ANNE KALELA

Factors Affecting Serum Matrix Metalloproteinase-9 with Special Reference to Atherosclerosis

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals I-V.

I Kalela A, Pönniö M, Koivu TA, Höyhtyä M, Huhtala H, Sillanaukee P, Nikkari ST (2000): Association of serum sialic acid and MMP-9 with lipids and inflammatory markers. Eur J Clin Invest 30:99-104.

II Kalela A, Koivu TA, Sisto T, Kanervisto J, Höyhtyä M, Sillanaukee P, Lehtimäki T, Nikkari ST (2002): Serum MMP-9 concentration in angiographically assessed coronary artery disease. Scand J Clin Lab Invest (in press).

III Kalela A, Koivu TA, Höyhtyä M, Jaakkola O, Lehtimäki T, Sillanaukee P, Nikkari ST (2002): Association of serum MMP-9 with autoantibodies against oxidized LDL. Atherosclerosis 160:161-165.

IV Kalela A, Laaksonen R, Lehtimäki T, Koivu TA, Höyhtyä M, Janatuinen T, Pöllänen P, Vesalainen R, Saikku P, Knuuti J, Nikkari ST (2001): Effect of pravastatin in mildly hypercholesterolemic young men on serum matrix metalloproteinases. Am J Cardiol 88:173-175.

V Sillanaukee P, Kalela A, Seppä K, Höyhtyä M, Nikkari ST (2002): Matrix metalloproteinase-9 is elevated in serum of alcohol abusers. Eur J Clin Invest 32:225-229.

ABBREVIATIONS

AAA(s)	abdominal aortic aneurysm(s)
AFOS	alkaline phosphatase
ALAT	alanine aminotransferase
AN(C)OVA	analysis of (co)variance
AP-1	activator protein-1
ASAT	aspartate aminotransferase
BMI	body mass index
CAD	coronary artery disease
CDT	carbohydrate-deficient transferrin
CHD	coronary heart disease
CRP	c-reactive protein
EC(s)	endothelial cell(s)
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FN	fibronectin
GGT	gamma glutamyl transferase
HDL	high density lipoprotein
IEL	internal elastic lamina
Ig	immunoglobulin
IL	interleukin
KGF	keratinocyte growth factor

LDL	low density lipoprotein
MCP-1	monocyte chemoattractant protein-1
MI	myocardial infarction
MMP(s)	matrix metalloproteinase(s)
MT-MMP	membrane type matrix metalloproteinase
natLDL	native low density lipoprotein
OD	optical density
oxLDL	oxidized low density lipoprotein
PDGF	platelet derived growth factor
PEA-3	polyomavirus enhancer A-binding protein-3
PG(s)	proteoglycan(s)
SH	sulfhydryl group
SMC(s)	smooth muscle cell(s)
TGF-β	transforming growth factor-β
TIMP(s)	tissue inhibitor(s) of matrix metalloproteinases
TNF-α	tumor necrosis factor-α
VLDL	very low density lipoprotein
VN	vitronectin

ABSTRACT

Atherogenesis starts early in life, but it usually takes several decades before clinical symptoms appear. Most acute complications of atherosclerosis are the result of rupture of an atheromatous plaque with intraluminal thrombus formation. Inappropriate extracellular matrix degradation has been implicated to play an important role in such plaque disruption. Matrix metalloproteinases (MMPs) are a family of enzymes which participate in this process. Increased expression of several MMPs and presence of MMP activity has been observed in the chronic inflammation of atherosclerotic arteries. There is active synthesis of MMP-9 by macrophages and smooth muscle cells (SMCs) in atherosclerotic plaques of patients with unstable angina, suggesting the role of this enzyme in acute coronary syndromes. Arterial inflammation may also increase serum MMP-9 concentration. However, little is known about the factors associated with serum MMP-9 levels. The aims of the present study were to investigate the association of serum MMP-9 concentration with blood leukocytes, serum CRP and oxLDL autoantibodies, which are indicators of inflammation, among healthy men and in patients with coronary heart disease. In addition, the effects of alcohol abuse and pravastatin treatment on serum MMP-9 concentration were examined. The main findings of this study were that serum MMP-9 associated positively with inflammation markers and with the severity of atherosclerosis. Serum MMP-9 concentration was decreased by pravastatin treatment and increased in alcohol abusers compared to social drinkers. These results suggest that serum MMP-9 concentration may reflect arterial inflammation and the severity of coronary artery disease.

INTRODUCTION

The rate of mortality in coronary artery disease (CAD) has declined considerably during past decades, but still remains the main cause of morbidity and mortality in Finland (Statistics Finland, 1999) and in other western countries. The decline in mortality from CAD can partly be explained by changes in the three main coronary risk factors, serum cholesterol concentration, blood pressure and smoking (Vartiainen et al. 1994).

Atherogenesis starts early in life, but it usually takes several decades before clinical symptoms appear (Stary et al. 1995). CAD is characteristically clinically silent until stenosis or thrombosis leads to impaired blood flow and myocardial ischemia. Clinically, this gives rise to angina pectoris, myocardial infarction (MI) and sudden cardiac death. Most cases of acute coronary syndromes are the result of rupture of an atheromatous plaque with overlying thrombus formation, which occludes or narrows the lumen (Falk et al. 1995). Plaque disruption occurs most frequently at the shoulder region between the plaque and the adjacent vessel wall where the fibrous cap is thinnest (Richardson et al. 1989). These regions have increased numbers of macrophages, indicating ongoing inflammation (Ross 1999). Macrophages are capable of secreting matrix metalloproteinases (MMPs), which are proteolytic enzymes that catalyze degradation of the extracellular matrix (ECM), thus predisposing the fibrous cap to rupture (Dollery et al. 1995, Galis and Khatri 2002). Atherosclerotic plaques in patients with unstable angina contain MMP-9 in addition to other MMPs (Galis et al. 1994b, Brown et al. 1995). MMP-9 is mainly expressed by macrophages and may seep from the arterial wall into the circulation. Its presence in

plasma may thus reflect arterial inflammation. This is supported by findings that, in primary arteritis, macrophages within the inflamed artery wall show increased expression of MMP-9, which is reflected also in increased serum levels of this enzyme (Nikkari et al. 1996b, Sorbi et al. 1996).

This thesis was designed to study different factors associated with serum MMP-9 level. Based on histological data, MMP-9 is suggested to be involved in the chronic inflammation of atherosclerosis (Brown et al. 1995). Serum levels of MMP-9 have been shown to be elevated as a result of arteritis (Sorbi et al. 1996). Since little is known about the factors associated with serum MMP-9 levels, special attention was paid to the possible role of serum MMP-9 concentration as a marker of arterial inflammation in atherosclerosis. Association between serum MMP-9 concentration and established markers of inflammation was investigated in two studies. The subjects in these studies included a population of clinically healthy men as well as patients with angina pectoris and their age-matched controls. The impact of coronary heart disease (CHD) on serum MMP-9 concentration was studied both in patients with clinical angina pectoris and in those with angiographically assessed coronary narrowing. The effect of pravastatin on serum MMP-9 concentration was examined in a double-blind placebo controlled trial. Finally, the effect of chronic alcohol abuse on serum MMP-9 level was examined.

REVIEW OF LITERATURE

1. Atherosclerosis

1.1 Formation of atherosclerotic plaques

The artery wall consists of three layers, the intima, the media and the adventitia (Stary et al. 1992). The intima includes the endothelium lining the lumen of the arterial wall and the underlying connective tissue. Particularly in large arteries the thickness of the intima varies greatly. The intima and media are separated by the internal elastic lamina (IEL), although this border is not clearly defined in some areas such as bifurcations, branch vessels, and curvatures (Stary et al. 1992). Layers of smooth muscle cells (SMCs) in the media maintain arterial tone. The external elastic lamina separates the media from the adventitia, which is rich in loose connective tissue and contains fibroblasts, vasa vasorum and nerves.

The pathogenesis of atherosclerosis is not completely understood. However, during the past decades increasing knowledge of this complex process has given rise to several hypotheses. These include the response to injury hypothesis, the lipid infiltration hypothesis, the oxidation hypothesis, the monoclonal hypothesis and there is also the inflammation hypothesis, which has recently gained considerable attention (Ross 1999, van Lente 2000, Kadar and Glasz 2001). It is generally viewed that atherogenesis begins early in life with accumulation of low density lipoprotein (LDL) cholesterol and monocytes in the intima of large elastic and muscular arteries (McGill et al. 1998). LDL is either trapped in the intimal matrix, principally in collagens through decorin or proteoglycans, or it passes through the IEL and moves further into

the media (Williams and Tabas 1995, Pentikäinen et al. 1997, Williams and Tabas 1998, Kadar and Glasz 2001, Skålen et al. 2002). Trapping of LDL in the intima lengthens its residence time and predisposes LDL to modifications, such as oxidation, acetylation or aggregation (Ross 1993). Oxidized LDL (oxLDL) shows enhanced uptake by macrophages via scavenger receptors, leading to foam cell formation. The earliest lesion, the fatty streak, is mainly due to the accumulation of lipid droplets in macrophages and in some extent in SMCs (Stary et al. 1994).

