Role of Matrix Metalloproteinases in the Development and Progression of Atherosclerosis

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Karina Di Gregoli and Jason Lee Johnson

Abstract

Atherosclerosis underlies the majority of cardiovascular diseases and is accepted as a primary cause of mortality worldwide. Matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) perform complex roles during the progression and development of atherosclerosis and subsequent plaque instability. Proposed actions of MMPs include extracellular matrix remodeling alongside regulation of vascular cell proliferation, migration and apoptosis including cell types such as monocytes, macrophages and vascular smooth muscle cells. As such, a large body of evidence from both in vitro and in vivo studies has shown that individual MMPs and TIMPs are utilized by distinct cell types to regulate their behavior. Consequently, it is now accepted that some MMPs promote the growth and development of advanced atherosclerotic plaques in experimental models whilst others do not. Similarly, human genetic and pathological findings reveal some MMPs correlate with vulnerable atherosclerotic plaque phenotypes, whereas others associate with stable lesions. Furthermore, broad-spectrum MMP inhibition in both mouse and man has proved ineffective at protecting from atherosclerotic plaque progression and instability. Considering the divergent effects MMPs exert on atherosclerotic lesions, selectively targeting individual deleterious MMPs may serve as a more efficacious therapeutic strategy. For example, our recent data demonstrate that a selective MMP-12 inhibitor retards atherosclerotic plaque progression in the apolipoprotein E (Apoe) mouse atherosclerosis model, whilst also promoting plaque stabilization through reducing monocyte recruitment into plaques whilst augmenting fibrosis. Similar studies have been conducted assessing MMP-13

K. Di Gregoli · J.L. Johnson (✉)
Laboratory of Cardiovascular Pathology, Faculty of Health Sciences, School of Clinical Sciences, University of Bristol, Level 7, Bristol Royal Infirmary, Marlborough Street, Bristol BS2 8HW, England, UK
e-mail: Jason.L.Johnson@bris.ac.uk

S. Chakraborti et al. (eds.), *Proteases in Human Diseases*,
DOI 10.1007/978-981-10-3162-5_20

inhibition. Accordingly, as our knowledge of the complex roles MMPs play during the development, progression and rupture of atherosclerotic plaques expands, new impetus is required for clinical trials evaluating the therapeutic potential of selective MMP inhibition, especially in the context of atherosclerosis.

Keywords
MMPs · Macrophages · Atherosclerosis · Vascular smooth muscle cells · Plaque rupture

1 Introduction

Atherosclerotic plaque development and progression is the principal underlying cause of cardiovascular disease, now reported as the primary cause of mortality and morbidity in developed countries [1]. Atherosclerosis is characterized by the accumulation of lipids (atheroma) and fibrous elements (sclerosis) within major arteries sustaining the heart (coronary arteries) and the brain (carotid arteries) [1]. It has been defined as a chronic, autoimmune-like disease, which develops in the presence of elevated circulating lipid levels [2]. Atherosclerotic plaque formation and progression is usually clinically silent. However, plaque rupture followed by thrombus formation and subsequent vessel occlusion can precipitate several clinical events including myocardial infarction, stroke, and peripheral vascular disease. The main underlying trigger for plaque rupture is ascribed to the loss of extracellular matrix (ECM) proteins, such as elastin and collagen, alongside decreased smooth muscle cells content within the plaques, which commonly corresponds to areas of marked inflammation [3]. These areas are characterized by the presence of foam cell macrophages, B- and T-cells, mast cells and smaller amounts of other white bloods cells [4]. Over the last quarter of a century, a large number of pathological and experimental studies have been conducted in this field to elucidate the pathophysiology of atherosclerotic lesion development, progression, and rupture. One of the principal goals in cardiovascular research is to find suitable targets to allow the development of new therapies, aimed at specific cell types or select molecules, attributed deleterious roles in atherosclerotic disease onset and progression. Matrix metalloproteinases (MMPs) have been implicated in all the stages of atherosclerosis, from plaque development to plaque rupture, through a large body of published work [5, 6]. Elevated expression levels of MMPs including MMP-1, -2, -7, -8, -9, -11, -12, -13, and -14 have been identified in human atherosclerotic plaques (see Table 1) [7–15]. Moreover, the majority of increased MMP expression within atherosclerotic lesions is specifically located to macrophages-rich areas (shoulder regions and around the lipid core) suggesting that macrophage-derived MMPs may

Table 1 MMPs up-regulated in human atherosclerotic plaques compared to normal arteries

MMP#	Cell type	Principal reference
MMP-1	Mø, VSMC, EC, and T cell	[7]
MMP-2	Mø and VSMC	[8]
MMP-3	Mø, VSMC, EC, and T cell	[9]
MMP-7	Mø	[10]
MMP-8	Mø, VSMC, and EC	[11]
MMP-9	Mø, VSMC, EC, and T cell	[7]
MMP-11	Mø, VSMC, and EC	[12]
MMP-12	Mø	[10]
MMP-13	Mø	[13]
MMP-14	Mø and VSMC	[14]
MMP-16	Mø and VSMC	[15]

Mø Macrophage, *VSMC* vascular smooth muscle cell, *EC* endothelial cell

play a key role in atherosclerotic plaque progression. Furthermore, considering MMPs have been proposed to induce plaque rupture in a dual manner: by direct degradation of ECM proteins (such as elastin and collagen) and by promoting the death of vascular smooth muscle cells (VSMCs), the main cell type responsible for ECM synthesis within the plaque [16]; many studies have focused their attention on inhibitors of MMPs as a therapeutic strategy to stabilize and perhaps induce regression of atherosclerosis [17].

