Atherosclerotic carotid disease, the vulnerable plaque in the vulnerable patient
Wallis de Vries, Bastiaan Melchior

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 26-09-2019
MULTISPECTRAL NEAR-INFRARED FLUORESCENCE MOLECULAR IMAGING OF MATRIX METALLOPROTEINASES IN A HUMAN CAROTID PLAQUE USING A MATRIX-DEGRADING METALLOPROTEINASE-SENSITIVE ACTIVATABLE FLUORESCENT PROBE

Authors:
Bas M. Wallis de Vries
Jan-Luuk Hillebrands
Gooltzen M. van Dam
René A. Tio
Johannes S. de Jong
Riemen H.J.A. Slart
Clark J. Zeebregts

Circulation 2009;119:534-536
Introduction
Formation of unstable atherosclerotic plaque in the internal carotid artery carries a high risk for emboli and subsequent cerebral ischemic events. The fibrous cap of such a plaque may become thin and rupture as a result of the depletion of matrix components through the activation of proteolytic enzymes such as matrix-degrading proteinases. Enhanced matrix breakdown has been attributed primarily to a family of matrix-degrading metalloproteinases (MMPs) that are highly concentrated in atherosclerotic plaques by inflammatory cells (eg, macrophages, foam cells), smooth muscle cells and endothelial cells. Elevated serum MMP-9 concentration is associated with carotid plaque instability and the presence of infiltrated macrophages. Furthermore, analysis of the presence of MMP-9 protein by ELISA within excised carotid plaques revealed high MMP-9 protein mass in calcified segments at or near the carotid bifurcation and in segments with intraplaque hemorrhage. Gelatin zymography showed an increased gelatinase activity of MMP-9 in these segments. These data favor the important role of MMP-9 in the pathogenesis of plaque instability.

Methods
We analyzed the topographic distribution of MMPs within an excised human carotid plaque by applying multispectral near-infrared fluorescence (NIRF) imaging (IVIS Spectrum, Caliper Life Sciences, Hopkinton, Mass). A surgical endarterectomy was performed on a 74-year-old women with a left-sided, symptomatic, 70% carotid stenosis. Immediately after endarterectomy, the plaque was placed in PBS and transported to the NIRF system. The plaque was then stretched out and fixed on a silicon plate with 25G needles.

Results
A PBS NIRF image was generated from both the intraluminal and extraluminal side of the plaque to determine the level of autofluorescence (background) (Figure 1). After incubation with a MMP-sensitive activatable fluorescent probe (MMPSense 680, VisEn Medical, Boston, Mass), we detected differential topographic NIRF signals within the specimen (Figure 1). Compared with the autofluorescence signal obtained without incubation with MMPSense, a 6- and 7-fold increase of the total NIRF signal was observed on the intraluminal and extraluminal side, respectively, after incubation with MMPSense 680. The NIRF signal was not homogeneously distributed across the plaque surface but rather resulted in the identification of areas with high NIRF intensity (denominated as “hot spots”) and areas with relatively low NIRF intensity (so-called “cold spots”)
Because MMP-9 activity has been positively correlated with plaque instability as described above, we next determined the contribution of MMP-9 to the NIRF signals as shown in Figure 1. To this end, in situ zymography was performed on tissue sections from excised hot and cold spots as indicated in Figure 1 by the framed areas.

After we had used dye-quenched gelatin as substrate on hot and cold spots, results indicated increased gelatinase activity within the hot spot relative to the cold spot (Figure 2). Both gelatinaseA (MMP-2) and gelatinaseB (MMP-9) are able to degrade, and thereby activate, dye-quenched gelatin. We therefore performed real-time reverse-transcriptase polymerase chain reaction analysis for MMP-2 and MMP-9 in order to discriminate between MMP-2 and MMP-9 as the gelatinase responsible for increased gelatinase activity in the hot spot. Results obtained revealed that the mRNA expression of MMP-2 decreased 14-fold and...
that of MMP-9 increased 8-fold in the hot spot compared with the cold spot. Therefore, the gelatinase most abundantly present and enzymatically active in the hot spot, as identified by in situ zymography, is therefore most likely MMP-9. Morphologically, the hot spot is characterized by reduced nuclear density (Figure 3A and 3B), reduced collagen deposition (Figure 3C and 3D), reduced numbers of invading smooth muscle actin (SMA)–expressing smooth muscle cells (Figure 3E and 3F) when compared with the cold spot. Real-time reverse-transcriptase polymerase chain reaction analysis for SMA expression confirmed the immunofluorescence staining data (30-fold decrease of SMA expression in hot spot compared with cold spot). Furthermore, a slightly increased level of CD68 mRNA expression in the hot spot was observed (=3-fold increase compared with cold spot) as also suggested by the CD68 immunofluorescent staining (Figure 3E and 3F).

