Localization of Tissue Transglutaminase in Human Carotid and Coronary Artery Atherosclerosis: Implications for Plaque Stability and Progression

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SUMMARY: Although atherosclerosis progresses in an indolent state for decades, the rupture of plaques creates acute ischemic syndromes that may culminate in myocardial infarction and stroke. Mechanical forces and matrix metalloproteinase activity initiate plaque rupture, whereas tissue inhibitors of metalloproteinases have an important (albeit indirect) role in plaque stabilization. In this paper, an enzyme that could directly stabilize the plaque is described. Tissue transglutaminase (TG) catalyzes the formation of $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptide bonds that are resistant to enzymatic, mechanical, and chemical degradation. We performed immunohistochemistry for TG in atherosclerotic human coronary and carotid arteries. TG was most prominent along the luminal endothelium and in the medium of the vessels with a distribution mirroring that of smooth muscle cells. Variable, often prominent, immunoreactivity for TG was also seen in the intima, especially in regions with significant neovascularization. Additionally, TG was detected in fibrous caps and near the "shoulder regions" of some plaques. A monoclonal antibody to the transglutaminase product $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptide demonstrated co-localization with TG antigen. Transglutaminase activity was found in 6 of 14 coronary artery atherectomy samples. Cross-linking of TG substrates such as fibrinogen, fibronectin, vitronectin, collagen type I, and protease inhibitors stabilized the plaque. Furthermore, the activation of transforming growth factor- β 1 by TG might be an additional mechanism for the promotion of plaque stabilization and progression by increasing the synthesis of extracellular matrix components. (*Lab Invest* 2001, 81:83-93).

The major manifestations of atherosclerosis, including stroke and myocardial infarction, are initiated by the formation of thrombus at the site of plaque rupture or erosion (Lee and Libby 1997; Shah, 1997). Disrupted plaques tend to have large lipid cores comprised of soft cholesteryl esters that redistribute the circumferential wall stress to the shoulder regions of the plaque (Davies, 1996; Falk, 1992; Richardson et al, 1989). This mechanical effect is exacerbated in plaques that have a thin fibrous cap (Loree et al, 1992) and in plaques that are only mildly to moderately stenotic because, according to Laplace's law, wall stress is directly proportional to the radius of the vessel (Lee and Kamm, 1994). The thinning of the fibrous cap may result from a reduced content of smooth muscle cells (SMC). The SMC reduction may be a result of decreased replication, reduced migra-

tion into the cap, or increased apoptosis (Geng and Libby, 1995; Libby et al, 1998). SMC are the source of collagen, elastin, and glycosaminoglycans in the cap. Consequently, a reduction in the SMC content leads to weakening of the fibrous cap through loss of these matrix constituents (Davies et al, 1994).

In addition to these physical forces, proteolytic factors also affect plaque stability. An infiltration of inflammatory cells is a common feature of disrupted plaques, especially in the "shoulder regions" (Lendon et al, 1991; van der Wal et al, 1994). These inflammatory cells, primarily macrophages, express zymogen forms of matrix metalloproteinases (MMP) that, when activated, degrade the collagen and elastin components of the plaque (Galis et al, 1994; Shah et al, 1995). Activated mast cells are also increased in vulnerable plaques and secrete enzymes such as tryptase and chymase that can activate pro-MMP derived from SMC and macrophages (Kaartinen et al, 1998; Kovanen et al, 1995).

The tissue inhibitors of metalloproteinases (TIMP) are a family of specific inhibitors that regulate MMP activity by forming 1:1 complexes with MMP (Dellery et al, 1995), thereby inhibiting proteolysis-mediated rupture. Although TIMP offer protection against proteolytic destabilization, they do not directly contribute to plaque stability. In contrast, the transglutaminases are a family of

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homologous enzymes that catalyze the cross-linking of proteins by forming $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptide bonds, thereby providing stability against chemical, mechanical, and proteolytic degradation to a variety of tissues (Greenberg et al, 1991).

