Local Generation of C-Reactive Protein in Diseased Coronary Artery Venous Bypass Grafts and Normal Vascular Tissue

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Background—Venous coronary artery bypass grafts (CABGs) are prone to accelerated atherosclerosis. In atherosclerotic diseases, serum C-reactive protein (CRP) levels have become an important diagnostic and prognostic marker. The origin of CRP in this setting remains to be elucidated.

Methods and Results—Monoclonal anti-CRP identified CRP expression in medial and intimal α-actin–positive smooth muscle cells (SMCs) of diseased CABGs with type V and VI lesions and also of native saphenous veins of atherosclerotic individuals. In addition, patent coronary arteries with type IV and V but not with type I through III lesions exhibited intense SMC staining for CRP. Calcified desobliterates of occluded coronary arteries with end-stage disease did not show SMC staining for CRP and were consistently negative for CRP mRNA, as detected by means of real-time polymerase chain reaction. However, CRP mRNA was expressed in 11 of 15 diseased CABGs and also in 10 of 15 native veins. By contrast, only 3 of 18 internal mammary and 4 of 12 radial arteries with virtually no atherosclerosis were positive for CRP mRNA.

Conclusions—CRP is produced by SMCs of atherosclerotic lesions with active disease but not in end-stage plaques. The role of CRP constitutively expressed by normal vascular tissue in vein graft disease has yet to be elucidated.

Key Words: atherosclerosis inflammation restenosis

In recent years, serum C-reactive protein (CRP) has become a powerful marker of future cardiovascular events. CRP is linked to vascular inflammation because it attracts monocytes and mediates LDL uptake by macrophages. Furthermore, CRP induces adhesion molecule expression but attenuates NO production of human endothelial cells. Histological investigations have described an association between intimal CRP deposition and the development of atherosclerotic plaques. None of these studies supposed that CRP immunoreactivity resulted from local expression of CRP. We speculated that elevated serum CRP levels do not reflect continuous hepatic CRP synthesis but rather represent local production. This hypothesis has recently been corroborated by findings of detectable CRP mRNA and protein in postmortem cases of aortosclerotic plaques. Yasojima et al described smooth muscle cells (SMCs) and macrophages as sites of CRP production. These data—together with our observation of CRP production by renal epithelial cells—argue against the liver as the only site of CRP formation. The aim of the present study was to examine local generation of CRP in human atherosclerotic lesions. Because we have been interested for years in the increased reocclusion rate of coronary artery venous bypass grafts (CABGs), we investigated samples of diseased CABGs for CRP mRNA and protein expression. The results were compared with patent atherosclerotic coronary arteries and calcified coronary desobliterates as well as with native saphenous veins, internal mammary arteries (IMAs), and radial arteries (RAs).

Methods

Sample Collection

For CRP mRNA detection, 10 desobliterates obtained from chronic coronary occlusions and samples of 15 diseased CABGs, 15 native saphenous veins, 18 left IMAs, and 12 RAs were immediately transferred into a guanidine isothiocyanate–containing lysis buffer and stored at −70°C. In 14 cases, saphenous veins and IMAs or RAs were obtained in the same patient. For immunohistochemistry, material of approximately half the samples was stored in RPMI medium, immediately fixed in formalin and embedded in paraffin. All specimens were obtained on coronary bypass surgery for patients with coronary heart disease. Furthermore, coronary arteries from 11...
patients with end-stage heart failure due to idiopathic cardiomyopathy who were undergoing allogeneic cardiac transplantation were examined by immunohistochemistry. None of these patients had coronary stenosis >50% on angiography. Samples were kindly provided by the German Heart Center, Berlin; however, no material for mRNA analysis was available. The local ethics committee approved the study.

**Immunohistochemistry**

Four-micrometer-thick serial sections were mounted on glass slides and deparaffinized in xylene. After rehydration and treatment with 0.01 mol/L citric acid, slides were preincubated with RPMI1640 + 10% AB serum. Monoclonal anti-CRP antibody (clone CRP-8, Sigma; 1:500) or monoclonal antibody against α-smooth muscle actin (α-SMA) (clone 1A4, Sigma; 1:9000) were used as primary antibody, each for 30 minutes. Secondary antibody administration (rabbit anti-mouse; DAKO; 1:25, 30 minutes) followed by alkaline phosphatase anti-alkaline phosphatase complex incubation (DAKO; 1:50, 30 minutes) was repeated for a total of 3 times each. Finally, substrate incubation (naphthole/neofuchsine, DAKO) and counterstaining with hemalaun were performed. Comparable sections were stained with hematoxylin and eosin as well as with Masson’s trichrome. The histology of each sample was assessed according to the Stary classification for advanced atherosclerotic lesions.12

**RNA Extraction and Reverse Transcription**

Total RNA was extracted from specimens of ~0.5 to 1 cm in length using RNeasy Mini Kit (Qiagen). Before spin column application, tissue was disrupted by using an automatic tissue disruption device (Braun, Melsungen, Germany) and by proteinase K digestion. RNA isolation followed the recommendations of the manufacturer, including DNase treatment. Reverse transcription was done by means of the TaqMan Gold RT-PCR Kit (Perkin Elmer (PE) Applied Biosystems).

