CHAPTER 8

Distribution of matrix metalloproteinase’s in human atherosclerotic carotid plaques and the role of smooth muscle cells and macrophage polarization in their production.


Submitted for publication
Abstract

OBJECTIVES Matrix metalloproteinases (MMPs) destabilize atherosclerotic plaques by promoting matrix destruction, angiogenesis and leukocyte infiltration. Macrophages and smooth muscle cells (SMCs) are involved in the development of vulnerable plaques, as they produce MMPs. AIM In this study we explored the potential of MMPsense as marker for atherosclerotic plaque instability using molecular imaging techniques. Secondly, presence of different MMPs in atherosclerotic plaques, and expression of MMPs by macrophage subtypes and SMCs was investigated.

METHODS Twenty-three consecutive plaques removed during carotid endarterectomy were incubated in a MMP-sensitive activatable fluorescent probe (MMPSense™ 680) and multispectral fluorescence imaging with IVIS® Spectrum was performed. mRNA levels of MMPs, macrophage markers, and SMCs were determined in specimens of plaques. mRNA expression of different MMPs was determined in in vitro differentiated M1 and M2 macrophages from healthy volunteers and in SMCs. Furthermore, MMP-9 protein was measured in supernatants of cultured macrophages with ELISA.

RESULTS There was a significant difference between autofluorescence signals and MMPSense signals, both on the intra- and extraluminal sides of plaques. MMP-9 and CD68 mRNA expression was higher in hot spots than in cold spots, whereas MMP-2 and αSMA expression was higher in cold spots. In plaques, MMP-9 mRNA expression was 100-1000 fold higher than other MMPs and correlated strongly with CD68 expression. In vitro M2 macrophages had higher mRNA expression of MMP-1, -9, -12 and TIMP-1 and lower MMP-2 and MMP-14 expression compared to M1 macrophages.

CONCLUSION MMP-9 is most dominantly MMP present in atherosclerotic plaques and is produced by M2 rather than M1 macrophages. MMPsense can be used to detect MMP-9 in plaques and might be a good marker to investigate plaque instability in the clinical setting.
Introduction

Atherosclerosis is a progressive inflammatory disease characterized by the accumulation of lipid-filled macrophages within the arterial intima. Continued inflammation may promote rupture of the atherosclerotic plaque’s protective fibrous cap causing subsequent clinical ischaemic events (1,2). The fibrous cap covering an advanced atherosclerotic plaque is typically composed of smooth muscle cells (SMC) and extracellular matrix (ECM) (3). Activated monocyte-derived macrophages and SMCs are critically involved in the development of high-risk vulnerable plaques by producing matrix metalloproteinases (MMP’s) (1,4,5).

A heterogeneous population of macrophages exists including a classically activated macrophage type (M1) as well as an alternatively activated macrophage population (M2) (6). The M1 macrophage is thought to have pro-inflammatory properties and polarization in vitro is driven by low concentration lipopolysaccharide (LPS) and interferon gamma (IFN-γ). Defined as classically however, M2 macrophages are anti-inflammatory and immune regulatory. Upon cytokine stimulation they modify development of atherosclerotic plaques. The M2 macrophage population can be divided in M2a, M2b and M2c subtypes, depending on the cytokine environment (IL-4, immune complexes and IL-10, respectively) (7). In contrast to its classical M2-properties, the function of M2a macrophages is wound-healing or tissue-repair functions, and the function of type M2c is matrix deposition and tissue remodeling; this last mentioned type might be most important in atherosclerosis (8). Recently, Wolfs et al. suggested that additional circumstances in the local microenvironment makes macrophage polarization in the atherosclerotic tissue even more complex than the typically described M1 and M2 macrophages distribution (9). So, not only cytokine environment, but also foam cell formation and CXCL4 (chemokine ligand 4), among other factors, play major roles.