Previous studies have suggested that tissue transglutaminase (TG) is present and active in the atherosclerotic plaque. Fibrinogen polymers, cross-linked through the α chain, have been detected in the intima (Shainoff and Page 1972; Valenzuela, 1992), providing indirect evidence for transglutaminase activity. The α chain of fibrinogen is the preferred substrate for TG, whereas factor XIIIa, a plasma form of transglutaminase that has also been found in the atheroma (Romanic et al, 1998), preferentially cross-links fibrinogen γ chains (Greenberg et al, 1991). In addition to fibrinogen, many of the other major substrates of the tissue form of the transglutaminase enzyme are prominent components of the extracellular matrix (ECM) of the atherosclerotic plaque. For these reasons, we performed a comprehensive analysis of the distribution and activity of TG in coronary and carotid artery atherosclerosis. The implications of transglutaminase expression in atherosclerotic tissues are discussed.

Results

Immunoblotting for TG Antigen

Homogenates of a segment of normal internal mammary artery, plaque obtained at carotid endarterectomy, and coronary artery tissue obtained by Directional Coronary Atherectomy were prepared as described in "Materials and Methods". Twenty-five micrograms of total protein was separated by 10% SDS-PAGE and immunoblotted with CUB 7402, a monoclonal antibody to guinea pig liver transglutaminase (GPTG). Similar results were obtained with TG100, another monoclonal antibody to TG (data not shown). Figure 1A shows the immunoblots of the homogenates from four carotid plaques (lanes 1 to 4) and the internal mammary artery (lane 5). In all cases, the TG migrated at the expected molecular mass of 85 kd (Gentile et al, 1991). Figure 1B shows the immunoblots of the three coronary atherectomy homogenates (lanes 1 to 3) and the internal mammary homogenate (lane 4). One sample (lane 2) demonstrated that the predominant TG antigen migrated at 65 kd, probably because of proteolysis that occurred *in vivo* or *in vitro* (despite the use of protease inhibitors).

TG and Isopeptide: Specificity of Immunohistochemistry

The distributions of TG antigen and isopeptide were similar in coronary and carotid arteries. Figure 2A shows immunohistochemistry (IHC) for the TG antigen. Figure 2B shows the distribution of the isopeptide. Figure 2C shows a specificity control for the IHC, with normal mouse serum substituted for the primary antibody. There was virtually no background immunoreactivity under the conditions used for IHC in this study.

Figure 1.

Immunoblots for transglutaminase in carotid and coronary artery plaques. Carotid artery plaque was obtained at the time of carotid endarterectomy and coronary artery tissue was obtained by Directional Coronary Atherectomy. Protein homogenates were prepared and 25 μg of total protein was separated by 10% SDS-PAGE as described in the "Materials and Methods" section. Immunoblotting was performed using an antibody to tissue transglutaminase (TG), CUB 7402. A, The immunoblot of four carotid plaques (lanes 1 to 4) and the homogenate of a normal internal mammary artery (lane 5). B, The immunoblot of three coronary atherectomy homogenates (lanes 1 to 3) and the internal mammary control (lane 4). TG antigen was detected in all cases at 85 kd, as expected, except for one sample (B, lane 2), where a 65-kd band, probably representing a proteolyzed sample, was seen.

TG Antigen Localization

TG was often detected in the fibrous cap overlying the necrotic core of a lipid-laden plaque, with TG antigen also present at the shoulders of the plaque. Figure 3 shows an example of a plaque obtained from a carotid endarterectomy that demonstrates these features. The fibrous cap, shown between the two arrows, contains TG antigen, which is especially prominent directly above the atheroma. TG antigen is also seen in the medium of the artery below the atheroma.

Figure 4 is a photomicrograph of TG and its product, $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptide ("isopeptide"), in a

Figure 2.

Specificity of antibody to transglutaminase and activity product (isopeptide). A cross section of an atherosclerotic coronary artery plaque is shown. Immunohistochemistry (IHC) for TG antigen (A) and the distribution of the isopeptide (B) are shown. C, Control for the specificity of the IHC, normal mouse serum was used in place of the primary antibody. The rectangular region of interest in C is shown at higher magnification in C'. Similar regions from A and B are shown enlarged as A' and B', respectively. This figure demonstrates that TG antigen and its isopeptide occur in similar distributions and that there is virtually no background reactivity under the conditions used for IHC in this study.

coronary artery plaque. TG antigen was prominent in the endothelium, within the intima, and in the medium of the vessel. The isopeptide was detected in a similar distribution to TG antigen (Fig. 4B). The TG distribution within the intima and the medium mirrored the distribution of SMC (Fig. 4C).