2 Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs), also named matrixins, are a large family of at least 24 proteolytic enzymes having a role is several physiological and pathological processes such as morphogenesis, angiogenesis, tissue repair, wound healing and remodeling. For these reasons, MMPs are involved in several pathologies including cancer progression and atherosclerosis, highlighting them as key therapeutic targets for medical research. The MMPs are multi-domain enzymes capable to degrade both ECM components and several non-ECM molecules. MMPs share similarity with two other proteinase families: ADAMs (a disintegrin and metalloproteinase family) and ADAMTSs (ADAM with thrombospondin motifs), as they all contain a zinc atom and a conserved methionine in the catalytic domain, and collectively consist the Metzincin family [18]. Due to its destructive capabilities, MMP activity is tightly regulated by a family of endogenous inhibitors named the tissue inhibitors of metalloproteinases (TIMPs) that, together with MMPs, are responsible the maintenance and balance of ECM homeostasis during physiological and pathological conditions. This equilibrium contributes to multiple other processes such as differentiation, growth, inflammation, migration, and apoptosis due in part to the

capacity of MMPs to target non-ECM substrates. MMP and TIMP expression, which exhibit distinct tissue/cell, temporal and spatial differences, are tightly regulated by numerous molecules including inflammatory cytokines, hormones, growth factors, and physical cell–cell and cell–matrix interactions (as reviewed by [19]).

3 MMP Classification and Structure

MMPs share some structural homology. Usually they present:

(i) ***Signal peptide*** *at the N-Terminus*: a hydrophobic sequence of 18–30 residues responsible for intracellular trafficking from the Golgi apparatus to the cell membrane which is cleaved during secretion [19].
(ii) ***Pro-peptide***: a highly conserved motif responsible for pro-MMP latency [19].
(iii) ***Catalytic Domain***: which contains a zinc-binding site responsible for the endopeptidase activity of MMPs.
(iv) ***Hinge Domain***: known also as a linker peptide, it is situated between the catalytic domain and the hemopexin-like domain. It stabilizes the collagenolytic activity due to the presence of several proline residues.
(v) ***Hemopexin-like Domain***: positioned at the C-terminus, it has strong sequence similarity to the serum protein hemopexin and an extensive range of roles amongst diverse MMPs [18].

Nevertheless, there are notable structural differences between MMPs that confer diverse biological properties. Based on their domain organization, MMPs can be divided into six groups [20] (see Fig. 1).

(i) MMPs presenting the pro-domain and the catalytic domain. This group includes MMP-7 and MMP-26, also known as Matrilysins.
(ii) MMPs containing the pro-domain, the catalytic domain, the hinge domain, and the hemopexin-like domain. This group contains several MMPs with diverse substrate specificities; MMP-1, -8, -13 (Collagenases), MMP-3, -10, -11 (Stromelysins), MMP-12 (Metalloelastase), MMP-20 (Enamelysin), MMP-19, MMP-22, and MMP-28.
(iii) MMPs comprising the pro-domain, a catalytic domain containing fibronectin-like repeats, the hinge domain and the hemopexin-like domain. In this group there are MMP-2 and -9, also named Gelatinases for their affinity to degrade gelatin.
(iv) The transmembrane type I MMPs are a group of MMPs that present, together with the pro-domain, the catalytic domain, hinge domain and the hemopexin-like domain, a transmembrane domain at the N-terminus. This domain allows this group of MMPs to localize on to the cell membrane,

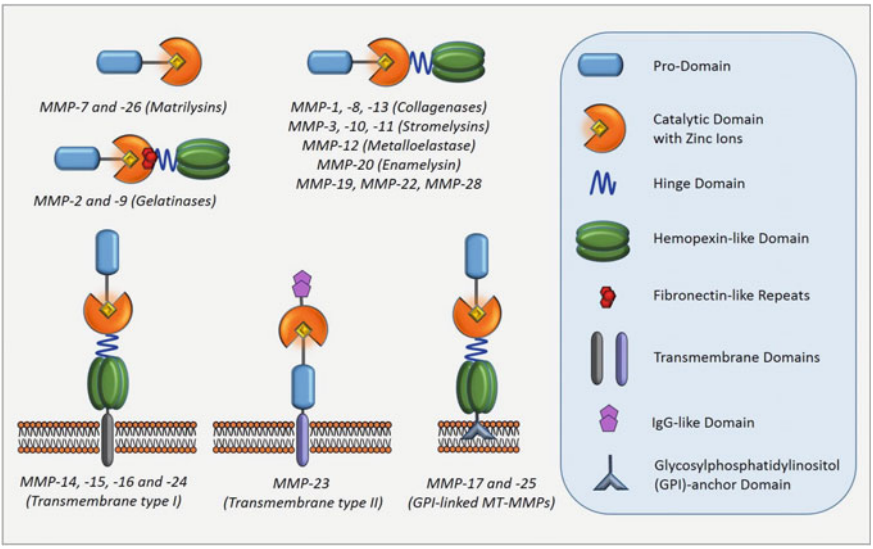

Fig. 1 Domain structure for the major classes of MMPs. Diagram illustrating the differing domain structures of the major MMP classes, including the pro-domain, catalytic domain with the active site zinc (Zn) bound to cysteine residues within this domain and "cysteine switch-residue" in the pro-domain, the hinge domain, the hemopexin-domain, the fibronectin-like type II repeats, and in some cases for MT-MMPs, either a transmembrane domain or a glycophosphatidylinositol (GPI)-anchor domain

projecting toward the extracellular space. This characteristic facilitates pericellular matrix degradation, and hence plays a prominent role in directing cell migration [21]. This group includes MMP-14, -15, -16, and -24.

(v) The transmembrane type II MMPs include one single MMP, MMP-23. Differently from the type I, type II MMP has the transmembrane domain at the C-terminus and additionally present an IgG-like domain.

(vi) There is a third group of membrane-type MMPs localized to the cell membrane via glycosylphosphatidyl inositol (GPI) anchor on the N-terminus. This group includes MMP-17 and -25.

