Nuclear Medicine and Biology 41 (2014) 562–569

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

# Gene expression levels of matrix metalloproteinases in human atherosclerotic plaques and evaluation of radiolabeled inhibitors as imaging agents for plaque vulnerability

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ARTICLE INFO

*Article history:*
Received 28 February 2014
Received in revised form 10 April 2014
Accepted 12 April 2014

*Keywords:*
Atherosclerosis
Matrix metalloproteinases
Tissue inhibitor of metalloproteinase-3
Carotid artery plaque
Inflammation
Autoradiography

ABSTRACT

*Introduction:* Atherosclerotic plaque rupture is the primary cause for myocardial infarction and stroke. During plaque progression macrophages and mast cells secrete matrix-degrading proteolytic enzymes, such as matrix metalloproteinases (MMPs). We studied levels of MMPs and tissue inhibitor of metalloproteinases-3 (TIMP-3) in relation to the characteristics of carotid plaques. We evaluated in vitro two radiolabeled probes targeting active MMPs towards non-invasive imaging of rupture-prone plaques.
*Methods:* Human carotid plaques obtained from endarterectomy were classified into stable and vulnerable by visual and histological analysis. MMP-1, MMP-2, MMP-8, MMP-9, MMP-10, MMP-12, MMP-14, TIMP-3, and CD68 levels were investigated by quantitative polymerase chain reaction. Immunohistochemistry was used to localize MMP-2 and MMP-9 with respect to CD68-expressing macrophages. Western blotting was applied to detect their active forms. A fluorine-18-labeled MMP-2/MMP-9 inhibitor and a tritiated selective MMP-9 inhibitor were evaluated by in vitro autoradiography as potential lead structures for non-invasive imaging.
*Results:* Gene expression levels of all MMPs and CD68 were elevated in plaques. MMP-1, MMP-9, MMP-12 and MMP-14 were significantly higher in vulnerable than stable plaques. TIMP-3 expression was highest in stable and low in vulnerable plaques. Immunohistochemistry revealed intensive staining of MMP-9 in vulnerable plaques. Western blotting confirmed presence of the active form in plaque lysates. In vitro autoradiography showed binding of both inhibitors to stable and vulnerable plaques.
*Conclusions:* MMPs differed in their expression patterns among plaque phenotypes, providing possible imaging targets. The two tested MMP-2/MMP-9 and MMP-9 inhibitors may be useful to detect atherosclerotic plaques, but not the vulnerable lesions selectively.



## 1. Introduction

Ischemic stroke represents a major health problem and is an important cause of long-term disability in several developed countries [1]. Mortality rate from stroke ranges from 10% to 30%, and its survivors remain at a high risk of recurrent ischemic events and mortality, both from myocardial infarction and repeated stroke [2]. Rupture of atherosclerotic plaques and consequent thrombus formation are considered as the crucial steps in the development of acute cardiovascular events, such as cerebral and myocardial infarctions [3,4]. High-grade internal carotid artery stenosis is, therefore, almost always an indication for immediate carotid endarterectomy (CEA) to avoid rupture of a potentially vulnerable plaque. Arterial stenosis is diagnosed by duplex ultrasound magnetic resonance angiography and computerized tomographic angiography. However, both techniques cannot assess plaque composition and vulnerability and, therefore, give no information on whether an immediate surgical intervention is imperative or not. Clinically important for an early selection and administration of an appropriate therapy would be the distinction between those atherosclerotic plaques that are at risk for rupture from those that are stable and do not require immediate intervention.

Much effort is put into the search for suitable radiotracers that allow non-invasive imaging of rupture-prone plaques by positron emission tomography (PET) or single photon emission computed tomography

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http://dx.doi.org/10.1016/j.nucmedbio.2014.04.085
0969-8051/

(SPECT) [5–7]. Recent advances in molecular imaging indicate that the key to distinguish vulnerable from stable plaques lies in immune processes. Among the most promising PET probes so far are those targeting activated immune cells. They include $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) [8,9], $^{18}$F-fluorodeoxymannose ($^{18}$F-FDM) [10] and folate-derived radiopharmaceuticals [11–13], targeting activated macrophages. We have recently evaluated in human CEA samples the folate receptor 2 and immune-stimulatory molecule CD80 as promising imaging targets [7,13].

The high load of atherosclerotic plaques with activated macrophages provides a prominent source of matrix metalloproteinases (MMP). MMPs play a central role in degradation of extracellular matrix (ECM), resulting in destabilization of the atherosclerotic plaque which may contribute to plaque rupture [14–18]. The integrity of the ECM depends on the balance of degradation and repair of collagen and other matrix components [19,20]. Macrophages and mast cells locally destabilize the plaque due to their ability to produce and secrete MMPs. The advantage of imaging active MMPs non-invasively by PET or SPECT could be the assessment of tissue degradation levels and thus allow detecting plaques in an advanced state of atherosclerotic lesion when they become harmful.

MMPs are a group of more than 20 structurally related proteases that share a zinc-based catalytic mechanism [21]. MMPs are transferred to the ECM as soluble or membrane-bound inactive zymogens (pro-MMPs). Once activated via the “cysteine switch” mechanism an active site exposing a zinc ion is provided that initiates its proteolytic activity. In tissue, MMP activity is counterbalanced by the tissue inhibitors of MMPs (TIMPs), which prevent excessive proteolytic activity. Among the many MMPs that have been identified, the two gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) contribute substantially to the degradation of type IV collagen and gelatin, the two main components of ECM. MMP-2 and MMP-9 are involved in a variety of diseases, e.g. cancer, Alzheimer’s disease, and myocarditis [22]. In the context of atherosclerosis, recent publications reported higher MMP-9 levels in macrophage-rich lesions and higher MMP-2 levels in the more stable smooth muscle cell-rich lesions [23,24].