TG antigen was often detected in microvessels in both the intima (Fig. 5A) and the adventitia (Fig. 5C). No TG was detected in the adventitia, except in adventitial microvessels. Figure 5C also demonstrates the prominent immunoreactivity of the medium for the TG antigen.

A summary of all cases is presented in Table 1. The arterial medium was prominently immunoreactive for TG

in all cases. The intima, plaque cap, and neovasculature exhibited variable intensities of TG immunoreactivity.

TG Activity

A standard activity assay was created using GPTG as a control (Fig. 6). The assay was linear between 0 and 1 $\mu\text{g/ml}$. TG activity was assayed in the homogenized coronary atherectomy tissue of 14 patients (Table 2). Eight of the patients had no detectable activity. The remaining six patients had activity equivalent to 0.223 to 0.669 $\mu\text{g/ml}$ of GPTG in 50 to 100 μg of total protein.

Figure 3.

IHC studies of a carotid artery plaque. A longitudinal section of a carotid artery plaque from a 65-year-old patient undergoing endarterectomy is shown after IHC for transglutaminase. The fibrous cap overlying a plaque is shown between the two arrows. TG antigen can be detected in the fibrous cap, as well as in the "shoulder regions" of the plaque. However, virtually no TG antigen can be seen inside the atheroma itself. TG antigen is also present in the medium of the vessel below the atheroma.

Discussion

In arteries with minimal or no atherosclerosis, transglutaminase antigen was detected exclusively in the medium and along the luminal endothelial border. In atherosclerotic arteries, TG antigen was detected at these sites as well as in the intima, within the fibrous cap, and in the endothelium of microvessels. In both normal and atherosclerotic arteries, the distribution of TG mirrored that of endothelial cells and SMC. The presence of TG in the fibrous cap and at the shoulder regions of atherosclerotic plaques could be effective in stabilizing the artery at these sites, rendering it less prone to rupture. The presence of transglutaminase on the luminal endothelium may prevent the dislodgment or erosion of endothelial cells that leads to coronary thrombosis without anatomical evidence of plaque rupture (Arbustini et al, 1999; Davies, 1997). The microvascular endothelial transglutaminase, on the other hand, could prevent the increased permeability and intra-plaque hemorrhage that often accompanies these vessels and that may predispose them to plaque rupture (Barger and Beeuwkes, 1990; McCarthy et al, 1999; Paterson, 1938; Zhang et al, 1993). All of the samples analyzed by immunohistochemistry showed similar anatomical distributions of TG antigen and isopeptide (Table 1). Our finding that 8 of 14 coronary atherectomy samples had no detectable TG activity (Table 2) could be a result of oxidant injury, nitrosylation (Melino et al, 1997), proteolysis, or the presence of histamine or other inhibitory substances in the plaque.

The mechanical and proteolytic forces that enhance atherosclerotic plaque rupture have been well characterized (Davies, 1996; Falk 1992; Lee and Kamm 1994; Lee and Libby, 1997; Loree et al, 1992; Richardson et al, 1989; Shah, 1997). TIMP enhance plaque stability

