Interaction of Oxidative Stress and Inflammatory Response in Coronary Plaque Instability
Important Role of C-Reactive Protein

Seiichi Kobayashi, Nobutaka Inoue, Yoshitaka Ohashi, Mitsuyoshi Terashima, Kiyoko Matsui, Takao Mori, Hideki Fujita, Kojiro Awano, Katsuya Kobayashi, Hiroshi Azumi, Junya Ejiri, Ken-ichi Hirata, Seinosuke Kawashima, Yoshitake Hayashi, Hiroshi Yokozaki, Hiroshi Itoh, Mitsuhiro Yokoyama

Objective—C-reactive protein (CRP), a predictor of cardiovascular events, localizes in atherosclerotic arteries and exerts proinflammatory effects on vascular cells. Reactive oxygen species (ROS) have been implicated in atherogenesis and plaque instability.

Methods and Results—Expressional pattern of CRP in directional coronary atherectomy specimens from 39 patients was examined. Characteristics of histological plaque instability and higher levels of serum CRP and fibrinogen were associated with the CRP immunoreactivity. In situ hybridization revealed the presence of CRP mRNA in coronary vasculature. Furthermore, the expression of CRP mRNA and protein was detected in cultured human coronary artery smooth muscle cells (CASMCS) by reverse transcriptase–polymerase chain reaction and Western blotting. In addition, CRP was frequently colocalized with p22phox, an essential component of NADH/NAPDH oxidase, which is an important source of ROS in vasculature. Moreover, the incubation of cultured CASMCSs with CRP resulted in the enhanced p22phox mRNA expression and in the generation of intracellular ROS.

Conclusions—The expression of CRP in coronary arteries was associated with histological and clinical features of vulnerable plaque, and it had a prooxidative effect on cultured CASMCSs, suggesting that it might play a crucial role in plaque instability and in the pathogenesis of acute coronary syndrome via its prooxidative effect. (Arterioscler Thromb Vasc Biol. 2003;23:1111–1117.)

Key Words: C-reactive protein † inflammation † oxidative stress † free radicals † coronary artery diseases

Atherosclerosis is a chronic inflammatory disease. This concept is supported by recent findings where systemic inflammatory markers such as C-reactive protein (CRP) and fibrinogen are regarded as strong predictors of cardiovascular complications in various clinical settings.1–3 Fibrinogen, a key coagulation factor, is considered to contribute atherogenesis by promoting platelet aggregation, fibrin formation, and plasma viscosity.4 However, the role of CRP in the pathogenesis of atherosclerotic vascular diseases remains unknown. Recent histological investigations have demonstrated that CRP is present in the human arterial intima at atherosclerotic lesions and is frequently colocalized with the terminal complement complex.5 Moreover, in vitro studies have shown that the stimulation of human endothelial cells with CRP induces the expression of adhesion molecules and monocyte chemoattractant protein-1 (MCP-1).6,7 These data suggest that CRP might have direct proinflammatory effects on vascular cells which might, in part, explain the involvement of inflammation in atherogenesis.

Reactive oxygen species (ROS) have been implicated in the pathogenesis of a variety of vascular diseases, including atherosclerosis. To date, many types of cells in vasculature have been shown to generate ROS. There are various potential sources that generate ROS in vascular cells: the mitochondrial electron transport chain, cyclooxygenase, lipoxygenase, xanthine oxidase, and NADH/NADPH oxidase.8–9 Recent evidences suggest that among them, NADH/NADPH oxidase plays a crucial role in the generation of ROS in vascular cells.10,11 This oxidase system was originally regarded as a defense against exogenous microorganisms in phagocytes. Phagocytic NADH/NADPH oxidase is composed of at least 6 components: plasma membrane spanning cytochrome b558 composed of gp91phox and p22phox, the 3 cytosolic components p67phox, p47phox, p40phox, and the small G protein rac.12,13 Although intense investigations have been conducted to identify vascular NADH/NADPH oxidase, its molecular characterization remains unclear. We have previ
ously reported that p22phox is expressed in not only inflammatory cells but also smooth muscle cells (SMCs), endothelial cells, and adventitial fibroblasts in atherosclerotic coronary arteries and that its expression increases with the progression of coronary atherosclerosis, indicating that p22phox is a common component between phagocytic and vascular NADH/NADPH oxidases and that the vascular oxidase system plays a crucial role in pathogenesis of coronary atherosclerotic disease.14

