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MMPs - Role in Cardiovascular Development and Disease

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1. ABSTRACT

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes important in the degradation and turnover of extracellular matrix (ECM) components. MMPs and their inhibitors play major roles not only in ECM degradation but also in mediating cell-cell adhesion, cell migration and invasion, cell proliferation and apoptosis, tissue remodeling, and growth factor and cytokine signaling. There is a vast amount of literature regarding changes in MMPs and MMP inhibitor levels during the progression of cardiovascular diseases but a paucity of information regarding their roles in the embryonic cardiovascular development. Yet, by studying cardiovascular development, much can be learned with regard to the pathophysiology and etiology of adult cardiovascular diseases. In fact, the development of many pathological conditions may reflect inappropriate recapitulation of embryonic events. The objective of this review is to provide an overview of what is known regarding the role of MMPs and their inhibitors during embryonic cardiovascular development and to relate these to the pathophysiology of adult cardiovascular diseases whenever possible.

2. INTRODUCTION

Matrix metalloproteinases are proteolytic enzymes whose function is primarily viewed as being the degradation and turnover of ECM components. However, MMPs and their inhibitors also play key roles in regulating many fundamental cell processes including regulation of cell growth, cell adhesion, cell migration and invasion, cell death, and tissue remodeling events. Because of their involvement in so many diverse processes, a better understanding of how this group of enzymes and their regulators interact and mediate these processes will be necessary in order to understand whole organism biology and pathology.

During the past decade, it has become recognized that MMPs and their inhibitors play significant etiological roles in the development of cardiovascular diseases including congenital heart defects, atherosclerosis, aneurysms, vascular remodeling, and myocardial ischemia and infarction. Many of these pathological conditions may stem from inappropriate recapitulation of embryonic and developmental events. This review will focus on what is known regarding MMPs and MMP inhibitors in

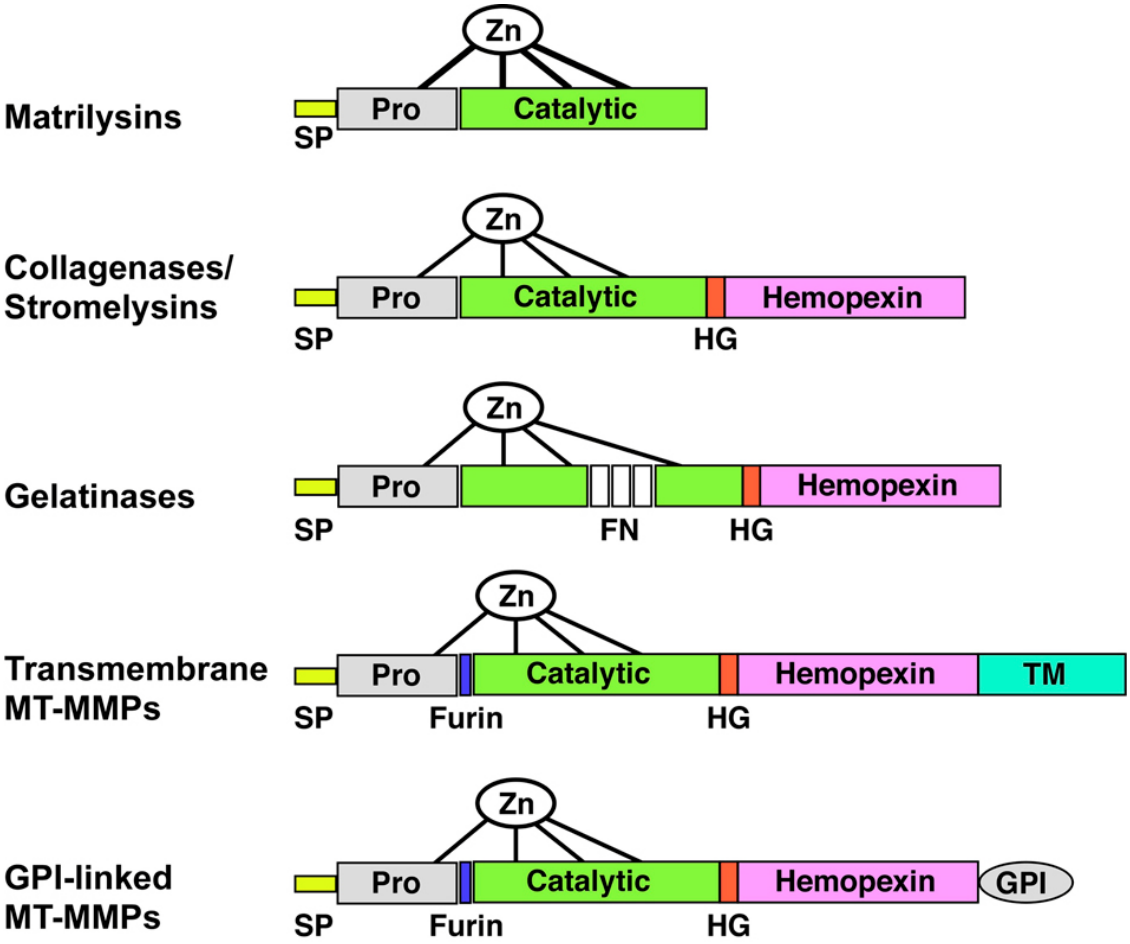

Figure 1. Domain structure for the major classes of MMPs. Major domains include the signal peptide (SP), prodomain (Pro), catalytic domain with the active site zinc (Zn) bound to cysteine residues within this domain and "cysteine switch-residue" in the prodomain, the hinge domain (HG), the hemopexin domain, and in some cases either a transmembrane domain or GPI-anchor domain (GPI). A furin cleavage site between the prodomain and the catalytic domain is found in some MMPs. In the gelatinases, fibronectin-like type II repeats (FN) are also present.

cardiovascular development and to relate these findings to various cardiovascular diseases whenever possible.

3. MMPS AND MMP INHIBITORS

MMPs are a family of zinc-containing endopeptidases that cleave almost every known component of the ECM. These enzymes were initially classified and given common names based on their substrates until it became clear that each has multiple, often overlapping, substrates. As the MMP genes became better characterized, MMP nomenclature moved toward a numerical system and the MMPs were grouped into classes based on their domain structure rather than substrate (Figure 1, Table 1, for reviews see, 1, 2).

The catalytic activity of MMPs is dependent on the presence of zinc bound to a conserved HEXXHXXGXXH motif found within the catalytic domain of MMPs (Figure 1). The catalytic domain forms a small cleft harboring the zinc and its 3-dimensional structure determines substrate cleavage-site specificity. For instance, the catalytic domain of MMP-2 and MMP-9 contains fibronectin type-II repeats making these MMPs particular effective in degrading multiple types of collagen. The pro-peptide domains of MMPs contain a conserved sequence, PRCXXPD. The cysteine residue within this sequence interacts with the catalytic zinc rendering MMPs inactive. MMPs (with the exception of MMP-7, MMP-23, and MMP-26) also have a hemopexin-like domain connected to the catalytic domain by an intervening hinge domain. The hemopexin domain influences substrate binding and specificity, membrane activation, and binding of MMP inhibitors.

Of particular recent interest are the membrane type-MMPs (MT-MMPs), of which six different ones have been described. MT-MMPs are anchored to the plasma membrane either through a GPI-tail or by a transmembrane domain (Figure 1). These MMPs are primarily activated by furins as they contain a conserved furin cleavage site

Table 1 Major MMPs and Their Principle Substrates

Group/Enzyme	MMP Designation	Principle Extracellular Matrix Substrates
Collagenases		
Interstitial collagenase	MMP-1	Collagens I, II, III, VII, and X, entactin, aggrecan, tenascin, proMMP-1, -2
Neutrophilic collagenase-2	MMP-8	Collagens I, II, and III
Collagenase-3	MMP-13	Collagens I, II, III, VI, X, aggrecan, fibronectin, laminin, tenascin, proMMP-9, -13
Gelatinases		
Gelatinase A	MMP 2	Collagens I, IV, V, VI, VII, X, and XI, fibronectin, laminin, vitronectin, entactin, proMMP-1, -9, -13
Gelatinase B	MMP 9	Collagens I, IV,V, VI, X, and XI, aggrecan, elastin, entactin, fibronectin, vitronectin
Matrilysin		
Matrilysin	MMP-7 (PUMP)	Collagens III, IV, IX, X, and XI, elastin, entactin, fibrin, fibronectin, laminin, tenascin, proMMP-2, -7, vitronectin
Stromelysins		
Stromelysin-1	MMP-3	Collagens III, IV, V, VI, IX, X, and XI, proMMP-1, -3, -7, -9, -13, osteonectin, tenascin, fibronectin, proteoglycans, laminin
Stromelysin-2	MMP-10	Collagens III, IV, V, and IX, fibronectin, laminin, proteoglycans
Stromelysin-3	MMP-11	Collagen IV, fibronectin, laminin, alpha1-proteinase inhibitor, aggrecan
Membrane type MMPs		
MT1-MMP	MMP-14	Collagens I, II, and III, fibrin, fibronectin, proMMP-2, -13, alpha1-proteinase inhibitor, vitronectin, proteoglycans, laminins, tenascin, aggrecan
MT2-MMP	MMP-15	ProMMP-2, fibronectin, laminin, proteoglycans, tenascin, entactin, aggrecan
MT3-MMP	MMP-16	ProMMP-2, collagen III, fibronectin, laminin, aggrecan, vitronectin
MT4-MMP	MMP-17	Fibronectin, fibrinogen
MT5-MMP	MMP-24	ProMMP-2, fibronectin, proteoglycans
MT6-MMP	MMP-25	Collagen IV, fibronectin, fibrinogen, fibrin, proteoglycans
Others		
Macrophage metalloelastase	MMP 12	Elastin, fibronectin, collagens I and V, osteonectin, alpha1-proteinase inhibitor, vitronectin
Enamelysin	MMP-20	Aggrecan, amelogenin

Abbreviations: MMP, matrix metalloproteinase and MT-MMP, membrane-type matrix metalloproteinase

between the prodomain and catalytic domain. MT-MMPs are critical to the functional activities of secreted MMPs as many proMMPs are converted to active forms by MT-MMPs (for reviews see, 3, 4-6). In addition to activating proMMPs, MT-MMPs directly degrade ECM components including collagens, fibronectin, vitronectin, laminin B chains, and proteoglycans (7, 8) and they can activate, release, and regulate turnover of cell-surface receptors and receptor ligands (for a review see 9). In addition, cells can actively redistribute cell-surface MT-MMPs thereby localizing and concentrating MMP activity to particular sites on their cell surfaces (10, 11).