A part of the fatty streaks are susceptible to progression and may proceed to intermediate lesions and further to advanced lesions (Stary et al. 1994). Whether this happens is to a large extent determined by mechanical forces, such as low shear stress, which lengthen the residence time of LDL in the intima, and also cause self-limited adaptive intimal thickening (Stary et al. 1992). Intermediate lesions are found in young adults. They are characterized by pools of extracellular lipid, that disrupt the coherence of intimal SMCs (Stary et al. 1994). When the intermediate lesion proceeds, pools of extracellular lipid increase to form a lipid core (Stary et al. 1995). This advanced lesion is called an atheroma, which per se does not narrow the vascular lumen. When the layer of collagen between the lipid core and the lumen of the vessel is thin, an atheroma is susceptible to rupture, leading to luminal thrombus formation (Stary et al. 1995). The connective tissue layer between the lipid core and the luminal surface, i.e. the fibrous cap, may gradually increase and cause narrowing of arteries. This type of advanced lesion, called the fibrous plaque, may also develop thrombus and/or hematoma. The fibrous plaque may also calcify. Complicated lesions are atheromas or fibrous plaques, which have disrupted or thrombosed (Stary et al. 1995). Progression of atherosclerosis is schematically shown in Figure 1.

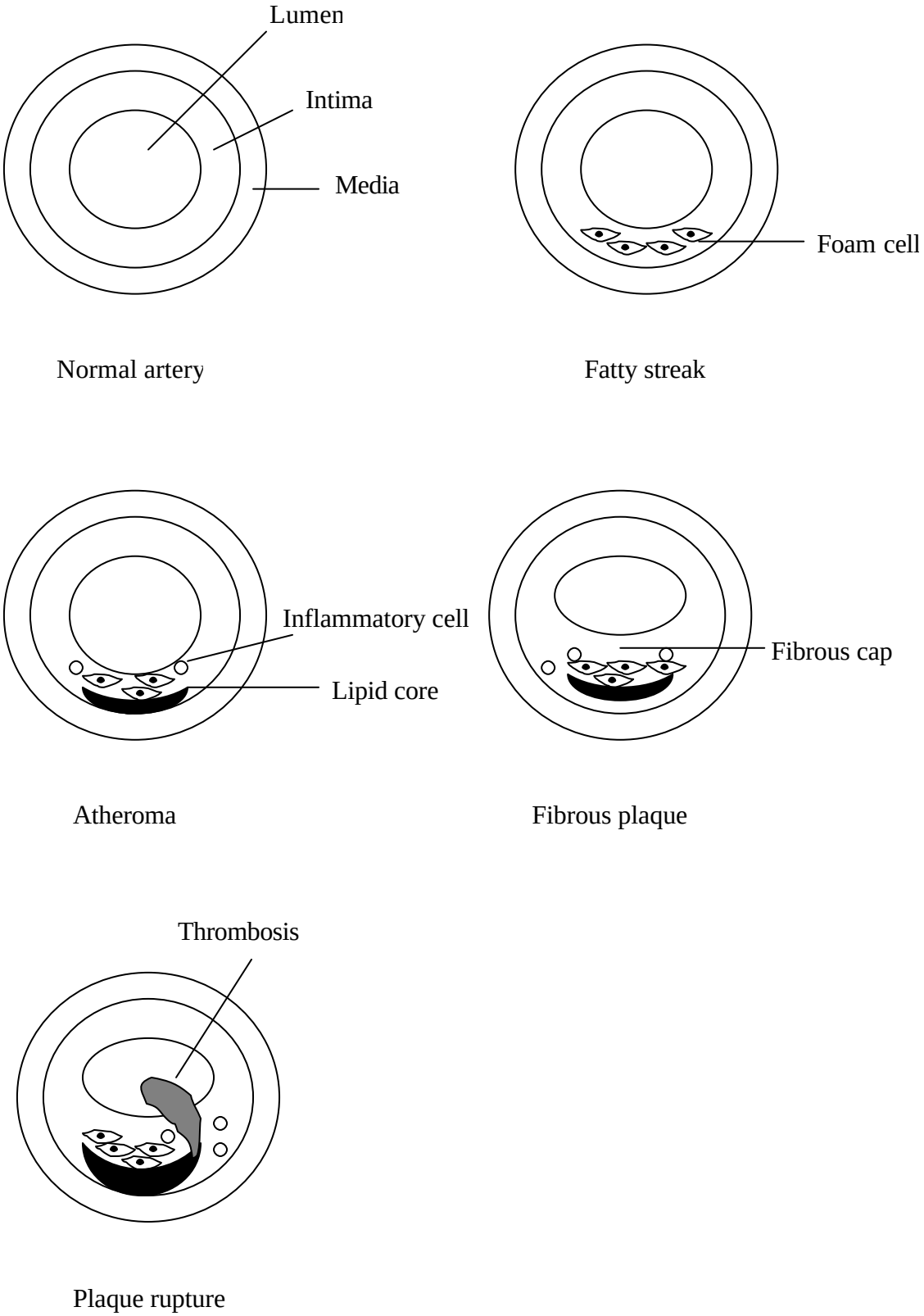

Figure 1. Progression of atherosclerosis

1.2 Rupture of atherosclerotic plaque

Morbidity and mortality from atherosclerosis is largely due to complicated lesions (Stary et al. 1995). Plaques are constantly disposed to mechanical forces, such as high circumferential stress, that may precipitate disruption of vulnerable plaques, giving rise to acute coronary syndromes: unstable angina pectoris, MI, and sudden death. Plaque vulnerability is related to its configuration and morphological complexity (Schroeder and Falk 1995). The vulnerability of the plaque has also been shown to increase with its size, and angiographic studies have shown a positive correlation between severity of stenosis and progression of coronary syndromes (Schroeder and Falk 1996). However, most acute coronary syndromes are thought to originate from disruption of plaques that have caused only mild to moderate luminal obstruction (Gutstein and Fuster 1999). Hard sclerotic plaques with a high content of SMCs and collagen, thick fibrous caps, and a low amount of extracellular lipid are fairly resistant to rupture (Falk et al. 1995). Vulnerable plaques consist of a large core of extracellular lipid, high amounts of lipid laden macrophages, reduced numbers of SMCs and a thin cap (Falk 1991). The core may be avascular, hypocellular, devoid of collagen, rich in lipid and very soft (Stary 1989, Davies 1990, Gertz and Roberts 1990, Falk et al. 1995). Rupture of a plaque usually occurs at the shoulder regions of the atheromatous plaque, where the fibrous cap is thinnest and heavily infiltrated with macrophages (Richardson et al. 1989). Macrophages produce MMPs and other proteolytic enzymes which catalyze degradation of ECM, and weaken the cap (Dollery et al. 1995, Galis and Khatri 2002).

1.3 Inflammation in atherosclerosis

The traditional risk factors of atherosclerosis include hyperlipidemia, smoking, hypertension and diabetes mellitus (van Lente 2000). However, they do not account for the overall risk of atherosclerosis (Hopkins and Williams 1981). Many inflammatory cells and molecules have been identified in association with plaque material, and this has led to the theory that inflammation plays a role in the development of atherosclerosis and to plaque disruption (Ross 1999).

An established inflammation marker, the leukocyte count, correlates with the extent of coronary atherosclerosis (Friedman et al. 1974, Kostis et al. 1984). It has also been shown to predict the recurrence of MI and major CHD events (Lowe et al. 1985, Phillips et al. 1992). C-reactive protein (CRP) is an acute phase protein, which has been shown to predict cardiovascular events both in patients with stable angina pectoris and in apparently healthy persons (Thompson SG et al. 1995, Kuller et al. 1996, Mendall et al. 1996, Ridker et al. 1997, Tracy et al. 1997). The rise of CRP after acute MI (Pietilä et al. 1993, and 1996, Ueda et al. 1996, Anzai et al. 1997) or during unstable angina pectoris (Berk et al. 1990, Liuzzo et al. 1994, Haverkate et al. 1997) correlates positively with their outcome. The principal source of CRP has been assumed to be the liver. However, it has recently been shown that arterial tissue itself produces CRP (Yasojima et al. 2001). In atherosclerotic plaques the major producers are smooth muscle-like cells and macrophages (Yasojima et al. 2001). CRP has also been suggested to have an active role in atherosclerosis e.g. by activating the complement and by promoting tissue factor production (Haverkate et al. 1997).

Activated endothelium secretes biologically active molecules such as monocyte chemoattractant protein-1 (MCP-1), which attract circulating inflammatory cells, monocytes and T-lymphocytes leading to their migration into the intima (Schroeder and Falk 1996). Recruited monocytes become activated themselves and differentiate into macrophages. The increased intimal population of macrophages and T-lymphocytes produce cytokines and growth factors, which attract medial SMCs into the intima (Weissberg 1999). It has recently been suggested that bone-marrow cells give rise to most of the SMCs that participate in the pathogenesis of atherosclerosis (Sata et al. 2002). Once in the intima, SMCs proliferate and produce ECM, which are important processes that contribute to both early and late atherogenesis (Wight 1989).

Memon et al. (2000) have recently suggested that inflammation induces oxidation of LDL. Whether this occurs in the circulation or in the vessel wall is not known. Oxidation of LDL has been thought to occur in the arterial wall on the basis that powerful antioxidant protection exists in the circulation (Steinberg et al. 1989). On the other hand there is also antioxidant protection in atherosclerotic plaques (Suarna et al. 1995).