4 MMP Activation and Inhibition

MMPs are produced as zymogens; the interaction between the pro-domain and the catalytic domain keep the MMP in an inactive conformation. In order to achieve full activation of these enzymes the pro-domain has to be cleaved, an essential regulatory step toward MMP activation [19]. Activation of the biologically inactive MMP (pro-form) follows a multi-step sequence of events also known as 'stepwise

activation'. First, the cleavage of a 'proteinase susceptible bait region' through the action of plasma or bacterial proteinases, destabilise the cysteine–Zn^{2+} negative interaction within the pro-domain, resulting in a MMP intermediate form. To achieve full activation, the *in-trans* activity of other intermediary or active MMPs is required in order to fully remove the inhibitory pro-domain [19]. Some MMPs are completely activated intracellularly by furin or other pro-protein convertases and then either translocated to the cell membrane or secreted, as active enzymes. MMP activity is closely regulated by endogenous inhibitors (such as TIMPs and α2-macroglobulin), proteolysis, or internalization and recycling [19]. In addition, other proteins with the ability to inhibit MMPs have been described, including the reversion-inducing cysteine-rich protein with Kazal motifs (RECK), tissue factor pathway inhibitor-2 (TFPI-2), and the pro-collagen C-terminal proteinase enhancer (PCPE). Nevertheless, TIMPs are the most potent endogenous inhibitors of MMPs and therefore considered key regulators in the physiological regulation of MMP activity. Four TIMPs have been identified within vertebrates, TIMP-1, -2, -3, and -4, which exhibit diverse inhibitory actions toward different MMP family members [22]. For example, TIMP-1 has a poor inhibitory effect on MMP-9, -14, -15, -16, and -24. TIMPs also harbor the ability to inhibit members of both the ADAM and ADAMTS family of proteinases [22]. TIMP expression is tissue specific and, similarly to MMPs, is finely regulated during development and remodeling. Most of their inhibitory capacity has been ascribed to the N-Terminal domain since it is able to form, when isolated, a stable native molecule with an inhibitory effect on MMPs [23]. TIMPs are normally secreted proteins, however they can localize to the cell membrane associated with membrane protein, including several MT-MMPs. Regulation of the equilibrium between MMPs and TIMPs is essential in homeostasis. Alterations in this balance can trigger patho-physiological conditions associated with atypical ECM turnover of the matrix and/or dysregulation of processes involved in wound healing, remodeling and inflammation. Cardiovascular disease, cancer, arthritis, and neurological disorders are all examples of pathologies where an imbalance between MMPs and TIMPs is apparent [23].

5 MMPs and Atherogenesis

5.1 Early Stage: Pathological Intimal Thickening

Atherogenesis is a multi-step sequence of events that leads to atherosclerotic plaque formation on the luminal side of major arteries. In humans, it is a process that develops and evolves over several decades, beginning with early lesions that can occur during childhood. The development of atherosclerotic lesions is dependent on multiple risk factors which can be genetic or modifiable in nature, including hypercholesterolemia, smoking, high blood pressure, sedentary lifestyle, and diabetes [24]. In man, the first event that generally occurs branch points within major arteries, is the formation of an early lesion, commonly termed pathological intimal

thickening (although sometimes referred to as fatty streaks) [25]. These early lesions are characterized by the accumulation of lipid-laden macrophages (also called foam-cell macrophages due to their appearance under the microscope), within a preexisting smooth muscle and ECM-rich intima. Raised levels of low density lipoprotein (LDL) within the blood stream, alongside alterations in shear stress, the presence of free radicals such as reactive oxygen species (ROS), or exposure to infection-related pathogens, can result endothelial damage. A damaged endothelium is subject to inflammatory activation that triggers expression of adhesion molecules (including vascular cell adhesion molecule-1; VCAM-1) that mediate leukocyte recruitment. Such adhesion molecules facilitate a transient contact, allowing leukocyte rolling at the luminal surface of the vessel wall. After firm adhesion to the endothelium, monocytes and lymphocytes transmigrate, penetrating into the *tunica intima* (the innermost layer of the artery) driven by a chemoattractant gradient, through molecules including monocyte chemoattractant protein-1 (MCP-1). Monocyte recruitment is considered a fundamental process during early lesion formation and atherosclerosis onset. In order to invade the arterial wall, monocytes are required to degrade the physical barrier represented by the ECM, therefore it is essential that they possess potent protease activity. Human monocytes constitutively express several MMPs and TIMPs including MMP-8, MMP-12, MMP-19, TIMP-1, and TIMP-2. Whereas upon adhesion and in response to inflammatory stimuli they can be activated and subsequently upregulate the expression of MMP-1, MMP-3, MMP-10, and MMP-14 via the stimulation of MAP kinase and NF-κB transcription factors [26]. Specifically, MMP-14 expression and activity is necessary for monocyte endothelial transmigration and invasion this process can be blocked by MMP-14 inhibition either by a neutralizing antibody, recombinant TIMP-2 or gene silencing in vitro [27–30]. Moreover, MMP14 inhibition of activated circulating monocytes by a neutralizing antibody, retards monocyte recruitment into existing atherosclerotic lesions in mouse model of atherosclerosis [30]. Once within the intima monocytes differentiate into macrophages in response to several stimuli, in particular the Colony Stimulating Factors (CSFs), which concomitantly drives the expression of scavenger receptors, growth factors, cytokines giving rise to a survival impulse [31]. Accordingly, recently recruited monocytes at sites within the artery where lipoproteins have accumulated and after their differentiation into macrophages, begin to internalize the modified lipoproteins from the surrounding areas, through their cell-surface scavenger receptors. This process results in transformation of macrophages into foam-cells macrophages (FCMs) [32]. Macrophages also interact with T-cells which are also recruited to developing plaques, inducing an array of immune and inflammatory responses including the expression of adhesion molecules, MMPs, cytokines, apoptotic mediators, and pro-thrombotic activities, which collectively drive an inflammatory amplification loop and therefore promoting atherosclerotic plaque progression [33]. Intra-plaque macrophages and foam-cells express a diverse range of MMPs and TIMPs [34]. In particular, it has been observed that MMP-7, MMP-9, and TIMP-3 expression is induced during macrophage differentiation in vitro [26], whereas MMP-1, MMP-3, and MMP-12 expression can be induced in macrophages

