C-Reactive-Protein-Associated Increase in Myocardial Infarct Size After Ischemia/Reperfusion

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ABSTRACT

C-Reactive protein (CRP), a marker for acute inflammation, is associated with increased risk of cardiovascular events. The mechanism underlying this association is uncertain. An acute inflammatory response was induced in rabbits by subcutaneous injection of croton oil (CO) 1 to 3 days before 30 min of regional myocardial ischemia/180 min of reperfusion. CO treatment increased plasma CRP from below the limit of detection to 2.5 ± 0.5 mg/dl and was associated with an increase in infarct size expressed as percentage of risk region [32 ± 6% vehicle controls (n = 7) to 47 ± 9% CO-treated rabbits (n = 7; P < 0.05)]. After 10 min of ischemia and 180 min reperfusion, no infarct was found in controls; however, an infarct of 7 ± 1% was found in CO-treated rabbits (P < 0.05; CRP, 2.3 ± 0.4 mg/dl). The CRP-related increase in infarct size was not observed in croton oil-treated, C6-deficient rabbits (n = 5/group), indicating the involvement of complement. In these rabbits, infarct size was 22 ± 2% (P < 0.05) despite having plasma CRP of 4.3 ± 0.4 mg/dl. The CRP-associated increase in infarct size was ameliorated by pretreatment with heparin (n = 7; infarct size 33 ± 3%; CRP, 2.3 ± 0.3 mg/dl; P < 0.05) or N-acetylheparin (n = 7; infarct size 23 ± 4%; CRP, 3.1 ± 0.5 mg/dl; P < 0.05). These observations may explain why increased serum CRP is associated with an augmented risk for cardiovascular events.

The pathophysiology of ischemic heart disease has an important inflammatory component (Yeh et al., 2001) in which an increased serum C-reactive protein concentration (CRP), commonly used as a marker for an acute inflammatory response, is associated with increased mortality due to cardiovascular events (Lagrand et al., 1999). This relationship holds true for asymptomatic individuals (Ridker et al., 2000) and patients with unstable angina (Lindahl et al., 2000) and acute myocardial infarction (Pietila et al., 1996).

It has been suggested that the epidemiological studies relating CRP to the incidence and outcome of ischemic syndromes are not simply due to CRP being a nonspecific marker of disease susceptibility or inflammation but rather that CRP might be involved directly in the pathogenesis of ischemic syndromes through a proinflammatory effect mediated by complement activation (Beranek, 1997). The primary evidence for this hypothesis is derived from studies of autopsy specimens showing CRP colocalized with activated complement components in infarcted myocardial tissue but not in normal myocardium (Lagrand et al., 1997; Yasojima et al., 1998b). CRP is also deposited in the ischemic rabbit myocardium (Kushner et al., 1963) and is closely correlated with the proportion of polymorphonuclear leukocytes in the inflammatory infiltrate (du Clos et al., 1981).

We sought evidence for a role for increased endogenous plasma CRP concentration in myocardial infarction in a rabbit model of ischemia/reperfusion injury. Studies assessed the effects of increased plasma CRP on infarct size after regional myocardial ischemia of different durations. Additional studies were carried out to determine whether the extent of myocardial injury could be altered through modulation of the complement cascade. We demonstrate that the endogenous increase in plasma CRP secondary to a remote inflammatory lesion is associated with an increase in myocardial tissue injury secondary to regional ischemia and reperfusion. The increase in myocardial injury is by a complement-dependent mechanism that can be ameliorated by pretreatment with heparin or N-acetylhheparin or prevented in rabbits deficient in C6 and incapable of forming the membrane attack complex.

ABBREVIATIONS: CRP, C-reactive protein; TTC, 2,3,5-triphenyltetrazolium chloride; C6, complement component 6; C5b-C9, membrane attack complex.
Materials and Methods

Guidelines for Animal Research. The procedures followed in this study were in accordance with the guidelines of the Internal Review Board of the University of Michigan Committee on the Use and Care of Animals and conforms to the standards in The Guide for the Care and Use of Laboratory Animals (NIH 86-23). Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine.

Inflammatory Stimulus. Male, New Zealand, white rabbits (2–3 kg) and C6-deficient, California, white rabbits (2.5–3.0 kg) were used for this study. An acute phase inflammatory response was induced by subcutaneous injection of four aliquots (0.5 ml each) of 1% croton oil (Sigma-Aldrich, St. Louis, MO) diluted in corn oil (Heuertz et al., 1993). The time course for the increase in plasma CRP was assessed in a pilot study. Plasma CRP was determined before and at selected intervals after subcutaneous injection of croton oil or vehicle control. Plasma CRP was measured by rate nephelometry (Beckman 360; Beckman Coulter, Inc., Fullerton, CA) using a goat anti-human CRP antibody (Beckman 449760; Beckman Coulter, Inc.) that cross-reacts with rabbit CRP. The limit of detection for the assay was 0.14 mg/dl.

