Tenascin-C Is Expressed in Macrophage-Rich Human Coronary Atherosclerotic Plaque

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Background—Tenascin is a large extracellular matrix glycoprotein generally found in adult tissues undergoing active remodeling such as healing wounds and tumors. To determine the potential role of tenascin-C (TN-C) in the pathophysiology of atherosclerosis, we investigated the pattern of expression of TN-C in human coronary atherosclerotic plaques.

Methods and Results—Immunohistochemical staining and in situ hybridization demonstrated minimal and random expression of TN-C in fibrotic but lipid-poor atherosclerotic plaques. In contrast, all plaques with an organized lipid core or ruptured intimal surface strongly expressed TN-C, which was preferentially concentrated around the lipid core, shoulder regions, and ruptured area of the plaques but not in the fibrous cap. TN-C was not detected in normal arterial tissue. To identify the cellular source of TN-C, the plaques were stained with smooth muscle cell- and macrophage-specific antibodies. TN-C expression correlated with the infiltration of macrophages. Northern blot and immunoprecipitation analysis showed that macrophages expressed 7.0-kb TN-C mRNA and 220-kDa protein. Reverse transcription–polymerase chain reaction of total RNA derived from macrophages showed that they express the small isoform of TN-C. Zymogram analysis revealed that macrophages markedly increased MMP-9 expression.

Conclusions—This study demonstrates that the level of TN-C expression correlates with the degree of inflammation present, not with plaque size. In addition, cultured macrophages have the capacity to express the TN-C gene. These findings suggest the significance of macrophages in the remodeling of atherosclerotic plaque matrix composition.

Key Words: atherosclerosis ■ remodeling ■ stenosis

Tenascin-C (TN-C) is a highly conserved, multifunctional protein implicated in cell proliferation,1,2 migration,3-6 differentiation,7 and apoptosis.8 TN-C all contains a cysteine-rich segment at their amino termini, through which 6 TN-C monomers link into a hexamer. This segment is followed by 13 epidermal growth factor–like repeats, and globular carboxyl terminus homologous to fibrinogen. The number of fibronectin repeats depends on mRNA splicing. The first 5 and the last 3 repeats are constitutively expressed, and the other repeats are either included or skipped. The expression of TN-C isoforms appears to be regulated during development.8,9 In this work, we studied the pattern of TN-C expression in lipid-poor and lipid-rich human atherosclerotic plaques. In addition, we investigated the expression of TN-C isoforms in cultured macrophages. Our findings suggest a new mechanism for the expression of TN-C in inflamed tissues.

Methods

Material
Twenty-seven human coronary arteries were obtained from 12 patients who underwent heart transplantation. As a normal control for arterial tissue, we used 5 sections from 3 human internal mammary arteries obtained during coronary bypass surgery. Staining was performed by the avidin-biotin complex immunoperoxidase procedure essentially as recommended by the manufacturer (Dako). The preimmune serum was used as negative control. In 9 cases, serial sections also were stained for collagen with Masson’s trichrome stain. In 17 cases, the distribution of macrophages was determined by immunohistochemical staining using antibodies to the CD68 antigen (Dako). From the Atherosclerosis Research Center, Divisions of Cardiology and of Anatomic Pathology (M.C.F.), and Burns and Allen Research Institute, Cedars-Sinai Medical Center, and UCLA School of Medicine, Los Angeles, Calif.

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Expression of TN-C in Different Categories of Human Coronary Plaques

TN-C was not found in the normal human mammary artery (Figure 1A). All 5 fibrous-rich plaques minimally expressed TN-C, which was randomly distributed throughout the plaque (Figure 1B). In contrast, examination of 12 lipid-rich plaques revealed high-level expression of TN-C, which was highly concentrated around the lipid core (Figure 1C). In 6 of the 12 sections, TN-C was also found within the lipid core. In the adventitial and medial cell layers of all plaques, TN-C staining was either minimal or absent. Serial sections stained with anti-CD68 antibodies showed that macrophages were highly concentrated around the lipid core, and some macrophages were found inside the lipid core (Figure 1D).

Eight plaques had a highly organized lipid core. The plaques were heavily infiltrated with macrophages, which were concentrated around the lipid core, at the plaque shoulders, and even within the fibrous cap. Strong TN-C positivity paralleled the distribution of macrophages (Figure 2A). Higher magnification of plaque shoulders showed strong expression of TN-C and accumulation of macrophages (inset of Figure 2A). TN-C positivity was not found in the media or adventitia of these plaques (Figure 2A). In the 2 ruptured lipid-rich plaques, there was a remnant of the thin fibrous cap, as determined by collagen staining (Figure 2B). Staining of these plaques with CD68 antibodies showed that the area of rupture was heavily infiltrated with macrophages (not shown). Strong and focal TN-C staining was observed around the ruptured area (Figure 2C). Substitution of anti–TN-C antibodies with preimmune serum control showed no staining (Figure 2D). The number of ruptured lesions is small and will limit our ability to accurately predict the potential role of TN-C in plaque rupture; however, we found consistent expression of TN-C in the ruptured area of these 2 plaques.