in response to inflammatory mediators and cytokines [34]. However, within atherosclerotic plaques, most macrophages are lipid-laden and therefore characterized as foam cell macrophages, therefore the accumulation of lipid within macrophages may exert the most dominant role on MMP and TIMP regulation. Indeed, immunohistochemistry (a valuable method for studying atherosclerotic plaque composition alongside macrophages and foam-cell macrophages in situ), has revealed the expression of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-11, MMP-12, MMP-13, MMP-14, and MMP-16 in foam-cell macrophages within lesions (Table 1). Moreover, the detection of matrix proteolysis (as assessed by in situ zymography) [35, 36] alongside the presence of cleaved collagen fibres at the corresponding sites [13], suggest that at least some of the MMPs expressed within these regions are in an active form. Recent direct evidence has demonstrated that MMP-1, MMP-3, and MMP-14 are over-expressed in foam-cell macrophages isolated from human plaques [37, 38] and that in vivo generated foam-cell macrophages from cholesterol-fed rabbits display heightened expression of MMP-1, MMP-3, MMP-12, and MMP-14, when compared to nonlipid-laden macrophages [39]. Additionally, increased expression and activity of MMP-14 in a sub-population of rabbit foam cell macrophages was associated with a concomitant loss of TIMP-3 expression, resulting in their increased invasiveness, proteolytic activity, and susceptibility to undergo apoptosis [40]. Therefore, the presence of foam-cell macrophage-derived MMPs within the atherosclerotic lesions may direct disease progression and predict future clinical outcome.

5.2 MMPs and Atherosclerotic Plaque Progression

One of the principal processes that determines the progression of a pathological intimal thickening toward the development of a mature atherosclerotic plaque is the formation of a fibrous cap which overlies a recently formed lipid core. The fibrous cap originates following the organized migration of vascular smooth muscle cells (VSMCs) from the *tunica media* (the middle layer of the artery that lies between the tunica intima on the inside and the tunica externa on the outside) toward the arterial lumen, alongside the continual growth of VSMCs already resident within the intimal thickening. The VSMCs overlying the lipid core proliferate and produce fibrous ECM components, such as collagen and fibronectin, providing a structural barrier that separates the thrombogenic lipid core from the blood stream, providing strength and hemodynamic stability to the developing lesion. In addition to the production of MMPs and TIMPs, macrophages within the plaque secrete numerous cytokines and mediators such as platelet-derived growth factor (PDGF), heparin-binding epidermal growth factor (EGF), and insulin-like growth factor (IGF) that facilitate the mobilization and recruitment of VSMCs [41]. In order to expedite their migration, VSMCs need to release themselves from their cell–cell and cell–matrix interactions which act as physical barriers—dysregulated MMP activity directs this process. Studies investigating the role of MMP activity on VSMC migration have focused their attention principally on MMP-2, MMP-9, and

MMP-14, presumably due to these MMPs harboring the ability to degrade the basement membrane protein collagen type IV [16]. MMP-2 has been shown to augment VSMC migration across basement membrane proteins in vitro [42, 43], and MMP-9 overexpression can also promote the migratory capacity of isolated VSMCs [44]. A comprehensive study revealed MMP-14 is critical during VSMC migration, facilitating VSMCs to first degrade and then infiltrate 3-D collagen barriers, including the arterial wall [45]. These findings have been substantiated through subsequent in vivo studies utilizing genetically modified mice lacking either MMP-2, MMP-9, or MMP-14 which all reported attenuated VSMC migration. MMP-3 has also been shown to promote VSMC migration, predominantly through the activation of MMP-9 [46]. A role for the collagenase MMP-13 in VSMC migration has been documented, induced through an Akt-ERK dependent pathway [47]. Additionally, MMPs can contribute to VSMC migration by cleavage of nonmatrix substrates. For instance, MMP-14 can cleave and shed from the cell membrane CD44 (a cell surface hyaluronan receptor), promoting increased cell motility [48]. Conversely, intact CD44 can serve as a docking station for secreted MMP-7 and MMP-9 on the VSMC membrane, localizing their proteolytic activity to the cell surface and potentially facilitating cell migration [49, 50]. MMP activity has also been linked to VSMC proliferation. Similarly to migration, proliferation requires the removal of cell–cell and cell–matrix interactions, which otherwise exert an inhibitory effect on cell division. Cadherins are a family of adhesion proteins involved in cell–cell contact regulation of proliferation, and have recently been identified as new substrates of MMP activity [51]. Cadherins also serve as membrane receptors for cell signaling transduction and their cleavage by MMPs can modulate β-catenin nuclear translocation (a member of Wnt/wingless signaling pathway), known to activate the transcription of several pro-proliferative genes [51]. Indeed, MMP-7 and MMP-12 can induce N-cadherin cleavage/shedding, and through β-catenin signaling, promote VSMC proliferation [52]. Taken together, MMP-directed VSMC growth and migration participates in fibrous cap formation and therefore plays a prominent role in atherosclerotic plaque formation—but is considered beneficial as it protects the developing plaque from instability.

5.3 MMPs and Unstable Plaque Development and Rupture

During atherosclerotic plaque progression, foam-cell macrophages undergo cell death via apoptosis or necrosis. Macrophage and foam-cell death promotes the establishment and expansion of an extracellular lipid-rich core, which is highly thrombogenic and harbors the potential to destabilise advanced atherosclerotic plaques. Unsurprisingly, a role for MMPs has been suggested in macrophage and foam-cell apoptosis [53]. For example, macrophage and foam-cell susceptibility to undergo apoptosis can be retarded by inhibition of MMP-12 or MMP-14 activity, through use of a selective inhibitor or a neutralizing antibody, respectively [40, 54]. Accordingly, TIMP-2 and TIMP-3 can both reduce foam-cell macrophage apoptosis, in part through inhibition of MMP-14-dependent N-Cadherin cleavage [30,