Inflammation is associated with an abnormality in the redox state in the vasculature. Various inflammatory cells such as macrophages and lymphocytes have a potency to generate ROS.15 The enhanced oxidative stress in inflammatory response might thus contribute to the pathogenesis of atherosclerosis. Systemic response to inflammation results in an increase in oxidized lipids in serum and in the enhancement of the oxidative modification of low-density lipoprotein.16 Given the significance of CRP in cardiovascular disease, it might be a key molecule that links inflammation to atherogenesis. To examine the role of CRP in the pathogenesis of atherosclerotic coronary diseases, we investigated its expression in atherosclerotic plaque obtained from directional coronary atherectomy (DCA) and, furthermore, examined its effects on p22phox and on the production of H2O2 in coronary artery SMCs (CASMCs).

Methods

Patients

This study was approved by the hospital ethical committee, and informed consent was obtained from all patients. Thirty-nine consecutive directional coronary atherectomy procedures were performed at Kobe University Hospital and Miki City Hospital. Clinical profiles are shown in the online Table (available at http://atvb.ahajournals.org).

Immunohistological Analysis

Immunohistochemistry and double-labeling immunofluorescence were carried out as described.14 The primary antibodies were rabbit polyclonal anti-human CRP antibody (DAKO), mouse monoclonal anti-human CD68 antibody (clone KP-1, DAKO), mouse monoclonal polyclonal anti-human CRP antibody (DAKO), sheep polyclonal anti-human CRP antibody (Wako), and rabbit polyclonal anti-human p22phox antibody against the synthetic peptide corresponding to its C-terminal region (residues 175 to 194). The total area of each section as well as the surface area occupied by CRP was outlined on computer-aided planimetry with image analysis software (Macscope, Mitani Co).

Cell Culture

Human CASMCs obtained from Clonetics were cultured with medium (Clonetics) supplemented with 10% FBS (Trace Scientific) and the manufacturer’s reagents (Clonetics). Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection. HUVECs were cultured to confluency in RPMI1640 medium supplemented with 20% FCS, 100 IU/mL heparin, 100 IU/mL endothelial cell growth supplement, 100 U/mL penicillin, and 100 μg/mL streptomycin. The confluent monolayers were incubated in serum-free medium for 24 hours and then incubated with or without human CRP (Sigma) for 24 hours.

Western Blot Analysis

Cell lysate (60 μg of protein) was separated on a 15% SDS acrylamide electrophoresis and then transferred onto a nitrocellulose membrane. The membrane was treated with rabbit anti-human p22phox antibody or anti-human CRP antibody and then with horseradish peroxidase–conjugated goat anti-rabbit IgG (Amersham). The signal was detected with a chemiluminescence kit (Immunostar, Wako).

Reverse Transcription–Polymerase Chain Reaction and Sequencing Reaction

The RT reaction was carried out with 4 μg of total RNA isolated from cultured CASMCs and HUVECs. The reverse transcription (RT) product of 1 μL was used as a template for polymerase chain reaction (PCR) amplification. PCR amplification was conducted for 1 minute at 94°C, 3 minutes at 61°C, and 3 minutes at 72°C. Amplified DNA was separated on 1% agarose gel with ethidium bromide and visualized under UV. The sets of primers used were as follows: 5‘-TCGTATGGCACAAGAGACAAAGACAC-3‘ (forward) and 5‘-AAACCTTGGCCTTGGCACTTCACT-3‘ (reverse). Purified PCR products were cloned into TA cloning vector (Invitrogen). The sequencing procedure was performed with an automated sequencer.

In Situ Hybridization

Gitoxigenin (DIG)-labeled 440-kb RNA probes were transcript from RT-PCR products amplified from CRP cDNA using a DIG RNA Labeling Kit (Boehringer-Mannheim) as described before.17 In situ hybridization was performed on frozen DCA specimens by using ISHR starting kit (Nippon Gene) following the protocol recommended by the manufacturer.18 The hybridized probes were detected using the component of the DIG nucleic acid detection kit (Roche Diagnostics) according to the manufacturer’s protocol.19

Detection of Intracellular ROS

Intracellular ROS was detected with 2′,7′-dichlorodihydrofluorescein diacetate (HDFDA, Molecular Probes). Briefly, confluent monolayer CASMCs were treated with H2DCFDA (10 μmol/L) for 30 minutes at 37°C in the dark. The fluorescent intensity was measured by a laser-scanning confocal imaging system (MRC-1024, BioRad Laboratories).

