C-Reactive Protein Induces Apoptosis in Human Coronary Vascular Smooth Muscle Cells

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Background—Accumulating evidence suggests that C-reactive protein (CRP), in addition to being a predictor of coronary events, may have direct actions on the vessel wall in the evolution of atherosclerosis. Although accumulation of vascular smooth muscle cells (VSMCs) in the intima is a key event in the development of arterial lesions, apoptosis of VSMCs also plays an important role in progression of atherosclerotic lesions and contributes to increased plaque vulnerability.

Methods and Results—In the present study we demonstrate that CRP induces caspase-mediated apoptosis of human coronary VSMCs. DNA microarray analysis was used to identify CRP-regulated genes. The growth arrest– and DNA damage–inducible gene 153 (GADD153) mRNA expression was prominently upregulated by CRP. As confirmed by Northern blot analysis, CRP induced a time- and dose-dependent increase of GADD153 mRNA expression. GADD153, a gene involved in growth arrest and apoptosis in vascular and nonvascular cells, is regulated at both transcriptional and posttranscriptional levels. CRP regulation of GADD153 mRNA expression in VSMCs occurs primarily at the posttranscriptional level by mRNA stabilization. Small interfering RNA (siRNA) specifically targeted to GADD153 reduced CRP-induced apoptosis. GADD153 also specifically colocalized to apoptotic VSMCs in human coronary lesions, further supporting a functional role for GADD153 in CRP-induced cell death.

Conclusions—These results demonstrate that GADD153 is a CRP-regulated gene in human VSMCs and plays a causal role in CRP-induced apoptosis. Pharmacological targeting of CRP expression or action may provide a novel therapy for atherosclerosis. (Circulation. 2004;110:579-587.)

Key Words: C-reactive protein ■ apoptosis ■ transcription factors ■ muscle, smooth, vascular

Many epidemiological studies have now shown that elevated levels of C-reactive protein (CRP), a classic acute-phase protein, correlate with increased risk of cardiovascular disease, myocardial infarction, and stroke among apparently healthy individuals. Furthermore, CRP levels have been found to predict the risk of recurrent coronary events among patients with stable and unstable angina in the acute phase after myocardial infarction and after revascularization procedures. In addition to being an independent cardiovascular disease risk factor, recent evidence implicates CRP as a mediator of atherosclerosis, although this issue is controversial. Inflammatory processes are key mechanisms in the initiation and progression of atherosclerosis. CRP has been localized directly within atherosclerotic plaques, where it induces the expression of genes involved in monocyte adhesion and recruitment such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, and monocyte chemoattractant protein-1. Furthermore, CRP has been shown to mediate LDL uptake by macrophages and to activate the complement system in vivo and in vitro, pathways that play important roles in atherogenesis.

Previous reports have shown that apoptosis in atherosclerotic plaques can involve all cell types in the lesion, including vascular smooth muscle cells (VSMCs), endothelial cells, T cells, and macrophages. The relative number of apoptotic cells depends on the stage of atherosclerotic plaque development and is generally higher in more advanced lesions. VSMCs synthesize most of the interstitial collagen fibers that stabilize the fibrous cap of the plaque. Excessive VSMC apoptosis in atherosclerotic plaques may compromise their integrity, render them vulnerable to proteolytic attack from inflammatory cells, and lead to plaque rupture. Apoptotic VSMCs can also increase thrombogenicity after plaque disruption, thereby promoting the occurrence of acute ischemic events. Flynn et al demonstrated that apoptotic human VSMCs produce active thrombin from its inactive precursor form, which is known to directly activate platelets to adhere,
aggregate, and release their α-granule contents. Thus, excessive apoptosis of VSMCs, through multiple mechanisms, may lead to increased plaque thrombogenicity and instability, resulting in an acute cardiovascular event.

GADD153 (growth arrest– and DNA damage–inducible gene 153), a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, is highly expressed by cells after treatment with a variety of growth arrest and/or DNA-damaging factors, such as oxidative stress. GADD153 has been linked to growth arrest and apoptosis in a number of biological systems. Microinjection of GADD153 into NIH-3T3 fibroblasts induces G1 arrest, whereas overexpression of GADD153 induces apoptosis in different cancer cell lines.

In the present study we demonstrate that CRP induces apoptosis of human coronary VSMCs.