The results of immunostaining data demonstrate a temporospatial correlation between distribution of macrophages and TN-C. However, this correlation is not absolute because of the tentative nature of immunohistochemical staining and the possibility that other intimal cells also express TN-C. The correlation between the expression of TN-C and the distribution of macrophages suggests that macrophages may express TN-C. It also is possible that TN-C was expressed in other muscle cells (SMCs) were isolated and cultured essentially as previously described. Oxidized LDL (ox-LDL) was a gift from Dr Navab (UCLA).

RNA isolation and Northern blot were performed essentially as previously described. Metabolic labeling and immunoprecipitation of media derived from cultured cells were performed as described.

Reverse transcription–polymerase chain reaction (RT-PCR) was performed essentially as described. First-strand cDNA synthesis was performed on total RNA extracted either from BHK cells permanently transfected by the small and long isoforms of TN-C (gift from Dr Erickson) or from cultured human macrophages treated with 50 μg/mL ox-LDL for 4 hours. The primers 5′-GGAATTCAGAAAGGCAGACACAAGAGCAAG-3′ and 5′-GGAATTTGAGTCNGTGATGTTGGCTNCTCA-3′ were used at a concentration of 1 mmol/L.

Zymography was performed by coating tissue culture plates (24 well) with 10 μg/mL fibronectin or tenasin substrates as described. The RAW264.7 cells were placed into aliquots on uncoated (control) and coated wells and incubated for 24 hours at 37°C. Conditioned media were collected and dialyzed, and protein concentrations were measured by Bradford assay. Protein (5 μg per lane) was resolved in a 10% SDS-PAGE gel containing 0.1% gelatin as described.

Results

The study set involved 27 coronary artery plaques. A cardiovascular pathologist (Dr Fishbein) independently classified each plaque. Five plaques were classified as fibrous, 12 were lipid-rich, 8 had active inflammatory infiltrates, and 2 were ruptured. The fibrous plaques were contained mostly collagen and a few inflammatory cells, and extracellular lipid was a minor component of the plaque. In lipid-rich plaques, most plaque is extracellular lipid with inflammatory cells concentrated around the lipid core. Macrophage-rich plaques are similar to lipid-rich plaque, but inflammatory cells are more numerous and more widely distributed around lipid core and in collagenous regions of the plaque. In ruptured plaques, the fibrous cap portions of the plaque are disrupted.
regions of the plaques and translocated into the macrophage-rich area. To distinguish between these 2 possibilities, serial sections from 4 coronary plaques with highly organized lipid core were hybridized to a digoxigenin-labeled TN-C cRNA probe. Consistent with our immunohistochemical staining, TN-C mRNA was distributed nonrandomly within the plaque and involved a subset of cells. These cells were most prominent in the subendothelial area of plaque shoulders (Figure 3A). No TN-C mRNA was detected in the media or adventitia. TN-C mRNA was not detected in the normal human mammary artery with the anti-sense riboprobe (not shown). To confirm the specificity of the TN-C anti-sense riboprobe, adjacent tissue sections were hybridized with the TN-C sense probe. No signal for TN-C was detected (Figure 3B). In these sections, macrophages were predominantly concentrated around the plaque shoulder (Figure 3C), a region that strongly stained for TN-C protein (Figure 3D). These data show a spatial correlation between distribution of TN-C mRNA and protein and localization of macrophages, suggesting that TN-C protein was predominantly retained at the site of synthesis.

Characterization of Intimal Cells
To identify the cellular source of TN-C, we stained the serial sections with SMC-specific antibodies. The intimal cells did not generally stain with smooth muscle α-actin antibodies except for a few pockets of cells near the media (Figure 4A). The fibrous cap and adventitia were generally negative, except for a few pockets of cells. In contrast, the medial cells strongly stained with anti-α-actin antibodies (Figure 4A). h-Caldesmon antibodies consistently stained medial cells, whereas intimal and adventitial cells did not stain (Figure 4B). Desmin antibodies predominantly stained medial cells, whereas intimal cells did not generally stain, except for a few cells (Figure 4C). Consistent with previous work, vimentin staining was distributed throughout the intima, media, and adventitia (Figure 4D), suggesting that the intima is cellular. These data suggest that the intimal cells exhibit a distinctive repertoire of cytoskeletal protein markers that distinguished them from medial SMCs.

