plated on cell binding fragments of fibronectin or on a surface coated with antibody binding to α5β1 integrin or mixed substrates of FN and tenascin.[67] The increase was detectable within 2-4 hours of plating the cells, suggesting that the regulation of collagenase gene expression was a direct response to integrin signaling. Binding of integrin α4β1 on rabbit synoviocytes to fibronectin suppresses the expression of MMP-1, MMP-3 and MMP-9.[26]

***Table 8.1. Regulation of matrix metalloproteinase gene expression by integrins.***

| MMP | Integrin | Ligand | Cell type | Reference |
|---|---|---|---|---|
| MMP–1 ↑ | α2β1 | type I collagen | osteogenic cells, human | Riikonen et al, 1995[8] |
| | | | fibroblasts, human | Langholtz et al, 1995[65] |
| | α5β1 | fibronectin (RGD) | synovial fibroblasts, rabbit | Huhtala et al, 1995[26] |
| MMP–1 ↓ | α4β1 | fibronectin (CS–1) | synovial fibroblasts, rabbit | Huhtala et al, 1995[26] |
| MMP–2 ↑ | α3β1 | anti–α3 MAb | rhabdomyosarcoma cells, human | Kubota et al, 1997[72] |
| | α3β1 | anti–α3β1 MAb | glioma cells, human | Chintala et al, 1996[71] |
| | α5β1 | anti–α5β1 MAb | glioma cells, human | Chintala et al, 1996[71] |
| | α5β1 | anti–α5β1 MAb | melanoma cells, human | Seftor et al, 1993[70] |
| | αVβ3 | anti–αVβ3 MAb | melanoma cells, human | Seftor et al, 1992[40] |
| | αVβ3 | vitronectin | melanoma cells, human | Seftor et al, 1992[40] |
| MMP–3 ↑ | α5β1 | fibronectin (RGD) | synovial fibroblasts, rabbit | Huhtala et al, 1995[26] |
| MMP–3 ↓ | α4β1 | fibronectin (CS–1) | synovial fibroblasts, rabbit | Huhtala et al, 1995[26] |
| MMP–9 ↑ | α3β1 | anti–α3 and β1 MAbs | mucosal keratinocytes, human | Larjava et al, 1993[69] |
| | α5β1 | fibronectin (RGD) | synovial fibroblasts, rabbit | Huhtala et al, 1995[26] |
| MMP–9 ↓ | α4β1 | fibronectin (CS–1) | synovial fibroblasts, rabbit | Huhtala et al, 1995[26] |

The fact that cellular response to fibronectin depends on the fibronectin receptor used in cell adhesion explains the need for several receptors to bind one ligand. Our results with collagen binding integrins are in agreement with this conclusion.[8] In the case of matrix degradation, this means that changes in cellular integrin pattern may precede altered MMP production.

### *Vitronectin Receptor Integrins and MMP Expression*

Cells bind to vitronectin through αV-integrins and when the small dermatan sulfate proteoglycan decorin is present with vitronectin, this binding is accompanied with induced MMP-1 expression.[68] This induction was also observed when decorin was present with the exposed cell binding domain of fibronectin, but not when intact fibronectin was used. αVβ3 heterodimer, is the most abignitions of all integrins and binds to vitronectin, fibronectin, fibrinogen, laminin, tenascin, denatured collagen, von Willebrand factor and osteopontin. It plays a significant role in angiogenesis, is expressed in both migratory and metastatic cells,[40,41] and in addition to cell motility can regulate matrix degradation. In vivo, αVβ3 is localized on the cell surface with MMP-2 in a functionally active form in vivo in angiogenic blood vessels and melanoma tumors.[76] It can not bind to native collagen, but may interact with denatured collagen, and therefore could possibly regulate collagen degradation and the organization of the resulting fragments. A positive correlation between β3 integrin and MMP-9 has already been suggested in human melanoma cells grown in nude mice.[74]

### *Antibody–Induced MMP Expression*

In our laboratory, we have used antibodies against α3 and β1 integrins in human keratinocytes to show that the expression of MMP-9 is induced.[69] Antibodies against α2 and α3 integrin have been reported to induce the activated form of MMP-2 and to enhance the secretion of proMMP-2 in human rhabdomyosarcoma cells.[72] The same antibodies enhanced the invasion of these cells through a reconstituted basement membrane.

Many different studies demonstrate the variety of antibodies capable of triggering MMP-2 expression. In melanoma cells it is antibodies against α5β1 and αVβ3 integrins,[70] in glioma cells antibodies against α3β1 and α5β1,[71] and in rhabdomyosarcoma cells anti-α2 and anti-α3 antibodies.[72]

When antibodies are employed to trigger MMP expression, it is often difficult to distinguish whether activation results from receptor activity or inhibition of ligand binding. It is also difficult to know beyond an intelligent guess which native ligand would have the same effect since integrins often recognize several ligand molecules.

## Integrin Signaling Leading to Altered Gene Expression

### *Integrin Induced Signals*

Integrin ligation leads to the formation of focal adhesion sites, whereas further signaling events, like protein phosphorylation require receptor clustering.[62,63,77] Second messengers involved in integrin signaling include intracellular $Ca^{2+}$ and phospholipid metabolite precussors to PKC activation.[78] Integrin mediated cell spreading is accompanied with $H^+$ influx, tyrosine phosphorylation of FAK and activation of at least c-Src, c-Fyn, c-Yes and c-Syk tyrosine kinases. Integrin signaling also leads to accumulation of signal transduction proteins such as Grb-2 and Sos, activation of Ras and MAP kinase, and the ERK and JNK pathways. These signals are relayed inside the cell and change in gene expression patterns and cellular events such as growth regulation.[62,63,77]

Integrin signals may converge with signals from other sources and as well and may function in additive or synergistic fashion with growth factors such as PDGF.[79,80] Adhesion

of fibroblasts to FN boosts $PIP_2$ synthesis and also modulates its hydrolysis in response to PDGF.[79] Mammary cells bound to basement membrane components through integrins resulted in milk protein secretion in response to prolactin, supporting the idea that integrin signals converge with signals from soluble factors rather than act alone.[81]

Neither the α or β subunit of the integrin heterodimer possess any intrinsic enzymatic activity, but a proposed model for integrin derived signaling suggests that cytoplasmic tails bind to specific molecules and initiate signaling upon integrin ligation or clustering. After integrin ligation, large protein complexes are formed as receptors, enzymes, enzyme substrates and cytoskeletal proteins gather together at the focal adhesion sites,[62,63,77] which form the framework for signaling cascades. FAK, also located at these structures, is one of the molecules closely connected to integrin derived signaling.[82] It is likely that signals from receptor tyrosine kinases and G-protein coupled receptors can also be mediated through FAK and synergize with integrin signals since FAK is also phosphorylated after treatment of cells with PDGF, neuropeptides or lysophosphatidyl acid (LPA).[83] It has also been proposed that PKC activity and calcium transients are needed as costimulates before integrin mediated FAK phosphorylation occurs.[84] The activation of epidermal growth factor receptor, or the activation of downstream PKC leads to increased αVβ5 integrin-mediated migration of human pancreatic carcinoma cells.[85] A small GTP binding protein named Rho is involved in this process and may also play a key role in integrating signals induced by integrins and growth factors.[85]

The cytoplasmic membrane proximal 13 amino acids of β1, β2 and β3 subunits bind to FAK.[86] The cytoskeletal protein paxillin is constitutively associated with FAK and the same proline rich region in FAK can be bound by the SH3 domain in c-Cas in an adhesion induced manner.[87,88] The aggregation of integrin receptors to focal adhesion sites must be accompanied with ligand binding occupancy in order for integrins to induce the recruitment of actin containing cytoskeleton. The induction of FAK requires intact cytoskeleton, and in addition to paxillin, cytoskeletal proteins talin and possibly also vinculin can bind to it.[88-90]

In addition to FAK, other β1-integrin cytoplasmic tail binding kinases have been found. Integrin linked kinase (ILK) colocalizes with β1 integrin in focal adhesions.[91] It phosphorylates the β1 integrin intracellular domain, and overexpression of it can disrupt epithelial cell architecture and inhibit cell adhesion. This indicates that it may play a role inside out signaling events rather than in integrin triggered signaling. Calreticulin is an intracellular $Ca^{2+}$ binding protein which regulates gene expression by interacting with the DNA-binding domains of nuclear hormone receptors. It can also bind to an amino acid motif in the intracellular domain of all α-subunits.[92] The downregulation of calreticulin inhibits the ability of several cells to attach and spread on extracellular matrix components, supporting the conclusion that it regulates the affinity state of integrins. Whether it mediates signals from integrins to the cell nucleus is unknown at this point. After cells are treated with PDGF or insulin integrin αVβ3 associates with two intracellular proteins. The function of p190 tyrosine phosphorylated protein[93] is not understood, but the tyrosine phosphorylated insulin receptor substrate-1 (IRS-1)[94] may be involved in the proliferation of cells exposed to vitronectin and treated with insulin.

Integrin ligation leads to autophosphorylation of tyrosine 397 on FAK, which then serves as a high affinity binding site for kinases including Csk, Fyn and Src. The Src family kinases may further induce the phosphorylation of paxillin, tensin and FAK.[95] The phosphorylation events recruit kinases and nucleotide exchange proteins leading to activation of the Ras and Rho signaling pathways. Another possible candidate for mediating the integrin derived signals leading to activation of Ras-pathway is the Shc adapter protein. When Shc is phosphorylated on its tyrosine residues, it recruits growth factor receptor-bound protein 2

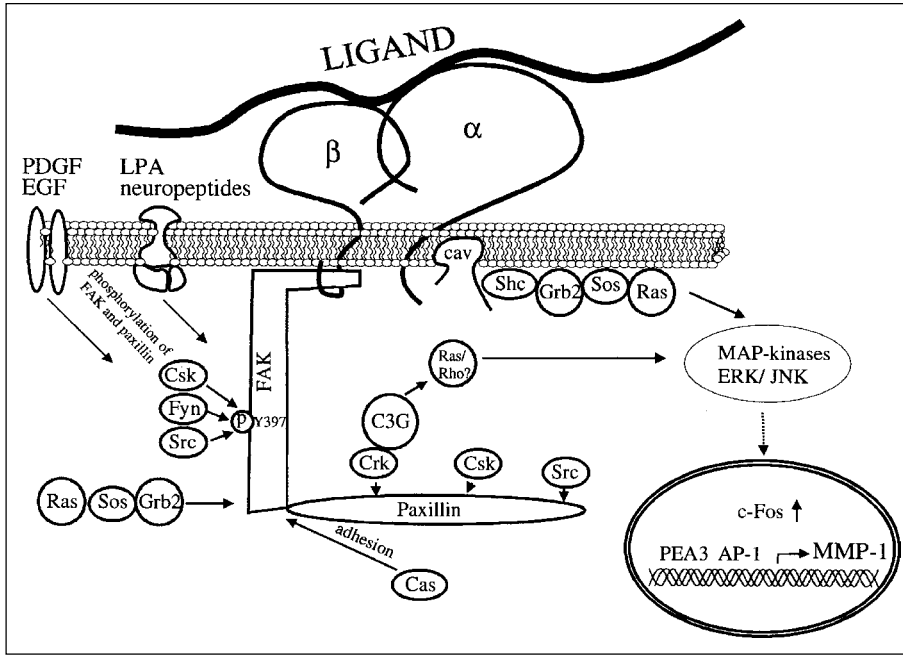


Fig.8.3. Putative signaling cascades leading to altered expression of MMP–1. After ligand binding and integrin clustering, FAK is autophosphorylated on tyrosine 397. This site may serve as a binding site for Src or Fyn kinases which in turn may phosphorylate the binding site for the Grb-2/Sos complex on FAK. Binding of Csk to autophosphorylated FAK may downregulate the activity of Src. The cytoskeletal protein paxillin is constitutively associated with FAK, whereas adhesion induces the binding of Cas to the same site on FAK. Treatment of cells with growth factors such as PDGF, FGF, or neuropeptides, or LPA induce signals from receptor tyrosine kinases or G–protein coupled receptors leading to phosphorylation of FAK and paxillin. The membrane protein caveolin links a subset of integrins to the Shc adapter protein which also may recruit the Grb–2/Sos complex and lead to in MAPK/ERK/JNK pathway activation. Possible candidates for Cas phosphorylation include Src, Crk and Abl. The activation of the Ras signaling pathway ending in MAPK/ERK/JNK activation and eventually to upregulation of c–Fos and MMP–1 may include signals derived from phosphorylation and signal complex formation from FAK, paxillin or caveolin. An additional stimulus by activated PKC may also be needed.

(Grb-2), a molecule constitutively associated with the ras GDP/GTP exchange protein Sos.[96] This complex promotes the activation of GTP-Ras activating serine/threonine kinase cascades, which include Raf, MAPKK and MAPK.

Cas was first described as a protein phosphorylated after cellular transformation by v-Crk and v-Src. The transformation associated phosphorylation of Cas is independent of cell adhesion. Its role in normal untransformed cells remains veiled, but it is known to bind to the proline rich region in the catalytic region of FAK.[97] The adhesion induced route through Cas- FAK association may involve c-Crk, c-Src or c-Abl binding to activated Cas, leading to further activation of C3G/Sos complex and the Ras signaling pathway.[98]

### *Integrin Signaling Leading to MMP Gene Activation*

Figure 8.2. summarizes signal transduction pathways and events that have putative importance in integrin mediated regulation of MMP expression. The molecular basis for

the specificity of individual integrins signaling is largely unknown. For example all integrins involved in regulation of MMP expression can activate FAK, and yet signals sent through different integrins leads to varied or even opposite effects on MMP expression.[6,26] Based on these variable effects, differential activation of FAK binding proteins or specific signals arising through integrin α subunits must be involved. The recently discovered association of a subset of β1 integrins with the cell surface membrane protein caveolin couples these integrins to the adapter protein Shc, and therefore to a distinct signaling pathway.[99] Integrins α1β1, α5β1 and αVβ3, but not α2β1, α3β1, or α6β1 associate with Shc through caveolin.[99] Caveolin is a membrane protein forming striated coatings in the flask shaped membrane invaginations known as caveolae purportedly important in membrane trafficking as well as signal transduction. These membrane structures have been seen in conjunction with several G-protein coupled receptors. However, whether the association to caveolin explains the fact that α1β1 integrin does not regulate MMP-1 expression in the same way as α2β1 integrin remains to be shown.

Integrin α2β1-dependent induction of MMP-1 in collagen coated cells may include both transcriptional activation of the MMP-1 gene and an increased half-life of its mRNA.[100] Experiments showing that chemical inhibitors of tyrosine kinases and protein kinase C can inhibit MMP-1 induction have yielded some clues about the signaling events involved in the process.[59,65,75] The three-dimensional collagen gel has been shown to induce the activity of a PKC-ζ isoform,[101] a component of the matrix stimulatory pathway which increases expression of α2β1 integrin and MMP-1 in cells cultured inside collagen gels.[101] Activation of PKC-ζ leads to activation of a transcription factor NF-κB may therefore be important regulators of integrin function inside collagen gels as well.

MMP-1 induction by α5β1 integrin binding to fibronectin is modulated by the PEA3 and AP-1 binding sites in MMP-1 gene promoter.[102] After plating the cells, the c-fos mRNA levels increased within 1 h, and c-Fos protein a correlated to collagenase expression, started to accumulate the nucleus after 10 min. Nuclear c-Jun was also substantially higher in cells on fibronectin fragments than in cells on intact fibronectin.[102] The expression of MMP-1 and MMP-2 was significantly decreased in cultures incubated with an antisense fos oligonucleotide,[102] demonstrating its importance in transcriptional activation. Data derived from reporter gene constructs suggest that integrin derived signals regulate the -1391/-67 segment of MMP-1 gene containing vital AP-1 and PEA3 sites. The fact that integrins can regulate the activity or expression of AP-1 makes it possible that integrin signaling pathways intersect with signals generated by other receptor pathways. With cells in suspension, overexpression of α5β1 integrin inhibits cell entry into S-phase and downregulates c-fos, c-jun and jun B. On the other hand, in cells adhering to fibronectin, α5β1 integrin enhances their expression.[103] This evidence leads us to believe that AP-1 nuclear factors are an integral part of the pathway triggered by integrin derived signals.

## Integrin Induced MMP Expression in Health and Disease

That extracellular matrix regulates cell behavior by integrin-mediated signals is a basic and well accepted principal. In cell culture models, the expression of at least four MMPs, namely MMP-1, -2, -3, and -9 seem to be modified by integrins. However, there is less information about in vivo conditions in which similar regulation could play essential role. In healing wounds MMP-1 is expressed by cells in contact with type I collagen.[73] It is also tempting to speculate that during embryogenesis migrating cells are able to pass through molecular barriers by targeted proteolysis preceded by recognition of the substrate.

MMPs are essential for cancer cell invasion and therefore also for metastasis formation. In tumors, MMPs are constantly produced by either the cancer cells themselves or stromal cells induced by the cancer cells. A third mechanism of matrix degradation is pro-

vided by the model that holds that matrix recognition by adhesion receptors leads to the temporary induction of MMPs. In tumor growth and metastasis formation, angiogenesis is essential, and both integrins and MMPs are strongly involved in this process as well.

To conclude, the cell culture experiments demonstrate the importance of integrins in the regulation of targeted matrix degradation. Given the complex nature of the phenomenon, which requires a complex relationship between three groups of proteins—integrins, MMPs and connective tissue components—it is not surprising that the process is incompletely characterized at the tissue level. Further studies directed at elucidating the MMP expression associated to specific cell-matrix contacts may revel new mechanisms in physiological cell migration as well as in cancer invasion.

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CHAPTER 9

# Pharmacological Inhibition of Collagenases

**Dennis H. Oh and Warren Hoeffler**

## Introduction

Collagen is the major structural protein in humans and other vertebrates, constituting the majority of the dry weight of tissues such as bone and the dermis of the skin.[1] The synthesis and degradation of collagen therefore is crucial to the homeostasis of connective tissue, and an imbalance of the two processes plays a role both in normal physiological processes such as developmental tissue remodeling and wound healing and in certain important pathological processes as well. Interstitial collagenase or matrix metalloproteinase-1 (MMP-1) catalyzes the essential and rate-limiting step in types I, II and III collagen degradation by site-specifically cleaving the collagen triple helix into three-quarter and one-quarter length fragments that subsequently denature and become substrates for collagenase and other proteases. The other collagenases—neutrophil collagenase or MMP-8, and collagenase-3 or MMP-13—also cleave collagen into similar fragments, but at slightly different sites.[2] When collagenases are abnormally expressed or activated or unchecked by their natural inhibitors, pathological connective tissue destruction can occur and is thought to participate in the pathogenesis of diseases such as rheumatoid arthritis and osteoarthritis,[3] periodontitis,[4] epidermolysis bullosa dystrophica and other chronic ulcerative diseases,[5] and malignant tumor invasion and metastasis.[6] Collagenases are therefore obvious therapeutic targets for controlling these diseases, and inhibitors which specifically decrease the activity of this class of enzymes may have important therapeutic benefits as well as offer insights into the biology of connective tissues.

### *Characteristics of the Ideal Inhibitor*

The ideal inhibitor of interstitial collagenase would possess the following properties: 1) Specificity for the enzyme. While nonspecific inhibitors of MMPs may be useful and have been advocated since multiple MMPs are usually activated simultaneously,[7] one can envision the eventual administration of a cocktail of inhibitors, each specific for one of the several MMPs known to be involved in a given disease. Because the collagenases have the most specific substrates among members of the MMP family, it may be reasonable to suppose that they will require a highly specific inhibitor. 2) Reasonable affinity for the target. While it is tempting to seek a high affinity interaction, this may not be desirable in all cases, especially those in which the inhibitor may have a catalytic role in inhibiting or degrading

*Collagenases*, edited by Warren Hoeffler. 

the target and should not be irreversibly bound to its target. 3) Bioavailability to the target site. This will in part be determined by the particular tissue being targeted. Some potential therapeutic indications for MMP inhibitors, such as rheumatoid arthritis, will likely require prolonged or chronic courses of treatment, making oral bioavailability desirable. Durability in serum, ability to cross into synovial spaces and possibly the blood-brain barrier are important for systemic effects and for treating joint and CNS disease, while the ability to be placed in an effective topical vehicle may be important in cutaneous applications for wound healing. Certain inhibitors which fall under the category of gene therapy also need access to the intracellular compartment. 4) Absence of nonspecific interactions which result in side-effects, including minimal antigenicity to the host. 5) A metabolism which does not render the inhibitor toxic or nonspecific. 6) A mechanism for removal of the inhibition when it is no longer needed. 7) Reasonably facile synthesis or availability from natural products, though it does not necessarily follow that this will reduce consumer cost.

**Steps in the Production of Active Collagenase**

The powerful tools of molecular and cellular biology have elucidated many of the afferent signals and mechanisms leading to collagenase gene activation, as well as the efferent cellular and extracellular events which ultimately lead to a functional protease, and are explored in depth elsewhere in this volume. Briefly, the major steps of this pathway are the signals which modulate gene activation, the synthetic pathways leading to secreted protein, and the activation and deactivation of protease. These steps involve specific processes which are schematized in Figure 9.1 and include: 1) Extracellular signals that induce MMP expression; 2) Signal transduction leading to gene activation; 3) Transcription, mRNA splicing and nuclear export; 4) Translation and post-translational processing (cleavage of the signal peptide and glycosylation); 5) Secretion of proenzyme; 6) Cleavage of the inhibitory propeptide to generate an enzyme with a functional active site; 7) Interaction with tissue inhibitors of metalloproteinases (TIMPs) and perhaps by nonspecific proteins such as $\alpha_2$-macroglobulin; 8) Degradation of the protease. In principle, each of the steps of the pathway or pathways involved in expression of the active protein, its natural inhibition, and its degradation are potential targets for externally inhibiting the activity of the enzyme. Each of the steps is conceivably manipulable in a manner that will specifically inhibit a particular MMP; some processes lend themselves more readily, both conceptually and technically, to achieving this goal.

Space does not permit discussion of all facets of collagenase inhibition in this chapter. Tremendous progress has been made in understanding how extracellular signals modulate collagenase expression. These processes, and specifically inhibition by retinoids, are discussed more fully in chapter 4. There are currently four known endogenous inhibitors or TIMPs which are responsible for naturally regulating the activity of MMPs. Their regulatory mechanisms, physiologic roles, and mechanisms of binding are important subjects for exploration, and may constitute a handle on collagenase expression which may eventually be exploitable by pharmacological means. The role of TIMPs is discussed more fully in chapter 11.

This review will focus on the use of exogenously derived, synthetic or semisynthetic pharmacological agents to inhibit interstitial collagenase in particular, though lessons learned from neutrophil collagenase, MMP-13, and other members of the MMP family will also be discussed. Moreover, discussion will focus on agents which directly inhibit transcription or post-transcriptional events. Three areas that are currently being explored or that seem particularly promising for development will be emphasized, though again, these have each been previously covered in considerable detail:[8-10] 1) Drugs that are typically small organic molecules commonly used for other indications but whose actions against collagenase activity

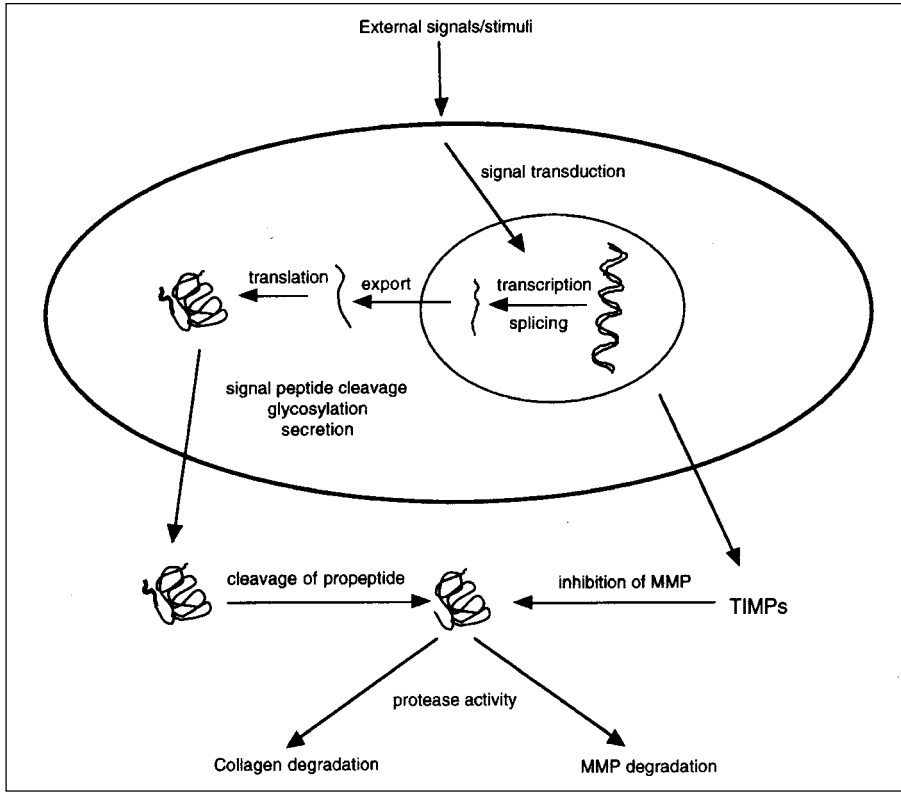



Fig. 9.1. Potential cellular and biochemical targets for inhibition of collagenases.

have been recently recognized, exemplified by the tetracycline family and its derivatives; 2) Novel active site peptide-mimetic protease inhibitors which have been recently refined based on atomic resolution structures of collagenase; 3) Antisense and antigene oligonucleotide inhibitors based on mRNA and DNA sequence data. These areas illustrate that further pharmacological developments in the future can and should be sought both in a re-exploration of novel properties of older, currently accepted drugs as well as in the rational design of pharmacological agents based on the newest science and technology.

## Familiar Drugs in Novel Applications

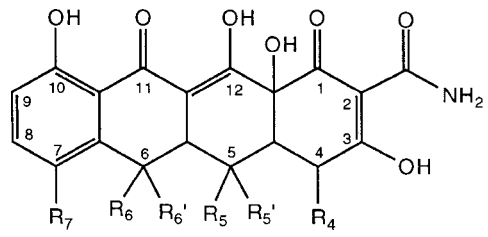
Pharmaceutical agents that are recognized for efficacy in one clinical application occasionally find new life in another. One well known example is aspirin which, originally used for its analgesic, antipyretic and anti-inflammatory actions, now also plays a major role in management of vaso-occlusive diseases due to its antithrombotic actions. A similar set of discoveries has occurred for several commonly used drugs whose anti-collagenase properties have only recently been explored. A significant advantage of these types of drugs is that there is already a considerable body of basic scientific data and clinical experience in using them, particularly with respect to side effects and idiosyncratic reactions. The most successful example has come from the tetracycline family, shown in Figure 9.2. Tetracyclines were originally derived from the soil bacteria, Streptomyces, and introduced clinically in 1948.

Since then, they have been an important class of antibiotics which treat a wide variety of infections in a number of different clinical settings. Other commercially available, semisynthetic members of the family include doxycycline and minocycline. The mechanism of their anti-bacterial action is by inhibition of protein synthesis through preferential and reversible binding to the 30S bacterial ribosomal subunit and subsequent inhibition of aminoacyl tRNA binding and polypeptide elongation.[11,12] In therapeutic use, the tetracyclines are well tolerated overall. The principal side effects are gastrointestinal irritation, particularly with oral administration, and vestibular symptoms in 30-90% of patients receiving minocycline. Rarely, hepatic and renal toxicity can also occur at high doses of the drug and with intravenous administration, often in the setting of pre-existing renal and hepatic insufficiency. Dermatological side-effects and complications include photosensitivity, onycholysis (particularly with demeclocycline), hyperpigmentation associated with minocycline, and gram-negative folliculitis. Other superinfections include Candidal overgrowth, and pseudomembranous colitis as a result of overgrowth of *Clostridium difficile*. Deposition of tetracyclines in teeth and bone, as well as pseudotumor cerebri with minocycline, are complications occurring primarily in infants and young children.[11,12]

Evidence has accumulated that the tetracyclines have anti-inflammatory actions which are distinct from their anti-microbial mechanisms and has been reviewed in detail elsewhere.[4,8,13,14] The periodontal and rheumatological literature has pioneered the documentation of the tetracyclines' anti-collagenase properties and efficacy in periodontal disease, and in rheumatoid and osteoarthritis.[4,15,16] Early evidence of a nonantimicrobial mechanism of action came with the reduction of alveolar bone loss in a diabetic rat model system and the associated inhibition of collagenase activity in the gingiva and skin of rats treated with minocycline, even under aseptic conditions.[17] Other nontetracycline antibiotics were unable to match the effect. Subsequently, minocycline was shown to inhibit human synovial collagenase and numerous reports of anticollagenase activity of the tetracycline family followed in diverse biochemical, cell culture, and animal model systems,[18-21] including studies focusing on inhibition of osteoclast-secreted collagenase,[22] cancer invasion and metastasis,[23] and aneurysmal degeneration.[24] Further evidence for a distinct nonantimicrobial mechanism of action came with a series of chemically modified tetracyclines (CMT) that possessed inhibitory effects on collagenase yet lacked antibacterial activity.[25] This effect also extended to other $Ca^{2+}$ and $Zn^{2+}$ dependent MMPs.

While it is clear that tetracyclines do possess distinct and even potent anti-inflammatory activities apart from their antibiotic properties, the principal mechanisms of action are still unclear. *A priori*, all of the processes diagrammed in Figure 9.1 are possible targets, and so far there is some evidence to support the existence of multiple direct targets of inhibition along the protease expression pathway. An early hypothesis suggested that the well-known ability of the tetracyclines to chelate metal ions could be involved in preventing proper calcium or zinc ion binding to collagenase, especially since addition of calcium ions reversed the inhibitory effect of minocycline on neutrophil collagenase in vitro.[17] In further support that tetracyclines inhibit cationic binding, a series of CMTs which typically lack the dimethylamino moiety at position 4 of tetracycline (4-dedimethylamino tetracycline) has been synthesized; the loss of anticollagenase activity in one, a pyrazole analog named CMT-5, was explained by the loss of moieties on the molecule that could chelate a divalent cation such as $Zn^{2+}$ or $Ca^{2+}$.[26] Crystallographic studies may help decide whether all or some divalent cation binding sites in collagenases are affected.

Tetracyclines have been shown to exert inhibitory effects earlier in MMP gene expression. They may inhibit proper activation of the neutrophil procollagenase, either by suppressing normal oxidative cleavage of the propeptide or increasing degradation of the proenzyme,[26-29] although this has not been observed by some workers.[20] Transcription can be




| | $R_4$ | $R_5$ | $R_5'$ | $R_6$ | $R_6'$ | $R_7$ |
|---|---|---|---|---|---|---|
| Tetracyclin | $N(CH_3)_2$ | H | H | $CH_3$ | OH | H |
| Doxycyline | $N(CH_3)_2$ | OH | H | $CH_3$ | H | H |
| Minocyclin | $N(CH_3)_2$ | H | H | H | H | $N(CH_3)_2$ |

Fig. 9.2. Representative members of the tetracycline family.

affected, as seen when keratinocytes treated with doxycycline and CMTs had lower levels of MMP-2 mRNA, though some nonspecific cytotoxicity and suppression of total RNA were also noted, possibly indicating a chelation of free divalent cations necessary for general cellular function.[30] More recently, tetracycline has been shown to inhibit induction of stromelysin mRNA by IL-1 while not affecting glycerol aldehyde phosphate dehydrogenase RNA levels, and this effect has been shown to require the presence of the AP-1 enhancer site upstream of the gene.[31]

Although tetracyclines have a well-documented direct inhibitory effect on collagenases and other MMPs, evidence also exists that these drugs have other mechanisms of action which may contribute to their in vitro, in vivo and clinical effects. Tetracyclines have been shown to inhibit production of inducible nitric oxide synthase, partially at the level of transcription and translation of this enzyme which produces nitric oxide, a molecule with pleiotropic biological actions, including up-regulation of MMP activity.[32] Inhibition of inducible nitric oxide synthase is in proportion to the tetracyclines' inhibitory potency against the collagenases.[33] Some other reported cellular effects of tetracyclines which could explain their anti-inflammatory and anti-collagenolytic properties include variable modulation of phagocytic activity of leukocytes,[34] inhibition of neutrophil motility,[35] modulation of intracellular calcium concentrations during T-cell activation,[36] and inhibition of type X collagen synthesis in chondrocytes that could not be reversed by addition of excess extracellular calcium ions.[37] In some cases such as angiogenesis, the role of tetracyclines in inhibiting angiogenesis by interacting with MMPs[38] has been questioned since these drugs inhibited angiogenesis in an in vitro model, while a synthetic peptide-mimetic inhibitor with activity against a broad spectrum of MMPs did not.[39]

Regardless of the precise mechanism of action, the tetracyclines have already been employed to treat a variety of inflammatory diseases. Although there was anecdotal clinical evidence for therapeutic efficacy of tetracyclines in periodontal disease[40] and arthritis,[13] a small double-blind study using a relatively low-dose of tetracycline failed to show any improvement in patients with rheumatoid arthritis.[41] However, an uncontrolled study using minocycline in conjunction with nonsteroidal anti-inflammatory drugs led to improvement in patients with rheumatoid arthritis.[42] Subsequently, two larger, randomized, double-

blind, placebo-controlled studies in Europe and the United States have shown modest to moderate improvement in patients with long-standing rheumatoid arthritis who were treated with minocycline 100 mg twice daily.[43,44] Most recently, a randomized, double-blind, placebo-controlled study of patients with early rheumatoid arthritis who had not been previously treated with so-called disease-modifying antirheumatic drugs (methotrexate, hydroxychloroquine, sulfasalazine or gold) showed improvement with minocycline 100 mg twice daily.[45]

A number of cutaneous disorders have been reported to respond to the tetracyclines, including acne vulgaris, acne rosacea, pyoderma gangrenosum, telangiectasias, pityriasis lichenoides et varioliformis acuta, panniculitides, pustulosis palmaris et plantaris, confluent and reticulated papillomatosis, mycosis fungoides, white sponge nevus, dystrophic epidermolysis bullosa, and bullous pemphigoid.[46-48] Of these, the response of dystrophic epidermolysis bullosa and bullous pemphigoid to tetracycline therapy may in part be rationalized in terms of collagenase inhibition. Dystrophic epidermolysis bullosa is a severe, sub-epidermal blistering skin disease with autosomal recessive and dominant inheritance types which has been reported to respond to minocycline in two patients.[49] Although interstitial collagenase was once considered to be a candidate for the primary defect in this disease,[5,50] the disease is now known to be due to defects in Type VII collagen which forms the anchoring fibrils which help in mediating dermal-epidermal adhesion.[51] However, Type VII collagen is a substrate for interstitial collagenase and gelatinase,[52] and it is conceivable that inhibition of these enzymes by a tetracycline could explain some of the effects, albeit in just two cases.[49] Bullous pemphigoid is an acquired blistering disease typically associated with autoantibodies against two hemidesmosomal proteins. In contrast to the single case report of tetracycline therapy in dystrophic epidermolysis bullosa, there have been numerous isolated reports of tetracyclines with or without nicotinamide used to treat bullous pemphigoid.[46] More recently, two unblinded clinical studies with twenty and seven patients,[53,54] respectively, reported that tetracycline plus nicotinamide was effective in the treatment of bullous pemphigoid, and comparable to systemic corticosteroids.[53] Since one of the bullous pemphigoid autoantigens (BP180) has multiple collagen-like domains and both 92 kD gelatinase (MMP-9) and collagenase (MMP-1) are elevated in blister fluid, and BP180 is a substrate for gelatinase in vitro,[51,55,56] it is tempting to speculate that collagenases are more intimately involved in the pathogenesis of bullous pemphigoid than conventionally thought, and that part of tetracycline's effect arises from its ability to inhibit these enzymes. So far, no attempt has been made to stratify patients based on their type of autoantibody. Both tetracycline and nicotinamide are also potential free-radical scavengers and electron acceptors and conceivably could prevent oxidative cleavage of procollagenase.[28] Moreover, recently reported studies in transgenic mice support the notion that matrix metalloproteinases mediate not only connective tissue metabolism, but also are somehow involved in communicating with overlying epithelial tissues and directing their properties.[57] Thus it is interesting to speculate that inhibition of collagenase may either directly help to prevent disruption of hemidesmosomes in the basement membrane, or indirectly aid by modulating dermal-epidermal interactions.