by preventing the degradation of the collagen and elastin components of the ECM (Dellery et al, 1995). TG could provide a mechanism for the direct stabilization of atherosclerotic plaque against chemical, proteolytic, and physical disruptions (Greenberg et al, 1991). The $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ isopeptide bond that is catalyzed by TG is particularly stable, even resisting degradation by lysosomal enzymes (Fesus et al, 1991). A variety of ECM components, including many that are expressed in the atheromatous plaque, are substrates for the transglutaminases. In particular, collagens type I and III, the major constituents of the fibrous cap (Libby et al, 1998; Rekhter et al, 1993), are cross-linked to fibronectin by TG (Mosher, 1984). Other types of collagen, including types II and V, are also substrates (Mosher, 1984). Entactin, a ubiquitous component of the basement membrane, is a substrate (Wu and Chung, 1991) that could be cross-linked by TG. This could potentially result in a more resilient barrier at the blood-vessel interface. Other substrates, such as vitronectin (Sane et al, 1990), osteopontin (Prince et al, 1991), thrombospondin (Bale and Mosher 1986), fibronectin (Martinez et al, 1994), dermatan sulfate proteoglycan (Kinsella and Wight, 1990), and apo(a) (Borth et al, 1991), are often increased in the atheromatous plaque (Dufourcq et al, 1998; Giachelli et al, 1995; Hajjar and Nachman, 1996; Riessen et al, 1994; Shekhonin et al, 1987; Wight et al, 1985).

In addition to directly cross-linking these matrix components, TG could also provide plaque stability by attaching protease inhibitors to specific plaque proteins. For example, $\alpha 2$ -antiplasmin is cross-linked to the α chain of fibrinogen (Greenberg et al, 1987), and elafin, an inhibitor of elastase, is cross-linked to a variety of matrix proteins (Schalkwijk et al, 1999). The covalent bonding of these protease inhibitors to the

Figure 4.

Transglutaminase is found in endothelium and smooth muscle cells (SMC) and co-localizes with isopeptide. The proximal left anterior descending coronary artery was excised from the explanted heart of a 45-year-old male undergoing transplantation for ischemic cardiomyopathy. The artery was fixed in neutral buffered formalin, paraffin-embedded, and processed for IHC. A, Distribution of TG antigen, which is similar to that of the isopeptide (B). C, Distribution of SMC (detected with anti-SMC α actin) and D, distribution of the luminal endothelium (detected with anti-von Willebrand factor). TG antigen was strongly immunoreactive along the luminal endothelial border (shown by the *black arrows*), throughout the intima, and in the medium of the vessel. The detection of TG antigen in the intima and medium of the vessel is similar to the distribution of SMC. The internal elastic lamina is indicated in all figures by the *white arrowheads*. Original magnification, $\times 400$.

Figure 5. Transglutaminase is detected in intimal and adventitial microvessels of an atherosclerotic coronary artery. TG antigen was often detected in microvessels, as demonstrated in these photomicrographs. A, Presence of TG antigen in intimal microvascular endothelial cells (as well as some other cells) is shown. B, Immunoreactivity for von Willebrand factor to identify the endothelial cells in a section adjacent to that shown in A. C and D, The medial-adventitial border. The external elastic lamina is identified by the *black arrowheads*. TG antigen (C) is present in the microvascular endothelium and in the SMC that are present in the medium (upper right-hand corner). Endothelial cells were identified using an antibody to von Willebrand factor (D). Original magnification, $\times 400$.

Table 1. Tissue Transglutaminase in Coronary and Carotid Atherosclerosis

Age	Vessel	Luminal Stenosis	Medium	Intima	Plaque Cap	Neovasculture
Coronary Arteries						
23	LAD	negligible	+++	—	N.A.	N.A.
41	LAD	50%	+++	+++	++	+++
45	LAD	90%	++	+++	N.A.	+++
57	RCA	10%	+++	—	N.A.	N.A.
59	LAD	75%	+++	++	+	N.A.
66	LAD	50%	+++	+	N.A.	+
68	LAD	90%	++	+++	++	+++
Carotid Arteries						
65	RIC	75%	+++	++	++	+
68	LIC	75%	++	++	+	N.A.
72	LIC	60%	+++	++	N.A.	+
74	RIC	60%	+++	++	+++	+++
75	RIC	75%	++	++	++	+++
78	LIC	75%	+++	++	++	++
78	LIC	60%	++	++	N.A.	++

LAD, left anterior descending coronary artery; RCA, right coronary artery; RIC, right internal carotid; LIC, left internal carotid; N.A., not applicable (plaque cap or neovasculture not visualized on sections).

The isopeptide was localized in all sections in a similar distribution to that of TG antigen.

plaque matrix could prevent plasmin-mediated activation of MMP and elastin degradation by elastase.