Quantification and Statistical Analysis

Data are expressed as mean±SD. Difference between 2 groups was analyzed by Student’s t test. Statistical comparison for >3 groups was analyzed by 1-way ANOVA and post hoc multiple comparison using Bonferroni test. Values of P<0.05 were considered significant.

Results

Histological Definitions of DCA Specimens

All atherectomy specimens were classified to 4 categories by H&E stain, as follows: (1) atheromatous tissue (n=34), (2) collagen-rich tissue (n=18), (3) neointimal hyperplasia (n=9), and (4) thrombotic tissue (n=11), as previously described.21 Lesions classified as atheromatous tissue contained fibrous connective tissue with or without inflammatory cells, cholesterol clefts, necrotic debris, and lipid cores (Figure 1A). Collagen-rich tissues consisted of predominantly collagenous component, contained SMCs (Figure 1B). Lesions classified as neointimal hyperplasia were defined as having proliferation of stellate to spindle-shaped cells in a loose myxoid stroma (Figure 1C). Thrombotic tissues were composed of fibrin deposition, plaque hemorrhage, or both with or without inflammatory cells (Figure 1D).

Immunoreactivity of CRP in DCA Specimens

The immunoreactivity of CRP was observed in all DCA specimens tested. To clarify the types of CRP-positive cells, double immunofluorescence of CRP and cell-specific mark-
ers was carried out. Infiltrating CD68-positive macrophages expressed intense CRP (Figures 2A through 2D). Vascular SMCs stained with α-actin expressed CRP protein (Figure 2E through 2H).

In atheromatous tissues of DCA specimens, the deposition of CRP was intensively observed (Figure 3A). In collagen-rich tissues, the CRP staining was observed only in a few SMCs in collagen tissue (Figure 3B). In neointimal hyperplasia, CRP expression was observed only in spindle-shaped cells (Figure 3C). In case of thrombotic tissues, its immunoreactivity was observed in inflammatory cells (Figure 3D). As negative control, there was no immunoreactivity when the primary antibody was omitted during staining procedure.

**Figure 1.** Light microscopic images of histological features of DCA specimens. All atherectomy specimens were classified into 4 categories, as follows: A, atheromatous tissue \((n=34)\), which contained fibrous connective tissue with or without inflammatory cells, cholesterol clefts, necrotic debris, or lipid cores; B, collagen-rich tissue \((n=18)\), which contained predominantly collagenous component, contained SMCs; C, neointimal hyperplasia \((n=9)\), which contained proliferation of stellate to spindle-shaped cells in a loose myxoid stroma; and D, thrombotic tissue \((n=11)\), which contained fibrin deposition, plaque hemorrhage, or both with or without inflammatory cells. H&E stain, scale bar=10 μm.

**Figure 2.** Double labeling immunofluorescence of CRP and cell markers. H&E stains show infiltrating inflammatory cells (A) and spindle-shaped cells (E) in DCA specimens. Green fluorescence signals show cell markers, anti-CD68 antibody for macrophages (B), and anti α-actin antibody for SMCs (F). Red fluorescence signals show the immunoreactivity of CRP (C and G). Colocalization of cell markers and CRP signals are shown by yellow fluorescence (D and H). Results are representative of 10 experiments (scale bar=10 μm).

**Semiquantitative Analysis of CRP in DCA Specimens**

The intensity of CRP expression was semiquantitatively analyzed according to the histological classification. The
immunoreactivity for CRP was pronounced in atheromatous and thrombotic tissues, and its immunopositivity rate of atheromatous tissue was significantly higher than that of collagenous tissue and neointimal hyperplasia (Figure 4A).

Next, the expression of CRP in coronary specimens was analyzed according to clinical profiles. The patients were divided into 2 groups according to serum level of CRP, ie, patients with or without elevated CRP level. CRP values >0.3 mg/dL were regarded as elevated. This limit was chosen because it is the upper level of normal in routine testing and previous investigations demonstrated that the level of CRP >0.3 mg/dL was associated with risk of coronary events.23,24 No significant differences by age, sex, or incidence of risk factors were observed between the 2 groups. As shown in Figure 4B, the immunoreactivity of coronary specimens from patients with elevated serum CRP level (high, n=17) was significantly higher than those without it (low, n=19). Thus, the level of serum CRP had influence on its immunoreactivity in coronary specimens. In addition, as shown in Figure 4C, significant correlation was observed between serum fibrinogen level and immunoreactivity of CRP in DCA specimens (R=0.58, P<0.01).