Oxidative modification of LDL makes it more atherogenic than its native form. OxLDL is taken up by macrophages via scavenger receptors leading to foam cell formation and fatty streak development (Steinberg et al. 1989). In addition, oxLDL increases synthesis and secretion of adhesion molecules by endothelial cells (ECs) and macrophages both *in vitro* and *in vivo* (Berliner et al. 1995). It also stimulates chemotaxis and recruitment of monocytes *in vivo* (Lehr et al. 1991), and induces macrophage and SMC proliferation *in vitro* (Yui et al. 1993, Björkerud and Björkerud

1996). OxLDL has also been shown to stimulate MMP-9 in macrophages by increasing the mRNA expression, protein synthesis, and gelatinolytic activity of this protease, thereby possibly predisposing the plaque to rupture (Xu et al. 1999).

OxLDL is immunogenic and thus induces the formation of autoantibodies, which have been detected in human and animal serum and atherosclerotic plaques (Palinski et al. 1989, Palinski et al. 1994, Ylä-Herttuala et al. 1994, Palinski et al. 1996). Elevated serum levels of oxLDL autoantibodies have been detected in patients with carotid atherosclerosis and CAD (Maggi et al. 1993, Maggi et al. 1994, Lehtimäki et al. 1999). OxLDL autoantibodies have also been suggested to predict MI and indicate an active atherogenic process (Salonen et al. 1992, Puurunen et al. 1994, Wu et al. 1997).

Contradictory results have also been reported. van de Vijver et al. (1996) did not find any association between oxLDL autoantibodies and the extent of coronary stenosis. The level of oxLDL autoantibodies was lower in elderly patients with ischemic stroke and in patients with acute MI compared to controls (Schumacher et al. 1995, Cherubini et al. 1997). This proposed antiatherogenic role of autoantibodies against oxLDL is strengthened by the finding that there was a suppression of atherosclerosis after immunization of hypercholesterolemic rabbits with oxLDL or malondialdehyde-modified LDL (Palinski et al. 1995, Ameli et al. 1996). In addition, an inverse relationship of oxLDL autoantibodies with carotid artery intima-media thickness has been reported (Fukumoto et al. 2000). Despite contradictory results concerning the involvement of oxLDL autoantibodies in the pathogenesis of atherosclerosis,

autoantibodies seem to be useful in the evaluation of the presence of oxLDL in the arterial wall (Tsimikas et al. 2001).

Infections have been suspected to initiate or promote arterial inflammation. One pathogen that has strongly been suggested to influence atherosclerosis is *Chlamydia pneumoniae* (Saikku et al. 1988). Other organisms implicated in atherosclerosis include herpes simplex virus, cytomegalovirus, and *Helicobacter pylori* (Mehta et al. 1998).

1.4 Ethanol and atherosclerosis

Several epidemiological studies have demonstrated a U-shaped or inverse association between alcohol consumption and the incidence of CHD (Klatsky et al. 1981, Rimm et al. 1991, Klatsky et al. 1992). It has been shown that CHD mortality is reduced by moderate alcohol consumption but increased with heavy drinking (Friedman and Kimball 1986, Doll et al. 1994). Excessive alcohol use and alcoholism have detrimental effects on the cardiovascular system and may result in increased incidence of stroke (Palomäki and Kaste 1993), cardiomyopathy (Knochel 1983), arrhythmias (Lange and Kinnunen 1987, Douds and Maxwell 1994) and hypertension (Criqui et al. 1981, Gordon and Kannel 1983). Heavy drinking may also cause excessive triglyceride synthesis and fatty liver formation, followed by hypercholesterolemia and decreased high density lipoprotein (HDL) cholesterol (Sabesin 1981). The degree of alcohol intake which would reduce all-cause mortality and risk of CHD is not clear

and seems to vary between men and women (McElduff and Dobson 1997, White 1999).

Several studies have demonstrated that different types of alcoholic beverages are equally associated with a lower CHD risk (Hennekens et al. 1979, Klatsky et al. 1986, Rimm et al. 1996). One of the several plausible mechanisms leading to a lower risk is an increase in HDL cholesterol by regular ethanol consumption (Barboriak et al. 1979, Ernst et al. 1980, Nishiwaki et al. 1994). In addition, LDL has been shown to be reduced in alcoholics compared to controls (Kervinen et al. 1991). Independently of the effects on lipoprotein metabolism, alcohol has also been suggested to have antithrombotic properties (Meade et al. 1979, Keller and Folts 1988, Demrow et al. 1995, Gorinstein et al. 1997).

Some studies have suggested a more pronounced cardioprotective effect of wine and beer compared to other beverages (Renaud and de Lorgeril 1992, Criqui and Ringel 1994, Gronbaek et al.1995). The particularly beneficial effects of wine, especially red wine, on CHD risk have been attributed to polyphenols because of their antioxidant activity (Frankel et al. 1993, Hayek et al. 1997, Nigdikar et al. 1998). This postulated mechanism involves quenching of free radicals leading to decreased oxidative damage of LDL, hence reducing its potential atherogenicity (Nigdikar et al. 1998). On the other hand, some studies have reported no change in, or even enhanced oxidizability of LDL cholesterol in wine consumers (de Rijke et al. 1996, van Golde et al. 1999, Caccetta et al. 2000). It is thus possible that a predominant pro-oxidant effect of alcohol itself may outweigh any antioxidant effect of beverage polyphenols (van Golde et al. 1999).

The association between alcohol intake and inflammatory markers of atherosclerosis has not been studied thoroughly. Moderate alcohol consumption has been suggested to decrease production of interleukin-6 (IL-6), the major cytokine of the acute-phase response (McCarty 1999). On the other hand, in alcoholic liver disease the concentrations of IL-6 and other cytokines are elevated (McClain et al. 1999). A U-shaped association between alcohol intake and established inflammatory markers, CRP and leukocyte count, has also recently been reported (Imhof et al. 2001). However, in the cirrhotic liver, production of most proteins, inflammatory or other, is reduced (Gopal and Rosen 2000).

1.5 Anti-inflammatory properties of statins

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) inhibit the rate- limiting enzyme of cholesterol synthesis in liver leading to decreased hepatic cholesterol production and upregulation of hepatic LDL receptor expression (Davignon and Laaksonen 1999). They have been shown to be effective in both primary (Shepherd et al. 1995, West Scotland Coronary Prevention Study Group 1998, Heart Protection Study Collaborative Group 2002) and secondary prevention (Scandinavian Simvastatin Survival Study Group 1994 and 1995, Jukema et al. 1995, Shepherd 1995, Heart Protection Study Collaborative Group 2002) of CAD. The clinical benefit of statins has been manifested early in the course of lipid lowering therapy, although the improvement in arterial morphology has been shown to occur slowly and only to a small extent (MAAS investigators 1994). The baseline or treated serum LDL cholesterol levels are only weakly associated with coronary angiographic

change or cardiovascular events. The beneficial effects of statins on clinical events may thus involve nonlipid mechanisms that modify endothelial function, plaque stability, thrombus formation, or inflammatory responses (Vaughan et al. 1996).

Statins attenuate inflammation in atherosclerosis both in the vessel wall and through changes in blood constituents. Statins have been shown to reduce the adhesion of monocytes to the endothelium (Weber et al. 1997, Teupser et al. 2001). They have been reported to diminish the expression of MCP-1 and abolish infiltration of macrophages into the arterial wall (Bustos et al. 1998). Macrophage scavenger receptors are downregulated by statins, which leads to lowered uptake of oxLDL (Pietsch et al 1996, Umetani et al. 1996, Draude et al. 1999). Statins possess antioxidant properties, which also reduce foam cell formation. Simvastatin inhibits the ability of macrophages to oxidize LDL by reducing their superoxide formation (Giroux et al. 1993), fluvastatin directly protects LDL from oxidation (Hussein et al. 1997, Suzumura et al. 1999), and CAD patients with statin therapy have lower oxLDL levels than those without lipid lowering drugs (Vasankari et al. 2001).

CRP, an inflammatory marker, has been considered as an independent risk factor for cardiovascular disease (Thompson SG et al. 1995, Mendall et al. 1996, Kuller et al. 1996, Ridker et al. 1997, Tracy et al. 1997). Statins have been shown to decrease serum CRP levels independently of changes in serum lipid levels (Jialal et al. 2001) mainly when the pretreatment value of CRP is elevated (Strandberg et al. 1999, Kluft et al. 1999, Ridker et al. 1999). Patients with elevated baseline CRP have greater reduction in morbidity or mortality of MI than those with normal CRP (Ridker et al. 1998, Horne et al. 2000). Statins have also been suggested to inhibit the production of

tumor necrosis factor alpha (TNF-α), a proinflammatory cytokine (Rosenson et al. 1999) as well as the secretion of MMP-9 by mouse and human macrophages (Bellosta et al. 1998) and by human peripheral monocytes (Ganne et al. 2000).

2. Matrix metalloproteinases (MMPs)

There are different classes of enzymes that participate in ECM degradation. These include serine proteases, cysteine proteinases, aspartic proteinases and MMPs. So far, 22 human MMPs have been identified, while there are also other MMPs restricted to other species (Sternlicht and Werb 2001). They are classified in five subgroups based on their substrate preference and structure: collagenases, gelatinases, stromelysins, membrane type MMPs (MT-MMPs) and other MMPs (Hoekstra et al. 2001) (Table I). MMPs may also be referred to by their molecular weight or the order of identification.