40, 55]. Furthermore, loss of TIMP-2 in vivo increases the number of apoptotic macrophages within atherosclerotic plaques of hypercholesteroleamic mice, whilst TIMP-1 depletion had no effect [30]. Therefore, uncontrolled cell death and subsequent lipid core enlargement contributes to plaque progression and is associated with plaque instability and propensity to rupture [56]. As can know be appreciated, the stability of atherosclerotic plaques is determined by its composition, specifically the VSMC and fibrous ECM content (which reflects the thickness and strength of the fibrous cap), together with the macrophage and lipid content (which reveals the size and possible rate of expansion of the lipid core) [57]. The vast majority of acute coronary events originate from atherosclerotic plaque instability, notably the rupture of the fibrous cap and ensuing leakage of the thrombogenic lipid core into the arterial lumen, triggering thrombosis [58]. As such, clinical symptoms, including myocardial infarction or stroke, are often a result of plaque rupture and subsequent thrombus formation, resulting in distal impairment of blood flow or embolization and consequent ischemia. Indeed, fibrous cap disruption leads to the exposure of highly thrombogenic plaque constituents such as tissue factor (TF), lipids or modified collagen fragments. The interaction of these factors with the flowing blood results in thrombus formation by triggering activation of the coagulation cascade [59]. As earlier discussed, mature atherosclerotic plaques are characterized by a soft and highly thrombogenic lipid-rich core and associated macrophages infiltration, which is encapsulated by a VSMC and ECM-rich fibrous cap that provides structural integrity [56]. However, atherosclerotic lesions are heterogeneous in nature and can vary in fibrous cap thickness and lipid-core size; different combinations of these two variables results in different plaque phenotypes and susceptibility to rupture, with diverse clinical outcome. Pathological studies of human coronary artery atherosclerotic plaques permit histological discrimination between stable and unstable (also defined as vulnerable or rupture-prone) atherosclerotic plaques [25, 56]. Characteristically, stable plaques constitute of a thick fibrous cap, particularly enriched with VSMCs and collagen, and a small lipid core with reduced macrophage accumulation. Plaques with thick caps (and nonstenotic) are generally clinically silent. However, unstable plaques typically present with a large lipid-core and a thin fibrous cap [5] and are characterized by a high number of macrophages plus other inflammatory cell types; and are commonly referred to as thin-cap fibro-atheromas (TCFAs). Histological and in vivo animal studies have demonstrated that inflammation (T-cells and macrophages) not only promotes atherosclerotic plaque formation, but also contributes to plaque destabilization [5]. Foam-cell macrophages produce several pro-inflammatory cytokines such as IFNγ, which in addition to mediating inflammatory responses, can also inhibit VSMC collagen synthesis [60]. As discussed earlier, within atherosclerotic plaques, macrophages are a major source of proteolytic enzymes, especially MMPs, alongside a plethora of inflammatory mediators in plaques, and are therefore considered to play a fundamental in ECM degradation (i.e., collagen and elastin) and subsequent fibrous cap weakening [5]. There is also evidence that macrophage-dependent MMP activity can promote fibrous cap thinning through potentiating VSMC death. For example, MMP activity may detrimentally affect VSMC survival by disrupting

cell–matrix interactions and therefore attenuating matrix-dependent survival signals [16]. The cleavage of death signal molecules and their receptors from the cell surface can trigger apoptosis through autocrine and paracrine processes. A number of MMPs including MMP-7 are able to generate the pro-apoptotic factor TNFα through proteolytic cleavage of pro-TNFα [16]. In addition, MMP-7 can cleave Fas ligand (FasL) to its pro-apoptotic soluble form (sFasL) [61]. Interestingly, MMP-7, TNFα, and FasL all co-localize in human atherosclerotic plaques, suggesting this apoptotic triptych may contribute to formation and expansion of the lipid-rich core [62]. The lateral aspects of an atherosclerotic plaque (commonly termed the shoulder regions) are the sites considered most prone to rupture, and reside between the lipid-rich core and the thinnest part of the fibrous cap. These areas are characterized by accumulations of macrophages and particularly foam-cell macrophages, alongside notable neovascularization [63]. Pathological studies of human atherosclerotic plaques have revealed that macrophages, VSMCs, lymphocytes, and endothelial cells within the rupture-prone shoulder regions express MMP-1, MMP-3, and MMP-9 [64]. MMP-2, MMP-7, MMP-11, MMP-12, MMP-13, MMP-14, and MMP-16 levels are also elevated at the shoulder regions of unstable plaques [8, 10, 12–15], where increased MMP activity and substrate cleavage has also been documented [7, 11, 13, 36]. These findings suggest that MMP expression and activity is strongly associated atherosclerotic plaque progression, highlighting them as therapeutic targets and predictors of clinical outcome in patients with advanced atherosclerotic disease.

6 MMPs as Therapeutic Target for Atherosclerosis

Animal models of atherosclerosis have been widely utilized to investigate the pathogenesis of plaque formation, progression, and instability with the objective of identifying novel therapeutic targets to prevent the clinical manifestations associated with atherosclerosis. Rabbits have been used in multiple studies as several strains spontaneously develop atherosclerotic plaques when fed a high-fat diet. However, most atherosclerosis in vivo studies are conducted in mouse models, despite the fact that wild-type mice atherosclerosis-resistant even after prolonged periods of high fat feeding. The two most commonly used mouse models of atherosclerosis are genetically modified where a key gene of the cholesterol transport pathway has been deleted; these genes are Apolipoprotein E (Apoe) or LDL receptor (Ldlr), thus rendering them hypercholesteroleamic [65–67]. These mice develop atherosclerotic lesions throughout the arterial tree including similar sites to plaque formation in man, even when fed on a normal diet [68]. However, on consumption of a high-fat diet, atherogenesis is significantly accelerated in either Apoe or Ldlr deficient mice, although the hypercholesterolemia is more marked in the Apoe deficient animals. There are also striking similarities in lesion development and progression between both models and humans, as early lesions closely resemble fatty streaks whilst longer periods of high-fat feeding produce complex

advanced lesions [68, 69]. A multitude of studies have been conducted in Apoe deficient mice (and to a lesser degree Ldlr knockout mice) to investigate the roles of MMPs in atherosclerosis. Such studies have utilized genetically modified mice which have global or cell specific knockout or over-expression of a single MMP/TIMP, or treated with a potential therapeutic agent that targets select or all MMPs. These studies have aided the elucidation of potential pathogenic roles of multiple MMPs and TIMPs in atherosclerosis plaque progression and stability (summarized in Table 2).