Figure 2. Hot spot identified using NIRF is characterized by increased gelatinase activity compared with cold spot. In situ zymography was performed on cryosections (7 µm) of cold (A) and hot spot (B). To this end, sections were incubated with DQ-gelatin (50 µg/mL, EnzCheck Gelatinase/Collagenase Assay Kit, Invitrogen-Molecular Probes, Breda, the Netherlands) overnight at room temperature. Nuclei were counterstained with DAPI. To control for specificity, sections of cold (C) and hot spot (D) were preincubated for 2 hours with 2 mmol/L 1,10-phenanthroline, a general inhibitor of MMPs. Sections were analyzed on a Leica DMLB fluorescence microscope (Leica Microsystems, Rijswijk, the Netherlands) equipped with a Leica DC300F camera and LeicaQWin 2.8 software. For clarity, the nuclear DAPI staining in the lower power magnifications is omitted. Original magnifications: A through D, × 80; insets in A through D, × 320.
Figure 3. Compared with the cold spot, the hot spot is characterized by reduced nuclear density, reduced collagen deposition, and reduced numbers of invading αSMA-expressing smooth muscle cells. Formalin-fixed cryosections (4 µm) of the hot and cold spot were stained with hematoxylin and eosin (A and B) and Masson trichrome (C and D) to evaluate general morphology and deposition of connective tissue (collagen), respectively. Sections were coverslipped in Depex mounting medium. Sections were analyzed on an Olympus BX50 microscope (Olympus Nederland BV, Zoeterwoude, the Netherlands) equipped with an Olympus DP70 camera and Cell B imaging software. Furthermore, immunofluorescent double-labeling for αSMA (clone 1A4, mIgG2a) and CD68 (clone KP-1, mIgG1) was performed on aceton-fixed cryosections to detect smooth muscle cells and infiltrating macrophages, respectively (E and F). Primary anti-αSMA and anti-CD68 monoclonal antibodies were detected with, respectively, Alexa488-conjugated goat anti-mouse IgG2a and TRITC-conjugated goat anti-mouse IgG1 polyclonal antibodies. Nuclei were counterstained with DAPI, and sections were coverslipped in Citifluor mounting medium. Sections were analyzed on a Leica DMLB fluorescence microscope (Leica Microsystems, Rijswijk, the Netherlands) equipped with a Leica DC300F camera and LeicaQWin 2.8 software. Original magnifications: A through D, ×20; E through F, ×100. Insets show high-power magnifications: A through D, ×400; E through F, ×630. H&E indicates hematoxylin and eosin.
Conclusion
The possibility to map the MMP distribution within an atherosclerotic plaque with high sensitivity by using state-of-the-art multispectral NIRF molecular imaging offers a number of potential benefits for the future once translational studies on the pathogenesis and treatment of the vulnerable plaque have been conducted. Histological and morphological analysis of atherosclerotic plaques can be carried out more accurately because specific (MMP) hot and cold spots can be identified by NIRF imaging, enabling different areas of vulnerability within a specimen. This may enable the basis for future noninvasive in vivo imaging tools to create the possibility to longitudinally measure the effect of cardiovascular drugs on atherosclerotic plaques, shifting the paradigm toward spatiotemporal functional molecular imaging as surrogate end point. Finally, if the relation between MMP expression and plaque instability is further established, in vivo NIRF imaging of carotid plaques opens the possibility of determining which plaques are vulnerable and which are not, thereby improving selection criteria for surgical or endovascular intervention.

ACKNOWLEDGMENTS

We thank Niels Harlaar, Manon van Riezen, and Mark Walther Boer for their technical assistance.

SOURCE OF FUNDING

The authors gratefully acknowledge funding granted by De Cock Foundation, Groningen, The Netherlands (No. 08-66).
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