The role of collagenolytic matrix metalloproteinases in the loss of articular cartilage in osteoarthritis

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TABLE OF CONTENTS

1. ABSTRACT

Digestion of cartilage collagen is a critical step in the loss of articular cartilage in osteoarthritis (OA). The hypothesis that matrix metalloproteinases (MMPs) are the primary enzymes involved in cartilage collagen digestion is supported by evidence that indicates that: 1) one or more MMP can digest the extracellular matrix components *in vitro*, 2) the enzymes are expressed in OA cartilage at the place and time of cartilage destruction, 3) specific digestion products of MMP are present in the OA cartilage, and 4) experimental strategies that alter the expression and/or the activity of MMP alter the progression of cartilage destruction in OA. These observations suggest that MMPs in general and collagenolytic MMPs in particular are promising targets for treatment of OA.

2. INTRODUCTION

Collagen digestion is a pivotal event in articular cartilage loss in osteoarthritis (OA). The collagen, which makes up approximately 70-80 % of the dry weight of cartilage extracellular matrix provides the structural framework of the cartilage, providing both tensile strength and form to the tissue, and once the integrity of the collagen meshwork is destroyed, restoration of articular cartilage structure rarely occurs. In OA, the digestion of extracellular matrix (ECM) is driven mainly by enzymes from the chondrocytes, although a role for enzymes from synoviocytes cannot be ruled out. Collagenolytic and gelatinolytic activity have been studied in OA cartilage from multiple animal models of early and late OA, and in late stage OA cartilage obtained at joint replacement. Most

Table 1. Collagens of the Articular Cartilage Fiber Meshwork

Type	Chain composition	Percentage of total collagen in articular cartilage	Reference
Type II	$\alpha1(II)_3$	~80% in immature cartilage, >90% in older cartilage	2,3
Type IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	~10% in immature cartilage, 2-3% in older cartilage	2, 6-9
Type XI	$\alpha1(XI)\alpha2(XI)\alpha3(XI)$	~10% in immature cartilage, 2-3% in older cartilage	2,5,8,9
Type V	$\alpha1(V)_2\,\alpha2(V)$	<1% in immature cartilage, 2-3% in older cartilage	2,8,9

evidence suggests collagen digestion is achieved through the action of matrix metalloproteinases (MMPs), a family of enzymes that contain zinc at the catalytic site and cleave components of the ECM at physiological pH and temperature.   In cartilage, the major MMPs involved in digestion of the extracellular matrix include the interstitial collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2 and MMP-9), stromelysin 1 (MMP-3), and membrane-type collagenases (MMP-14 and 16).

Four lines of evidence support a role for the MMPs in the loss of cartilage in OA. MMPs have the capability to digest the collagen components of the matrix, are expressed at the right place and at the right time to affect matrix digestion, and the appropriate digestive products of MMP are present in the tissues. Finally, procedures that alter MMP expression or activity also alter the progression of OA in several animal models.   The combined evidence suggests a major role for MMP activity in digestion of collagen in the ECM of cartilage in OA. Thus, inhibition of activity and expression of specific MMPs shows promise for therapeutic intervention in OA progression.

3. COLLAGENS OF CARTILAGE ECM

One target of the MMPs in OA is the collagen meshwork of articular cartilage.   Organization of the cartilage collagen into a "meshwork" is implied from the ability of the collagen component to retain proteoglycans within the matrix and to resist the significant osmotic pressure generated by these proteoglycans. However, the nature of the meshwork, including the contribution of direct links between collagen fibers to its integrity, is complex and not fully understood.   The meshwork may be dependent on entanglement of anchored fibers, direct covalent crosslinks between collagen molecules, or direct attachments mediated by additional molecular components (1).   Covalent crosslinks between collagen molecules within a fiber are well-characterized, and the potential of crosslinks between collagen molecules in different fibers is supported by studies of the crosslinked peptides involving type IX collagen [see (2) for a review].

Collagens are classified into three major groups, the fiber forming collagens, the aggregate forming collagens, and the fibril associated collagens with interrupted triple helices, or FACIT collagens (2,3). The major collagens expressed in cartilage are the fiber forming collagens, but FACIT collagens and aggregate forming collagens are also represented (Table 1). The fibers of collagen within cartilage are heterotypic, i.e., each fiber incorporates molecules of three or more different genetic types of collagen (4). In cartilage, fiber-forming collagens type II and type XI, and the prototype of the FACIT

collagens, type IX are covalently crosslinked within the fibers In young animals, roughly 80% of the cartilage matrix collagen is type II collagen, with roughly equal amounts of type IX and type XI accounting for most of the remaining matrix collagen (5). All three of these collagen types are present in fibers isolated from cartilage after mechanical disruption (6).

Type II collagen molecules $[\alpha1(II)_3]$ are composed of three identical peptide chains that form a triple helical molecule. Each of the α chains has a central region of ~1000 amino acid residues with a unique primary structure rich in proline and hydroxyproline residues and in which every third amino acid residue is glycine. These unusual features allow the chains to form a tight triple helix, resulting in a rigid, rod-like molecule.   The central portion of the native collagen molecule, the triple helix, is roughly 280 nm long and about 1.5 nm in diameter. The triple helical domain is resistant to digestion by most proteases, but can be digested by several members of the MMP family (see below). The amino- and carboxyl-termini contain short, non-helical telopeptide regions that are involved in cross-linking. As extensively studied in type I collagen, the type II collagen molecules form fibers by a process of self-assembly. The result is a fiber with the 64 nm banding pattern characteristic of collagen.

