Short Communication

Calcification Locates to Transglutaminases in Advanced Human Atherosclerotic Lesions

Hanke L. Matlung,* Harald C. Groen,^{†‡}
Judith de Vos,* Theo van Walsum,[§]
Aad van der Lugt,[‡] Wiro J. Niessen,^{§¶}
Jolanda J. Wentzel,[†] Ed vanBavel,*
and Erik N.T.P. Bakker*

From the Department of Biomedical Engineering and Physics,* Academic Medical Center, Amsterdam; the Department of Biomedical Engineering,[†] the Biomedical Imaging Group Rotterdam,[‡] and the Department of Radiology,[§] Erasmus MC, Rotterdam; and the Faculty of Applied Sciences,[¶] Delft University of Technology, Delft, The Netherlands

Transglutaminases play an important role in vascular smooth muscle cell-induced calcification *in vitro*. In this study, we determined whether these enzymes are also involved in human atherosclerotic calcification using nine carotid artery specimens obtained at endarterectomy. Sections of the carotid artery specimens were registered to micro-computed tomography images and stained for tissue-type transglutaminase, plasma transglutaminase factor XIIIa (FXIIIa), the N^ε(γ-glutamyl)lysine cross-link, and the macrophage marker CD68. *Ex vivo* micro-computed tomography revealed extensive calcification, which significantly correlated with the cross-link. FXIIIa was found to be the dominant transglutaminase, rather than tissue-type transglutaminase, although staining of both transglutaminases correlated with the cross-link. Staining for FXIIIa colocalized with CD68 at both the cellular and tissue level. In conclusion, areas of calcification locate to the presence and activity of transglutaminases in human atherosclerotic arteries. FXIIIa seems to be the dominant transglutaminase and may be derived from local macrophages. These results are consistent with the hypothesis that transglutaminases participate in the calcification process of human atherosclerotic arteries. (Am J Pathol 2009, 175:1374–1379; DOI: 10.2353/ajpath.2009.090012)

These cells may be derived from stem cells or differentiate from smooth muscle cells or pericytes. Recent work from Johnson et al² suggests a role for transglutaminases in the process of arterial calcification. On the basis of *in vitro* experiments on cultured healthy arterial segments and smooth muscle cells, these authors proposed a novel mechanism of arterial calcification, for which tissue-type transglutaminase (TG2) is central in the chondro-osseous differentiation and calcification of smooth muscle cells. Transglutaminases form a class of enzymes with pleiotropic function³ that consists of nine known members. Among these, TG2 is expressed in endothelial cells, smooth muscle cells, and monocytes/macrophages. The plasma transglutaminase, factor XIIIa (FXIIIa) is expressed in monocytes/macrophages.⁴ TG2 has been implicated in an animal model for atherosclerotic lesion formation,⁵ whereas both TG2 and FXIIIa are involved in vascular remodeling.^{6,7} TG2 and FXIIIa are also involved in normal bone formation, where they act in concert to promote chondrocyte maturation.⁸ Although these data suggest an important role of transglutaminases in the development of atherosclerotic calcification, evidence of their role in human atherosclerotic lesion development is lacking. Therefore, we investigated the presence of transglutaminases, ie, TG2 and FXIIIa, the transglutaminase-induced cross-link and macrophages in human atherosclerotic carotid arteries. These markers were correlated to calcified areas that were detected *ex vivo* with micro-computed tomography (μCT) imaging.

H.L.M. and E.N.T.P.B. are supported by the Netherlands Heart Foundation (grant 2001T038). H.C.G. is supported by the Interuniversitair Cardiologisch Instituut Nederland (Grant 44.138). T.v.W. and W.N. is supported by the Stichting voor de Technische Wetenschappen of The Netherlands Organization for Scientific Research (NOW). A.v.d.L. is recipient of a fellowship from the Netherlands Organisation for Health Research and Development (NWO-KF Grant 907-00-122).