Expression of CRP in Vascular Cells

To examine whether vascular cells in coronary arteries generate CRP, in situ hybridization and RT-PCR were performed. In situ hybridization with the CRP antisense probe showed a significant staining in inflammatory cells as well as spindle-shaped cells in atheromatous lesion, whereas there was no signal using sense probe (Figures 5A through 5F). As shown in Figures 5G and 5H, CRP mRNA and protein were detected in cultured CASMCs and HUVECs. Sequencing of the CRP complementary DNA obtained from human CASMCs obtained by RT-PCR revealed identity to its sequence from hepatocytes (data not shown). Thus, the CRP

Figure 3. Immunostaining of CRP in DCA specimens. CRP immunopositivity was intensively observed in atheromatous tissue (A). In collagen-rich tissue, the CRP staining was observed only in SMCs contained in collagen tissue (B). In neointimal hyperplasia, CRP expression was observed only in spindle-shaped cells (C). In case of thrombotic tissues, CRP was observed in inflammatory cells (D). Scale bar=10 μm.

Figure 4. A, CRP immunopositivity rates in 4 types of tissue were compared semiquantitatively. AT indicates atheromatous tissues; CO, collagen-rich tissue; NE, neointimal hyperplasia; and TH, thrombotic tissue (*P<0.01). B, Relation between CRP-positive rates in DCA specimens and serum CRP levels. Patients were divided into 2 groups according to serum CRP concentration (cut point, 0.3 mg/dL). CRP immunopositivity of DCA specimens from patients with elevated serum CRP level (high, n=17) was significantly higher than those without it (low, n=19). C, Correlation between serum fibrinogen level and CRP-positive rates.
molecule expressed on vascular smooth cells is identical with the classical CRP expressed in the liver.

Colocalization of CRP and NADH/NADPH Oxidase p22<sub>phox</sub>
To examine the relation between CRP and oxidative stress, double staining of CRP and p22<sub>phox</sub> was carried out. Considerable overlapping of CRP with p22<sub>phox</sub> in mononuclear inflammatory cells as well as spindle-shaped smooth muscle-like cells was observed (Figure 6A).

CRP Increased p22<sub>phox</sub> Expression and ROS Production in Cultured Coronary Artery Smooth Muscle Cells
The direct effect of CRP on the expression of p22<sub>phox</sub> in CASMCs was examined by Western blotting. Incubation of CASMCs with CRP for 24 hours resulted in an increase in the expression of p22<sub>phox</sub> protein in a dose-dependent manner (Figure 6B). Furthermore, the experiments with H<sub>2</sub>DCFDA, an intracellular fluorescence probe, showed that the upregulation of CRP expression was accompanied by an increase in intracellular H<sub>2</sub>O<sub>2</sub> concentration (Figure 6C).
lated p22\textsubscript{phox} was associated with the production of intracellular ROS (Figure 6C).

**Discussion**

The present study demonstrated that the immunoreactivity of CRP was observed in DCA specimens, especially in atherosomatous and thrombotic tissue, suggesting that the histological characteristics had profound influences on the immunoreactivity of CRP. In addition, DCA specimens obtained from patients with higher serum levels of CRP or fibrinogen had more intensive immunoreactivity of CRP, suggesting that clinical markers of cardiac risk are correlated with deposition of CRP in the vascular bed. Taken together, histologically as well as clinical features of instability of coronary plaque are implicated in enhanced immunoreactivity of CRP in DCA specimen. Furthermore, the localization of CRP was closely associated with NADPH oxidase p22\textsubscript{phox}, and CRP directly enhanced the expression of NADPH oxidase p22\textsubscript{phox} protein in cultured vascular cells. These results suggest that CRP accumulating in coronary beds might enhance vascular oxidative stress via p22\textsubscript{phox}-based NADH/NADPH oxidase. Thus, local as well as systemic inflammation might play important roles in plaque instability and coronary risk with increased oxidative stress in the vascular bed.

CRP, an acute-phase protein, acts as a primitive defense against exogenous microorganisms. CRP binds phosphocholine on the surface of invading microorganisms and marks them for killing by activating complement and phagocytes.25,26 CRP also interacts with FcyR II receptors on phagocytic cells and acts as an opsonin.27 We found that CRP increased p22\textsubscript{phox} protein expression in cultured SMCs, and although their precise mechanism remains undetermined, there are several possible mechanisms that exert a direct effect on vascular SMCs. First, SMCs might have a specific receptor, like leukocytes, for CRP. Second, the observed effects of CRP might be mediated by its direct binding to cell membranes, because it has affinity for phosphocholine. Recently, it was shown that CRP directly activates the intracellular signaling, including ERK and PI-3 kinase, in several cell types.28,29 Additional investigation is needed to clarify the signal transduction responsible for induction of p22\textsubscript{phox} by CRP.