2.1 Structure of MMPs

MMPs have several common features in their structure. The domains of MMP-9 are shown as an example in Figure 2. A typical MMP has an N-terminal signal sequence, which is removed after secretion, a pro-domain that is lost upon activation, and a catalytic domain containing the Zn^{2+} binding region (Woessner 1991, Birkedal-Hansen et al. 1993). All MMPs, except MMP-7, MMP-26 and MMP-23, contain a hemopexin- or vitronectin-like domain in the C-terminus, which is linked to the catalytic domain by a hinge region (Woessner 1995, Velasco et al. 1999, Park et al.

2000). MMP-7 and MMP-26 lack these domains, whereas MMP-23 has a unique cysteine-array and an immunoglobulin (Ig)-like domain replacing the hinge as well as hemopexin-like domains (Velasco et al. 1999, Pei et al. 2000). The hemopexin-like domain regulates the substrate specificity of MMPs and is involved in the binding of tissue inhibitors of MMPs (TIMPs) to gelatinases (MMP-2 and MMP-9) and MMP-13 (O´Connell et al. 1994, Knäuper et al. 1997). The two gelatinases have an additional domain composed of three fibronectin type II-like repeats in the catalytic domain, which confer the high affinity binding of these enzymes to gelatin and collagen, enhance their proteolytic activity and are crucial for the elastolytic activity (Collier et al. 1992, Murphy et al. 1994, Shipley et al. 1996, Rowsell et al. 2002). In addition, MMP-9 has in its hinge region a sequence homologous to α2 chain of type V collagen, which is suggested to mediate binding to type V collagen (Wilhelm et al. 1989).

Table I. Classification of human matrix metalloproteinases.

Name	**Number**	**Substrate**
Collagenases		
Interstitial collagenase	MMP-1	Collagens I, II, III, VII and X, gelatin, PGs
Neutrophil collagenase	MMP-8	Collagens I, II and III, PGs
Collagenase 3	MMP-13	Collagens I, II and III
Collagenase 4	MMP-18	Collagen I
Gelatinases		
Gelatinase A (72 kDa)	MMP-2	Collagens IV, V, VII, X and XI, gelatin
Gelatinase B (92 kDa)	MMP-9	Collagens IV, V and XIV, elastin, gelatin, PGs
Stromelysins		
Stromelysin 1	MMP-3	Collagens III, IV, IX and X, FN, gelatin, laminin, PGs
Stromelysin 2	MMP-10	Collagens III, IV, IX and X, FN, gelatin, laminin, PGs

Metalloelastase	MMP-12	Collagen I and IV, elastin, FN, gelatin, laminin, VN
Matrilysin	MMP-7	Collagens III, IV, IX and X, gelatin, PGs, laminin, elastin, entactin, tenascin, versican
Membrane-type MMPs		
MT1-MMP	MMP-14	Collagens I, II and III, FN, laminin, VN
MT2-MMP	MMP-15	Aggrecan, FN, laminin, tenascin
MT3-MMP	MMP-16	Collagen III, FN, gelatin
MT4-MMP	MMP-17	Gelatin
MT5-MMP	MMP-24	PGs
MT6-MMP	MMP-25	Collagen IV, fibrin, FN, gelatin
Others		
Stromelysin 3	MMP-11	Serine protease inhibitors
RASI-1	MMP-19	Collagen IV, entactin, FN, gelatin, laminin, tenascin
Enamelysin	MMP-20	Aggrecan, amelogenin
	MMP-23	Not identified
Matrilysin 2	MMP-26	Collagen IV, FN, gelatin, VN
Epilysin	MMP-28	Not identified

Modified from Nagase 1997, English et al. 2001, Hoekstra et al. 2001, Sternlicht and Werb 2001. FN=fibronectin, PG=proteoglycan, VN=vitronectin.

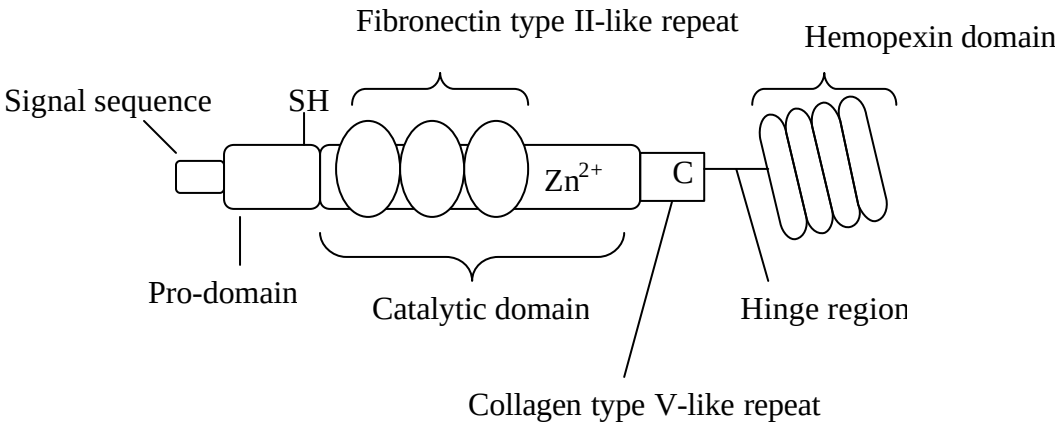

Figure 2. Domain structure of MMP-9 (modified from Nagase 1997, Sternlicht and Werb 2001).

2.2 Regulation of MMP activity

Since activated MMPs can variously degrade essentially all ECM components, their activity has to be kept under tight control. The regulatory mechanisms include 1) regulation of transcription, 2) activation of latent proenzymes and 3) inhibition of the active enzymes by TIMPs, the naturally occuring specific inhibitors of MMPs.

2.2.1 Transcriptional regulation

Most MMPs are regulated at transcriptional level, although modulation of mRNA half-life with cytokines and growth factors may also influence MMP expression (Overall et al. 1991, Delany and Brinckerhoff 1992, Tamai et al. 1995). Regulators of MMP gene transcription include growth factors, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), fibroblast growth factor-2 (FGF-2), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β) as well as inflammatory cytokines like TNF-α and IL-1 (Frisch and Ruley 1987, Overall et al. 1989, Circolo et al. 1991, Mitchell and Cheung 1991, Putnins et al. 1995, Pickering et al. 1997). In addition, some hormones, oncogenes, and tumor promoters, as well as heparin and contact to ECM can induce or repress the expression of MMPs (Matrisian et al. 1986, Kerr et al. 1988, Offringa et al. 1988, Birkedal-Hansen et al. 1993, Mauviel 1993, Kenagy et al. 1994).

Several MMPs contain in their promoter region an activator protein-1 (AP-1) binding site (Benbow and Brinckerhoff 1997). The extracellular stimuli result in activation of

the nuclear AP-1 transcription factor complex (composed of members of Fos and Jun families), which then binds to the AP-1 site in the promoter and contributes to transcription of the MMP gene (Karin et al. 1997).

Several other putative regulatory elements, such as polyomavirus enhancer A-binding protein-3 (PEA-3), have been identified within various MMP gene promoters, and many have been shown to regulate cell- and circumstance-specific gene expression (Benbow and Brinckerhoff 1997). Thus, the organization of the transcriptional promoter of a MMP and other signals like cellular context, determine how it responds to a given stimulus.

Basal and inducible levels of MMP gene expression can also be influenced by genetic variations. Common bi-allelic single-nucleotide polymorphisms that affect the rate of transcription have been identified within several MMP gene promoters (Ye 2000). The polymorphisms may influence the development and progression of different diseases. A polymorphism in MMP-1 promoter results in 37-fold enhancement of transcription and is present in tumor cell lines and more often in ovarian cancer patients than in the general population (Rutter et al. 1998, Kanamori et al. 1999). MMP-3, MMP-9 and MMP-12 contain polymorphisms which have been associated with progression (MMP-3) or severity (MMP-9, MMP-12) of CAD, although the influence on transcription is modest (Ye et al. 1995, Zhang et al. 1999, Jormsjö et al. 2000). The promoter polymorphism of MMP-9 gene has also been associated with the development of complicated coronary lesions (Pöllänen et al. 2001).

2.2.2 Posttranscriptional regulation

MMPs are synthesized and most of them also secreted as inactive proenzymes or zymogens. The latency of the proenzymes is maintained by an interaction between sulfhydryl (SH) group of cysteine in the proregion and the Zn^{2+} in the active site preventing the association of the Zn^{2+} with a water molecule, which is required in the catalysis (Springman et al. 1990). This interaction can be destabilized by proteinases (such as plasmin, trypsin, mast cell chymase and other already activated MMPs), SH-reactive compounds (iodoacetate, organomercurials), denaturants (urea, SDS) and by heat treatment (Saarinen et al. 1994, Nagase 1997). Disruption of the Cys-zinc bond leads to opening of the switch and subsequent autocatalytic cleavages, finally resulting in the generation of a catalytically competent enzyme. *In vivo*, plasmin and other already activated MMPs have been suggested to be the most significant posttranscriptional regulators of MMP activation (Lijnen 2001).

2.2.3 Inhibition of MMP activity

Active MMPs are inhibited by a naturally occurring specific inhibitors, TIMPs, α2-macroglobulin and exogenous substances such as heparin, tetracyclins and synthetic inhibitors (Birkedal-Hansen et al. 1993, Kenagy et al. 1994, Dollery et al. 1995). The TIMPs represent a family of four inhibitors with 37 – 51 % overall sequence identity (Sternlicht and Werb 2001). They inhibit MMPs reversibly in a 1:1 stoichiometric fashion, and the N-terminal domain is necessary for the inhibitory effect (Brew et al. 2000).