The tetracyclines have also been reported to have efficacy in a variety of other human inflammatory diseases which have no clear relationship to bacterial infection, such as persistent corneal ulcerations[58] and recurrent aphthous ulcers.[59] In addition, inhibition of MMPs has been postulated as one mechanism to explain the successful use of tetracycline as a sclerosing agent in chemical pleuredesis.[60]

Other drugs have also been shown to inhibit collagenase in vitro. Cephalothin, but not doxycycline, tetracycline or gentamicin, was able to inhibit MMP activity in extracts from tissue around loose total hip arthroplasty prostheses.[61] This curious result was explained by

the relative absence of neutrophil collagenase in such tissue extracts, though further studies and confirmation are required. Structurally related to the tetracyclines, anthracyclines such as aranciamycin have been reported as inhibitors,[62] as have anthracene carboxylic acids, anthraquinones and coumarins,[9] suggesting that not all rings of the tetracycline template are essential for activity. Of the nonantibiotic agents, phenytoin, a commonly used antiepileptic, has been the most carefully studied. Following the initial observation that collagenase is elevated from fibroblasts of patients with recessive dystrophic epidermolysis bullosa, and is inhibited in vitro by phenytoin at the level of synthesis,[63,64] anecdotal case reports and an open study reporting efficacy in patients with RDEB were reported.[65,66] However, a multicenter, placebo-controlled, double-blinded study subsequently showed no improvement in clinical outcome of RDEB patients as measured by numbers of blisters and erosions.[67] Since collagenase levels in blister fluid from these patients were not assayed, it is unclear if there is a subgroup of RDEB patients which may benefit from such therapy. Moreover, since increased collagenase is now known to be a secondary phenomenon in the pathogenesis of RDEB which is caused by defects in type VII collagen, the role of phenytoin in treating this particular disease may be minimal, although type VII collagen is a substrate for collagenase.[52] Phenytoin may yet have beneficial uses in other diseases in which over-expressed collagenase plays a more prominent if not primary role.

Finally, while our discussion has focused on pharmacological inhibition of events downstream from gene activation, it is worth noting that various pharmacological agents such as Tenidap and methotrexate are capable of intercepting the IL-1 stimulated cascade,[68,69] specifically inhibiting collagenase induction while not affecting TIMP-1 or stromelysin mRNA levels,[69] and deserve further exploration in the future.

## Novel Active Site Protease Inhibitors

The search to develop an effective MMP inhibitor was initially modeled on the success of angiotensin converting enzyme (ACE) inhibitors, another class of zinc-containing endopeptidase inhibitors, which have since become commonly used to treat hypertension and heart failure.[70] Inhibitors of the zinc-containing neutral endopeptidase also provided a precedent.[71] However, prior to any detailed three-dimensional structural information of the collagenases, the rational design of specific low-molecular weight peptide inhibitors relied on biochemical data that demonstrated different MMPs had different substrate affinities and catalytic efficiencies, and utilized crystallographic data from related enzymes such as thermolysin.[72] For example, human fibroblast and human neutrophil collagenases were shown to have different activities against collagen substrates with varying single amino acid substitutions in the $\alpha$1 chain.[73,74] Figure 9.3 shows the standard structural nomenclature for describing the natural substrate and its inhibitors in the active site.[75] Residues on the N-terminal side of the scissile bond of collagen or its inhibitors are designated P1, P2, and P3, while residues on the C-terminal side of the scissile bond are designated P1', P2' and P3'. The P1' residue has been an especially useful handle in manipulating the specificities of inhibitors.[9,72] The enzyme's active site pocket at each residue is similarly designated as S or S' with a number corresponding to that residue. In addition to studies which have manipulated the substrate, the construction of truncated neutrophil collagenase, chimeric proteins composed of N-terminal neutrophil collagenase and C-terminal stromelysin,[76] truncated interstitial collagenase and chimeras composed of N-terminal interstitial collagenase and C-terminal stromelysin,[78] and chimeras composed of interstitial collagenase and stromelysin[77] have all suggested that substrate specificity is determined by a complex interaction between the active site and the carboxy terminal hemopexin domain.

The peptide-mimetic inhibitors have as a general feature a functionality that is capable of coordinating to the catalytic zinc ion at the site of the scissile bond in collagen, surrounded

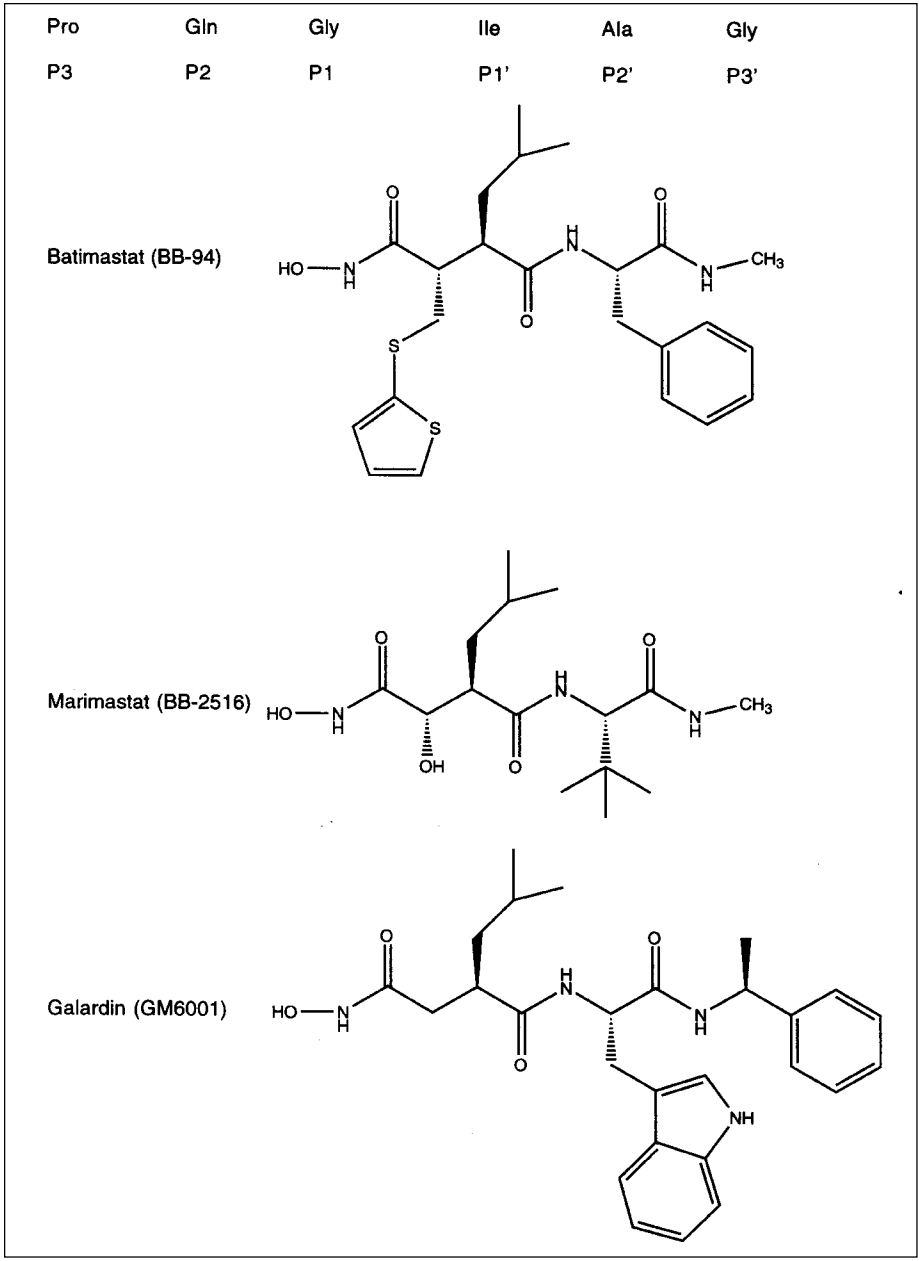



Fig. 9.3. Nomenclature of the collagen substrate and some representative active site inhibitors for matrix metalloproteinases

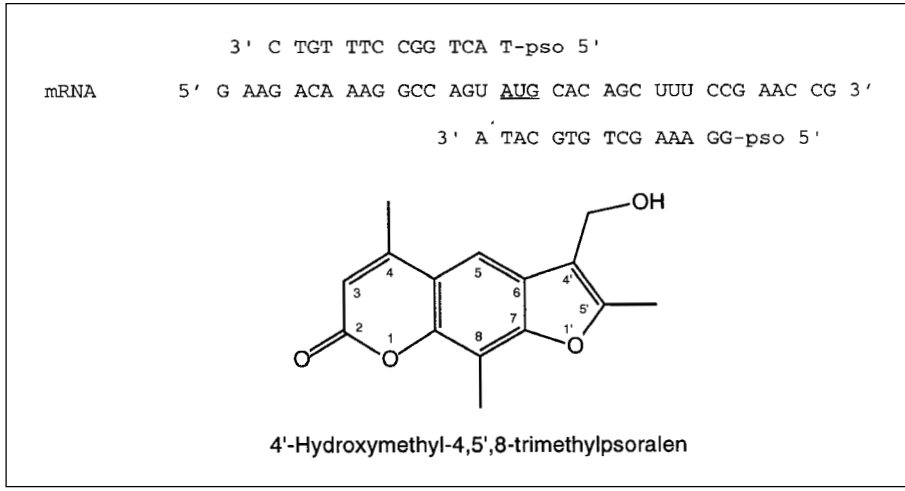



Fig. 9.4. Nucleotide sequence around the initiation codon of MMP–1 mRNA, and two possible antisense oligonucleotides conjugated to psoralen.

on either side by residues capable of fitting and binding to the substrate pocket. So far, peptide fragments on the P' side of the substrate cleavage site have had more success in conferring specificity with typical inhibition constants in the nanomolar range. The inhibitors can be subdivided by their zinc binding groups into hydroxamate, β-mercaptocarbonyl, N-carboxyalkyl, and phosphorus-containing (phosphoramidate, phosphinic acids, phosphonic acids, and phosphonoalkyl) compounds.[9] Even before the solution of the crystal structures of stromelysin and collagenase, it was evident that stromelysin was capable of accommodating a wider range of substituents at the various surrounding peptide positions than was collagenase which tended to bind substrates with smaller amino acid side chains.[79] On the other hand, some inhibitors which are relatively nonspecific contain an electron-rich phenolic ether moiety which, although seemingly too bulky to fit in the usually more restrictive active site of human fibroblast collagenase, nevertheless binds well to MMP-1 and has been proposed to form a π–π electronic interaction with the guanidium moiety of Arg 214.[9,80] However, an inhibitor which has been designed to specifically form a salt bridge with this arginine has been orders of magnitude less potent than the best inhibitors.[81]

### *Inhibitors Based on Structural Data*

Rational efforts to inhibit collagenases and MMPs have benefited greatly from the rise of structural biology. In the last several years, the increasing number of atomic resolution x-ray crystallographic structures and NMR structures has provided insight into the mechanism of binding of known inhibitors, and these have suggested optimizations and new modes of binding previously unsuspected. The detailed structure of collagenases is discussed fully in the first chapter of this volume. Briefly, there are now a number of crystallographic structures of the catalytic domains of human fibroblast collagenase[82-87] and neutrophil collagenases,[88-92] each bound to a variety of inhibitors, as well as a structure of full-length collagenase from porcine synovial fibroblasts.[93] These data have been supplemented by the three dimensional structures of other MMPs such as stromelysin.[94-96] Crystal structures of fibroblast collagenase in two crystallographic forms not bound with an inhibitor have also been

described and have the interesting feature that the active site is occupied by a stretch of the amino terminus from its symmetry-related neighbor.[85] The difficulty in obtaining a crystal structure of the full length human protein has been due to the relative instability of collagenase during the purification and crystallization process, resulting in autocatalytic cleavage of the C-terminal hemopexin domain. Whether this relative stability of structures can be exploited in designing future inhibitors remains to be seen and will probably require additional data from protein chemistry and folding studies.

The cumulative and consensus result of these structures is that the catalytic domain of the collagenases is composed of twisted five-stranded β sheets and three long α helices whose general topology is similar to that of thermolysin. The active site's floor contains a zinc atom that is coordinated by three histidines. A water molecule is the fourth ligand in the free enzyme, although this water is typically displaced in the structures which have been obtained with inhibitors and, in the case of the hydroxamates, is coordinated by the two hydroxamate oxygens in configurations which have been described as either an approximate square pyramidal[83] or trigonal bipyramidal geometry.[85] A shared methionine residue in a 1,4-tight turn adjacent to the active site and the overall architectural similarity to other zinc-containing peptidases such astacin, adamalysin and serralysin families has led to the proposal that these families be grouped under the label "metzincins".[86] However, in contrast to these other families, the MMPs contain a second, noncatalytic zinc which is tetrahedrally coordinated and a calcium octahedrally coordinated that probably serve a structural role, though biochemical studies of full-lengh and truncated stromelysin and gelatinase suggest that the second zinc may not be necessary for structural integrity in the full-length enzyme.[97] Indeed, other biochemical studies of the inhibitory role of tetracyclines were consistent with the presence of a second zinc ion.[20] Additionally, there has been a second[85,88] and third[82,92] calcium described in some of the structures. MMP-1, in which Arg 144 replaces Ile in other MMPs at the mouth of the S1' pocket, has a limited number of small amino acid residues that can be accommodated in an inhibitor at this site. Further crystallographic studies have illustrated the possibility of other unanticipated modes of inhibitor binding under more extreme conditions, for example, where the methylamide group of batimastat ligates to the zinc and the phenylalanine residue occupies the S1' pocket when the enzyme is cocrystallized with inhibitor at high salt concentrations.[94] In another contrast to inhibitor interactions with thermolysin, the inhibitors of collagenase and stromelysin appear to interact with the active site by hydrogen bonding to the backbone alone.[84,93] The complete structure of porcine synovial collagenase has been solved with its C-terminal hemopexin domain which has approximate four-fold symmetry with each unit comprised of a β sheet formed by four antiparallel β stands, forming a four-bladed structure which has been likened to the "flights of a dart".[93] The linker peptide between the catalytic and hemopexin domains appears to lack any secondary structure, apparently explaining its propensity for autolytic cleavage; this observation, long a source of frustration for those attempting to prepare crystals of the full length enzyme, may potentially be exploitable in designing a new class of inhibitors. Other mysteries include the means by which a collagen triple helix is recognized by the full-length enzyme so specifically, and how this structure is accommodated into the active site.[87-93]

### *Hydroxamate Inhibitors for Blocking Tumors*

While active site inhibitors of collagenase would be of interest in treating any of the diseases in which the enzyme is over-active, one focus of clinical drug development and trials has been in oncology. The elaboration of various MMPs by malignant cells and their role in breaching basement membranes and degrading connective tissue is one mechanism by which tumor invasion, local spread and metastasis are thought to occur.[99] In this context,

there is little evidence to support the notion that these inhibitors alone could serve as monotherapy against malignancy since they have not been shown to be cytotoxic to a variety of malignant cells grown in cell culture. Rather, they may have a role in adjuvant therapy, delaying or preventing tumor growth, invasion and metastasis. Of the inhibitors which have been developed so far, the hydroxamates have been the most extensively studied in vitro and in vivo, and several are currently in clinical trials. Batimastat (BB-94, 4-(N-hydroxyamino)-2(R)-isobutyl-3(S)-[(2-thienylthomethyl)succinyl]-L-phenylalanine-N-methylamide) is the prototype of the majority of hydroxamate inhibitors. It possesses a broad spectrum of inhibition with $IC_{50}$ values for collagenase (MMP-1), both 72 and 92 kilodalton gelatinases (MMP-2 and MMP-9), and matrilysin (MMP-7) in the 2-6 nM range, and for stromelysin (MMP-3) at 20 nM.[6] Although it is poorly soluble in aqueous solution (< 3 μg/ml), depot and intraperitoneal injections have nevertheless been demonstrated to result in systemic levels of the drug well within the range of the major MMPs.[6] A second generation of hydroxamate inhibitors such as Marimastat (BB-2516) has been developed and possesses improved solubility and are orally bioavailable. Marimastat is as effective as Batimastat in inhibiting MMP-1, MMP-2, MMP-7 and MMP-9 in vitro, and an order of magnitude worse at inhibiting stromelysin.[96]

In animal models, batimastat has been reported to inhibit tumor growth and spread (modest effect in the latter) of B16-BL6 melanoma cells injected in mice,[6,101] inhibit angiogenesis and reduce tumor doubling time in hemangiomas in mice by inhibiting recruitment and organization of vascular cells and structures,[102] human breast cancer regrowth and metastasis in a nude mouse xenograft model,[103] reduce human colon cancer growth and local, regional and distant spread as well as reduce ascites volume and improve survival in a mouse model,[104-106] and in human ovarian cancer.[107] However, the drug was ineffective in preventing colonization in multiple organs following intraperitoneal injection of a Burkitt lymphoma cell line into SCID mouse model, using PCR to detect the presence of the malignant clone.[6] Recently, AG3340, a nonpolypeptide MMP inhibitor whose design was based on the crystallographic data accumulated so far, has shown picomolar $K_i$s against MMP-2, MMP-3, and MMP-9. In a Lewis lung carcinoma murine model, this inhibitor was associated with modest reductions in the sizes of primary tumors and a more impressive reduction in metastases following intraperitoneal administration.[108] Interestingly, other similarly designed inhibitors with in vitro inhibition of MMPs had a wide range of effects in the animal model, including enhanced tumor growth. AG3340 is orally bioavailable and a phase I study in healthy volunteers has been announced in abstract form.[109]

Some hydroxamate-based inhibitors have also been developed for other clinical applications. Galardin or GM6001, another hydroxamate inhibitor, is one of the most potent peptide inhibitors of human skin fibroblast collagenase with a $K_i$ of 0.4 nM.[110] While it is effective in inhibiting MMP-1, it is not specific since similar $K_i$ values have been measured for neutrophil collagenase, and gelatinases A and B.[111] GM6001 has been shown to inhibit angiogenesis in rats and chick chorioallantoic membranes,[112,113] corneal ulceration due to chemical and infectious insults in rabbits[113] and phorbol ester-induced skin inflammation and thickening when applied topically to the ears of hairless mice.[111] Interestingly, GM1489, in which a carboxylic acid group replaces the hydroxamate and a phenylmethyl group replaces methyl at the P3' position, was as effective as GM6001 in reducing skin thickening, although GM1489 is specific for MMP-1 over MMP-2, MMP-3, MMP-8 and MMP-9 by at least several orders of magnitude.[111] Another inhibitor, Ro-31-7467, inhibits collagenase with an $IC_{50}$ of 16.8 nM, compare to 208.8 nM for gelatinase A, and 238.8 nM for stromelysin.[114] Ro-31-7467 was able to inhibit bone resorption in a cultured neonatal mouse calvarial system at $10^{-8}$ M assayed in a bone resorption model, although not completely at concentrations at which its specificity for collagenase was retained.[114] Ro 32-3555 inhibits

collagenases fairly specifically with $K_i$ values of 3-4 nM, one to two orders of magnitude better than its inhibition of stromelysin or either of the gelatinases.[115] In a rat monoarthritis model in which Propionibacterium acnes was injected intra-articularly, Ro 32-3555 administered orally was capable of inhibiting cartilage degradation.

Few human trials of the synthetic peptide inhibitors have been reported so far in the peer-reviewed literature. Batimastat (BB-94) has been in Phase II clinical trials for malignant pleural fluid effusions and ascites[6] and a phase I trial in patients with various advanced malignancies without ascites has recently been reported.[116] This trial involved 9 patients stratified into three dose levels 600, 1200 and 1800 mg/m$^2$; batimastat was administered every four weeks. The principal side effects were abdominal discomfort and cramping at 1200 and 1800 mg/m$^2$, which were severe and prolonged at the highest dose requiring premedication with analgesics, including narcotics. Due to the side effect profile, the poor bioavailability necessitating administration as a suspension, and the development of marimastat, batimastat has not been pursued as systemic therapy and has reportedly been discontinued as a treatment for cancer.[107,117] Marimastat, a second generation, orally bioavailable hydroxamate inhibitor which followed Batimastat, is now in clinical trials for lung, ovarian, colorectal, pancreatic, and small cell lung cancers and glioblastomas.[9,116] Galardin or GM6001 has been in phase III trial for treatment of corneal ulceration.[9] The complete and published results of the various clinical trials of these inhibitors are awaited with anticipation.

In contrast to inhibitors based on similarities to the collagen substrate of MMPs, an alternative approach has exploited the fact that the prodomain sequence of MMPs contains a highly conserved twelve amino acid sequence, MRKPRCGVPDVG, containing a cysteine residue whose free thiol moiety is thought to form a coordinating ligand with the catalytic zinc, thus preventing collagenolytic activity.[114,115] The sequence interestingly is found to be oriented in the active site of the enzyme running in the opposite direction than the collagen substrate and the peptide analog inhibitors discussed above.[86] A screen of various peptides based on this sequence resulted in the identification of the hexapeptide Ac-RCGVPD-$NH_2$ and the pentapeptide Ac-RCGVP-$NH_2$ with $IC_{50}$ values of 4.5 and 10 $\mu$M, respectively. These compounds were active against both stromelysin and collagenase. Subsequently, it was shown that only Cys-75, Gly-76 and Val-77 are essential for inhibition, and that replacement of Cys-75 with isocysteine improved potency by 5-fold. These complexes suffer a potential problem with stability due to autoxidation, and because the sequences are common to both stromelysin and collagenase, they are not completely specific for a single type of MMP. The details of interactions will have to await a high-resolution structure with the prodomain sequence, and it may be possible to substitute other residues or moieties which will both allow discrimination between the large S1' pocket of stromelysin and the more restrictive collagenase as well as enhance stability of the molecule.

In addition to active site inhibitors based on oligopeptide derivatives, nonpeptide compounds have also been developed which retain the hydroxamate moiety as a zinc-coordinating ligand and have been shown to inhibit various MMPs, including MMP-1. These include aryl sulfonamides, alkyl sulfonamides and futenones.[9]

In addition to crystallography and NMR, other methods are being developed which promise to aid in the design and optimization of active site inhibitors. Molecular dynamics simulations and other computational methods which search conformational space will be important as additional structural information is provided by more traditional methods.[120] The contribution of molecular dynamics may be particularly relevant in understanding the time-dependent aspects of collagenase activity and pointing attention to residues or parts of the protein which may significantly determine key vibrational or phonon modes necessary for catalysis. It may also possibly explain how an entire collagen triple helix is cleaved

by an active site which appears to be able to accommodate only a single polypeptide at a time. In a further acknowledgment that our present understanding of protein structure-function relationships is incomplete, it is worth noting that there is evidence from random mutagenesis studies that sites of enzymes and proteins which are not obviously related to their functional sites are indeed important for function.[121] Because these may be in more exposed surfaces of the protein, they may constitute another, more accessible target for therapeutic peptides, antibodies and small molecules.

## Oligonucleotides

Active site inhibitors and TIMPs bind to the activated collagenase in a 1:1 stoichiometry and face the nontrivial challenge of molecule-to-molecule combat with every single enzyme secreted by the cell. In inflammatory states such as a healing wound or an arthritic joint where cells are stimulated to increase transcription and the half-life of mRNA is increased,[122] or in milieus such as tumor invasion where the cells are inherently over-expressing collagenases, the number of enzyme targets can be considerably amplified. Potentially large numbers of inhibitors may be needed to achieve a therapeutically measurable effect, possibly at the expense of toxicity if the inhibitor is not particularly bioavailable to the target tissue. To minimize this problem, an alternative approach is to target steps in the expression pathway which are more proximal, prior to the amplification which occurs with transcription of a gene and translation of messenger RNA.

In contrast to designing inhibitors of proteins which typically requires some consideration if not high-resolution knowledge of the tertiary structure, the rational design of oligonucleotides which bind to DNA or RNA and inhibit their function has usually relied on the primary structure or linear sequence of nucleotides of the target. The wealth of nucleic acid sequence information that is now available has facilitated the development of both antisense and, to a much lesser extent, antigene studies based on oligonucleotide technologies. With respect to collagenase, the sequences of cDNAs and the entire gene for human fibroblast collagenase are now available.[123-125] Unfortunately, in designing a therapeutically useful agent, difficult questions remain after the relatively straightforward task of finding a oligonucleotide that is capable of binding to its target: 1) Is the intended interaction the only one occurring? Ideally, not only should the target sequence be unique to ensure specificity, but the possibility of forming higher order nucleic acid structures (triple helices, quartets, parallel strands) as well as binding to other types of macromolecules introduces the potential for alternative side-reactions which may compete with the desired interaction. 2) What is the best method and vehicle for delivery? Unlike active site inhibitors and probably most small molecules, oligonucleotide agents have the nontrivial challenges of reaching the target tissue and cell, entering into a cell efficiently, and somehow journeying to the nucleus and possibly the cytoplasm intact, in addition to binding the target itself. 3) How can a durable response be produced? Before binding to a specific target, and once bound, how can the oligonucleotide resist degradation and perhaps go on to inhibit more targets? These challenges are not entirely unique to oligonucleotides agents, but we have only recently begun to understand the facets of the problem unique to oligonucleotides and some potential strategies for overcoming them.

The most mature and successful oligonucleotide-based approach to repressing gene expression is antisense technology.[10,126] This strategy utilizes oligonucleotides complementary to a target mRNA which bind antiparallel to each other via conventional Watson-Crick base pairs. In the case of oligonucleotides composed of DNA, a DNA:RNA hybrid forms that then becomes a substrate for RNAse H in the nucleus. In principle, this enzyme cleaves the RNA component and the DNA oligonucleotiede is unaffected, free to rebind a new message. Another, probably less important, mechanism of action is a steric block to binding

or procession of the translational complex. Other mechanisms are conceivable, such as blocking export out of the nucleus, disruption of normal splicing and actual binding to the coding strand of the gene itself, but are not well-substantiated.

### *Enhanced Oligonucleotide Binding Using Psoralens*

In addition to purely sequence-dependent aspects of the oligonucleotide (e.g., length and percentage GC:AT content) other factors can affect the efficacy of the antisense effect. In many cases, targets which include the initiation codon AUG are the most effective in blocking expression.[10] Modifications to the oligonucleotide, such as adding an intercalating group to one or both termini, can enhance binding; if the added group has reactive functionality, a permanent covalent modification is then possible. For example, psoralens are tricyclic furocoumarins that have the ability to intercalate into double-stranded DNA and, at sites which contain the sequence 5'-TA-3', form either monoadducts or interstrand crosslinks with the thymidines of one or both strands upon absorption of ultraviolet light in the UVA range (320-400 nm).[127] When psoralens are conjugated to oligonucleotides that bind to a complementary mRNA target and place the psoralen adjacent to a reactive pyrimidine and are then irradiated with UVA light, the adducts formed are extremely stable and fix the antisense oligonucleotide to its target. The potential advantages of this approach include the possibility of enhanced uptake by the cell and an inherent 5' cap resistant to exonuclease activity, the potential ability to initiate antisense activity with a light trigger, an enhanced binding profile which may reduce the amount of oligonucleotide required for the task, and a permanent block to translation. A disadvantage is that while these covalent hybrids are still substrates for RNase H, they are now suicide substrates and the potential for regenerating the antisense oligonucleotide is lost.

### *Phosphorothioate Oligos*

The mechanisms by which oligonucleotides enter cells are not entirely clear. In addition to the uptake of naked oligonucleotides into cells,[128] it is also possible to enhance incorporation by the use of various vectors, including lipids, polycations, adenoviral proteins, and electroporation.[129,130] Regardless of how the oligonucleotide enters the cell and journeys to the appropriate intracellular and perhaps intranuclear compartment, stability of the oligonucleotide is another issue that has been challenging. Commonly, chemical modifications to the oligonucleotide's backbone have been made which confer resistance to endogenous endonucleases. Of these, the phosphorothioates have been among the most popular, though concerns about their ability to produce nonspecific effects continue to be raised.[131,132] Phosphorothioates have replaced one oxygen of the DNA phosphodiester with a sulfur. The consequence is a doubly anionic charge per residue (whereas the phosphodiester has only one negative charge), and the creation of a chiral center and $2^n$ possible diastereomers result. While this has not been a significant problem in designing antisense agents, it has limited the applicability of phosphorothioates to triple-helix forming oligonucleotides where stereochemical considerations appear to be more crucial.[133] In addition to internal modifications, capping the 5' end, the 3' end, or both with a variety of moieties, some of which possess useful functionality has also been popular and reasonably successful.

Many applications of antisense oligonucleotides in MMP biology have focused on suppressing the events leading to induction of MMP gene expression. For example, the use of antisense c-fos and c-jun RNA and phosphorothioate oligonucleotides has demonstrated that fos and jun are required for induction of collagenase and stromelysin gene expression,[134,135] and phosphorothioate oligonucleotides antisense to IL-6 mRNA were capable of inhibiting collagenase expression following ultraviolet A irradiation.[136] Similarly, antisense molecules have been used to study other steps that modulate MMP activity. For example,

the transfection of vectors designed to produce antisense RNA has become increasingly popular for the study of function of a target and if capable of stable replication, offers a means of sustained gene therapy. For example, 3T3 cells modified to produce an antisense RNA complementary to TIMP mRNA became invasive, tumorigenic and metastatic in athymic mice,[137] and transfection of a vector containing an antisense sequence to c-jun mRNA in keratinocytes was able to inhibit basal and TGF-β induction of the collagenase promoter.[138] However, antisense targets which affect processes involved in induction and TIMP inhibition are likely to have multiple effects since different MMPs often share common signaling events in induction and TIMP binding.