The FACIT collagen, type IX, is cross-linked to the type II molecules at the surface of the fibers and can be visualized by electron microscopy of mechanically isolated fibers (6). Type IX collagen consists of three different α chains, which form a molecule with four non-helical domains (NC1, NC2, NC3, and NC4) and three collagen domains (col 1, col 2, and col 3). Each of the type IX α chains, $\alpha1(IX)\alpha2(IX)$ and $\alpha3(IX)$, is a unique gene product. The molecule formed from these chains contains 3 collagen domains in the form of triple helices that are rigid and inflexible, but the full-length molecule is flexible and bends easily at the non-collagen domains. In one model, the col 1 and col 2 domains are arranged parallel to the axis of the cartilage fiber, with the col 1 domain folded back in the hole region of the type II collagen fiber (10). Crosslinks between type IX and type II occur between the amino terminal telopeptides of col 1 and type II molecule, and between the carboxy terminal telopeptides of col 2 and lysyl residue 980 within the triple helical domain of type II collagen.   The remaining triple helical domain, col 3, extends outward, away from the axis of the fiber. Two of the non-collagen domains of type IX have unusual features that are potentially important to cartilage matrix organization.   A glycosaminoglycan (GAG) chain is attached at NC3 of the $\alpha2(IX)$ chain (11). The GAG chain aligns with the repeat unit of the collagen fiber, and it appears to lie within the hole region of the type II collagen of the fiber. The other unusual feature is $\alpha1$ (IX) NC4, the

Table 2. Matrix metalloproteinases in OA cartilage

MMP	Common Name	Potential Substrate(s) in Cartilage ECM	Expression in Cartilage	References
MMP-1	Collagenase 1, Interstitial collagenase	Type II collagen	Expressed in normal, early OA, late OA cartilage	18-21
MMP-8	Collagenase 2, Neutrophil collagenase	Type II collagen	Expressed in late OA, but not in all cases.	18-20
MMP-13	Collagenase 3	Type II collagen, gelatin, telopeptides	Strongly expressed in early, moderate and late OA	16-21
MMP-2	Gelatinase A	Type XI , V collagens, gelatins, telopeptides, type II collagen	Expressed in normal cartilage, strongly upregulated in OA	19, 20
MMP-3	Stromelysin 1	Type IX collagen, Aggrecan, telopeptides, proMMP-1	Highly expressed in normal cartilage and early OA. Downregulated in late OA	19, 20
MMP-9	Gelatinase B	Type XI and V, gelatins, telopeptides	Not expressed in normal cartilage. Expressed in moderate and late OA.	19, 20
MMP-14	Membrane type MMP-1	Type II collagen, gelatin, proMMP-2, -13	Expressed at high levels in normal, early and late OA cartilage.	19, 20, 22
MMP-16	Membrane type MMP-3	proMMP-2	Expressed at high levels in late OA cartilage	20

globular domain at the carboxyl terminus of type IX. This globular peptide is very rich in basic amino acid residues, resulting in an isoionic pH higher than 10 and a very strong positive charge at neutral pH (7). As indicated above, type IX bends at NC3, and Col 3 and NC4 extend outward, away from the axis of the fiber. This brings NC4 into proximity with the negatively charged PG of the matrix, offering the potential for strong electrostatic binding between the matrix collagen and the PG.

Type XI collagen is a fiber-forming collagen that is usually found in association with type II collagen. The component α chains are α1(XI), α2(XI), and α3(XI). The α1 and α2 chains share many properties with the α1(V) and α2(V), while α3(XI) is identical to α1(II) (12). In fetal calf cartilage, type XI molecules have the chain composition α1(XI)α2(XI)α3(XI) (5). The location of type XI in the collagen fibers is less certain. Some evidence suggests that type XI resides in the core of the fiber. Antibodies against type XI do not stain most intact fibers, but do stain the disrupted ends of fibers exposed during mechanical disruption (13).

Several additional collagen types have been demonstrated in articular cartilage from various mammals. Type III collagen has been reported in the fibers of human articular cartilage, where it apparently participates in formation of the heterotypic collagen fiber with type II.

In older animals, type V collagen accumulates in cartilage, and the type XI collagen fraction is somewhat reduced (2). In older dogs, type V and type XI are present in articular cartilage in roughly equal amounts (Smith and Mickler, unpublished results). Although these collagens are very similar in chemical properties, it is not clear that they perform the same function in the cartilage matrix. Another prominent collagen component in normal and OA cartilage is type VI collagen, which is localized in the pericellular matrix of chondrocytes. Type VI is an aggregate-forming collagen, and does not appear to have a structural role in the collagen meshwork of cartilage extracellular matrix. Type VI contains large non-helical domains at both ends of a short, helical portion. Although it is actively synthesized in OA cartilage (14,15), it does not form fibers. Type VI collagen is not digested by MMP -1, -2, -3, or -9, but is digested by serine proteases (16). Several additional collagens have been identified in various

studies of cartilage structure and development, but will not be considered in this review because the evidence for their function in maintenance of the collagen meshwork of articular cartilage is missing or scanty.