Although different TIMPs bind tightly to most MMPs, some differences have been reported (Murphy and Willenbrock 1995, Liu et al. 1997). For example, TIMP-3 appears to be a more potent inhibitor of MMP-9 than are the other TIMPs (Sternlicht and Werb 2001). Furthermore, matrilysin (MMP-7), which lacks the C-terminal hemopexin-like domain, is more resistant to inhibition by TIMPs (Baragi et al. 1994).

α2-Macroglobulin is an abundant plasma protein, and it is the major inhibitor of MMPs in tissue fluids (Birkedal-Hansen et al. 1993). Synthetic inhibitors like 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA) interact with or remove Zn^{2+} at the active site, but they show little if any selectivity and are therefore of limited analytical or therapeutic potential (Birkedal-Hansen et al. 1993).

2.3 MMPs in atherosclerosis

Expression of MMPs is generally found at low levels in normal vascular tissue, upregulation is seen during certain physiological and pathological processes (Dollery et al. 1995). Interaction of monocytes with type I collagen and laminin induces their MMP-9 expression, which is thought to help their migration (Khan et al. 1997, Wesley et al. 1998). Once in the intima, monocytes transform into macrophages and accumulate lipid to become foam cells (Ross 1999). Foam cells release cytokines and superoxides, which induce cellular MMP-1, MMP-3 and MMP-9 production and also MMP-2 and MMP-9 activation (Yanagi et al. 1991, Hanemaaijer et al. 1993, Galis et al. 1994a, Rajagopalan et al. 1996). MMPs have a role also in SMC migration and proliferation in relation to intimal thickening, as documented in a rat carotid balloon

injury model (Bendeck et al. 1994, Zempo et al. 1994, Webb et al. 1997). Increased activities of MMP-2, MMP-3 and MMP-9 have been detected in SMCs during periods of migration and proliferation (Bendeck et al. 1994, Zempo et al. 1994).

The rupture of coronary plaques occurs mainly in the shoulders of the overlying fibrous cap containing accumulations of macrophages (Dollery 1995, Schroeder and Falk 1996). The overall matrix degrading activity is increased in these rupture-prone shoulder areas (Galis et al. 1994b, Nikkari et al. 1995) with elevated levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9 and MMP-12 in macrophages (Henney et al. 1991, Galis et al. 1994b, Brown et al. 1995, Nikkari et al. 1995, Halpert et al. 1996, Li et al. 1996, Nikkari et al. 1996a). The capability of macrophages to degrade ECM has been confirmed by the *in vitro* findings that lipid-laden macrophages from atherosclerotic plaques constitutively express MMP-1 and MMP-3 and induce ECM breakdown (Galis et al. 1995b, Shah et al. 1995). Some production of MMPs is also present in mast cells, SMCs, lymphocytes and ECs (Galis et al. 1994b, Brown et al. 1995, Halpert et al. 1996, Kaartinen et al. 1998, Nelimarkka et al. 1998). Vascular cells also produce TIMP-1 and TIMP-2 (Galis et al. 1994a, 1994b, 1995a, 1995b, 1995c). The way by which secreted MMPs become activated is unknown, but reactive oxygen radicals released by foam cells, and chymase and tryptase released by mast cells may be involved (Frears et al. 1996, Rajagopalan et al. 1996, Johnson et al. 1998).

2.4 MMP-9 in arterial inflammation

MMP-9 is associated with different disorders involving arterial inflammation. It has been shown to be highly expressed in the disruption-prone regions of atherosclerotic plaques (Galis et al. 1994b, Brown et al. 1995, Zaltsman and Newby 1997). Non-atherosclerotic arteries do not contain detectable amounts of MMP-9 (Brown et al. 1995). Macrophages are the main source of MMP-9, although lymphocytes, mast cells, SMCs and ECs also express this protease (Galis et al. 1994a, Brown et al. 1995, Kaartinen et al. 1998, Nelimarkka et al. 1998). In temporal arteritis, an accumulation of macrophages containing MMP-9 has been reported especially in regions of IEL disruption (Nikkari et al. 1996b). This primary arteritis seems to seep out MMP-9 protein into the circulation, since also raised serum levels of the protease have been shown concomitantly to vascular expression (Sorbi et al. 1996). Serum levels of MMP-9 have been reported to be elevated in patients with MI and unstable angina (Kai et al. 1998) as well as in stable angina (Inokubo et al. 2001, Noji et al. 2001). The growth and rupture of an aneurysm have been proposed to be partly mediated by MMP-9, since human abdominal aortic aneurysms (AAAs) produce 10-fold more of this enzyme than normal aorta, and MMP-9 activity increases with size of the aneurysm (McMillan et al. 1995, Thompson RW et al. 1995). Macrophages are a central source of MMP-9 both in temporal arteritis and AAA.

MMP-9 has a broad substrate specificity, being particularly active against gelatins and type IV collagen (Birkedal-Hansen et al. 1993). It also possesses proteolytic activity against elastin and core protein of some PGs (Table 1). Growth factors and cytokines capable of up-regulating the expression of MMP-9 *in vitro* include IL-1, TNF-α, and

PDGF (Barath et al. 1990, Ross et al. 1990, Clinton et al. 1991). MMP-9 may also be activated by cytokines that stimulate plasmin activity, which has been speculated to activate MMP-9 proteolytically *in vivo* (Lijnen 2001).

2.5 Serum levels of MMP-9

MMP-9 is known to be a regular serum component (Vartio and Baumann 1989). MMP-9 is present in serum as a zymogen, an active protease and it is bound to TIMP-1. There has been some speculation as to where the circulating MMP-9 is derived from (Figure 3). As outlined above, arterial inflammation raises serum MMP-9 concentration (Sorbi et al. 1996). Increased serum levels have been detected both in stable and unstable CAD (Kai et al. 1998, Inokubo et al. 2001, Noji et al. 2001). Elevated serum MMP-9 has also been reported in cancer (Sonnante et al. 2000, Hrabec et al. 2001), asthma (Belleguic et al. 2002) and rheumatoid arthritis (Ahrens et al. 1996). Thus, in addition to vascular cells including macrophages, SMCs, lymphocytes and ECs, a variety of other cell types have been shown to express MMP-9 (Galis et al. 1994a, Brown et al. 1995, Nelimarkka et al. 1998). Polymorphonuclear leukocytes have also been shown to be able to produce MMP-9 (Zaoui et al. 1996), but whether they secrete the protein in the circulation or in the inflamed tissue is unclear.

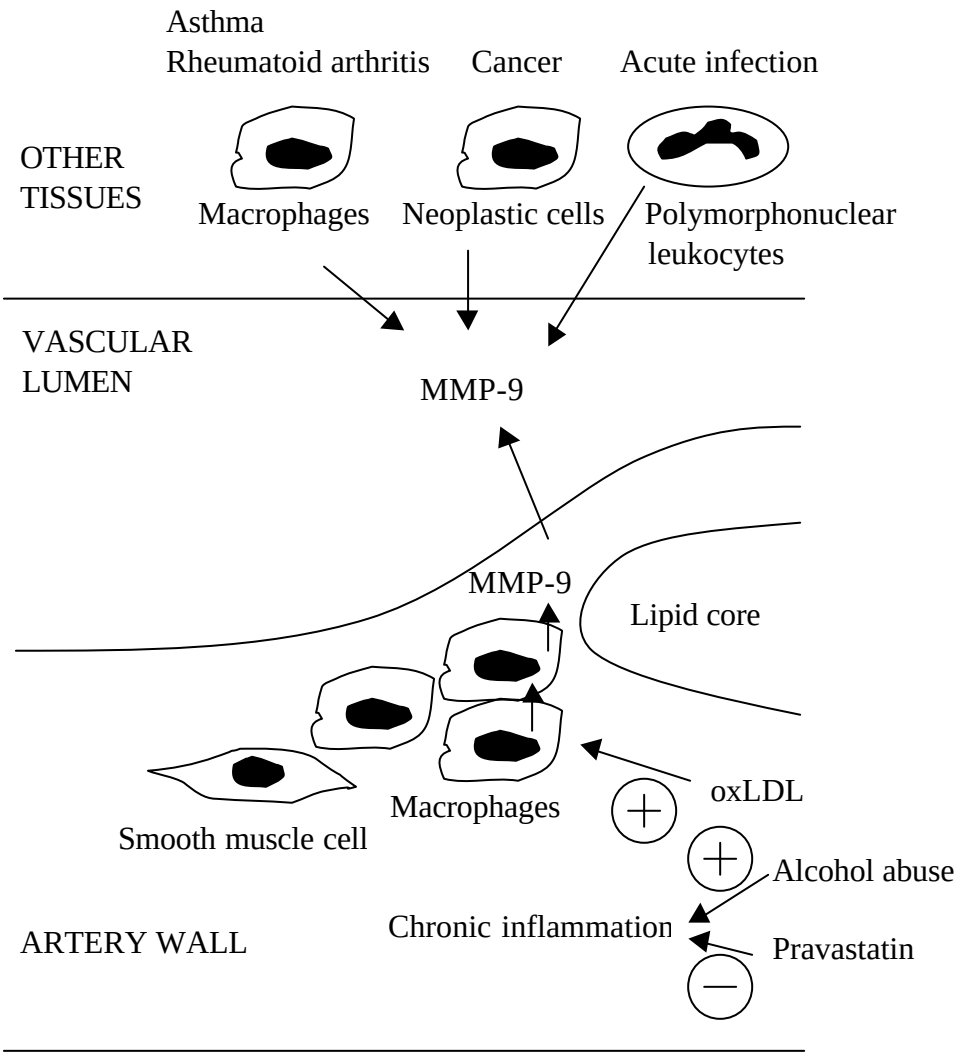

Figure 3. Proposed mechanisms contributing to serum MMP-9 concentration.