Therefore, it may be desirable to develop antisense inhibitors against each MMP target with the eventual goal of mixing and matching agents to a particular therapeutic goal. Antisense oligonucleotides targeted directly against MMP mRNAs have been reported now for several MMPs. Antisense methylphosphonate oligonucleotides have been used against MMP-2 or MMP-9 to selectively inhibit their activity in HT1080 fibrosarcoma cells.[139] The MMP-2 antisense oligonucleotide was directed at the initiation codon while the MMP-9 antisense oligonucleotide targeted the upstream 5' untranslated region. Specificity for each target was maintained at concentrations from 5-50 μM, and was lost at 100 μM where growth inhibition was also observed after 120 hours of exposure the antisense agent. MIM human melanoma cells have been stably transfected with a plasmid vector expressing a 777 nucleotide frangment complementary to MMP-1 mRNA.[140] In two clones, MMP-1 mRNA expression was blocked 90-96% with concomitant decreases in protein synthesis. Invasion assays through type I collagen and surprisingly Type IV collagen showed inhibition in these transfected clones, in spite of the presence of functional MMP-2. The use of vectors to deliver antisense sequences has also been utilized against MMP mRNA. Transfection of an episomal vector expressing an antisense RNA against MMP-2 into cultured mesangial cells resulted in decreased MMP-2 and a cellular morphology more characteristic of a quiescent state rather than an activated state as seen in untreated controls.[141]

### *Inhibition With Antisense Oligos*

Our own approach to inhibiting interstitial collagenase has been to apply the work of others who have shown that psoralens which are covalently attached to short antisense oligonucleotides can be targeted to a mRNA site in a highly specific fashion, and indeed confer additional stability to the DNA:RNA hybrid.[142,143] One caveat in utilizing psoralens or any UV-activatable compound in the inhibition of collagenase is that UVB and, to a lesser extent, UVA also induce collagenase expression.[144-146] The process perhaps involves oxidative processes occurring at the cell's plasma membrane, eventually leading to activation of the collagenase gene at the AP-1 site.[147] Therefore, it is was necessary to optimize the system such that the concentration and quantum efficiency of the psoralen effectively competes with any MMP-1 induction. We used antisense phosphorothioate oligomers directed against the region around and including the initiation AUG codon of human collagenase mRNA, as shown in Figure 9.4. One sequence positioned the psoralen at a 5' UA 3' site within the initiation codon in which a psoralen monoadduct or crosslink could form once the complementary strand binds. Another sequence positioned the psoralen at a downstream 5' CG 3' site in which no significant adducts can form. Following incubation with synthetic complementary DNA sequences in vitro and irradiation with broadband 15 J/cm$^2$ UVA light, 80% of the target sequences were covalently crosslinked to the oligonucleotide which positioned psoralen at a 5' TA 3' site. In contrast, the oligonucleotide which positioned psoralen in a 5' CG 3' site had no detectable covalent complex formation, even when the antisense oligonucleotide concentration was elevated a hundred-fold over the initial test concentration. A random-sequence oligonucleotide also did not result in covalent hybrids, confirming that

binding of the antisense agents and that reactivity of the psoralen were both sequence-specific. Antisense oligonucleotides were subsequently added to collagenase mRNA synthesized in vitro, and the inhibitory effect was assayed in a cell-free rabbit reticulolysate translation reaction. Addition of the antisense oligonucleotide positioning psoralen at a 5' UA 3' site resulted in no detectable difference in the quantity translated product when the reactants were unirradiated. However, upon irradiation with UVA, the level of translation dropped over 50% relative to the untreated control. The inhibition in translation was probably entirely due to a steric block since RNase H is absent in the reticulocyte lysate. On the other hand, none of the suspected mechanisms for inducing collagenase expression by UVA exists in a cell-free system. Nonetheless, the observed difference was impressive, and was not seen with a random sequence oligonucleotide coupled to psoralen in the absence or the presence of light.

These results were extended to cell and tissue culture systems. When fibroblasts grown as monolayers in cell culture were treated with the antisense oligonucleotide-psoralen conjugate, no effect on collagenase expression was detected in unirradiated cells when assayed by Western immunoblotting. As expected, upon UVA irradiation, cells which were not treated with antisense agents showed a slight increase in collagenase expression with each increment in UVA dose. However, upon irradiation and addition of the antisense oligonucleotide which positioned psoralen at a 5'UA3' site, a dose-dependent decrease of protein occurred at 5 and 10 J/cm$^2$. This effect was not observed with a random sequence oligonucleotide which also showed no inhibition of UVA-induced increase in collagenase expression. This important result demonstrated that the inhibitory effect of the psoralen-antisense agent was sufficient to overcome the inductive effects that UVA has on collagenase gene expression. To further explore the effects of antisense agents in a more realistic tissue model, a "dermal equivalent" was constructed by dispersing fibroblasts in a collagen gel, allowing the mixture to contract, and then treating the resulting three-dimensional "dermis" with antisense-psoralen conjugates.[148] The system creates an additional challenge for antisense inhibition in that fibroblasts in collagen matrices synthesize several-fold higher levels of collagenase than cells grown in monolayer due to the presence of increased amounts of collagenase mRNA.[149] Surprisingly, the antisense-psoralen conjugate was able to inhibit collagenase production in dermal equivalents which had been unirradiated as well as irradiated with 5 J/cm$^2$ UVA light. This was in contrast to the results of in vitro translation and monolayer cell culture which showed no inhibitory effect unless UVA light activated the psoralen. Thus we concluded that psoralen-antisense oligonucleotides are able to diffuse through a collagen matrix, enter fibroblasts and inhibit collagenase synthesis, in spite of the fact that fibroblasts under these conditions normally synthesize more collagenase than in monolayer culture. The presence of an inhibitory effect even in the dark suggests that in the context of a 3-D collagen matrix, fibroblasts respond to antisense oligonucleotides in a quantitatively if not qualitatively different way. Perhaps the uptake of oligonucleotides is enhanced, giving rise to higher intracellular antisense concentrations. Alternatively, cells under these conditions may have a more efficient mechanism for identifying and degrading antisense: mRNA hybrids, either by elevated levels of RNase H or through some as yet unidentified mechanism.

While antisense methods have shown promise in basic research and are now being evaluated in clinical trials, the use of triple-helix forming oligonucleotides (TFOs) to target the genes themselves has become increasingly popular in recent years, and may someday provide a comparable if not improved method for gene therapy.[150] This approach may potentially offer a permanent change in gene expression if point mutations and recombination occur as a result of processing of the oligonucleotide which may be treated as a lesion itself by the cellular repair machinery.[151] So far, this technology has only been applied to a

homopurine sequence within the 92 kDa gelatinase gene using a psoralen-conjugated oligonucleotide containing 8-oxo-adenine to stabilize the complex at physiologic pH which allowed binding and thermostable complex formation in vitro.[152] It remains to be seen whether this approach as well as mutagenesis induced by chimeric RNA-DNA oligonucleotides[153] can be extended to living cells and tissues, and whether the mechanisms of repair of these complexes will prove to be a stumbling block in the search for a durable inhibitory response, or can be harnessed to produce therapeutically useful site-specific mutations and recombination in vivo.

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CHAPTER 10

# Collagenase in Embryonic Development and Postnatal Remodeling of Connective Tissues

**Stephen M. Krane and Weiguang Zhao**

## MMPs with Collagenase Activity

In the process of remodeling of bone and other connective tissues, components of the extracellular matrix (ECM) such as collagens and proteoglycans are degraded and removed and new components are synthesized and deposited. The extent and intensity of remodeling is regulated by specific cells in each tissue that respond to signals from other cells and the matrix itself.[1] Three main classes of enzymes have been implicated in the degradation of collagen, the most abundant component of the ECM. The first includes members of the matrix metalloproteinase (MMP) family.[1-7] The second includes lysosomal cysteine proteinases such as cathepsins B and L which cleave collagens in the telopeptide domain.[8,9] Another cysteine proteinase is cathepsin K (also designated cathepsin OC2, X, O or O2 by various investigators) which is highly expressed in osteoclasts[10,11] and is the site of mutations in human pycnodysostosis,[12] a disorder characterized by osteosclerosis. The third class includes serine proteinases such as plasmin, generated through the plasminogen activator system.[13,14] Plasmin and cathepsin B have been implicated in the activation of MMP zymogens.[15,16] After activation, certain MMPs are capable of degrading different types of collagen substrates. It is likely that such MMPs have a role in the physiological resorption of collagen in the uterus, in embryonic development and postnatal remodeling and in pathological processes such as local invasion by malignant tumors, resorption of periodontal structures in periodontal disease or the destruction of joints in rheumatoid arthritis.[17-19]

The MMPs or matrixins are members of a large subfamily of proteinases that have many common structural features[6](see chapter 1). There have been more than 20 different MMP gene products described, 15 of which have been described in humans. Included in the MMP subfamily[2-7] are human genes that encode at least three collagenases, three stromelysins and several gelatinases among which are cell-bound forms with a transmembrane domain.[20] Although there is considerable conservation of amino acid sequence motifs among the human MMPs, only the collagenases ("fibroblast" collagenase [MMP-1 or collagenase-1],"neutrophil" [MMP-8 or collagenase-2] and the rodent-type interstitial collagenases[21-23] which are homologous to human collagense-3 or MMP-13)[24] can cleave native, undenatured, "interstitial" collagens (types I, II, III, X) within the triple helical domain at neutral pH. According to one report, the 72kDa gelatinase also has collagenolytic activity if purified free

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from the Tissue Inhibitor of Metallo Proteinases (TIMP) and by this criterion could also be considered as a collagenase.[25] One of the transmembrane MMPs, MT1-MMP, when expressed as a soluble protein using a construct in which the transmembrane sequence was deleted also had collagenase activity.[26] Nevertheless, only one interstitial collagenase has been identified so far in the mouse, the human collagenase-3 homologue.[59]

In native type I collagen, collagenase cleavage occurs between a Gly/Ile in the α1(I) chain and a Gly/Leu in the α2(I) chain, 3/4 the distance from the N-terminus yielding a larger (A) fragment and a smaller (B) fragment.[27] In other "interstitial" collagens, e.g. type II collagen, the cleavage site is at a corresponding locus. There are many Gly/Ile and Gly/Leu sequences distributed throughout the component chains of type I collagen, yet only the bonds between residues 775-776 are cleaved by collagenases. Valuable information about collagenase specificity had been obtained through studies of small synthetic peptides that could be cleaved by collagenase but with a relatively high $K_M$. Nevertheless, the results of these studies did not explain how collagenases cleave the natural triple helical substrate with a much lower $K_M$ and cleave denatured collagens more slowly than native collagens.[28,29]

## Creating Mutations in Collagen that Confer Resistance to Collagenase

Our approach to this problem has been to generate mutated α1(I) chains of type I collagen that are resistant to cleavage by collagenases in the helical domain. We first constructed several collagenase-resistant mutations in the mouse Col1a-1 gene and tested them in cultured Mov13 cells.[30,31] We found with substitution of Pro for Ile776 at the P1' position that the collagen was totally resistant to collagenase cleavage (Fig. 10.1). Another collagen encoded by a mutation (Mut IV) that had a double substitution of Pro for Gln774 and Ala777 at P2 and P2', a Met for Ile776 and a double substitution of Ala for Val782,783 was also not cleaved. Of additional interest was our observation that cleavage of the wild type α2(I) chains in the type I heterotrimer is dependent on the presence of cleavable sequences in the α1(I) chains, suggestive of a dominant negative effect.

Although there was strong circumstantial evidence indicating a role for collagenases in resorption of the ECM in different tissues, particularly in pathological conditions, we reasoned that rigorous proof for such a role would require evidence obtained through additional techniques such as those of molecular genetics. We used two different strategies. The first was to introduce a transgene containing one of the mutations described above (Mut IV). The second involved targeting the same mutation to the endogenous Col1a-1 gene[32,33] via homologous recombination in embryonic stem cells using the "hit-and-run" strategy of Hasty et al.[34] We chose to target the substrate rather than the enzyme(s) because of the potential redundancy in collagenase genes in the mouse analogous to the human. During the period since our studies were initiated, however, there have been no reports indicating the presence of collagenase gene products other than the interstitial collagenase.[59]

## Transgenic Mice with Collagenase Resistant Collagen

### *Introduction of a Mutant Transgene*

In the first approach, the murine genomic clone encoding the amino acid substitutions around the collagenase cleavage site in Mut IV were microinjected into the pronuclei of mouse embryos.[33] Few transgenic animals were recovered; only one of the 13 live embryos dissected at day 13 and one of 18 animals analyzed at weaning carried the transgene. The latter animal, a male, was bred with females to derive transgenic offspring. Transgenic mice carrying Mut IV in multiple copies did produce type I collagen that was highly resistant to cleavage by collagenase, consistent with the conclusion that collagenase recognizes a single

**EFFECTS OF AMINO ACID SUBSTITUTIONS IN THE COLLAGEN α1(I) CHAIN ON SUSCEPTIBILITY TO COLLAGENASE CLEAVAGE**

| | | Collagenase Cleavage |
|---|---|---|
| | ↓ 775-776 | |
| WT | -G-P-Q-G-I-A-G-Q-R-G-V-V- | + |
| Mutant | | |
| I | -.-.-.-.-P-.-.-.-.-.-.-.- | 0 |
| II | -.-.-P-.-.-P-.-.-.-.-A-A- | 0 |
| III | -.-.-.-.-.-.-.-.-.-.-A-A- | slow |
| IV | -.-.-P-.-M-P-.-.-.-.-A-A- | 0 |
| V | -.-.-.-.-M-.-.-.-.-.-A-A- | slow |

Fig. 10.1. Effects of amino acid substitutions in the collagen a1(I) chain susceptibility to collagenase cleavage. Mutations were created in a Col1a-1 genomic clone and expressed in Mov 13 cells as described.[30] The enzyme used was a purified preparation of human rheumatoid synovial collagenase.

site in native collagen.[33] Furthermore, point mutations of the mouse α1(I) collagen chain such as those in Mut IV which would be predicted to affect cleavage and stabilize the triple helix around the cleavage site[30] rendered the protein resistant to degradation.[33] With the exception of the founder, however, animals carrying the transgene invariably died. Since the transgene constructs carried all of the known regulatory elements required for tissue specific expression, it is likely that ectopic expression of the transgene was not the cause of the lethality. The S1 nuclease analysis as well as protein analysis indicated that the transgene was highly expressed, resulting in the accumulation of collagenase cleavage-resistant type I collagen. Since the ratio of α1(I):α2(I) peptide chains of type I collagen extracted from transgenic mice was much higher (3:1 to 15:1) compared to the wild-type mice (2:1), α1(I) homotrimers must have been deposited in excess (Fig. 10.2). Furthermore, the α1(I) homotrimeric molecules were completely resistant to collagenase cleavage. Therefore, overexpression of collagen and the deposition of type I homotrimers lacking the α2(I) chains, rather than the expression of the mutant collagen, per se, was the probable explanation for the embryonic lethal phenotype of the transgenics. This conclusion was confirmed by the lack of a lethal phenotype in mutant mice carrying the same mutation in the endogenous gene, as will be discussed. These observations support the notion that embryonic lethality may be caused by overexpression of a transgene rather than by the mutation introduced.

Attempts have been made to analyze the function of specific genes in development by expressing in transgenic mice mutant forms of proteins that behave as dominant negative mutations.[35] The results of our reported study[33] emphasize the caution that must be used in interpreting data in animals carrying mutant transgenes since the phenotypes may not always be due to the expression of the mutant form of the protein but rather to overexpression or ectopic expression of the transgene. This caveat is particularly relevant when the

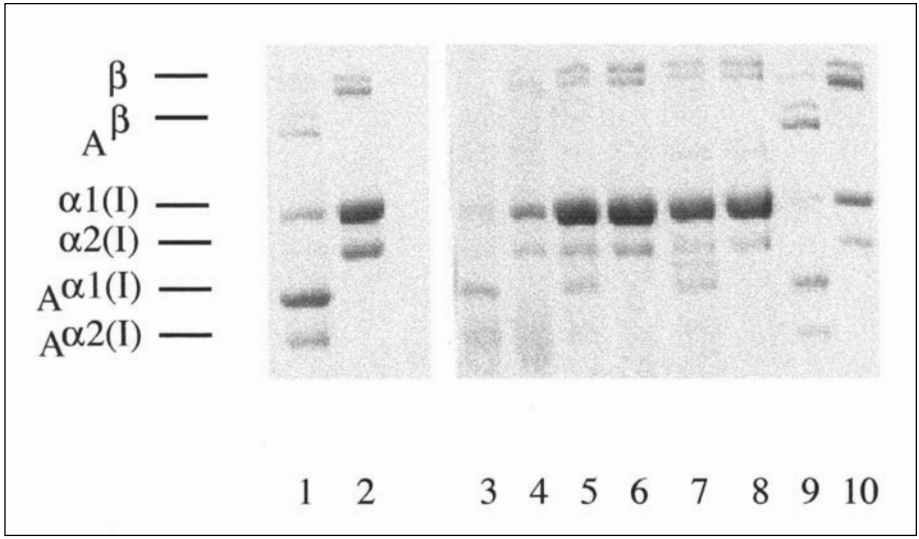


Fig. 10.2. Effects of Human Fibroblast Collagenase on Collagens from Wild-type and Transgenic Embryos. Transgenic mice were produced[33] in which the Mut IV mutation encoding amino acid substitutions around the cleavage site depicted in Figure 10.1. Collagen was extracted from E14 embryos from a different litter than those shown previously and incubated with rheumatoid synovial collagenase under identical conditions.[33] Lanes 5,6,7,8: transgenics; Lane 3,4,9,10: wild-type litter mates. Samples in Lanes 1,3,5,7, and 9 were incubated with typsin-activated human collagenase for 18 hr at 20°C (sufficient collagenase was used to digest 25 μg collagen); samples in Lane 2,4,6,8, and 10 were incubated in buffer without collagenase. Note the high ratio of a1(I): α2(I) in Lanes 6 and 8 consistent with the presence of α1(I) trimers (compare with Lanes 2 and 10). Since cleavage was incomplete, these trimers must have contained collagenase-resistant a1(I) chains encoded by the Mut IV transgene.

stoichmetry of a multimeric protein such as collagen is altered by overexpression of one component of the multimer.[35-38]

### *Homologous Recombination of a Mutant Transgene*

In view of the difficulties in the interpretation of the results from introduction of the collagenase-resistance Mut IV transgene, in our second strategy we employed targeted mutagenesis to introduce the same mutation into the endogenous Col1a-1 gene.[33] In contrast to the transgenic mice, mice generated by the gene-targeting technology that carried the same mutation in their endogenous Col1a-1 gene (the mutant allele[r] of the Col1a-1 gene was named Col1a1$^{tm1\ Jae}$) developed normally to young adulthood and only then displayed alterations compatible with impaired collagen turnover. Nevertheless, collagen extracted from the skin, tendon and bone was not cleaved in the helical domain by human MMP-1 (collagenase-1) or murine or human MMP-13 (collagenase-3) at what was previously considered to be the single site at which these enzymes cleave undenatured type I collagen. An example is shown in Figure 10.3. We had demonstrated earlier that MMP-8 (neutrophil collagenase or collagenase-2) also did not cleave this mutant collagen produced by fibroblasts in cell culture.[31] Our data on the effects of collagenases on cleavage of triple helical collagen that contained the targeted mutations in the α1(I) chains around the helical cleavage site indicated that all three chains in the type I heterotrimer must have the proper cleavable

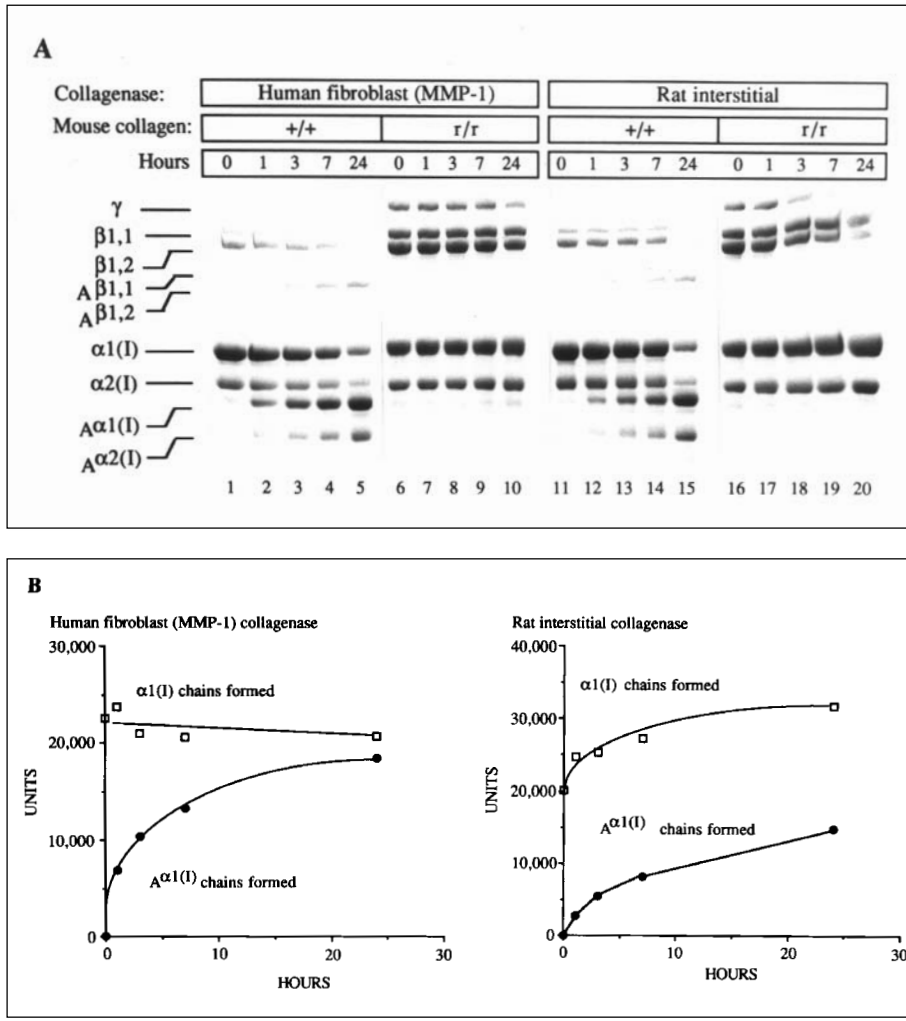


Fig. 10.3. Time course of cleavage at the helical and aminotelopeptide sites in mouse type I collagen by purified rat interstitial collagenase and human fibroblast (MMP-1) collagenase. (A) SDS-PAGE (5% acrylamide) showing reaction products after incubation with +/+ (Lanes 1-5 and 11-15) or r/r collagens (Lanes 6-10 and 16-20) and sufficient purified human fibroblast (MMP-1) collagenase (Lanes 1-10) or rat (lanes 11-20) interstitial collagenase to digest ~50% of the pepsinized +/+ collagen after 24 hr at 20°C. The substrates were collagen extracted from skin of +/+ mice with pepsin and from skin of r/r (Col1a1$^{tm1Jae}$) mice in 0.5M acetic acid without pepsin as described.[56] Parent solutions containing enzyme and substrate were prepared at 0°C and incubated at 20°C to start the reaction. Aliquots calculated to contain 25 μg of collagen were withdrawn and the reaction stopped by the addition of 1/10 vol of 500mM EDTA at the indicated intervals. After the reactions were stopped by the addition of EDTA to final concentration of 50mM, the samples were stored at 4°C until analysis by SDS-PAGE. (B) Rates of cleavage of helical region ( conversion of pepsinized +/+ type I collagen to A$^{\alpha 1[I]}$ components) and aminoterminal region ( conversion of β components in acid-extracted r/- type I collagen to α1[I] chains) analyzed by densitometry of samples shown in (A).

sequences.[30,33] Even a single mutated α1(I) chain prevented the cleavage of the other two +/+ chains (i.e., another α1(I) chain and an α2(I) chain). The $K_M$ for the triple helical type I collagen molecules had been previously shown to be several orders of magnitude lower than that for the corresponding gelatins.[28] It had been also proposed that collagenases are able to attack triple helical collagen because the region around the cleavage site is relatively poor in Pro (hydroxyproline [Hyp]) in the (-Gly-X-Y-) triplet-Y-position as seen in Figure 10.1 and since Pro/Hyp stabilize the triple helix, the Pro-poor area is susceptible to unwinding 39. In this regard, in preliminary results using circular dichroism (in collaboration with Dr. H.P. Bachinger Shriners Hospital, Portland, OR), we found that substituting Pro in only 2/333 triplets would have such a profound effect on structure.

### *Mouse Phenotype*

Although embryonic development of the r/r mice appeared to be normal, as early as approximately 4 weeks of age they began to develop thick skin and patchy hair loss accounted for by dermal fibrosis. In older mice (> 5-6 months) skin abnormalities consisting of thickening and roughening, associated with patchy hair loss and small ulcerations were regularly observed.[33] Indeed, in our initial report, we had not yet systematically examined the younger mice. Examination of skin sections revealed that the dermis from the r/r mutant mice was significantly thicker than that from control (r/r) mice and was filled with dense collagen fibers (Fig.10.4). The collagen fibers were irregular in form and penetrated deeply into the hypodermis. The overall increased thickness of the skin in the homozygous r/r mice, extending from the epidermis to the muscular layer, was accounted for by the increase in thickness in the dermis. The hair follicles appeared to be buried within the dense collagenous deposits whereas in control mice the hair follicles had their origin in the hypodermis. It is presumed that interference with the function of the hair follicles embedded in the dense collagenous matrix accounted for the hair loss. Several of these findings resemble those in the skin of patients with systemic sclerosis (scleroderma). Analysis of pepsin digests of mutant skin protein by SDS-PAGE and delayed reduction indicated that ~95% of the collagen was type I collagen with only traces of type III collagen.[33] Interestingly, homozygous females and heterozygous male and female mice developed similar but milder skin abnormalities compared to those of homozygous males at the same age.

Another defect resulting from the mutant collagen became evident in the impaired reproductive ability of mutant females. In mammals, the uterine mass changes considerably during pregnancy and postpartum involution.[40,41] In mice, the total uterine mass accounted for by collagen accumulation increases up to 20-fold during pregnancy, and after parturition the uterus rapidly recovers from prepregnant size.[42] This postpartum involution is accomplished within the first two days after giving birth and involves transcriptional activation of the collagenase gene and increased release of collagenase extracellularly followed by the massive degradation of most of the collagen deposited in the uterus within a few days.[40-42] In previously pregnant mutant r/r females, degradation of collagen was severely disturbed, leading to the accumulation of nodules in the uterine wall. These nodules consisted of large collagen aggregates, reflecting the impaired collagen degradation during the postpartum period (Fig. 10.5). The maintenance of a high collagen content in the postpartum uterus is presumably responsible for the reduced number of litters and decreased litter size of mutant females. Our results suggest that the massive degradation of collagen at the time of parturition is critically dependent on the presence of a functional cleavage site between Gly775 and Ile776 in the α1(I) chains of type I collagen. Our observations that there was a diffuse as well as nodular pattern to the collagen disposition in the r/r postpartum uterus suggests that the intensity of the normal resorptive process initiated at the time of parturition is not uniform throughout the uterus. So far we have only observed these changes in

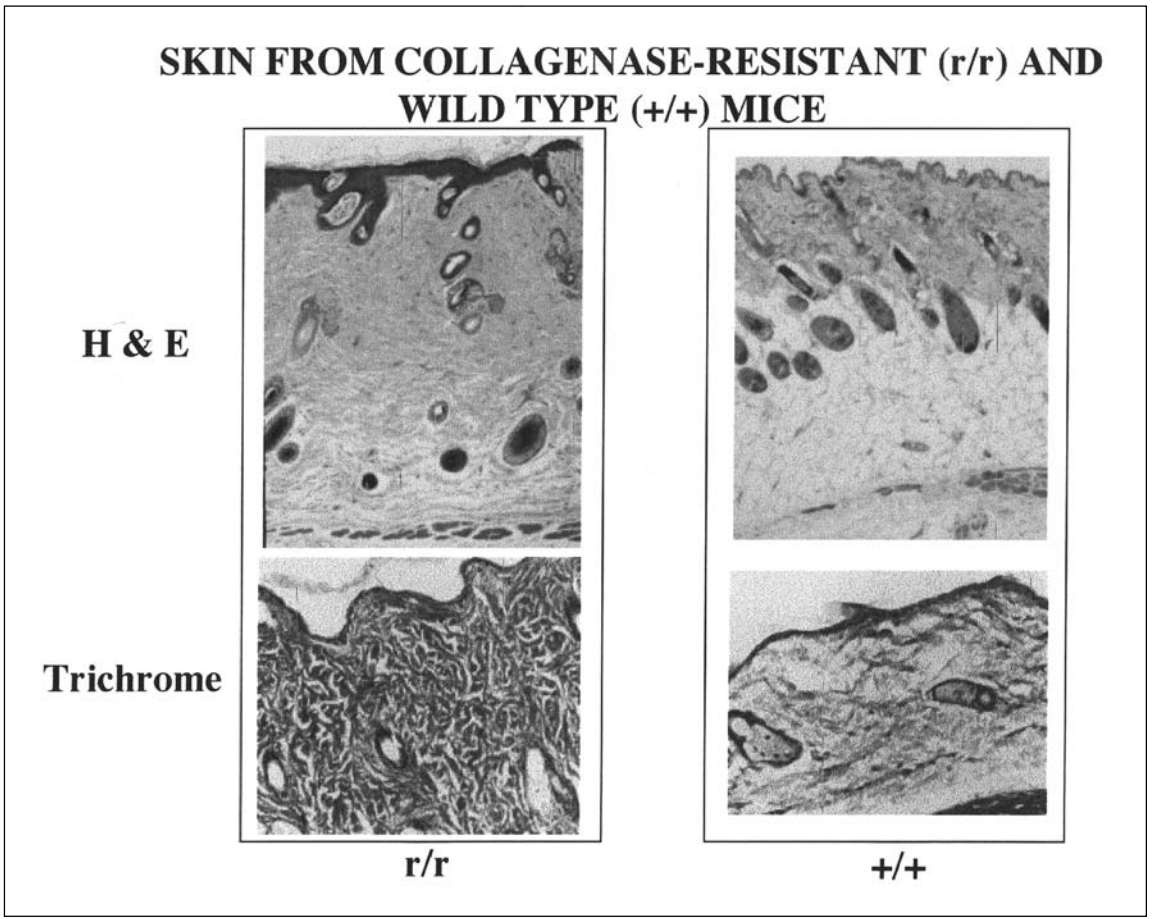


Fig. 10.4. Histology of skin from collagenase-resistant (r/r) and wild-type (+/+) mice at ~6months of age. Paraffin-embedded sections were stained with hematoxylin and eosin (upper panels) and trichrome (lower panels). Photomicrographs were taken at same magnification. Note accumulation of collagen in the dermis from the r/r mice.

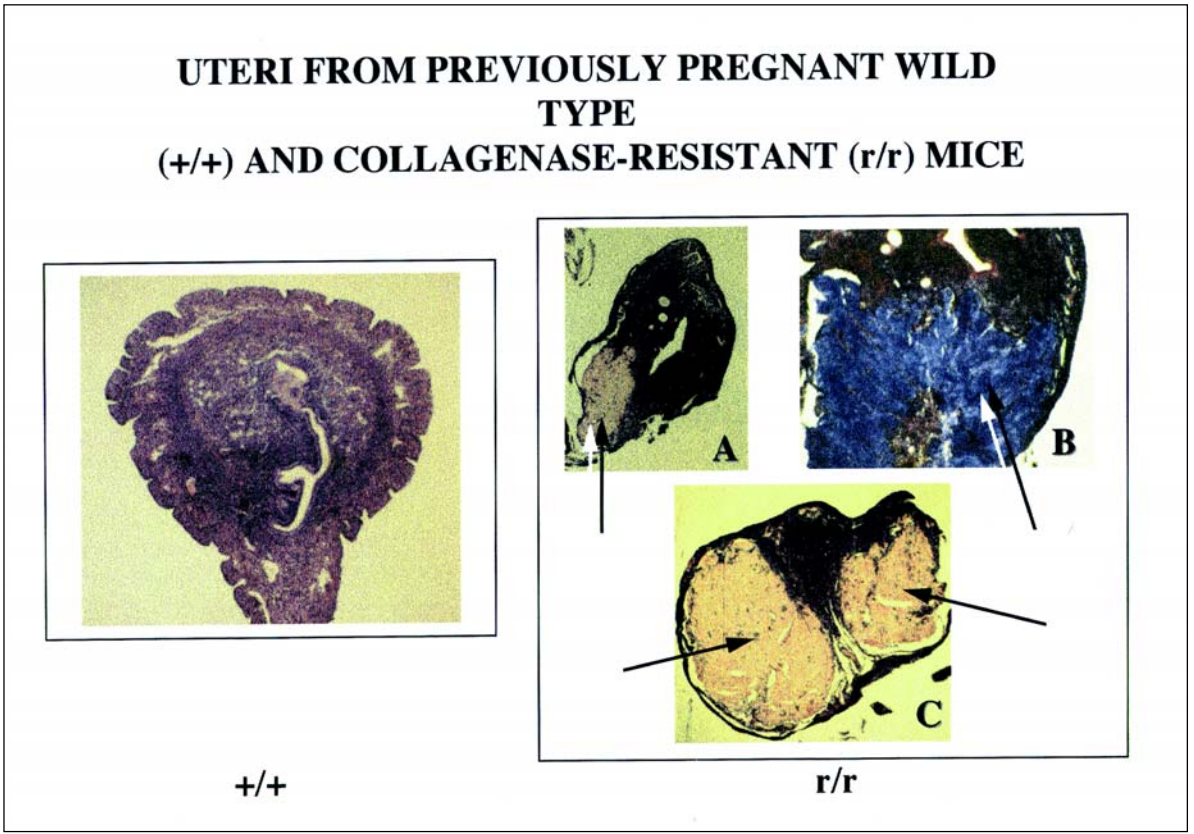


Fig. 10.5. Histology of uteri from previous pregnant collagenase-resistant (r/r) and wild-type (+/+) mice. Sections were stained with hematoxylin and eosin with the exception of the sample from the r/r mouse labeled (B) which was stained with trichrome.

the uterus of previously pregnant homozygous (r/r) females and not the heterozygous (r/+) females.