Using DNA microarrays, we identified the growth arrest–and DNA damage–inducible gene GADD153 as a CRP-regulated gene and established a functional role for GADD153 in apoptosis by using small interfering RNA (siRNA) technology. Our findings suggest that CRP induction of VSMC apoptosis through a GADD153-dependent pathway may contribute importantly to the progression and/or stabilization of atherosclerotic lesions.

**Methods**

**Cell Culture**

Primary human coronary artery VSMCs were obtained commercially from Cambrex. Cells (passages 4 to 9) were grown to 70% to 80% confluence in medium supplemented with 5% FBS and the manufacturer’s reagents (Cambrex). Troglitazone was kindly provided by Parke-Davis. Highly purified CRP (from human fluids) was obtained from Trichem Resources. The endotoxin level of CRP was below 0.06 U/mL and resuspended in 50 μL ice-cold lysis buffer (50 mmol/L HEPES, 1 mmol/L dithiothreitol, 0.1 mmol/L EDTA, 0.1% CHAPS, pH 7.4). After centrifugation at 10 000 g for 10 minutes at 4 °C, caspase-3 activity in supernatants was measured according to the manufacturer’s instructions.

**cDNA Array Assay**

CRP-induced regulation of gene expression was analyzed with a commercially available cDNA array corresponding to 96 human genes indicative for stress and toxicity and 12 housekeeping genes (SuperArray) according to the manufacturer’s instructions. Two micrograms of total RNA was used to synthesize biotin-16-DUTP-labeled probes with the use of the AmpoLabeling-LPR method (30 cycles).

**RNA Isolation and Northern Blotting**

Total RNA was isolated with the use of TRIzol reagent (Life Technologies). Northern blotting was performed as previously described with GADD153 cDNA. Blots were rehybridized with cDNA encoding the constitutively expressed housekeeping gene GAPDH to assess equal loading of samples. GADD153 mRNA levels were normalized against GAPDH mRNA.

**RNA Stability Assay**

Human VSMCs, cultured for 48 hours in the presence or absence of CRP (20 μg/mL), were treated with actinomycin D (5 μg/mL) and harvested at indicated time points. RNA was isolated, and GADD153 expression was analyzed by Northern blot. GADD153 expression was quantified by densitometry and normalized against GAPDH signal, and GADD153 mRNA half-life was calculated by the equation $C = C_0 e^{-kt}$, where $C$ is the mRNA remaining at time $t$, $C_0$ is the mRNA at time of actinomycin D addition, and $K_d$ is the mRNA decay constant.

**Small Interfering RNA**

SiRNA for GADD153 exon sequence and nonsilencing control siRNA were purchased from Ambion. The following GADD153 sequence was used: sense 5' GGUGCCUGUCUCAUGAAGATTT 3'; antisense 5' UCUAUCUGAAAgACGAGCCTC 3'. Transfections, performed with a complex of siRNA (100 nmol/L) and 24 μL siPORT Lipid (Ambion) per 100-mm dish, were performed under serum-free conditions with the use of OPTI-MEM 1 (Invitrogen). Complete growth medium was added 5 hours after transfection. Eighteen hours after transfections, cells were washed in PBS and treated with CRP as indicated.

**Annexin V Flow Cytometry**

Apoptotic VSMCs were detected and quantified with a commercially available annexin V–fluorescein isothiocyanate (FITC) assay (Oncogene Research Products) as previously described.

**TUNEL Assay and Immunofluorescent Staining**

DNA fragments generated in response to apoptotic signals were labeled with the use of a commercially available kit from Oncogene Research Products (Fluorescein-FragEL DNA Fragmentation Detection Kit) according to the manufacturer’s instructions. VSMCs were immobilized onto glass slides with the use of a Cytoospin. Paraffin-embedded human coronary artery sections were deparaffinized with xylene, rehydrated, and permeablized with proteinase K. After terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay, sections were blocked (1% BSA, 1% cold-water fish gelatin, 1 mol/L glycine, 3% normal goat serum; Sigma) and stained with a GADD153 antibody for 1 hour, followed by a secondary goat anti-rabbit IgG (1:200; Texas red; Molecular Probes Inc). Cells were visualized with a confocal microscope, and colocalization of fluorescein and Texas red or DAPI staining was performed by overlay projections.
Results

CRP Induces Apoptosis of Human Coronary VSMCs

Exponentially growing VSMCs were exposed to increasing concentrations of CRP for 72 hours in the presence of 5% FBS. CRP induced VSMC apoptosis as revealed by typical morphological changes, annexin V binding, and TUNEL analysis. As shown in Figure 1A, CRP treatment (1 to 100 μg/mL) was associated with a dose-dependent increase of annexin V–FITC binding to phosphatidylserine residues, which are externalized to the cell surface as cells undergo apoptosis. Compared with untreated control cells, CRP at 20 μg/mL induced a 3.86±0.54-fold increase in annexin V–FITC–positive apoptotic cells (P<0.05). CRP treatment was associated with characteristic morphological features of apoptosis such as cell shrinkage, retraction from neighboring cells, increased refractile ability, and retraction from neighboring cells. Staining with hematoxylin-eosin shows condensed or fragmented chromatin in CRP-stimulated VSMCs.