AIMS OF THE STUDY

MMP-9 has been shown to be up-regulated in unstable angina. It is expressed by macrophages located especially in vulnerable regions of the atherosclerotic plaque. In primary arteritis, there is increased expression of MMP-9 at the sites of ruptured IEL, and serum levels are elevated as well. The published results suggest that MMP-9 is associated with an inflammatory process in the vascular wall and diffuses to the circulation. However, the association of serum MMP-9 concentration with inflammatory markers and its role in reflecting arterial inflammation are not yet clear. The aims of this study were to investigate the association of serum MMP-9 concentration with blood leukocytes, serum CRP and oxLDL autoantibodies, which are indicators of inflammation, among healthy men and in patients with CHD. In addition, the effects of alcohol abuse and pravastatin treatment on serum MMP-9 concentration were examined.

In detail, the aims were to elucidate:

1. the association of serum MMP-9 concentration with the established inflammatory markers, namely leukocyte count and CRP, in healthy men.
2. the association of serum MMP-9 concentration with oxLDL autoantibody level, and to compare the levels between patients with angina pectoris and controls.
3. the serum levels of MMP-9 in patients with angiographically assessed CAD.
4. the effect of pravastatin on serum MMP-9 concentration.
5. the effect of chronic alcohol abuse on serum MMP-9 level.

SUBJECTS AND METHODS

For more detailed information on the study subjects and methods, please refer to the original articles I-V.

1. Subjects

1.1 Men from an occupational health survey (I)

Sixty-five male employees (mean age 45 years, range 37 – 58 years) were invited to participate in the study in connection with an occupational health survey. All subjects were examined and interviewed by a physician. Subjects with diagnosed CHD, diabetes, cancer, liver disease, infectious disease or autoimmune disease and those who had been operated within half a year were excluded from the study. The study was approved by the Ethical Committee of the University Hospital of Tampere.

1.2 Patients with angiographically assessed CAD (II)

The subjects were patients referred to the Tampere University Hospital for coronary angiography because of chest pain or otherwise suspected CAD. The 80 study participants were divided into three groups: 34 patients (27 men, 7 women) with ≥50 % narrowing in three coronary arteries, 27 patients (20 men, 7 women) with ≥50 % narrowing in one or two coronary arteries, and 19 controls (9 men, 10 women) without pathological findings in angiography. Each patient was interviewed by a physician regarding smoking habits, medication, diabetes mellitus and other diseases.

The study protocol was approved by the Ethical Committee of the Tampere University Hospital.

1.3 Men with angina pectoris from the 1997 Finrisk study (III)

The National Public Health Institute of Finland has perfomed large cross-sectional population surveys related to the risk factors of CAD in five geographic areas every five years since 1972. The present population originated from the 1997 FINRISK study, which had a total sample size of 11500 subjects. From this population 243 males with angina pectoris and 238 age-matched controls were selected for the substudy. The study was conducted according to the Helsinki Declaration of 1975 on Human Experimentation and was approved by the Ethical Committee of Primary Health Care Clinics in Finland.

1.4 Men in a placebo-controlled pravastatin study (IV)

The study was randomized, double-blind, placebo-controlled with treatment groups of pravastatin (40 mg/day for 6 months, n=24) and placebo (n=26). The mean age of the subjects was 35±4 years. At baseline, serum total cholesterol levels were normal or mildly elevated (mean 5.5±0.8 mmol/l). Participants did not receive any other drug therapy or antioxidants, and they were instructed to adhere to their normal diet during the study.

1.5 Alcohol abusers (V)

Serum samples were obtained from 40 male alcoholics with an ethanol consumption of >1000 g/week (>87 standard units containing 11 g pure ethanol each) before entering detoxification. The alcoholics were examined and interviewed by a physician. All alcoholics had a well documented history of chronic alcoholism and none of them had a history of liver disease or showed clinical signs of liver disease at the time of interview. No one had any symptoms of clinically manifest cardiovascular disease. Serum samples from 40 social drinker males with ethanol consumption of <200 g/week (<17 standard units containing 11 g pure ethanol each) were used as controls. The study was approved by the Ethical Committee of the University Hospital of Tampere.

2. Lipid analyses

In the study I, blood was drawn after 10-12 hours' fast. Total cholesterol and HDL cholesterol were determined by an enzymatic colorimetric method (CHOD-PAP, Labsystems, Finland). Triglycerides were determined by enzymatic hydrolysis of triglycerides with subsequent determination of liberated glycerol by colorimetry (Boehringer Mannheim, Germany). In the study II, the subjects fasted overnight before sample collection. Total cholesterol and triglycerides were determined by enzymatic methods (Nycotest, Nycomed AS, Norway) using a Monarch 2000 analyzer. HDL cholesterol was measured with the same enzymatic method after precipation of LDL and very low density lipoprotein (VLDL) with polyethylene glycol. In the study III, total and HDL cholesterol were determined from fresh serum

samples by an enzymatic method (CHOD-PAP, Roche Diagnostics, Germany). Triglycerides were measured by a fully enzymatic method (GPO-PAP, Roche Diagnostics, Germany) using the Olli-C analyzer (Konelab, Finland). In the study IV, blood samples for biochemical analyses were collected after an overnight fast before and after the treatment period. Plasma triglycerides, total cholesterol and HDL cholesterol were analyzed colorimetrically using a Cobas Integra 700 automatic analyzer using reagents and calibrators recommended by the manufacturer (Hoffmann-La Roche Ltd., Switzerland). LDL concentration was calculated using Friedewald's formula (Friedewald et al. 1972).

3. Measurement of serum CRP (I, IV)

In the study I, serum CRP levels were determined by means of particle-enhanced immunonephelometry using the Behring Nephelometer analyzer II (Marburg, Germany). In the study IV, serum CRP was measured with a two-site ultrasensitive assay in the form of prototype reagents (InnoTrac Diagnostics Oy, Turku, Finland). The dynamic range was from 0.1 to 300 mg/l.

4. Determination of serum MMP-9 concentration

Rapid sampling and processing of blood is essential for the accurate assessment of MMPs. All serum samples were collected in tubes without clot accelerator. Blood was sentrifuged within one hour after sampling. Serum was immediately separated, frozen and stored at –70 °C without freeze-thaw cycles until analysis. Quantitation of

immunoreactive MMP-9 was carried out by an enzyme-linked immunosorbent assay (ELISA). Recombinant MMP-9 was used as standard. The microtiter plate was coated with the monoclonal antibody against MMP-9 (code GE-213), and samples and standards were added. The bound proteins were detected with a secondary polyclonal antibody produced in chicken against MMP-9. A peroxidase-labeled anti-chicken-IgG (Chemicon, USA) was used for the detection of the bound secondary antibody. O-phenylenediamine (OPD) tablets (KemEnTec) were used to visualize the peroxidase label. The color formation was measured at 450 nm (Anhos 2000 microplate reader) and calculations were done using a Multicalc program (Wallac, Finland). The monoclonal antibody recognizes both the free MMP-9 and that bound to its inhibitor, TIMP-1.

5. Determination of autoantibodies against oxLDL (III)

Antigens for this assay included native LDL (natLDL) prepared from a pooled plasma of three donors and protected against oxidation by 0.27 mM EDTA and 20 μM butylated hydroxytoluene (BHT) in phosphate – buffered saline (PBS), and oxLDL obtained after 24-h oxidation of the natLDL with 2 μM $CuSO_4$. For ELISA, half of the wells were coated with 50 μl of natLDL and the other half with 50 μl of copper-oxidized LDL antigen in PBS for 16 h at 4°C. The remaining non-specific binding sites were saturated using 2 % human serum albumin in PBS containing 20 μM BHT and 0.27 mM EDTA for 2 h at 4°C. After washing, 50 μl of the serum samples were added and incubated overnight at 4°C. After incubation, peroxidase conjugated goat anti-human-IgG monoclonal antibody (Organon, USA no. 55220 Cappel), diluted

1:4000 in buffer containing 0.27 mM EDTA, 20 μM BHT, 1 % human serum albumin, 0.05 % Tween in PBS, was added and incubated for 4 h at 4°C. After incubation and washing, 50 μl of freshly made substrate [0.4 mg/ml OPD (Sigma) and 0.045 % H_2O_2 in 100 mM citrate buffer, pH 5.0] was added. The optical density (OD) was measured at 492 nm using a microplate reader (Wallac 1420 Victor™, Wallac Oy, Turku, Finland).

6. Determination of MMP-9 genotype (IV)

A cytosine (C) to thymidine (T) promoter polymorphism at position -1562 relative to the start of transcription in MMP-9 gene was determined by polymerase chain reaction and restriction enzyme *BbuI* digestion. Thirtyfive cycles of amplification were carried out in the following conditions: denaturation at 97 °C for 1 min, annealing at 65,7 °C for 1 min and extension at 72 °C for 1 min. After the *BbuI* enzyme digestion, fragments were separated by electrophoresis on 2.5 % agarose gel and visualized with ultraviolet light.

