Inflammatory Cytokines Stimulated C-Reactive Protein Production by Human Coronary Artery Smooth Muscle Cells

Paolo Calabró, MD; James T. Willerson, MD; Edward T.H. Yeh, MD

**Background**—Serum C-reactive protein (CRP) levels are good predictors of the development of cardiovascular events in apparently healthy men and women. CRP has been believed to be produced exclusively by hepatocytes during the acute-phase response. Several lines of evidence have suggested that atherosclerotic arteries can also produce CRP. However, the cell types that produce CRP locally in the atherosclerotic arterial wall have not been clearly identified.

**Methods and Results**—Human coronary artery smooth muscle cells (HCASMCs) and human umbilical vein endothelial cells (HUVECs) were incubated with interleukin-1β (IL-1β), IL-6, their combination, tumor necrosis factor-α (TNF-α), or lipopolysaccharide (LPS) at different concentrations. The supernatants were concentrated and analyzed by a high-sensitivity enzyme-linked immunosorbent assay specific for human CRP. RNA was extracted from the HCASMCs for reverse transcriptase–polymerase chain reaction (RT-PCR) using specific primers for the CRP. Maximal CRP production was observed in HCASMCs after 48 hours of incubation with the combination of 25 ng/mL of IL-1β and 10 ng/mL of IL-6, whereas incubation with IL-1β or IL-6 alone only modestly induced CRP. Incubation with TNF-α (50 ng/mL) or LPS (1000 EU/mL) resulted in an increase in CRP production comparable to the IL-1β and IL-6 combination. The induction of CRP in HCASMCs was independently confirmed by RT-PCR comparing the relative CRP mRNA levels. The induction of CRP production by HCASMCs was not reproduced in HUVECs, however.

**Conclusions**—These results demonstrated that HCASMCs, but not HUVECs, could produce CRP in response to inflammatory cytokines. The locally produced CRP could directly participate in atherogenesis and the development of cardiovascular complications. (*Circulation*. 2003;108:1930-1932.)

**Key Words:** atherosclerosis • inflammation • muscle, smooth • interleukins • risk factors

C-reactive protein (CRP), a marker of inflammation, is an important predictor of future cardiovascular events in apparently healthy men and women and could directly participate in the pathogenesis of atherosclerosis through activation of endothelial cells. CRP, named for its capacity to bind to the C-polysaccharide of *Streptococcus pneumoniae*, was the first acute-phase protein to be described. CRP, like other acute-phase proteins, is synthesized by the liver in response to microbial infection, tissue injury, and autoimmune disorders. It had been shown that interleukin-1β (IL-1β) and IL-6 strongly induced the expression of CRP in human hepatocytes and hepatoma cells. Recently, human neuronal cells were found to produce CRP in Alzheimer’s disease. In addition, renal cortical tubular epithelial cells were shown to produce CRP after inflammatory stimuli. Interestingly, CRP has also been found in human atherosclerotic plaques, which could be the result of indirect deposit from circulating cells or direct production by cells in the arterial wall. We show that human coronary artery smooth muscle cells (HCASMCs), but not human umbilical vein endothelial cells (HUVECs), can synthesize CRP after stimulation by inflammatory cytokines.

**Methods**

**Cell Culture**
HCASMCs, HUVECs, and endothelial cell supplements were purchased from Cascade Biologics; penicillin, streptomycin, medium 231, medium 199, and smooth muscle cell growth supplement were from Gibco BRL; and fetal bovine serum, human serum, heparin, and gelatin were obtained from Sigma. HCASMCs were plated onto 0.1% gelatin-coated culture dishes from Corning, Inc, and grown in 231 medium with growth supplement and 1% penicillin/streptomycin; HUVECs were plated onto 0.1% gelatin-coated culture dishes and grown in 199 medium with endothelial growth supplement, heparin, antibiotics, and 15% fetal bovine serum. Cells were used at passage 5 to 7.
CRP Assays
CRP level in the cell supernatant was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit specific for human CRP (Diagnostic System Laboratories) according to the manufacturer’s directions. The minimum detectable concentration of the assay was 1.6 ng/mL. All the experiments were performed in triplicate. Cells were cultured in 6-well plates until 80% to 90% confluent and were incubated for 48 hours with recombinant human IL-1β (R&D Systems) (25 ng/mL), recombinant human IL-6 (R&D Systems) (10 ng/mL), their combination, recombinant human tumor necrosis factor-α (TNF-α) (R&D Systems) (50 ng/mL), or lipopolysaccharide (LPS) derived from Escherichia coli O113:H10 (Associates of Cape Cod, Inc) (1000 EU/mL); the culture supernatants were then concentrated (~10 times) using centrifugal filter units (Millipore) and assayed for CRP.