ADAMs (A Disintegrin And Metalloproteinase) may be considered as an extended family of the MMPs (for reviews see, 12, 13). ADAMs are integral membrane glycoproteins containing a disintegrin domain (related to snake-venom integrin-binding ligands that disrupt integrin/ligand interactions) and a metalloprotease catalytic domain (that may or may not exhibit MMP-like activity). Over 30 have been described. Many ADAMs regulate cellular behavior through their cell-surface convertase and sheddase activities and by mediating cell-signal transduction activities of certain receptors. ADAMs have also been implicated in mediating angiogenesis and cardiovascular development and disease (14-16).

The degree of MMP activity depends, in part, on levels of local inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) bind MMPs, block MMP activity, and regulate MMP-dependent cell migration, invasion, and tissue remodeling (for reviews see 17, 18-21). Four TIMPs have been identified in vertebrates and they share similar functional motifs. The amino-terminal portion is responsible for the MMP inhibitory capacity. Structurally, this inhibitory capacity is dependent on maintaining the integrity of particular disulfide bonds within the TIMPs (22, 23). The carboxyl-domain is primarily responsible for TIMP binding to ECM molecules. In the case of TIMP-1 and TIMP-2, the carboxyl domain is responsible for binding proforms of MMPs and in the case of TIMP-3, it is responsible for the binding of TIMP-3 to sulfated ECM components like chondroitin and heparan sulfate proteoglycans (24).

TIMPs play major roles in regulating many biological events through their ability to mediate MMP activity. Studies show TIMPs inhibit the migration of endothelial cells (25, 26), formation of 3-dimensional endothelial tubule structures (27, 28), and smooth muscle cell invasion *in vitro* (29, 30). *In vivo* for instance, exogenous TIMP-3 blocks basic fibroblast growth factor-stimulated angiogenesis in chorio-allantoic membrane assays (28) and overexpression of TIMP-2 inhibits formation of experimental hemangiomas in mice (31).

Alpha2-macroglobulin (an abundant plasma protein) is also a major irreversible inhibitor of MMPs (see review by 32). Alpha2-macroglobulin binds active MMPs, traps them, covalently bonds with the MMPs, and blocks their catalytic activity. Once bound, the alpha2-macroglobulin/MMP complex is removed by the LRP scavenger-receptor complex and cleared through endocytosis (33, 34). Extracellular MMPs levels can also be regulated by similar clearance mechanisms, such as what occurs when thrombospondin-2/proMMP-2 complexes are removed by internalization via binding to the LRP scavenger-receptor complex (35).

The discovery of a GPI-linked membrane MMP inhibitor, RECK (REversion-inducing-Cysteine-rich

protein with Kazal motifs), has garnered recent attention. RECK plays an important role in embryonic neurogenesis and vasculogenesis, tumor angiogenesis, and metastasis (for reviews see 36, 37, 38). Knockout mice for RECK die by embryonic day 10.5, have a smaller body size, and exhibit more abdominal hemorrhages than their heterozygotic litter mates (39). In addition, the vascular networks formed in RECK-deficient embryos are not organized as tight tubules. RECK inhibits proMMP-2 activation and the enzymatic activity of active MMP-2, MMP-9, and MT1-MMP (36, 39, 40). It is also expressed in normal adult tissue, including vascular smooth muscle cells of large vessels. When RECK synthesis is stimulated or is over expressed, RECK attenuates tumor growth by limiting endothelial cell migration and angiogenic sprouting (26, 39).

4. MMP ACTIVATION

Most MMPs are synthesized and released as inactive pro-enzymes requiring proteolytic cleavage of the prodomain before exhibiting their full proteolytic capacity. For example, MMP-2 is secreted as a 70-72 kDa pro-molecule that is reduced to 62-63 kDa when processed to its mature active form. Once converted, MMP-2 can degrade type-IV collagen, interstitial collagens, and several other ECM components (3, 41-43). Hence, levels of MMP activity not only depend on the levels of synthesis and secretion by cells but on their conversion to functionally active forms.

ProMMPs can be activated in at least four ways: autolytically, by plasmin, by MT-MMPs, or through the actions of other active MMPs. Autolytic MMP activation can be initiated by chemical means. 4-Aminophenylmercuric acetate, thiols, sodium dodecylsulfonate, and chaotropic agents can all initiate proMMP activation by perturbing the disulfide bond between the cysteine residue in the prodomain and the zinc ion (the so called "cysteine switch", 44, 45, 46, and for review see 47). This permits the zinc ion to bind water and hydrolyze peptide bonds including the intramolecular peptide bond linking the prodomain to the rest of the MMP molecule. Interesting, recent studies suggest chemical activation mechanisms may also occur *in vivo* as nitric oxide generated by living cells can activate proMMP-2 (48).

Plasmin generated from plasminogen is also capable of initiating proMMP activation (49-51). Plasmin is thought to activate proMMPs by proteolytically cleaving a portion of the MMP prodomain, thereby disrupting the catalytic zinc/proMMP cysteine bond responsible for enzyme latency. However in the case of proMMP-2 activation, activation by plasmin requires MT1-MMP as a co-factor, although the catalytic activity of MT1-MMP is not required (52). Why the MT1-MMP is required for plasmin to activate MMP-2 is still unclear.

MT-MMPs are thought to be the primary activators of proMMPs. Some MT-MMPs directly activate MMPs, as is the case for MT2-MMP activation of proMMP-2 (53). Although TIMPs inhibit proteolytic activity of MMPs, MT-MMP activation of many proMMPs require TIMPs (3, 54-59). In the case of proMMP-2, activation usually requires either TIMP-2 or TIMP-3 and MT1-MMP or MT3-MMP (for reviews see 5, 60-63). The N-terminal domain of the TIMP binds the catalytic domain of the MT-MMP forming a TIMP/MT-MMP complex on the cell surface (Figure 2). While this inhibits the activity of the occupied MT-MMP, the C-terminal domain of the TIMP retains the ability to bind proMMP-2, effectively recruiting proMMP-2 to the cell surface forming a "ternary complex". Once on the cell surface, an adjacent TIMP-free MT-MMP then cleaves the prodomain and activates proMMP-2. However, excess TIMP interferes with this activation by sequestering all the available TIMP-free MT-MMP molecules on the cell surface. Therefore, TIMPs can either facilitate or inhibit the activation of proMMP-2 depending on the relative levels of TIMP (54, 55, 64).

Activation of proMMP-2 or other MMPs, however, does not exclusively require TIMPs. For instance, MT1-MMP can activate proMMP-2 in the absence of TIMP-2 if the cells express alphaV beta3 integrins. This integrin directly binds proMMP-2, docking it to the cell surface for activation by MT1-MMP (65). Blocking the interaction between alphaV beta3 integrin and proMMP-2 inhibits proMMP-2 activation and inhibits angiogenesis and tumor growth (65, 66). Consequently, there are numerous means available to cells for activating proMMPs.

5. OVERVIEW OF MMP FUNCTIONS

5.1. Cell-ECM Adhesion, Migration, and Invasion

Much of our understanding regarding MMP and TIMPs stems from work in the area of cancer biology. It was long ago recognized that ECM turnover was important for cell metastasis and angiogenesis and that high levels of MMPs are correlated with increased tumor invasion capacity and their ability to recruit blood vessels. MMPs also have major roles in controlling the migratory behavior of both normal and transformed cells (for reviews see, 67, 68, 69). For example, selectively blocking MMP-2 or MMP-9 activity with synthetic peptides or inhibiting their synthesis using antisense mRNAs, blocks tumor and endothelial cell migration (70-74). MMPs also modulate the way cells interact with their extracellular environment. For instance, alphaV beta3 integrins expressed by melanoma cells do not bind native fibrillar type I collagen unless the collagen is first proteolytically cleaved by MMPs (75). And, MT-MMPs exhibit integrin convertase activity generating mature alphaV beta3 integrin subunits capable of transmitting outside-in signaling when bound by their ligand (76, 77).

