Differential Accumulation of Proteoglycans and Hyaluronan in Culprit Lesions
Insights Into Plaque Erosion

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Objective—The importance of the extracellular matrix molecules versican, biglycan, decorin, and hyaluronan in plaque instability has not been recognized.

Methods and Results—Coronary lesions with acute thrombi and stable plaques were examined for the accumulation and distribution of specific proteoglycans and hyaluronan at culprit sites. The cell surface receptor for hyaluronan, CD44, and smooth muscle (SM) cell maturation markers were also assessed. Proteoglycans and hyaluronan accumulated in distinct patterns depending on plaque type. The fibrous cap of stable lesions was enriched in versican and biglycan, with considerably less staining for decorin and hyaluronan, whereas picrosirius red revealed a heavy accumulation of collagen type I. In contrast, intense staining for hyaluronan and versican was found in erosions at the plaque/thrombus interface, with weak staining for biglycan and decorin; collagen content was predominantly type III. Rupture sites showed little immunoreactivity for proteoglycans or hyaluronan. CD44 was localized along the plaque/thrombus interface in erosions, whereas in ruptures and stable plaques, it was mostly confined to inflammatory cells. Positive immunostaining for immature SM cells (SM myosin heavy chain SM1 and SMemb) was present in stable and eroded plaques, whereas the presence of SM2 and smoothelin was weak or nonexistent.

Conclusions—Specific accumulation of versican, hyaluronan, and CD44 at the sites of plaque erosion implicates an involvement of these molecules in events associated with acute coronary thrombosis. (Arterioscler Thromb Vasc Biol. 2002;22:1642-1648.)

Key Words: culprit plaques † proteoglycans † hyaluronan † CD44 † smooth muscle cells

Sudden coronary deaths mostly arise from acute thrombi precipitated by unstable plaque. Plaque rupture, the most frequent cause of thrombosis, has been implicated in the episodic progression of coronary stenosis, as supported by sequential angiography/intravascular ultrasound imaging and, to a greater extent, pathological studies. Rupture-prone atherosclerotic plaques have a dense infiltrate of macrophages within a thin fibrous cap interspersed with lymphocytes, overlying an acellular mass of lipids. Plaque erosion, recently described in our laboratory, is another significant cause of acute coronary thrombosis and sudden death. Eroded plaques are distinct from ruptures in that they are strikingly rich in smooth muscle (SM) cells (SMCs) and proteoglycans, with relatively few inflammatory cells. Furthermore, the overlying thrombus is confined to the luminal surface with no established communication with the deep underlying plaque. In a large series of >240 sudden coronary deaths studied by our laboratory, ∼52% of the lesions demonstrated acute thrombi; of these, 60% were ruptures, whereas eroded plaque occurred at a frequency of 40%. Stable plaques (lesions of ≥75% cross-sectional luminal narrowing without acute or organized luminal thrombi) constitute ∼26% of sudden coronary deaths.

Proteoglycans (versican, biglycan, and decorin) and hyaluronan are extracellular matrix (ECM) molecules that accumulate in topographically distinct patterns within developing atherosclerotic and restenotic lesions (see reviews). Not only do these molecules contribute to plaque burden, they also influence fundamental cellular and extracellular events associated with the pathogenesis of vascular lesions, such as thrombosis, lipid metabolism, and vascular proliferation and migration. Furthermore, the ability of proteoglycans to interact with other components of the ECM contributes to their propensity to regulate, in part, the biomechanical properties of vascular lesions and the ability of plaques to resist rupture.

Although previous studies have confirmed the involvement of proteoglycans and hyaluronan in chronic atherosclerotic...
disease, it is not clear whether the accumulation of specific types of proteoglycans discriminate among lesion types associated with sudden coronary events. Therefore, we sought to determine whether specific proteoglycans and/or hyaluronan differentially accumulate at culprit lesion sites.