MMP’s are proteolytic enzymes that can degrade ECM proteins such as gelatin (MMP-2 and -9), collagen (MMP-1, -8 and -13), elastin (MMP-12) and fibrin (MMP-3 and -10). They can be inhibited by tissue inhibitors of metalloproteinases (TIMP’s). The ever growing MMP family now consists of more than 20 known proteins (10). There is conflicting evidence about the role of MMP-9 in atherosclerosis. In carotid atherosclerotic disease MMP-9 is mostly associated with the development of unstable plaques. In patients with symptomatic carotid disease, increased MMP-9 levels have been shown both in plasma and in plaque tissue (11-16). Other studies showed an inverse relation between plaque activity and MMP-9 plasma levels, or even evidence for a plaque stabilizing role for MMP-9 (17,18). At the moment, MMP’s cannot be visualized with conventional imaging modalities, such as duplex ultrasound, computerized tomography scanning, and magnetic resonance imaging. Although these conventional imaging techniques have improved and do have the ability to image cardiovascular anatomy and physiology on a macroscopic scale, they lack the possibility to detect biological processes at the cellular or molecular level (19). In contrast,
molecular imaging has the possibility to target molecular components of atherosclerotic disease on a microscopic scale using smart activatable probes (20). As such, MMPs may be targeted with a MMP-sensitive probe (MMPsense) and can be visualized by fluorescence imaging (21-23). MMPsense is a protease activatable fluorescent imaging agent that is activated by MMP -2, -3, -9 and -13. MMPsense is optically silent in its inactivated state and becomes highly fluorescent following protease-mediated activation.

In this study, we analyzed the presence of MMPs in human carotid plaques by using fluorescence and investigated the differences in intensity of fluorescence signals. Furthermore, macrophages were differentiated and polarized in vitro into M1, M2a and M2c macrophages to investigate expression of MMPs in these subtypes, as well as in smooth muscle cells. In this way the potential of MMPsense as marker for atherosclerotic carotid plaque vulnerability was explored, and the relation to MMP expression of macrophage subtypes and smooth muscle cells throughout atherosclerotic plaques.

**Materials and Methods**

**Study Design**

Patients presenting with symptoms (i.e. with a history of recent cerebrovascular accident (CVA)), transient ischaemic attack (TIA) or amaurosis fugax) and a stenosis of the common carotid artery of 70-99% as detected by duplex ultrasound examination underwent open carotid surgery at the University Medical Center Groningen (UMCG). Additionally, asymptomatic patients with a stenosis of 80-99%, found by routine control were also eligible for surgical treatment. Therefore, a total of 23 carotid specimens were obtained by means of carotid endarterectomy (CEA) of the carotid bifurcation using standard techniques (24). Risk factors such as hyperlipidemia, hypertension, smoking status, obesity (body mass index, BMI) and diabetes mellitus were recorded. Hyperlipidemia and hypertension was defined as described before by our group (25). To measure MMP expression in differentiated macrophages, ten healthy volunteers were included without known cardiovascular disease or risk factors. The study was approved by the Institutional Review Board (IRB) of the UMCG and informed consent was obtained from all patients and healthy volunteers.

**Carotid Endarterectomy Sample Collection and Timepath of Study**

Plaques were obtained immediately after endarterectomy. Following endarterectomy all specimen were washed with PBS to remove blood and debris. After storage in PBS, samples were put on ice and taken to the laboratory for fluorescence imaging. The plaques were cut open longitudinally and pinched on a silicone plate with non reflective black paper (XPB-24 black paper, Caliper Life Sciences, Hopkinton, MA, USA) in between to reduce autofluorescence. Then, autofluorescence signals were recorded on the intra- and extraluminal sides of the plaque. The mean time between excision of the plaque and
determination of autofluorescence was 31 minutes (16-45). It took 9 minutes (2-26) to complete the autofluorescence recordings. After that, the plaque was incubated with a MMP-sensitive activatable fluorescent probe (MMPSense™680, VisEn Medical, Boston, MA, USA), which was diluted 1:10. Specimens were incubated for 66 minutes (60-72) before imaging.