Table 2 Effect of MMP modulation on atherosclerotic plaque development and stability in animal models

Modulation	Model (species)	Site	Size	VSMCs	Mø	References
MMP-1 Tg	Apoe KO (Ms)	Aorta and root	↓	↔	↔	[70]
MMP-2 KO	Apoe KO (Ms)	Aorta and root	↓	↓	↔	[74]
MMP-3 KO	Apoe KO (Ms)	Aorta, BCA	↑/↑	↓/ND	↓/↔	[76, 77]
MMP-7 KO	Apoe KO (Ms)	BCA	↔	↑	↔	[77]
MMP-8 KO	Apoe KO (Ms)	Aorta	↓	↔	↓	[80]
MMP-9 KO	Apoe KO (Ms)	Aorta, BCA	↓/↑	ND/↓	↓/↑	[77, 78]
MMP-9 Tg	Apoe KO (Ms)	Arch, collar	↔	↔	↔	[71, 72]
MMP-12 KO	Apoe KO (Ms)	Aorta, BCA	↔/↓	↔/↑	↔/↓	[77, 78]
MMP-12 Tg	kbt:JW (Rb)	Aorta	↑	↑	↑	[73]
MMP-13 KO	Apoe KO (Ms)	Root	↔	↔	↔	[81]
MMP-14 KO	Ldlr KO (Ms)	Root	↔	↔	↔	[82]
Non selective MMP inhibitor	Ldlr or Apoe KO (Ms)	Aorta, BCA	↔	↔	↔	[87–89]
MMP-12 inhibitor	Apoe KO (Ms)	Aorta, BCA and root	↓	↑	↓	[54]
MMP-13 inhibitor	Apoe KO (Ms)	Carotid	↔	↔	↔	[92]
miR-24 inhibitor (*MMP-14 over-expression*)	Apoe KO (Ms)	BCA	↑	↔	↑	[84]
TIMP-1 KO	Apoe KO (Ms)	Aorta and root	↔/↓	↔/ND	↔/↑	[30, 83]
TIMP-2 KO	ApoE KO (Ms)	BCA	↔	↓	↑	[30]
TIMP-3 KO	ApoE KO (Ms)	Aorta and root	↓	ND	↓	[85]
TIMP-1 RAd	Apoe KO (Ms)	BCA and root	↓/↔	ND/↔	↓/↔	[55, 86]
TIMP-2 RAd	Apoe KO (Ms)	BCA	↓	↑	↓	[55]
miR-712 inhibitor (*TIMP-3 over-expression*)	Apoe KO (Ms)	Carotid, aorta and aortic arch	↓	ND	↓	[103]

Results of in vivo animal studies evaluating the effects of modulating matrix metalloproteinases (MMP) or tissue inhibitors of MMPs (TIMP) on atherosclerotic plaque size and cellular composition, using transgenic (Tg) or adenoviral (Rad) over-expression, gene knockout (KO), pharmacological inhibitors of MMPs, or microRNA (miR) inhibitors.
VSMC Vascular smooth muscle cell, *Mø* macrophage, *BCA* brachiocephalic artery, *root* aortic root, (↓) decreased, (↑) increased, (↔) no change, and *ND* not determined

6.1 Overexpression Studies

Dissimilar to humans, mice do not constitutively express MMP-1. However, when human MMP-1 was over-expressed exclusively in macrophages of Apoe deficient mice, an unexpected reduction in plaque size, and collagen content was observed [70]. In contrast, macrophage-specific over-expression of pro-MMP-9 did not affect atherosclerotic plaques [71]. However, in a collar-induced carotid artery model of atherosclerosis in Apoe deficient mice, local over-expression of pro-MMP-9 promoted intra-plaque hemorrhage [72]. Furthermore, the transplantation of transduced stem cells permitting the over-expression of an active form of MMP-9, increased plaque progression [71]. Similarly, macrophage-specific over-expression of active of MMP-12 in transgenic rabbits, augmented plaque size and markers of inflammation [73], suggesting that MMP-9 and MMP-12 activation may promote atherosclerosis progression (summarized in Table 2).

6.2 Knockout Studies

A number of studies have been conducted in Apoe knockout mice which are also deficiency for a single MMP or TIMP to elucidate the roles for a selected MMP/TIMP in atherosclerotic plaque formation. Interestingly, these studies have revealed that MMPs exert protective and detrimental effects on atherosclerosis. For instance, Mmp2 knockout mice exhibits a reduction in plaque size, attributed in part to a reduction in VSMC content and implying that plaque stability is compromised in the absence of MMP-2 [74], as MMP-2 is necessary for VSMC migration and intimal formation in vivo [75]. Equally, although Mmp3 deletion resulted in larger aortic and brachiocephalic plaques, a reduction in VSMC number was observed, associated with an increased number of buried fibrous layers (a surrogate marker of plaque instability), suggesting that MMP-3 may promotes plaque stability through promoting VSMC accumulation [76, 77]. Indeed, after carotid ligation, Mmp3 knockout mice shows decreased VSMC migration and associated neo-intimal formation [46]. Likewise Mmp9 deficient mice develop larger plaques with an increased number of buried fibrous layers, and a concomitant reduction in VSMC content [77]. Taken together with findings from an arterial injury model demonstrating MMP-9 promotes VSMC migration and concomitant neo-intimal formation [46], these studies support a beneficial role for MMP-9 in promoting plaque stability through favouring VSMC accumulation. However in another study assessing aortic plaques in mmp9 KO studies, revealed no change in plaque area and a reduced number of lesions, although they also suggested that plaque VSMC number was lowered in Mmp9 deficient animals [78].