Cells localize MMP activity to specific sites (*e.g.*, focal contacts and invadopodia) and as such, are capable of differentially regulating substrate adhesiveness at discrete regions on their cell surface. This is essential for the formation of functional invadopodia and cellular invasion (10, 11, 78-81). MMPs can be localized and concentrated on the cell surface by binding to integrins. For instance, the clustering of alphaV beta3 integrins in response to proMMP-2 or ECM binding results in co-clustering of MT1-MMP by virtue of specific interactions between this

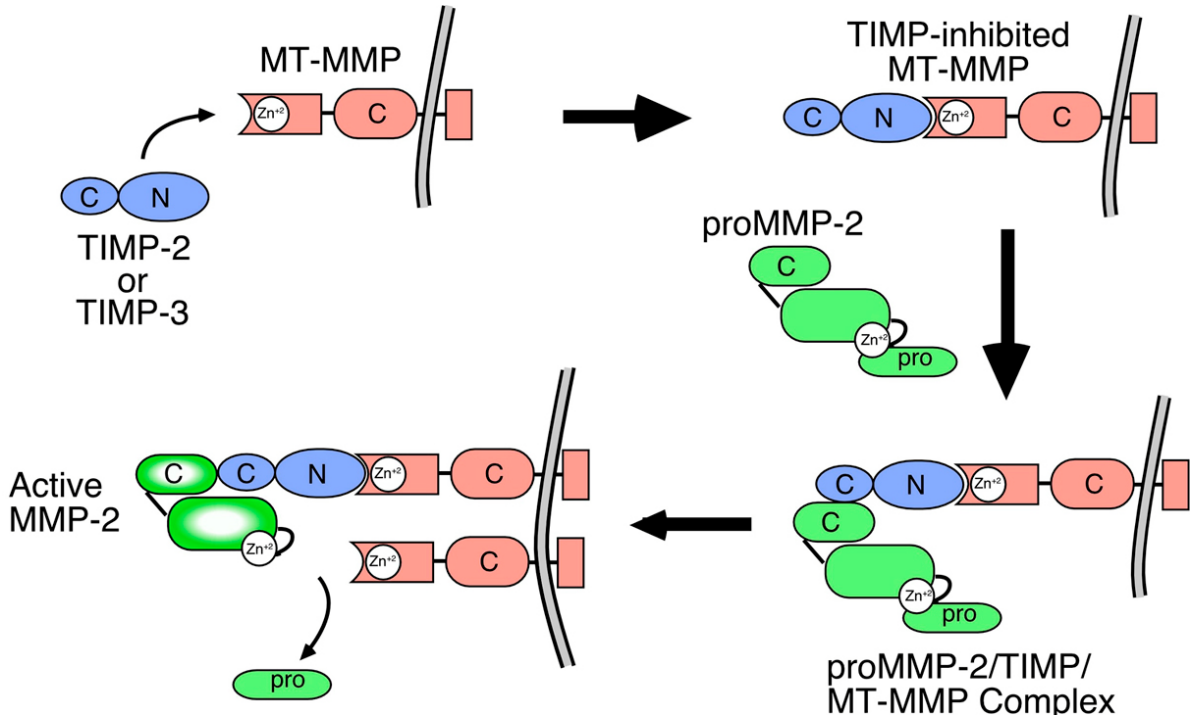

Figure 2. Simple model illustrating the basic steps of proMMP-2 activation through the formation of a proMMP-2/TIMP/MT-MMP ternary complex. In this model, TIMP-2 or TIMP-3 binds the catalytic domain of MT1-MMP or MT3-MMP forming a TIMP/MT-MMP complex on the cell surface. While this inhibits the activity of the occupied MT-MMP, TIMP-2 and TIMP-3 retain their ability to bind proMMP-2 effectively recruiting proMMP-2 to the cell surface. Once on the cell surface, an adjacent TIMP-free MT-MMP cleaves and activates proMMP-2.

integrin and the hemopexin domain of MT1-MMP. Blocking the interaction between MT1-MMP and alphaV beta3 inhibits angiogenesis (65, 66). The formation of proMMP/TIMP/MT-MMP complexes also enable cells to localize MMP-2 activity to specific sites on the cell surface (10, 11, 72, 80).

Cells can also cluster MMPs through the interactions between MMPs and cell surface CD44. CD44 is a multifunctional cell surface molecule that mediates cell-cell and cell-ECM adhesion (82, 83). CD44 is the principle receptor for hyaluronan, a large polysaccharide that plays an important role in cell migration and invasion. CD44 also serves as a receptor for other ECM components including fibronectin, collagen type-I, and heparan and chondroitin sulfate proteoglycans. CD44 binds MMP-9, promoting tumor migration and angiogenesis. CD44 also recruits MT1-MMP into lamellopodia via binding to MT1-MMP's hemopexin domain. MT1-MMP then proteolytically releases the ectodomain of CD44 from the cell surface, a prerequisite for migration of many cell types (84-87). Therefore, through specific interactions with cell surface receptors, cells can localize proMMP activation and MMP activity to particular areas of the cell surface where it is needed for directed cell migration and invasion.

Degradation products generated from ECM by the proteolytic action of MMPs also mediate cell migratory and invasive responses (Table 2). Cleavage of laminin-5 by MMP-2 generates gamma2-chain laminin fragments that bind epidermal growth factor (EGF) receptors and induce breast epithelial cell motility (88, 89). Angiostatin, generated from plasminogen by the actions of MMP-2, MMP-3, MMP-7, MMP-9, or MMP-12, reduces endothelial cell invasion by blocking MT1-MMP and MMP-2 activity (90-92). Likewise, expression of MMP-12 inhibits angiogenesis by cleaving and shedding urokinase receptors required for endothelial cell invasion (93). MMP-9 generates a cleavage fragment from the alpha3 chain of type-IV collagen that binds alphaV beta3 integrins and attenuates tumor angiogenesis and tumor growth (94). Therefore, MMPs can regulate cell migration by generating bioactive cryptic fragments of ECM components.

5.2. MMPs and Epithelial-to-Mesenchymal Transitions

MMPs have been implicated in regulating epithelial-to-mesenchymal transitions (EMTs). Exogenous inhibitors of MMPs block EMT during tumor metastasis while reducing endogenous MMP inhibitor levels increases metastasis (30, 95, 96). Blocking MMP activity during embryonic heart development also inhibits the EMT responsible for endocardial cushion cell formation and inhibits the subsequent migration of cushion cells *in vivo* and *in vitro* (97). Studies also suggest MMPs alter cell-cell adhesion interactions mediated by cadherins during EMTs. For instance, MMP-3 cleaves the ectodomain of E-cadherin (98).

Overexpressing MMP-3 in mammary epithelial cells increases degradation of E-cadherin (99). Upon release, the ectodomain of E-cadherin perturbs the function of intact

Table 2. Bioactive Molecules Released or Degraded by MMPs

MMP	Growth Factor and Receptor Activation or Release	Cytokine Activation	Adhesion Molecules/Bioactive peptides
MMP-1	IGF-BP, proTNF-alpha, VEGF	proIL-1beta, monocyte chemoattractant protein-3	
MMP-2	proTGF-beta, IGF-BP, VEGF, proTNF-alpha, FGFR1, endothelin-1	proIL-8, monocyte chemoattractant protein-3	PEX, angiostatin,, endostatins, laminin-5 gamma2 fragments
MMP-3	proTNF-alpha, proHB-EGF, IGF-binding protein, proTGF-beta, basic FGF	proIL-1beta	E-cadherin, laminin-5 gamma2 fragments, osteonectin fragments
MMP-7	proTNF-alpha, proHB-EGF, proTGF-beta, FasL		E-cadherin, beta4-integrin, endostatin
MMP-9	VEGF, proTGF-beta, proTNF-alpha, basic FGF, kit ligand, endothelin-1	proIL-1beta, proIL-2R alpha, interferon-beta, IL-8	ICAM-1, alpha3 collagen, type-IV fragments
MMP-12	urokinase receptors		laminin-5 gamma2 fragments
MMP-13	basic FGF, proTGF-beta		laminin-5 gamma2 fragments
MMP-14	proTGF-beta, basic FGF, VEGF, proTNF-alpha		CD44, alphaV-integrin, tissue transglutaminase

Abbreviations: FGF, fibroblast growth factor; HB-EGF, heparin binding-epidermal growth factor; ICAM, intercellular adhesion molecule; IGF, insulin growth factor; IL, interleukin; MMP, matrix metalloproteinase; PEX, hemopexin-like domain; TGF, transforming growth factor; TNF, tumor necrosis factor; and VEGF, vascular endothelial growth factor.

membrane-anchored E-cadherin and stimulates the invasion of these cells into 3-dimensional collagen matrices (98, 99). Conversely, increasing E-cadherin expression reduces MMP activity and metastasis (100, 101). Collectively, these studies suggest MMPs play important roles in mediating cell-cell adhesion and EMTs.

5.3. MMPs and Cell Signaling

Regulation of growth factor and growth factor-receptor expression, activation, and turnover are also important in the development and maintenance of the cardiovascular system. MMPs release membrane-anchored growth factors and cytokines, and release and activate latent growth factors sequestered within the ECM (Table 2). For example, MMP-2, MMP-9, and MT1-MMP release and activate latent transforming growth factor-beta (TGF-beta), basic fibroblast growth factor (FGF), and vascular endothelial growth factors (VEGFs) deposited within the ECM and make available other growth factors, such as insulin growth factor (IGF), by degrading their binding proteins (85, 102, 103). MMP-7, by forming a complex with CD44 and proheparin-binding epidermal growth factor (proHB-EGF), proteolytically cleaves proHB-EGF and this cleavage is a prerequisite for the trans-activation of the erbB receptors by this ligand (104-106). Blocking MMP activity has also been shown to attenuate growth factor-related apoptosis in endothelial cells (107, 108). Therefore, controlling MMP activity is instrumental in regulating normal and pathological growth factor-mediated processes.