Ischemia/Reperfusion Studies. Rabbits were pretreated with either croton oil or vehicle control 1 to 3 days before initiating the final phase of the experimental protocol. On the day of the experiment, rabbits were anesthetized with a mixture of xylazine (3 mg/kg) and ketamine (35 mg/kg), followed by pentobarbital (90 mg/kg) intramuscularly. Additional pentobarbital was administered as required to maintain anesthesia. After tracheotomy, rabbits were ventilated with room air, and the heart was exposed via a left thoracotomy. The heart was supported in a pericardial cradle and a 3-0 silk ligature was placed around the left circumflex coronary artery. The artery was occluded for 30 min by exerting traction on the ligature and subsequently reperfused for 180 min. After completing the protocol, a venous blood sample was obtained for determination of plasma CRP.

Determination of Infarct Size and Area at Risk. At the completion of the reperfusion phase of the protocol, the hearts were removed and cannulated by the aorta on the Langendorff perfusion apparatus. The left circumflex coronary artery was ligated in the same area as it was ligated during the surgical preparation. The hearts were perfused with a modified Krebs-Henseleit buffer for 5 min (20–25 ml/min). The perfusion pump was stopped, and 0.2 ml of an India ink colloidal suspension (KOH-I-NOOR; Rapidograph, Inc., Bloomsbury, NJ) was injected slowly into the hearts through a side-arm port connected to the aortic cannula. The colloidal suspension was allowed to distribute through the heart for 10 s. At the conclusion of this time, the perfusion pump was turned on (20 ml/min) to insure equal distribution of ink through the heart tissue. Presence of the colloidal suspension demarcates normal myocardium with a black color. After 1 min of perfusion, the heart was removed from the perfusion apparatus and submerged in modified Krebs-Henseleit buffer to remove excess ink from the hearts. The hearts were cut into six transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. Sections of the left ventricle were incubated in 0.4% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 10 min at 37°C. TTC demarcates the noninfarcted myocardium (area at risk) with a brick-red color indicating the presence of a formazan precipitate resulting from the reduction of TTC by dehydrogenase enzymes present in the viable myocardial tissue. Irreversibly injured tissue is unable to form the formazan precipitate and therefore appears as pale yellow in color. Tissue demarcated by a purple-black color represents the region perfused by the noninfarct-related coronary vascular distribution. Both surfaces of each transverse section were traced onto clear acetate sheets that were scanned with a Macintosh IICI computer interfaced with an Apple flatbed scanner and digitized using MacDraft (Macro Enter Corporation, Boca Raton, FL) to calculate infarct area. Total area at risk is expressed as the percentage of the left ventricle. Infarct size is expressed as percentage of the area at risk.

Determination of Threshold Duration of Ischemia for Myocardial Infarction. The duration of ischemia was varied in this series of experiments, which was otherwise carried out as described above. Ischemia was induced by occlusion of the left circumflex artery for 5 min (n = 3) or 10 min (n = 7) in rabbits treated with vehicle or croton oil. Sham occlusion experiments (n = 3) were included to assess the degree of TTC staining in naive hearts. In these experiments, infarct size was determined by perfusing the heart with 30 ml of 0.4% TTC solution. Using this approach, a small area of the myocardium (6 ± 1%) was found to be TTC negative. This background was subtracted from the other groups to determine the amount of TTC staining that could be attributed to the ischemic episode.

C6-Deficient Rabbits. California white rabbits genetically deficient in the C6 component of complement were used to assess the importance of the membrane attack complex (C5b-C9) in the CRP-associated increase in infarct size. After confirming complement deficiency (see below), rabbits were anesthetized, and the myocardial ischemia/reperfusion study was carried out as described above (30 min of ischemia/180 min of reperfusion).

Pharmacological Inhibition of the Complement Cascade. As demonstrated previously (Friedrichs et al., 1994; Black et al., 1995), heparin and N-acetylheparin suppress activation of the complement cascade. Therefore, we assessed the effects of these pharmacological interventions on infarct size 1 to 3 days after induction of an acute phase inflammatory response. Heparin (300 U/kg; Elkins-Sinn, Cherry Hill, NJ) or N-acetylheparin (2 mg/kg, Sigma-Aldrich) were administered intravenously 2 h before the ischemia/reperfusion protocol.

Statistics. Data are expressed as the mean ± S.E.M. Differences between groups were determined by analysis of variance followed by a Tukey test for differences. All tests were two tailed, and P < 0.05 was considered statistically significant.

Results

Time Course for Elevation of Plasma CRP. Subcutaneous injection of croton oil caused a local inflammatory lesion having the characteristics of a wheal containing large amounts of extravasated fluid. The development of the localized skin lesion was accompanied by an acute phase inflammatory response. Plasma CRP increased from below the limit of detection (0.14 mg/dl) to ~3.5 mg/dl 1 to 3 days after administration of croton oil (Fig. 1).