In contrast, an increase in VSMC content was reported within the brachiocephalic plaques of mmp7 knockout mice [77], in agreement with a pro-apoptotic role attributed to MMP-7 on VSMCs [79], and indicating a deleterious role for this MMP in atherosclerosis. Mmp8 deficient mice show reduced plaque size and macrophage number but increased collagen content, suggesting MMP-8 promotes

plaque progression [80]. Several lines of evidence have strongly indicated a detrimental role for MMP-12 in plaque progression and instability. Mmp12 deficiency results in smaller brachiocephalic artery plaques, with a reduced number of macrophages and buried fibrous layers [77] and diminished indicators of elastin degradation [78]. Moreover, the ratio between macrophages and VSMCs within the plaques of Mmp12 knockout mice are favourably increased toward VSMCs, in part due to reduced monocyte/macrophage invasion and apoptosis [54], suggesting MMP-12 promotes plaque instability. Further studies in a rabbit model of atherosclerosis have confirmed a detrimental role for MMP-12 in atherosclerosis [73]. Collectively these findings strongly imply that MMP-12 promotes plaque progression and instability. Whilst exerting moderate effects on plaque size, macrophage and VSMC content, mice with either global deletion of MMP-13 or macrophage-specific loss of MMP-14 exhibit a marked increase in plaque fibrillar collagen content, indicating significant roles for these two MMPs in collagen degradation and consequently plaque destabilization [81, 82].

Consequently, these studies imply that some MMPs, such as MMP-2, -3, and -9, exert a protective effect on atherosclerotic plaque progression by promoting VSMC growth and consequent fibrous cap formation. Contrastingly, other MMPs including MMP-7, -8, -12, -13, and -14, may promote plaque instability via increased inflammation, matrix degradation and apoptosis, therefore increasing the propensity of plaque rupture (summarized in Table 2).

6.3 Inhibitor Studies

Although Timp1 deficient mice had larger aortic atherosclerotic lesions with enhanced MMP activity, accompanied with heightened macrophage and lipid content [83], Timp2 knockout mice display a more unstable plaque phenotype than their Timp1 deficient counterparts [30]. These plaques were characterized by increased necrotic core size, buried fibrous layers, macrophage number, and macrophages undergoing apoptosis and proliferation; they also presented reduced collagen and VSMC content, indicative of reduced stability [84]. Equally, Timp3 deficiency in Apoe knockout mice increased lesion size within the aorta and at the aortic root, associated with heightened macrophage accumulation [85]. As therefore expected, systemic over-expression of TIMP-1 or TIMP-2, via adenovirus-mediated gene transfer, reduced lesion development and plaque progression in Apoe knockout mice [55, 86]. Additionally, gene transfer long term over-expression of TIMP-2, but not TIMP-1, arrested progression of established plaques at least in part by constraining monocyte/macrophage invasion and their susceptibility to apoptosis [55]. These findings lend robust support for MMP inhibition as a therapeutic strategy to prevent plaque progression and destabilization. Accordingly, there have been numerous endeavours by academia and industry to develop and deploy synthetic inhibitors of MMPs. Nevertheless, broad spectrum inhibitors containing zinc-chelating groups (such as thiol or hydroxamate groups, or tetracycline derivates) have given inconsistent results. Administration of hydroxamic acid-based,

nonselective MMPs inhibitors to either Lldr knockout or Apoe KO deficient mice revealed no beneficial effects on plaque development or progression [87, 88]. Likewise, doxycycline (a commonly used antibiotic with known nonspecific MMP inhibitory ability) failed to prevent atherosclerosis development in Apoe deficient mice [89]. Furthermore, two independent, randomized, double-blind, and placebo controlled clinical trials involving treatment with of patients with symptomatic coronary and carotid artery disease with doxycycline, did not favourably influence plaque composition or clinical outcome [90, 91]. In contrast, use of a highly selective MMP-12 inhibitor, RXP470.1, arrested plaque progression and improved stability in Apoe deficient mice with preexisting atherosclerosis [54]. In response to MMP-12 inhibition, lesions exhibited reduced lipid core expansion and macrophage apoptosis, increased VSMC to macrophage ratio, decreased plaque calcification, and attenuated elastin degradation [54]. These results, together with a reduction of buried fibrous layers, reflected those observed previously in Mmp12/Apoe double knockout mice [77]. Similarly, a second study where a highly specific MMP-13 inhibitor was deployed, revealed intra-plaque collagenolytic activity was reduced and associated with preservation of fibrillar collagen content within plaques [92], mirroring the effects also witnessed in Mmp13 deficient mice [81]. Taken together, considering broad spectrum MMP inhibition failed to exert any striking benefits on atherosclerosis in either clinical or animal studies, whilst selective MMP inhibition was beneficial in mice, support the tenet that individual MMPs (and therefore possibly TIMPs) play divergent roles in disease development and progression. Consequently, these proof-of-principle studies in mice provide an incentive to translate selective MMP inhibitor treatment into human atherosclerotic patients (summarized in Table 2).

6.4 microRNA Regulation of MMPs

microRNAs (miRs) are small noncoding RNA molecules of approximately 22 nucleotides in length which have the ability to post-transcriptionally regulate gene expression. They are transcribed by polymerase II in the nucleus and are initially produced as primary miRs (pri-miRs). These pri-miRs are processed to miR precursors (pre-miRs) by RNAse III Drosha before they can be exported to the cytoplasm where they are eventually processed into mature and biologically functional miRs through the action of another RNAse III named Dicer. Mature miRs are able to target and bind the 3′ untranslated regions (3′-UTR) of messenger RNA (mRNA) and modulate their expression. It has been predicted that miRs may modulate up to 90% of mammalian genes and therefore play fundamental roles in regulating cellular function [93]. Numerous studies have recently investigated that ability of miRs to regulate MMP expression. For instance, the 3′UTR region of MMP-1 is targeted and regulated by miR-526 [94], which could have potential implications for collagenolysis in plaques. MMP-2 is a direct target of miR-29b, and consequently miR-29b over-expression can inhibit VSMC migration and proliferation and subsequent neo-intimal formation [95]. MMP-3 has been