**Methods**

**Selection of Cases**
The hearts from sudden coronary death victims consisted of consult cases provided by the Chief Medical Examiner of the State of Maryland. Coronary deaths were defined as natural deaths without extracardiac causes and in which at least 1 epicardial coronary artery had $\geq 75\%$ cross-sectional luminal narrowing by an atherosclerotic plaque or a plaque with a superimposed thrombus. An acute plaque rupture consisted of a luminal platelet-fibrin-rich thrombus continuous with an underlying lipid-rich core through a disrupted thin fibrous cap. Plaque erosion was defined as an acute thrombus in direct contact with the intimal plaque. Stable plaque was defined as cross-sectional luminal narrowing $\geq 75\%$ in the absence of a luminal thrombus.

**Selection and Histological Preparation of Culprit Lesions**
Forty-nine culprit plaques from sudden coronary death patients were identified (plaque rupture in 11 patients, plaque erosion in 20, and stable plaque in 18). All those who experienced plaque rupture were males (aged 45.8 $\pm$ 10.5 [mean $\pm$ SD] years). Of the 20 patients with eroded lesions, there were 11 males (aged 42.0 $\pm$ 7.6 years) and 9 females (aged 40.0 $\pm$ 9.3 years), whereas of the 18 patients with stable plaques, there were 13 males (aged 47.2 $\pm$ 11.0 years) and 5 females (aged 47.3 $\pm$ 8.6 years). Coronary segments were either fixed in 10% neutral buffered formalin or cryopreserved. Paraffin or cryostat sections were incubated with primary antibodies chosen on the basis of the successful identification of the proteoglycans in these regions (Figure 3B).

**Immunohistochemistry**
Paraffin or cryostat sections were incubated with primary antibodies against human SM $\alpha$-actin (Sigma Chemical Co), the macrophage marker CD68 (Dako), and the T-cell marker CD45RO (Dako). Monoclonal antibodies directed against CD61 (Beckman Coulter) and fibrin II (Accurate Chemical & Scientific Corp) were used to recognize platelets and fibrin, respectively. The adhesion receptor CD44 was identified by use of a specific monoclonal antibody (The Binding Site Ltd). Markers for SMC differentiation included human SM $\alpha$-actin (HHF-35, Dako), human SMC myosin heavy chains (MHCs) SM1 and SM2 and nonmuscle-type HMC SMemb (Yamasa Corp), and smoothelin, a novel marker for the contractile SMC phenotype (MONOSAN). The labeling of primary antibodies was achieved by using a biotinylated link antibody, and positive staining was visualized by using a 3-amino-9-ethylcarbazole substrate- chromogen system; the sections were counterstained with Gill’s hematoxylin.

**Antibodies to Proteoglycans and Detection of Hyaluronan**
Rabbit polyclonal antisera for the core proteins (amino-terminal peptides) of human biglycan (LF-51) and decorin (LF-136) were generously provided by Larry Fisher, National Institute of Dental Research, Bethesda, Md. A rabbit antibody specific for the poly E region of human versican, VC-E, was kindly provided by Richard LeBaron (University of Texas at San Antonio). The biotinylated hyaluronan-binding protein region of aggrecan was used as a specific probe for the detection of hyaluronan (4 $\mu$g/mL) and was kindly provided by Charles Underhill (Department of Anatomy and Cell Biology, Georgetown University, Washington, DC). Specificity of hyaluronan staining was verified by abolition of staining by pretreatment of the sections with Streptomyces hyaluronidase (data not shown).

**Quantification of Immunostaining**
Quantification of immunostaining was confined to the area of the rupture site and, in the case of erosion or stable plaque, within 200 $\mu$m of the lesion interface. For ruptures and erosions, care was taken to avoid counting areas of inflammatory cells entrapped within the thrombus. Immunohistochemical quantification for macrophages, SM $\alpha$-actin, SM1, SM2, SMemb, and smoothelin was assessed by computer-assisted color image analysis (Bioquant, R&M Biometrics), as previously described. T cells were counted individually and expressed as a percentage of the total number of cells per millimeter squared.