MMP inhibitors themselves exhibit growth regulatory activities. In fact, TIMP-1 and TIMP-2 were initially identified as erythroid colony-stimulating factors (109, 110). Recent findings show both TIMP-1 and TIMP-2 mediate cell proliferation independent of their MMP inhibitory activity (25, 111-114). TIMP-2 binds directly to alpha3 beta1 integrins and initiates a transduction signal within cells that is independent of its MMP inhibitory activity (111, 112). Consequently, TIMP-2 alters the proliferative response of cells to several growth factors including EGF, platelet-derived growth factor (PDGF), basic FGF, and VEGF-A. Addition of a single alanine to the N-terminal end of TIMP-2 renders it incapable of inhibiting MMP activities (22, 23), yet, this TIMP is still capable of binding proMMPs, binding alpha3 beta1 integrins, and eliciting signal transduction responses in cells (111). In fact, alanine-modified TIMP-2 or the C-terminal domain of TIMP-2 (non-MMP inhibitory domain) both have the ability to block growth factor-dependent angiogenesis as effectively as wild-type TIMP-2 (111, 114). Hence, not only do these inhibitors block MMP-mediated activation of growth factors and cytokines, but they are also capable of directly eliciting or altering receptor-mediated signal transduction in cells independent from their MMP inhibitory capacity.

5.4. MMP-Deficient Animals

Several mutant mice with genetic deficiencies in various MMPs have been generated (115). However, mice have a considerable amount of compensatory capacity for the genetic loss of a particular MMP during development. Despite numerous studies implicating MMPs in mediating important developmental events, MMP deficient mice are generally normal in appearance and overall growth. For instance, MMP-2 deficient mice, while smaller in size, are otherwise normal and fertile (116). Because MMPs have overlapping substrates and functional roles, other MMPs likely compensate for the genetic lose of an MMP. However, detailed examination of cardiovascular development in MMP deficient animals has not been performed. One MMP that does exhibit a developmental phenotype when knocked out is MT1-MMP. MT1-MMP deficient mice exhibit craniofacial and skeletal defects, are growth restriction, and have higher mortality rates than their wild-type littermates (117). Recently, MT1-MMP expression has also been knocked down in zebrafish embryos using antisense mRNAs and these embryos also exhibit axial deformities and cranial defects (118). While the overall embryonic development of most MMP deficient mice is phenotypically normal, the same can not be said for these mice as they age or are challenged by disease. As discussed later, responses to vascular injury, myocardial ischemia, and vascular disease in MMP deficient mice are quite different from their wild-type littermates.

6. MMP AND TIMPS IN CARDIOVASCULAR MORPHOGENESIS

Cardiovascular morphogenesis requires dynamic changes in cell-cell adhesion, cell migration, selective

proliferation and apoptosis, and tissue remodeling. Many adult cardiovascular diseases may reflect a recapitulation of embryological events. For instance, it has been proposed that intimal thickening in response to hyperplasia after balloon angioplasty or during restenosis may reflect an abnormal recapitulation of the EMT responsible for the formation of endocardial cushion cell tissue during embryonic cardiac septation (119-123). In response to injury or increased work load/stress, the heart undergoes remodeling events eliciting changes in MMP activity and ECM turnover as a part of the response to functionally compensate for the extra load. Such responses may mirror MMP-driven remodeling events that occur during embryonic cardiac morphogenesis (*e.g.*, cardiac looping, trabeculation of the primitive ventricles, and myocardialization of the conal outflow tracts). Unfortunately, there is a profound lack of understanding regarding the precise role of MMPs or their inhibitors during cardiovascular morphogenesis and development.

6.1. Embryonic Vasculogenesis and Angiogenesis

Vasculogenesis is the process of making de novo blood vessels directly through cell determination of angioblasts (the endothelial cell precursors) and differentiation. This is a distinct process from angiogenesis. Angiogenesis, by strict definition, is the process of making new blood vessels from preexisting blood vessels either through sprouting or remodeling of existing vessels (124). Unfortunately, the two terms are often used interchangeably. Very little is known regarding the role of MMP and MMP inhibitors in the process of vasculogenesis because it begins so early in embryogenesis. Both vasculogenesis and angiogenesis require that endothelial cells (or their precursors) form 3-dimensional aggregates and networks through directed cell migration, invasion, and ECM remodeling. Hence, much of what is known regarding angiogenesis likely applies to embryonic vasculogenesis as well.

The earliest embryonic site for vasculogenesis occurs during blood island formation in the extraembryonic yolk sac wall where vasculogenesis is coupled with hematopoiesis (for review see 125). These blood islands coalesce forming a primitive vascular network that becomes connected to vascular networks forming within the embryo (intraembryonic vasculogenesis is thought to be uncoupled from hematopoiesis although recent studies suggest otherwise, 126, 127). Fibroblast growth factors, VEGFs, and their receptors play essential roles in the formation and maintenance of blood vessels during embryogenesis (128-131). MMPs, particularly MMP-9, release bioactive basic FGF and VEGFs sequestered within the ECM (102, 132). In turn, active FGFs and VEGFs stimulate the expression of several MMPs necessary for angiogenesis (133-136). MMP-9-deficient mice are fertile but these mice exhibit abnormal or delayed angiogenesis (137). In this case, MMP family members likely compensate for the loss of MMP-9.

Vasculogenesis and angiogenesis requires cell migration, proliferation, and the formation of branching endothelial chains and concomitant lumen formation (138). MMPs also play important roles in regulating these events. For instance, invasion and remodeling of endothelial cells into 3-dimensional networks requires MMP activation of proMMP-2 and MMP activity (139-141). MT1-MMPs rather than secreted MMPs appear to be more important in the formation of these 3-dimensional networks (141, 142). Recombinant TIMP-1 and synthetic MMP inhibitors attenuate microvascular endothelial cell migration and increase VE-cadherin and PECAM-1 expression and accumulation at cell-cell junctions (25). Migration of endothelial cells during angiogenesis also requires breakdown of collagenous components in the ECM as substitution of wild-type collagen type-I with one that is collagenase resistant, blocks new vessel formation (143).

Integrins play major roles in vasculogenesis and angiogenesis. When the function of beta1 integrin is blocked in avian embryos, angioblasts still form cord-like assemblies resembling blood vessels but these cords do not develop lumens (144). In contrast, neutralizing antibodies to alphaV beta3 integrin prevent the angioblasts from organizing into blood vessels (145, 146). PEX (a natural-occurring proteolytic fragment of MMP-2) inhibits proMMP-2 binding to alphaV beta3 integrin resulting in a decrease in MMP-2 activation and angiogenesis (65). As mentioned earlier, MT1-MMPs exhibit integrin convertase activity generating mature alphaV beta3 integrin subunits capable of eliciting intracellular transduction signals when bound by ligand (76, 77). Therefore, MMP activity may be an upstream prerequisite enabling integrin-mediated signaling necessary for blood vessel formation.

In the past, endothelial cell precursors were thought to be only present in the embryo and fetus. However, evidence shows endothelial cell precursors exist in adult bone marrow and peripheral blood. VEGF, granulocyte-monocyte colony-stimulating factor, basic FGF, and IGF-1 all stimulate endothelial-precursor cell mobilization and differentiation (for review see 147, 148, 149). Both MMPs and plasmin stimulate angiogenesis by liberating these growth factors from ECM (150-152). However, excessive MMP activity inhibits angiogenesis. MMP-mediated degradation of plasminogen generates angiostatin and endostatins, potent inhibitors of angiogenesis (153), thereby providing a braking mechanism for MMP-driven angiogenesis. Endothelial cell migration in adult blood vessels requires loosening of inter-endothelial cell contacts and weakening of peri-endothelial cell support. In embryos lacking the angiogenic factor, angiopoietin-1, endothelial cells fail to associate with the underlying ECM and do not recruit peri-endothelial support cells resulting in the formation of leaky vessels (154). Overexpression of Ang-1 results in the formation of non-leaky vessels. Angiopoietin-1 increases plasmin generation and MMP-2 secretion in adult porcine pulmonary arterial endothelial cells while suppressing TIMP-2 secretion (155). Hence, the angiopoietin-1-dependent recruitment of peri-endothelial cells and degree of endothelial cell adhesion and blood vessel permeability may depend on particular levels of MMP and plasmin activity.

The decision of circulating endothelial cells to integrate into blood vessel walls may involve the

ephrin/EphB family. Ephrins are transmembrane ligands for a family of EphB receptor-tyrosine kinases (156, 157). The binding of ephrins to Eph receptors stimulates transduction signals in the EphB-expressing cells but can also transduce a reverse signal into the ephrin/ligand-expressing cell. Such interactions and signaling events have been shown to play major roles in the development of the vascular system including blood vessel remodeling and specifying artery or vein differentiation (158, 159, for review see, 160). EphB4 receptors promote microvascular endothelial cell migration and proliferation (161). Stimulation of the EphB4 receptor by ephrin-B2 increases levels of both activated forms of MMP-2 and MMP-9 in cultured human microvascular endothelial cells and stimulates the migration and proliferation of these cells (161). In ephrinB2- or EphB4-deficient mouse embryos, there is a complete arrest of angiogenesis (159, 162). In addition, ephrin-B2 reverse signaling is required for proper development and remodeling of the embryonic cardiac valves (162-164). These observations show ephrins and ErbB receptors likely have important roles in regulating MMP activity during blood vessel development.

6.2. MMPs and TIMPs in Heart Morphogenesis

The first morphological evidence of embryonic heart formation is the organization of bilateral epithelial-lined compartments within the lateral plate mesoderm. Within the anterior cardiogenic field of this mesoderm flanking the developing foregut, cells begin segregating and forming endocardial-lined tubes that fuse in the midline and then become surrounded by developing cardiomyocytes (165). Thus, heart formation begins much like intraembryonic vasculogenesis elsewhere in the embryo. Because congenital heart and great vessel defects occur with a frequency of almost 1:200 live births and they comprise the most common life-threatening birth defect, future preventative or intervention measures will rely on a better understanding of normal cardiac development.