Accepted for publication June 18, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Erik N.T.P. Bakker, Ph.D., Department of Biomedical Engineering and Physics, Academic Medical Center, PO Box 22700, 1100 DE Amsterdam, the Netherlands. E-mail: n.t.bakker@amc.uva.nl.

Vascular calcification is considered a tightly regulated process of matrix deposition by osteoblast-like cells.¹

Materials and Methods

Tissue Specimens

Atherosclerotic plaque tissue was collected from patients undergoing carotid endarterectomy (eight males and one female, aged 51 to 80 years). Detailed patient characteristics are found in Supplemental Table 1 (see <http://ajp.amjpathol.org>). All patients displayed cerebrovascular symptoms. From the nine symptomatic patients, eight of the atherosclerotic plaques were collected from the symptomatic artery and one from the asymptomatic carotid artery in a patient with an occlusion of the symptomatic artery. Specimens were kept in a tissue bank at -80°C until further processing. The study was approved by the medical ethics committee, and all patients gave written informed consent.

Ex Vivo μCT

After storage, specimens were defrosted before scanning with a μCT scanner (Skyscan 1072, Skyscan, Kontich, Belgium) operating at 80 kV and 100 μA to image calcifications inside the plaques. The samples were scanned in air as a whole during 15 to 30 minutes, depending on their length, varying from 8 to 25 mm. After reconstruction, the image resolution of the sample was $18 \times 18 \times 18 \mu\text{m}$.

Immunohistochemical Staining

After μCT scanning the carotid arteries were fixed in formalin for 24 to 48 hours and decalcified with EDTA for an additional 24 to 48 hours before routine processing and embedding in paraffin. Adjacent sections (5 μm) were deparaffinized, and antigen retrieval was performed by boiling in 0.01 mol/L citrate buffer. Thereafter slides were blocked with 5% goat serum, and primary antibodies were incubated either for 60 minutes at room temperature (TG2) or overnight at 4°C (FXIIIA and cross-link); overnight incubation with the antibody against TG2 yielded similar results (data not shown). Secondary antibodies were applied for 30 minutes at room temperature. Bound antibodies were visualized using AEC chromogen (Sigma-Aldrich, St. Louis, MO). Sections were counterstained with hematoxylin. Negative controls were obtained by omission of the primary antibody. For detection of TG2, a rabbit polyclonal antibody was used (concentration 1:10, Labvision, Runcorn, UK) in combination with a horseradish peroxidase-conjugated polyclonal goat anti-rabbit antibody (concentration 1:100, DakoCytomation, Glostrup, Denmark). FXIIIA was detected by a rabbit polyclonal antibody against FXIIIA (prediluted, Labvision) in combination with a horseradish peroxidase-conjugated polyclonal goat anti-rabbit and mouse secondary antibody (prediluted, Abcam, Cambridge, UK). The product of transglutaminase activity was detected by a mouse monoclonal antibody against the $N^{\epsilon}(\gamma\text{-L-glutamyl})\text{-L-lysine}$ isopeptide cross-link (concentration

1:100, Covalab, Villeurbanne, France) together with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (concentration 1:100, Southern-Biotech, Birmingham, AL). Macrophages were detected by a mouse monoclonal antibody against human CD68 (concentration 1:1600, DakoCytomation) in combination with the Dako REAL EnVision Detection System, Peroxidase/DAB+. Digitized images were made with a NanoZoomer Digital Pathology system (C9600, Hamamatsu, Hamamatsu City, Japan) set up at $\times 40$ magnification.

Double immunofluorescent staining was performed by overnight incubation of slides with a rabbit polyclonal antibody against FXIIIA (prediluted, Labvision) and a mouse anti-human monoclonal antibody against CD68 (concentration 1:100, Abcam). Slides were then incubated for 1 hour at room temperature with secondary antibodies: goat anti-rabbit-Cy3 (concentration 1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-mouse-fluorescein isothiocyanate (concentration 1:100, Abcam). Nuclei were stained with 4,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were made with a confocal microscope (Leica, Wetzlar, Germany), using sequential scanning to avoid cross talk of the dyes.