**Fluorescence imaging and data analysis**

Fluorescence images were obtained with a commercial imaging system with an ultra-sensitive charge-coupled device camera mounted on a light-tight black chamber (IVIS® spectrum, Caliper Life Sciences, Hopkinton, MA, USA). The charged coupled device (CCD) camera was cooled to -90°C Celsius. The excitation and emission filter were set at Cy5.5. By dividing the mean radiance (p/s/cm²/sr) from the MMPSense signal by the autofluorescence signal the target-to-background ratio (TBR) was calculated. The imaging data were analyzed using Living Image® 3.0 software (Caliper Life Sciences, Hopkinton, MA, USA).

**Immunohistochemistry**

Slices of plaques were embedded in paraffin, and sections of 4 μm were cut. Macrophages were identified by incubation with monoclonal mouse anti-human CD68 (1:50; mo876 clone PG-M1 DAKO, Glostrup, Denmark). For detection of MMP-9, a goat anti-human antibody (AB911, R&D systems, Minneapolis, USA) was used. Appropriate secondary antibodies labeled with horseradish peroxidase (HRP) were used. Color reaction was developed using DAB staining with chromogen, and sections were counterstained with hematoxylin.

**In Vitro Differentiation and Polarization of M1, M2a and M2c Macrophages**

From ten healthy donors peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep (Axis Shield PoC As, Oslo, Norway). Subsequently, monocytes were allowed to adhere to culture plates. The adherent cells were maintained for 5 days in RPMI 1640 medium (Lonza, Walkersville, MD, USA) supplemented with 10% filtered fetal calf serum (FCS) and 20 ng/ml macrophage colony stimulating factor (M-CSF, R&D systems) for differentiation into macrophages. Macrophages were directed towards M1, M2a and M2c phenotypes by use of 48 hours stimulation with 100 U/ml IFNγ (PeproTech, USA) and 1 ng/ml LPS (Sigma, Germany), 20 ng/ml IL-4 and/or 10ng/ml IL-10 (PeproTech), respectively. MMP-9 protein was measured in supernatants of cultured macrophages with ELISA (duoset, R&Dsystems) according to manufacturer’s description.

**RNA expression in vitro in macrophage subtypes and SMCs and ex vivo in plaques**

To measure MMP expression, RNA was extracted from above named macrophage subtypes and from smooth muscle cells as described before (25). From four imaged plaques areas
with high intensity (hotspots) and low intensity (cold spots) were excised and mRNA was isolated from these specimens. Four un-imaged plaques were divided in equal slices of 5 mm, and mRNA was also isolated from these slices. cDNA samples were added in duplicate for amplification by the Taqman real time PCR system (ABI Prism 7900HT Sequence Detection system, Applied Biosystems, Foster City, CA, USA). mRNA expression of MMP-1, -2, -3, -8, -9, -12, -13, -14 and -16, and TIMP-1 was measured by using Taqman primer/probes sets. In slices of plaques, also mRNA expression of CD68 (pan macrophages), CD86 (M1 macrophage marker), CD163 (M2 macrophage marker) and αSMA (ACTA2) was investigated. Ct (threshold cycle) values were determined using the software program SDS 2.4 (Applied Biosystems). Relative gene expression was normalized to the expression of GAPDH and calculated by the following formula: relative gene expression = $2^{-\Delta Ct}$ ($\Delta Ct = Ct$ gene of interest – Ct GAPDH).

