Production of the Long Pentraxin PTX3 in Advanced Atherosclerotic Plaques

Michael S. Rolph, Sabine Zimmer, Barbara Bottazzi, Cecilia Garlanda, Alberto Mantovani, Göran K. Hansson

Abstract—Elevated plasma levels of the pentraxin protein family member C-reactive protein (CRP) are associated with increased risk of cardiovascular disease in both healthy and high-risk subjects. The long pentraxin family member, pentraxin 3 (PTX3), was recently described. Like CRP, PTX3 is induced by acute inflammatory stimuli and is increased in the blood of patients with acute myocardial infarction. Unlike CRP, it is expressed in a wide range of cell types, but not in hepatocytes. In this study, we have investigated the expression of PTX3 in atherosclerosis. Immunohistochemical staining of advanced atherosclerotic lesions revealed strong expression of PTX3. In contrast, no PTX3 expression was observed in nonatherosclerotic internal mammary arteries. By staining serial sections with cell type– and PTX3-specific antibodies, we observed that PTX3 was produced principally by macrophages and endothelial cells. Infrequent expression by smooth muscle cells was also observed. Our results suggest that PTX3 may contribute to the pathogenesis of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:· · · · · · · ·)

Key Words: pentraxin 3 ▪ atherosclerosis ▪ macrophage ▪ immunohistochemistry ▪ C-reactive protein

Cardiovascular disease is the major cause of death in developed countries. Atherosclerosis is the predominant cause of this high incidence of cardiovascular disease. Intimal cholesterol accumulation is a major pathological feature of atherosclerosis, but in recent years there has been a growing understanding of the inflammatory nature of the disease. Indeed, the very earliest stages of lesion formation are characterized by an influx of macrophages and T lymphocytes, which become progressively activated during lesion development. The precise nature and role of the inflammatory response in atherosclerosis are not fully understood, but they are currently an area of intense research interest because of their potential as diagnostic and therapeutic targets.

Studies in animal models have suggested that the inflammatory response associated with atherosclerosis promotes lesion development. This is supported by epidemiological studies in humans. For example, elevations in the plasma concentration of the acute phase protein C-reactive protein (CRP), which is widely used as an indicator of systemic inflammation, correlate with increased risk of cardiovascular disease in both healthy and high-risk subjects. CRP deposition has been detected in atherosclerotic lesions and may enhance lesion development through its ability to activate the complement pathway.

CRP belongs to the pentraxin protein family, members of which are structurally distinguished by a characteristic pentameric structure. In recent years, a number of new pentraxins have been discovered, including pentraxin 3 (PTX3), neuronal pentraxin 1, and neuronal pentraxin 2. These molecules are known as long pentraxins and are approximately twice the size of the prototypic pentraxins CRP and SAP. PTX3 was the first long pentraxin to be discovered, and its expression is induced in response to inflammatory stimuli, including tumor necrosis factor (TNF)α, interleukin (IL)-1β, and lipopolysaccharide (LPS). PTX3 shares homology with CRP at the C-terminal end but has no homology to any known protein at its N-terminal end. PTX3 is produced by a range of cell types, including monocytes/macrophages, endothelial cells; and fibroblasts, but is not produced by hepatocytes, which are a major source of CRP. Like CRP, PTX3 is able to bind the C1q complement component, and it has been proposed that PTX3 may play the same function in the periphery as CRP does in the circulation.

Two of the major cellular components of atherosclerotic lesions, namely macrophages and endothelial cells, are potent producers of PTX3 (but not of CRP) in response to inflammatory stimuli. Given the inflammatory nature of the atherosclerosis disease process and the previously identified involvement of the prototypic pentraxin CRP in atherosclerosis, this suggests that PTX3 may be involved in the pathogenesis of atherosclerosis. In this study, we have investigated PTX3 expression in advanced human atherosclerotic lesions. Immunohistochemical analysis demonstrated strong PTX3 expression in the lesions, whereas PTX3 expression was not...
detected in healthy, nonatherosclerotic internal mammary arteries. By staining serial sections with appropriate cell type–specific antibodies, it was found that endothelial cells and macrophages were the principal producers of PTX3, while a minor fraction of smooth muscle cells (SMCs) also produced PTX3.