6.2.1. MMPs and Cardiac Tube Formation and Looping

Very little is known regarding the role of MMPs and TIMPs during the early phases of heart development. To date, the earliest MMP known to be expressed during heart development is MMP-2. In the avian embryo, MMP-2 expression first appears within the lateral plate mesoderm and becomes increasingly restricted to the splanchnic mesoderm adjacent either side of the developing cranial foregut (166). Within this splanchnic mesoderm, angioblasts organize into two primitive endocardial-lined tubes that eventual fuse forming a single inner endocardial tube surrounded by differentiating cardiomyocytes this single heart tube is suspended from the foregut into the primitive thoracic cavity by dorsal mesocardium. The dorsal mesocardium eventually ruptures permitting looping of the primitive heart tube. During the process of making a single heart tube, MMP-2 is expressed in the endocardium, early differentiating cardiomyocytes, and dorsal mesocardium but is soon lost within the myocardium (166, 167). Blocking MMP-2 activity prevents midline fusion of the primitive heart tubes leading to cardiac bifida (167). TIMP-2 is also expressed within the endocardium of the single heart tube and in the dorsal mesocardium just prior to the rupture of the dorsal mesocardium and the onset of cardiac looping (167, 168).

Cardiac looping converts the single, straight tubular heart into a S-shaped tube and re-positions the primitive heart chambers into their adult anatomical positions before cardiac septation is complete. Looping is thought to be driven by elongation and remodeling of the heart tube at the cranial end (169). As the heart tube continues to lengthen during the looping process, TIMP-3 expression appears within the endocardium and in the myocardium of the outflow region (168). The cardiac outflow region is where the most pronounced remodeling and heart-tube lengthening occurs (170, 171). The expression of TIMP-3 within this myocardium is precisely where one would expect it if it were involved in the lengthening and remodeling of cardiac tube.

The direction of cardiac looping may be driven by differing rates of proliferation, not in the heart tube itself, but rather in the dorsal mesocardium and adjacent splanchnic mesoderm (greater on the left side rather than the right, 167). MMP-2 is expressed within the dorsal mesocardium and in both the right and left adjoining splanchnic mesoderm whereas TIMP-2 expression is more prevalent in the left side of the dorsal mesocardium and adjoining left splanchnic mesoderm. This suggests the possibility that more proMMP-2 is activated on this side as TIMP-2 plays an important role in proMMP-2 activation. Interestingly, cell proliferation is also more pronounced within the left splanchnic mesoderm and left dorsal mesocardium. Blocking MMP-2 activity not only disrupts this asymmetric pattern of proliferation, it also randomizes the direction of cardiac looping (167) and increases the incidence of dextrocardia (reversal of the normal anatomical position of the heart, *i.e.*, right-sided heart). Therefore, MMP-2 mediated growth appears to be involved in orchestrating the direction of cardiac looping.

Recently, HB-EGF expression has also been reported to be more prevalent in the left side of early embryos than in the right while its receptors, erbB1 and erbB4, are expressed symmetrically (172). Interestingly, in iv/iv mouse mutants that exhibit situs inversus, HB-EGF expression is also reversed. Mice with non-MMP cleavable proHB-EGF exhibit heart failure, myocardial hypoplasia, and enlarged heart valves (173) and HB-EGF-deficient mice have similar defects and abnormal semilunar and AV valves (174, 175). Therefore, one could speculate that temporally- and spatially-restricted MMP activation may dictate where bioactive HB-EGF is available.

6.2.2. Heart Septation

Defects in cardiogenesis during the first three weeks of gestation are usually lethal (*i.e.*, during heart tube formation and early cardiac looping stages), and therefore, spontaneously aborted. However, anomalous events occurring later in embryonic development often permit the embryo and fetus to make it to term. These anomalies most often manifest themselves as great vessel or cardiac septal defects in neonates.

The septation of the atria and ventricles and division of the cardiac outflow tract into the aorta and

pulmonary artery requires the migration, proliferation, and differentiation of two distinct mesenchymal populations, endocardial-derived cushion cells and invading neural crest (NC) cells (176, 177). MMPs have been implicated in regulating of EMTs responsible for forming both populations of cells. MMP-2 is expressed by endocardial cells prior to and during the EMT of the endocardium in both the atrioventricular and outflow tract regions of the developing heart (166, 178). MT3-MMP (although based on primer sequences listed in the publications, it is mistakenly refer to as MT1-MMP) is expressed just prior for endocardial EMT suggesting proMMP-2 activation is a prerequisite to the EMT (97, 178). Collagen type-IV integrity is lost within the endocardial basement membrane just prior to endocardial EMT and blocking MMP activity not only decreases levels of active MMP-2 in the heart, it prevents the loss of basement membrane integrity and inhibits endocardial EMT (97, 179). In addition, TGF-beta, a growth factor known to enhance endocardial EMT (180, 181), increases MMP-2 and MT3-MMP expression in migrating endocardial-derived cushion cells (97). Activation of proMMP-2 requires TIMP-2 or TIMP-3 via formation of a ternary complex with either MT1-MMP or MT3-MMP. Both TIMP-2 and TIMP-3 are expressed in the endocardium of the atrioventricular and outflow tract regions prior to and during EMT of the endocardium (168). Therefore, endocardial cells undergoing EMT and migrating cushion tissue cells not only can activate proMMP-2 necessary for migration but they have the means to direct this activity to the leading edges of their invadopodia.

Hyaluronan, an abundant ECM component of the pre-mesenchymal heart (182), is an important mediator of cell migration and invasion. Mice lacking hyaluronan synthase die *in utero* because of failed cushion tissue formation in both the atrioventricular and outflow tract regions (183). Hyaluronan is essential for heart development because it augments heregulin-1 activation of erbB receptors essential for proper heart development (184). Heregulin (also known as neuregulin-1) up-regulates the expression of multiple MMPs in several cell types and directly induces EMT of the endocardium. Like hyaluronan-deficient mice, heregulin-deficient mice and mice with nonMMP-cleavable proHB-EGF exhibit heart malformations and die *in utero* (184-187). Hence, MMPs play pivotal roles in modulating hyaluronan and erbB signaling necessary for EMT of the endocardial and cardiac septation.

Endocardial cushion tissue formation requires turnover of the cell-cell adhesion molecules NCAM, N-cadherins, and PECAM-1, in a subset of endocardial cells. PECAM-1, a cell adhesion molecule expressed in the endocardium, is lost in endocardial cells undergoing EMT. In mice, hyperglycemic conditions decrease VEGF-A released from the myocardium, decrease endocardial MMP-2 expression, and cause a retention of PECAM-1 in endocardial cells, thereby, inhibiting the endocardial EMT (179). Blocking the bioavailability of endogenous VEGF mimics the effect of hyperglycemia on endocardial EMT whereas exogenous recombinant VEGF-A reverses the deleterious effects of hyperglycemic conditions (179). Moreover, the effect of hyperglycemia on endocardial EMT is lost in cultures of atrioventricular explants derived from PECAM-1 deficient mice. PECAM-1 is normally shed from endothelial cell surfaces during EMT through the action of MMPs (188). Thus under hyperglycemic conditions, the reduction in VEGF-A released from the myocardium would decrease MMP-2 expression resulting in retention of PECAM-1 within the endocardium. Consequently, cell-cell separation necessary for endocardial cushion tissue formation would be insufficient for proper embryonic cardiac septation.

Many congenital heart and great vessel defects stem from aberrant neural crest (NC) morphogenesis (for review see,189). A perturbation of NC cell migration into the pharyngeal arches or NC cell proliferation resulted in congenital heart defects such as persistent truncus arteriosus, transposition of the great vessels, ventricular septal defects, double-outlet right ventricle, and others. MMPs and their inhibitors may have important roles in mediating NC cell emigration from the neural tube (also an EMT event) and regulating NC cell migration and invasion through embryonic ECM. In the trunk axial level, NC progenitor cells transiently express MMP-2 and blocking this expression inhibits NC cell EMT (190). During the early emigration of cranial NC cells, NC cells encounter MMP-2 protein localized within the ectodermal and neural tube basement membranes and within the interstitial ECM through which they migrate (166). Blocking MMP activity inhibits NC cell migration both *in vivo* and *in vitro* (166, 190, 191). *Patch* mice, which exhibit NC-related craniofacial and cardiac defects (192, 193), have deficiencies in MMP-2 and MT1-MMP expression and NC-derived mesenchyme from these embryos have decreased migratory capacity *in vitro* (194). ADAM-13, a disintegrin with MMP activity, is also required for proper migration of NC cells into the branchial (pharyngeal) arches of *Xenopus* embryos (195).

In addition to the dorsal mesocardium and early endocardium, TIMP-2 mRNA is also expressed in the neural epithelium during early neurulation and only in NC cells that will enter pharyngeal arches III, IV, and VI (often referred to as cardiac NC cells as a subset of these cells participate in cardiac septation and valve formation, 196). *In vitro*, cardiac NC cells express MT1-MMP and TIMP-2 and secrete and activate proMMP-2. Antisense TIMP-2 oligonucleotides block proMMP-2 activation *in vitro*, decrease cardiac NC cell migration from explants, and inhibits cardiac NC formation and migration *in vivo* (196). Because TIMP-2 is required for activation of proMMP-2 by MT1-MMP, this suggests TIMP-2 expression by cardiac NC cells initiates proMMP-2 activation necessary for their EMT and subsequent migration. These studies show MMPs and their inhibitors play important roles in enabling cardiac NC mesenchymal cells to reach the cardiac primordia and participate in cardiac septation.