CRP Induces Apoptosis Through a Caspase-Mediated Mechanism

Activation of caspases is a key element in apoptotic cell death. VSMCs were pretreated with various concentrations...
of the cell-permeable irreversible pan-caspase inhibitor Z-VAD-FMK for 3 hours, then stimulated with CRP (20 μg/mL) for 72 hours and analyzed by annexin V–FITC flow cytometry (Figure 3A). Z-VAD-FMK at 50 μmol/L partially blocked CRP-induced apoptosis (4.50 ± 0.64-fold increase of annexin V–FITC–positive cells versus 2.77 ± 0.35; P < 0.05), whereas 200 μmol/L completely abolished CRP-induced cell death. Furthermore, CRP treatment substantially increased VSMC caspase-3 activity compared with unstimulated cells (Figure 3B). Taken together, these data indicate that CRP induces coronary VSMC apoptosis through a caspase-dependent mechanism.

**DNA Microarray Identifies GADD153 as a CRP-Regulated Gene**

A biased gene array, containing 96 genes implicated in cellular stress or death and 12 housekeeping genes, was used to identify CRP-regulated genes in human VSMCs. Growing cells were cultured in the presence of 20 μg/mL CRP, and RNA was isolated after 48 hours and subjected to DNA array analysis. As shown in Figure 4, CRP at 20 μg/mL resulted in a substantial upregulation of GADD153 mRNA expression compared with untreated cells (5.71 ± 0.32-fold; P < 0.05).

**GADD153 mRNA Level Is Increased by CRP**

GADD153 is prominently expressed after treatment of cells with a variety of growth arrest and/or DNA-damaging factors and has been shown to directly mediate apoptosis. Northern blot analysis revealed that basal expression of GADD153 in exponentially growing cells was low but substantially increased after treatment with CRP (20 μg/mL), reaching maximum levels after 48 hours (Figure 5A). These results are consistent with the GADD153 mRNA upregulation observed by gene array. Data presented in Figure 5B demonstrate a dose-dependent increase of GADD153 mRNA levels after treatment with CRP, reaching a maximum at 20 μg/mL (5.1 ± 0.65-fold increase; P < 0.05). These data demonstrate that induction of apoptosis in VSMCs by CRP is accompanied by a prominent increase in the expression of GADD153, a member of a family of genes linked to several proapoptotic pathways.

**CRP Induces GADD153 mRNA Stabilization**

Depending on the cell type and specific mechanism of induction, mRNA levels of GADD153 are regulated either through an induction of GADD153 promoter activity, through enhanced mRNA stabilization, or through a combination of
both mechanisms. To determine the mechanism by which CRP regulates GADD153 mRNA expression, VSMCs were transiently transfected with a human GADD153 promoter construct containing 900 bp of a 5'-flanking DNA inserted upstream of a luciferase reporter gene. Exposure to CRP (1 to 20 μg/mL) for 12 to 48 hours did not significantly increase GADD153 promoter activity, thus indicating that CRP increases GADD153 mRNA expression at the posttranscriptional level (data not shown). To directly demonstrate a posttranscriptional mechanism of GADD153 regulation by CRP, we next investigated its effect on GADD153 mRNA stability. The decline of GADD153 mRNA levels in CRP-treated (20 μg/mL, 48 hours) and untreated cells was examined after the addition of the transcription inhibitor actinomycin (5 μg/mL). RNA was extracted at subsequent time points from each group, and GADD153 mRNA levels were normalized to those of the housekeeping gene GAPDH. As shown in Figure 6, the half-life of GADD153 mRNA was longer in CRP-stimulated cells than in untreated cells (354 versus 59 minutes, respectively). Together, these results demonstrate that CRP regulation of GADD153 mRNA expression occurs mainly at the posttranscriptional level by stabilizing mRNA turnover.