**Statistical Analysis**

Values are presented as mean ± standard deviation or median (range), unless stated otherwise. For correlations, Pearson’s and Spearman’s correlation coefficients were used when appropriate. A two-tailed, paired student T-test was used for parametric distributions (i.e. fluorescence signal and TBR)). Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. For comparing more than two independent samples, the Kruskal-Wallis test was used (i.e. four types of macrophages). A two-sided P value < 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 20.0, SPSS inc®, Chicago IL, USA).
Results

Patient Demographics
A total of 15 men and eight women with a mean age of 70 ± 9 years were included. Baseline characteristics of participants are shown in Table 1.

| Table 1. Baseline characteristics and risk factors for atherosclerosis |
|--------------------|------------------|
| Patients (n=23)     |                  |
| Men, n (%)          | 15 (65)          |
| Age, years          | 70 ± 9           |
| Symptomatic, n (%)  | 21 (91)          |
| Transient ischemic attack (TIA), n (%) | 9 (39) |
| Cerebro vascular accident (CVA), n (%) | 8 (35) |
| Amaurosis fugax, n (%) | 4 (17) |
| BMI, kg/m²           | 27 ± 3           |
| Smoking status, n (%)| 10 (43)          |
| > 1 pack a day, n (%)| 6 (26)           |
| ≤ 1 pack a day, n (%)| 4 (17)           |
| None, smoked in last 10 years (%) | 6 (26) |
| Hypertension, n (%)  | 19 (83)          |
| Controlled with single drug, n (%) | 5 (21) |
| Controlled with 2 drugs, n (%) | 5 (21) |
| Requires > 2 drugs or uncontrolled, n (%) | 9 (39) |
| Systolic blood pressure, mmHg | 146 ± 22 |
| Diastolic blood pressure, mmHg | 80 ± 12 |
| Hyperlipidemia, n (%)  | 14 (61)          |
| Use of lipid-lowering drugs, n (%) | 9 (39) |
| Diabetes mellitus, n (%) | 7 (30)* |

* Four patients had diabetes controlled by diet or oral agents, three patients where on insulin. Data are expressed as mean ± standard deviation; percentages between brackets.

Fluorescence imaging
Fluorescence signal of each plaque was recorded before and after incubation with MMPSense™680. MMP signals were heterogeneously distributed throughout plaques (Figure 1). The mean TBR was considered appropriate, and did not significantly differ between intraluminal and extraluminal sides (7.15 vs 6.43; P=0.53). Fluorescence signals clearly augmented after incubation with MMPSense compared to autofluorescence, showing highly significant differences on both intraluminal (6.34 vs 1.09; P<0.0001) and extraluminal sides (6.12 vs 1.04; P<0.0001) (Figure 2).
Figure 1. Sections of fluorescence image of an atherosclerotic carotid plaque specimen after MMPSense incubation (as well as autofluorescence) showing heterogeneous accumulation on intra- and extra luminal side.
Figure 2. Fluorescence signals after incubation with MMPSense compared to autofluorescence. (A) intraluminal (6.34 vs 1.09; p<0.0001) and (B) extraluminal side (6.12 vs 1.04; p<0.0001).

**Ex vivo MMP, αSMA and macrophage expression in plaques**

MMP-2, MMP-9, αSMA and CD68 mRNA expression was investigated in hot and cold spots from 4 imaged plaques. As can be seen in figure 3A, MMP-9 and CD68 expression was up regulated in hot spots, whereas MMP-2 and αSMA were down regulated in hot spots. To investigate the expression and distribution of MMPs, and their relation to M1 and M2 macrophages in plaques, mRNA expression of MMPs was compared to CD68 (pan macrophages), and to an M1 marker (CD86) and an M2 macrophage marker (CD163). Also, MMP expression was compared to αSMA expression. The strongest correlation was found between MMP-9 and CD68 mRNA expression (P< 0.001, Figure 3B). Furthermore, MMP-9 expression was 100 to 1000 times higher compared to mRNA expression of other MMPs (data not shown) None of the other MMPs showed a significant correlation with macrophage markers, except for MMP-2 and MMP-14 which both correlated significantly with CD86 and CD163. There was a significant correlation between MMP-2 and αSMA expression (Figure 3B). Immunohistochemical staining of plaques for CD68 and MMP-9 showed overlap of CD68 and MMP-9 as can be seen in a representative picture in figure 3C.
Figure 3. Expression of MMPs in atherosclerotic plaques. (A) Fold change mRNA expression in hot spots versus cold spots. (B) Hot spots and cold spots from 4 imaged plaques were excised and mRNA expression of MMP-2, MMP-9, αSMA and CD68 was determined with RT-PCR. Correlation between MMP-9 and CD68 mRNA expression and between MMP-2 and αSMA mRNA expression in slices of plaques. (C) Co localization of immunohistochemical staining of CD68 and MMP-9 in plaques.