Several research groups are currently working on the development and evaluation of MMP inhibitor-based radiotracers targeting MMPs in their active form [25–28]. MMP inhibitors have been evaluated as diagnostic and therapeutic tools in cancer, autoimmune disease, and cardiovascular disease [29,30]. Despite the over 3000 entries under “MMP inhibitors” in the data base Integrity (Thomson Reuters), of which many have been tested both experimentally and clinically, only one or two, including the antibiotic doxycycline, are approved for systemic use as MMP inhibitor by the Food and Drug Administration. The low success rate is mainly due to the undesirable side effects of MMP inhibitors in particular on the musculoskeletal system. Most are broad spectrum MMP inhibitors or at least not selective for one specific MMP-type. This is not only a drawback for therapy but is also a limitation for the imaging approach with respect to the specificity of the resulting signal.

We have recently introduced the radiosynthesis of a selective low molecular weight MMP-9/MMP-2 inhibitor as a potential PET probe for the two gelatinases [27]. In the present study, excised human artery wall segments from CEA classified into normal arteries, stable and vulnerable atherosclerotic plaques were investigated regarding their gene expression levels of various MMPs. We further characterized this tissue regarding the catalytically active forms of MMP-2 and MMP-9, the targets of our radiotracer [27]. We tested in vitro the ability of the tracer as an imaging agent for atherosclerotic plaques and further evaluated the $^{3}$H-labeled selective MMP-9 inhibitor methyl 4-[3-(formylhydroxyamino)-4-(4-(4-trifluoromethoxy-phenoxy)phenylsulfonyl)butyl]benzoate ($^{3}$H-**2**, compound **19 g** in reference [31]) as a potential probe to specifically target active MMP-9 in vulnerable atherosclerotic plaques.

## 2. Material and methods

### 2.1. Clinical data and human tissue banking

Patients were referred to the Clinic for Cardiovascular Surgery of the University Hospital of Zurich (Zurich, Switzerland) for CEA between 2010 and 2012. Written informed consent was obtained from all patients. Before surgery, all patients underwent Duplex ultrasound (to define the degree of stenosis and the morphologic characteristics of plaques) or computed tomography angiography, as well as a computed tomography or magnetic resonance brain scan. The atherosclerotic plaques were removed from the internal, external and common carotid artery by bifurcation advancement [32]. All excised specimens were photographed for macroscopic re-evaluation, transferred within 5 min into RNAlater® solution (Sigma, St. Louis, MO, USA), stored at 4 °C overnight and at −80 °C until further use.

### 2.2. RNA isolation and quantitative polymerase chain reaction

Total RNA was extracted from plaque specimens using the Isol-RNA Lysis Reagent (5 Prime, Gaithersburg, USA) and the bead-milling TissueLyser system (Qiagen, Hilden, Germany). QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) was used to generate cDNA. Primers were synthesized by Microsynth (Balgach, Switzerland) or purchased from Qiagen (Hilden, Germany). Primer sequences are listed in Supplemental Table 1. Quantitative polymerase chain reaction (qPCR) was performed with the DyNAmoTM Flash SYBR® Green qPCR Kit (Thermo Scientific) using a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Beta-actin (ACTB) primers were used as internal control to assess amplification efficiency. The amplification signals were detected in real-time, which permitted accurate quantification of the amounts of the initial RNA template during 50 cycles. All reactions were performed in duplicates and in three independent runs. Quantitative analysis was performed using the SDS software v2.4 (Applied Biosystems, Carlsbad, CA, USA) and a previously described quantification method [33]. The specificity of the PCR products of each run was determined and verified with SDS Software's dissociation curve analysis feature.

### 2.3. Immunohistochemical stainings

For histology and immunohistochemistry, plaques were embedded in Neg-50 Frozen Section Medium (Thermo Scientific, Runcorn, UK) and cut in 7 µm serial slices on a cryostat (Cryo-Star HM 560 MV; Microm International GmbH), thaw-mounted onto coated glass slides (Superfrost Plus, Thermo Scientific, Runcorn, UK), and stored at −80 °C until further use. For histological characterization of the plaque morphology hematoxylin and eosin (HE; (Sigma, St. Louis, MO, USA) staining was applied.

Immunohistochemistry was performed according to the protocol of Dako REAL™ EnVision™ Detection System, Peroxidase/DAB, Rabbit/Mouse (Dako, Carpinteria, CA, USA) using mouse anti-CD68 (1:50) (ab955, Abcam, Cambridge, UK), rabbit anti-MMP-2 (1:100) (ab79781, Abcam, Cambridge, UK) or mouse anti-MMP-9 (1:300) (ab3195, Abcam, Cambridge, UK) antibodies. Sections were counterstained with hematoxylin solution, Gill No. 1 (Sigma, St. Louis, MO, USA). Corresponding immunoglobulin G (IgG) antibodies served as internal negative control.

All sections were covered using Eukitt mounting medium (Sigma) and analyzed under a microscope (Zeiss AxioImager A1, Jena, Germany or digital slide scanner Pannoramic 250, Sysmex, Horgen, Switzerland).