6.2.3. Embryonic and Fetal Cardiac Remodeling

One of the earliest reports showing MMP expression during heart development was based on

immunocytochemistry using an antibody directed against a collagenase. Nakagawa et al. (197) showed a collagenase was present within the cardiac trabeculae, ventricular and atrial walls, and in mesenchymal cells of the developing cushion tissues of embryonic day 11.5 and 12.5 rat embryos. Since then, other MMPs and their inhibitors have been shown to be temporally and spatially expressed during stages of embryonic and fetal heart remodeling. The developing valve leaflets surrounding the atrioventricular orifices express MMP-2 (166, 178). During the formation and remodeling of the muscular septa, cells of septum primum adjacent ostium primum express MMP-2, TIMP-2, and TIMP-3. TIMP-3, but not TIMP-2, is also expressed within remodeling myocardium (166, 168). With the onset of cushion cell formation, the atrioventricular and conal myocardium begin expressing TIMP-3 in a pattern suggesting TIMP-3 participates in separating the atrial myocardium from the ventricular myocardium and in realigning the atrioventricular canals with the developing ventricles (168). The overlapping expression of TIMP-2 and MMP-2 within the epicardium and developing coronary vasculature also suggests MMP activity is important in the development of this tissue layer as well (166, 168).

Remodeling events responsible for transfiguring the primitive ventricular myocardial wall into a compact layer and inner trabecular layer also involve MMPs. In the mouse embryo, MMP-2, MMP-3, MMP-9, and MMP-13 are all expressed in prenatal hearts, particularly over the trabeculae and epicardial tissue, as early as embryonic day 12, and continue to be expressed there during the remainder of fetal period (198). Based on zymography, levels of active MMP-13 increase in mouse hearts beginning embryonic day 12 and plateau a few days before birth while levels of active MMP-2 peak at embryonic day 16 and then decline, suggesting differing roles for these two MMPs during late stages of embryonic heart development (198). Activation of the erbB2/erbB4 receptor complex by endocardial-derived heregulin is required for trabeculation and remodeling of the primitive ventricle (186). MMPs release bioactive VEGFs from the ECM (102, 199) and active VEGF-A increases the expression of several MMPs (200, 201). Therefore, if MMP processing is required for erbB signaling in the developing heart, the specific temporal and tissue-specific expression of MMPs and TIMPs could dictate where and when particular growth factors modulate cardiac remodeling events. This may be exemplified by the observation that expansion of the trabecular layer occurs at the expense of the ventricular compact layer with modest overexpression of VEGF-A, as VEGF-A-driven increases in MMP expression could lead to an abnormal increase in heregulin signaling (202).

7. MMP AND TIMPS IN CARDIOVASCULAR DISEASE

7.1. MMPs and Atherosclerosis

Atherosclerosis, a pathological vascular remodeling event, is initiated by a chemical and/or mechanical-induced injury of the endothelium leading to an accumulation of lipid beneath the endothelium. Circulating monocytes then infiltrate the vascular intima. Here, these monocytes become activated, take up lipid, and release a variety of cytokines and growth factors. Eventually, a lipid core develops within the intima forming a plaque surrounded by a thin fibrous capsule. In response to monocytic invasion, VSMCs also migrate from the tunica media into the intima and undergo hyperplasia thereby contributing to stenosis of the vessel (203-205). Clinical complications and symptoms are often triggered by rupture and release of unstable plaques leading directly to obstructions or to development of thrombi compromising the circulation to vital organs. Clinical complications also result from thinning and weakening of the vascular wall promoting the development of aneurysms.

7.1.1. Plaque Development, Intimal Thickening, and Plaque Stability

Inflammation and the associated increase in MMP activity are implicated in playing major roles in plaque-forming events and plaque destabilization (205). Circulating monocytes exhibit little or no MMP activity, but once adhered to the endothelium and in contact with underlying ECM, there is a marked up-regulation of MMP expression (206-209). Activation of these vascular monocytes (*i.e.*, macrophages) results in the release of several different inflammatory cytokines and growth factors (*e.g.*, interleukin-1, interleukin-6, tumor necrosis factor-alpha, EGF, PDGF, basic FGF, macrophage migration-inhibitory factor) that induce expression of several types of MMPs (210-215). Still, others are inhibitory for MMP expression (*e.g.*, interleukin-4, interleukin-10, and interferon-gamma) or increase TIMP expression (216-218).

Many acute cardiovascular events related to atherosclerosis may be due to occlusive thrombi formation occurring after a partial disruption of an atherosclerotic plaque (219, 220). Several lines of evidence support the idea that infiltration and activation of macrophages within plaques induce breakdown of the fibrous capsule leading to an increased likelihood of plaque rupture. For instance, lipid-laden macrophages from human atherosclerotic plaques secrete MMP-1 and MMP-13 (221) and culturing macrophages with fibrous caps of human plaques induces MMP-dependent collagen degradation via macrophage-derived MMP-3 (222). MMP-3, MMP-7, and MMP-12 mRNA expression in coronary lesions also co-localize with clusters of lipid-laden macrophages found in shoulder areas of plaques and along the capsular border of human carotid plaques (223, 224). Based on immunostaining, the distribution of MMP-2, TIMP-1, and TIMP-2 is uniform within lesion-free arteries whereas MMP-1, MMP-3, and MMP-9 levels are higher within the endothelium overlying plaques, in the plaque core, and in vascular smooth muscle cells (VSMCs) adjacent the fibrous cap and shoulders of the lesions (225, 226). In addition, MMP-8, a collagenase, is more prevalent in shoulders of plaque lesions from patients exhibiting symptoms than in plaque shoulders of non-symptomatic patients (227). Oxidized low-density lipoproteins within the developing plaque also induce MT1-MMP and MMP-9 expression in macrophages while reducing TIMP-1 expression in these cells (213, 228).

Macrophage migration-inhibitory factor released by macrophages and VSCMs within plaques, increases MMP-9 expression and is elevated in both cells types in vulnerable plaques but not in plaques having thicker fibrous capsules (214). MMP-2, MMP-9, MMP-12, and MMP-13 levels increase in plaques forming in ApoE-deficient mice fed a high-fat diet but this does not occur in ApoE-deficient mice fed a low-fat diet or in wild-type mice (229). Collectively, these and other studies show plaque development and plaque instability are associated with increases in overall MMP activity in areas occupied by macrophages. However, it should be noted that increases in macrophage MMP expression do not always correlate with increases in plaque formation. For example, macrophage-specific miss-expression of MMP-1 in ApoE-deficient mice fed a high-fat diet form fewer atherosclerotic lesions then those with normal macrophages (230).

Inflammatory cytokines and growth factors released by macrophages entering the site of a developing plaque up-regulate MMP expression in VSMCs as well (including MMP-1, MMP-2, MMP-3, and MMP-9, 231, 232-234). As a consequence, VSMCs migrate from the tunica media into the intima and undergo proliferation, thereby narrowing the vascular lumen (235, 236). The expression of several MMPs in macrophages and VSMCs also increase in several different vascular injury models (221, 224, 237, 238) as well as in injuries associated with surgical removal and grafting of saphenous veins (239). In a focal endothelial injury model leading to intimal thickening, MMP-2 and MMP-9 expression increase within the neointima (240). VSMC of mice lacking MMP-2 do not migrate into the intima nor do they exhibit hyperplasia after experimentally-inducing vascular remodeling (241, 242). VSMC from mice lacking MMP-9 also have a reduced capacity to migrate, proliferate, and invade the intima (243, 244). While both MMP-2 and MMP-9 appear to be required for neointimal formation, it appears that MMP-9, together with CD44, may be primarily responsible for the ongoing reorganization of fibrillar collagen occurring during neointimal formation (242).

In vivo, the principle mechanism for proMMP activation is through the proteolytic action of MT-MMPs, and in the case of proMMP-2 and proMMP-9, it involves MT1-MMP or MT3-MMP (60, 245, 246). MMP-2 is rapidly converted to its active form in injured arterial walls and wounded VSMC cultures (237, 238, 247). Studies show both macrophages and VSMC within human plaques express MT1-MMP and MT3-MMP and that cytokines released by activated macrophages increase MT1-MMP and MT3-MMP expression in VSMCs (213, 215). Hence, MT-MMPs, in addition to their own ability to degrade ECM, likely mediate the degree to which MMP-2 and MMP-9 functionally contribute to plaque formation, stability, and intimal thickening. However, few studies have yet to investigate the role of this family of MMPs in plaque formation and stability.

Given the evidence that MMP activity is an important determinant in plaque development and stability, recent studies are exploring the possibility that MMP inhibition or limiting MMP bioavailability may slow the progression of plaque development and help stabilize plaques. Systemic delivery of non-selective synthetic MMP inhibitors does not reduce plaque mass in LDL-deficient mouse models but it does attenuate aortic elastin degradation and ectasis in these mice (248). In contrast, blocking MMP activity with native inhibitors or eliminating synthesis of particular MMPs, genetically, does restrict neointimal formation. As mentioned, VSMCs of mice lacking MMP-2 do not exhibit hyperplasia after vascular injury (241, 242). Mice deficient in both MMP-9 and ApoE and fed a high-fat diet develop significantly fewer advanced atherosclerotic lesions than their wild-type MMP-9, ApoE-deficient counterparts (249). This is not true for mice deficient in both MMP-12 and ApoE, showing differential roles for these two MMPs in plaque development (249). In TIMP-1-deficient mice, intimal thickening induced by vascular injury is significantly greater compared to wild-type mice (250). However, mice with MMP-11 deficiency have enhanced neointimal formation 2-3 weeks after injury (251). Why this occurs is unknown but could be related to the fact MMP-11 cleaves alpha1-proteinase inhibitor (252), an important inhibitor of elastase activity (253, 254). Therefore, one might speculate that because of the MMP-11 deficiency, levels of alpha1-proteinase are higher thereby restricting elastase activity. Regardless, the consensus from these studies is that MMPs play important roles in enabling VSMC invasion into the intima and in stimulating VSMC hyperplasia after vascular injury.