**Proteoglycans and Hyaluronan**
The intensity of staining was graded semiquantitatively by 2 independent observers (F.D.K. and R.V.) on a scale from 0 to 3, with 0 indicating undetectable staining; 0.5, variably detectable staining; 1.0, detectable staining; 2.0, moderate staining; and 3.0, strong staining similar to that reported by Evanko et al. Areas of measurement were restricted to the plaque rupture site or superficial layers in the case of erosion or stable plaque, as described above. The mean scores for each lesion type are reported.

**Statistical Analysis**
Data are presented as mean $\pm$ SD. Continuous variables were compared by ANOVA ($t$ test with Dunnett correction). Differences between the 3 groups were considered significant at a value of $P<0.05$.

**Results**

**Topographical Patterns of Proteoglycan and Hyaluronan Accumulation in Culprit Plaques**
Analysis of staining was focused on the fibrous cap, the plaque/thrombus interface in the case of erosion, or the site of plaque rupture. The different lesion types exhibited a distinct pattern of ECM proteins. In stable plaques, the reaction to versican was intense, whereas staining for hyaluronan was much less prominent (Figures 1A, 1B, 2C, and 2D). The intensity of staining for biglycan paralleled that of versican, and in comparison, the staining for decorin was weak to mild (Figures 1C, 1D, 2E, and 2F). Stable plaques demonstrated a mixture of collagen types I and III, with the latter being present at healed rupture sites (Figure 2B).

In erosions, the pattern of proteoglycan and hyaluronan staining was remarkably different relative to stable plaque. Although the reaction to versican was equally intense, the staining for hyaluronan was very prominent and the most notable among the 3 culprit lesions (Figures 1B and 3D). Biglycan immunostaining was barely detected, whereas the reaction to decorin was mild (Figures 1C, 1D, 3E and 3F). Picrosirius red staining revealed a predominance of collagen type III in these regions (Figure 3B).

At plaque rupture sites, staining for versican, hyaluronan, decorin, and biglycan was generally weak to nonexistent (Figure 1 and online Figure IC through IF, available at www.ahajournals.org). Plaque rupture sites contained mostly...
attenuated strands of collagen type I in regions of fibrous cap thinning (online Figure IB).

**Lesion Composition in Culprit Plaques**

Notable differences in the distribution of cellular infiltrate were apparent regarding the various lesion types. In stable and eroded plaques, macrophage density was minimal and generally <3%, whereas in ruptured plaques, CD68-positive macrophages were the predominant cell type, having a density of 28.8±4.7% (P<0.001 versus stable or eroded plaque). In contrast, α-actin–positive SMCs were the predominant cell type in stable (23.7±4.2%) and eroded (21.0±3.5%) lesions, whereas at rupture sites, SMCs were rare (0.6±0.4%, P<0.0001). T lymphocytes were the least represented cell type and were sparsely noted in stable (3.9±1.7) and eroded (1.3±0.8) lesions. At rupture sites, T cells were most remarkable (6.4±1.3) and were significantly higher than in erosions (P=0.008).

**Identification of CD44 in Culprit Plaques**

We performed immunohistochemical analysis to determine whether CD44, a cell surface receptor for hyaluronan, was present in culprit plaques. In stable lesions, CD44 staining was mostly associated with inflammatory cells. In contrast, CD44 in eroded plaques appeared to be mostly localized to a subset of SMCs at the plaque/thrombus interface in areas rich in hyaluronan (Figure 4). Occasional staining was seen in platelets and inflammatory cells within the thrombus as well. In ruptures, CD44 staining was mostly confined to inflammatory cells, in particular, those associated with the necrotic core and the thrombus; there was no demarcation of CD44 staining as found in erosions.
SMC Phenotype in Stable and Eroded Plaques