siRNA Reduces CRP-Induced GADD153 mRNA Expression and Apoptosis

To establish a functional role for GADD153 in CRP-mediated apoptosis of human VSMCs, siRNA was used to inhibit GADD153 expression. A nonsilencing RNA duplex was used as a negative control. As shown in Figure 7A, GADD153-specific siRNA attenuated CRP-induced (20 μg/mL, 48 hours) GADD153 mRNA expression by 46.7%, whereas nonsilencing siRNA had no effect. GADD153

Figure 4. CRP induces GADD153 mRNA expression in human VSMCs. Cells were treated with CRP (20 μg/mL) and harvested after 48 hours. Total RNA was isolated, reverse transcribed into single-strand cDNA, and labeled with biotin-16-dUTP. After hybridization with cDNA array membranes containing genes indicative for stress and toxicity, chemiluminescence was visualized by autoradiography. GADD153 is indicated in the boxed area. The autoradiogram depicted is representative of 3 separate experiments.

Figure 5. CRP induces GADD153 mRNA expression in human VSMCs. Cells were cultured in the presence of 5% FBS and growth factors and treated with 20 μg/mL CRP for the indicated time points (A) or with the indicated CRP concentrations (B) for 48 hours. Total RNA was isolated, and level of GADD153 mRNA was detected by Northern hybridization. The results are representative of 3 independently performed experiments. *P<0.05 vs untreated control (Con); data are expressed as mean±SEM.
siRNA (100 nmol/L) reduced CRP-induced cell death by 36.4% compared with cells transfected with nonsilencing siRNA (100 nmol/L), whereas siRNA or nonsilencing siRNA alone had no effect on cell apoptosis (Figure 7B). Taken together, these data demonstrate a causal role for GADD153 gene expression in CRP-induced cell death.

**CRP and GADD153 Colocalization in Atherosclerotic Lesions**

As previously reported, in early atherosclerotic lesions CRP is localized in the deep intima adjacent to the media (Figure 8A). In advanced atherosclerotic lesions we also found CRP deposition in the shoulder region of the plaque (Figure 8A). Staining of serial sections with α-smooth muscle actin antibody revealed that CRP-positive cells represented VSMCs of the neointima, as well as macrophages and foam cells. Double-immunofluorescence staining of serial sections demonstrated the presence of DNA fragmentation (TUNEL-positive cells) in areas containing VSMCs and CRP, which colocalized in cells immunopositive for GADD153. Figure 8B shows a representative example of GADD153 staining of TUNEL-positive apoptotic VSMCs. CRP or GADD153-specific staining could not be detected in normal coronary sections. Serial sections treated with secondary antibody alone yielded negative results (data not shown). The colocalization of TUNEL-positive cells with GADD153 immunoreactivity in human atherosclerotic lesions further supports a functional role for GADD153 in CRP-induced apoptosis in vivo.

**Discussion**

The present study demonstrates that CRP induces changes in cultured human coronary VSMCs characteristic of apoptosis: cell rounding, detachment from the culture plate, increased annexin binding, and DNA fragmentation. The induction of apoptosis was accompanied by a prominent induction of GADD153 mRNA expression, regulated at a posttranscriptional level. SiRNA targeting GADD153 partially blocked CRP-induced apoptosis, suggesting a causal role for GADD153 in programmed VSMC death. In human atherosclerotic lesions, CRP was expressed in the vicinity of VSMCs expressing GADD153 and undergoing apoptosis. These results suggest that CRP accumulating in atherosclerotic lesions might induce VSMC apoptosis via GADD153 gene expression. Thus, presence of CRP in the vessel wall may ultimately have important implications for the progression of atherosclerosis and stability of organized atherosclerotic plaques.

Inflammatory processes play a key role in the pathogenesis of atherosclerosis. Large epidemiological studies have shown that even modest increases in CRP serum levels are associated with a higher risk of future cardiovascular events in both apparently healthy individuals and patients with coronary heart disease. The principal source of circulating CRP is the hepatocyte, which synthesizes CRP under the transcriptional control of inflammatory cytokines, in particular interleukin-6. However, CRP itself has been detected in atherosclerotic lesions of human coronary arteries but was
not present in the normal vessel wall. Recent data suggest that CRP is also synthesized within atherosclerotic lesions by VSMCs and macrophages. Direct effects of CRP on vascular cells include the following: recruiting monocytes and lymphocytes; inducing expression of adhesion molecules on endothelial cells; participating in foam cell formation by mediating LDL uptake by macrophages; and inducing monocyte chemoattractant protein-1 production by endothelial cells. Thus, besides being a marker for atherosclerosis risk, CRP may play a direct role in the development of atherosclerotic lesions.