### *Effects on Collagen Homeostasis*

These findings illustrate aspects of the dynamic relationship in a specific tissue between collagen synthesis, on one hand, and collagen degradation on the other. We have noted, in unpublished observations, using riboprobes for the mouse interstitial collagenase and in situ hybridization, that there is expression of the collagenase mRNA of a spotty nature in clusters of basal keratinocytes in the skin (also see chapter 12). Some cells in the dermis, presumably fibroblasts, also show the presence of collagenase mRNA. Collagenase-1 (MMP-1) is also expressed in human keratinocytes in inflammation and wounding.[43-45] Data from several laboratories indicate that epithelial cells such as keratinocytes as well as various mesenchymal cells bind to type I collagen matrices using $\alpha 2\beta 1$ integrins on the cell surfaces[46,47](see chapter 8). In wounded epidermis, migrating keratinocytes continue to express these collagen-binding $\alpha 2\beta 1$ integrins but then redistribute them such that they are concentrated on the frontobasal portions of the cell.[47] Ligation via this integration is associated with induction of expression of collagenase, in human keratinocytes, MMP-1;[46,47] antibodies to the $\alpha 2\beta 1$ integrin blocks the collagenase induction. Interestingly, the human keratinocytes require active collagenase for migration and although collagen from the r/r mice can induce collagenase gene expression, the collagen is not cleaved and the cells do not migrate.[47] In other systems, e.g. utilizing melanoma cells, binding to type I collagen also induces collagenase gene expression; cleavage of the collagen then "reveals" sites for binding by the $\alpha v\beta 3$ integrin, ligation of which results in sustained viability of the cells through inhibition of programmed cell death (apoptosis).[48]

We thus envision a sequence of focal remodeling events in the dermis in which keratinocytes and fibroblasts are repeatedly stimulated through injury such as UV irradiation or mechanical trauma to release various cytokines that stimulate matrix degradation and synthesis; these responses also involve cell contacts with extracellular matrix components (Fig. 10.6). Normally, the extra collagen accumulated is eventually degraded through the action of collagenases and the remodeling cycle subsides. In the case of the r/r mice, the proteolytic degradation of the deposited collagen cannot take place because of the targeted mutations around the cleavage site and thus collagen accumulates. Failure to degrade the type I collagen results in retention of some of the other excellular matrix components intimately associated with type I collagen. The remodeling sequences suggested for the skin are based not only on our observations in the r/r mice but also on concepts utilized for years as a framework to consider remodeling events in bone.

## Characterizing Effects on Joints and Bone

In our original report,[33] we mentioned the occurrence in r/r mice of joint contractures and deformed bones beginning at ~3-6 months of age. In a more detailed examination, we have now detected contractures at the ankle joint in r/r mice as young as four weeks of age. Histologically in the joints involved in contractures, we observed excessive collagen deposition in dermis and subcutaneous areas as well as thickened tendons and ligaments. We also have indications that tendon migration is impaired in r/r mice. A model was developed for examination of these mice based on techniques described for use in the rabbit.[49] Metallic markers were inserted into the tibia and patellar tendon and the relative change in position during growth were monitored by serial X-rays over the period from 4-15 weeks of age. Preliminary results indicate a decrease in tendon migration in the few r/r mice examined compared to the +/+ mice.[50] We showed further by in situ hybridization using mouse interstitial collagenase $^{35}$S-riboprobes, that collagenase transcripts were found in cells in the

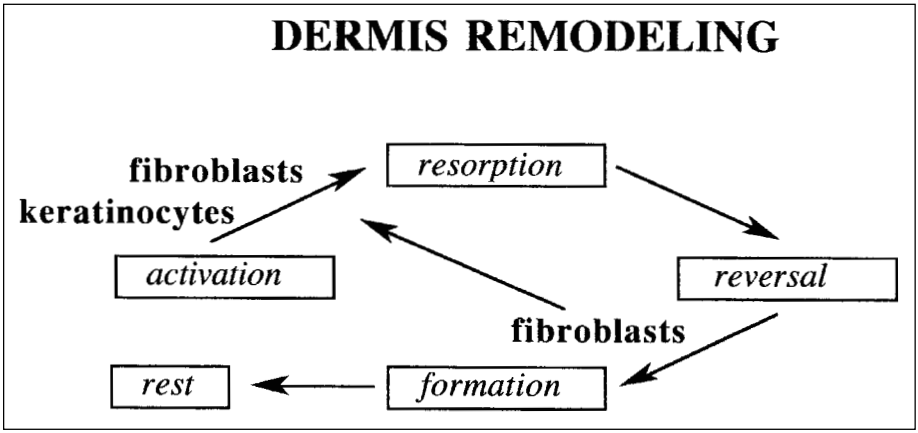


Fig. 10.6. A possible sequence of remodeling events in the dermis.

periosteum and at tendon/bone interfaces involving the expression and action of collagenases possibly mediated by trauma of repeated flexion/extension and other stresses at the joints. Inability of collagenase to act on the type I collagen substrate results in accumulation of the collagen in the locations described. Cleavage of type I collagen in the helical domain by mouse collagenase expressed in fibroblasts and osteoblasts at tendon/bone interfaces may be necessary to prevent the development of joint contractures and permit tendons to migrate normally during linear bone growth. Failure of tendon migration and development of contractures during growth may also contribute to bone deformities and fracture in the r/r mice.

We had also noted that whereas overall bone development was normal in the r/r mice, deformities of the tibia and increased deposition of trabecular and cortical bone in femurs and tibias were frequent after the age of 6 months, suggesting that bone remodeling might also be affected.[33] This was based on radiographs of a few mice at different ages and examination of a limited number of bones by H & E staining. In the spine, we observed the development of kyphosis associated with the presence of excessive woven bone that could possibly represent healing fractures. The most consistent finding in long bones, particularly the tibias, was the presence of anterior bowing deformities and suggestive evidence of old fractures. The volume of bone was excessive, but not to the degree observed in the forms of osteopetrosis in rodents and there was not a persistence of dense bone in the metaphysis at the expense of the marrow, a characteristic feature of osteopetrosis. It is possible, however, that the excessive bone could be accounted for by accumulation of woven bone from incomplete fracture healing. An increase in the volume of bone in the medullary canal of the femur was also seen, but this was mostly woven bone and could also be due to fractures.

Recently, we have begun to quantitatively analyze the calvaria from +/+ compared with r/r mice at 4 and 6 weeks of age for the extent of osteoclastic resorption in the basal state.[51] In addition, we have begun to measure the responses of these bones to parathyroid hormone (PTH). PTH is thought to stimulate bone resorption through the direct action on osteoblasts and stromal cells and then through an indirect action to increase the differentiation and function of osteoclasts. PTH acting on osteoblasts and stromal cells increases collagenase gene transcription and synthesis. In order to assess the potential role of collagenase in the bone resorptive actions of PTH, we utilized the homozygous collagenase resistant (r/r) mice and the protocols of Boyce and co-workers.[53,54] Human PTH (1-34) was injected

subcutaneously over one side of the calvaria in wild type (+/+) or r/r mice four times daily for three days. Osteoclastic bone resorption was then measures on histological sections. Preliminary results indicated that osteoclast numbers and the size of the bone marrow spaces were increased and prominent osteoblastic proliferation was present in bones from PTH-treated wild type (+/+) mice.[51,60] In striking contrast, in r/r mice, resorporation in response to PTH was markedly reduced inside the calvariae and few osteoclasts were identified. The responses of periosteal osteoblasts were also significantly suppressed in the r/r mice. Nevertheless, bone cells responded to PTH as demonstrated by in situ hybridization using interstitial collagenase riboprobes. Abundant collagenase mRNA was detected in the periosteum of PTH-treated r/r as well as +/+ mice. Thus cleavage of collagen at the helical site by collagenase appears to be necessary for the induction of osteoclastic bone resorption by PTH. Several possible mechanisms might explain how collagenase could be involved in the bone resorptive actions of PTH. For example, Chambers and colleagues[54] have proposed that the action of collagenase produced by osteoblasts or stromal cells might be required for denuding of collagen on bone surfaces in order for osteoclasts to attach. We have also considered that the degradation of this surface layer of collagen might also release stored matrix-bound growth factors or that cleavage products resulting from the action of collagenase on type I collagen might also be biologically active on osteoclasts. The latter is supported by the findings in a recent report indicating that collagen fragments produced by interstitial collagenase can activate osteoclastic bone resorption.[55] Finally, based on the findings described earlier,[47,48] collagenase cleavage of type I collagen could expose cryptic integrin binding sites involved in osteoclast migration or prevention of osteoclast apoptosis.

## Characterization of Collagenase Cleavage

In analysis of collagenase cleavage, we first used highly purified preparations of rat fibroblast collagenase and human MMP-1, the latter derived from cultures of rheumatoid synovium, to obtain data on cleavage at the two sites in type I collagen. Subsequently, we showed that purified rat interstitial collagenase, which converted the collagen $\alpha$ chains from wild type mice (+/+) to the 3/4 length ($A^{\alpha}$) fragments, also decreased the content of $\beta$ components without proportionately increasing the content of 3/4 length ($A^{\beta}$) components.[33] These highly purified preparations of rat interstitial collagenase 40 (97% aminoacid sequence identity with the mouse)[23] made an additional cleavage between Gly/Val in the amino(N)-telopeptide beginning with the putative crosslinking Lys, i.e., LysSerAlaGlyValSerValPro. The cleavage is C-terminal to the crosslinking Lys, eight residues before the start of the major collagen helix containing the triplet GlyProMet. There is considerable conservation of these sequences in collagens from different species (Fig. 10.7). In contrast to the effects of the rodent collagenases, partially purified preparations of human fibroblast collagenase (MMP-1) converted the a chains to $A^{\alpha}$ and the $\beta$ components to $A^{\beta}$. None of the collagenases, however, cleaved the collagen extracted from the skin of homozygous collagenase-resistant mice (r/r) at the helical locus. The relative rates at which the human and rat enzymes cleaved at the helical and N-telopeptide sites was determined by measuring the formation of $A^{\alpha 1(I)}$ chains from a1(I) chains of pepsinized +/+ collagen and the formation of a1(I) chains from acid-extractable r/r collagen, respectively, and quantitated by densitometric analysis of the Coomassie blue-stained bands after SDS-PAGE. In several experiments, the rate of N-telopeptide cleavage of purified rodent interstitial collagenase approximated that of the helical site. Purified sheep and rabbit MMP-1 (~50% amino acid sequence identity with the rat collagenase but ~85-90% identity with the human enzyme and each other), activated with APMA or stromelysin-1, also readily cleaved at the helical site but at low rates or not at all at the N-terminal site.[56] Purified rat collagenase was also found to cleave the crosslinked components in human, acid-extracted collagen to $\alpha$

chains, based on the relative amounts of $A^{\beta}$ and $A^{\alpha}$ products. The N-telopeptide cleavage activity is thus a property shared by rodent fibroblast collagenases but not by human MMP-1 or the MMP-1-related enzymes from other species. Nevertheless, human type I collagen is a substrate for this cleavage by the rodent collagenases.

We have expressed in *E. coli* human MMP-1 collagenase, (HCL1 [called CLH in ref. 56]) and mouse interstitial collagenase (MMP-13 [MCL3 or CLM]) cDNAs and chimeric constructs in pET-3d, juxtaposing mouse collagenase (MMP-13) sequences N-terminal to the Zn-binding site in the catalytic domain and MMP-1 sequences C-terminal (MCL3/HCL1 or CLMH) and vice versa (HCL1/MCL3 or CLHM).[56] MMP-13 (MCL3 or CLM) and chimeric molecules that contained the MMP-13 sequences N-terminal to the Zn-binding site (MCL3/HCL1 or CLMH) cleaved (+/+) collagen at the helical locus and cleaved crosslinked (r/r) collagen in the N-telopeptide (β components converted to α chains). Human MMP-1 (HCL1 or CLH) and chimeric MMP-1/MMP-13 with MMP-1 sequences N-terminal to the active site (HCL1/MCL3 or CLHM) cleaved collagen at the helical locus but not in the N-telopeptide. All activities were inhibited by TIMP-1, 1,10-phenanthroline and EDTA. Thus, sequences in the distal 2/3 of the catalytic domain appear to determine the N-telopeptide degrading capacity of MMP-13[56] (Fig. 10.8).

In preliminary experiments, we also expressed human collagenase-1 and -3 in the pET-3d vector in *E. coli* as described above[57] and found that the recombinant collagenases cleaved wild type I collagen at the previously documented helical site as examined by SDS-PAGE. Recombinant collagenase-3, as predicted, based on the sequence identity with the mouse collagenase, made an additional cleavage at an N-telopeptide site, demonstrated using acid soluble collagen containing abundant crosslinks extracted from the r/r mice as substrate; recombinant collagenase-1 did not cleave at this site. Collagenase-3 could thus function in the destruction of the extracellular collagenous matrices in disease by cleaving crosslinked collagen at this additional site (Fig. 10.9). We have also prepared other constructs in pET-3d that create chimeric molecules of the human collagenase-1 (HCL1) and collagenase-3 (HCL3) analogous to the murine interstitial collagenase/ human collagenase-1 chimeric molecules. Our data confirm what we have found using human/mouse collagenase chimeric molecules and suggest that the specificity for the N-telopeptide cleavage resides in sequences N-terminal to the Zn-binding site.

## Concluding Remarks

The results of the studies described clearly demonstrate the necessity for collagenase cleavage at the helical sites in the type I collagen for selected processes in tissues such as skin, bones, joint structures and uterus that are critical for connective tissue remodeling in postnatal life. Our observations that the homozygous (r/r) mice developed normally to adulthood pose an interesting problem as to how type I collagen turnover is accomplished during embryogenesis in the absence of a functional collagenase helical cleavage site. The identification of the second site at the N-telopeptide domain of the protein recognized by the rodent fibroblast collagenases and human collagenase-3 (MMP-13) but not by human MMP-1 or related enzymes, suggests the possibility that cleavage by collagenases or their enzymes at this previously unrecognized site (Fig. 10.9) could be sufficient to achieve collagen degradation during embryonic development. Cleavage at this site alone, however, seems not to be adequate to assure full equilibrium between collagenase synthesis and degradation during later life. Our results are consistent with the concept that enzymes with different specificity utilizing two distinct cleavage sites are involved in collagen degradation. The two sites may be of different significance for type I collagen degradation in prenatal and postnatal life. It will be necessary to demonstrate that collagenase cleavage at the N-telopeptide site takes place in vivo. This will require methodology such as the use of monoclonal

**COLLAGEN N-TELOPEPTIDE AMINO ACID SEQUENCES***

| | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| human a1(I) | Y | D | E | K | S | T | G | G | | | I | S | V | | | | | | P | G | P | G | P | M |
| mouse a1(I) | Y | D | E | K | S | A | G | | | | V | S | | | | | S | | | | P | G | P | M |
| mouse a2(I) | Y | S | D | K | | | G | | | | V | S | | | | | S | | | G | P | G | P | M |
| human a2(I) | Y | D | G | K | | | G | | | | V | G | L | | | | | | | G | P | G | P | M |
| human a1(II) | F | D | E | K | | A | G | G | A | Q | L | G | V | | | M | Q | | | | | G | P | M |
| mouse a1(II) | Y | D | E | K | | A | G | G | A | Q | M | G | V | | | M | Q | | | | | G | P | M |
| human a1(III) | Y | D | V | K | | S | G | | | | V | A | V | G | G | L | | | | A | | G | Y | P |

* data from SwissPro

Fig. 10.7. Amino acid sequences in the N-telopeptide region of component α chains of types I, II and III collagens from several species.

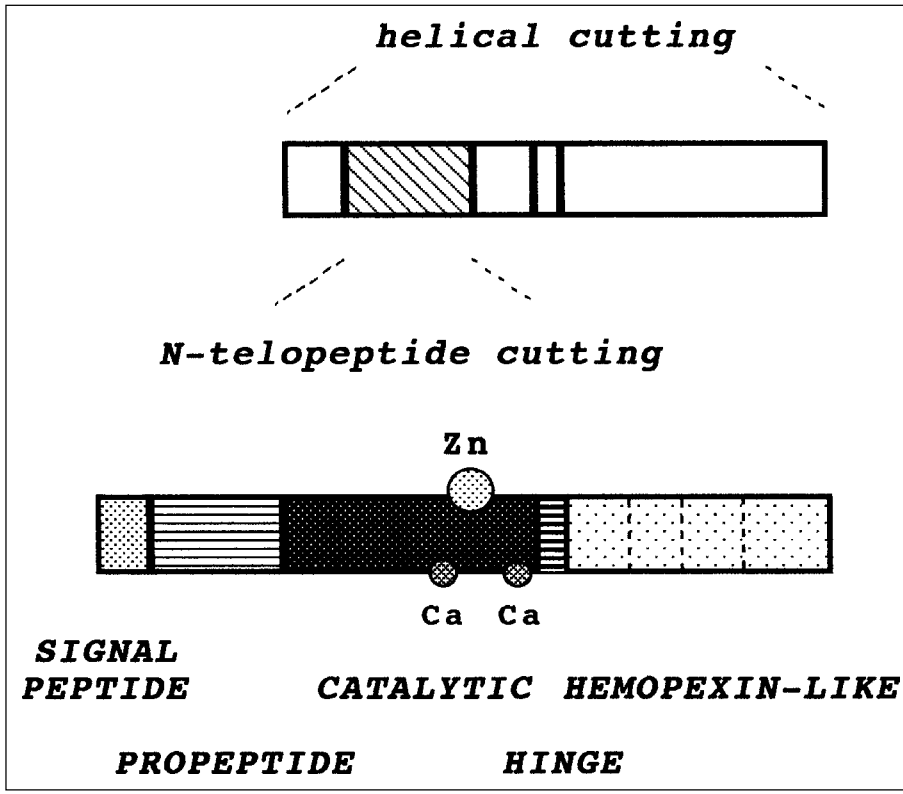


Fig. 10.8. Domains within collagenases that determine N-telopeptide and helical cutting activity determined by expressing chimeric molecules of human collagenase-1 (MMP-1) and mouse interstitial collagenase, homologous to human collagnease-3 (MMP-13)56. Those sequences in the catalytic domain of mouse interstitial collagenase that conferred N-telopeptide cutting activity are shown in the cross-hatched area. When the corresponding sequences in human collagenase-1 (MMP-1) were present, N-telopeptidase activity was not observed.

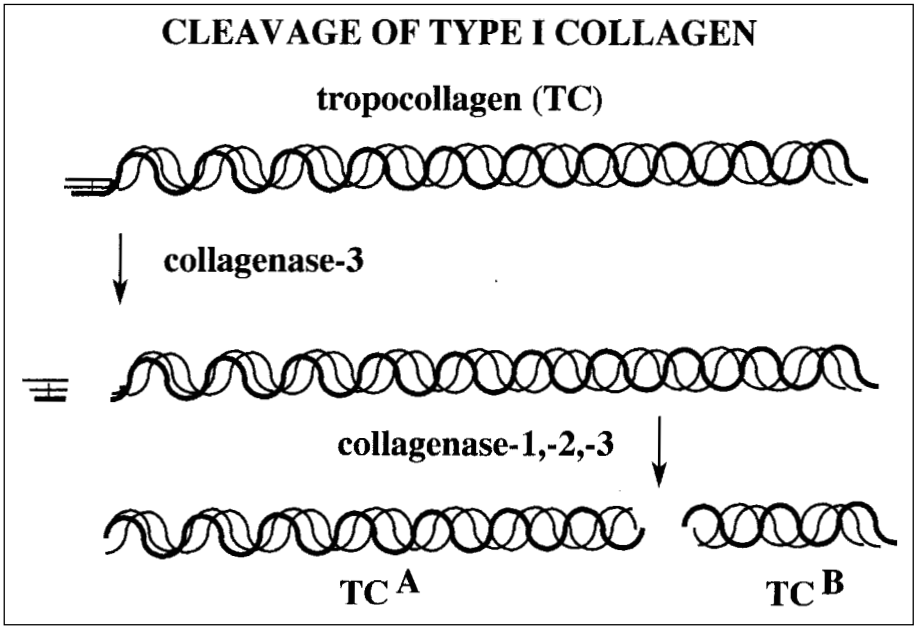


Fig. 10.9. A scheme for cleavage of the different domains of tye I collagen by collagenases. Only collagenase-3 cleaves the N-telopeptide domain. It has not been established, however, whether the N-telopeptide region is cleaved before, during or following cleavage of the helical domain.

antibodies that specifically recognize epitopes on the collagen chains only after collagenase cleavage in the N-telopeptide. Antibodies that recognize epitopes at the helical site only after collagenase cleavage have been successfully applied in other studies.[58] An alternative approach would be to target mutations to the N-telopeptide that would inhibit cleavage at this site.

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# Chapter 11

# The Role of Interstitial Collagenases in Tumor Progression

**Eric W. Howard**

## Introduction: Tumor Progression and the Role of Interstitial Collagenase

Collagenases and other members of the matrix metalloproteinase (MMP) family are involved in normal and developmental processes in vertebrates, but it is their roles in pathological processes that have thrust these enzymes into the scientific spotlight in recent years. In particular, their participation in tumor growth and metastasis, and the potential for blocking them therapeutically, has driven much of the research on MMP structure, function, expression, and inhibition. In the past few years, there has been a tremendous increase in MMP-related studies with a focus on tumor biology. Several factors have contributed to this growth. First, research conducted for the past three decades has firmly established the role of MMPs in tumor invasion and growth. Cellular transformation is often associated with increased invasiveness, and this process is often MMP-mediated. As a consequence, a great effort has been expended learning about the regulation of MMP expression. Second, MMPs are enzymes that can be inhibited by naturally occurring proteins such as the tissue inhibitors of metalloproteinases (TIMPs), as well as by a growing array of synthetic inhibitors. It is becoming apparent that blocking MMP activity can block tumor growth and metastasis, and it is not surprising that a number of pharmaceutical companies are actively pursuing MMP inhibitors as therapeutics. Finally, a growing arsenal of investigative tools have become available, making the study of MMPs realistic for people new to the field. Some of the assays for MMP activity, such as zymography, are quite straightforward, and numerous substrates and other activity assay components are available commercially. This, coupled with the commercial availability of antibodies, ELISA kits, and cDNA probes, has contributed to the high level of interest and participation in the MMP field.

The steps involved in tumor progression are generally agreed upon, but many of the details are only partially understood. It is known that tumor growth is dependent on the development of a tumor vasculature which enables tumors to expand beyond the limits of diffusion of nutrients.[1,2] It is also agreed that tumors enter and exit the vasculature in order to migrate to distant sites. Finally, it is accepted that tumor invasion and metastasis is associated with proteolysis of extracellular matrix. How this proteolysis is regulated, what cells produce the proteinases, and what proteinases are involved is not entirely clear.

Tumor growth and invasion is dependent on localized factors that determine how a tumor will grow and what invasion routes are likely to be utilized.[3] Among other things, it

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has been reasoned that a tumor's ability to digest extracellular matrix barriers is a key factor in the progression of the tumor. Given that fibrillar collagens are prevalent throughout tissues, and likely serve as barriers to tumor cell migration, it is likely that tumor invasion is linked to the expression of interstitial collagenases. Early evidence for this was found by Robertson and Williams, who added tumor extract to a fibrillar collagen solution, then analyzed the degradation of the collagen over time.[4] They found a heat inactivatable component in the tumor extract that digested the collagen, and reasoned that the putative enzyme responsible for this was collagenase. Similarly, Taylor et al demonstrated that tumor biopsies from several sources could digest collagen films, while Dresden et al showed similar results using several soluble assays.[5,6] This group further showed that this activity was inhibitable by EDTA, a property of the zinc-dependent MMPs. McCroskery et al subsequently purified collagenase from rabbit tumor samples, and found it to be similar to other collagenases previously identified.[7] These and other early studies demonstrated that tumor cells do express and activate collagenolytic enzymes significantly above the levels expressed by normal tissue.

At present, with an ever-increasing number of MMPs linked to various aspects of tissue function in both normal and disease processes, we are faced with the task of assigning critical roles in tumor progression to each of our favorite enzymes. As a result, the literature is now teeming with studies linking virtually every MMP with every imaginable form of cancer. In a way, these studies highlight the redundancy built into cellular processes, a fact made particularly clear by recent MMP gene knockout studies showing surprisingly few phenotypic changes, considering their ubiquitous expression during development.[8] That said, the numerous studies published on collagenase involvement in tumor progression have led to several general conclusions. First, tumors associated with increased collagenase expression tend to be more invasive, and are often faced with a fibrous, collagen matrix barrier through which they must pass. Second, a significant amount of the collagenase in tumor-laden tissue is expressed by nontumor stromal cells. This has led to the conclusion that tumor cells can alter their matrix environment by inducing adjacent cells to produce proteinases. Third, the proteinases expressed by tumor cells and stromal cells combine in proteolytic cascades leading to their mutual activation. Collagenase is expressed as a zymogen, as are most extracellular proteinases. Without the cooperation of various other proteinases, no matrix turnover can occur, simply because most of the proteinases would be inactive. Similarly, it has traditionally been thought that the initial and critical cleavage of fibrillar collagens by collagenase is a prerequisite for collagen's subsequent degradation by other MMPs such as the gelatinases. This would be another level of proteolytic cascade. This review focuses on the role of interstitial collagenases in tumor progression, and demonstrates some of the evolution in our thinking about the first member of the MMP family.

### *Stromal and Basement Membrane Barriers*

Tumor growth and metastasis involves several steps in which collagenases likely play a role. This includes extracellular matrix digestion to allow tumor cells to migrate and invade.[9-11] Interstitial collagenases have traditionally been thought to play a role in digesting underlying stromal extracellular matrix, but not to degrade the major basement membrane components lining blood vessels and epithelial layers. Nonbasement membrane extracellular matrix components include the fibrillar collagens type I and III, fibronectins, proteoglycans, and numerous other protein components that vary with the tissue source. Fibrillar collagen networks are thought to act as barriers to cells. This barrier function may take the form of a physical block to cell migration, depending upon the tissue-specific architecture and composition of the interstitium. A number of tumors grow to the point of compressing surrounding tissue, suggesting that they are limited by surrounding matrix. In

tissues containing a high percentage of collagenous stroma or surrounded by a collagenous sheath, interstitial collagenases would be expected to play a major role in both normal remodeling and in tumor invasion.

On the other hand, the nature of the collagen barrier may be more subtle. Tumor cell adhesion to different extracellular matrix components is considered a critical step in invasion and metastasis.[9] Cell migration, however, involves transient adhesion and de-adhesion, and these are critically influenced by the surrounding matrix through which the cell must travel. Proteolytic action alters the physical and chemical properties of extracellular matrices, thereby altering cellular adhesion to the matrix. Several events can lead to cellular de-adhesion from matrix. Signaling in response to growth factors can lead to changes in cell-matrix adhesion by changing the activation state of integrins. More important to this discussion, proteolysis can change extracellular matrix properties, leading to altered cellular responses to that matrix. For example, proteolysis can alter the physical strength of a matrix, causing increased matrix malleability. This can then lead to the loss of tension, which can in turn lead to the disassembly of cellular stress fibers and focal adhesions. This change in cell shape has been linked to the increased expression of collagenases, as discussed in previous chapters. While changes in cell-matrix tension probably do not drive most instances of cellular de-adhesion, more subtle changes in matrix integrity, particularly as a result of the actions of proteinases, can significantly regulate cell motility. This has recently been demonstrated with keratinocytes, which utilize interstitial collagenase to digest their underlying collagen substratum in order to migrate in a wound bed[12] (see chapter 12). Re-epithelialization during normal wound repair is critically dependent on the expression of collagenase-1 by keratinocytes. Blocking collagenase activity with TIMPs, peptide inhibitors, or with antibodies blocks keratinocyte migration on a type I collagen substrate. In addition, culturing these cells on noncleavable collagen prevents cell migration, but does not block the $\acute{a}_2\hat{a}_1$ integrin-mediated induction of collagenase-1 expression.[12] Basement membrane components do not induce collagenase-1 expression by these cells.

As demonstrated in the examples below, there are numerous cases where collagenous stroma or capsules serve as barriers to tumor invasion. Interstitial collagenase, the primary mediator of fibrillar collagen turnover, thus has a clear role in tumor progression. However, as the examples below will emphasize, it seems that many well-accepted paradigms ultimately crumble under the weight of new evidence. In this case, the idea that collagenase is not involved in basement membrane degradation may soon be made obsolete if evidence recently presented by Durko et al proves to be the rule rather than the exception. This group recently demonstrated that melanoma cells down-regulated for collagenase expression through transfection with anti-sense collagenase cDNA were unable to invade through basement membrane preparations or type IV collagen, in spite of their normal expression of gelatinase A.[13] While further study into this phenomenon is certainly needed, these results are sure to make those of us working with gelatinases very nervous. On the other hand, it has also recently been shown that tumor cells introduced into mice readily extravasated even when expressing high levels of TIMP.[14,15] This would lead one to assume that transit through the endothelial basement membrane either does not require proteolysis, or involves other classes of proteinases other than MMPs. The same study showed that, without active MMP-mediated proteolysis, these same cells failed to grow in their new location. Perhaps blocking interstitial collagenases prevents the ability of tumor cells to transit through tissue stroma. Thus, while it is still not clear how or when extracellular matrices actually serve as barriers to invasion, it is evident that MMP-mediated matrix turnover is important for tumor progression.

### *Tumor and Stromal Expression of Collagenase*

What is not clear is the origin and regulation of collagenase in different tumor environments. Localization of collagenase protein to tumor or stromal cells using immunological methods does not provide insights into which cells actually expressed the collagenase, since collagenase will bind to what ever cell possesses an appropriate receptor or binding protein. However, based on the findings by a number of laboratories, it now appears that collagenase expression associated with tumors includes both tumor and stromal cell components. While a number of tumor cells have the ability to express MMPs both in vitro and in vivo, a significant amount of the collagenase associated with tumors is expressed by stromal cells such as fibroblasts, as well as by infiltrating eosinophils and macrophages. This implies that tumor cells induce collagenase expression by stromal cells, and this does seem to be the case. The tumor-mediated induction of stromal cell collagenase expression may involve inflammatory cytokines, growth factors, extracellular matrix fragments, or any number of other cell mediators. For example, extracellular MMP inducer (EMMPRIN) (see chapter section 6.3) is expressed on the surface of tumor cells, and will induce collagenase expression by fibroblasts.[16] Coculture of tumor cells with fibroblasts will also induce collagenase expression by the nontumor cells.[17] Other tumor-secreted factors, as discussed in previous chapters, may be produced, depending upon the type of tumor.

A number of tumor cells themselves express collagenase in addition to inducing this expression in other cells. Direct evidence for this was demonstrated, for example, by Gavrilovic et al, who transplanted a rabbit carcinoma into mice, then immunolocalized rabbit collagenase expression with an antibody that was specific for the rabbit enzyme. This group also identified tumor-derived stromelysin, suggesting that at least part of a collagenase activation cascade was expressed by tumor cells.[18] Collagenase-3, which was originally cloned from breast cancer tissue (see chapter 3), also appears to be expressed primarily by tumor cells.[19,20] In addition, hepatocellular carcinomas were found to express collagenase, based on immunolocalization[21] and in situ hybridization.[22] Transfection of an oral squamous cell carcinoma-derived cell line with anti-sense oligonucleotides to E1AF, an ets-family transcription factor which regulates collagenase expression, resulted in decreased collagenase, stromelysin, and gelatinase B expression.[23] Introduction of these cells into nude mice demonstrated a decreased invasive potential compared to control cells, suggesting that tumor-derived MMPs can mediate invasiveness. Macdougall and Matrisian have hypothesized that cell types that would normally express a particular MMP during their growth and development would, when transformed, express this MMP. Thus, mesenchymal cell-derived tumors would be expected to express collagenase, while epithelial cell-derived tumors would not.[17] However, collagenase expression has been demonstrated in cultured tumor cell lines isolated from a variety of cell types, including breast carcinoma,[24-26] pancreatic carcinoma,[27] bladder carcinoma,[28] prostate carcinoma,[29] gliomas,[30] and fibrosarcoma and melanoma cells.[31] Moreover, keratinocytes, which are epithelial cells, can express collagenase, but this expression is regulated by the composition of the matrix to which these cells adhere.[12] In normal situations, epithelial cells do not invade into their substrata, but this changes during malignant transformation. Migration of epithelial cells through a basement membrane would expose them to underlying collagenous stroma, potentially inducing the expression of collagenase-1. This normal response to specific extracellular matrices, which is critical for successful wound healing, would clearly be undesirable in a tumor. Keratinocyte-derived tumors invading through a basement membrane and into collagenous stroma would thus be expected to express collagenase once they interact with fibrillar collagen; however, the number of invading cells doing so may be small and thus difficult to detect. In a number of cases, collagenase expression has been localized to both tumor cells and the adjacent

stroma, suggesting that both sources may be involved in the production of matrix-degrading enzymes.

## Participation of Collagenase in Proteolytic Cascades

Tumor cell invasion, depending upon the individual tumor, involves numerous proteinases in addition to various MMPs.[32-34] Different tissue environments require different proteinase capabilities. As individual tumor cells migrate through different layers of tissue, they encounter different extracellular matrices. Because a tumor is composed of cells adjacent to numerous micro-environments, both cellular and matrix, the topology of collagenase expression, localization, and activation may be quite variable in a given tumor. Thus, while collagenase, based on its relatively high expression, may play a more prominent role in tumor invasion in some of the cases described below, it may play an important role in many tumors in which its detection has either not been studied or its expression levels are below the sensitivity of current assays. It is also likely that multiple proteinases representing several proteinase families act in concert to enable cells to migrate and invade through different tissue environments. It is well established that collagenases are expressed as latent enzymes, and that some participation in proteolytic cascades is required for them to perform their biological functions. Similarly, its activation is dependent on the presence of other proteinases such as matrilysin and stromelysin. As described in previous chapters, a number of proteolytic pathways lead to collagenase activation. In addition, collagenase, like any other enzyme, acts catalytically, and its ability to degrade substrate is dependent on the presence of its inhibitors. Small imbalances can lead to significant matrix degradation.