For SMC phenotype in plaques, please see online Figure II (available at www.ahajournals.org). The expression of SMC differentiation and maturation markers was examined in stable and eroded plaques to determine whether modulation of the SMC phenotype was associated with the differential accumulation of proteoglycans in these lesions. Rupture sites were not examined because they contain so few SMCs. The percentage of positive areas for myosin isoforms was remarkably similar in stable and eroded plaques, in that there was decreased expression of SM2 (stable plaque 7.4±2.7%, eroded plaque 5.2±1.9%) relative to MHC isoforms SM1 (stable plaque 16.6±3.7%, eroded plaque 10.7±1.4%) and SMemb/MHC-B (stable plaque 17.8±3.5%, eroded plaque 12.1±2.6%; P<0.05). Although smoothelin expression was negative in erosions, it was detected in 3 of 7 stable plaques without evidence of healed plaque rupture.

Discussion

The present study of fatal coronary artery disease demonstrates clear differences in the accumulation patterns of proteoglycans and hyaluronan among varying culprit lesions. Most intriguing was the intense immunostaining pattern for versican and hyaluronan at the plaque/thrombus interface, whereas staining for decorin was weak, and that for biglycan was negative.

Figure 3. Plaque erosion: picrosirius red staining and identification of proteoglycans. A, Low-power (×20) and high-power (×200) micrographs of an eroded plaque (Movat pentachrome stain). Black box outlines a region at the plaque/thrombus interface. SMC- and proteoglycan-rich (blue-green) surface is seen adjacent to the thrombus. LP indicates lipid pool; Th, thrombus. B, Corresponding picrosirius red staining showing a plaque surface rich in collagen type III. C through F, Immunohistochemical identification of the ECM molecules versican, hyaluronan (bHABR), biglycan, and decorin, respectively. Note intense staining for versican and hyaluronan at the plaque/thrombus interface, whereas staining for decorin was weak, and that for biglycan was negative.

Figure 4. Role of hyaluronan and its CD44 ligand in plaque erosion. A, Illustration suggesting a functional role of hyaluronan (HA) in promoting plaque erosion. At the top right is a magnified view of the boxed area of the thrombus/plaque interface. Green indicates hyaluronan and versican; yellow, lipid pools; blue circles, macrophages; and Th, thrombus. Red border represents CD44 receptors, and white circles in the insert depict the CD44 ligand on hyaluronan. The selective accumulation of hyaluronan near the luminal surface may promote deendothelialization, resulting in CD44-dependent platelet adhesion. Furthermore, the presence of CD44 on activated SMCs may stimulate proliferation and migration. Hyaluronan can directly promote the polymerization of fibrin, which may also facilitate SMC migration. B, Immunohistochemical identification of endothelium by von Willebrand factor (vWF), demonstrating the loss of surface endothelium (arrowheads). Note positive reaction of the intraplaque capillaries in the deeper layers of the lesion (arrows). C, Intense reaction to CD44 localized to the plaque/thrombus interface. D and E, Thrombus showing CD61-positive platelets and fibrin staining, respectively. The layering (arrowheads) of platelets and fibrin is suggestive of ongoing thrombosis. Original magnification ×200 (B through E).
erosion; there was little hyaluronan detected at the luminal surface of stable plaques. These differences occurred despite similarities in SM phenotype between the 2 lesions. At plaque rupture sites, there was relatively little overall accumulation of proteoglycans and hyaluronan within the ECM compared with stable or eroded lesions. The relative absence of SMCs and degradation of proteoglycans may, in part, account for the reduction of ECM proteins in the fibrous cap. The excessive accumulation of versican and hyaluronan in erosion implicates an involvement of these molecules in events associated with acute coronary thrombosis.