Plaque rupture and subsequent thrombosis are key events in the onset of coronary syndromes. Numerous studies have documented VSMC apoptosis in human atherosclerotic and restenotic lesions. The loss of cellular mass in the atheroma may contribute to a weakening of the intimal plaque texture and to a reduced deposition of extracellular matrix proteins, both increasing plaque vulnerability. This hypothesis is supported by the observation that VSMCs derived from atherosclerotic plaques show increased rates of apoptosis in culture compared with cells from normal coronary arteries. Furthermore, VSMC apoptosis is increased in unstable versus stable angina plaque tissue, supporting the involvement of VSMC cell death in lesion instability.

The formation of a lipid-rich necrotic core is an important hallmark of the transition of the fatty streak into an advanced atherosclerotic lesion. Previous studies have associated both macrophage and VSMC cell death with the formation and progression of the necrotic core and conversion from a hypercellular lesion to a more cytopenic fibrotic atheroma.
Inefficient removal of apoptotic cells from the plaque could be the main source of calcifying matrix vesicles and may accelerate atherosclerosis by inducing inflammatory responses. In the present study CRP was present in early atherosclerotic lesions and fibrous cap atheroma containing GADD153 expressing VSMCs undergoing apoptosis. These findings suggest that CRP in vessel walls may directly contribute to the progression of atherosclerosis and increased plaque vulnerability. This hypothesis is supported by the findings of Kobayashi et al., who demonstrated an association of CRP expression in coronary arteries with histological and clinical features of plaque instability. Moreover, in CRP overexpressing apolipoprotein E knockout mice, lesion formation was markedly accelerated.

Previous large prospective studies have shown that high sensitive CRP serum levels >3 μg/mL are associated with an increased risk of cardiac events in patients with stable or unstable angina. In our study we used CRP doses between 0.1 and 20 μg/mL. It remains controversial whether increased CRP levels simply reflect the intrinsic inflammation and tissue damage within the arterial lesion or whether they directly promote plaque instability and contribute to progression of atherosclerosis. Although our data demonstrate a proapoptotic effect of CRP, other studies have shown that it can stimulate VSMC proliferation through an AT1 receptor–dependent pathway. Because VSMC proliferation and apoptosis coexist in atherosclerotic lesions, the overall effect of CRP on VSMC content in atherosclerotic lesions therefore likely results from the ultimate balance between these processes.

GADD153 is a member of a large family of transcription factors known as bZIP (basic region leucine zipper) proteins. GADD153 dimerizes with and inhibits the binding of C/EBP-related transcription factors to their consensus DNA target sequence. Beside induction of gene transcription, GADD153-C/EBP heterodimers have been shown to recognize novel DNA target sequences, thereby activating gene transcription. GADD153, highly expressed after treatment of cells with a variety of growth arrest and/or DNA-damaging factors, has been implicated in regulating cell growth and apoptosis in different cell types. A direct relationship between GADD153 gene expression and apoptosis was demonstrated by studies of GADD153−/− mice in which cultured fibroblasts are more resistant to apoptosis compared with those of wild-type animals. GADD153 protein expression was also highly induced in apoptotic VSMCs observed in neointimal lesions of balloon-injured carotid artery. In combination, these data suggest a potentially important role for GADD153 in CRP-induced apoptosis of VSMCs.

Induction of apoptosis in human VSMCs by CRP is accompanied by an increase in GADD153 mRNA expression. SiRNA specifically targeting GADD153 partially blocked CRP-induced cell death, indicating a functional role of GADD153 in CRP-mediated apoptosis. The mechanism(s) linking upregulation of GADD153 expression to downstream proapoptotic signaling events, however, remains to be elucidated. The pathway by which CRP stabilizes GADD153 mRNA also remains to be determined.

In summary, data presented in this study demonstrate that CRP induces caspase-mediated apoptosis in cultured human VSMCs, coupled to an increase of GADD153 mRNA expression. SiRNA specifically targeting GADD153 reduced CRP-induced cell death, suggesting that GADD153 may be a novel molecular mediator of CRP-induced VSMC apoptosis. In atherosclerotic lesions, GADD153 colocalized with TUNEL-positive VSMCs. These findings further support the emerging hypothesis that CRP may play a direct role in the development of atherosclerosis and its thrombotic complications and constitutes a novel target for the treatment of atherosclerosis.

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