TG could also indirectly stabilize the plaque by activating latent transforming growth factor- β -1

(TGF- β 1) (Kojima et al, 1993). TGF- β 1 is secreted in an inactive complex comprised of latent TGF- β 1 and the latency associated peptide. TG-mediated activation of TGF- β 1 requires cross-linking of latency associated

Figure 6.

TG activity standard curve. A colorimetric assay for transglutaminase was established, using guinea pig liver transglutaminase (GPTG) as the standard enzyme. The assay is based on the incorporation of 5-biotinamidopentylamine into immobilized N, N'-dimethylcasein, as described in "Materials and Methods." The assay was linear between 0 and 1 μg/ml of GPTG. This assay was used to determine the activity of transglutaminase in coronary artery atherectomy tissue (Table 2).

peptide to components of the ECM (Kojima et al, 1993; Nunes et al, 1995, 1997). Once activated, TGF-β1 enhances the expression of a variety of ECM components and induces inhibitors of matrix degradation (plasminogen activator inhibitor type 1 [PAI-1] and TIMP). TGF-β1 also inhibits the expression of matrix degrading enzymes (plasminogen activators and MMP) (Massagué, 1990). Thus, increased TGF-β1 activity, mediated by TG activation of the latent complex, could strengthen the plaque against rupture. The increased ECM content could also promote plaque progression.

In addition to its ability to stabilize the plaque by cross-linking important ECM substrates, TG has other roles that could affect the stability of the atheromatous plaque and its response to injury. These include a direct cell adhesive action (Gentile et al, 1992), roles in apoptosis (Fesus et al, 1989, 1991), the regulation of cell growth (Birckbichler and Patterson, 1978), and wound healing (Bowness et al, 1988). TG in the intracellular compartment also has a recently recognized role as a G protein in signal transduction (Nakaoka et al, 1994). Thus, transglutaminase is able to alter many of the processes that are thought to regulate plaque stability (Davies, 1996; Falk, 1992; Lee and Kamm, 1994; Lee and Libby, 1997; Loree et al, 1992; Richardson et al, 1989; Shah 1997), and it may have other roles that are important to endothelial and SMC functions.

A variety of factors could affect the activity of TG in the plaque. TG is sensitive to oxidant stress, presumably through oxidant modification of an active-site cysteine (residue 276) (Greenberg et al, 1991). TG has 16 additional free cysteine residues that are potential targets for oxidation (Folk, 1983). The water-soluble gas phase extracts of cigarette smoke, in concentrations as low as 0.5%, can inhibit the TG content of 10^7 human alveolar macrophages by 40% (Roth et al,

1986). Nitric oxide can inhibit transglutaminase activity through nitrosylation of the active-site cysteine (Bernassola et al, 1999; Catani et al, 1998; Melino et al, 1997) or through the reaction of nitric oxide with $O_2^{\cdot-}$ to form peroxynitrite, because cysteine residues are particularly sensitive to oxidation with peroxynitrite (Berlett and Stadtman, 1997). Thus, the generation of oxidant stress from smoking (Pryor et al, 1984; Tully et al, 1969), hyperlipidemia (Ohara et al, 1993), diabetes (Horie et al, 1997; Schmidt et al, 1995), and hypertension (Harrison, 1997; Scheidegger et al, 1997), as well as the health status of the endothelium, which is influenced by these and other risk factors (Harrison, 1997), could modulate TG activity.

The content of glycosaminoglycans could also modulate the ability of TG to form plaque-stabilizing protein cross-links. Both midkine (Kojima et al, 1997) and vitronectin (Sane et al, 1990) are much more readily cross-linked by TG in the presence of sulfate-rich glycosaminoglycans. Interestingly, the content of sulfated glycosaminoglycans is reduced in the cap of the unstable plaque (Davies et al, 1994).