Genetic diversity in MMP genes can have an impact on the progression of disease. A common polymorphism in the MMP-9 promoter region increasing MMP-9 transcription is associated with increased severity of coronary artery disease (255). Higher plasma levels of MMP-9 appear to be a prognostic indicator of cardiovascular mortality in patients with coronary artery disease (256, 257). The link between coronary disease and higher MMP-9 levels is consistent with studies showing overexpression of MMP-9 using an adenoviral vector, increases intravascular thrombi formation in porcine coronary arteries after balloon injury; the effect being inhibited by TIMP-1 (258). In mice, the level of MMP expression in atherosclerotic lesions also depends on genetic backgrounds. C3H/HeJ mice deficient in ApoE show increased MMP-2 and MMP-12 levels in their aortas and macrophages when compared to ApoE-deficient C57B1/6 mice. While MMP-9 activity levels in the aortic tissues are comparable in these mice, MMP-9 activity is higher in the macrophages of C3H/HeJ mice (259). Although C3H/HeJ mice develop smaller atherosclerotic plaques than C57B1/6 mice, the outer elastic tunics are more disrupted, eroded, and fragmented than in C57B1/6 mice (259). These studies show MMP-related genetic differences play important roles in the development of pathophysiological defects.

7.1.2. Restenosis

Balloon angioplasty followed by stent implantation is a common procedure used to restore blood flow, particular coronary blood flow. Unfortunately there is

a 25-40% reoccurrence of symptoms within 6 months because of restenosis by VSMC migration and intimal hyperplasia (260, 261). Therefore, development of therapies directed toward preventing restenosis would be of great value.

Many studies have focused on VSMC migration and growth and the synthesis and turnover of ECM within the intima because these are key characteristic features of restenosis after balloon angioplasty (262-264). Using a double injury model in rabbits, Li et. al. (265) reported increases in both MMP-2 and MMP-9 levels and cell proliferation in stented vessels. MMP-2 activity increases in the days following balloon injury and correlates positively with increases in the degree of collagen degradation and VSMC migration (237, 266). In patients, blood collected from the coronary sinus show increases in MMP-2 levels within 4 hr of balloon angioplasty with no change in TIMP-2 levels (267) and MMP-9 levels increase significantly within coronary sinus blood collected immediately after 60 seconds of balloon expansion (268). Increases in MT1-MMP protein levels and active MMP-2 (with no change in proMMP-2 protein levels) also occur in the tunica media in response to balloon-induced vascular injury and these increases depend on phosphatidylinositol 3-kinase signaling (247). Inhibitors of phosphatidylinositol 3-kinase prevent the increase in MT1-MMP and MMP-2 activity and blocks VSMC migration and neointimal hyperplasia (247). MMP-3 may also play an important role in restenosis. MMP-3 degrades many ECM components and activates other secreted proMMPs (269-271). MMP-3 is prevalent in human plaque material and is expressed by both macrophage foam cells within plaques and intimal VSMCs (223, 225, 272). MMP-3 levels quickly rise and VSMC migration and proliferation ensues following mechanical injury of VSMCs. These effects are inhibited by MMP-3 antisense oligonucleotides (273). TIMP-2 gene transfer also inhibits VSMC migration and delays neointimal development in balloon-injured rat carotid arteries (274) further supporting the idea that MMP activity plays an integral role in neointimal development after angioplasty.

Studies suggest the intimal hyperplastic response due to stent implantation is greater than that of balloon angioplasty alone. In rabbit iliac vessels, MMP-9 mRNA levels rapidly increase after vascular injury but they are almost twice as high in stented vessels than in those subjected to balloon angioplasty alone (275). Moreover, active MMP-9 is detected within 1 day after injury but only in the stented vessels where it remains elevated for up to 60 days. In contrast, active MMP-2 is first detect 7 days after balloon angioplasty with or without stenting and remains elevated for up to 60 days (275). Moreover, cell proliferation is nearly 50% greater in stented vessels, mostly in cells surrounding the border of the stent, compared to those subjected to balloon angioplasty alone (265). These data suggest the vascular response to stent implantation is different from balloon angioplasty alone, at least with regard to MMP-2 and MMP-9 activities.

Several different approaches have been used to try and prevent restenosis including the use of stents delivering immunosuppressive drugs (*e.g.*, rapamycin, cyclosporine, sirolimus, etc.), anti-inflammatory drugs (*e.g.*, dexamethasone), anti-proliferative drugs (*e.g.*, paclitaxel), tyrosine receptor kinase inhibitors, and MMP inhibitors. Marimastat and GM6001, both non-selective synthetic MMP inhibitors, block restenosis in stented vessels without changing cell proliferation or intimal collagen content (265, 276). In culture, the migration and proliferation of rat aortic smooth muscle cells is inhibited by Batimastat, a non-selective synthetic MMP inhibitor (277). However, Batimastat does not significantly influence the degree of neointimal thickening following stenting of atherosclerotic porcine femoral arteries (278). In primates with genetic propensities for developing atherosclerosis, the non-selective synthetic MMP inhibitor, RO113-2908, also fails to prevent intimal thickening and restenosis in response to angioplasty (279). These conflicting results regarding the effectiveness of MMP inhibitor treatment may stem from the use of different animal models or differences in the ability of each synthetic MMP inhibitor to effectively block specific MMPs (*e.g.*, MMPs versus ADAMs having MMP-like activity). In addition, VSMC heterogeneity and embryological origin may generate different injury reactions and responses to MMP inhibitor treatments (280, 281).

Recent studies suggest blocking alphaV beta3 integrin signaling inhibits neointimal hyperplasia and restenosis by altering MMP levels. An antibody antagonist to the beta3 integrin subunit decreases proMMP-9, proMMP-2, and active MMP-2 levels and inhibits VSMC migration in injured rat carotid arteries compared to untreated injured vessels without changing TIMP-1 and TIMP-2 levels (282). In a balloon injury model, either Batimastat or alphaV beta3 integrin-inhibitory peptides can inhibit neointimal formation but they are much more effective in preventing constrictive remodeling of balloon injured vessels when combined (283). Moreover, the degree of restenotic inhibition correlates with a decrease in MMP activity (283). *In vitro*, a cyclic peptide antagonist for the alphaV integrin subunit blocks migration of VSMCs and this effect is accompanied by decreases in focal adhesion kinase activity and MMP-2 secretion (284). Not only does alphaV beta3 integrin signaling increase MMP-2 synthesis in some cells (285), this integrin also binds proMMP-2 enabling proMMP-2 activation and provides a mechanism by which cells can localize MMP-2 activity to particular regions of its cell surface (286). This suggests at least two possible routes by which alphaV beta3 antagonists could inhibit neointimal hyperplasia, *i.e.*, preventing proMMP binding, activation, and directed proteolysis, and/or decreasing MMP synthesis induced through the alphaV beta3-signaling cascade.

The use of endogenous MMP inhibitors has shown promise in limiting restenosis and neointimal thickening in animal models. Neointimal formation is much more pronounced after vascular injury in TIMP-1-deficient mice (250). Local overexpression of TIMP-1 in virally-transfected VSMC cells of balloon-injured rat carotid

arteries decreases the number of VSMC found with the intima and inhibits neointimal hyperplasia (287-291). A peptide-targeted adenovirus encoding TIMP-1 also inhibits restenosis induced by balloon endothelial denudation in rabbits (292). In addition, adenoviral delivery of TIMP-1 into ApoE-deficient mice fed a high-fat diet reduces atherosclerotic lesion size, reduces the number of macrophages in the lesions, and increases the collagen, elastin, and VSMC alpha-actin content of the tunica media (293). Adenoviral delivery of TIMP-2 at the time of balloon-induced injury also inhibits VSMC migration into the intima and attenuates neointimal thickening in rat carotid vessels (274). These studies suggest that manipulating TIMP levels may offer a better therapeutic approach to limiting restenosis after angioplasty than non-selective synthetic MMP inhibitors.

Late graft failure occurring because of neointima formation and subsequent atherosclerosis complicates the use of autologous saphenous vein or internal thoracic arterial grafts in coronary bypass surgery. Overexpression of either TIMP-2 or TIMP-3 via an adenoviral system prevents neointimal formation and VSMC cell migration in organ cultures of saphenous vein graphs (294). Similarly, overexpression of TIMP-1 in human saphenous vein explants inhibits neointimal formation by more than ~50% (295). However, only TIMP-3 prevents this from occurring in vein-to-carotid artery grafts *in vivo* (296). The mechanisms behind graft failure are further complicate by the fact the vessels taken from different regions have inherently different of levels MMPs and extracellular MMP-inducing proteins (297). With a better understanding of these differences and appropriately manipulating the MMP-to-TIMP stoichiometry, we may be able to reduce the incidence of graft failure.

7.2. MMPs and Aneurysms

Aneurysms may be considered as a pathological type of expansive remodeling and MMPs play a major role in aneurysm formation. Tissue remodeling is a prominent feature of degenerating vascular walls and the loss of structural integrity is associated with a decrease in collagen and elastin content (298, 299). Studies in MMP-deficient mice show MMPs play key roles in the development of aneurysms. MMP-2- and MMP-9-deficient mice do not develop aneurysms under experimental conditions that normally induce aortic aneurysms in wild-type mice (300, 301). Loss of MMP-9 and MMP-12 gene expression in ApoE-deficient mice also protects against atherosclerotic-related media thinning and ectasis (249). Interestingly, re-infusion of active macrophages or bone marrow transplants from wild-type mice into MMP-9-deficient mice re-establishes the development of aneurysms in these deficient mice (300, 301).