CRP mRNA Expression
Cells cultured in 60-mm plates were incubated for 48 hours with 25 ng/mL IL-1β, 10 ng/mL IL-6, their combination, 50 ng/mL TNF-α, and 1000 EU/mL LPS, and total cellular RNA was extracted by Trizol reagent. Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed with the Access RT-PCR System (Promega) according to the manufacturer’s directions. For each reaction, 1 μg of total RNA served as a template. For amplification, a primer pair specific for human CRP (forward, TCGTATGCCACCCAGAGACA; reverse AAACCTTCGGCCTTGCACTTCAT ACT; GenBank accession No. M11725) was used. These primers were designed to yield a product of 440 bp after 40 amplification cycles. In all experiments, control reactions were performed substituting sterile nuclease-free water for the RNA template in the reaction. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was amplified as a reference for quantification of CRP mRNA. The RT-PCR products were visualized on 1% agarose gel with ethidium bromide.

Results
CRP Production by HCASMCs, but Not by HUVECs
The results of CRP released into the media and the CRP mRNA levels in HCASMCs after treatment with inflammatory cytokines were shown in Figures 1 and 2, respectively. As shown in Figure 1A, CRP production was minimal without stimulation, and incubation of HCASMCs with 50 ng/mL of IL-1β or 10 ng/mL of IL-6 alone led to a small but significant induction. Maximal CRP production was observed after the combination of the 2 cytokines (Figure 1B). TNF-α or LPS also induced a similar level of CRP production and showed a dose-responsive relationship (Figure 1C). In contrast, CRP production could not be detected in HUVECs after similar stimulation protocols (data not shown). To confirm the results of CRP protein production in HCASMCs, we also assayed the mRNA levels in HCASMCs by RT-PCR. Figure 2 shows CRP mRNA levels in HCASMCs after the different treatments. IL-1β plus IL-6 combination caused a significant increase in CRP mRNA level compared with baseline. Treatment with LPS and TNF-α also upregulates the CRP mRNA levels. The RT-PCR amplified band was confirmed to be authentic CRP by direct sequencing.

Discussion
CRP has been shown to be an excellent predictor of future cardiovascular events in apparently healthy men and women.\(^3,4\) This could be in part the result of some of the biological properties of CRP such as its stability, lack of diurnal variation, and lack of influence of gender and age.\(^10\) However, accumulating evidence also points to the possibility that CRP is a direct participant in vascular inflammation.\(^16\) One of the outstanding unresolved issues in this field is the source of CRP production in humans. It has been previously assumed that hepatocytes are the only source of CRP production during the acute-phase response. During the acute-phase reaction, serum CRP levels often increase up to 100 or 200 μg/mL; however, the level of serum CRP that is useful for predicting cardiovascular risk is 1 to 3 μg/mL. In fact, patients with serum CRP levels >10 μg/mL should have the test repeated at a late date to exclude infection, autoimmune diseases, or malignancy.\(^17\) Thus, we sought to identify another source of CRP production that could help explain the lower level of CRP useful for cardiovascular risk prediction.

We show that CRP is produced by HCASMCs, but not by HUVECs, after exposure to inflammatory cytokines. This locally produced CRP could play an important role in the activation of endothelial cells.\(^5,9\) Two studies have shown that both epithelial cells of the respiratory tract and renal epithelium produce CRP.\(^14,18\) Moreover, neuronal cells also
CRP seem to be capable of synthesizing acute-phase reactants involved in the pathogenesis of neurodegenerative disease. These studies expand the variety of cell types that could participate in CRP production; however, relevance of these observations to the pathogenesis of atherosclerosis is doubtful. CRP has been observed to colocalize with the terminal complement complex in atherosclerotic plaques. In this report, however, the authors suggested that CRP is deposited into atherosclerotic plaques by the liver instead of local synthesis. In contrast, work by Yasojima et al. suggested that cells in the arterial wall synthesize CRP. Using in situ hybridization techniques, the authors showed that elongated muscle-like cells inside the atherosclerotic plaque were positive for CRP. Our findings, thus, provide a direct demonstration that HCASMCs, but not HUVECs, are a source of locally produced CRP in the arterial wall. The locally produced CRP can directly participate in atherogenesis and the development of cardiovascular complications.

Isolated human hepatocytes can produce 10 times more CRP compared with control after stimulation. Human hepatoma cell line HepG2 is able to produce CRP 4-fold compared with control in conditions similar to those used in our experiments. Another hepatoma cell line Hep3B stimulated with conditioned medium (generated by stimulating peripheral blood mononuclear cell with LPS at a dose of 1 µg/mL for 24 hours) showed an approximately 50-fold increase in CRP mRNA compared with unstimulated cells. Thus, CRP productions by human coronary artery smooth muscle cells is less robust than those produced by the liver. This could partly account for the lower serum level of CRP (1 to 3 mg/L) useful for cardiovascular risk prediction and the high level of CRP (>10 mg/L) observed during the acute-phase response.

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References

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