For localization of the stainings and μCT images, every image was subdivided in sections of $1.6 \times 1.6 \text{ mm}$. These subsections were presented in a random manner and orientation to two blinded observers. Hereby, subsections were selected randomly from the complete dataset including all patients and stainings and analyzed for the percentage of positive staining and the presence of calcium per area of tissue present in that section. Only sections with minimal folding and distortion of tissue were used for analysis ($n = 2$ to 3 locations per patient, 76 cross sections in total stained for either TG2, FXIIIA, the cross-link, or CD68 and were subdivided into 304 sub-

Figure 1. Appearance of calcifications throughout the length of a plaque. A two-dimensional representation of the μCT data is shown here; red indicates the lumen of the vessel, and the outer wall of the vessel is shown in gray. White areas indicate calcifications. Cross sections were used to correlate μCT images to accompanying histological sections, in this case stained for the cross-link. Scale bar = 1 mm. CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; CL, cross-link.

sections). Averaged scores were converted to binary measurements (negative staining, positive staining) with a cutoff value of 4%.

Matching of μ CT and Immunohistochemical Images

To correlate the immunostainings with μ CT data, a matching (registration) of the images between both modalities is needed. This matching requires the localization of the histological sections in the three-dimensional μ CT data. We included 7 degrees of freedom for the registration of these image modalities. Thus, with respect to the volumetric μ CT data, the sections were translated in three dimensions, rotated in three dimensions, and isotropically scaled. These registrations were done manually, using landmarks such as the internal and external contours of the vessel. Applying these procedures directly to the histological sections is not so straightforward

because of the many degrees of freedom. Therefore, as an intermediate step, during the microtome slicing process, *en face* images of the remaining embedded tissue were made at 1-mm intervals. From these *en face* images a three-dimensional stack was created, which was first registered with the μ CT data as described above. This provided estimates for position along the length axis, for tilting and overall shrinkage of the tissue during embedding. Subsequently, each individual histological section was registered to the corresponding *en face* image, using translation, rotation, and isotropic scaling. The full registration is the combination of the registration of the *en face* images to the μ CT data, and the registration of histology images to the *en face* images. Finally, small errors in this two-stage registration procedure, such as slice-dependent deformations resulting from microtome sectioning, were corrected by minor manual adjustments in the positioning of the histological section within the three-dimensional μ CT data.

Figure 2. Example of immunohistochemical and double immunofluorescent staining of a carotid atherosclerotic plaque section. **A:** H&E staining, $\times 5$ magnification. **B:** Cross-link staining. **C:** Corresponding μ CT image; bright spots indicate calcium. **D:** FXIIIa staining. **E:** CD68 staining. Scale bar = 300 μ m. **F:** TG2 staining. **G–I:** Confocal images of double immunofluorescent staining of FXIIIa (red) and CD68 (green). Nuclei are stained with 4,6-diamidino-2-phenylindole (blue).

In addition, an interobserver study was used to validate the registration process. Hereby, two patients were randomly selected and registered by a second observer, blinded for the previous results. The three-dimensional orientation of the histological slices in the μ CT domain was compared between the two observers, and differ-

ences were found to be well within the grid size used for the data analysis (1.6×1.6 mm).

Statistics

The associations between calcium and the cross-link and between the TG2, FXIIIa, CD68, and cross-link staining were analyzed using cross tabulation and χ^2 tests. Associations were considered statistically significant at $P < 0.05$.