4. THE COLLAGENOLYTIC MMPS

The MMP are a family of enzymes that digest components of the extracellular matrix in the extracellular environment. The MMPs contain zinc at the catalytic site, and require calcium for maintenance of structure and activity. Most secreted MMP have three domains, a prodomain, responsible for maintaining the proenzymes in the latent state, the catalytic domain, which contains the active site responsible for hydrolysis of substrates, and a large hemopexin-like domain, which apparently determines the substrate specificity of the enzyme (17). Of more than 20 family members that exhibit unique substrate specificities and catalytic properties, at least 10 specific MMPs are potentially important in the digestion of articular cartilage collagen in OA (Table 2). The list is not exhaustive, and one or more of the newly described MMPs may emerge in the future as important catalytic agents in OA. In addition, several members of the growing ADAM (A Disintegrin and Metalloproteinase domain) family are clearly important in cartilage metabolism and catabolism, and are expressed in OA. The most extensively characterized ADAMs in cartilage are the aggrecanases and the procollagen peptidases. These and other enzymes must be considered future studies in destruction of the ECM, particularly in studies on cleavage of non-helical domains of collagen. Despite these caveats, 1 or more of the MMPs in Table 2 has the capability to digest the entire collagen array of the extracellular matrix of cartilage, and the preponderance of evidence suggests that the MMPs are primary agents involved in cartilage damage in OA.

Four sub-families of MMP may have roles in the catalytic events in OA cartilage. The members of the first group, the "true collagenases" (MMP-1, MMP-8, and MMP-13), cleave native types I, II, and III collagen at a single site within the triple helical domain of the collagen molecule, producing characteristic ¾-¼ digestion products called TC[A] and TC[B]. In their native configuration, the major fiber-forming collagens--types I, II, and III--are not susceptible to digestion by most proteolytic enzymes, but

can be digested by the collagenases, MMP-1, -8, and -13 (17,23). Each of these enzymes cleaves the triple helical collagen molecule at a single locus. Cleavage of one chain is followed immediately by cleavage of the other two chains at the same site. The major collagen of cartilage, type II collagen $[\alpha 1(II)]_3$, is cleaved between residues gly 775 and leu 776, yielding TC ^{A} and TC ^{B}, the ¾ and ¼ length helical fragments. The fragments are diagnostic indicators of collagenase activity. Once the helix is cleaved by collagenase, the resultant fragments have lower melting points than the full-length molecule, and denature at physiological pH and temperature. After they are denatured, the component chains of collagen (gelatin) are susceptible to digestion by a variety of enzymes, including the gelatinases, MMP-2 and MMP-9.

The second group includes 2 members (MMP-2 and MMP-9) which exhibit extremely strong activity against gelatins from all collagen types, but also cleave native type IV collagen, the primary collagen of basement membranes; type V collagen, a collagen associated with type I collagen in a variety of connective tissues; type XI collagen, a type V-like component of cartilage collagen fibers; and several non-collagenous substrates, including laminin (24), elastin (25), and FAS ligand (26). Recently MMP-2 has been shown to function as a "true collagenase", cleaving soluble type I and type II collagens at the primary collagen cleavage site, as well reconstituted fibers of type I or II (27,28). We have shown that MMP-2 is able to cleave type II collagen within native fibers isolated mechanically from cartilage, but MMP-13 cleaves this substrate at a higher rate than MMP-2 (Smith and Mickler, unpublished).

The third subfamily of MMPs of potential importance for the catabolism of cartilage matrix collagen is the membrane-type MMP subfamily. There are 6 family members, MMP-14, -15, -16, -17,-24, and -25 (17, 29). These MMP contain a basic prohormone convertase cleavage sequence within the prodomain. This sequence is thought to be cleaved within the cell by the furin family of enzymes, so that these MMP are secreted in the active form. Four of the membrane-type MMPs (MMP-14, -15.-16, and -24) have a hydrophobic membrane insertion domain and a cytoplasmic domain at the COOH-terminus (on the carboxyl side of the hemopexin-like domain). The other 2 membrane-associated MMP are retained at the cell membrane by a glycosylphosphatidal-inositol anchor, and lack a cytoplasmic tail (MMP-17 and -25). MMP-14 cleaves native collagen types I, II, and III and is able to activate MMP-2 and MMP-13. None of the other membrane-type MMPs has been shown to digest native type II collagen (17). The role of the cytoplasmic tail of the membrane-type MMPs is apparently involved in dimerization of the enzymes, which may be important in the cell surface activation of MMP-2 (30).

The final group of MMPs, the stromelysins, include 3 members, Stromelysin 1 (MMP-3), Stromelysin 2 (MMP-10), and Stromelysin 3 (MMP-11), with the ability to cleave a wide variety of ECM and non-ECM substrates. MMP-3 is strongly expressed at the protein and RNA level in normal cartilage and in early OA. It can cleave a number of ECM components, including aggrecan, but its major activities may be its role in activation of other MMP, in cleaving telopeptides of type II and other collagens, and in cleaving type IX collagen at non-helical sites (17).

5. CONTROL OF MMP ACTIVITY

MMP activity within the tissues is controlled at multiple levels within the OA cartilage. First, the amount of latent proenzyme expressed is controlled at the level of transcription by cytokines and other mediators. Then, enzyme activity is initiated by activation of the latent enzymes, and finally, the active enzyme is subject to inhibition by endogenous inhibitors. Each of these mechanisms has a role in the control of MMP activity during the progression of OA.

5.1. Control of MMP expression

Expression of MMPs varies widely in response to a number of extracellular stimuli. Most of the MMPs are strongly expressed by connective tissue cells *in vitro* in response to inflammatory mediators such as IL-1 and TNFa (31-33). Other agents that modify expression include the retinoids and glucocorticoids which repress MMP transcription (34, 35), and growth factors such as TGF-ß which can either repress or promote MMP expression depending on cell type and physiological state (36, 37). In addition to these factors, it is clearly established that altered mechanical stress strongly affects MMP expression in chondrocytes and other connective tissue cells (38). Mechanical stimuli can act through altered cytokine expression (39, 40) but also alter the tissue response to cytokines (41).