Expression of TN-C by Cultured Monocyte-Derived Macrophages
The intimate association of TN-C with the infiltration and distribution of macrophages in the plaques suggests that plaque macrophages may be the source of TN-C. To explore this possibility, human-derived monocytes were isolated, cultured, and allowed to differentiate into macrophages. Because cells located around the lipid core strongly expressed TN-C, we reasoned that lipid components may stimulate TN-C expression by macrophages. To assess this possibility, cultured macrophages were treated with ox-LDL for 1, 2, 4, and 24 hours. In a parallel experiment, the U937 monocytic cell line was treated identically. Cultured rat arterial SMCs were used as positive control. As shown in Figure 5A, both monocyte-derived macrophages and U937 monocytic cells expressed the 7.0-kb TN-C mRNA transcript, and addition of ox-LDL did not significantly change the level of TN-C mRNA. Cultured SMCs expressed 2 TN-C mRNA transcripts when treated with 1 nmol/L platelet-derived growth factor (PDGF)-BB: the small (7.0 kb) and large (8.4 kb) isoforms of TN-C (Figure 5A). To control for loading, the blots were reprobed with cyclophylin cDNA (Figure 5B). Densitometric analysis of the blots revealed that cultured rat SMCs expressed the 7.0-kb TN-C mRNA transcript, and addition of ox-LDL did not significantly change the level of TN-C mRNA. Cultured SMCs expressed 2 TN-C mRNA transcripts when treated with 1 mmol/L platelet-derived growth factor (PDGF)-BB: the small (7.0 kb) and large (8.4 kb) isoforms of TN-C (Figure 5A). To control for loading, the blots were reprobed with cyclophylin cDNA (Figure 5B). Densitometric analysis of the blots revealed that cultured rat SMCs generally expressed 40% to 70% higher levels of TN-C mRNA than the cultured macrophages, when normalized for RNA loading. These differences in the pattern of TN-C expression by human monocyte-derived macrophages, the U937 cell line, and SMCs have been consistent throughout multiple isolates of macrophages and passages of cultured SMCs. We conclude that macrophages have the ability to express the TN-C gene.
To determine the presence of TN-C in conditioned media, macrophages and U937 cells were labeled with $^{35}$S-methionine in the absence and presence of ox-LDL. The SMCs were used as positive control. As shown in Figure 6, the anti–TN-C antibody immunoprecipitated the small isoform of tenascin (220 kDa) from the conditioned media of either macrophages or U937 cells. In contrast, the same antibody recognized 2 tenascin proteins from cultured SMCs: the small (220 kDa) and large (280 kDa) isoforms. These data demonstrate that macrophages synthesize primarily the small form of TN-C.

We used RT-PCR to further investigate the expression of TN-C isoform by macrophages. The PCR primers were designed to flank the alternatively spliced region of TN-C. The upstream primer was 46 bases upstream from the 5'-splicing junction, whereas the downstream primer was localized 315 bases downstream from the 3'-splicing junction. The PCR products were analyzed by 1% agarose gel. Deletion of primers (P) and template (T) in PCR reaction mixture was used as negative control. Expected sizes of small (450 bp) and large (2340 bp) isoforms are shown in left margin.

**Figure 6.** Synthesis of TN-C protein by cultured macrophages. Cultured human monocytes and U937 cells were labeled with $^{35}$S-methionine in the absence and presence of ox-LDL. Quiescent cultures of SMCs (2nd and 7th passages) were treated with 1 nmol/L PDGF-BB or 1 μmol/L Ang II in the presence of $^{35}$S-methionine. Media were collected, precleared, and used for immunoprecipitation with anti–TN-C antibodies. Large (L) and small (S) isoforms of TN-C are marked in left margin.

To determine the potential role of TN-C in human atheroma, we determined its effect on the metalloproteinase activity in the conditioned media of macrophages that have been exposed to TN-C. As shown in Figure 8A, we found a consistent increase in the amount of gelatinolytic activity associated with pro–MMP-9 released after 24 hours by macrophages cultured on tenascin substrate compared with those cultured on uncoated dishes. Fibronectin was less effective (Figure 8A). Faint gelatinolytic bands running at lower molecular weights most likely represent the processing of zymogen to activated forms. Densitometric analyses of 3 separate experiments showed that exposure of cells to fibronectin or TN-C increased the activity 6- and 13-fold, respectively, compared with uncoated dishes (Figure 8B).