It is currently accepted that efficient, directed proteolysis by cells requires that the proteinases involved in matrix turnover be localized to the plasma membrane or adjacent matrix.[35] Zucker et al demonstrated that tumor cells contain a significant amount of collagenolytic activity associated with their plasma membranes, while Omura et al have identified a putative, cell-surface receptor specific for interstitial collagenase.[36,37] Proteinases likely involved in collagenase activation cascades, including plasminogen activators, are similarly localized.[35] This makes sense, since collagenase activation would be significantly more efficient catalytically if it were restricted to one- or two-dimensional migration rather than free in solution.

The following examples demonstrate some of the findings that have linked the expression of collagenase with specific tumors.

## Collagenases in Carcinomas

### *Collagenases in Skin Carcinomas*

Tumors associated with skin often exhibit an increased expression of collagenase, and provide a good example of some of the topics discussed above. Keratinocyte-derived tumors which acquire an invasive phenotype must transit their underlying basement membrane in order to metastasize. Further cell migration must then occur through the underlying collagenous stroma. Basal cell carcinomas are often surrounded by a fibrous collagen matrix, and appear to utilize interstitial collagenase to invade through this matrix.[38] Indeed, while these tumors are slow to metastasize, they are associated with significant, MMP-mediated tissue destruction.[39] Studies using in situ hybridization have shown a correlation between collagenase mRNA levels and tumor volume, and this expression was concomitant with that of stromelysin, suggesting that an activation cascade was present.[40] In other words, the ability of a tumor to expand into tissue may require collagenolytic activity to either make room for increased tumor volume or to allow for cell migration. As appears to be the case with numerous tumors, most of the collagenase expression was localized to fibroblastic

cells, and several studies of collagenase localization in basal cell carcinomas detected no expression in tumor cells.[40,41] This confirmed earlier results reported by Bauer et al in 1977.[42]

Squamous cell carcinomas similarly show collagenase expression, and can be highly invasive. Earlier studies demonstrated that collagenolytic activity, as measured by collagen degradation, was significantly increased in squamous cell carcinoma tissue samples[43,44] and cultured explants.[45] More recently, collagenase-1 was detected in all oral cavity tumor samples analyzed by Muller et al, with the majority expressing high levels. No collagenase-1 was detected in normal oral mucosal samples. In that study, high levels of collagenase-1 expression correlated with higher degrees of tumor differentiation.[46] In addition, the presence of collagenase-1 was often accompanied by the presence of stromelysin-2, suggesting the possibility that an activation cascade was present. As with basal cell carcinomas, much of the collagenase expression associated with squamous cell carcinomas has been primarily localized to stromal cells.[39,47] This suggests that tumor factors induce collagenase expression by stromal cells. Indeed, Huang et al showed that cultured tumor cell conditioned medium will induce collagenase expression by cultured fibroblasts.[48] This was also demonstrated recently by Charous et al in which a set of squamous cell carcinomas was shown to express collagenase-1 in over half the tissue samples.[49] Most mRNA in these samples was detected in the adjacent stroma, but with some collagenase expression detected in the tumor cells.

The study by Charous et al showed no significant correlation between MMP expression and clinical stage, but clearly demonstrated a significant overall increase in MMP expression in tumors versus normal tissue.[49] In addition, the presence of a given MMP did not correlate with tumor stage or even the presence of a tumor. This has been found in a number of studies with various tumors. Unfortunately, tumors comprise a diverse set of microenvironments where localized differences in extracellular matrix, matrix-bound factors, and cells can significantly influence the expression of MMPs. Thus, localized differences in matrix composition within a tumor environment may result in poorly detectable yet significant differences in collagenase expression within a sub-population of tumor cells. This is based on the idea that extracellular matrix composition can dictate if tumor cells are able to express proteinases. In vitro, many tumor cells show high levels of MMP expression, suggesting that they have the innate capability to produce matrix degrading proteinases. For example, tumor cell lines derived from squamous cell carcinomas can themselves be very invasive due to their expression of MMPs. Transfection of such cells with antisense constructs designed to block the expression of the transcription factor E1AF, which itself regulates collagenase-1 production by these cells, will block tumor invasion in vivo and collagen gel invasion in vitro.[23] More recently, it was demonstrated that collagenase-3 in squamous cell carcinomas is predominantly expressed by the tumor cells themselves, with only limited stromal expression.[50,51] Thus, it would appear that both tumor-derived and stroma-derived collagenase is involved in tumor progression, depending on various complex environmental interactions. It is also clear that a significant amount of work is needed before we understand the complex regulatory processes governing tumor invasion.

### *Collagenases in Gastrointestinal Carcinomas*

Gastrointestinal carcinoma represents another set of common epithelial-derived tumors associated with increased collagenase degradation. As with other invasive tumors, colorectal tumors express a variety of MMPs and other proteinases useful in degrading both basement membranes and underlying stroma. Like skin-associated tumors, collagenase expression is typical with advanced-stage gastrointestinal tumors, and it is now thought to be prognostic. This idea took time to develop. Early work by Irimura et al showed that both tumor extracts and conditioned medium from colon tumor samples expressed type I collagenolytic activity, but that this expression did not correlate with tumor stage.[52] In fact, a

significant amount of enzyme was associated with uninvolved mucosal tissue. On the other hand, van der Stappen et al found that colorectal tumor extracts contained significantly more collagenolytic activity than did normal intestine.[53] This group also found a good correlation between tumor stage and collagenase expression. Differences in tissue extraction and assay conditions were cited as possible sources of the discrepancy between the findings of these two studies.[53] As also cited in this study, the methodologies utilized could not distinguish between different MMPs. One additional problem involves the potential presence of TIMPs, which, if present in varying amounts, would significantly alter the apparent levels of collagenase activity in collagen degradation assays. In addition, tumors are complex, and analysis of tumor extracts for enzymatic activity would likely miss highly localized foci of collagenase expression.

As new assay formats became available, particularly mono-specific antibodies and cDNA probes, several groups were able to associate the presence of interstitial collagenase with malignancy in colorectal tumors. Using immunolocalization, Hewitt et al showed a significant presence of collagenase in the tumor stroma, but not in the epithelium, suggesting that the tumor cells themselves were, with a few exceptions, not producing enzyme.[54] However, no correlation between apparent collagenase levels and tumor stage was found. As the authors pointed out, immunolocalization detects both active and latent enzyme, while substrate assays utilized in the previous studies require active collagenase, and this remains a problem in the field. Interestingly, this group also detected collagenase associated with the vasculature, a subject which will be addressed below. Subsequent studies have shown a correlation between tumor stage and the presence of collagenase mRNA. Urbanski et al showed that the percentage of cells expressing several MMPs, including collagenase, significantly increased with advancing colonic neoplasia. In particular, interstitial collagenase, as well as stromelysin 3, were the best indicators of malignancy.[55] Murray et al have recently correlated the expression of collagenase in colorectal tumors with survival rate, and showed that increased collagenase levels can act as an independent prognostic factor.[56] In that study, the median survival time of the collagenase-positive group and the collagenase-negative group, 11 versus 48 months, respectively, was significantly different. This presents us with a complete turn-around, from earlier work showing little correlation between collagenase levels and tumor stage to the present, where collagenase may prove to be a reliable marker for malignancy.

Interestingly, Sakurai et al demonstrated collagenase mRNA and protein expression in all gastric cancer specimens studied, and showed that collagenase expression levels correlated well with eosinophil infiltration.[57] Gray et al localized collagenase mRNA expression in colorectal carcinoma to fibroblasts, endothelial cells, and, particularly, eosinophils.[58] In fact, in that study, eosinophils produced the most collagenase message as detected by in situ hybridization. Production of collagenase by eosinophils has been reported in several cancers, suggesting that these cells may play a significant role in tumor invasion and normal matrix remodeling.

### *Collagenases in Breast Cancer*

Breast neoplasia, which are often quite invasive, is also associated with MMP-mediated matrix degradation. The most common form of breast cancer, invasive ductal carcinoma, involves the formation of a palpable mass of fibrous tissue consisting of fibrillar collagens. While a number of MMPs have been identified in breast cancer tissues and cells derived from these tissues, the presence of a fibrous stroma suggests the need for interstitial collagenase expression for tumor cell invasion and extracellular matrix remodeling. Indeed, Iwata et al have recently shown that, in breast tumors, 87% of carcinoma cells immunostained positively for collagenase, higher than that found for gelatinase A, gelatinase B, or

stromelysin.[59] Early studies with mouse tumor-derived cells demonstrated that cells with a high colonization potential expressed significantly more interstitial collagenase, and particularly active collagenase, than low colonization potential cells, based on collagen degradation assays.[60] In human tissue explants, collagenase expression was similarly distributed, with metastatic samples having significantly higher levels.[61]

As with other tumors, there is evidence for both tumor-derived and stroma-derived collagenase expression in breast neoplasms. A number of mammary-derived tumor cells express MMPs when cultured in vitro, providing some evidence that tumor cells can express collagenase. Alessandro et al have demonstrated that the human breast carcinoma cell line 8701-BC expresses collagenase, but not its activator, stromelysin.[34] Nutt and Lunec have similarly shown collagenase expression by MDA-MB-231 cells.[25] On the other hand, Wolf et al identified collagenase in adjacent stromal fibroblasts in breast carcinomas by in situ hybridization.[62] Interestingly, while MCF-7 breast adenocarcinoma cells in culture do not express interstitial collagenase themselves, they will induce its expression in fibroblasts in coculture.[63] On the other hand, collagenase-3, which was originally isolated from breast cancer RNA samples, has been localized to the tumor cells, and appears to be expressed primarily by tumor cells versus stromal cells.[19,50,51] Thus, both tumor and stromal cells may be involved in collagenase expression in breast cancer.

### *Collagenases in Other Tumors*

A number of tumors become encapsulated by fibrillar collagens. A good example of this is seen in thyroid cancer, which is not typically invasive, but is associated with the expression of collagenase by host cells, not by tumor cells.[64] Liver tumors, which are often delimited by collagen fibrils, also express interstitial collagenase. Terada et al showed that all cholangiocarcinoma biopsies studied expressed collagenase as detected by immunostaining.[21] In all cases, expression was localized to both tumor cells and tumor stroma. Interestingly, Okazaki et al recently demonstrated that, in hepatocellular carcinoma, only early lesions with well-differentiated tumor cells expressed collagenase mRNA, as detected by in situ hybridization.[22] More advanced lesions showed no collagenase expression. Early lesions were characterized by tissue destruction and the lack of a fibrous capsule. In more advanced tumors, cells were less differentiated, showed high proliferative rates, and demonstrated fibrous capsules. These observations are consistent with the decreasing expression of collagenase during hepatic tumor progression, since it would be expected that high levels of collagenase expression would prevent the formation of a collagenous capsule. Several tumors not typically associated with the formation of a fibrous capsule are often associated with collagenase expression. This is exemplified by some brain tumors. In a recent study by Nakagawa et al, interstitial collagenase was immunocytochemically detected in all glioblastomas studied, and in four out of six anaplastic astrocytomas.[65] Astrocytes have previously been shown to express both MMPs and TIMPs in culture,[30] particularly interstitial collagenase. These cells are invasive both in vitro and in vivo; significantly, transfection of an astrocytoma cell line with TIMP-1 decreased its invasiveness in vitro.[66] Thus, it appears that, while different tumors vary with respect to the development of collagenous capsules, their ability to invade and metastasize is very often correlated with their expression or their induction of collagenase. Fibrous capsules seem to block invasion only if collagenase expression is low.

Most of the evidence presented in the above studies is correlative; however, there is very compelling evidence that collagenase and other MMPs are intimately involved in invasion and metastasis. For that, we must look at the ability of MMP inhibitors to block these processes.

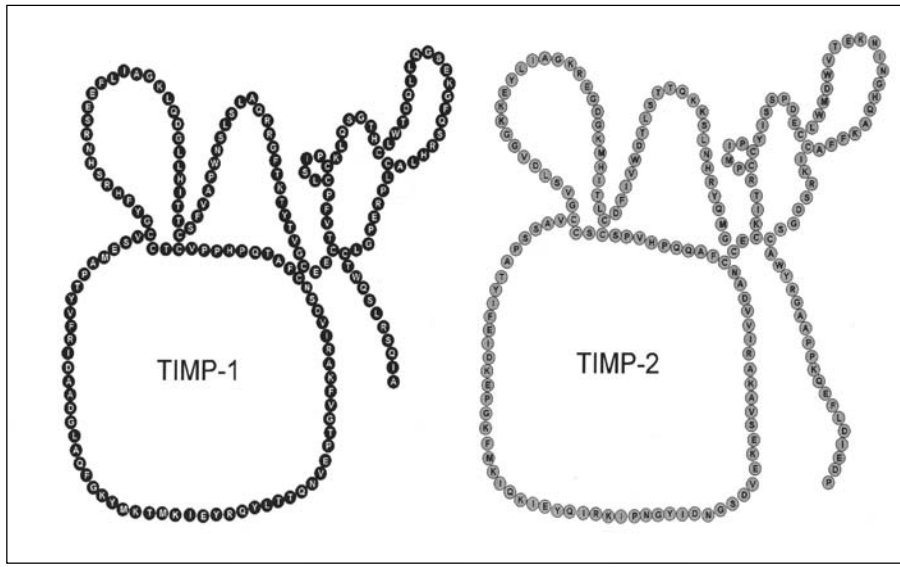


Figure 11.1. TIMP secondary structures. TIMPs all share a similar structure, including the presence of six disulfidelinkages. Depicted here are TIMP-1 and TIMP-2, the two most commonly encountered TIMPs. Both are very potent inhibitors of collagenases.

## Regulation of Collagenase Inhibition–The Role of TIMPs in Cancer

TIMPs are the primary inhibitors of collagenases, as well as all other MMPs.[67] To date, there have been four human TIMPs identified (TIMPs 1-4), all with similar secondary structure but with somewhat different inhibitory capabilities against the various MMPs (Fig. 11.1). TIMPs bind MMP active sites, forming stoichiometric, noncovalent complexes. By doing so, TIMPs comprise the third part of the MMP regulatory triad, the other parts consisting of MMP expression and activation. The best evidence that MMPs play a critical role in tumor progression is the ability of TIMPs to block tumor progression. This was demonstrated by Schultz et al, who used recombinant TIMP-1 to block tumor invasion in vitro and in vivo.[68] Similarly, Khokha et al used antisense cDNA constructs to block TIMP expression in mouse 3T3 cells, and found that decreased TIMP expression correlated with increased invasiveness in vitro and in vivo.[69] TIMP levels in metastatic cells lines have repeatedly been shown to inversely correlate with invasiveness,[70-72] and increasing TIMP levels in such cells by transfection inhibits invasiveness.[73,74] Interestingly, Koop et al have demonstrated that melanoma cells overexpressing TIMP-1 extravasate as efficiently as parent cells, but show significantly reduced tumor growth after extravasation.[15] This suggests that transit through the endothelial basement membrane may not require MMP activity, but subsequent steps in metastasis do. This is consistent with a possible role for interstitial collagenase involvement in digesting stromal extracellular matrix. In contrast, Kawamata et al recently showed that TIMP-overexpressing bladder carcinoma cells extravasate poorly, but grow, invade, and metastasize similarly to parental cell lines.[28] These discrepancies between the two studies may be due to differences in TIMP cDNA expression levels, relative MMP/TIMP ratios, and tumor environments. In addition to TIMPs, a number of synthetic MMP inhibitors have been successfully used to block tumor invasion and metastasis.[75] Taken together, these studies strongly support the role of MMPs in various aspects of tumor growth,

invasion, and metastasis. While some of this inhibition may result from the ability of TIMPs and synthetic inhibitors to block tumor migration through tissue, it is now clear that angiogenesis, which is a critical factor for tumor survival and growth, is itself dependent on MMP activity.

## Role of Collagenase in Angiogenesis

One of the critical steps in tumor growth and metastasis is the development of a tumor vasculature. Without vascularization, a tumor's growth is limited by its ability to obtain nourishment by diffusion of metabolites into the tumor mass. This would constrain the tumor to an estimated size of not more than a few $mm^3$ in volume; any larger, and the cells within the tumor mass would undergo necrosis.[1,2] It is for this reason that tumor cells express a wide array of angiogenic factors that lead to the proliferation and migration of endothelial cells. In response to these factors, endothelial cells themselves produce the factors necessary to sprout from existing vessels, invade tissue, and assemble tubular networks that mature into capillaries. These factors include activated MMPs, which are linked to several events associated with angiogenesis. Perhaps the best evidence for the critical role of MMPs in angiogenesis is the potent inhibition of this process by both TIMPs and synthetic MMP inhibitors. After stimulation, endothelial cells mediate the dissolution of adjacent basement membrane, which frees them to migrate towards the stimulus, where they encounter stromal extracellular matrix. While basement membrane digestion requires MMPs that degrade type IV collagen and laminin, a process linked to gelatinase A expression, further invasion may require collagenolytic activity to enable endothelial cells to migrate through stromal matrix. Endothelial cells are known to express interstitial collagenase and other MMPs in response to stimulatory factors such as phorbol esters and cytokines.[76-80] This expression may be a requirement for endothelial cell migration and morphogenesis, particularly in vivo. It is thought that the dynamics of capillary sprouting depend upon the tight balance between endothelial cell differentiation and basement membrane integrity versus cell proliferation and matrix turnover. An imbalance between active MMPs and TIMPs would likely result in localized basement membrane degradation.

If MMPs are critical for angiogenesis, then TIMPs and other MMP inhibitors should block this process. In fact, MMP inhibitors are extremely potent at blocking angiogenesis. Endothelial cells express TIMPs, and it has been demonstrated that this expression can mask the presence of MMPs in cultured endothelial cells as measured by fibrillar collagen degradation assays.[80,81] Addition of TIMPs or synthetic MMP inhibitors will also block angiogenesis in vivo.[82-84] An interesting example is the lack of vascularization in cartilage, which has been attributed to the presence of TIMP-1.[85] Because the angiogenic process is complex, much work has been done in vitro with cultured endothelial cells. While such models represent only a small part of the angiogenic process, they have been useful in understanding some aspects of endothelial cell biology.

Endothelial cells combine to form tubular structures, and tubes assemble to form patent vessels. As they progress from a differentiated, quiescent state to a migrating, proliferative state, and back again, endothelial cells express different sets of genes, though most of these transcriptional changes remain unknown.[86-88] In vitro, endothelial cells will form tubular networks when grown on basement membrane preparations such as Matrigel, which is composed of laminin, type IV collagen, and proteoglycans, as well as growth factors and proteinases.[89,90] Tube formation occurs rapidly, and can be at least partially blocked with specific antibodies to Matrigel components such as laminin.[91] In most models, this process relies on a malleable substratum which can be physically manipulated by cells, and it has been proposed that cells on a two-dimensional matrix exert tractional forces that create lines of force between cells, leading to the creation of cell networks. This rearrangement is

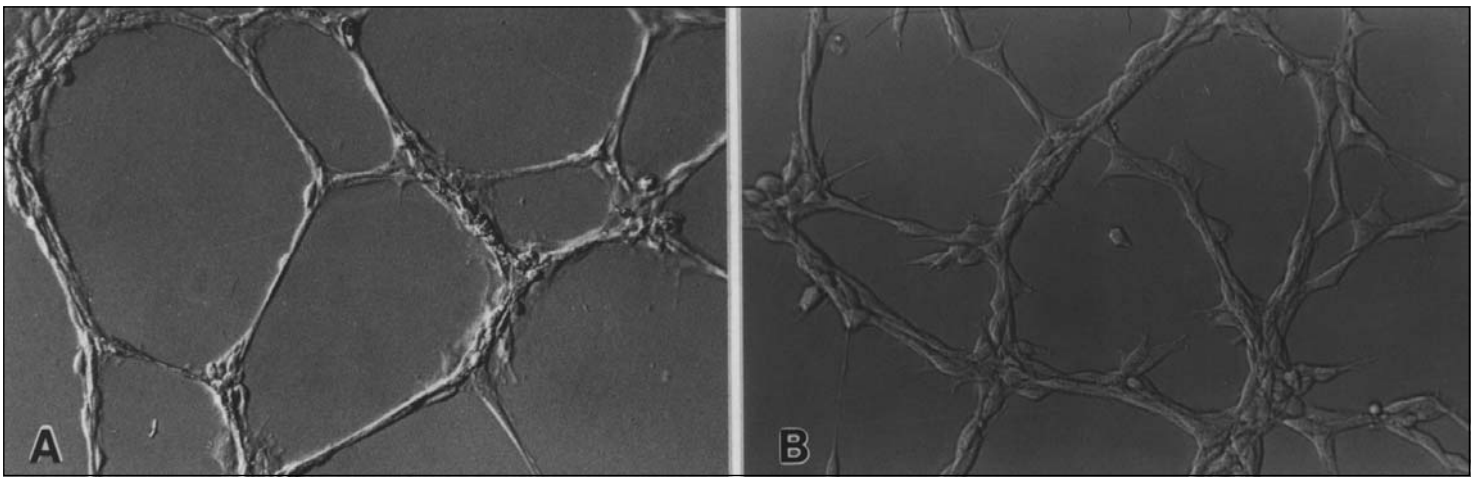


Figure 11.2. In vitro endothelial cell morphogenesis. Given the right environment, endothelial cells will assemble into multi-cellular tubular networks. In this case, microvascular endothelial cells isolated from spontaneously hypertensive rats are shown cultured on a thick layer (12 mg/ml) of Matrigel (panel A) and on plastic (panel B). The cells on plastic were first allowed to attach in 0.5% serum overnight, were serum-starved two days, and then stimulated with 5 ng/ml EGF in serum-free medium. On both Matrigel and on plastic, these cells lost stress fibers and matrix attachments, enabling them to assemble in networks within 24 hours. Interestingly, in both cases, treatment with either TIMP-1 or the synthetic MMP inhibitor, GM6001, did not block this morphogenesis. In vivo, both of these inhibitors are very potent at blocking angiogenesis, demonstrating the limitations of in vitro systems as models for significantly more complex organismal processes.

dependent on the malleability of the matrix on which cells attach, and does not occur on thin, nonmalleable substrata. This traction-mediated formation of tubular networks, in which endothelial cells exert tractional forces and physically manipulate their substrata during tubular network formation, has been demonstrated on defined thicknesses of type I collagen, fibronectin, and Matrigel.[92-96] Some endothelial cells spontaneously form tube-like structures when cultured on plastic.[97] These spontaneous tube formers first establish a confluent monolayer of cells, and a small portion of the cells establish a network on top of this monolayer. It has been demonstrated that the monolayer cells assemble a network of type I collagen cables, and that this collagen matrix influences the subsequent tubular network formation. As with the Matrigel system, this appears to involve tractional remodeling of a planar substratum. Type I collagen may be a critical matrix component synthesized by these cells during angiogenesis, and may be a marker for endothelial cells expressing the angiogenic phenotype.[98] Inhibition of type I collagen synthesis, or degradation of collagen itself, inhibits angiogenesis, supporting the idea that endothelial cells actively manipulate their extracellular environment both physically and biochemically.[99-101] Mechanistically, traction-mediated morphogenesis of cells may involve changes in cytoskeletal arrangement, focal adhesion assembly, as well as specific binding interactions to matrix components, all leading to signal transduction and transcriptional regulation.

One of the important features of all of these models seems to be the loss of stress fibers and the cellular de-adhesion caused by matrix deformability. For example, we have recently developed a model in which certain endothelial cell clones can assemble tubular networks very rapidly, but on nonmalleable substrata (Fig. 11.2). These cells can do this is because they respond to epidermal growth factor by disassembling stress fibers and focal adhesions, allowing them to migrate and form multicellular structures. In other in vitro models, the actions of collagenase or other MMPs may alter the adhesive properties of a given matrix, leading to the same morphogenesis. In vitro, the mechanical properties of a matrix can be manipulated experimentally by altering the concentrations of different matrix components. In vivo, this is accomplished by a balance between proteolysis and matrix deposition. Fisher et al have demonstrated that endothelial cells grown in collagen gels, when stimulated with phorbol esters, express interstitial collagenase and degrade the fibrillar collagen over time.[102] Blocking this process with MMP inhibitors blocks tubular morphogenesis of these cells, suggesting the critical role of collagenolysis to angiogenesis. There is also indirect evidence that blocking collagenase activity is part of the program leading to vessel stability. Endothelial cells interact with smooth muscle cells and pericytes to yield active TGF-$\alpha$, which would decrease collagenase expression while increasing TIMP-1 expression.[103] This would suggest that, in stable vascular structures, maintaining basement membrane integrity is an active process. As with tumor invasion, collagenase may function to remove physical and adhesive matrix barriers to allow for the migration of endothelial cells. Because of the importance of angiogenesis for tumor biology, and because collagenases and TIMPs are so intimately involved in the angiogenic process, there is likely to be a continued interest in determining how changes in collagenase activity regulates blood vessel dynamics.

## Conclusions

Much progress in the collagenase field has been made in the past 30 years. From a single enzyme, collagenase, MMPs have developed into a large family involved in many aspects of normal and disease processes. Our task is now to understand how collagenases function at the molecular level to modify cell behavior, particularly with regard to tumor invasion and metastasis, as well as tumor angiogenesis. To date, several key questions remain. It is not yet clear how collagenases are regulated by the interaction of different cell types with different extracellular matrix components. Because tumor cell migration and

invasion is dependent on cell-matrix interactions, and because proteolytic modification of matrix can profoundly alter cellular responses to a given matrix, we must determine the mechanisms of matrix-mediated signaling of cells leading to changes in collagenase expression. At the same time, we need to further evaluate the ability of collagenases to cleave different substrates in a physiologically relevant environment. If indeed interstitial collagenases are able to digest basement membranes, then our ideas regarding their roles in cell migration and invasion will certainly change. Similarly, because collagenase-mediated proteolysis appears to be involved in various aspects of tumor growth and invasion, then understanding its inhibition will continue to garner significant interest. A considerable effort and, presumably, money is being expended by a number of pharmaceutical firms toward the development of new generations of synthetic MMP inhibitors.[104] With the number of members of the MMP family still increasing, it is reasonable to predict that a certain amount of redundancy exists. While each MMP may vary with regard to its transcriptional regulation, substrate specificity, and cellular origin, it is likely that multiple MMPs participate in many of the biological events in which they participate. Thus, a major goal is to identify inhibitors with high specificity towards individual MMPs. Such inhibitors, be they TIMP-based or peptide-based, will finally enable us to determine an individual MMP's role in different biological processes. It would also be useful to determine, at the tissue level, whether specific MMPs are latent or active. There is thus a great interest in developing substrates and immunoreagents specific for individual MMPs. The next generation of biological tools directed towards collagenases will further enable us to characterize tumor stages based on collagenase expression and activation. At the same time, with increasingly specific and therapeutically useful inhibitors of collagenase activity and expression, we may be able to block its role in tumor invasion and angiogenesis. Surely the next 30 years in the collagenase field will be at least as exciting as the last.

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CHAPTER 12

# The Role of Collagenase in Wound Healing

**Mona Ståhle-Bäckdahl**

## Introduction

### *Trading Perfection for Promptness-Repair Versus Regeneration*

At first glance, the ultimate goal in the process of wound repair would seem to be the complete restoration of injured tissue. However, the capacity to fully regenerate lost tissue has been strongly selected against during evolution, and this ability, with little exception, now remains only in fishes and amphibians. It appears that the transition from aquatic to terrestial life correlates with a decline in regenerative potential. In fact, the ability to rapidly and effectively accomplish wound closure constitutes a critical step in evolution. Achieving full replacement of injured body parts, a time-consuming task, was simply not compatible with a successful struggle for survival in mammals. An important consideration may be that stresses on weight bearing limbs of animals living on dry land make regeneration of appendages impossible. The only mammalian tissue capable of complete regeneration is the deer antler[1] and while other related animals developed horns, one can only speculate about the selective advantages of antler evolution.

How does wound healing relate to the phenomenon of epimorphic regeneration? Are they similar processes with tissue repair representing only a blunted, compromised version of complete tissue replacement; or are they biologically distinct? There are important similarities: both processes involve cell migration, proliferation and redifferentiation, however there are also differences, the major one being that epimorphic regeneration entails the impressive capacity to develop entirely new structures such as bone and muscle in their appropriate locations. And yet epimorphic regeneration must be regarded as a luxury in nature, while the universality of wound healing testifies to its critical role in survival.

### *The Phases of Normal Wound Healing*

The purpose of tissue repair is to reinstate the functional and structural integrity of the damaged organ. To this end, normal wound healing follows a sequential pattern involving a series of closely regulated, coordinated, and overlapping events. In response to injury and blood vessel disruption, there is acute inflammation which activates the coagulation cascade and leads to hemostasis. This process releases chemotactic agents which serve as migration signals for adhering neutrophil leukocytes, the key participants in the innate immune system and the first line of defense against potentially harmful microorganisms that

*Collagenases*, edited by Warren Hoeffler. 

may invade the host.[2] Although the repair mechanisms are generally applicable to most organs, the present review will focus mainly on skin and epidermal wound closure.

Following after neutrophils, other cells such as monocytes/macrophages and lymphocytes arrive at the site of the wound, starting the complex process of repair and remodeling. A multitude of different growth factors and proteolytic enzymes are released to degrade damaged tissue and debris and stimulate synthesis of new matrix. Granulation tissue is composed of a provisional matrix containing fibronectin, vitronectin, fibrinogen, hyaluronic acid and types I and III collagen. Collagen is the major structural component of the skin, and digestion of this protein is critically dependent upon the proteolytic activity of interstitial collagenases produced by resident and migratory cells. In the process of wound repair, fibroblasts proliferate and assume a more contractile phenotype, becoming myofibroblasts to accomplish wound contraction.[3] New blood vessels form and sprout through the wound bed.

Shortly after injury, sedentary basal keratinocytes at the wound edge detach from the underlying substratum and assume a migratory cell phenotype. This begins the crucial process of wound re-epithelialization, which forms a protective barrier against the potentially harmful exterior environment. The keratinocytes lose their apical-basal polarity stretch out and move towards the free edge. While initial re-epithelialization begins with cell migration, within 1-2 days epidermal cells behind the advancing front begin to proliferate, generating additional migrating cells. Once the tissue defect is covered and re-epithelialization completed, the keratinocytes resume their normal quiescent phenotype and again firmly anchor to the basement membrane. This apparently programmed sequence of events evidently requires precise spatial and temporal control. Mechanisms regulating this dynamic and important process are the focus of current research in wound healing biology, but are as yet incompletely understood.

The final phase of wound healing involves matrix restructuring. The composition of the connective tissue is gradually altered: fibronectin and type III collagen levels decrease while type I collagen synthesis increases to provide additional tensile strength to the skin. This is a rather slow process, as by three weeks the wound has only an estimated 20% of the strength of the uninjured skin.[4] To impart additional strength to the wounded skin, collagen is synthesized at a high rate until it returns to baseline within six to twelve months. Collagen remodeling depends on both increased synthesis and collagen catabolism, controlled by collagenases from granulocytes,[5,6] macrophages,[7] fibroblasts,[8] and epidermal cells.[9] The tensile strength of mature scar tissue is estimated at about 70% and thus, once wounded, skin never regains preinjury strength.[4]

## *The Collagenases*

Within the metalloproteinase (MMP) gene family, the collagenases constitute a distinct subgroup of enzymes sharing the unique ability to specifically cleave interstitial collagen fibers (see chapter 1). Metalloproteinases have somewhat overlapping substrate specificities, with most enzymes capable of digesting fibronectin and gelatin. Unique among these is interstitial collagenase with the exclusive ability to cleave collagen fibers. This specific catalytic event has been studied in greater detail than the other MMP-mediated reactions[10] and involves cleavage of a single peptide bond.[11] At physiologic temperature the two digestion fragments of collagen loosen their triple helical structure and denature into gelatin peptides which are then susceptible to subsequent digestion by a variety of proteases.[12] Because it is responsible for performing the initial and rate-limiting step in collagen fiber degradation,[13,14] collagenase activity is critical in the remodeling of the dermal matrix.

To date, three different collagenases have been identified: Collagenase-1 (fibroblast collagenase, MMP-1)[15,16](see chapter 1), neutrophil collagenase (MMP-8)[17,18] (see chapter

2) and, the most recently characterized, collagenase-3 (MMP-13)[19](see chapter 3). Neutrophil collagenase has only been detected in neutrophil leukocytes, whereas collagenase-1 is expressed by a variety of cell types other than fibroblasts, such as keratinocytes,[20,21] endothelial cells,[22] monocytes/macrophages,[16,23] chondrocytes and osteoblasts.[24,25]

The fact that there are at least three distinct collagenases sharing the particular ability to cleave fibrillar collagens appears a luxurious redundancy likely developed to protect a key biological function. However, detailed analysis of the enzymatic properties of the respective enzymes reveals difference in their substrate specificities. Collagenase-1 preferentially cleaves type III collagen, a prominent component of early wound matrix.[26] Neutrophil collagenase is more active against type I collagen, and collagenase 3 cleaves type II collagen more efficiently than types I or III.[27,28] This pattern of substrate preferences suggests that the collagenases evolved as specialized enzymes to remodel tissues with different collagen composition.

Apart from different substrate specificities, the collagenases also differ profoundly in gene regulation (see chapter 4). Briefly, neutrophil collagenase is transcribed in bone marrow precursor cells and the circulating mature neutrophils store the preformed protein in cytoplasmic granules to be released at the proper signal. On the other hand, collagenase-1 and collagenase-3 are subjected to tight transcriptional regulation and are readily induced by phorbol esters, cytokines and growth factors. These enzymes contain enhancer elements in their promoter regions, like AP-1-binding sites and a PEA3 consensus sequences, which control the responsiveness of these genes to a host of transactivating factors.[29-31] The AP-1 site confers inducibility by cytokines and growth factors, and the PEA3 binding site is recognized by the transcription factor c-ets. Interestingly, the stromelysin-1 and the urokinase plasminogen activator genes, both implicated in the activation of procollagenase, share the AP-1/ PEA3 motif,[32,33] suggesting that all three genes may be subject to coordinate expression to create a proteolytic cascade operative in vivo.