MMP expression in macrophage subtypes and SMCs in vitro
mRNA levels of GAPDH (household gene), MMP-1, -2, -3, -8, -9, -12, -13, -14 and -16, and TIMP-1 were determined in M1, M2a, M2c and IL-4 / IL-10 differentiated M2 macrophages from 10 healthy volunteers. mRNA expression of MMP-1 was significantly increased in all three M2 macrophage types compared to M1 macrophages (P<0.05, Paired T-test for all,
TIMP-1 expression was significantly decreased in M1 macrophages compared to M2 macrophages (P<0.001, Figure 4B). MMP-9 and MMP-12 mRNA was undetectable in SMCs. MMP-9 expression was higher in M2 macrophages compared to M1 (Figure 4C). Only the difference between M1 and M2c macrophages was statistically significant (Paired T-test, P=0.02). MMP-12 was significantly higher expressed in M2 compared to M1 macrophages (P<0.001, Figure 4D). On the contrary, MMP-2 and MMP-14 were significantly higher in M1 macrophages compared to all types of M2 macrophages (P<0.02, Figure 5). Of note, MMP-1 and MMP-2 expression was high in SMCs. MMP-3, -8, -13 and -16 were undetectable in *in vitro* generated macrophages and in SMCs. From mRNA data it was shown that expression of MMP-9 was 100 to 1000 times higher compared to mRNA expression of other MMPs (figure 4C). Therefore MMP-9 protein secretion was investigated in supernatants of cultured macrophages. As can be seen in figure 6, all different types of macrophages can produce MMP-9 although M2 macrophages produce more than M1 (not significantly different). So protein levels of MMP-9 are comparable to the mRNA expression of MMP-9 in different subtypes of macrophages.

**Figure 4.**

![Figure 4](image)

Figure 4. Relative expression of MMP mRNA levels in macrophages from 10 healthy volunteers and in SMCs.

Significantly higher relative expression in M2-like macrophages (black dots) compared to M1-like macrophages (open dots) was revealed by quantitative reverse transcription polymerase chain reaction in (A) MMP-1, (B) TIMP-1 and (D) MMP-12. MMP-9 was also higher (C).
Figure 5. Relative expression of MMP mRNA levels in macrophages from 10 healthy volunteers and in SMCs. Significantly lower relative expression in M2 macrophages (black dots) compared to M1-like macrophages (open dots) was revealed by quantitative reverse transcription polymerase chain reaction in (A) MMP-2, and (B) MMP-14.

Figure 6. MMP-9 protein levels in supernatants of cultured M1 and M2 macrophages measured by ELISA.
Discussion