In humans, the MMP content in the regions of aneurysms is much higher compared to healthy vessels and likely contributes to the decrease in collagen and elastic content of the vascular tunic in these regions (302-309). Plasma levels of MMP-9 are also higher in patients with aortic aneurysms (310-312). Genetic polymorphisms in the MMP-9 promoter also suggest differences in MMP-9 activity within the vessels comprising the circle of Willis thereby leading to increased susceptibility to intracranial aneurysm formation (313). Studies are ongoing to determine if MMP-9 and other MMP polymorphisms might serve as useful diagnostic tools for aneurysms (256, 311).

TIMP-1-deficient mice exhibit aortic medial ruptures and disrupted elastic fibers and much of the MMP activity is associated with macrophages found within the media (314). In ApoE-deficient mice, deletion of TIMP-1 gene reduces plaque size but increases the potential for aneurysm formation (315). In contrast, local overexpression of TIMP-1 prevents aortic aneurysm degeneration and rupture in a rat model (316). As TIMP-1 is an effective inhibitor of MMP-9 activity, this suggests a miss-balance between MMP-9 activity and TIMP levels plays a key role in the development of aneurysms.

While non-selective synthetic MMP inhibitors have been relatively ineffective in preventing atherosclerosis, studies suggest synthetic MMP inhibitors may be more useful in preventing the formation or slowing the progression of aneurysms. In femoral-arteriovenous shunt models that stimulate flow-mediated enlargement of iliac vessels, MMP-2 activity increases in iliac vessels within 3 days and the degree of iliac enlargement is attenuated by synthetic MMP inhibitors (317-320). Aneurysmal dilation and aortic medial elastin destruction usually occurring in LDL-receptor-deficient mice or induced by brief endothelial elastase treatment, is also attenuated by MMP inhibitors (248, 299, 321, 322). Doxycycline, an antibiotic and nonspecific inhibitor of MMPs, has no significant effect on development of atherosclerosis in LDL-receptor-deficient mice but markedly decreases the frequency of aortic aneurysm formation in these mice (323). Doxycycline also inhibits aneurysm development in experimentally-induced aneurysm models at concentrations within the dose range used in humans as an antibiotic (324). Doxycycline decreases levels of both latent and active forms of MMP-2 and MMP-9 released from explants of human aortic aneurysm vessels (325) but only MMP-9 levels decrease in aneurysm tissues taken from patients receiving preoperative treatment with doxycycline (but it has little or no effect on MMP-2, 326). Doxycycline is well tolerated by patients even after prolonged treatment and is capable of reducing the higher MMP-9 plasma levels found in patients with small asymptomatic abdominal aortic aneurysms, back to normal (327). Recently, it has been shown that a derivative of doxycycline lacking antibiotic characteristics but retaining MMP inhibitory activity, still inhibits VSMC migration into the intima and prevents intimal thickening in a rat carotid injury model (328). Therefore, doxycycline and other MMP inhibitors offer some promise as possible therapeutic agents for preventing or treating aneurysms.

7.3. MMPs and Progressive Heart Failure

In response to hypertension, elevated ventricular wall stress, or injury, the heart attempts to functionally compensate by remodeling. One of the earliest responses to an acute myocardial infarction or unstable angina is an increase in the expression and activity of MMP-1, MMP-2, and MMP-9 (329-333). MMP-2, MMP-13, MT1-MMP, and TIMP-2 levels are also up-regulated in congestive heart failure (334, 335). The type of overload (*i.e.*, pressure overload versus volume overload) associated with

hypertension and congestive heart failure have differential effects on MMP and TIMP expression within the myocardium (336). MMPs (*e.g.*, MMP-9) are usually expressed at low levels in normal myocardium but are up-regulated with acute pressure and volume overloads and eventually return to baseline levels with chronic pressure and volume overloads (337). MMP-3 levels increase with acute volume and chronic pressure overloads (337) while MMP-1 levels decrease with volume overload but do not change with pressure overload (337). Interestingly, unloading the heart with a ventricular-assist device also down-regulates MMP levels and increases TIMPs levels in human heart tissue biopsies (338). Hence, levels of specific MMPs and MMP inhibitors expressed within the heart depend on the type of overload it is experiencing.

The increases in active MMPs levels associated with cardiac overloading remain elevated for several days (337, 339). Spontaneously hypertensive rats exhibit significant left ventricular hypertrophy, dilation, increase chamber compliance, and eventually succumb to congestive heart failure by 9-13 weeks. During the early phase, significant elevations in MMP activity occur in both spontaneously hypertensive rats and in chronic overload models of hypertension (334, 340-343). This is followed by a loss of collagen content and integrity and eventually leads to progressive heart dilation (344-346). In response, cardiac fibroblasts increase the deposition of several ECM components including collagen type-I, collagen type-III, collagen type-IV, collagen type-VI, fibronectin, and laminin and cardiac myocyte increase their actin content (347, 348), all in an attempt to compensate for the dilation (349-352). While these compensatory events are somewhat effective at first, after several weeks, right and left ventricular hypertrophy recommences even though collagen levels and activity of some MMPs (particularly MMP-2) return back to normal (353, 354). Without any intervention, this hypertrophy continues and the heart eventually fails when again, MMP levels become elevated and myocardial fibrosis increases (342, 354). In both spontaneous hypertensive rat models and chronic overload models, transition into the decompensation (failure) phase is slowed by treatment with MMP inhibitors if given before this phase begins (340, 341). Synthetic non-selective MMP inhibitors also limit left ventricular dilation and reduced wall stress during development of congestive heart failure in experimental porcine models (355). In addition, the degree of ECM degradation in the ventricular walls is less in the MMP inhibitor-treated animals (355). MMP inhibitors also attenuate left ventricular dilation after induction of myocardial infarcts in mice (356). Unfortunately, loss of MMP activity or treatment with MMP inhibitors delays infarct healing (357).

Disruption of the normal equilibrium between ECM synthesis and degradation plays a major role in cardiac remodeling and pathophysiology. Constitutive expression of human MMP-1 in the hearts of mice (there is no MMP-1 gene homolog in mice, 358) results in a 20% mortality rate within 6 months and a loss of interstitial collagen associated with compensatory cardiac hypertrophy (359). After experimentally-induced myocardial infarction, MMP-9- and MMP-2- deficient mice exhibit less left ventricular enlargement and cardiac rupture than wild-type mice (357, 360, 361). Loss of TIMP expression is also associated with progression of heart failure (362, 363). Deletion of either the TIMP-1 or TIMP-3 gene results in the development of left ventricular dilation and hypertrophy (362, 364) and accelerates post-myocardial infarct remodeling (365). These studies suggest that TIMPs are necessary for limiting MMP activity under normal conditions and show that many of the detrimental changes observed with angina and myocardial infarction are likely due to a shift favoring increased MMP activity.

8. PERSPECTIVES

It is obvious MMPs and their inhibitors play important roles in cardiovascular development and progression of cardiovascular disease. Much has been learned regarding the spatial and temporal changes in MMPs and their activities during the development and progression of cardiovascular disease. However, there is profound lack of understanding regarding the precise relationship between MMP and MMP inhibitors and the cause and effect of these diseases. For example, are the observed changes in MMP levels responsible for the development of cardiovascular disease or are they secondary consequences of the disease process? If changes in MMP activity are the cause, what are the targets or initial consequences of this activity and how do the various cell types in the affected areas respond? How does this translate into functional pathological changes within the organ? The functional roles for MMPs during embryonic development and the development cardiovascular diseases are generally attributed solely to the catalytic properties of MMPs. Little attention has been given to the possibly that other domains within MMPs and MMP inhibitors might have important functional properties or consider that bioactive peptides generated from their own turnover might have roles in these processes. The importance of these considerations are evident from recent studies showing TIMP-2 inhibits angiogenesis independently from its MMP inhibitor function (111, 114) and that naturally-generated peptide fragments of MMP-2 lacking catalytic activities block angiogenesis (366).

Important clues to the onset and development of cardiovascular disease and therapeutic intervention will stem from work investigating the role MMPs and TIMPs during embryonic development. Many of the same basic biological processes necessary for establishing a functioning cardiovascular system *in utero* are recapitulated during the development of cardiovascular disease. For instance, the decision as to whether a blood vessel becomes an artery or vein during development and the subsequent functional alterations, mimic the remodeling events occurring during adult vascular pressure overloading and expansive remodeling in response to vascular stenosis. Angiogenic responses to chronic cardiac ischemia require the same basic cell-biological steps as takes place during embryonic angiogenesis. Determining the functional and responsive differences in VSMCs with different embryological origins may elucidate why vascular grafts from taken from different regions have different MMP and

MMP inhibitor characteristics and respond differently when used in coronary bypasses. Using genetically-modified animals with altered MMP and TIMP stoichiometries, genetically-modified domains, and temporal and tissue-specific alterations in MMP expression will greatly enhance our understanding of the complex interplay between cells and tissues mediated by these molecules. Identification of new polymorphisms within genes of MMPs, TIMPs, and their transcriptional regulators and studying their effects on cardiovascular development and disease will help us better understand the etiological basis of these diseases and provide invaluable diagnostic tools.

Even with our current limited understanding, experimental and clinical studies indicate that by managing MMP and MMP inhibitor levels, we will be able to someday prevent or delay the detrimental changes accompanying cardiovascular disease. But before effective therapeutic agents can really be developed, we need a much better understanding of the cause and effect relationship between MMPs and congenital cardiovascular defects and cardiovascular diseases.

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