The signaling mechanisms that control these changes in expression are complex, interrelated, and to some extent, cell specific (for reviews see (32, 42, 43)). They involve multiple phosphorylation-dependent signaling pathways that regulate gene expression including the mitogen activated protein kinase (MAPK) pathways. The MAPK family includes the cJun N-terminal kinases (JNKs), the extracellular signal-regulated kinases (ERKs), and the p38 kinases, and each of three groups of MAP kinases appear to be involved in expression of one or more MMPs (44). In addition, nuclear factor ?B activation is necessary for increased expression of MMP-1, -3, - 9, and -13 (45).

Studies of inducible MMPs (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13)have implicated a conserved AP-1 site approximately -70 bp upstream of the transcription initiation site as essential for basal expression (42). Other cis acting elements including one or more PEA-3 sites, AP-2 sites, and additional AP-1 sites have been identified farther upstream and have been shown to contribute to control of MMP expression (44, 45). In contrast, the promoters for MMP-2 and MMP-14 lack AP-1 sites, and presumably these MMP are regulated by different pathways (46). It is important to note that while MMP-2 is constitutively expressed in normal cartilage, its expression (20) and activity are significantly increased in OA cartilage (47) .

5.2. Endogenous inhibitors of MMPs

A family of four tissue inhibitors of metalloproteinases (TIMPs) has been identified in multiple tissues of mammals (17, 48). The TIMPs inhibit MMP activity in a 1:1 stoichiometry, binding at the catalytic site of most of the active MMP. All four TIMPs can inhibit all MMP, but, TIMP-1 is a poor inhibitor of the membrane-type MMPs, including MMP-14 (48). In addition to binding to the catalytic site in active MMPs, TIMPs 2 and 4 bind to the hemopexin-like domain of proMMP-2, TIMP 1 binds to proMMP-9 and TIMP 3 binds to both MMP-2 and -9. The biological consequences of these associations have not been fully examined, but the binding of TIMP-2 to proMMP-2 is involved in the cell surface activation of this MMP (see below).

RECK (reversion-inducing cysteine-rich protein containing Kazal domains), a glycoprotein inhibitor of activity and/or expression of MMP-9, MMP-14, and of MMP-2 activation (49) is expressed in multiple tissues including the synovium (50). RECK expression is significantly down regulated in RA synovium, but is widely variable in OA synovium. To date, there are no data concerning the expression of RECK by chondrocytes from OA cartilage. Similarly, SPOCK (sparc/osteonectin, CWCV and kazal-like domains proteoglycan precursor) or testican-1 has been shown to inhibit the activation of MMP-2 by MMP-14 and -16 (51). Although this inhibitor was initially described from testicular tissue and has been associated with the adult brain and the developing nervous system, it is also expressed in mouse and human OA cartilage(52, 53).

5.3. Activation of latent MMPS

The secreted MMPs are released as latent proenzymes that require activation in the extracellular space, providing an additional control level for expression of MMP activity. Activation of the latent MMPs can be achieved by physiological agents such as various proteases and reactive oxygen species, or by non-physiological agents such as organomercurials and SDS. These disparate mechanisms have been resolved by the observation that the secreted proMMP molecules are maintained in a latent state by the interaction of a highly conserved, unpaired cysteine residue in the prodomain with the catalytic zinc at the active site in the catalytic domain. The resulting "cysteine switch" allows the latent proenzymes to be activated by any agent that disrupts the cysteine-zinc association (54).

Activation *in vivo* generally results from cleavage and loss of the prodomain, and presumably, in most cases, occurs through the action of proteases. Proteases proposed for activation of various MMPs include the plasminogen activator/plasmin system(55-57), thrombin (58), and other MMP, especially MMP-3 (stromelysin) and the membrane type proteinases(59, 60). Alternatively, two pathways involving reactive oxygen species have been described (61, 62). Studies of human and bovine cartilage in explant culture indicate that activation of proMMPs is a rate limiting step in cartilage collagen digestion, and implicate plasmin as a significant part of the activation pathway (63). Cartilage samples incubated with IL1a/oncostatin M accumulate significant quantities of procollagenase in the tissues well before the onset of collagen digestion. The endogenous procollagenases can be activated by addition of APMA or active MMP-3. Addition of plasminogen to the cultures initiated early collagen release from the tissue. The release was blocked by serine protease inhibitors (63).

Many MMPs can be activated *in vitro* by other MMPs. Stromelysin-1 (MMP-3) activates proMMP-1, -13, -8, and others. The membrane-type MMPs activate several secreted proMMPs. Activation of proMMP-2 is the prototype mechanism of an activation pathway that involves MMP-14 and TIMP-2. MMP-2 has an important role in migration of many tumor cells, due to its ability to digest type IV collagen and laminin, two important components of basement membranes (24, 58). MMP-2 activation in a number of cells, including the highly invasive tumor cells, occurs at the cell surface, and putatively involves a ternary complex of TIMP-2, MMP-14 (membrane type 1- MMP), and proMMP-2 (58, 64). In the proposed mechanism, activation of latent MMP-2 at the cell surface is initiated by binding between a site on the hemopexin-like carboxyterminal domain of proMMP-2 and a site on the carboxyterminal domain of TIMP-2. In the resultant complex, the inhibitor domain of TIMP-2, at the amino terminal end of the molecule, is exposed and able to inhibit active MMPs by binding to the exposed catalytic site. MMP-14 is activated within the cell during transport to the cell surface, where it is inserted in the membrane with the active catalytic domain exposed. The TIMP-2 binds to an active MMP-14, at the cell surface to form a complex containing latent MMP-2, inhibited MMP-14, and TIMP 2. An adjacent "free" active MMP-14 then cleaves the proMMP-2 to yield active enzyme (58, 64). In the presence of excess TIMP 2, the MMP-2 is inhibited, so the ratio of MMP-2 to TIMP 2 within the tissue is critical to the action of this enzyme.