## Collagenase Expression in Skin Wounds

### *Localization of Collagenase-1 in Skin Wounds*

In healthy, noninflamed and uninjured skin collagenase-1 is not present, at least not in levels detectable by immunohistochemistry and in situ hybridization assays.[34,35] However, it has been demonstrated from the first studies that wounded skin is associated with collagenolytic activity.[36,9] However, the cell types involved and the time-course of collagenase production during tissue repair were uncertain until recently when a series of papers have elucidated the pattern of collagenase expression in injured skin. A brief examination of their content shows that in response to wounding, there is a distinct and prompt induction of collagenase mRNA and protein in epidermal cells bordering the site of injury.[35] In addition, collagenase expression has been detected in dermal cells scattered throughout the wound bed matrix. Although not fully characterized, the location and gross cellular morphology of these cells suggests that they are fibroblast-and macrophage-related.[34,35,37]

One outcome of this research has been the realization that collagenase-1 production in wounded skin is a universal phenomenon. Regardless of etiology, collagenase-1 seems to be expressed in all types of wounds: acute, chronic, inflammatory, traumatic, infectious etc, indicating that the underlying pathogenesis is of minor significance in this respect.[34,38,39] Evidently, the signal initiating collagenase-1 transcription is the epidermal insult per se. This raises the pressing questions of how this occurs, and what the role(s) of collagenase is in both the initiating the process, and during the ensuing injury.

### *Expression of Collagenase-1 in Keratinocytes is Closely Related to Re-epithelialization*

Studies from our own laboratory demonstrate that collagenase-1 expression in wound edge keratinocytes is rapidly turned on following injury.[35] The experimental procedure that we used involves normal human skin obtained from plastic surgery, wounded ex vivo, kept in culture, and harvested at different time-points. The earliest time point studied shows evidence of collagenase mRNA at 4 hours after wounding (Fig. 12.1), perhaps collagenase expression is an immediate response to injury that occurs even earlier. Examining these healing wounds we found that the peak expression of collagenase-1 occurred at 12-24 hours and remained high until day 2, when expression began to decrease. Over the next few days collagenase expression was less intense but persisted until the migrating keratinocytes had covered the unprotected surface. Thus, our studies demonstrate that in these acute wounds, collagenase-1 levels mirror the re-epithelialization process, which is complete in 5-7 days. In fact, when the thin sheet of keratinocytes had migrated across the injured surface and contacted the keratinocytes moving from the opposite wound edge collagenase-1 mRNA was no longer detectable. The results obtained by in situ hybridization were confirmed on the protein level by immunohistochemistry, demonstrating that collagenase protein levels reflected mRNA levels at corresponding locations. This temporal pattern of collagenase expression is compatible with data obtained from surgical pig wounds,[39] human burn wounds,[38] experimental suction blisters[40] and our own human surgical wounds[35](Fig. 12.2). These data indicate that collagenase-1 production in epidermis is primarily, albeit not exclusively,[41] a feature of the migratory keratinocyte phenotype.

## Regulation of Collagenase During Re-epithelialization

### *Site Specific Regulation of Collagenase-1 in Skin Wounds*

The consistent and localized expression of collagenase-1 in wound edge epithelium and its close temporal and spatial relation to re-epithelialization indicates that this process is precisely regulated. There is a large body of evidence demonstrating that the production of collagenase-1 is primarily controlled at the level of transcription,[29,42] although modulation of mRNA half-life may play some role.[43] A multitude of cytokines and growth factors along with proto-oncogenes such as members of the fos/jun family and c-ets have the capacity to induce transcription of collagenase-1 through binding to enhancer elements in the collagenase promoter[44-47] (see chapter 4). In addition, various signals that can be provided to cells in culture have been shown to upregulate collagenase transcription, such as alterations in cytoskeletal conformation and cell shape changes,[48-50] ultraviolet light,[51] and heat-shock treatment.[52]

What mediators induce collagenase expression in the migrating front of epithelial cells in vivo? Several aspects of the pattern of collagenase expression in this setting suggest that its control resides in the immediate microenvironment of the injured epithelium; collagenase production is confined to a subset of epithelial cells, the induction occurs very rapidly following injury, and is shut off only when the epithelial cells have made contact across the wounded surface. Furthermore, as shown by us, in skin wounds undergoing re-epithelialization ex vivo and thus lacking inflammatory stimuli, collagenase-1 is expressed in a pattern mimicking that observed in vivo.[35] Taken together, these findings strongly suggest that local, and possibly autocrine factors, are most likely inducing collagenase in wound-edge epidermis.

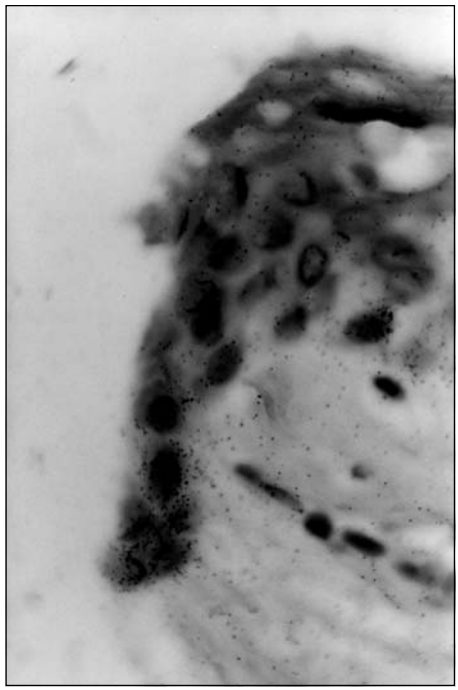

Fig. 12.1. Expression of collagenase-1 is rapidly induced in wound edge keratinocytes after acute ex vivo injury in human skin. Bright-field photomicrograph shows positive signal for collagenase-1 mRNA appearing as black dots in migrating wound edge keratinocytes.

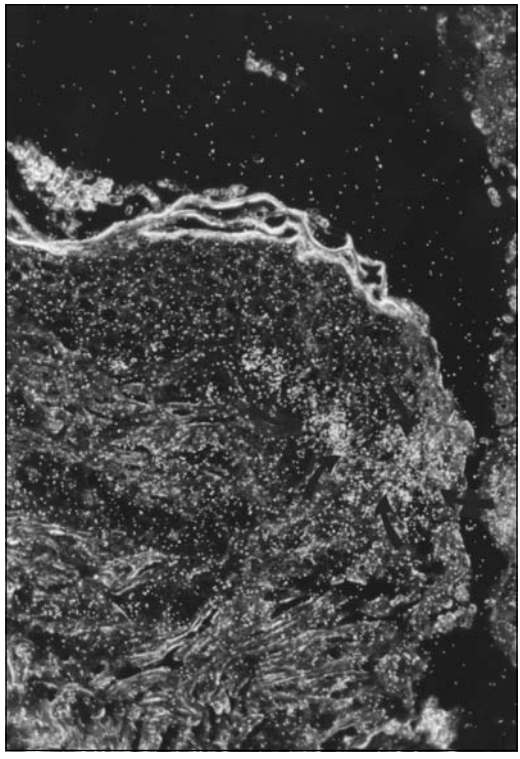

Fig. 12.2. Abundant signal for collagenase-1 is seen in vivo in an acute human skin wound. Expression of collagenase-1 mRNA at 24 hours following injury. Strong signal for collagenase-1, appearing as white dots in this dark-field photomicrograph, is detected in basal keratinocytes bordering the ulcer (arrows).

### *Integrin Receptor Modulation During Re-epithelialization Affects Collagenase Expression*

Within the rapid sequence of events ensuing injury, the disruption of keratinocyte cell-cell contacts leaves a free edge where there once was a neighboring cell, an event that can greatly effect the adhesion apparatus. Pertubation of adhesion is associated with changes in cell shape which may constitute a signal to induce collagenase.[48-50] Integrin molecules are rearranged, and cells at the very margin of the wound are dislodged from the basement membrane and stretch out towards the free edge.

Integrins are heterodimeric adhesion molecules composed of a cytoplasmic domain interacting with the cytoskeleton, a transmembrane region, and an extracellular domain that binds to one or more ligands. Thus, integrin structure is well suited to mediate communication between the intracellular and extracellular compartments, being key players in signal transduction pathways. There is accumulating evidence that integrin-ligand interplay may modulate cell behavior through tyrosine phosphorylation of intracellular proteins.[53] The alterations in keratinocyte integrin expression pattern associated with wound healing have been thoroughly studied.[54-56] The cells moving off from the wound edge start expressing a different repertoire of integrin receptors, for example, there is a switch from the $\alpha 6\beta 4$ laminin receptor to $\alpha 5\beta 1$ which mediates attachment to fibronectin in the provisional matrix. This pattern of integrin expression persists until re-epithelialization is completed, and $\alpha 6\beta 4$ reappears.[55] Quiesent basal keratinocytes constitutively express collagen-binding $\beta$ 1-integrins present predominantly on the lateral cell surface. Although they are still expressed in migratory keratinocytes during wounding, these integrins are processed differently, rearranged and relocate to the frontobasal surface of the cells.[54,55,57-61] Re-epithelialization is a dynamic process where the migrating cells move over a continuously changing substratum expressing a shifting set of integrins, and creating a variety of interactions between these molecules during wound healing. Thus, it is plausible that the altered integrin pattern associated with wounding is responsible for inducing a new repertoire of proteins in migrating epidermal cells. In fact, it has been demonstrated that a pertubation of integrin adhesion by adding blocking antibodies to $\alpha 2$ and $\alpha 3$ collagen-binding integrin subunits effectively inhibits collagen-mediated collagenase production by keratinocytes in vitro.[62,63]

### *Cell-Matrix Interactions Influence Collagenase Expression During Wound Healing*

The distinct localization of collagenase-1 in marginal keratinocytes bordering the wound raises the possibility that these cells are signaled to produce collagenase by losing contact with the basement membrane. When liberated from the basement membrane, wound edge keratinocytes are exposed to a different milieu and encounter components of the underlying provisional matrix. Contact with extracellular matrix molecules can influence cell behavior both directly through adhesion interactions and indirectly by serving as a reservoir for growth factors.[64-65] Thus, work from several groups has shown that keratinocytes cultured on types I and IV collagen[66-68] produce significant levels of collagenase, whereas keratinocytes grown on laminin and other basement membrane components do not.[37,69] These regulatory mechanisms are likely to differ between different cell types. For example, in fibroblasts collagenase induction is reported after exposure to fragments of fibronectin[70,71] and a combination of fibronectin and tenascin, characteristic of remodeling tissue.[64] Evidently the alterations of extracellular matrix composition is a powerful regulator of cell phenotype.

Consistent with these in vitro observations, it was shown that in chronic wounds expression of collagenase-1 colocalized with $\alpha 5\beta 1$ integrin receptor in wound edge keratinocytes, indicating that these cells were no longer associated with the basement mem-

brane but exposed to the interstitial matrix of the wound bed[37](see chapter 8). In addition, in lesions of dermatitis herpetiformis—a blistering skin disease characterized by inflammatory destructive changes in the basement membrane—basal keratinocytes exhibited a prominent signal for collagenase-1[41] irrespective of their state of migration. Based on such data, it was hypothesized that contact with the main component of the dermal matrix, type I collagen, provides a mechanism for induction of collagenase-1 in wound edge epithelium. It was further proposed that collagenase is not only invariably present but also critical for keratinocyte migration and re-epithelialization to occur. The research group led by H.G. Welgus and W.C. Parks have in this model linked the induction of collagenase in keratinocytes plated on collagen to a mechanism which would provide the keratinocytes with a sense of direction during re-epithelialization. To function in this apparently complicated process, collagenase would simply have to perform its standard although unique proteolytic action, i.e., cleave fibrillar collagen.[63] This model is attractive because it offers explanations for many observations made over the past several years. However, even though collagenase expression seems most prominent in keratinocytes that have moved beyond the limit of the basement membrane and are exposed to dermal collagen, collagenase mRNA can be detected in epithelial cells still in contact with the basement membrane. In skin wounds the strongest signal for collagenase was seen at the very edge of the wound, but was also detected in cells behind the migrating front.[35,37] Does this mean that a signal to induce collagenase can be communicated between cells, so that nearby cells not in direct contact with the primary stimulus also produce collagenase?

Naturally, mechanisms for inducing collagenase may be distinct under different conditions. For example, in a variety of blistering skin diseases, strong signal for collagenase mRNA was consistently seen in actively regenerating keratinocytes. These cells were primarily associated with histologically intact basement membrane, suggesting that contact with the dermal matrix may not be a prerequisite for collagenase production in epidermal cells.[40] As discussed earlier, cytokines and growth factors are potent inducers of collagenase transcription in vitro and it is conceivable that cytokines from migratory leukocytes and/or keratinocytes themselves, via autocrine pathways, have a direct role in mediating collagenase expression in inflammatory disorders.

### *Autocrine Regulation of Collagenase in Keratinocytes*

As mentioned above, a variety of cytokines and growth factors have the capacity to induce transcription of collagenase. In the specific conditions of skin wounding, the mechanisms regulating collagenase production are likely to emanate from the confined environment of the wound itself, implicating autocrine factors as important contributors to this process. I will discuss a few of the cytokines and other factors present at the wound site, although this list is obviously not complete.

Keratinocytes are a major source of cytokines,[72] some of which modulate other keratinocyte-derived cytokines via autocrine pathways.[73] Of special interest in this context is epidermal growth factor (EGF), which is reported to enhance epidermal regeneration and wound healing[74,75] and induce collagenase-1.[76] In vitro, treatment with EGF stimulates keratinocyte migration on collagen via upregulation of $\alpha 2\beta 1$-collagen-binding integrin. The EGF receptor is a transmembrane tyrosine kinase which also binds transforming growth factor alpha (TGF-$\alpha$) and, interestingly, certain viral growth-factor-like proteins. In skin, the known effects of EGF/TGF-$\alpha$, including the rise in intracellular calcium, are mediated through this receptor and determined by the localization and the tyrosine-kinase activity of the receptor.[77,78] The functional status of the receptor and the binding of ligands are regulated by protein kinase phosphorylation of the receptor. The activated EGF-receptor in turn phosphorylates other cellular proteins and indirectly activates protein kinase C. Thus, the

EGF/TGF-α system is likely to be involved in signal transductions pathways regulating keratinocyte responses to wounding. Indeed, it was recently proposed that collagenase production in keratinocytes in contact with collagen requires a functional EGF receptor autocrine loop. By blocking the keratinocyte EGF-receptor in vitro, collagen induced expression of collagenase[79] was completely abolished.

A potential modulator of collagenase production in response to injury is the recently identified extracellular MMP inducer (EMMPRIN), (see chapter 6). EMMPRIN, which was previously named tumor-cell derived collagenase stimulatory factor (TCSF), is a cell surface glycoprotein initially isolated from tumor cells,[80,81] but now known to be present also on the surface of normal keratinocytes.[82] In vitro, EMMPRIN has the capacity to stimulate the production of at least three members of the MMP family: collagenase-1 (MMP-1), stromelysin-1(MMP-3[82] and 72kDa gelatinase (MMP-2).[80,83] In skin, EMMPRIN is expressed constitutively throughout the living layers of the epidermis, but not in the dermis or in most other normal tissues. Interestingly, and in contrast to adult tissues, EMMPRIN appears to be widely distributed in the developing embryo, suggesting that it participates actively in processes associated with tissue remodeling.[82] To date nothing is known about the possible role of EMMPRIN in epidermal regeneration during wound healing, but judging from its appearance in keratinocytes, it is likely to play a role in MMP regulation.

### *Activation of Procollagenase*

Like the other members of the MMP gene family, collagenase is secreted in a proenzyme form that needs activation to be catalytically competent. Latent metalloenzymes can be activated in vitro by many mechanisms including: treatment with organic mercurides, chaotropic agents, or proteases. Activation involves the disruption of a cysteine-zinc interactive site and subsequent autoproteolytic cleavage removing the prodomain to generate the fully processed form of the enzyme. The necessity of activating latent enzymes constitutes an important step in regulating extracellular metalloproteinase activity. Unfortunately, whereas in vitro activation of metalloproteinases is standard laboratory procedure, in vivo activation mechanisms have remained elusive.

Researchers have suggested that the plasminogen/ plasmin system may serve as a mechanism to generate active collagenase in vivo.[84] Plasmin can activate both procollagenase and prostromelysin, the latter itself an activator of procollagenase. In this way one can envision a proteolytic cascade initiated by the conversion of plasminogen to active plasmin through the action of urokinase plasminogen activator (uPA). Like collagenase, expression of uPA is controlled transcriptionally through AP-1/PEA3 regulatory elements, suggesting that the two genes may be subject to coordinate expression. In the mouse, uPA is detected in migrating keratinocytes during wound healing,[85] and experimental data show that disruption of the plasminogen gene results in impaired healing of skin wounds in this species.[86] Furthermore, in humans, uPA is detected in clinical ulcers[87] and is coexpressed with collagenase-1 and stromelysin -1 in basal keratinocytes in the blistering skin disease dermatitis herpetiformis.[41]

Possible candidates for in vivo activators of procollagenase are membrane-type matrix metalloproteinases (MT-MMP), a growing subgroup of enzymes within the MMP gene family and today comprising at least four members.[88-92] These proteases have the capacity to enhance the expression of other MMP enzymes, although recent data indicate that they cooperate with other factors to achieve full effect.[93] So far, these enzymes have been studied solely in association with tumor conditions,[94] and at this point the potential role for MT-MMPs associated with wound healing has not been extensively investigated.

## Role of Collagenase in Wound Healing

It is reasonable to assume, based on the research partly reviewed here, that collagenase-1 has a key role in the wound healing process. Expression of collagenase in dermal cells, consistently found in repairing skin wounds seems more logical than the finding of collagenase in epithelial cells. Tissue repair involves the turnover of dermal fibrillar collagen, a process requiring the proteolytic activity of collagenases. Increased collagenolysis is needed throughout the remodeling phase leading to the formation of a mature scar, a process requiring months. During this period, there are marked changes in collagen composition, and the amount of type III collagen. Although transiently present in elevated levels in the early wound matrix, type III collagen expression decreases, and shifts towards the formation of type I collagen and the subsequent maturation of collagen into thicker and more compact fibers. Collagenases are obvious participants in this process and collagenase-1 seems to be the principal enzyme involved, although neutrophil collagenase released from migratory inflammatory cells may assist in collagen degradation. The potential role for collagenase 3 in this context is only beginning to be studied. Our own data do not show evidence of collagenase 3 expression in acute skin wounding (Stähle-Bäckdahl, unpublished observations). However, Saarialho-Kere recently showed that collagenase 3 was expressed by dermal fibroblasts in chronic wounds, suggesting that collagenase 3 may be involved in matrix remodeling associated with wounding.[95]

There is no doubt that the principal function of epidermal cells expressing collagenase is in closing the wound by cell migration and proliferation. What is the specific role for collagenase-1 in this setting, and is it critical for cell immigration? A hypothesis recently advanced is that collagenase activity is necessary for the keratinocyte to achieve re-epithelialization. In support of this idea Pilcher et al have shown that keratinocyte migration in vitro is dependant upon catalytically functional collagenase.[63] As discussed earlier, it is well-established that keratinocytes migrating on type I collagen produce collagenase in vitro. Indeed, when blocking collagenase activity in this system, the keratinocytes failed to migrate. It was further hypothesized that keratinocyte migration would only occur in association with collagenase cleaving collagen molecules. Consistent with this, it was shown that the cells did not migrate on mutant collagen lacking the collagenase cleavage site, a finding strongly linking collagenase catalytic activity to keratinocyte movement. Thus, there is substantial evidence supporting a direct role for collagenase in keratinocyte migration. Moreover, in this model the authors proposed that by cleaving collagen fibers, collagenase actually serves to direct the keratinocytes during migration; the keratinocytes are provided fresh collagen substrate ahead and leave behind digested gelatin fragments no longer attracting collagenase. Admittedly, it is tempting to speculate that the critical role for collagenase in re-epithelialization utilizes its unique biological role. However, even though collagenase may be required for cell migration and epithelial regeneration, its precise functions in the wounded epidermis are more elusive. The assumption is that re-epithelialization in different tissues follows similar if not identical mechanisms. Consistent with this are experimental data on mucosal healing showing that analogous to the situation in skin, contact with type I collagen stimulates epithelial cell migration more than does contact with fibronectin and laminin. Therefore, it is somewhat surprising that healing of ulcers in the gastrointestinal mucosa does not seem to involve collagenase-1. In such lesions there is distinct expression of matrilysin (MMP-7) but not collagenase-1 in the wound edge epithelium, the latter detected only in the stromal tissue underneath. Matrilysin has a broad substrate specificity but it does not cleave type I collagen, indicating a different role in re-epithelialization that does not involve collagen.

Another interesting question concerns the role of type III collagen in this setting. Type III collagen is the preferred substrate for collagenase-1 in vitro and one might infer that

collagenase-1 may have evolved to serve a unique role in tissues containing by a high collagen III content. In fresh wounds the deposition of granulation tissue occurs in an orderly sequence lead by fibronectin, followed by type III collagen, and then by type I collagen. This time course seems to match the appearance of collagenase-1 expression in wound edge keratinocytes and it is tempting to connect the two. Does type III collagen induce collagenase-1 in migrating keratinocytes?

A clinically relevant issue concerns chronic skin ulcers. By their chronic nature, these ulcers have a problem in the process of healing. Despite overexpression of collagenase-1 in such wounds, at levels even more pronounced than in acute wounds,[37] these ulcers do not re-epithelialize effectively. So, even though collagenase-1 may be required for re-epithelialization, it is not sufficient for keratinocyte migration to take place. Despite ample collagenase-1 production, epithelial cells in chronic wounds lose their migratory phenotype and "forget" that they are wound edge keratinocytes. These keratinocytes can temporarily reassume a migratory phenotype if challenged by additional injury. In clinical practice, use is made of this empirical observation when performing surgical revision of a wound, which sometimes enhances re-epithelialization. What is the mysterious signal elicited by the injury that can stimulate the keratinocytes to migrate? One can speculate about the signals involved, but clearly it is not solely the upregulation of collagenase-1.

In summary, research over the past few years has shown us that collagenase-1 is a key component of skin wound healing. Besides participating in the remodeling of the wounded dermal tissue, collagenase-1 serves a distinct role in epithelial wound closure. Many questions remain, but additional insight into the mechanisms of keratinocyte migration will provide us with powerful tools to effectively intervene in and facilitate the wound healing process.

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CHAPTER 13

# Matrix Metalloproteinases in the Pathogenesis of Lung Injury

**Annie Pardo and Moisés Selman**

## Cell Types and Extracellular Matrix in the Normal Lung

The mammalian lung is a highly specialized and complex organ constituted by an exquisite system of conducting airways and, in the respiratory zone, by millions of alveolar-capillary units responsible for gas exchange. Evidence is presented that certain pathologies of the lung involve matrix metalloproteinases, and specifically collagenase. The pathologies considered in this chapter mostly involve the lung parenchyma, the transitional and respiratory zone of the lung, which provoke severe disarrangement during gas exchange, and in the biomechanical properties of this organ.

In general, the lung parenchyma is constituted by the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. The alveolar wall is essentially a three-compartment system consisting of a specialized epithelium, a closely opposed network of capillary endothelium, and a complex extracellular matrix supporting them. This interstitial matrix is bounded by the epithelial and endothelial basement membranes.

In areas where gas exchange occurs, the respective basement membranes fuse in such a way that the distance between air and blood is very small, i.e., providing a thin air/blood barrier for efficient gas exchange. In the other areas of the alveolar septa, these membranes are separated by an interstitium or extracellular matrix composed of collagens, elastic fibers, fibronectin, and proteoglycans, etc. Although the extracellular matrix plays an essential structural role, it also has a dynamic role in harmonizing the events that determine the structural and functional integrity of the lung under normal conditions, or after injury.

### *Lung Cell Populations*

Two types of alveolar epithelial cells have been described; squamous or type I pneumocytes, and cuboidal granular or type II pneumocytes.[1] Nearly 95% of the normal alveolar surface is lined by flattened, largely expanded type I epithelial cells. The geometry of these cells is ideally suited for gas exchange because they provide an extremely thin barrier between alveolar air and capillary blood. Type I epithelial cells represent the end-stage of alveolar epithelial cell differentiation, with little if any replicative potential, and are a very vulnerable target to injury by inhaled and blood borne agents.

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Type II pneumocytes are cuboidal cells projecting into the lumen, often occupying a niche in the corner of the alveoli. These secretory cells are metabolically very active and cover the remaining 5% of alveolar surface. Along with other molecules, type II cells synthesize and secrete surfactant, a complex surface-active compound formed by a mixture of several specialized proteins and phospholipids that covers the alveolar lining, lowering the surface tension at the alveolar air-liquid interface.[2] Type II pneumocytes are also stem cells capable of proliferation and differentiation into type I pneumocytes. Concerning the extracellular matrix, alveolar type II cells synthesize a variety of matrix components in vitro, including fibronectin, type IV collagen, laminin, and proteoglycans.[3] In addition, these cells are capable of in vitro production of interstitial collagenase and gelatinases A and B, suggesting that they actively participate in basement membrane and connective tissue remodeling.[4]

Alveolar capillaries do not run independently of one another but form an integrated network of short cylindrical segments. The capillary wall consists of a complete and nonfenestrated endothelium resting on basement membrane. Pulmonary endothelial cells are highly sensitive to injury, but unlike type I epithelial cells, have the capacity to migrate and proliferate. In vitro, alveolar capillary endothelial cells are able to produce an underlying extracellular matrix consisting of collagens, fibronectin, laminin and glycosaminglycans. They are also metabolically active cells able to activate or inactivate a number of bioactive amines and peptides.[5,6]

In the interstitial matrix, the cell component is represented by mesenchymal cells consisting of a heterogeneous population of cells including fibroblasts, contractile interstitial cells (myofibroblasts and lipid-laden interstitial cells), and pericytes.[7] Furthermore, based on differences in collagen production, proliferation rates, response to specific mediators, and a variety of cell surface receptors, evidence of the functional heterogenity of fibroblasts is emerging. Fibroblasts are the predominant cellular source of type I and III collagens, elastin, and fibronectin as well as other matrix macromolecules, and are anchored to the extracellular matrix in the interstitial space. Fibroblasts actively participate in extracellular matrix degradation by secreting members of the metalloproteinase family, such as interstitial collagenase1, gelatinase A, and stromelysin.[8,9] Working together with the components of the extracellular matrix, fibroblasts provide both structural and functional integrity to the lung parenchyma. In addition, pericytes, and probably myofibroblasts, regulate air/blood flow by their contractile properties.

Besides the resident cell population, the lung parenchyma contains a variety of migratory cell types such as interstitial and alveolar macrophages, and a few lymphocytes responsible for local defense mechanisms. Under different physiological and pathological conditions, each resident cell type that contributes to the synthesis and breakdown of matrix macromolecules is strongly influenced by autocrine and paracrine acting cytokines. Integrins, a highly conserved family of heterodimeric transmembrane glycoproteins, also participate in the regulation of the lung matrix turnover program through mediation of bi-directional cell-matrix and cell-cell interactions.

### *Extracellular Matrix*

The extracellular matrix (ECM) participates in both structural and dynamic roles in the different physiologic zones of the lung: the proximal conducting airways and vasculature, the distal gas-exchange zone (alveoli), and the intervening transitional zone (respiratory bronchioles). In addition to information regarding possible novel structural proteins, a growing body of evidence indicates that growth factors, proteinases, proteinase inhibitors, and similar molecules are typically present in the ECM. In the alveoli and respiratory bronchioles, the dynamic ECM accommodates constant fluctuations in alveolar volume during

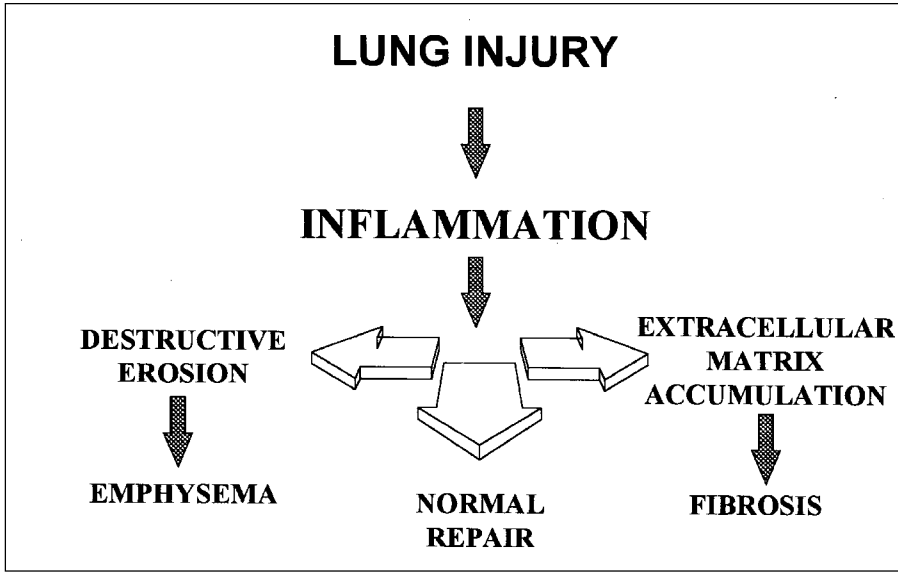


Fig. 13.1. Lung injury is usually followed by diffuse alveolitis. Subsequently 3 possible responses may occur: precise extracellular matrix remodeling ensure ad integrum restitution of the lung parenchyma. Exaggerated degradation of extracellular matrix results in destruction of the alveolar walls. Otherwise, lung response to inflammation is characterized by excessive deposit of extracellular matrix, mainly collagens resulting in progressive fibrosis.

inspiration and expiration by providing a strong and expansible framework that supports the attenuated alveolar epithelial-capillary interface. The ECM also elicits a variety of cellular responses including adhesion, spreading, differentiation, proliferation and migration, induction of polarity and gene expression, and the outgrowth of cell processes.[10]

The interstitial matrix of the alveolar walls is composed of fibrillar collagens types I, III, V, and VI, elastin, proteoglycans, and fibronectin. Elastic fibers consisting of elastin and microfibrils, and collagen fibers are the major elements of the interstitial ECM in the lung parenchyma, and their spatial distribution gives the lung its characteristic elastic properties during ventilation.[11]

The ECM comprises components of the alveolar and capillary basement membranes as well as those present in the interstitial connective tissue of the alveolar septa.[12,13] Basement membrane contains nonfibrillar collagens, primarily of type IV collagen, laminin, entactin, fibronectin, and heparin sulfate/chondroitin sulfate proteoglycans. ECM assembly appears dependent upon the concentrations of component molecules and seems to occur by mass action with the type IV collagen network serving as a scaffold for the other molecules.[5]

Degradation of ECM macromolecules occurs through a variety of proteinases. A growing body of evidence suggests that matrix metalloproteinases (MMPs) are responsible for degrading matrix components in both normal physiological processes and pathological conditions. Besides their pivotal role in maintaining extracellular matrix homeostasis, MMPs actively participate in the immune and inflammatory responses to injury. A hypothetical scheme covering lung response to acute and chronic injury is presented in Figure 13.1. This line of thought makes clear the concept that a precisely regulated turnover of the extracellular matrix is critical for normal repair and maintenance of lung physiology after injury.

Modifications to this finely regulated program are implicated in a number of acute and chronic lung diseases, and MMPs are believed to have a major role in their pathogenesis.

## Matrix Metalloproteinases in Acute Lung Injury

Acute lung injury is a nonspecific reaction of the lung to a multitude of injurious causes, including noxious environmental or endogenous agents. Regardless of etiology, the common denominator is severe endothelial and alveolar lining cell injury leading to lung edema, diffuse inflammation, and fibroblast proliferation. This ultimately results in severe disruption of the gas exchange alveolar-capillary structures, and, in the worst of cases, respiratory failure.

An important feature of the inflammatory response is the immediate delivery of polymorphonuclear leukocytes, monocytes, and lymphocytes from the circulatory system to the site of injury. Concomitant alterations in both epithelial and endothelial cells result from the effects of a variety of cytokines, that are temporally and spatially regulated, and directly influenced by inflammatory cells.[14,15] All these events control the remodeling of the surrounding extracellular matrix, which in turn influences the functioning of surrounding cells.

Despite many studies performed in vitro and in vivo, the pathological processes occurring during the development of acute lung injury are not completely understood. An early diagnostic feature of acute lung injury is an interstitial and intra-alveolar edema involving a compromise of the lung endothelial/epithelial barrier, via disruptions of the basement membranes.[16,17] In addition, the rapid inflammatory cell infiltrate, alveolar lining cell hyperplasia, and fibroblastic proliferation induce a remodeling of the extracellular lung matrix. All these pathological changes suggest the participation of proteolytic enzymes, specifically those related to basal lamina and interstitial matrix turnover.