**Real-Time Polymerase Chain Reaction**

Quantitative real-time polymerase chain reaction (PCR) was performed with the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems), as described previously.10 Primers were designed to amplify only cDNA by using an exon junction–spanning reverse primer. Correct amplification was proven by (1) conventional PCR and gel electrophoresis identifying a single band at ~150 bp, (2) sequencing of 2 different PCR products showing a base pair composition identical to that published (GenBank accession number NM_000567), and (3) failure to amplify genomic DNA. The PCR A through C. Immunohistochemistry of diseased CABG with type VI lesion, magnification ×100, showing identical staining patterns for α-SMA and CRP. Lumen toward the lower side. D and E. Immunohistochemistry for CRP of coronary artery from a heart failure patient with advanced atherosclerosis (50% stenosis on angiography), magnification ×100, showing enlarged intima with SMC proliferation and foam cell generation. Medial SMCs (▲) and foam cells (▼) with cytoplasmic CRP staining (detail). F. CRP staining of a coronary artery with type V lesion from another heart failure patient (<50% stenosis on angiography), magnification ×100. Note staining of medial (▲) and spindle-like intimal SMCs (detail, ▼). Arrows (detail, ▼) mark extracellular deposits of CRP within the lipid core. G and H. CRP and control immunostaining of native saphenous vein, magnification ×100. I and J. Immunohistochemistry for CRP of left IMA, magnification ×50.
assay was established as a multiplex PCR amplifying within the same tube CRP cDNA as well as hypoxanthine phosphoribosyltransferase cDNA for standardization. Each sample was measured 5 times.

**Results**

Immunohistochemistry revealed intense CRP staining of diseased CABGs with severe intimal thickening (neointima) (Figure, A through C). Staining of adjacent sections with anti-α-SMA indicated that CRP-positive cells represented SMCs of the neointima (lower parts) as well as the media (upper parts). In addition, we observed CRP staining of spindle-like and foam cells within the proliferating intima of coronary arteries with advanced atherosclerotic lesions (Figure, D through F). The origin of CRP from coronary artery SMCs was most obvious in the media of arteries with type IV and V lesions (Table), where virtually all α-SMA–positive cells were also stained by anti-CRP. Coronary desobliterates with heavy calcification and a very low cell content typically showed extracellular CRP staining within the intima but almost no staining of CRP-positive cells; accordingly, we did not find CRP mRNA in desobliterates (Table). However, transcription of CRP mRNA was seen in most diseased CABGs at levels comparable to those of maximally stimulated Hep3B cells. Interestingly, “native” saphenous veins, which exhibited a pattern of medial CRP immunoreactivity similar to that of diseased CABGs (Figure, G through H), preponderantly expressed CRP mRNA as well. On the other hand, IMAs (elastic artery; Figure, I through J) and RAs (SMC rich) showed no apparent CRP staining of medial SMCs. CRP mRNA transcription was observed in 3 IMAs and 4 RAs. The number of positive IMAs significantly differed from native veins (χ² = 8.57, P = 0.005 for IMA; Pearson’s χ² and Fisher’s exact test, 2-tailed). One patient was positive for both IMA and saphenous vein samples; 13 other patients only showed CRP mRNA expression of either venous or arterial material, which argues against systemic factors such as hyperlipidemia being responsible for CRP expression of normal vascular tissue.

**Discussion**

In the present study, we detected CRP protein and/or mRNA in atherosclerotic lesions of diseased CABGs and coronary arteries but not in coronary desobliterates with fibrous cap formation and heavy calcification. It was noteworthy that 2 CABGs with no detectable CRP mRNA also exhibited changes of advanced atherosclerosis but histologically showed preponderance of fibrous caps with very few neointimal SMCs. We conclude that local CRP expression is limited to active sites of disease with large cellular expansions and is not found in end-stage plaques. SMCs were identified as the predominant site of CRP expression. Our findings clearly suggest that CRP immunoreactivity of SMCs results from local biosynthesis, although we cannot rule out a contribution from serum CRP. However, a recent publication provided evidence that the extent of serum CRP elevation in patients with abdominal aortic aneurysms is associated with aneurysmal size and that—in at least some patients—CRP is produced locally within the inflamed vessel wall. 13

Our results might be limited by the fact that we were not able to investigate CRP mRNA transcription in coronary atherosclerosis (with the exception of one case with positive CRP testing in extensive coronary artery disease [data not shown]) and normal coronary arteries. Moreover, our data on patients with end-stage heart failure and nonsignificant coronary lesions (<50% stenosis) may not be extrapolated to patients with significant lesions (>70% stenosis) or to apparently healthy subjects with early atherosclerotic lesions.

The most intriguing finding of our study was the strong CRP expression by normal saphenous veins and—to a significantly lesser extent—by normal arteries. This is in accordance with a recent publication that described the immunohistochemical localization of CRP in normal vascular tissue. 14 It remains to be elucidated whether our findings in native veins can be extended to individuals without generalized atherosclerosis or might be related to varicosis. Interestingly, we observed strong CRP expression in varicose veins of 4 young patients without any cardiovascular risk factors (data not shown). Whether our results are related to the increased reocclusion rate of venous compared with arterial CAGB needs to be further characterized. As a future perspective, we suggest that local CRP expression of atherosclerotic lesions might contribute to the distinct serum CRP elevations that are seen in atherosclerosis and correlate to the extent of the disease.

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References
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