Although the above mechanism of activation for cell surface proMMP-2 is supported in many studies, other mechanisms may exist within the connective tissues. Both thrombin and activated protein C can activate MMP-2 directly in microvascular and human umbilical vein endothelial cells. This activation occurs very quickly (within 2 hours for thrombin, vs. 24 hours for phorbol myristic acetate activation) and does not require MMP-14 or generate the intermediate 62 kDa form seen with MMP-14 (65). Other enzymes have also been reported to activate MMP-2, including MMP-13.

ProMMP-13 is activated by membrane preparations containing MMP-14, but TIMP-2 is not required for activation of this MMP as it is for activation of proMMP-2 (66). Excess TIMP 2 and TIMP 3 block activation, but TIMP1 allows partial activation (to intermediate, partially activated forms) (66- 67).

6. EVIDENCE THAT MMPS DIGEST COLLAGEN IN OA CARTILAGE

There are 4 lines of evidence that suggest that MMPs in general, and the collagenolytic MMPs in

particular, have a major role in digestion of cartilage collagen in OA. They are: 1) One or more MMPs can digest all of the cartilage collagens *in vitro*; 2) The MMPs are expressed in the correct tissue location and time to affect collagen digestion during the pathogenesis of OA; 3) Products of collagen digestion are present in the OA tissue; and 4) procedures that alter the expression and/or activity of MMPs also alter the progression of OA.

6.1 . MMPS can digest cartilage collagen

The first line of evidence is the demonstration *in vitro* that the MMPs have the capability to digest the major collagenous components of the cartilage extracellular matrix. As described above, in Section 4, the collagenases (MMP-1, MMP-8, and MMP-13) are able to cleave the native collagen molecule, in solution or as reconstituted fibrils.

Recent studies indicate that 2 additional MMP family members can cleave collagen types I, II, and III at the classical collagenase cleavage site. MMP-14, the membrane-type MMP implicated in the cell surface activation of MMP-2 also cleaves a wide spectrum of substrates including collagen types I, II, and III. In addition, recent studies indicate that gelatinase A (MMP-2), cleaves native collagens at the collagenase cleavage site (27-28). The relative activity of the secreted MMPs against type II molecules in solution is MMP-13>>MMP-1>MMP-8=MMP-2(28, 68-69). When the expression data (see below) is combined with the relative efficiency data, MMP-13 appears to be the most likely enzyme to affect collagen digestion in OA.

Minor collagens of the matrix are resistant to digestion by the collagenases, but susceptible to digestion by other MMPs. Type XI and type V collagen resist digestion by the "true collagenases" but are digested by the gelatinases MMP-2 and MMP-9 (47, 70-73). Extracts of OA cartilage have high levels of activity against type XI collagen which can be inhibited by EDTA and 1,10 phenanthroline and is removed from the extract by affinity chromatography on gelatin-sepharose. However, evidence that these activities result in cleavage of type XI or type V collagen within the cartilage is lacking. Native type XI and type V are cleaved into multiple smaller peptides by the gelatinases, making the identification of cleavage products more difficult. In addition, it is not clear that these collagens are exposed for digestion in the cartilage, as they may be sequestered within the heterotypic fibers of articular cartilage.

Digestion of cartilage collagen also involves non-helical domains within collagen molecules that are extremely important to matrix integrity. Examples include the interruptions of the helix in FACIT collagens, and the telopeptide regions of fiber-forming collagens that are commonly involved in intra- and intermolecular covalent crosslinks. These sites are potentially targets for the stromelysin 1 (MMP-3), as has been demonstrated for cleavage of type IX collagen in the NC2 domain (74). However, non MMPs including the cathepsins, serine proteases, and ADAM-TS family are also candidate

enzymes. Several ADAMs are expressed in cartilage, included pro-collagen peptidases and aggrecanase(s) (19-20).

The digestion of non-helical regions of type IX is likely of paramount importance in OA. Amongst the earliest changes in the canine anterior cruciate transection model of OA is an increase in water content of the articular cartilage. This is presumed to be a consequence of "loosening" the collagen meshwork, possibly through the cleavage of telopeptide crosslinking regions of type II and type IX collagen. The primary cleavage site in type IX collagen is in NC2, the non helical domain between Col2 and Col3, is cleaved by stromelysin (8, 74). Another cleavage site has been identified in type IX collagen in or near NC3 which releases the NC3-Col4-NC4 fragment from developing chick sternum (75). The enzyme responsible for this cleavage has not been identified. Cleavage of collagen type IX or type II at these cleavage sites, as well as cleavage of type II at the classical cleavage site would be expected to disrupt the collagen network. The crucial crosslink between NC2 and residue 930 in the type II triple helix would be affected by cleavage of either type II or type IX.

6.2. MMPS are expressed in articular cartilage in OA

The second line of evidence for a role of MMP in OA progression is the demonstration that the MMP are produced by the tissue in the right place and the right time. From the early 70s many laboratories working in model systems and with human OA cartilage obtained at joint replacement surgery have demonstrated that OA cartilage contains elevated levels of activity against native collagen, and that the collagenolytic activity is blocked by agents that inhibit MMPs (EDTA or 1,10 phenanthroline), but not by inhibitors of other proteases (47, 55, 76-79). Many of these studies, and a number of additional studies from many laboratories have been performed to evaluate pharmaceutical agents designed to delay the destruction of articular cartilage in OA by targeting MMP activity. Research to identify the appropriate target MMP(s) expressed in OA cartilage was intensified by the identification of collagenase 3 (MMP-13) (Table 3) (80) and the demonstration that this MMP is produced by chondrocytes from OA cartilage and that it cleaves type II collagen at a significantly higher rate than MMP-1 (68, 81).