**Methods**

**Antibodies**
The following antibodies were used in this study: mouse anti-α-smooth muscle actin (Dako), mouse anti-CD68 (Dako), rabbit anti-von Willebrand factor (Dako), and rat anti-PTX3 (clone MNB415). The MNB4 antibody does not cross-react with other pentraxin family members, CRP, and SAP (B. Bottazzi, unpublished observations, 2001). Secondary antibodies, biotinylated horse anti-mouse IgG and biotinylated rabbit anti-rat IgG were both from Vector.

**Specimens**
Advanced atherosclerotic lesions (n=9) were obtained from tissue removed during carotid endarterectomy procedures. Internal mammary arteries (n=2) were obtained from tissue removed during coronary artery bypass grafting. All surgical specimens were obtained by protocols approved by the Karolinska Hospital Human Ethics Review Board.

**Immunohistochemistry**
Frozen sections were fixed in acetone, air-dried, and stained with a standard immunohistochemical protocol. Briefly, the sections were blocked for 30 minutes with 5% serum (goat serum for PTX3 and von Willebrand factor staining; horse serum for CD68 and α-actin staining). The sections were then incubated overnight at 4°C with appropriately diluted primary antibody. The sections were rinsed in phosphate-buffered saline and incubated for 30 minutes at room temperature with biotinylated horse anti-mouse IgG or biotinylated rabbit anti-rat IgG. After quenching endogenous peroxidase for 30 minutes with 0.3% peroxidase, the sections were incubated for a further 30 minutes with avidin/biotinylated peroxidase complex (Vector). Color was developed using 3,3′-diaminobenzidine (Vector), and the sections were counterstained with hematoxylin. Between each incubation, the sections were washed in phosphate-buffered saline. Except where indicated, all incubations were performed at room temperature.

**SMC Culture**
The human SMC line CRL-1999, derived from the aorta of an 11-month-old-child, was obtained from American Type Culture Collection. Cells were maintained in DMEM medium supplemented as described by the provider at 37°C in the presence of 5% CO₂. Confluent cells were incubated with supplemented DMEM medium without fetal calf serum for 24 hours and then stimulated with LPS (Sigma) or IL-1β (Peprotech) for 4 hours. All reagents were tested for endotoxin contamination by using the Limulus Amebocyte Lysate from BioWhittaker.

**RNA Extraction and Northern Blot Analysis**
RNA was extracted and purified with TRIzol (Sigma) as described by the manufacturer. Ten micrograms of total cellular RNA was electrophoresed on a standard formaldehyde-agarose gel, blotted onto Hybond N+ nitrocellulose membrane (Amersham), and fixed by cross-linking. The probe for human PTX3 was the full-length cDNA. After prehybridization for 2 hours at 65°C, the [α-32P]dCTP-labeled probe was hybridized overnight at 65°C. The hybridized membrane was washed twice in 2×SSC, 0.05% SDS at room temperature and twice in 0.1×SSC, 0.1% SDS at 55°C. Blots were exposed to x-ray film (Eastman Kodak) at −80°C.

**Results**

**PTX3 Expression in Advanced Atherosclerotic Plaques**
To examine PTX3 expression in atherosclerosis, we performed immunohistochemistry on advanced atherosclerotic plaques obtained from carotid endarterectomy specimens (n=9). PTX3 staining was observed in all atherosclerotic plaques on the surface of the lumen as well as within the body of the lesion (Figure 1A through 1D). In contrast, no PTX3 staining was detected on sections from nonatherosclerotic internal mammary arteries (Figure 1E and 1F). In some areas of the plaque, a diffuse PTX3 stain was observed, which
is consistent with an extracellular localization. As a negative control, the primary antibody was omitted from the staining procedure (Figure 1A, 1C, and 1D), or the staining was blocked by the addition of excess recombinant PTX3 to the primary antibody (not shown). In both cases, no DAB product was observed, except in foam cells, which gave a very weak granular staining (Figure 1A).

Identification of Endothelial Cells and Macrophages as the Major Producers of PTX3 in Atherosclerotic Lesions

To identify the cells responsible for PTX3 production, we stained serial sections for PTX3 and specific cell types, namely macrophages, endothelial cells, and SMCs. Staining with von Willebrand factor confirmed that endothelial cells are major producers of PTX3 (Figure 2A through 2C). Staining of endothelial cells was observed in all lesions but was patchy and not consistently localized in relation to any specific area of the artery or lesion.