7. Zymography (V)

For zymography, gelatin was incorporated into 10 % polyacrylamide gels. Equal volumes of serum from controls and alcoholics were loaded in loading buffer. After electrophoresis, the gels were rinsed for 30 min with 2.5 % Triton X-100, incubated in 50 mM Tris, 10 mM CaCl, pH 7.8 at 37 °C for 20 h and stained with Coomassie blue

R. Prestained low range SDS-PAGE molecular weight standards were used to estimate the average sizes of MMPs (Bio-Rad, USA).

8. Western blot analysis (V)

Serum samples and prestained molecular weight standards (BioRad) were separated on 10% SDS-PAGE and transferred electrophoretically to nitrocellulose sheets (Sigma). The blots were blocked with 1 % BSA in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween-20 (TBST). Incubations were done with the monoclonal antibody GE-213 against MMP-9 diluted in TBST. A biotinylated anti-mouse IgG (1 µg/ml) (Vector, USA) diluted in TBST was used as the secondary antibody. Subsequently, Vectastain ABC-reagent (Vector) was added, and peroxidase activity was visualized using diaminobenzidine (DAB) and H_2O_2 as substrate.

9. Statistical methods

Statistical analyses were done with a desktop computer using Statistica for Windows version 5.1 (Statsoft, Inc., Tulsa, OK, USA) (I-V) and BMDP (Statistical Software, USA) (I). T-test for independent samples was used to compare the means in the study groups. The difference in frequences was analyzed by χ^2 test. The trend of background characteristics was analyzed by analysis of variance (ANOVA). Multiple regression was used to identify the independent parameters associated with oxLDL autoantibodies. Relations between the tertiles of serum MMP-9 levels with lipid risk

factors (serum total cholesterol, serum HDL cholesterol, serum triglycerides) and markers of inflammation (serum CRP and blood leukocyte count) were determined using stepwise polychotomous logistic regression analysis (BMDP, Statistical Software, USA). The effect of pravastatin treatment on CAD risk factors was analysed by t-test for dependent samples. ANOVA for repeated measurements was used to determine the difference in CAD risk factors in both the pravastatin and placebo groups. P-values of <0.05 were considered as being statistically significant.

RESULTS

1. Association of serum MMP-9 concentration with inflammatory markers (I, III)

The results from 65 clinically healthy men were divided into tertiles according to their serum MMP-9 values (I). Correlations between MMP-9 tertiles and lipid risk factors (serum total cholesterol, serum LDL cholesterol, serum HDL cholesterol, serum triglycerides) and markers of inflammation (serum CRP, blood leukocyte count) were determined. In these men serum MMP-9 was positively associated with blood leukocyte count (P=0.001, ANOVA). There was also an increasing trend of serum CRP with increasing serum MMP-9 level, but it did not reach statistical significance. There was no correlation between serum lipids and serum MMP-9 level. In the polychotomous logistic regression analysis (including age, body mass index [BMI], blood leukocytes, serum CRP, serum total cholesterol, serum HDL cholesterol and serum triglycerides), blood leukocyte count was the only factor significantly associated with serum MMP-9 tertiles.

The 1997 Finrisk substudy with 243 men with angina pectoris and 238 controls investigated the association of serum MMP-9 concentration with oxLDL autoantibodies. In the whole study population (n=481), serum MMP-9 concentration (β=0.200, P<0.001) and smoking (β=0.179, P<0.001) were significantly associated with oxLDL autoantibodies in multiple regression analysis. The model included age, diastolic blood pressure, serum cholesterol, serum HDL cholesterol, serum

triglycerides, BMI, smoking and serum MMP-9 concentration as independent variables.

2. Serum MMP-9 concentration in CAD patients (II, III)

Serum MMP-9 concentration was not significantly higher in patients with angina pectoris than in control subjects (54.2±29.9 μg/l and 50.6± 23.1 μg/l, respectively, P=NS) (III). On the other hand, in serum of patients with advanced atherosclerosis MMP-9 concentration correlated with the extent of angiographically determined disease (II). Serum MMP-9 level was highest in patients with 3-vessel CAD (P=0.001, ANOVA). After adjustment for age, sex and diabetes the result remained statistically significant (P=0.025, ANCOVA). Serum MMP-9 concentration was higher in patients with 3-vessel CAD (57.3±39.1 μg/l) than in those with 1- or 2-vessel CAD (40.4±25.1 μg/l, P=0.044) or in control subjects (32.2±16.1 μg/l, P=0.007). The difference between the group with 1- or 2- vessel CAD and controls was not statistically significant. The frequency of statin treatment was 11 % in controls, 67 % in patients with 1- or 2-vessel CAD, and 82 % in those with 3- vessel CAD (P<0.001, ANOVA).

3. Effect of statin therapy on serum MMP-9 concentration (IV)

There were no statistically significant differences in baseline values between the pravastatin and placebo groups. Compared to placebo, pravastatin decreased serum

LDL cholesterol and total cholesterol significantly (P<0.001) during the 6 months' treatment, while there were no significant changes in serum HDL cholesterol and serum triglycerides. When the changes in serum MMP-9 concentrations in the treatment groups were compared, pravastatin decreased serum MMP-9 concentration significantly (-5.2 μg/L, -13%) compared to placebo (+5.6 μg/L, +16%) (P=0.033). This difference did not change after adjusting for serum total cholesterol and LDL cholesterol. Changes in blood leukocytes and serum CRP concentration were not statistically significant between the groups.

4. Serum MMP-9 concentration in alcoholics (V)

An association between alcohol abuse and serum MMP-9 concentration was studied in 40 male alcoholics with ethanol consumption more than 1000 g/week (>87 standard units containing 11 g pure ethanol each) and 40 male social drinkers with an ethanol consumption of less than 200 g/week (<17 standard units containing 11 g pure ethanol each). The average serum MMP-9 concentration was 1.6-fold in sera of male alcoholics compared to control subjects (70.9 ± 47.7 μg/l and 43.1 ± 19.2 μg/l, respectively, p=0.001). Both zymography and Western blotting confirmed the increase of serum MMP-9 concentration in alcoholics. Gelatin substrate zymography of sera from alcoholics and controls showed a major band at 80–92 kD. The band was confirmed to be formed of a doublet of pro- (92 kD) and activated (80 kD) forms of MMP-9 by Western blotting of serum samples with a monoclonal antibody against MMP-9 (GE-213). This antibody recognizes both the pro- and active forms of MMP-9 and also that bound to its inhibitor TIMP-1.

Serum gamma glutamyl transferase (GGT) activity was significantly induced in alcoholics compared to that in controls (121 ± 126 U/l vs 45 ± 46 U/l, respectively, P=0.0007). The mean values of serum carbohydrate-deficient transferrin (CDT), serum aspartate aminotransferase (ASAT) and serum alanine aminotransferase (ALAT) were above the normal range in alcoholics, while serum alkaline phosphatase (AFOS) level was normal. Within the alcoholic group, serum MMP-9 concentration did not correlate with age, serum GGT, serum CDT, serum ASAT, serum ALAT or serum AFOS.

DISCUSSION

1. Serum MMP-9 concentration as an inflammatory marker

MMP-9 has been shown to be present in human atherosclerotic lesions and suggested to have a role in unstable angina pectoris (Galis et al. 1994b, Brown et al. 1995). Arterial inflammation has also been shown to be reflected in increased serum MMP-9 concentrations (Nikkari et al. 1996b, Sorbi et al. 1996). The aim of study I was to examine the association of serum MMP-9 concentration with the blood leukocyte count and serum CRP, which are established inflammatory markers.

Early studies have suggested that total blood leukocyte count correlates with the severity of coronary stenosis and predicts MI (Friedman et al. 1974, Kostis et al. 1984). In the Framingham Study, a significant association between the onset of CAD and total blood leukocyte count was demonstrated (Kannel et al. 1992). In elderly men, the total blood leukocyte count predicted CAD and all-cause mortality (Weijenberg et al. 1996). Serum CRP has been evaluated as a risk predictor in subjects without known CAD (Ridker et al. 1997, Ridker et al. 2000), in those at risk of CAD (Kuller et al. 1996), and in patients with stable angina (Haverkate et al. 1997), unstable angina (Ferreiros et al. 1999, Lindahl et al. 2000) or MI (Tommasi et al. 1999).

Our finding of the association between blood leukocyte count and serum MMP-9 concentration has not been reported before. There was also an increasing trend in serum CRP with rising serum MMP-9 concentration, but it did not reach statistical significance. Serum MMP-9 concentration might prove to be a sensitive but not

necessarily a specific marker of arterial inflammation, since raised levels of serum MMP-9 have also been reported in several other inflammatory disorders, such as rheumatoid arthritis (Ahrens et al. 1996), and asthma (Belleguic et al. 2002). However, if elevated serum MMP-9 levels reflect increased expression of MMP-9 in the arterial wall by activated macrophages, blood leukocyte count may be raised as a consequence of the ongoing inflammation or they may directly contribute to the serum MMP-9 concentration at the sites of acute inflammation.