Results

A volume rendering of a carotid artery including atherosclerotic plaque and calcifications, visible as bright spots in the μ CT images, is shown in Figure 1 (a three-dimensional representation of Figure 1 is available in Supplemental Movie 1, see <http://ajp.amjpathol.org>). The lumen was segmented manually from the μ CT image for visualization. There was a diverse appearance of calcification, ranging from isolated spots to large, complex dense structures. On the basis of the μ CT images, we found that calcification was present in 24% of the total plaque area analyzed. Histological cross sections were registered to corresponding reformatted slices from the μ CT image to colocalize calcifications and cross-link staining. This revealed that calcified areas stain heavily for the cross-link, as shown in a typical example in Figure 2, A–C. Staining for the cross-link was abundant, but not restricted to calcified areas. In total, the cross-link was present in 62% of the analyzed areas. Staining for FXIIIa was found in 38% of the areas and colocalized with calcifications (Figure 2D). Macrophages, as identified by CD68 staining (Figure 2E), also colocalized with calcification and with FXIIIa, which was shown by the double immunofluorescent staining for FXIIIa and CD68 (Figure 2, G–I; $n = 3$). In contrast, minimal positive staining for TG2 was seen throughout the plaques (Figure 2F). In total, only 7% of the scored areas stained positively. Positive control staining for TG2 as well as negative controls for the cross-link, FXIIIa, and CD68 (immunohistochemical and double immunofluorescent staining) can be found in Supplemental Figure 1 (see <http://ajp.amjpathol.org>).

Cross-tabulation (Table 1) showed that calcifications are strongly associated with the cross-link (Pearson χ^2 , $P < 0.001$). Thus, of the calcified areas 88% showed positive staining for the cross-link. Both transglutaminases, TG2 and FXIIIa, are associated with the cross-link (Pearson χ^2 , $P = 0.002$ and $P < 0.001$). However, TG2 was present in only 10% of the cross-link-positive areas, whereas 50% of the cross-link-positive areas showed positive staining for FXIIIa. Macrophages, a possible source of FXIIIa, are strongly correlated with FXIIIa in the plaques (Pearson χ^2 , $P < 0.001$). Staining for the cross-link and FXIIIa outside calcified areas was located mainly in the necrotic core, rather than in the fibrous cap or shoulder regions of the plaque (Supplemental Figure 2, see <http://ajp.amjpathol.org>).

Table 1. Cross-Tabulation Tables

		CL		Total
		Negative	Positive	
Calcium				
Negative				
Observed	106	124	230	
Expected	87	143	230	
Positive				
Observed	9	65	74	
Expected	28	46	74	
Total				
Observed	115	189	304	
Expected	115	189	304	
Pearson χ^2 $P < 0.001$				
		CL		Total
		Negative	Positive	
FXIIIa				
Negative				
Observed	94	94	188	
Expected	71	117	188	
Positive				
Observed	21	95	116	
Expected	44	72	116	
Total				
Observed	115	189	304	
Expected	115	189	304	
Pearson χ^2 $P < 0.001$				
		CL		Total
		Negative	Positive	
TG2				
Negative				
Observed	114	170	284	
Expected	107	177	284	
Positive				
Observed	1	19	20	
Expected	8	12	20	
Total				
Observed	115	189	304	
Expected	115	189	304	
Pearson χ^2 $P = 0.002$				
		CD68		Total
		Negative	Positive	
FXIIIa				
Negative				
Observed	104	84	188	
Expected	81	107	188	
Positive				
Observed	27	89	116	
Expected	50	66	116	
Total				
Observed	131	173	304	
Expected	131	173	304	
Pearson χ^2 $P < 0.001$				

Top to bottom: cross-tabulation tables between calcium and cross-link (CL), FXIIIa and CL, TG2 and CL, and FXIIIa and CD68. Indicated are observed numbers and their expected numbers on the basis of absence of correlation.

Discussion

In this study we tested the hypothesis that transglutaminases are associated with calcification in human atherosclerosis. The main findings of the current study are that i) calcified areas in human atherosclerotic plaque stain positively for the transglutaminase-induced cross-link, ii) this $N^{\epsilon}(\gamma\text{-glutamyl})\text{lysine}$ cross-link correlates significantly with staining for TG2 and FXIIIa, both members of the transglutaminase family, iii) FXIIIa staining is dominant over TG2, which is only marginally present, and point iv) FXIIIa staining correlates with CD68 staining and also colocalizes with CD68-positive cells as shown by double immunofluorescent staining, suggesting that macrophages represent the major source of FXIIIa.