Since matrix metalloproteinases are physiologically relevant mediators of matrix remodeling,[8,18,19] both we and others have hypothesized that this family of zinc dependent endopeptidases are strong candidates as contributors to the cell and interstitial tissue pathological features observed in acute lung injury. Results supporting this hypothesis have recently been obtained in experimental animal models,[20-22] and as the result of studies on adult respiratory distress syndrome (ARDS), the human prototype of acute lung injury.[23,24]

### *Evidence for a Role of MMPS in Animal Models of Acute Lung Injury*

Rats exposed to 100% oxygen experience severe lung oxidant damage, characterized by alterations in the alveolar capillary barrier, increasing permeability to solutes, with resultant interstitial and intra-alveolar edema and respiratory failure. In this well characterized model of short-term lethal hyperoxic lung injury, we demonstrated a remarkable upregulation of rat interstitial collagenase (MMP-13), as well as of gelatinases A (MMP-2) and B (MMP-9).[20] Regarding interstitial collagenase, we found increased collagenolytic activity in bronchoalveolar lavage fluid (BALF) as compared to controls. In situ hybridization, and immunohistochemistry revealed that interstitial collagenase transcript and protein, respectively, were diffusely distributed throughout the lung parenchyma. The cellular source of interstitial collagenase were free alveolar macrophages and alveolar epithelial cells, although in some areas, interstitial cell staining was also observed. Positive staining was also seen in bronchiolar nonciliated epithelial cells, whereas vessels were consistently negative.

Analogous findings have been reported by Devaskar et al, who observed an increased expression of type I collagenase with the associated high levels of type I collagenolytic activity in newborn lung rats with hyperoxic lung injuries.[21]

Interstitial collagenases initiate the first step in the catalytic cleavage of collagen fibrils and have been implicated in a number of physiologic and pathological processes in which

the collagen network is widely and actively remodeled.[8,18,19] As mentioned above, acute lung injury causes severe damage to the parenchymal structures, with a variety of abnormalities in the interstitium and alveolar spaces. An increased expression of interstitial collagenase in hyperoxic injuries strongly suggest that an active degradation of interstitial collagens might be occurring in the early phases of acute lung damage and contributing to changes in epithelial permeability.

In our model of hyperoxic injury in rats, gelatinolytic activity was also significantly increased.[20] Zymographic analysis of BALF revealed that control rats expressed gelatinase A, whereas animals exposed to 100% oxygen exhibited both gelatinase A and B activity. Similar findings were reported by D'Ortho et al[22] in a model of lipopolysaccharide (LPS)-induced acute lung injury in guinea pigs. BALF from LPS-treated animals displayed an increase in the major gelatinase species of 68 and 92 kDa (gelatinases A and B respectively), when compared with control animals not expressing 92 kDa gelatinase. Interestingly, neutrophil elastase, which is increased in patients with ARDS,[25] seems to activate gelatinase B in acute hamster lung injury induced by intratracheal administration of LPS.[26]

In our model of 100% oxygen toxicity, the cellular localization of the gelatinases was evaluated by in situ hybridization and immunohistochemistry.[20] The injured lungs exhibited a clear increase in both transcripts, but while gelatinase A mRNA was usually diffusely distributed in the lung parenchyma, a more focused pattern was observed with gelatinase B. Numerous free alveolar macrophages expressed both type IV collagenases, and in areas of septa thickening, an intense interstitial distribution of gelatinase A transcript was noticed, suggesting the participation of pulmonary fibroblasts and interstitial inflammatory cells in the synthesis of this enzyme.

The gelatinases and interstitial collagenase were also expressed by putative type II alveolar epithelial cells located in the corner of alveoli and protruding into the lumen. In this context, we have recently demonstrated that in vitro, type II pneumocytes produce interstitial collagenase and both gelatinases.[4] Comparable findings concerning type IV collagen breakdown have been reported in fetal type II epithelial cells, implying that these cells function in basement membrane disruption during late fetal lung development.[27] Likewise, increased expression of MMPs by alveolar epithelial cells may link the alveolar epithelium to the pathogenesis of oxidative injury, and lead to increased epithelial permeability and lung edema.

These enzymes are secreted as zymogens that must be activated, and this proteolytic processing, along with the action of active MMPs in the microenvironment, are subject to rigorous control by specific TIMPs (see chapter section 11.4). It has been reported that hyperoxia induces a large increase in the mRNA encoding TIMP-1 in rabbits and mice.[28,29] Therefore, the excessive expression and synthesis of a MMP is not necessarily related to excess MMP activity in vivo. In order to test the presence of local gelatinase activity in hyperoxic injured tissue, we developed lung in situ zymography (Fig. 13.2). With this method, we observed intense areas of gelatinolytic activity following the alveolar septa, suggesting that gelatinases are active in vivo.

### *Adult Respiratory Distress Syndrome (ARDS)*

ARDS is a common and lethal condition resulting from nonspecific damage to the alveolar capillary membrane that produces a rapid disruption of gas exchange by an explosive inflammatory reaction in the lung parenchyma.[30] The damaged membrane's permeability vastly increases and the alveolar spaces are flooded with inflammatory cells and plasma proteins. In general, human acute lung disease is characterized by high microvascular permeability, low pressure pulmonary edema, refractory hypoxemia, and respiratory failure.

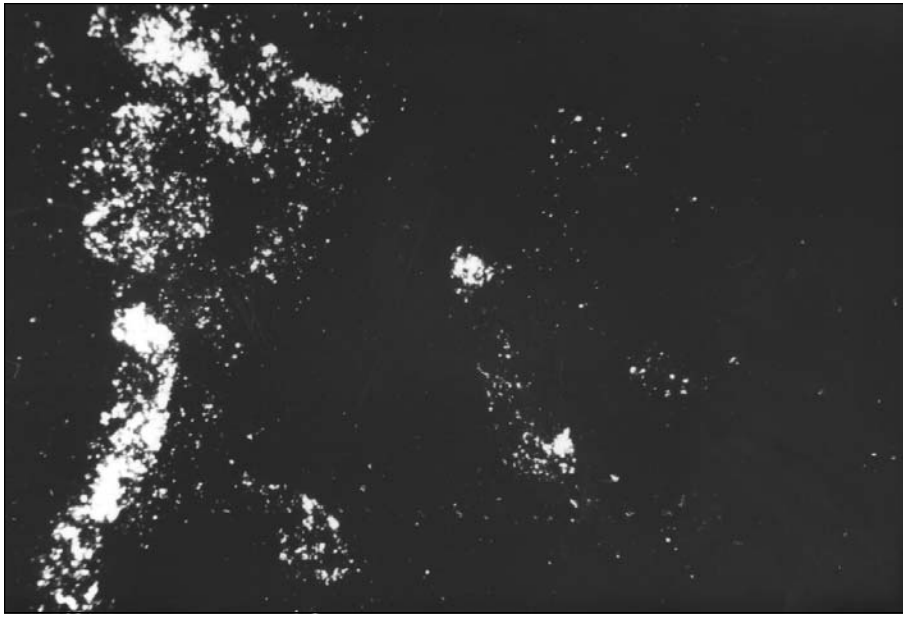

Fig. 13.2. In situ zimography. Frozen tissue section from 100% oxygen exposed lung was immersed into NTB 2 photographic emulsion whose main component is gelatin. Slides were incubated into humidified chambers at room temperature for 5 days, processed by photographic development, and examined with a light microscope. Areas of gelatinolytic activity following the alveolar septa are observed as clear spots over the black substrate background.

The possible participation of MMPs in the pathogenesis of ARDS has been explored mostly through BALF. In a first approach, Christner et al[31] found increased type I and type III collagenase activity in the majority of patients with ARDS. However, no significant correlation was detected between collagenolytic activity and the clinical and cellular parameters studied. More recently, Ricou et al,[23] using ELISA assessed the presence of gelatinase B and TIMP-1 in the BALF and plasma of 33 intensive care unit patients, and analyzed the time course of both factors over several days or weeks with respect to its relation to the clinical evolution of ARDS. During the early phase of disease, they found that gelatinase B and TIMP-1 increased in comparison to controls. In short course ARDS, gelatinase B levels decreased rapidly, but remained elevated in prolonged ARDS, while TIMP-1 tended to increase with the severity of lung injury. Similar findings were reported by Torii et al,[24] who found that the increased levels of MMP-9 correlated with increased concentration of 7s collagen, an aminoterminal noncollagenous portion of type IV collagen used as a marker of basement membrane disruption. Interestingly, both groups[23,24] detected a close correlation among gelatinase B and neutrophils, which led them to propose that in ARDS, this enzyme originates from neutrophils in the alveolar space. This is an interesting idea because a growing body of evidence supports this critical role for neutrophils in the pathogenesis of acute human lung injury. For example, neutrophil influx into air spaces occurs before the development of acute lung injury, and after the development of ARDS, the severity of lung damage correlates with the extent of neutrophil influx into the lungs. Furthermore, in some patients, the persistence of the initial neutrophilic inflammatory response is associated with

higher mortality.[32] However, it will be necessary to corroborate the real cellular source of gelatinase B in ARDS in situ since this enzyme has only been located in alveolar macrophages and alveolar epithelial cells during the development of experimental acute lung injury.[20]

In addition to MMP-9, Torii et al[24] evaluated the levels of gelatinase A and its respective tissue inhibitor, TIMP-2, using a one-step sandwich immunoassay. In BALF obtained from 17 patients with ARDS, MMP-2 was significantly higher than healthy control subjects, while TIMP-2 was under the detection in both sets of subjects.

As with MMP-9, gelatinase A correlated with increased concentration of 7s collagen, but also increased alongside laminin, used as a marker of basement membrane disruption. This interesting finding is supported by an immunohistochemical study performed by Hayashi et al,[33] who found strong reactivities for gelatinases A and B in the lungs of patients with diffuse alveolar damage, both during the early stage as well as the organizing phase of the disorder. Particularly, MMP-2 showed focal colocalization with disrupted epithelial basement membranes. However, both TIMP-1 and TIMP-2 were upregulated, underscoring the complexity of the relationships between MMPs and their inhibitors in the lung microenvironment.

Gelatinases have substrate specificity for denatured collagens, basement membrane type IV collagen, and elastin.[34-36] These enzymatic activities might play a significant role in the pathogenesis of acute lung injury by contributing to: a disruption of basement membranes; the cell migration characteristic of the inflammatory process; and an unregulated remodeling of the extracellular matrix. Therefore, the excessive production and activity of gelatinases and interstitial collagenase could promote the increased alveolar-capillary barrier permeability, favoring alveolar edema and inflammation. Our current knowledge on the pathogenic mechanisms operating in acute lung have allowed the identification of numerous injury mediators which contribute to the pathologic response. Advances in understanding the possible role of matrix metalloproteinases in lung remodeling should stimulate experimental and subsequent clinical trials to investigate new ways of stabilizing or reducing the severity of acute lung injury.

## Metalloproteinases in Chronic Lung Injury

### *Lung Emphysema*

Lung emphysema is a major pathological condition usually associated with cigarette smoking. The disease is characterized by abnormal and permanent enlargement of respiratory regions of the lung distal to the terminal bronchioles, accompanied by the destruction of the alveolar septa.[37] The disease provokes the disappearance or severe disturbance of the orderly structure of the pulmonary acinus where gas exchange occurs, leading to progressive and irreversible respiratory insufficiency. Corresponding to their locations in the secondary pulmonary lobule, two major forms of emphysema have been described: panacinar and centriacinar. Panacinar emphysema, generally seen in patients with $\alpha$1-antiprotease deficiency, is less common, and is characterized by air space enlargement throughout the acinus. Centriacinar emphysema is strongly associated with cigarette smoking, and results from destruction of alveoli around the proximal respiratory bronchiole.

The most prevalent theory about the pathogenesis of emphysema involves an abnormal balance between proteases and antiproteases in the lung, where emphysema develops as a result of excessive proteinase burden—mainly neutrophil elastase—in the lower respiratory tract.[37,39] The hypothesis linking cigarette smoke, increased elastolytic activity, and the development of emphysema attributes the recruitment of elastase-producing neutrophils in smokers' lungs and the inactivation of the $\alpha$1-proteinase inhibitor to tobacco compounds.

However, several crucial questions challenge this hypothesis: Are neutrophils primary participants in lung destruction? Is neutrophil elastase the only enzyme capable of degrading lung elastin? And are the elastic fibers the only extracellular matrix molecules affected during the development of the disease?

**Is Neutrophil the Main Culprit Cell?**

Although much attention has focused on neutrophil elastase as a mediator of the lung destruction observed in emphysema, the accumulated evidence is mostly circumstantial, and in fact, studies attempting to find increased tissue levels of this enzyme in emphysematous lesions have yielded controversial results.[40,41] The concept that increased numbers of neutrophils and macrophages are present in the lungs of smokers is mainly based on findings in BALF, but few morphologic studies have attempted to describe the types of inflammatory cells present in the alveolar walls of smokers. Nevertheless, macrophages, and not neutrophils, are the most abundant inflammatory cells found in the BALF of cigarette smokers, as well as in the respiratory bronchioles where emphysematous changes first manifest themselves.[42,43]

But most importantly, a recent morphometric study performed by Finkelstein et al[44] held that the extent of emphysema, determined by the volume density of the lung parenchyma, was directly related to the number of alveolar macrophages and T-lymphocytes present in the lesions. Moreover, a negative correlation was found between the number of neutrophils and the amount of lung destruction. Therefore, macrophages are the most abundant inflammatory cell in areas of lung destruction, and alveolar disruption is associated with the presence of this cell type.

Macrophages are a major source of proteases capable for lung destruction, and in addition to interstitial collagenase-1 (MMP-1), they produce at least four different metalloproteinases also able to degrade insoluble elastin: metalloelastase (MMP-12), matrilysin (MMP-7), and gelatinases A and B (MMP-2, MMP-9).[32,45-47] Interestingly, several preliminary reports suggest that in both human emphysematous lung tissue specimens and experimentally induced emphysema, the number of cells expressing 92 kDa gelatinase increases.[48-50] In these studies, the cells were located along alveolar walls, spaces, and interstitium, and most likely represented activated alveolar and interstitial macrophages along with some epithelial cells.

In this way, these inflammatory cells may also contribute to the disruption of elastic tissue. A complication is that alveolar macrophages in the lower respiratory tract of cigarette smokers are a potential source of oxidants capable of inactivating the active site of $\alpha$1-antiproteinase and rendering it ineffective as an inhibitor of neutrophil elastase.[51] Therefore, macrophages, by virtue of their increased numbers in the site of lesions and exaggerated production of metalloproteinases and oxidants, are ideal candidates as agents eroding the morphological and functional integrity of smoker's lungs.

**Could the Elastic Fiber Rupture Alone Explain the Emphysematous Lesion?**

Alveolar walls are constituted of a dynamic and complex connective tissue framework including interstitial collagens (the predominant component), elastic fibers, proteoglycans, fibronectin, and other glycoproteins.[52]

In emphysema, the terminal respiratory unit is often completely demolished and moreover, the emphysematous spaces may coalesce into larger bullae which in some cases are several centimeters in diameter. It is difficult to conceive of such damage to the alveolar septa without the action of proteases capable of degrading interstitial collagen—the main component of lung parenchyma extracellular matrix. The collagenases, a subgroup of the metalloproteinases gene family have as members with specific substrate specificity for fibrillar

collagen,[53-55] interstitial collagenase, neutrophil collagenase, and collagenase 3. In other words, lung destruction occurring during the development of emphysema should necessarily affect more than elastic tissue and involve the multiple action of proteolytic enzymes like interstitial collagenase released into the local milieu.

The first important contribution showing a possible role for interstitial collagenase in the pathogenesis of this disease was published by D'Armiento et al.[56] They demonstrated that transgenic mice expressing a collagenase transgene in their lungs developed morphological changes strikingly similar to human emphysema. Histological analysis of the lungs revealed disruption of the alveolar walls and coalescence of the alveolar spaces with no evidence of fibrosis or inflammation. Moreover, those mice expressing the highest levels of the transgene developed the most severe emphysematous lesions. The enzyme used in these transgenic mice was interstitial collagenase (MMP-1), the collagenase present in macrophages and fibroblasts. In support of this finding, lungs showed a conspicuous decrease in collagen fibers whereas elastic fibers seemed to be normal. From this evidence, it was concluded that, the upregulation of interstitial collagenase, an enzyme that does not degrade elastin, provoked lung emphysematous changes.

This finding was later confirmed in our laboratory using an experimental model of lung disease induced by tobacco smoke in guinea pigs.[57] During cigarette smoke exposure, lungs exhibited progressive inflammatory lesions of mononuclear predominance, and after 6 to 8 weeks, varied degrees of emphysematous changes were observed. Coinciding with the progression of bronchiolar and alveolar inflammation and development of emphysematous lesions, an increased expression and synthesis of interstitial collagenase in alveolar macrophages was identified. Furthermore, these findings were accompanied by an elevation of endogenous collagenolytic activity. The increase in collagenase activity occurred simultaneously with a higher degree of inflammation and alveolar rupture, suggesting that active collagen breakdown takes place during the progression of emphysema. In addition to alveolar macrophages, alveolar epithelial cells also expressed interstitial collagenase. Interestingly, type II pneumocyte cells seem very active in matrix remodeling after injury, complementing the observation that they also produce a number of MMPs in acute lung injury.[20] Recent reports have confirmed the upregulation of MMP-1 in human emphysematous lung tissues and the presence of collagenolytic activity in the BALF of patients with pulmonary emphysema.[50,58,59]

Taken together, the findings in transgenic mice, the model of tobacco-induced damage, and in human disease strongly suggest that emphysematous lesions involve more than elastic tissue disruption, and also that the degradation of interstitial collagens contributes significantly to the pathogenesis of this disease.

**An Unrestrained Positive Feedback Between Enzymes and Inhibitors?**

In dealing with this system, it is important to realize that excessive collagenolytic activity may affect more extensively than normal the lung connective tissue metabolism because both macrophage/fibroblast collagenase and neutrophil collagenase are able to hydrolyze and inactivate $\alpha_1$-proteinase inhibitor.[60,61] Evidence like this indicates that MMP-1 and MMP-8 display expanded substrate repertoires and support theories about the existence of a new interface between interstitial matrix turnover and serine proteinase inhibitors. Excessive secretion of interstitial collagenases in the lung microenvironment could conceivably perturb the serine-proteinase/$\alpha_1$-proteinase inhibitor balance and contribute to the recorded increase in elastolytic activity. Similarly, other metalloproteinases such as stromelysins 1 and 3 can cleave $\alpha$1-antiproteinase and consequently may also potentiate the activity of neutrophil elastase.[62,63]

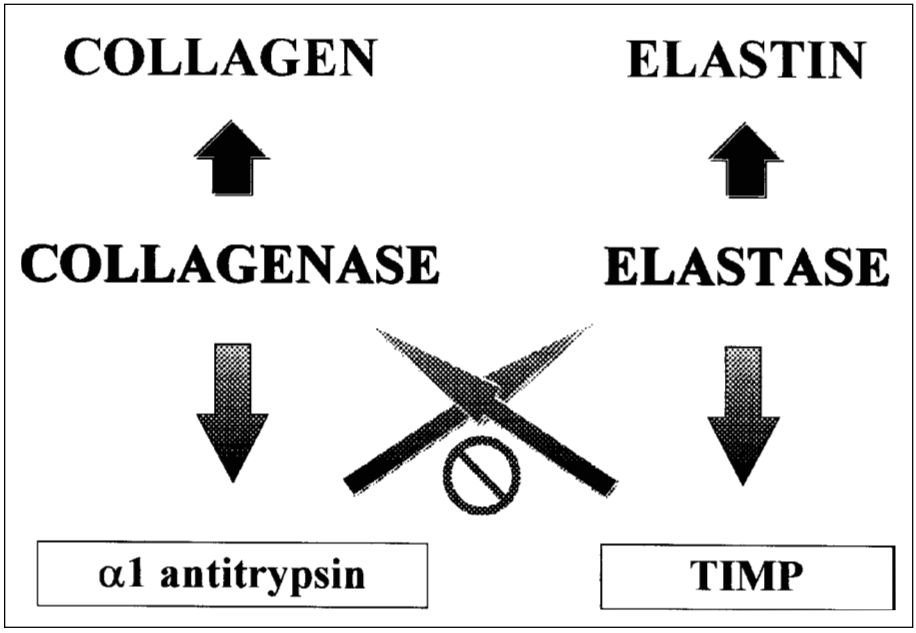


Fig. 13.3. Interstitial collagenase and neutrophil elastase may contribute to the development of emphysema both through degradation of matrix molecules as well as by degradation of specific enzymatic inhibitors.

Because of these related activities, it has also been shown that neutrophil elastase and other serine proteases degrade TIMP, thus increasing collagenolytic and other metalloproteinases activities[64] (Fig. 13.3).

In this scenario, we postulate that a complex network of interrelated proteinases — serine proteases and metalloproteinases— capable of degrading different extracellular matrix molecules and proteinase inhibitors, are pivotal members in the pathogenesis of pulmonary emphysema.

### *Diffuse Pulmonary Fibrosis*

Pulmonary fibrosis is a consequence of a large number of diseases and a variety of lung injuries.[65] In general, lung injury may produce transient and mild pathological changes that are quickly repaired, allowing the lung to return to normal condition. By contrast, if the injury is severe or repetitive, or if it occurs in a "susceptible" individual, the lesion may evolve to diffuse interstitial and intra-alveolar fibrosis.

When fibrosis occurs, these diseases progress slowly into irreversible and lethal conditions and until now, there has been no therapy capable of reverting the pathological process. Idiopathic pulmonary fibrosis (IPF), a disease of unknown etiology, is the prototype of one of the most frequent and aggressive fibrotic disorders of the lung, and is usually fatal, with the survival period averaging four to five years.

Regardless of the etiology, the pathogenesis of diffuse lung fibrosis generally presents the following sequence: initial lung damage, interstitial and intra-alveolar inflammation (alveolitis), fibroblast proliferation, and finally the endstage lung, with its abnormal

accumulation of interstitial collagens. In other words, these diseases evolve from an initial cellular inflammatory reaction (early phase), to extensive fibrosis (advanced phase), often ending in terminal honeycomb change (end-stage lung).

**The Early Stage. Rupture of the Basement Membranes, a Role for Gelatinases?**

Several studies performed in human fibrotic lung disease and in experimental models have furthered the conclusion that, if during the initial injury and subsequent inflammation there is extensive destruction of lung scaffolding and damage to the integrity of the basement membrane repair by epithelial cells becomes impossible. This situation results in a fibrotic response, and in these areas where fibroblast proliferation and fibrosis becomes prominent, hyperplastic type II pneumocytes replace type I alveolar cells and line residual air spaces. For alveolar repair and correct re-epithelialization to occur following injury,[66-69] intact basal lamina is essential. Furthermore, intra-alveolar fibrosis, a frequent feature of the fibrotic lung disorders, requires migration of interstitial fibroblasts and deposition of extracellular matrix outside of the epithelial basement membrane. Since mesenchymal cells are confined to the interstitial space bounded by the alveolar basal lamina, focal disruption of this membrane should be required for airspace fibrosis.

Toward this line, Raghu et al[16] demonstrated by immunohistochemical methods that early in the course of fibrotic diseases, basement membrane is disrupted by an interstitial collagen invasion of the alveolar spaces. Disruption was signified by gaps in the distribution of type IV collagen and laminin, and in these areas, there was continuity between the interstitial ECM and the ECM occupying the alveolar spaces.

Increased levels of gelatinases A and B, which degrade several components of basement membrane including type IV collagen, seem to be associated with lysis of basal lamina in lung disorders such as acute lung injury, cancer and bronchiectasis.[20,70,71] However, their role in fibrotic lung disorders is presently unknown.

Recently, Hayashi and colleagues[33] have approached, through immunohistochemical and confocal microscopic techniques, the localization in lung tissues from patients with diffuse alveolar damage and idiopathic pulmonary fibrosis, of gelatinases A and B, TIMP-1 and TIMP-2, and type IV collagen. In the former, the injury initiates inflammatory and fibroproliferative processes that either progress to end-stage fibrosis or resolve themselves completely, whereas IPF appears to be a continuing pathological process with progressive deposition of extracellular matrix and remodeling of lung parenchyma culminating in end-stage fibrosis. However, when evolved to fibrosis, the entire process of lung injury and diffuse alveolar damage matures during a period of a few weeks while the process in IPF is a more gradual phenomenon, taking due course in the range of several months to years. The authors found that myofibroblasts and alveolar epithelial cells lining thickened septa had increased reactivity for MMPs and TIMPs in both disorders, although the expression was stronger in diffuse alveolar damage.

Myofibroblasts and type II pneumocytes seem important in the pathogenesis of pulmonary fibrosis. After lung injury, myofibroblasts proliferate and often emerge as the predominant mesenchymal component of the fibrotic tissue. In addition to their contractil capacity, myofibroblasts are active collagen producing cells and have been implicated in lung architectural distortions, contributing to the decreased lung compliance observed in pulmonary fibrosis.[72] According to the study of Hayashi et al,[33] myofibroblasts may also participate in the fibrous remodeling of extracellular space and basement membrane by secreting MMPs and TIMPs.

On the other hand, failure to replace damaged type I epithelium by proliferation and differentiation of type II pneumocytes appears to be an important determinant of whether or not progression to fibrosis ensues.[73,74] When appropriate re-epithelialization does not

occur after injury, type II pneumocytes proliferate and line the airspaces, synthesizing several cytokines, growth factors, and MMPs.[4,75-77] Interestingly enough, in both diffuse alveolar damage and IPF, the expression of MMP-2 by myofibroblasts and type II pneumocytes showed focal colocalization with type IV collagen, suggesting that activation of this metalloproteinase contributes to subsequent proteolysis of basement membrane components.

In general, these findings show some similarity with those in hyperoxia-induced acute lung injury, where increased gelatinolytic activity accompanied increased expression of MMP-2 and MMP-9 in situ.[20] Furthermore, it has been shown that the use of recombinant TIMP-2 significantly reduces immune complex-induced acute alveolitis in vivo, supporting the pathogenic nature of gelatinases.[78] In this context, a potential hypothesis is that following lung injury, gelatinases A and B are overexpressed, provoking focal disruptions of the alveolar basement membrane and deteriorating normal re-epithelialization, enhancing fibroblast migration to the alveolar spaces. In light of these properties, it is probable that a rapid response mediated by TIMPs would revert this process.

However, given their pleomorphic functions, MMP and TIMP function in basement membrane turnover during lung fibrosis should be considerably complex, and obviously further studies are necessary to pinpoint the enzyme-inhibitor in vivo relationship.

**The Fibrotic Phase. A Role for Interstitial Collagenases?**

Independently of etiology, the final common pathway in pulmonary fibrosis is the inappropriate and uncontrolled accumulation of collagenous extracellular matrix in the distal respiratory tract, which eventually leads to destruction of the lung parenchyma. An important aspect of pulmonary fibrosis development is the altered metabolism of several components of the extracellular matrix. However, the role of increased deposits of elastin, proteoglycans, and fibronectin in the pathogenesis of lung fibrosis and in the loss of lung architecture remains unclear. Nonetheless, the absolute increase of collagens, taken together with the abnormalities in their spatial distribution, seem culpable for the disorganization and distortion of the normal lung parenchyma during fibrosis progression. In this way, collagen metabolism regulation is central to the pathogenesis of fibrosis that follows injury and inflammation.

Collagen accumulation appears to be progressive because of demonstrations that the concentration of this protein is usually higher in postmortem lung tissues than in biopsy samples taken in the same patients months or years before.[79,80] All interstitial collagens increase in the lung parenchyma, but the temporal patterns of deposition differ among them. Thus, a predominance of the type III and VI collagens associated with loose, reticular matrix, can be observed in sites of early or active fibrogenesis, while an abundance of dense connective tissue composed mainly fibrillar type I collagen is observed in areas of advanced fibrosis.[16,81]

In a normal adult, the net matrix production is zero, the concentration of collagen per unit lung mass remains constant, and matrix synthesis and degradation are balanced.[82] Therefore, collagen accumulation in pulmonary fibrosis represents an imbalance in the collagen turnover rates favoring degradation, and resulting in an excessive deposit of this protein. This increase in collagen content must result from an increase in synthesis, a decrease in degradation, or the sum of both processes.

Rates of synthesis are usually increased in experimental lung fibrosis, although the two studies performed in humans have failed to provide direct evidence for increased lung collagen production.[79,83] Figures obtained from animal models indicated a significant but transient increase in collagen synthesis, whereas collagen accumulation is usually constant.[84,85] Since human studies have been done in advanced stages of the disease, increased collagen

biosynthesis could be an early and transient stage during the development of pulmonary fibrosis, perhaps representing the activation or selective expansion of high collagen-producing fibroblasts or myofibroblasts. Indirect evidence for this hypothesis is given by an immunohistochemical study performed by Kuhn et al,[86] who corroborated that in the lungs of patients with early stage fibrosis, a large population of collagen-producing cells was typical. By contrast, in biopsies of patients with chronic pulmonary fibrosis, none or only a few type I collagen-synthesizing fibroblasts were identified. Moreover, foci of highly activated fibroblasts expressing type I collagen were seen in areas of active fibrogenesis whereas no type I procollagen expression was observed in dense fibrotic areas of the lung.[76]

On the other hand, several lines of evidence indicate that changes in collagen degradation are an integral part of the fibrotic process. Normally, a significant amount of collagen synthesized by mesenchymal cells is intracellularly degraded. Therefore, the amount of procollagen secreted might be modulated by alterations in this intracellular degradative pattern. In this context, increased collagen secretion might be at least partially associated with a decrease in the proportion of procollagen degraded intracellularly.[87]

However, once the lung collagen deposit forms, it is mainly modulated by the extracellular collagenolysis, of interstitial collagenases, which are unknown entities in the pathogenesis of lung fibrosis. One possibility is that collagen degradation is crucial to in the abnormal remodeling observed during fibrosis. Evidence for this hypothesis is largely related to the discovery of comparatively higher levels of active collagenase in BALF from patients with interstitial lung diseases.[88-90] However, other studies have demonstrated that collagenase and collagenolytic activity can be present or absent in BALF without any association with the activity and prognosis of the disease or with tissue collagenolytic activity.[91-93]

Therefore, in accordance with the results obtained in a number of studies in our laboratory, we postulate that a decrease in collagen degradation is an essential mechanism for collagen accumulation in the fibrotic lung. As an example in support, patients with IPF usually exhibit a remarkable decrease in lung collagen degradation.[79] Furthermore, in chronic hypersensitivity pneumonitis, a disease in which most patients improve or heal, we have demonstrated that healing appears associated with higher levels of lung collagenolytic activity, while progression to fibrosis seems related to significantly lower collagenase activity.[92]

Though regulated by complex mechanisms, a decrease in collagenolytic activity, is probably related to a decreased expression and synthesis of interstitial collagenases or with an upregulation of TIMPs. In support of this point of view, we have found that the TIMP-1/collagenase molar ratio is higher in fibroblasts derived from human fibrotic lungs than in normal ones.[94] This finding makes possible the presence of lung fibroblast subpopulations with different abilities to produce collagenase and TIMP. In fibrotic lungs, low-collagenase, high TIMP producing subsets predominate. Likewise, collagenase inhibitory activity appears significantly higher in the lung parenchyma of patients with IPF and chronic hypersensitivity pneumonitis.[95] Furthermore, in the fibrotic phase of both diffuse alveolar damage and IPF, TIMP-1 and TIMP-2 increase in areas of dense fibrosis, suggesting that upregulation of these inhibitors contributes to the stability of collagen and the other matrix components.

Sequential studies of experimental lung fibrosis induced in rats using paraquat and oxygen or silica, have consistently revealed the dual nature of collagenolysis. During the early inflammatory process, experimental rats generally display a clear increase in lung collagen degradation.[96,97] However, in more advanced phases that coincide with active fibrogenesis, nearly all rats displayed a significantly lower rate of collagenase activity. While increased collagenolytic activity is one outcome, the initial increase of matrix degradation at sites of parenchymal inflammation could also contribute to matrix reorganization through more subtle means. For example, preliminary studies performed in our laboratory have

shown increased collagenase-3 mRNA lung expression in experimental silicotic rats, principally in areas of developing silicotic granulomas.[98] On the other hand, and analogous to human studies, an upregulation of TIMP-1 appears to occur in experimental lung fibrosis.[99]

The conclusion to be drawn from this data is that a decrease in collagenase activity is a general mechanism of tissue fibrotic processes, and has been well documented in cirrhotic livers, in the skin of patients with Progressive Systemic Sclerosis, and in keloid lesions.[100-102]

**Regulatory Cytokines. A Key Role for Transforming Growth Factor Beta (TGF-β)?**

A great variety of cytokines capable of affecting every part of the pathological processes are upregulated and released by inflammatory, connective tissue, and epithelial cells during the development of the diffuse lung fibrotic disorder. These include TNF-α, PDGF, and TGF-β, all considered profibrotic cytokines.[75-77] In particular, TGF-β, secreted first by macrophages and in more advanced stages by fibroblasts and epithelial cells, maximizes collagen accumulation by getting at several points in the pathway of collagen synthesis and degradation. It stimulates procollagen gene transcription, increases α-chain mRNA stability, decreases procollagen intracellular degradation, inhibits collagenase expression, and stimulates TIMP production.[103-105] Furthermore, TGF-β stimulates the synthesis of 72 kDa type IV collagenase and consequently may participate in basement-membrane restructuring.[106] In addition of enhancing a fibrotic response, TGF-β may also contribute to lung damage by inhibiting both the proliferation and differentiation of alveolar epithelial cells.[107] This cytokine has also been connected to the emergence of myofibroblasts in the lung microenvironment during fibrotic processes.[108] Taken together, these fragments of evidence support a central role for TGF-β in the pathogenesis of diffuse lung fibrosis. However, it is important to consider that the fibrotic response represents an imbalance between the profibrotic and antifibrotic cytokines released, where profibrotic prevail and provoke exaggerated collagen accumulation. This phenomenon was recently confirmed by the finding that aFGF, as profibrotic cytolcine, and its receptors are upregulated during the entire evolution of a pulmonary fibrosis induced by paraquat plus hyperoxia.[109] The expression of this factor seemed to be stronger in areas where the lung architecture was better preserved. Recent evidence suggests that aFGF appears to exert an antifibrotic role since downregulates α1 (I) collagen gene expression and upregulates interstitial collagenase expression by lung fibroblasts.[110]

Further research and analysis of the regulation of MMPs and TIMPs in progressive fibrosis are required for the satisfactory understanding of detailed mechanisms of excessive extracellular matrix accumulation.