Another influence on TG activity could be the presence of amine compounds that are known to competitively inhibit the enzyme (Ishitani and Suzuki, 1989; Martinez et al, 1989; Stenberg et al, 1975). Degranulating mast cells, which release histamine, an inhibitor of TG (Martinez et al, 1989), are a component of the cellular inflammatory response in the plaque (Kaartinen et al, 1998; Kovanen et al, 1995; Lendon et al, 1991; van der Wal et al, 1994). Recently, it was shown that the mast cell content of unstable plaques is increased compared with stable plaques (Kaartinen et al, 1998). Histamine is released from mast cells during myocardial ischemia (Frangogiannis et al, 1998) and is found in significantly higher levels (two-fold increase) in coronary arteries from patients who died from coronary heart disease compared with controls (Kalsner and Richards, 1984).

In summary, TG is present in the atherosclerotic plaques of coronary and carotid arteries. Although the enzyme is active in at least some plaques, a variety of factors that are involved in the pathogenesis of atherosclerosis could inhibit its activity. The inhibition of TG at anatomically critical sites, such as the "shoulder" region of the plaque could contribute to plaque rupture.

Materials and Methods

The monoclonal antibody to GPTG, CUB 7402, was a gift from Dr. Paul J. Birckbichler (Birckbichler et al, 1985), Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, and was also purchased (NeoMarkers, Lab Vision Corporation, Fremont, California). CUB 7402 cross-reacts with TG from a variety of species, including human (Greenberg et al, 1987). Another antibody to TG, TG100, was also obtained from this source. Combinations of TG100 and CUB 7402 (MS-300; NeoMarkers, Lab Vision Corporation) were also used for immunoblotting. The previously characterized IgM κ murine monoclonal antibody to the Ne(γ-glutamyl)lysine isopeptide,

Table 2. Transglutaminase Activity in Coronary Artery Atherectomy Tissue

Patient Number	Age	Sex	Target Vessel	ACIS Type	TP Analyzed (μ g)	Equivalent GPTG Activity (μ g/ml)
1	70	M	LAD	UAP	100	N.D.
2	87	F	RCA	UAP	80	N.D.
3	73	F	RCA	UAP	100	0.641
4	63	M	CIRC	MI	100	N.D.
5	45	M	RCA	UAP	50	N.D.
6	70	M	RCA	MI	50	N.D.
7	66	F	LAD	MI	50	0.395
8	48	M	CIRC	UAP	100	0.288
9	87	M	LAD	MI	50	0.243
10	51	M	LAD	MI	80	0.223
11	61	M	RCA	MI	80	0.669
12	64	F	RCA	MI	80	N.D.
13	44	M	RCA	MI	80	N.D.
14	65	F	RCA	UAP	80	N.D.

RCA, right coronary artery; CIRC, circumflex coronary artery; LAD, left anterior descending coronary artery; ACIS, acute coronary ischemic syndrome; UAP, unstable angina pectoris; MI, myocardial infarction; TP, total protein; GPTG, guinea pig liver transglutaminase; N.D., no detectable activity.

814 MAM (Roem et al, 1991), was obtained from CovalAb Oullins (Cedex, France). A murine monoclonal antibody to smooth muscle actin, clone 1A4, was purchased from DAKO (Carpinteria, California) and a rabbit polyclonal antibody to von Willebrand's factor, RB-281-P, was purchased from NeoMarkers, Lab Vision Corporation. GPTG was purchased from Sigma Chemical (St. Louis, Missouri); N, N'-dimethylcasein from Calbiochem (San Diego, California), and 5-biotinamidopentylamine from Pierce Chemical Company (Indianapolis, Indiana). Alkaline phosphatase substrate was purchased from BioRad (Richmond, California). Chemiluminescence reagents (SuperSignal) were purchased from Pierce Chemical Company (Indianapolis, Indiana).

IHC

IHC was performed using procedures described by Sternberger et al (1970) and Hsu et al (1981). Briefly, paraffin embedded tissues were sectioned (5-microns thick) and antigen retrieval was performed using citrate buffer (Biogenex, San Ramon, California). Tissues were treated with the primary antibodies described above. To control for non-specific binding, normal mouse serum was substituted for the primary antibody on some sections. Secondary and tertiary antibodies were provided in a kit (314 KLD; Innovex, Richmond, California) and the reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Slides were counterstained with hematoxylin (Sigma).