Role of Transglutaminases in Atherosclerosis

Previous work from others addressed the expression of TG2 and the cross-link in human atherosclerotic specimens.^{9–11} These publications showed that transglutaminases, through cross-linking of extracellular matrix proteins, could contribute to increased plaque stability. However, in the current study we found transglutaminases and the cross-link to be absent from cap and shoulder regions and mainly associated with calcified areas. Whereas the present study reveals a strong correlation of transglutaminases with calcification in human atherosclerotic specimens, detailed mechanistic insight is provided by the recent *in vitro* experimental work performed by Johnson et al.² These authors found transglutaminases to be crucial for the switch of smooth muscle cells toward a chondro-osseous phenotype and subsequent mineralization. Work on osteoblast differentiation also supports the role of transglutaminases in tissue mineralization. Thus, cross-linking activity of transglutaminases affects cell differentiation and ultimately mineralization of osteoblast cultures by the formation and stabilization of a fibronectin-collagen network.¹² In addition, osteoblasts differentiate faster on a TG2-cross-linked type I collagen matrix than on native collagen.¹³ It is, however, not clear whether transglutaminases contribute to calcification through cross-linking activity only. Transglutaminases also accelerate the maturation of chondrocytes, which relates to the engagement of transglutaminases with integrins,⁸ a process that does not require transglutaminase-catalyzed transamidation.

FXIIIa in Atherosclerosis

Our previous work showed that in vascular remodeling, FXIIIa can substitute for the lack of TG2. This was also shown to be the case for *in vitro* calcification of smooth muscle cells and arteries,² in which exogenous FXIIIa could rescue the phenotypic switch of $TG2^{-/-}$ smooth muscle cells toward the chondro-osseous phenotype by phosphate donor treatment, which may also explain why the TG2 knockout mouse does not display an overt skeletal phenotype. FXIIIa may originate from plasma, platelets, and monocytes/macrophages. In the current study

we found a significant correlation between FXIIIa and CD68 staining, suggesting that within the plaque, macrophages are a major source of FXIIIa. An alternative possibility is that osteochondrogenic trans-differentiated smooth muscle cells produce FXIIIa, because in endochondral ossification FXIIIa is released by chondrocytes.¹⁴ However, the double fluorescent immunostaining for CD68 and FXIIIa clearly shows that macrophages are the main source of local FXIIIa synthesis. We found FXIIIa and CD68 staining outside calcified areas also. It is not clear whether these sites reflect early stages in calcification.

The currently found colocalization of transglutaminase activity and calcification adds to a variety of transglutaminase effects that may aggravate atherosclerosis. Thus, FXIIIa is considered to be a marker of alternatively activated (T helper 2) macrophages and plays a role in the phagocytosis of erythrocytes and other particles.¹⁵ In addition, FXIIIa may act in an autocrine manner on angiotensin receptors. Thus, FXIIIa is able to induce dimerization of angiotensin type 1 receptors on monocytes. These angiotensin type 1 receptor dimers show increased activation, resulting in increased attachment to endothelial cells. This process could ultimately affect atherosclerosis, because blockade of receptor dimerization resulted in decreased atherosclerotic lesion development in apolipoprotein-deficient mice.¹⁶

Transglutaminases have also been found to mediate inward remodeling in small arteries under a variety of conditions.^{6,7} In those vessels, transglutaminases mainly affect remodeling through cross-linking the media and adventitia. It remains to be established whether transglutaminases are also involved in large vessel media remodeling during atherosclerosis. Yet it is conceivable that media cross-linking would counteract the Glagov phenomenon.¹⁷

In summary, transglutaminase activity may determine the outcome of atherosclerotic lesion development through an array of mechanisms, including calcification. The challenge for future work will be to further unravel these mechanisms and to explore possibilities for beneficially affecting local transglutaminase activity.