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CHAPTER 14

# Collagenase and Aging

**Michael D. West**

## Introduction to Aging

In the course of human development, one observes a progressive increase in body size and maturation until sexual maturity is attained. Having reached this summit, the body then shows a progressive degeneration with the passage of time, a phenomenon frequently referred to as aging. The reason for the maturation phase may seem obvious, but why we age has remained a conundrum. As G.C. Williams once observed, "It is indeed remarkable that after a seemingly miraculous feat of morphogenesis a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed."[1] In this chapter, we will explore one model for the cellular basis of this decline in connective tissues. We will focus in on a candidate mediator of age-related connective tissue degeneration, interstitial collagenase, to which we will hereafter refer to as "collagenase".

The aging of skin provides an important model for biological gerontology. The connective tissue of the dermis is readily accessible for studies from individuals of all ages. Generally a small punch biopsy is sufficient to supply cultures of fibroblasts, keratinocytes, or sections for histology. Such studies demonstrate pronounced changes in histology with age, alterations that share many features with similar changes occurring in other tissues in the body.[2] These observations suggest that the study of aging skin could yield important clues to the pathogenesis of other age-related connective tissue disorders, and perhaps, aging in general.

Skin, however, is unique it its exposure to relatively high levels of ultraviolet radiation. Therefore, a distinction is usually drawn between those changes in the skin attributed to long-term exposure to solar radiation (actinic skin damage) and those independent of such extrinsic causes (intrinsic skin aging). Much of this discussion will focus on intrinsic mechanisms, although, there may be considerable overlap between the two pathways.[3] We will begin by reviewing what is known of the histological alterations in aging dermis and then discuss the possible role of the aging fibroblast in this process. We will then review recent insights regarding the altered gene expression of senescent cells, and examine the transcriptional regulation of collagenase. Finally, we will examine possible targets for pharmaceutical intervention.

### *Histological Changes in the Aging Dermis*

The aging of connective tissues is associated with the destruction of histological architecture (morpholysis) often resulting in an associated loss of function (physiolysis). Morphological changes during cutaneous aging include: dermal atrophy, wrinkling, elastolysis,

*Collagenases*, edited by Warren Hoeffler. 

and a loss of subcutaneous fat. Dermal atrophy, as opposed to epidermal, is believed to play the most significant role in the visible changes, with critical alterations occurring in several components of the extracellular matrix (ECM).

The dermis is comprised of a complex of ECM molecules, of which collagen is the most abundant component.[4,5] There is a striking loss of collagen with age. The collagen content of the dermis is estimated to decrease at about 1% per year during adult life.[6] In particular, there is a loss of the fascicular collagen fibrils and an increase in fibrils that show a disorganized and granular morphology. The altered appearance of the collagen fibers has also been attributed to the loss of intercalated proteoglycan[7] in particular, a loss of hyaluronic acid and dermatan sulfate.[8] However, since collagen alone imparts most of the tensile strength to the skin, the degeneration of structural integrity of collagen fibers may underlay the pronounced fragility of elderly skin.

In addition, elastin comprises some 2% of the protein content of the dermis.[9] It is a fibrous structural protein that supplies the skin and other connective tissues with the capacity for elastic recoil. Elastin fibers are markedly disintegrated in the course of both actinic and intrinsic aging.[10,11] By the age of 70, most fibers show a decrease in number and diameter, and appear fragmented, especially in the dermo-epidermal region.[12] In addition, the fibers are reported to be distinctly "fuzzy" suggesting that the margins of the fibers are damaged.[7,11] As is the case with collagen, alterations in elastin are seen in both intrinsic and actinic aging, although the actinic changes are more severe.[13] It is thought that elastolysis plays an important role in not only the aging of skin, but also of the arteries, lungs, and other tissues.

Genetic perturbations in elastin biology are observed in cutis laxa, a family of metabolic defects in elastin maintenance. The syndrome is best known for the pronounced progeroid facies, with affected individuals looking many years older than their actual age, and the presence of aneurysms, emphysema, and other elastic disorders.[14-17] It is believed that the disease has at least three genotypes, an autosomal dominant, an autosomal recessive, and an X-linked dominant form. While generally considered a defect in elastin synthesis or degradation,[18] cells from the autosomal recessive form of the disorder have been reported to show a five-fold elevation in collagenase mRNA.[19] This highlights what is probably a degree of overlap in elastin and collagen maintenance pathways.

Since the aging of skin is characterized by degenerative changes in the dermis, the next question we might ask is what accounts for the altered behavior of the cells that are responsible for maintaining the ECM. A cell generically referred to as the connective tissue fibroblast is believed to have the primary responsibility of maintaining these proteins. We will now explore the regulation of fibroblast activity in conditions of stress, such as inflammation, wounding and aging.

### *Connective Tissue Maintenance*

The above description of the ECM in aged tissues speaks more to the steady-state status than to the specific mechanisms of the degeneration. The mere existence of damaged ECM could be attributed to deficiencies in either the synthetic or proteolytic pathways or some combination of the two. Indeed the ECM is normally maintained through a continuous process of turnover with a temporally-regulated synthesis of structural proteins as well as proteolytic enzymes. Therefore, to find the cause of the altered status of the dermis during aging, one needs to consider the dynamic role of the fibroblast in maintaining the dermis.

In order to study the function of fibroblasts, and in particular, how that function may be altered in aging, an additional parameter must be considered. Fibroblasts respond in a dynamic fashion to a variety of extracellular signals such as platelet-derived growth factors

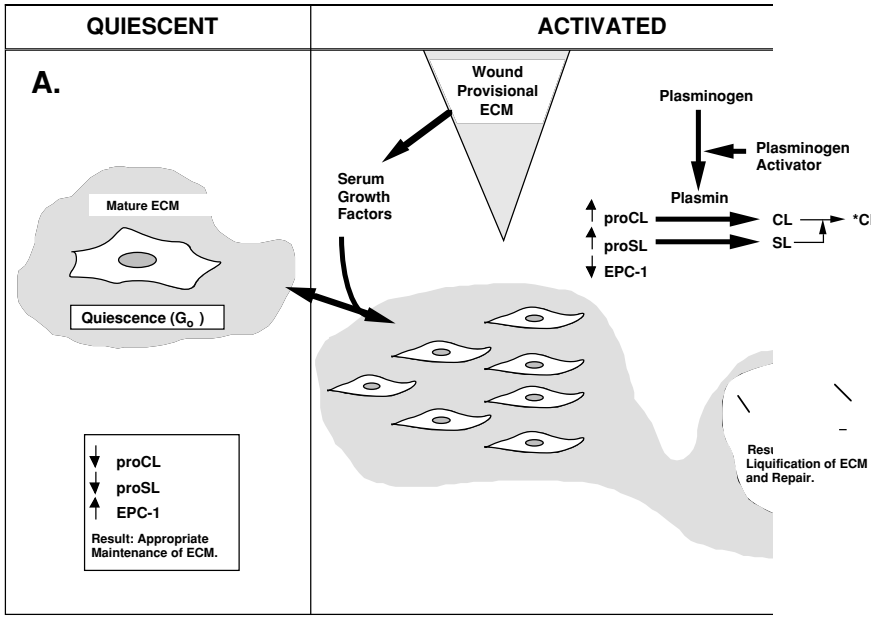


Fig. 14.1. Functional Dynamics of Young Fibroblasts. A.) Young proliferation—competent fibroblasts, may reside in a quiescent state characterized by a lack of cell division and by the secretion of proteins that maintain ECM. B.) In the presence of exogenous activators, for instance, those resulting from inflammation or wounding, the cells re-enter the cell cycle and up-regulate the production of proteins such as collagenase that transiently degrade the ECM to initiate tissue remodeling.

triggering a wound repair response, IL-1 an inflammatory response, IGF-1 a growth response, and so on. In the absence of such signals, the cells generally rarely divide and display a quiescent pattern of gene expression that facilitates the maintenance of the ECM. The fibroblast remodels the ECM through a clastic or destructive phenotype in which the cells destroy the extracellular proteins, and a blastic phenotype in which they synthesize new protein. A simplified characterization of this responsiveness is shown in Figure 14.1. The fibroblast in its normal maintenance state of "quiescence", proliferates at a low rate and produces relatively small quantities of proteolytic enzymes. In the presence of appropriate stimuli, the cells can re-enter the cell cycle, and increase the expression of proteolytic enzymes such as collagenase. This "activated" phenotype is typically a transient state such as postoperative day one in sutured incisions and day five of large defect full-thickness wounds and decreases back to quiescence thereafter.[20]

It is interesting to speculate why the up-regulation of genes like collagenase are nearly a universal characteristic of fibroblasts activated by a wide range of signals of tissue damage. Why would it be advantageous to degrade collagen in the case of a recent wound, or in the case of a local infection? One possibility is that many repair processes require the recruitment of accessory cells such as macrophages, to digest dead cells and tissues, cells that would normally be impeded in reaching the site in a rapid manner by the dense packing of the ECM components.

Matrix metalloproteinases (MMPs) play a critical role in the remodeling of connective tissues. Collagenase activity is the rate-limiting event in the degradation of collagen.[21] This

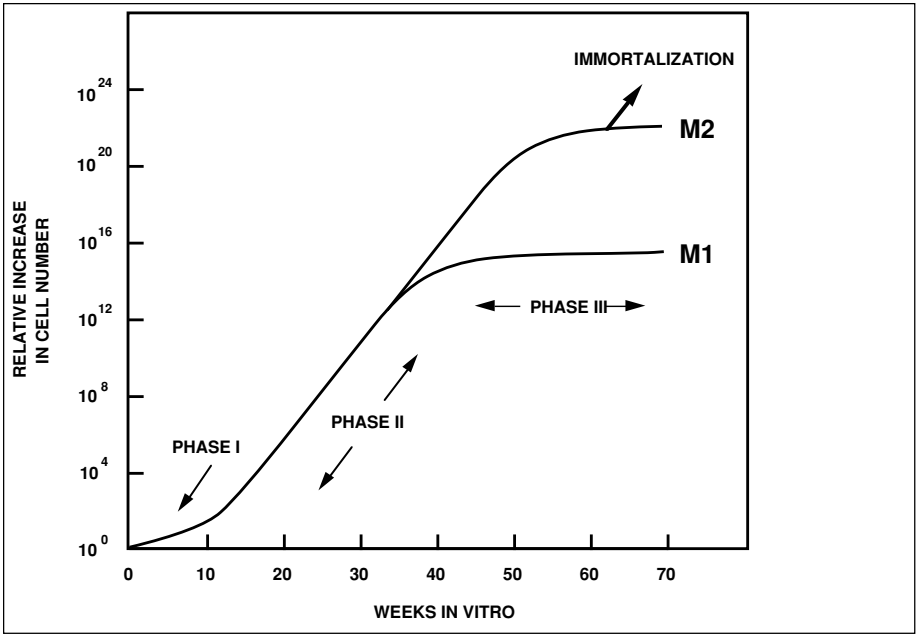


Fig. 14.2. Replicative Senescence In Vitro. The curve shows the replicative history of a culture of mortal cells. Phase I, represents the cells growing from the original tissue explant, a period of active growth commences—termed phase II. After about 50-100 doublings, cell proliferation ceases in what is called phase III or Mortality-1 [M1]). In the presence of viral oncoproteins, such as SV40-Tantigen, M1 may be bypassed which allows the cell to proliferate up to 40% longer to a later horizon designated Mortality-2 (M2). Rare clones rising from populations of M2 cells may then "immortalize". That is, attain a capacity for indefinite growth (arrow).

initial proteolytic event allows it to "unwind" and it is subsequently degraded by gelatinases. Collagenase is believed to play an important role in such diverse degenerative disorders as; arthritis, gingivitis, and bone resorption.[22] Other metalloproteinases such as stromelysins 1 and 2 target a broader array of proteins.[23,24] These targets include: fibronectin, proteoglycan core protein, the nonhelical regions of elastin, collagen types II, IV, and IX, laminin, procollagens I and III, and gelatin. Collagenase and stromelysin must be activated from their proenzyme precursors by other proteases, one being plasminogen activator.[25-27]

## The Molecular Biology of Cellular Aging

The field of cellular gerontology owes it's origin to the pioneering work of Leonard Hayflick who demonstrated that when cultured in vitro, human cells show a finite replicative capacity.[28-30] Subsequent studies demonstrated that fibroblasts from normal tissues replicate 50-100 population doublings depending upon the age of the donor.[31,32] As shown in Figure 14.2, cells taken from a donor and grown in vitro, enter a period of logarithmic growth. With time, they slow their rate of division and finally arrest their growth, despite the presence of growth factors normally adequate to stimulate cell division. The remarkable aspect of this phenomenon is its universality. All normal diploid human cells described to date are mortal.[33] The exception being malignant tumor cells that show an immortal phenotype. Early studies demonstrated that the clock of cellular aging is linked to a mitotic

clock rather than a metabolic clock, that is, cells age only when they are allowed to divide. Simple metabolism, or the passage of time did not advance cells along their life span. An important question, therefore, is how a cell can count and "remember" how many doublings it has undergone.

An important unifying principle that may explain many of these observations is the telomere hypothesis of cell aging and immortalization.[34,35] This model proposes that differentiated somatic cells such as fibroblasts lack the enzyme telomerase. As a result, each round of cell division results in a loss of DNA from the lagging strand of the 3' linear end, perhaps due to the end replication problem. This is in line with numerous reports that cultured mortal cells lose telomeric DNA at a rate of about 50-200bp per cell doubling.[36] When perhaps only a single chromosome end has lost repeat sequences, the cell may detect a linear DNA end that is indistinguishable from a double strand break. As a result, the cell may exit the cell cycle in a DNA damage checkpoint arrest, similar to that seen in cells exposed to ionizing radiation.[37] This arrest is dependent on p53 and is designated Mortality-1 (M1).[38]

In support of this model of M1 cell cycle arrest, viral oncoproteins, such as simian virus 40 large T-antigen that is believed to bind and inactivate p53 and RB, allow cells to replicate past M1 and continue to lose telomeric repeats for typically about a 40% extension of life span. The cells now arrest a second time in a horizon designated Mortality-2 (M2). This arrest is typified by a marked increase in dicentric chromosomes, consistent with a greater number of chromosomes lacking telomeres. Rarely, foci of cells that have reactivated telomerase appear, leading to clones of cells that are capable of immortal growth.

The correlation of telomerase expression with the immortal phenotype is striking.[39] Up to 98% of immortal tumor cell lines of many sources display stable telomere length and the presence of telomerase activity, and up to 85-90% of malignant tumors show telomerase activity. Normal cells and tissues generally lack telomerase activity. Nevertheless those cell lines lacking observable telomerase, show an extension of telomere length, suggesting that maintenance of telomere length is essential for immortalization, though the mechanisms of extension may vary.

The telomere hypothesis has aided both cancer and cell aging research in providing valuable markers of disease progression. Telomerase activity provides an important marker for the progression of malignancy. In addition, since telomere length predicts the replicative capacity of cells,[40] telomere length is a useful marker for the extent of replicative senescence in a tissue sample. Dermal fibroblasts from neonates have a typical TRF length of about 10 kbp, while the length when those cells reach senescence in vitro is in the range of 5.0-7.0 kbp. In a study of telomere length in samples of skin (dermis and epidermis) of individuals of various ages, TRF length was observed to decrease at a rate of about 20bp per year.[41] In a similar study of fibroblasts explanted from the dermis, the rate was calculated at about 15 bp loss per year (Allsopp et al, 1992). In striking contrast, explanted fibroblasts from donors with Hutchinson Gilford syndrome (Progeria) showed TRF lengths of about 5.5 kbp compared to age-matched controls of about 9.0 kbp.[40]

The above data would suggest that while there is a moderate degree of cell turnover in the dermis with age, there is no evidence of the majority of cells reaching replicative senescence. Even if a majority of cells were to reach replicative senescence in the latter decades of life, one could argue that the remaining "young" cells would possess the capacity to respond to trauma or other injury by entering the cell cycle. However, as we pointed out earlier, the senescent cells would not be expected to do so. Inasmuch as they are present in a wound bed, one would expect a blunting of the proliferation response compared to young tissue. Perhaps a more significant question is what is the pattern of gene expression in senescent cells, and are there any alterations that may explain age-related morpholysis even if only a minority of the cells in a tissue were senescent.

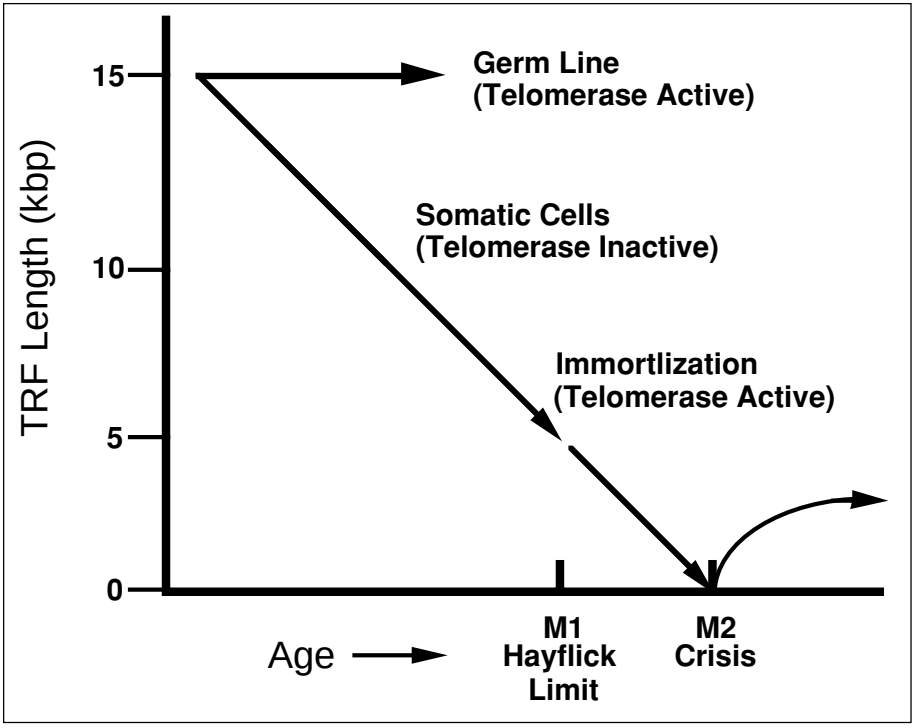


Fig. 14.3. The Telomere Hypothesis. Human germ line cells such as sperm display terminal restriction fragments (TRFs) averaging 15 kbp that are maintained despite rapid cell division. This is presumably due to abundant telomerase activity.. In contrast, somatic cells express little to no telomerase activity and the telomeric repeat sequences are progressively lost with cell division. By eliminating the gene products of the tumor suppressor genes p53 and RB, an extension of the replicative life span of cells occurs such that they now enter a second horizon of growth arrest designated Mortality-2 (M2).

### *The Role of Replicative Senescence in Skin Aging*

The dermal fibroblast is a discontinuously replicating cell. That is to say, it is normally nondividing (quiescent), and may divide as infrequently as on average every one to five years. However, in response to wounding or inflammation, the cells possess the capacity to rapidly re-enter the cell cycle and divide to replace damaged cells. Following the resolution of the repair response, the cells can again enter quiescence and begin a pattern of gene expression to maintain the ECM. In Figure 14.3 these states are designated as before, with the example of activated gene expression being collagenase, and quiescent-specific expression being EPC-1.[42]

An important alteration occurring in replicative senescence is also shown in Figure 14.3. Whereas young cells display the capacity to respond to extracellular signals in the dynamic fashion mentioned, senescent cells are unresponsive to growth factors not only in regard to entering the cell cycle, but also in regard to regulating extracellular gene expression. Indeed, the majority of genes altered in the course of cellular aging are ECM or extracellular proteolytic proteins.[43] Interestingly, the senescent cell is not locked in the quiescent state but the activated state. That is to say, the senescent cell is, for the most part, a constitutively

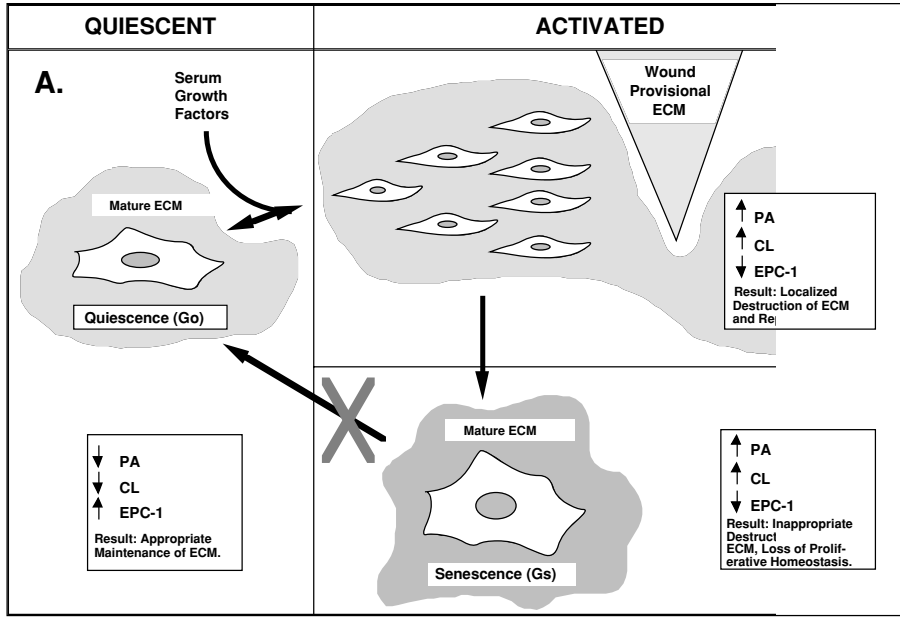


Fig. 14.4. Dynamics of Young and Senescent Fibroblast Function. A.) This panel illustrates a young fibroblast that has the capacity to enter quiescence (Go) in the absence of tissue damage and low growth factors concentrations. B.) This panel illustrates that young fibroblasts can be induced by serum growth factors or other stimuli to become activated. C.) This panel illustrates M1 activation. Senescent fibroblasts lose responsiveness to growth factors in terms of both cell division and ECM metabolism. Senescent fibroblasts also lose the capacity to become quiescent and instead are blocked in a cell cycle phase distinguishable from Go designated Gs. The progressive shift from Go to Gs during aging may result in an inappropriately degenerative activity toward mature ECM.

activated cell, at least in regard to most of extracellular gene expression. The phenotype has been designated M1 activation.[3]

In regard to synthetic pathways, the expression of collagen α1(I) mRNA decreases during the activation of skin fibroblasts and replicative senescence results in a constitutive decrease.[43-46] A similar decrease in collagen expression and an increase in collagenase has been seen in senescent rat skin in vitro and in vivo.[47] In regard to collagen synthesis during in vivo aging, there is evidence of a down-regulation of collagen synthesis during the progression from fetal to adult development. However, from 30-40 years of age and thereafter, there is no measurable decrease.[48]

In the context of pathogenesis, the proteolytic arm of the balance of anabolism and catabolism is often pivotal. In this regard, collagenase has been shown to be inappropriately overexpressed in senescent cells.[49,50] Because collagenase expression is altered with the growth state of the cell, it is critical to study its expression in the cells in the spectrum of growth conditions, from quiescence in which the cells reside in the $G_o$ phase of the cell cycle, through the various stages of re-entry into the cell cycle. Carefully performed experiments that control for serum activation effects reveal the interesting phenomenon that senescent cells differ largely in that they express genes such as collagenase more constitutively.[49] That is, the senescent cell doesn't just overexpress collagenase, it expresses it under conditions a young

cell would not, i.e. under conditions that normally induce quiescence. Since these are the conditions within which the fibroblast normally resides and maintains tissue, one could conclude that the senescent cell may be profoundly different from the young cell in its normal maintenance capacity. In addition to collagenase expression, fibroblasts secrete inhibitors of collagenase such as the tissue inhibitor of metalloproteinases I (TIMP-1). Interestingly, TIMP-1 expression is constitutively low in senescent cells[49,51] and decreased earlier than the increase in collagenase expression.[51] A TIMP-related protein designated mitogen-inducible gene 5 (mig-5) has also been observed to be down-regulated during cellular aging, though the role of this putative metalloproteinase inhibitor is less clear.[52] A related metalloproteinase (MMP-3) or stromelysin is also overexpressed by senescent cells.[51] Stromelysin overexpression would be predicted to have deleterious effects of its own, though since it is necessary for maximal activation of collagenase under physiological conditions,[53] it would be predicted to increase the activated collagenase to even higher levels.

In summary, senescent skin fibroblasts display a constitutively activated phenotype designated M1 activation, accompanied by an unbalanced overexpression of collagenase activity that, while appropriate under conditions of wounding or inflammation to dissolve the extracellular matrix for the recruitment of accessory cells, is inappropriate, especially when the condition is chronic rather than transitory. Another important implication from these observations is that the total increase in collagenase activity in the senescent cell compared to a young cell in conditions that induce quiescence can be as high as 50-fold. Therefore, it is possible that only a minority of senescent cells could, over a period of time, cause a dominant effect of "action at a distance", explaining how a tissue containing only a minority of senescent cells could nevertheless be profoundly affected.

Elastin is produced by skin fibroblasts.[54,55] The observed loss of elastin could be explained either by the decreased elastin expression seen in old cells[55] or by the down-regulation of protease inhibitors. These inhibitors include TIMP family members that inhibit proteases, such as stromelysin, that are in the elastin proteolytic pathway. In addition, there is a marked increase in both urokinase-type and tissue-type plasminogen activator and their associated inhibitors in senescent cells, with there being a large increase in the net plasminogen activator activity.[87] Plasminogen activator activates plasmin, which is a wide-spectrum protease capable of cleaving numerous substrates as well as activating other proteolytic enzymes. Therefore, while the exact pathway leading to elastolysis in aging skin is not known at present, it is reasonable to assume that alterations in the proteolytic pathway may have a dominantly deleterious effect on the architecture of the elastin network.

Studies of fibroblasts cultured from skin of donors of various ages suggests that these results obtained from cells aged in vitro may parallel those aged in vivo. and Jarisch et al, 1996 reported a significant increase in collagenase mRNA expression in response to IL-1β induction in cells from old (> 60 years) compared to young (< 20 years). In addition, Burke et al[55] reported that fibroblast cultures derived from old patients showed higher basal levels of collagenase expression, higher levels of inducibility with phorbol esters, and markedly higher levels of expression of a reporter gene driven by the collagenase promoter in transient transfection assays. Further support for a role of collagenase up-regulation in aging comes from the observation that fibroblasts taken from the skin of Werner Syndrome patients (a premature aging disorder) express senescent levels of collagenase expression soon after being placed in culture.[51]

The loss of replicative capacity with age, all by itself and apart from the ancillary effects of altered ECM gene expression, could account for some important changes in skin function, such as impaired or delayed wound repair.[57-60] A striking example of an age-dependent loss of replicative capacity of cultured fibroblasts correlating with delayed wound repair was reported in hamsters.[61] There are numerous facets of the senescent phenotype that

may contribute to impaired wound healing response. These include diminished proliferation and migration[62-64] and alterations in the synthetic and proteolytic pathways, as described above.

### *Transcriptional Mechanisms*

Genotoxic stress induces a unique pattern of gene expression that induces intracellular repair enzymes, cell cycle inhibition, and causes a parallel alteration of extracellular gene expression to aid in the removal and replacement of damaged cells. Interestingly, this is very similar to the pattern of gene expression seen in senescent cells. For instance, ionizing radiation induces a p53/p21-mediated cell cycle arrest and in parallel results in an increase in the expression of plasminogen activator and collagenase[65-67] (see chapter 7). Other DNA-damaging agents such as UV have been shown to induce MMP production as well (see chapter 7). Herrmann et al[68] reported that UVA exposure induced MMP1, 2, and 3 expression in cultured fibroblasts.

The altered gene expression is primarily at the level of transcription.[69,70] The signal transduction pathways that mediate this altered gene expression, however, are poorly understood at present. The fact that the mechanism of cell cycle arrest in M1 is believed to be mediated by DNA damage pathways recognizing telomeric DNA damage, makes it an attractive possibility that the altered ECM gene expression of senescent cells is regulated by similar pathways as well.

The transcriptional regulation of collagenase in response to serum growth factors is relatively well characterized.[71] This region includes the AP-1 element (-66 to -72) and the PEA3/ets element (-82 to -89)[72] (see chapter 4). A similar combination of AP1 and PEA3/ets elements has been shown to regulate other metalloproteinases, such as MMP-9(92 kDa Gelatinase B) expression by ras.[73]

The region of the collagenase promoter that drives collagenase expression in senescent cells has been called the "senescence-responsive element" (SnRE) and has been localized to the region of -34 to -100 and includes three known transcription factor binding sites: AP-1, CdxA, and PEA3/Ets. The transient transfection of various combinations of wild type and mutant AP-1, CdxA, and PEA3/ets elements suggests that eliminating the AP-1 site decreases senescent transcription by 90%, while eliminating the CdxA site decreases activity by approximately 80%, and finally, the removal of the PEA3/ets site decreases activity by about 40%. The evidence suggests that these elements may all play a role in senescent-specific collagenase expression, though other uncharacterized sequences may contribute as well.[74]

Interestingly, AP-1 binding is markedly down in senescent cells,[75] suggesting that there may be unique properties associated with the factors binding the AP-1 site in senescent cells. Indeed, while the serum-mediated induction of c-jun, c-myc, and c-ras expression are similar in young and old cells, c-fos expression is markedly diminished.[76] Since c-fos/c-jun heterodimer forms an important activator of the AP-1 element,[77] the loss of c-fos may play a role in the decreased binding but it does not clarify why the AP-1 element is critical for expression in senescent cells. Choi et al,[78] reported that senescent cells had higher basal levels of collagenase. However, in response to UV or methanesulfonate (MMS) exposure, c-fos was induced in senescent cells without a corresponding AP-1 binding or induction of collagenase. It is tempting to speculate that a modification of the c-fos/c-jun complex inhibits its binding in senescent cells, and that this modification inhibits c-fos induction in response to serum, but not UV or MMS. Since UR and MMS stress responses appear to be mediated by RacÆPAKÆJun kinase pathway[79] posttranscriptional modifications in c-jun may play an important role in transcription in senescent cells, though this remains to be demonstrated.

The fact that the pattern of gene expression in response to genotoxins closely resembles that of phorbol ester led to the investigation of the role of intracellular signal transducers, such as protein kinase C, in the regulation of nuclear events. In this regard, Hallahan et al[80] showed that the X-ray inducibility of EGR1 and jun occurred within 0.5-3 hours and involved the protein kinase C pathway. Other studies have shown that UV induces altered gene expression via membrane tyrosine kinases and the MAP kinase cascade.[81] Again however, convincing data on the status of these pathways during cell senescence are lacking.

Other proposed mediators of senescent cell gene expression are secreted lymphokines such as IL-1 and IFN-gamma. Both lymphokines have been shown to be upregulated during cellular aging.[44,82] Furthermore, both are observed to induce an activated pattern of gene expression, such as the induction of collagenase expression.[83,84] Furthermore, an elevation in IL-1x and β has been observed during aging in vivo. Perhaps even more significant are reports that antisense to IL-1x extends the proliferative life span of cells.[82] Despite these observations, a clear elucidation of the role of IL-1 or IFN-gamma in senescent cell gene expression is lacking.

### *Pharmaceutical Targets*

The pattern of gene expression in M1 activation, in particular the constitutive overexpression of collagenase, would suggest that agents that down-regulate collagenase gene expression or up-regulate TIMP-1 would have a beneficial effect on aging skin. In this regard, it is interesting to note that retinoids repress collagenase expression[83-86] and also increase the expression of TIMP-1 mRNA.[86] In addition, there is mounting evidence that they have a beneficial effect on aging skin.

If the aging of cells through actinic or intrinsic mechanisms shares the common features of DNA damage leading to checkpoint arrest of the cell cycle and M1 activated gene expression, then an intriguing approach at intervention would be a strategy to target central regulators of the signaling pathway with small molecules. One such possible target is the enzyme Poly (ADP-Ribose) Polymerase (PARP). PARP is believed to detect the presence of DNA damage and to modify numerous proteins to signal DNA damage checkpoint and DNA repair. Inhibitors of PARP have been shown to increase the replicative life span of cultured fibroblasts,[37] suggesting that such compounds may have utility to prevent the accumulation of cells in the state of M1 activation in aging skin or other tissues.

Final certainty as to the ideal pharmaceutical target for altering the deleterious pattern of gene expression observed in aging connective tissues awaits clarification as to the pivotal signal transducers of age-related genotoxicity. Telomere shortening may be one important example, but clearly actinic damage is important as well, and the pathways signaling UV damage may be quite distinct.

## Conclusions

The aging of connective tissues is characterized by a progressive degeneration of ECM components, such as type I collagen. The cause of this degeneration may be traced to alterations in the function of fibroblasts. While these cells may temporarily express an activated state accompanied by the induction of destructive genes like collagenase, these responses are generally transitory, functioning to dissolve the tightly packed ECM to allow accessory cells such as macrophages access to the site of injury to phagocytose dead cells and pathogens. With aging, a progressive increase in the percentage of fibroblasts with a critical loss of telomeric DNA may result in cells with a DNA damage checkpoint phenotype, in which the cells exit the cell cycle and constitutively overexpress activation-specific genes such as collagenase. Research into the signaling pathway and the transcriptional mechanisms regulating

this senescent-specific pattern of gene expression may yield important new insights into novel targets for pharmaceutical intervention in age-related disease.

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