**Morphological Diversity of Proteoglycans and Hyaluronan in Culprit Lesions**

**Stable Plaques**

Previous immunohistochemical studies of coronary atherosclerosis from human and nonhuman primates suggest that proteoglycans and hyaluronan accumulate in distinct regions of the plaque dictated by certain structural features and lesion stage. In the present study, the organization of ECM as reported previously was most similar to that found in stable plaques. These lesions generally represent healed ruptures identified by breaks in the fibrous cap with a surrounding repair reaction. Although many studies have focused on chronic atherosclerotic disease, there was no previous attempt to determine whether proteoglycans and hyaluronan differentially accumulate in culprit plaques, particularly in those lesions with thrombi.

**Plaque Rupture**

In the present study, rupture sites contained few α-actin–positive SMCs and little ECM; most notable was the near absence of proteoglycan and hyaluronan staining and the small amount of collagen type I. The paucity of SMCs at rupture sites is consistent with previous reports from our laboratory. Although collagen degradation within the fibrous cap has received much attention as a critical factor in provoking rupture, this is the first report highlighting the concomitant loss of proteoglycans and hyaluronan in this region. The degradation of proteoglycans in advanced human atheroma (in particular, versican) has been attributed to the expression of matrix metalloproteinase-12/metalloelastase and matrix metalloproteinase-7/matrilysin by resident macrophages as well as serine protease plasmin and the ADAMTS family of metalloproteinases. There is little current knowledge of the relevance of proteoglycans and hyaluronan in maintaining the integrity of the fibrous cap.

**Plaque Erosion**

The mechanism(s) of erosion is poorly understood. The appearance of “activated” SMCs and the enrichment of proteoglycans at the plaque/thrombus interface are the most striking aspects of this lesion. The appearance of increased hyaluronan at the plaque/thrombus interface in erosion is unique compared with other culprit lesions. Thus, it is tempting to speculate that increased accumulation of hyaluronan may provide a high-risk substrate for thrombosis development. For example, hyaluronan may interfere with the integrity of normal vascular endothelium. In general, endothelial cells display a marked heterogeneity with respect to hyaluronan receptor expression and function depending on their vascular origins. Endothelium isolated from larger vessels exhibits a lower potential for adherence to hyaluronan relative to that of endothelium of microvascular origin. Furthermore, endothelial cells in culture demonstrate decreased cell growth and an increased propensity to apoptosis induced by serum deprivation when maintained on hyaluronan substrates. The influence of hyaluronan on endothelial integrity in relation to erosion requires further study.

Although ex vivo biocompatibility studies describe the thromboresistant properties of hyaluronan, there are significant data in the literature suggesting that hyaluronan may play a supportive role in the development of thrombosis. For example, hyaluronan binds to specific cell receptors, such as CD44, and the receptor for hyaluronan-mediated motility, RHAMM, as well as to other proteins, such as TSG-6, collagen, and proteoglycans. In particular, CD44 receptors have been shown to mediate the adhesion of platelets to hyaluronan. The deendothelialized surface in erosion may expose hyaluronan, thereby promoting platelet attachment via a CD44-dependent mechanism. Furthermore, CD44 is thought to promote atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. In the present study, CD44 was highly prominent at the plaque/thrombus interface of eroded lesions, particularly in a subset of SMCs. The ligation of hyaluronan to its CD44 receptor may cause the activation of SMCs through specific cell signaling. Thus, in erosion, the expression of CD44 may be critical for the migration of SMCs to the wounded edge represented by the loss of endothelium.

Another important aspect of hyaluronan, which may have an impact on erosion, is its ability to modulate fibrin formation. The rate of thrombus-induced fibrin polymerization has been shown to be accelerated >500% in the presence of 60 μmol/L hyaluronan. In addition, hyaluronan can alter the architecture of fibrin clots, thereby further promoting cell proliferation and migration activity. Polymerized fibrin in the presence of hyaluronan results in gels with larger fiber mass-to-length ratios and pore diameters. These factors may become critical in organizing thrombi of eroded plaques; acute erosions are often superimposed on what appears to be repeated episodes of thrombosis and healing, which may represent a mechanism of plaque progression. Approximately 75% of erosions show a layering of platelets and fibrin deep within the intima (authors’ unpublished data, 2002).