Acknowledgments

We thank Kim van Gaalen for excellent technical assistance.

References

1. Johnson RC, Leopold JA, Loscalzo J: Vascular calcification: pathological mechanisms and clinical implications. *Circ Res* 2006, 99:1044–1059
2. Johnson KA, Polewski M, Terkeltaub RA: Transglutaminase 2 is central to induction of the arterial calcification program by smooth muscle cells. *Circ Res* 2008, 102:529–537
3. Lorand L, Graham RM: Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* 2003, 4:140–156
4. Bakker EN, Pistea A, VanBavel E: Transglutaminases in vascular biology: relevance for vascular remodeling and atherosclerosis. *J Vasc Res* 2008, 45:271–278
5. Boisvert WA, Rose DM, Boullier A, Quehenberger O, Sydlaske A,

- Johnson KA, Curtiss LK, Terkeltaub R: Leukocyte transglutaminase 2 expression limits atherosclerotic lesion size. *Arterioscler Thromb Vasc Biol* 2006, 26:563–569
6. Bakker EN, Buus CL, Spaan JA, Perree J, Ganga A, Rolf TM, Sorop O, Bramsen LH, Mulvany MJ, VanBavel E: Small artery remodeling depends on tissue-type transglutaminase. *Circ Res* 2005, 96:119–126
7. Bakker EN, Pistea A, Spaan JA, Rolf T, de Vries CJ, van Rooijen N, Candi E, VanBavel E: Flow-dependent remodeling of small arteries in mice deficient for tissue-type transglutaminase: possible compensation by macrophage-derived factor XIII. *Circ Res* 2006, 99:86–92
8. Johnson KA, Rose DM, Terkeltaub RA: Factor XIIIa mobilizes transglutaminase 2 to induce chondrocyte hypertrophic differentiation. *J Cell Sci* 2008, 121:2256–2264
9. Auld GC, Ritchie H, Robbie LA, Booth NA: Thrombin upregulates tissue transglutaminase in endothelial cells: a potential role for tissue transglutaminase in stability of atherosclerotic plaque. *Arterioscler Thromb Vasc Biol* 2001, 21:1689–1694
10. Haroon ZA, Wannenburg T, Gupta M, Greenberg CS, Wallin R, Sane DC: Localization of tissue transglutaminase in human carotid and coronary artery atherosclerosis: implications for plaque stability and progression. *Lab Invest* 2001, 81:83–93
11. Sumi Y, Inoue N, Azumi H, Seno T, Okuda M, Hirata K, Kawashima S, Hayashi Y, Itoh H, Yokoyama M: Expression of tissue transglutaminase and elafin in human coronary artery: implication for plaque instability. *Atherosclerosis* 2002, 160:31–39
12. Al-Jallad HF, Nakano Y, Chen JL, McMillan E, Lefebvre C, Kaartinen MT: Transglutaminase activity regulates osteoblast differentiation and matrix mineralization in MC3T3–E1 osteoblast cultures. *Matrix Biol* 2006, 25:135–148
13. Chau DY, Collighan RJ, Verderio EA, Addy VL, Griffin M: The cellular response to transglutaminase-cross-linked collagen. *Biomaterials* 2005, 26:6518–6529
14. Nurminskaya M, Magee C, Nurminsky D, Linsenmayer TF: Plasma transglutaminase in hypertrophic chondrocytes: expression and cell-specific intracellular activation produce cell death and externalization. *J Cell Biol* 1998, 142:1135–1144
15. Töröcsik D, Bardos H, Nagy L, Adany R: Identification of factor XIII-A as a marker of alternative macrophage activation. *Cell Mol Life Sci* 2005, 62:2132–2139
16. AbdAlla S, Lother H, Langer A, el Faramawy Y, Quitterer U: Factor XIIIa transglutaminase crosslinks AT1 receptor dimers of monocytes at the onset of atherosclerosis. *Cell* 2004, 119:343–354
17. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Koletts GJ: Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med* 1987, 316:1371–1375