With the recognition that membrane-type MMPs with the ability to digest a wide spectrum of extracellular substrates are expressed in connective tissues including cartilage, and the observation that MMP-2 also possesses "true collagenase" activity, it has become increasingly important to identify and quantify the specific enzymes produced in the tissues.

Identification of the array of MMPs expressed within OA cartilage has been achieved at the mRNA and the protein expression level. At the RNA level MMP-13, MMP-1, and MMP-8 are each expressed in samples of human OA cartilage (18). Recently, quantitative PCR techniques have been utilized in several laboratories to evaluate the expression of multiple MMPs and TIMPs in

Table 3. Critical Studies of Expression of Collagenase in OA

Critical observations of MMP expression in osteoarthritic cartilage	Publication Date	References
Hydroxyproline released from OA cartilage by an unidentified MMP	1977-78	76, 77
Collagenase activity and digestion products measured in cartilage extracts from human (84) and canine OA cartilage	1983	84, 85
Collagenase mRNA quantified in cartilage and chondrocytes	1989-1992	86, 87
MMP-8 (Collagenase 2) cloned from neutrophils	1990	88
MMP-13 (Collagenase 3) cloned from breast carcinoma	1994	80
MMP-13 expressed in OA cartilage	1996	68, 81
MMP-8 expressed in OA cartilage	1996	89
MMP-1, MMP-8, MMP-13 expressed in cartilage lesions in human OA	1997	18
MMP-14 expressed in OA cartilage	1997	90
Differential expression profile of 5000 ESTs in OA and normal human cartilage reveals high levels of MMP-3 expression in OA cartilage	2001	91
Relative MMP expression profile of most collagenolytic MMP characterized in human OA and in cartilage from the STR/ort mouse	2002	19, 82
Complete relative expression profile of known MMPs and ADAMTSs characterized in late stage human OA cartilage	2004	20

human articular cartilage (19-20) and in several animal models (82). The consensus of multiple studies in these systems is that MMP-13 is the primary collagenolytic enzyme, but that MMP-1 is also expressed. In the canine anterior cruciate ligament transection model of OA, MMP-2 is strongly expressed (83). It is present in synovial fluid of the unstable knee from the first day after surgery, and remains high for up to 8 months (Smith and Mickler, unpublished). In addition, it is strongly expressed in articular cartilage from dogs with experimental OA and patients with late stage OA (73, 83). MMP-9 expression is elevated in OA cartilage as well (38), but is not uniformly present—there is little in early OA, and in some dogs none is detectable at 3 months or 8 months after surgery.

6.3. MMP digestion products are present in OA cartilage

The third line of evidence supporting a role for MMPs in digestion of collagen in cartilage matrix is the identification of specific digestion products of collagen within the tissue. As indicated above, the working hypothesis for digestion of collagen in the tissue is that the collagen fragments, TC^A and TC^B, generated by collagenase cleavage denature at normal body temperature, and then are digested by gelatinases and other proteases. In fact, these fragments do not survive in the circulation in homoeotherms, and an assay temperature from 7-20° below 37°C is generally used to demonstrate the TC^A and TC^B fragments considered diagnostic of collagenolytic activity in culture medium or tissue extracts. However, studies utilizing 2 newly devised approaches have demonstrated that articular cartilage retains at least a portion of collagenase-cleaved type II collagen. The primary assay tools are antibodies raised against peptide epitopes that are sequestered within the triple helix in native collagen, but exposed after cleavage of the molecule at the classical collagenase cleavage site (92-94). Immunohistological studies utilizing the neoepitope antibody raised against the carboxyterminus of a1(II) TC^A reveals a significant amount of cleaved type II collagen that is retained in OA cartilage (95-96). In addition, the cleaved collagen can be quantified by digestion of cartilage with a-chymotrypsin, which digests denatured collagen but not native, triple helical collagen. The cleaved collagen in the extract is quantified by an ELISA assay directed against collagenase generated epitopes.

Neoepitopes generated by collagenase have been identified in samples from cartilage in a variety of animal models of OA as well as from patients with joint disease. One study of human articular cartilage samples obtained at knee arthroscopy, autopsy, or joint replacement surgery indicates that normal cartilage contains from 2-4% cleaved collagen. The values are considerably higher in patients with anterior cruciate ligament (ACL) rupture (~12 %) and in patients with late stage OA (~14 %) (97). Similar studies in Taft-Hartly guinea pigs, which develop spontaneous OA with age, reveal significant levels of these collagenase-specific epitopes in OA cartilage (98). In the guinea pig cartilage, the collagenase digestion products co-localize with MMP-13 and/or MMP-1. The values for canine experimental OA are also similar (99).

Confirming evidence that one or more MMPs digest type II collagen in OA cartilage was obtained using *in situ* zymography of human OA cartilage. In these studies, FITC- labeled type II collagen was applied to microscope slides and frozen sections of OA cartilage were mounted over the film (100). The slides were incubated at 37°C and were observed after 24 and 48 hours. Areas of lysis developed over chondrocytes at lesion areas within the cartilage. Lysis of the collagen film was completely blocked by addition of EDTA to the reaction buffer, indicating that the activity was due to MMPs. The pattern of digestion matched the pattern of MMP-13 expression determined by *in situ* hybridization. Unfortunately, no specific inhibitor of MMP-13 activity was used in these studies and the site of expression of other MMPs was not determined, so the investigators could not confirm that collagenolysis was due solely to MMP-13 activity.