Study III showed an association between oxLDL autoantibodies and serum MMP-9 concentration. Circulating oxLDL autoantibodies and serum MMP-9 both originate partly from the arterial wall (Nikkari et al. 1996, Sorbi et al. 1996, Tsimikas et al. 2001). There may be a common causative mechanism between the two, since oxLDL has been shown to upregulate MMP-9 expression, protein synthesis and gelatinolytic activity by macrophages *in vitro* (Xu et al. 1999). It is possible that oxLDL upregulates MMP-9 expression by increasing the production of TNF-α, which is known to stimulate MMP-9 production (Jovinge et al. 1996, George 1998). In addition, oxLDL may contribute to elevation of serum MMP-9 concentration by increasing infiltration of monocytes into the arterial wall (Chen et al. 1999), proliferation of macrophages (Sakai et al. 1996) and foam cell formation (Quinn et al. 1987). Taken together, our results suggest that oxLDL may be associated with MMP-9 secretion by inflammatory cells in atherosclerotic plaques.

In the multiple regression analysis (age, diastolic blood pressure, serum cholesterol, serum HDL cholesterol, serum triglycerides, BMI, smoking and serum MMP-9 concentration as independent variables) serum MMP-9 concentration and smoking

were independently associated with oxLDL autoantibodies. Plasma MMP-9 concentration has been shown to be elevated in healthy smokers compared to non-smokers (Nakamura et al. 1998). Smoking has also been related to increased oxidative stress (Reilly et al. 1996, Marangon et al. 1998), and smokers have been reported to have significantly higher oxLDL autoantibody levels compared to nonsmokers (Fickl et al. 1996), although this may be true only in smokers with CAD (Lehtimäki et al. 1999) or hypercholesterolemia (Heitzer et al. 1996). Smoking may thus elevate both serum oxLDL autoantibody titer and MMP-9 concentration.

2. Serum MMP-9 concentration and autoantibodies against oxLDL in CAD patients

In the substudy of the 1997 Finrisk study (III), serum MMP-9 concentration did not differ between subjects with angina pectoris and controls. In study II, serum MMP-9 concentration correlated with the extent of angiographically assessed CAD, but statistical significance was reached only between patients with 3-vessel CAD and those with 1- or 2-vessel CAD or controls. It thus seems that severe CAD is associated with the elevated serum MMP-9 concentration.

Coronary angiography measures the narrowing of the artery lumen, but it has been criticized for its inadequacy to evaluate the severity of CAD, since initially the plaque material grows into the vessel wall maintaining a constant lumen and acute coronary events usually occur at sites of previously minor lesions (Ambrose et al. 1988, Little et al. 1988, Hong et al. 1994, Nissen 2001). Detection of stenosis may however

indirectly measure also minor lesions, since the angiographic severity (number of stenosed vessels) has been shown to correlate with the percentage of the coronary artery length involved by atheroma (Nakagomi et al. 1996). The angiographic severity may in fact indicate advanced atherosclerosis with stable stenosed plaques, but also unstable plaques with excessive inflammation.

The serum autoantibody level against oxLDL was significantly higher in subjects with angina pectoris than in controls (III). This is in line with several studies, which have documented raised levels of oxLDL autoantibody titers in patients with CAD (Maggi et al. 1993, Lehtimäki et al. 1999, Erkkilä et al. 2000). Since the presence of antigen is necessary for the production of antibodies, these results also suggest that oxLDL levels were elevated.

Few methods for direct measurement of oxLDL have been developed. Monoclonal antibodies against oxidatively modified LDL have been used in ELISA procedures (Kohno et al. 2000, Toshima et al. 2000, Ehara et al. 2001). The problem of the immunological methods is the heterogeneity of the oxidation products of LDL. The antibodies against oxLDL have been reported to recognize also epitopes on proteins other than LDL (O´Brien et al. 1996). Another method for measuring LDL oxidation is the determination of the baseline level of conjugated dienes in lipids extracted from LDL (Ahotupa et al. 1996). The level of diene conjugation and the autoantibody titer against oxLDL have been shown to correlate (Ahotupa et al. 1998).

3. Effect of pravastatin on serum MMP-9 concentration

Treatment with statins has demonstrated an improvement in clinical outcomes in both primary (Shepherd et al. 1995, West Scotland Coronary Prevention Study Group 1998, Heart Protection Study Collaborative Group 2002) and secondary prevention (Scandinavian Simvastatin Survival Study Group 1994 and 1995, Jukema et al. 1995, Shepherd 1995, Heart Protection Study Collaborative Group 2002) of CHD. The rapid reductions in coronary events without evidence of substantial regression of atherosclerotic plaque have raised interest in the effects of statins beyond lipid modification (MAAS investigators 1994). Pravastatin decreased serum MMP-9 concentration significantly compared to placebo in mildly hypercholesterolemic men (IV). This study agrees with the previous *in vitro* results that statins inhibit MMP-9 secretion by monocytes and macrophages (Bellosta et al. 1998, Ganne et al. 2000).

The decrease in serum MMP-9 concentration was independent of the reduction in serum lipid values. The mechanism may be a direct one on MMP-9 secretion or it may involve anti-inflammatory effects suggested for statins, such as reduction of monocyte adhesion (Weber et al. 1999), decrease of macrophage infiltrates (Shiomi et al. 1995, Bustos et al. 1998), and reduction of foam cell formation (Giroux et al. 1993, Pietsch et al 1996, Umetani et al. 1996, Hussein et al. 1997, Suzumura et al. 1999, Draude et al 1999). The decrease of serum MMP-9 concentration by pravastatin did not result from the decrease in blood leukocyte count, known to be associated with serum MMP-9 concentration (study I), since pravastatin did not decrease blood leukocyte values.

4. Alcohol abuse and serum MMP-9 concentration

Several epidemiological studies have demonstrated a U-shaped correlation between alcohol consumption and the incidence of CHD (Klatsky et al. 1981, Rimm et al. 1991, Klatsky et al. 1992). The postulated mechanisms also involve effects on the inflammatory response. Moderate alcohol consumption has been shown to decrease production of IL-6 (McCarty 1999), and a U-shaped association between alcohol intake and serum CRP and blood leukocyte count has been reported (Imhof et al. 2001).

In the study V raised serum levels of MMP-9 were found in alcoholics, and we suggest that the elevation of serum MMP-9 concentration in alcoholics may be associated with inflammation. Since heavy drinking has detrimental effects on the cardiovascular system (Moore and Pearson 1986, Klatsky et al. 1990, Bengtsson et al. 1991), it is possible that the circulating MMP-9 is derived from arterial inflammation. Another candidate for MMP-9 production would be the liver, but the serum MMP-9 concentrations did not correlate with markers of liver injury (serum ASAT, serum ALAT). This makes it unlikely that the increased serum MMP-9 concentration would be liver-derived. In fact, serum levels of MMP-9 are decreased rather than increased in hepatitis and cirrhosis (Lichtinghagen et al. 2000). Since MMPs synthesized in tissues seep into the bloodstream, the increase of MMP-9 concentration in serum may thus represent a marker for pro-inflammatory effects of chronic alcohol abuse.

SUMMARY AND CONCLUSIONS

Based on histological data, MMP-9 is suggested to be involved in the chronic inflammation of atherosclerosis. Serum levels of MMP-9 have been shown to be elevated as a result of arteritis. However, little is known about the factors affecting serum MMP-9 levels, especially in regard to arterial inflammation (Figure 3). The present study examined associations of serum MMP-9 concentration with factors involved in atherosclerosis. The findings and conclusions are:

1. Serum MMP-9 level was shown to correlate with blood leukocyte count in healthy men. There was a trend of increasing serum CRP with increasing serum MMP-9 concentration, although it did not reach statistical significance. OxLDL autoantibodies were positively associated with serum MMP-9 concentration, which is in line with previous *in vitro* results that oxLDL upregulates MMP-9 expression. Since the level of serum MMP-9 did not associate with lipids (serum total cholesterol, serum LDL cholesterol, serum HDL cholesterol, serum triglycerides), it may prove to be a sensitive marker of inflammation. Furthermore, if oxLDL regulates the expression of MMP-9 in vivo, serum levels of this protease may be mostly derived from the vasculature.
2. Serum MMP-9 level did not differ between men with angina pectoris and controls from a population survey. It is possible that in these men the disease is in the stable state and is not advanced to the level where the inflammatory response is excessive.
3. In hospitalized patients with angiographically assessed CAD, serum MMP-9 concentration was significantly higher in those with 3-vessel CAD compared to those with 1- or 2-vessel CAD or controls. Angiography has been criticized for its

inability to detect vulnerable plaques, but nevertheless associates with the extent of the disease. Serum MMP-9 concentration may thus reflect the excessive inflammation in patients with severe atherosclerosis.

4. Pravastatin decreased serum MMP-9 concentration independently of changes in lipid values in mildly hypercholesterolemic but otherwise healthy men. The changes were moderate but statistically significant when compared to the placebo group. Together with a previous *in vitro* study, which showed that statins inhibit the secretion of MMP-9 by human macrophages, these results are in agreement with suggestions that statins possess anti-inflammatory properties.
5. Serum MMP-9 concentration was significantly higher in chronic alcohol abusers than in social drinkers. A U-shaped association between alcohol consumption and serum CRP and blood leukocyte count has been suggested. Elevated serum MMP-9 concentration in alcoholics may thus reflect arterial inflammation resulting from the effects of chronic alcohol abuse on the cardiovascular system.

In conclusion, serum MMP-9 concentration may be derived from the vasculature and reflect arterial inflammation. It is possible that serum MMP-9 measurements may be used to assess the extent of atherosclerosis and arterial inflammation.

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