On the basis of the pathological findings of the present study, we propose a working hypothesis of potential critical events leading to the primary event of plaque erosion. The selective accumulation of hyaluronan, loss of surface endothelium, and expression of CD44 may promote thrombosis and the proliferation and migration of SMCs (Figure 4). Although the study of hyaluronan has been mostly restricted to wound healing as a component of tissue repair, our observations imply a more active role for hyaluronan in the induction of deendothelialization and thrombosis in erosion.

**SMC Phenotype in Culprit Plaques**

We determined whether differences in SMC phenotype could explain the differential accumulation of proteoglycans and
hyaluronan in eroded and stable lesions. Reduced expression of selective SM myosin isoforms such as SM2 represents the phenotypic modulation of SMCs toward an immature state.\(^2\) In contrast, smoothelin, a constituent of the cytoskeleton, is a marker of highly differentiated contractile SMCs.\(^19\) In the present study, SMCs at the lumen interface of stable plaque or at the boundary of the plaque/thrombus in the case of erosion displayed characteristics of immature or dedifferentiated SMCs. Smoothelin-positive cells were absent in erosion and only positive in a few cells in those stable plaques without evidence of healed plaque rupture. Thus, with the present markers, the selective accumulation of proteoglycans and hyaluronan in stable and eroded plaques appeared to be independent of SMC phenotype.

**Study Limitations**
A retrospective analysis of autopsy tissue cannot identify mechanisms of lesion progression and thrombosis because the study material represents static observations. Specific processes involved in erosion are difficult to define without a relevant animal model for testing. It is conceivable that alterations in proteoglycans and hyaluronan content may result from the thrombotic process rather than representing an initiating event(s).\(^26\) Although it is difficult to discern whether hyaluronan is a pathogenic factor or a consequence of thrombus evolution, ruptured plaques with acute or healing thrombi showed little accumulated hyaluronan (authors’ unpublished data, 2002). Therefore, this finding supports a role of proteoglycans and hyaluronan in plaque evolution, ruptured plaques with acute or healing vascular disease. Thus, with the present markers, the selective accumulation of proteoglycans and hyaluronan in stable and eroded plaques appeared to be independent of SMC phenotype.

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Figure I. Plaque Rupture: picrosirius red staining and identification of proteoglycans. A, Low- (×20) and high-power micrographs (×200) of a ruptured plaque; the black box outlines the rupture site. Abbreviations: FC= fibrous cap, NC= necrotic core, Th= thrombus. B, Picrosirius red staining of the same artery as A showing an attenuated fibrous cap consisting predominantly of collagen type I (red/white birefringence). C-F, Immunohistochemical identification of versican, hyaluronan, biglycan, and decorin, respectively; reactions to these markers were negative or weak.
Figure II. Smooth muscle phenotypes in eroded and stable plaques. Movat stain with immunohistochemical detection of SM MHC (SM1 and SM2), NM MHC (SMemb/MKHC-B) isoforms and smoothelin in serial cryosections of eroded (A-F) and stable (G-L) plaque, ×200 magnification. 

A, Micrograph shows an eroded surface with a superimposed thrombus (Th). B, α-Actin staining at the same site as in A. C, Smoothelin expression was negative. D & E, Numerous cells are positive for SM1 while SM2 expression was sparse. F, SMemb was equally positive as SM1. G, Micrograph of the luminal surface of a stable plaque. H, α-Actin staining of the same site as in A. I, A few superficial SMCs were positive for smoothelin. J, Abundant SM1 positive SMCs are noted. K, Shows reduced expression of SM2 relative to SM1 and SMemb. L, SMemb is heavily expressed in stable plaque.