6.4. Changing MMP activity changes OA progression

The fourth line of evidence supporting a role for MMPs in cartilage matrix digestion is derived from studies of genetic or pharmaceutical modification of MMP expression or activity. The pharmaceutical studies have been used to test agents designed to modify the progression of OA by reducing MMP activity or expression, while the genetic studies have been used to identify therapeutic targets for drugs or for possible gene therapy strategies. Agents that have been proposed for use in OA therapy have been tested in animal models or in cultured OA cartilage obtained at the time of joint replacement surgery. A number of these agents affect the expression or activity of one or more MMPs, and also affect the progression of OA. Whether the change in MMP expression and activity is the cause or the result of the change in progression is

problematic—clearly any thing that alters the progression of OA will alter stage-specific enzyme activity as well. However, several studies suggest that changes in MMP activity and expression directly cause changes in the progression of OA pathology. Three approaches are commonly attempted—genetic manipulation of MMP and/or TIMP expression using knockouts or transgenes, inhibition of MMP activity by exogenous inhibitors, or inhibition of expression of MMP through manipulation of cytokines or signaling pathways.

6.4.1. Genetic alteration of MMP expression

Mice expressing an inducible transgene for a spontaneously active form of human MMP-13 were generated to demonstrate that MMP-13 was a potential causative agent in OA progression (101). The mice had 2 genes inserted. One inserted gene, the tetracycline-regulated transcriptional activator (tTA), was under the control of the type II collagen promoter so that it was expressed only in cartilage. The second gene, under the control of the tet07 promoter, coded for a mutant MMP-13 protein with a proline for valine substitution in the prodomain, which prevented the formation of a latent proMMP. The mutant MMP-13 is spontaneously active. In the absence of doxycycline, the tTA gene-product binds to the tet07 promoter, inducing expression of the MMP-13 gene. Doxycycline prevents this interaction, so that the mutant MMP-13 gene is turned off in the presence of the drug. This combination of transgenes allowed the development and growth of the transgenic mice to occur normally in the presence of doxycycline. When the doxycycline was withdrawn, the mutant MMP-13 gene was expressed in cartilage as a spontaneously active MMP-13 When it was activated in adult mice, the cartilage synthesized an excess of active MMP-13 which resulted in clearly demonstrable collagen cleavage that was detected by immunohistochemistry, using a neo-epitope antibody. In addition to collagen cleavage, the cartilage exhibited significant changes of OA, including loss of safranin O staining, which indicates loss of proteoglycan, and the development of cartilage surface damage and fissures extending to the bone. The cartilage from those mice expressing MMP-13 also expressed type X collagen throughout the matrix, an observation consistent with the development of OA.

Knockout mice have been developed for a number of catalytic factors, but these deletions have resulted in non-intuitive, unexpected phenotypic effects. In one notable study, 4 knockouts that targeted well documented catabolic agents—interleukin-1 ß (IL-1ß), stromelysin 1 (MMP-3), inducible nitric oxide synthase (iNOS), and IL-1 ß converting enzyme (ICE)—and wild type mice were subjected to medial collateral ligament transection and partial medial meniscectomy (102). OA developed in all groups, but lesions occurred earlier and were more severe in the mice lacking any one of the known catabolic agents. Mice lacking ICE, iNOS, or IL-1ß developed OA in the contralateral knee. This study suggests that these agents are important in normal cartilage physiology, and indicate the utility of conditional gene deletions (as described in the previous paragraph for the MMP-13 insertion) for examining catabolic factors thought to be important in development of OA.

6.4.2. Pharmacologic inhibition of MMP activity.

Studies directed at inhibition of MMPs by pharmaceutical agents include studies of agents that inhibit MMP activity and agents that inhibit MMP expression. Direct inhibition of MMP activity has been tested with broad-spectrum inhibitors and with specific inhibitors. Broad spectrum inhibitors such as Batimastat and Marimastat have been tested in oncology, but were found to cause stiffness and pain in joints (103). Long term administration of Marimastat causes a number of musculo-skeletal problems in rats, suggesting that future studies of MMP inhibition will require more selective MMP inhibitors (104).

One collagenase selective MMP inhibitor, Ro 32-3555, slows progression of OA in the SRT/ort mouse (105) and in rats following surgical tear of the meniscus (106) . This inhibitor has not been fully tested in human OA, but clinical trials of Ro 32-3555 in humans with rheumatoid arthritis were halted due to lack of efficacy, and testing in human OA was halted as well (107). The only MMP inhibitor that has been shown to be effective in slowing OA progression (joint space narrowing) in human OA is doxycycline, which also inhibits MMP expression and is discussed in the following section (108).

6.4.3. Pharmacologic inhibition of MMP expression

One common treatment for OA is administration of non-steroidal anti-inflammatory drugs (NSAIDS), a therapy that affords some pain relief and improvement in measures of joint function. In addition, these drugs generally reduce the activity of some of the MMPs in cartilage *in vitro* and *in vivo*. (78, 109-113). Diacerhein is thought to block the action of cytokines such as IL-1 and/or TNFa, and consequently to inhibit MMP activity and expression (114). Alternatively, agents that interfere with signaling activities of cytokines are also effective modifiers of cartilage MMP activity. For example, inhibition of components of the MAP kinase (115), iNOS (116-117), or NF-?ß pathways (118) strongly inhibits cartilage degradation in models of OA or of inflammatory arthritis.