Our study shows that fluorescence imaging with a smart MMP-sensitive activatable probe clearly reveals a heterogeneous distribution of MMPs across the atherosclerotic carotid plaques. The signals of ex vivo human carotid plaques were significantly enhanced after incubation with the fluorescent probe accounting for a 6- to 7 fold increase of signals. MMP-9 was found to be highest expressed in plaques and in different subtypes of macrophages. Signal enhancements such as with MMPsense have been described using other protease probes, both in ex vivo carotid specimens (22), and in vivo rabbits (26). Typically, there are more intense signals (hot spots) near the carotid bifurcation. In a previous study, segments at or near the bifurcation and segments with intraplaque hemorrhage contained higher MMP levels and activity (especially MMP-9) compared to segments distant from the bifurcation. Histologically stable plaques contained lesser amounts of MMPs, which were predominantly MMP-2. TIMPs were highly abundant in fibrotic and necrotic segments (27). In a previous study by Wallis-de Vries using MMPsense in atherosclerotic plaques, and also in the present study it was shown that in areas with high intensity (hot spots) mRNA expression of MMP-9 was increased compared to areas with low intensity (cold spots) and accompanied with a slight increase in CD68 mRNA expression (23). mRNA expression of MMP-2 was decreased in hot spots compared to cold spots, and also αSMA expression. Also good correlations in plaques between mRNA expression of MMP-9 and CD68, and between MMP-2 and SMCs were found. These data are supported by another study, where macrophage rich lesions showed higher MMP-9 activity while SMC-rich lesions showed higher MMP-2 activity (28). Also, SMCs were found in stable plaques in other studies (28,29). MMP-9 protein is abundantly produced by macrophages and is abundantly present in plaques as shown by immunohistochemical staining. In a study by Loftus et al. the character, level, and expression of MMPs in carotid plaques was correlated to clinical status of patients undergoing carotid endarterectomy. The MMP-9 concentration was significantly higher in patients developing symptoms within one month compared to asymptomatic patients (11). Also in a study done by Heo et al. plaque rupture was significantly associated with the development of vascular events in carotid atherosclerotic disease, and with immunohistochemical expression of MMP-2 and -9 (30). One explanation for this might be that MMP-2 and -9 are capable of degrading collagen type IV which is the major component of the basement membrane (14). No difference in the levels of MMP-1, -2, or -3 was found between symptomatic and asymptomatic patients (11). It was also anticipated that serum levels of MMP-9 and MMP-2 were significantly higher in symptomatic patients compared to patients without symptoms (12,13). However, another group found serum MMPs where not predictive of local events, in a group of eighteen patients (31). In the in vitro study mRNA expression of MMP-2 was highest in pro-inflammatory M1 macrophages and in SMCs. MMP-9 and MMP-12 mRNA was highest in M2 macrophages and could not be found in SMCs. This was supported by other groups who found an over expression of MMP-9 in M2 macrophages (32), and
differentiation towards M2 macrophages up regulated MMP-12 expression (4). However, Newby et al. also suggest that each macrophage subtype (not only M2) can be acted on by pro-inflammatory mediators leading to activated states (4). Further research is needed to fully understand the mechanism of MMPs produced by macrophages in the process of an atherosclerotic plaque becoming vulnerable. Applications for non-invasive optical imaging of fluorescent signals could be of less clinical value in coronary atherosclerosis because of the limited penetration depth of only a few millimeters. Therefore we started testing a radiolabeled MMP tracer in ex vivo atherosclerotic plaques recently, from which the results look promising.

We found MMP-9 mRNA expression in plaque tissue was 100 to 1000 times higher compared to other MMPs. These findings strongly suggest that in hot spots MMPsense most likely is activated by MMP-9. Taken everything into account it seems that MMPsense can be used to detect areas of plaque instability primarily by detection of MMP-9 produced by M2 macrophages.

**Conclusion**

It is feasible to image MMP-9 in atherosclerotic tissue ex vivo using a smart activatable fluorescence probe and fluorescence imaging. MMP-9 is produced by macrophages and is abundantly present in plaques as shown by immunohistochemical staining and qRT-PCR. Furthermore, areas with high intensity (hot spots) have a higher mRNA expression of MMP-9 compared to areas with low intensity (cold spots). Also, MMP-9 expression was highest in M2 macrophages and could not be found in SMCs. In conclusion, MMPsense can be used to detect MMP-9 in plaques and might therefore be a good marker to investigate plaque instability.

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MMP IMAGING AND THEIR PRODUCTION BY SMCS AND MACROPHAGE SBTYPES IN PLAQUES

References