In coronary vasculature, a significant overlapping of CRP with p22\textsubscript{phox} was observed, although it was not complete. In our previous investigation using coronary arteries obtained from autopsied cases, synthetic type, but not contractile type, of SMCs preferentially expressed p22\textsubscript{phox}.14 The characteristics of CRP-expressing SMCs are still unknown; however, their phenotypic changes may be important for expression of CRP. In the present study, there was overlapping of CRP with \(\alpha\)-actin or CD68. RT-PCR and Western blotting showed the expression of CRP in cultured SMCs and HUVECs. However, we failed to show the overlapping of CRP with endothelial makers. During the procedure of percutaneous coronary arterectomy, large amounts of vascular endothelial cells were likely lost or damaged.

Although the major source of CRP is hepatocytes, other extrahepatic sources are reported; neurons in the brain produced CRP, and human arterial tissue is capable of generating CRP.17,30 In the present study, DCA specimens obtained from patients whose serum CRP levels were more than 0.3 mg/dL had more CRP immunoreactivity. From these findings, it is speculated that CRP generated in the liver may deposit in the vasculature, and macrophages and vascular cells might uptake it. In our investigation, however, in situ hybridization revealed that CRP mRNA was detected. In addition, the results of RT-PCR showed that cultured SMCs expressed CRP mRNA. Thus, we speculate that the CRP molecules expressed are originated from both the circulating blood and vascular cells themselves. The sequence of CRP expressed in cultured SMCs was identical with that expressed in the liver. Thus, coronary CRP is identical with classical CRP expressed in the liver. However, a proportion of liver-derived and vascular cell-derived CRP is not determined in the present study. Also, additional investigations are necessary to determine what cell types preferentially generate CRP mRNA.

Inflammation plays an important role in the pathogenesis of atherosclerosis and plaque instability. Inflammatory cytokines could mediate plaque instability by various mechanisms, including expression of matrix metalloproteinases, induction of apoptosis in SMCs, and activation of prothrombotic properties.31–33 Clinical investigations support the concept that inflammatory processes are involved in pathogenesis of acute coronary syndrome. For example, prospective epidemiological studies indicate that high levels of serum CRP are associated with increased risk of coronary events, and the association becomes stronger as CRP levels increase.34–36 Furthermore, statins, which have been approved to reduce coronary risk, are reported to decrease the serum level of CRP.34,35 Recent reports suggest that CRP might directly participate in various processes of atherosclerosis: CRP mediates low-density lipoprotein uptake in macrophages via CD32 and induces MCP-1 and adhesion molecules.6,7,36 In our investigation, CRP enhanced p22\textsubscript{phox} expression as well as ROS generation in cultured SMCs. These findings suggest that CRP in vessel walls is not just a bystander of inflammation but probably contributes directly to the pathogenesis of atherosclerosis.

Various coronary risk factors, including hyperlipidemia and diabetes, are associated with enhanced vascular ROS.37,38 Enhanced ROS activate redox-sensitive signal transduction pathways, which induce expression of atherogenic gene products such as adhesion molecules and other vascular proinflammatory gene products.39 Furthermore, generated ROS activate MMPs in cultured vascular cells.40 Thus, ROS play a pivotal role in not only atherogenesis but also plaque instability. In the present investigation, the expression of p22\textsubscript{phox} was colocalized with CRP in DCA specimens; furthermore, CRP directly enhanced the expression of p22\textsubscript{phox} and ROS generation in SMCs. These results suggest that the ROS derived from p22\textsubscript{phox}-based NADH/NADPH oxidase play an important role in the instability of atherosomatous plaque and that CRP itself may take part in the pathophysiology of acute coronary syndrome.

In conclusion, histological as well as clinical features of plaque instability are associated with expression of CRP in...
DCA specimens, and the expression of CRP was colocalized with p22phox. CRP directly induced p22phox expression and generated ROS in cultured CASMCs. Given the importance of prooxidative effects, CRP is likely a key molecule linking inflammation and oxidative stress in the pathogenesis of coronary artery disease, including acute coronary syndrome.

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References

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HT indicates hypertension; HL, hyperlipidemia; DM, diabetes mellitus; BMI, body mass index; LAD, left anterior descending coronary artery; RCA, right coronary artery; Cx, circumflex