Tissue Specimens

Coronary artery sections were obtained from autopsies and from the explanted hearts of patients undergoing heart transplants at North Carolina Baptist Hospital and from the Cooperative Human Tissue Network

of the National Cancer Institute. Sections of coronary arteries from seven patients (five from autopsies, two from explanted hearts of patients undergoing heart transplant), ages 23 to 88 years old, with varying extents of coronary artery disease, ranging from negligible to severe were processed for IHC against TG. Four of the patients were male and three were female. Coronary artery tissue was obtained from 17 patients, ages 44 to 87, undergoing Directional Coronary Atherectomy. Fourteen of these samples were used for TG activity assays and three were used to prepare homogenates for immunoblotting. Carotid artery tissue was obtained at the time of endarterectomy from 11 patients, ages 65 to 79 years old. Sections of seven of these carotid arteries were used for IHC and four were used to prepare protein homogenates for immunoblotting. A segment of a left internal mammary artery that was trimmed at the time of bypass grafting was obtained and a protein homogenate was prepared for use as a normal control with immunoblotting. Tissue procurement was approved by the Institutional Review Board (IRB).

Immunoblotting

All fat was removed from the internal mammary artery using a dissecting microscope. Control artery, carotid endarterectomy plaque, and coronary atherectomy tissue were rinsed in ice-cold Tris-buffered saline (150 mM NaCl, 10 mM Tris, pH 7.4) and homogenized in Tris-buffered saline containing a protease inhibitor cocktail for general use (P2714; Sigma Chemical). Particulate debris was removed by centrifuging at $10,000 \times g$ for 10 minutes. The protein concentration of the supernatant was determined using the BioRad protein assay. Twenty-five micrograms of protein was mixed with sample buffer (5% 2-mercaptoethanol, 1% sodium dodecyl sulfate, 1.5 M urea, 2.5 mM EDTA, 30 mM Tris-HCl pH 7.5, and 0.001% bromophenol blue),

then subjected to SDS-PAGE on a 10% Laemmli gel. Immunoblotting was performed using a 1:1000 dilution of TG antibody followed by an anti-mouse IgG conjugated to horseradish peroxidase at a dilution of 1:5000. Bound antibodies were detected with chemiluminescence (SuperSignal, Pierce Chemical Company).

Coronary Artery Transglutaminase Activity Assay

Coronary artery atherectomy samples were frozen in liquid nitrogen immediately after retrieval from the patient, then pulverized and lysed in 10 mM Tris-HCl, pH 7.5, containing 1 mM MgCl₂, 1 mM EGTA, 5 mM β -mercaptoethanol, 0.5% CHAPS, 0.1 mM AEBSF, and 10% glycerol. The lysates were centrifuged at $16,000 \times g$ for 30 minutes at 4° C, and the supernatant was used in an ELISA-based assay for transglutaminase, as previously described (Slaughter et al, 1992), with minimal modifications. This assay is based on the incorporation of the TG substrate 5-biotinamidopentylamine (5-BPA; Pierce Chemical Company) into immobilized N, N'-dimethylcasein, with subsequent detection using streptavidin-alkaline phosphatase. Homogenates of coronary artery atherectomy tissue (50 to 100 μ g) were added to reaction buffer (100 μ l of 0.1 M Tris-HCl pH 8.5, 10 mM 5-BPA, and 10 mM dithiothreitol), and added to each well of an ELISA plate that had been coated with N, N'-dimethylcasein and blocked with BSA as described (Slaughter et al, 1992). Control for nonspecific binding was performed by omitting 5-BPA. The homogenized atherectomy samples were incubated with the immobilized N, N'-dimethylcasein for 1 hour and the plate was washed as described (Slaughter et al, 1992). To each well, 100 μ l of a 1:500 dilution of streptavidin-alkaline phosphatase solution in 100 mM Tris pH 8.5, 150 mM NaCl, 0.05% Tween-20, and 1% BSA was added for 1 hour at room temperature. After washing, color was developed using p-nitrophenylphosphate in diethanolamine buffer (BioRad). Purified TG (Sigma Chemical) was used to establish a standard curve.

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