**Discussion**

In this study, we have demonstrated that the expression of TN-C increases with ascending levels of plaque instability, from fibrous through lipid-rich to ruptured plaque. We also
have shown that the induction of TN-C mRNA and protein is associated with progressive accumulation of macrophages in the plaque and spatial correlation between TN-C expression and macrophage accumulation. These features were absent in the control arterial tissue. On the basis of these data, we hypothesized that macrophages express TN-C in advanced human atherosclerotic plaques. This hypothesis was supported by cell culture experiments. We found that macrophages express only the small isoform of TN-C. The same cDNA probe detected 2 TN-C mRNA transcripts in cultured SMCs, suggesting that the lack of detection of the large isoform in cultured macrophages was not due to the inability of the cDNA probe to detect the large isoform. Immunoprecipitation studies supported the Northern blot data and showed that macrophages synthesize and secrete the small 220-kDa isoform, whereas SMCs secrete at least the small and the large 280-kDa isoform. RT-PCR analysis of total RNA derived from macrophages confirmed the Northern and immunoprecipitation data. In addition to the major small isoform, we detected 2 larger PCR products, which were 720 and 990 bp. Because each fibronectin type III repeat of TN-C is 270 bp long, we think that these 2 additional PCR products may be the D and A1A2 isoforms that we have previously described. Because we detected only the small isoform by Northern blot and immunoprecipitation, it is possible that either the other larger isoforms did not translate into the proteins or their expression levels were very low.

Theoretically, macrophages might increase the level of TN-C in inflamed tissues through 2 different mechanisms: through secretion of various growth factors and cytokines that activates other cells or by expression of the TN-C gene directly. In our study, we observed that TN-C expression and macrophages codistributed around the lipid core, suggesting that lipid core constituents may induce TN-C expression in macrophages. Our cell culture studies, however, do not support this notion because ox-LDL did not affect TN-C mRNA levels. It is possible that other lipid constituents induce TN-C expression or that macrophages in atheroma constitutively express TN-C. Our in situ hybridization data support the later possibility because TN-C mRNA and macrophages colocalized in the region of the plaque that was lipid poor, ie, plaque shoulders. This notion was further supported by our cell culture studies in which macrophages maintained under serum-free media expressed TN-C mRNA. We suggest that the expression of TN-C by macrophages may have constitutive and inducible components and that the presence of macrophages per se is sufficient to increase the level of small isoform of TN-C in tissue.

Our finding of a close correlation between macrophage infiltration and TN-C expression is supported by others. Satta et al noted a close relationship between TN-C expression and distribution of mononuclear inflammatory cells in abdominal aortic aneurysms. Le Poole et al reported that the level of TN-C in skin biopsies from inflammatory vitiligo patients correlated with the degree of inflammation. Finally, immunohistochemical analysis of muscle biopsies from Duchenne/Becker muscular dystrophy and myositis patients revealed a close relationship between distribution of TN-C and the presence of macrophages. These studies, however, did not determine which cell type is the source of TN-C in these tissues.

There is limited information about the biological activity of the small isoform. The soluble form of the small but not the large isoform inhibited neuronal outgrowth. In addition, the small isoform bound more strongly to fibronectin than the large isoform and presumably incorporated more efficiently into the extracellular matrix. This suggests that the small isoform may not diffuse away from its site of synthesis and may explain the strong focal distribution of TN-C that we observed in the plaques.

The addition of TN-C most likely alters the engagement pattern of matrix receptors of intimal cells, which in turn cause rearrangement of the cytoskeletal network and intracellular cascade of signal transduction, leading to changes in gene expression and tissue remodeling. We observed that the composition of extracellular matrix affects metalloproteinase expression of macrophages. Interaction of macrophages with fibronectin and TN-C markedly induced the expression of 92-kDa gelatinase severalfold. TN-C was more effective in the induction of the gelatinase activity than fibronectin. Similarly, Khan and Falcone observed that when TN-C was used as a substrate, there was a marked induction of metalloproteinase-9 gene expression in mouse macrophage cell line. Tremble et al reported that TN-C but not fibronectin upregulated synthesis of collagenase, stromelysin, and gelatinase in rabbit synovial fibroblasts. The ability of TN-C
to modulate gene expression of metalloproteinases could affect the stability of atherosclerotic plaque because the increased metalloproteinases activity stimulates collagen degradation in the fibrous cap of human atherosclerotic plaque. Taken together, it is conceivable that the presence of TN-C in the vulnerable region of the plaque increases the activity of metalloproteinases, which in turn promotes the breakdown of extracellular matrix and destabilizes the plaque.

The introduction of small isoform of tenasin into the matrix may generate a dynamic interactive networks of matrix proteins that create a microenvironment that affects macrophages and other intimal cells. We hypothesize that this microenvironment promotes progression and destabilization of atherosclerotic plaques rather than maintenance and stability.

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