Macrophages in the lesion were identified by CD68 expression. By examining serial sections stained with CD68, it was apparent that numerous lesional macrophages expressed PTX3 (Figure 2D through 2F). Foam cells within lipid-rich areas of the plaque consistently stained for PTX3, although the staining was of low intensity. Macrophages within the fibrous cap stained less consistently with PTX3, but the staining intensity was greater than that observed in macrophages within the lipid-rich core.

The majority of SMCs, as detected by α-smooth muscle actin staining, were negative for PTX3. In a minority of sections, we were able to detect some PTX3 staining by SMCs (Figure 2G through 2I). This was mostly detected on subendothelially located SMC.

PTX3 Expression in Cultured Human Aortic SMCs: Inducibility by Inflammatory Mediators

Expression of PTX3 by human SMCs has not been previously described. To confirm the in vivo findings, we examined expression of PTX3 mRNA in cultured human SMCs. Little or no PTX3 expression was detected in unstimulated cultures. In contrast, strong PTX3 expression was induced in response to LPS or IL-1β (Figure 3). These results confirm that SMC can express PTX3 mRNA, and that, as previously described for other cell types, it is induced by inflammatory stimuli. The second, upper band observed in the PTX3 Northern blot (Figure 3) has been previously described and may represent a precursor RNA molecule.

Discussion

A range of cell types, including macrophages and endothelial cells, produce PTX3 in response to inflammatory stimuli such as bacterial endotoxin, IL-1, and TNF. Because atherosclerosis is an inflammatory disease, and because macrophages and endothelial cells are major cellular constituents of atherosclerosis, we hypothesized that PTX3 would be expressed in
atherosclerotic lesions. We found strong PTX3 staining in macrophages and endothelial cells in advanced atherosclerotic lesions. In contrast, sections from nonatherosclerotic internal mammary arteries did not express PTX3.

Expression of PTX3 by both endothelial cells and macrophages was patchy in all lesions examined, whereas only a small minority of the SMCs stained for PTX3. It will be interesting to identify the factors that regulate PTX3 expression in atherosclerosis. IL-1 and TNF are both expressed in advanced atherosclerotic lesions, and these molecules are major candidates for regulating PTX3 expression. Another candidate is oxidized LDL, because many of the proinflammatory effects of oxidized LDL are mediated through activation of NF-κB, a transcription factor known to be required for PTX3 expression. Interferon-γ is known to suppress the transcription of PTX3 and is produced by T cells within the fibrous cap of advanced atheromas. Few T cells are located within the lipid-rich core. Differential expression of interferon-γ within the different regions of the plaque may contribute to the reduced frequency of macrophages staining for PTX3 in the fibrous plaque compared with the lipid-rich core.

Few studies have examined PTX3 expression in vivo. After intraperitoneal LPS injection in mice, strong PTX3 mRNA expression was detected in endothelial cells of the heart, lung, and skeletal muscle. In contrast, glial cells are the major source of PTX3 after intracerebral LPS administration. In rheumatoid arthritis, PTX3 expression was detected in endothelial and synovial cells, whereas increased serum PTX3 was detected in the blood of patients with acute myocardial infarction. The present study extends our knowledge of PTX3 in human disease by demonstrating strong PTX3 staining in advanced atherosclerotic plaques. In addition, our finding that a minority of SMCs stained for PTX3 is the first description of PTX3 production by this cell type. Preliminary in vitro studies of cultured human SMCs confirmed this finding and revealed a similar pattern of inducibility to that for other cell types such as monocytes and endothelial cells.

To date, there is little functional information about PTX3. It has been proposed that PTX3 may play the same function in the periphery as CRP does in the circulation. Like CRP, PTX3 can bind to the C1q complement component. CRP is able to activate the complement pathway and in atherosclerotic lesions is frequently colocalized with components of the complement pathway. Recent studies have also identified a direct proinflammatory effect of CRP. It will be interesting to examine the role of PTX3 in complement activation during atherogenesis. Another function of PTX3 that could conceivably be involved in atherogenesis is its ability to bind dead, especially apoptotic, cells. In conclusion, our data suggest that PTX3 may be involved in the pathogenesis of atherosclerosis. We are currently conducting in vitro and in vivo studies to more closely define the regulation of PTX3 production and its role in atherosclerosis.

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References


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