identified and validated as a putative target of miR-93, as such miR-93 over-expression in human nucleus pulposus cells promoted collagen accumulation [96]. In osteocarcinoma, miR-539 plays a key role in inhibiting osteosarcoma cell invasion and migration through regulating MMP-8 expression in osteosarcoma cells [97]. Direct targeting of MMP-9 by miR-204 can suppress trophoblast-like cell invasion, contributing to the development of pre-eclampsia [98]. Furthermore, MMP-9 expression may also be indirectly regulated by miR-497 via direct targeting of MEK1 in endothelial cells, in response to the anti-hyperlipidaemia drug probucol [99]. Another study conducted in chondrocytes revealed miR-320 was able to directly target and down-regulate MMP-13 expression during chondrogenesis, and vice versa during inflammatory osteoarthritis [100]. Numerous microRNAs have been identified and predicted to target and regulate the expression of MMP-14. miR181a-5p has been shown to downregulate MMP-14 expression by direct targeting of its 3′UTR, reducing cancer cell invasion, and angiogenesis [101]. Similarly, miR-9 can inhibit neuroblastoma cell invasion, metastasis, and angiogenesis by targeting of MMP-14 mRNA [102]. With regard to atherosclerosis, MMP-14 protein expression can be directly modulated by miR-24 in macrophages in response to GM-CSF, influencing the invasive capacity of macrophages [84]. Consequently, administration of a locked nucleic acid (LNA)-miR-24 inhibitor significantly exacerbated preexisting atherosclerosis in Apoe deficient mice, through increasing lesion size, macrophage content, and MMP-14 expression [84]. Moreover, miR-24 expression correlate with more stable coronary plaques in humans, suggesting a protective role of miR-24 in atherosclerosis, presumably through decreased MMP-14 activity [84]. Finally, miR-712 is induced in response to shear stress in endothelial cells of Apoe deficient mice, and through targeting of TIMP-3, exerts a detrimental effect on atherosclerosis via promotion of endothelial inflammation [103]. Collectively, these findings suggest modulation of microRNA may serve as a valuable tool for regulating MMP and TIMP expression in atherosclerosis, highlighting these important and powerful molecules as significant targets for medical intervention.

7 Conclusions

Through studies conducted in isolated cells and animal models, alongside human pathological and clinical findings, MMPs have been established to play a fundamental role in cardiovascular diseases, especially the development, progression, and rupture of atherosclerotic plaques. Seminal studies utilizing animal models that permit genetic modulation of individual MMPs or TIMPs has allowed the identification of specific roles select MMPs exert on all vascular cell types, and the ensuing significance to atherosclerosis. Collectively, this large body of work has demonstrated that modulation of MMP expression/activity can halt and even reverse atherosclerosis, whilst disappointingly broad-spectrum MMP inhibition does not replicate these effects, presumably due perturbation of both beneficial and

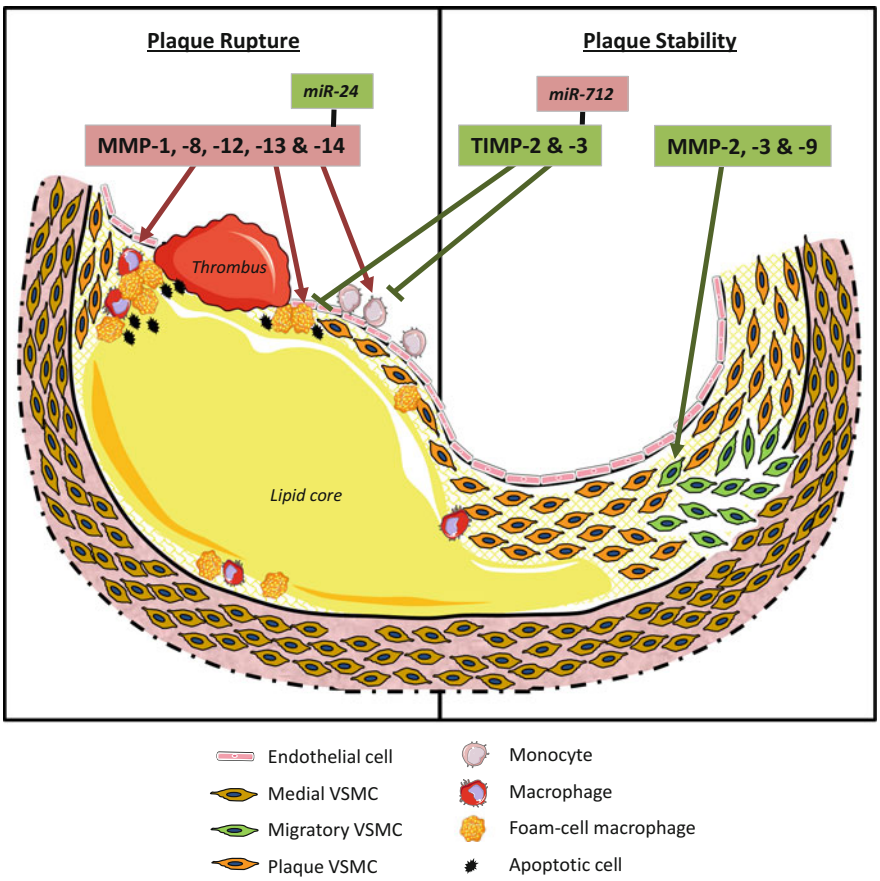

Fig. 2 Divergent roles of MMPs in atherosclerotic plaque progression and stability. Hypothetical model of the potential beneficial and deleterious roles of MMPs and TIMPs during atherosclerotic plaque progression and rupture. Matrix metalloproteinase (MMP)-2, -3, and -9 can facilitate vascular smooth muscle cell (VSMC) migration from the media into the developing atherosclerotic plaque where they participate in fibrous cap formation and maintenance, thus promoting plaque stability. In opposition, MMP-1, MMP-8, MMP-12, MMP-13, and MMP-14 can degrade extracellular matrix proteins present in the fibrous cap whilst also encouraging the recruitment and accumulation of monocytes and macrophages, and their subsequent susceptibility to apoptosis as foam cells—which collectively enhance lipid core expansion, thrombogenicity of the plaque, and thinning of the fibrous cap. Consequently, the stability of the plaque is compromised and vulnerable to plaque rupture and ensuing thrombus formation. More recently, microRNA (miR) have been identified which can regulate MMP and TIMP expression/activity, exerting direct effects on plaque progression

detrimental MMPs (summarized in Fig. 2). Therefore, it is acknowledged and necessary to generate and deploy inhibitors which harbor restricted specificity towards selected MMPs, including MMP-12 and MMP-13, to facilitate transition to man—particularly in the context of atherosclerotic plaque stabilization.

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