Because tetracyclines were shown to inhibit MMP activity *in vitro,* we tested doxycycline in a canine ACLT model of OA (83). Daily oral administration of a single dose (~2 mg/kg) strongly delayed the progression of cartilage damage in OA, and significantly reduced collagenolytic and gelatinolytic activity in extracts of articular cartilage from the OA knee (83). However, evaluation of the results suggested an effect of doxycycline on canine OA in addition to simple inhibition of enzyme activity. Total collagenase and gelatinase levels were measured in cartilage samples taken 24 hours after the last doxycycline dose and after extraction of the enzymes from the tissues. After extraction, TIMPs in the extracts were destroyed by reduction and alkylation, a procedure that involves exhaustive dialysis (55). Finally, the total enzyme activity was measured after activation of latent enzyme with APMA. The total enzyme activity was reduced,

relative to cartilage from placebo-treated control animals, by 82% for collagenase and 75% for gelatinase. Activity reduction of this magnitude requires relative high concentrations of doxycycline *in vitro* —higher than the peak serum concentration expected at the dose administered. Further, no doxycycline would be expected to be retained in the samples after the multiple dialysis steps utilized in the extraction procedure. The results suggest that doxycycline reduced the amount of total MMP in the canine OA cartilage.

Although the reduction of enzyme protein could conceivably be the result of reduction in the rate of disease progression by an alternative mechanism, a study of patients with end-stage OA indicates that direct reduction of MMP expression is a better explanation. Patients who were scheduled for total hip replacement because of end-stage OA were given doxycycline or placebo capsules for 5 days before surgery. Cartilage damage assessed by radiography and confirmed at surgery was severe in all groups. Collagenolytic and gelatinolytic activity from the cartilage of the head of the femur was assayed against native or denatured type II collagen. The cartilage from the doxycycline-treated patients yielded significantly lower concentrations of collagenase and gelatinase in the extracts than extracts from cartilage of untreated patients (119). As in the dog studies, no doxycycline was likely to be present at the time of assay, and the brief treatment of long-standing and severe OA was unlikely to significantly change the extent of cartilage destruction.

Normal human chondrocytes in culture express MMP-1, MMP-8, and MMP-13 at low levels, but these enzymes are expressed at 10 fold higher levels by chondrocytes from lesion areas of the OA joint. Chondrocytes from apparently normal cartilage adjacent to the lesions of the OA joint express these MMPs at 3-5 fold higher levels than control chondrocytes. Incubation of the lesion chondrocytes in the presence doxycycline strongly inhibits the expression of MMP-13 at both the mRNA and the protein level in a dose-dependent manner. MMP-13 expression was reduced at the transcription level in the presence of 10 fold lower concentrations of doxycycline (1 µg/ml) than is necessary for similar inhibition of enzyme activity (37, 120).

The above results obtained *in vitro* and in the canine ACLT model were followed by a clinical trial of doxycycline in human OA (108). Women with knee OA were treated with 100 mg doxycycline twice a day for 30 months (n=218; 149 completed the 30 month study) or with placebo capsules (n=213, 158 completed the study). The joint space width was measured at baseline, 16 months, and 30 months. Joint space narrowing in the doxycycline group was reduced by 40% at 16 months and by 33% at 30 months. Thus, the progression of structural damage in OA was reduced by doxycycline, an agent selected because it reduced MMP activity in OA cartilage.

7. CONCLUSIONS AND CAVEATS

The available data suggest strongly that MMPs in general, and MMP-13 in particular, have a pivotal role in destruction of cartilage matrix in OA. Specifically, these enzymes have the capability to digest the collagenous components of the ECM, they are produced at the right place and time to affect digestion, OA cartilage contains diagnostic digestion products of collagen, and alteration of MMPs alters the progression of matrix destruction in OA. It appears likely that inhibition of one or more of the MMPs is likely to slow the progression of OA, and could become an effective therapy for OA. There are several important questions that remain to be answered.

A strong case has been made for MMPs as the agents of digestion of the collagen meshwork in articular cartilage matrix in OA, but there are important limitations on the data. Although the lines of evidence cited in this review are consistent with the hypothesis that MMPs are responsible for digestion of cartilage collagen in OA, they do not rule out redundant, alternative mechanisms. For a number of years, stromelysin (MMP-3) was thought to be the major enzyme that digested aggrecan in the ECM of cartilage. The 4 lines of evidence that support the role of collagenases in digestion of type II collagen also support a role for MMP-3 in digestion of aggrecan. The enzyme is able to digest the substrate, cartilage contains significantly higher levels of MMP-3 than normal cartilage in early and moderate OA, aggrecan digested at the stromelysin digestion site is present in the tissue, and the rate of aggrecan digestion is significantly slowed by agents that reduce activity of MMP-3. However, when the primary cleavage product of aggrecan present in OA and RA synovial fluid was identified, it was not the fragment produced by digestion of aggrecan with stromelysin. An "aggrecanase" enzyme was postulated, and was ultimately identified as one or more members of the ADAMTS family of proteinases [see (121) for review]. Although both aggrecanase and MMP digestion products of aggrecan are present in OA cartilage, the consensus is that aggrecan is the major enzyme involved in cartilage catabolism in OA.

Although it does not appear likely that a stromelysin-aggrecan scenario might apply to the digestion of triple-helical collagen in OA, it is possible that a similar scenario might apply to cleavage at other sites within the collagen meshwork. For example, cleavage of the telopeptides of type II, IX, and XI by several MMPs and/or the cleavage of type IX within the Col2 domain by MMP-3 have been identified as potentially critical steps in the initial disruption of the collagen meshwork in experimental OA. However, these sites, or adjacent sites non-helical sites are likely to be susceptible to digestion by other proteases as well as by MMPs.

8. ACKNOWLEDGEMENTS

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