Inflammation-Induced Elevation in Plasma CRP Is Associated with Increased Myocardial Infarct Size. Subcutaneous injection of croton oil induced an acute inflammatory response that was associated with a major increase in infarct size after regional ischemia/reperfusion (Fig. 2A).
CRP was increased only in rabbits treated with croton oil. Correlation analysis revealed a significant relationship between plasma CRP and myocardial infarct size ($r = 0.55; P < 0.01$; Fig. 2B). The size of the risk region did not differ between groups (Fig. 2C). Thus, differences in this variable cannot explain the significant increase in infarct size in croton oil-treated rabbits compared with vehicle controls. The mean overall risk region for all rabbits included in the study was $42 \pm 1\%$ ($n = 65$).

Threshold Duration of Ischemia to Cause Infarction. We examined the possibility that rabbits undergoing an acute inflammatory response would develop an infarct after a short episode of myocardial ischemia that was not capable of inducing irreversible injury in vehicle controls. Evidence of myocardial infarction was not observed in normal rabbits subjected to a 5- or 10-min period of coronary artery occlusion followed by 180 min of reperfusion compared with sham-occluded rabbits (Fig. 3). In rabbits treated with croton oil (CRP, $2.3 \pm 0.4 \text{ mg/dl}$), a 10-min period of myocardial ischemia followed by 180 min of reperfusion resulted in the development of an infarct that averaged $7 \pm 1\%$ of the risk region ($P < 0.05$).

CRP-Associated Increase in Infarct Size Is Dependent on Complement. To determine whether the complement system was involved in the mechanism linking the acute inflammatory response with increased infarct size, rabbits genetically deficient in C6 were compared with normal rabbits. The presence or absence of an intact complement system was determined with the use of the red blood cell lysis assay before the ischemia/reperfusion study. Incubation of plasma from normal rabbits with antibody sensitized sheep erythrocytes resulted in lysis and release of hemoglobin. The concentration of plasma from normal rabbits required to produce 50% lysis ($EC_{50}$) was $2.5 \pm 1\%$, with maximal lysis occurring between 10 to 30% plasma. In contrast, plasma samples from the rabbits genetically deficient in C6 did not

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**Figure 1.** Time course for the increase in plasma CRP after subcutaneous injection of croton oil. Values represent the mean $\pm$ S.E.M. ($n = 3$).

**Figure 2.** Increased plasma CRP concentration is associated with increased myocardial infarct size after ischemia/reperfusion. A, croton oil treatment increases plasma CRP and infarct size after ischemia/reperfusion. Values are the mean $\pm$ S.E.M. ($n = 7$). The * indicates $P < 0.05$ from vehicle control. B, correlation between plasma CRP and infarct size ($r = 0.55; P < 0.01$). In vehicle controls, CRP was below the limit of detection ($<0.14 \text{ mg/dl}$). Data from Figs. 1 and 5A are included in the correlation. C, croton oil treatment increased infarct size independent of the size of the risk region.
lead to hemolysis (5 ± 1% lysis with 60% plasma), confirming that the plasma from the C6-deficient rabbits lacked the ability to generate the terminal membrane attack complex (C5b-C9).

C6-Deficient and C6-competent rabbits displayed similar increases in plasma CRP after the subcutaneous administration of croton oil. In C6-deficient rabbits, CRP was 4.3 ± 0.4 mg/dL, whereas in vehicle controls, CRP remained below the limit of detection (<0.14 mg/dL). In contrast to complement-sufficient rabbits with an acute inflammatory response, croton oil-treated C6-deficient rabbits had significantly smaller infarcts after ischemia/reperfusion (P < 0.05; Fig. 4). Infarct size in croton oil- and vehicle-treated rabbits was 23 ± 4 and 22 ± 2%, respectively (P < 0.05).

Pharmacological Inhibition of the Complement Cascade. Additional studies were performed to determine whether the increased infarct size associated with elevated plasma CRP could be suppressed by glycosaminoglycans such as heparin and the nonanticoagulant heparinoid N-acetylheparin. Both heparin and N-acetylheparin, administered intravenously 2 h before the induction of ischemia, ameliorated the increase in infarct size (Fig. 5). In croton oil-treated rabbits, infarct size was reduced from 43 ± 4% in vehicle controls to 31 ± 3 and 23 ± 4% in rabbits pretreated with either heparin or N-acetylheparin, respectively (P < 0.05). Plasma CRP was increased to a comparable degree in all groups (~2.9 mg/dL; Fig. 5).

Discussion

C-Reactive protein represents one of several acute phase reactants (Pepys and Baltz, 1983). The two major inducible acute phase proteins are CRP and serum amyloid A, which are normally present in plasma in only trace amounts and undergo rapid increases in rates of synthesis and plasma concentrations (Volanakis, 1982), attaining concentrations >1000 times normal in response to inflammatory insults (Westhuyzen and Healy, 2000).

Differences exist among species in the expression of acute phase proteins. CRP is prodigiously expressed in humans and rabbits but is minimally induced by inflammatory stimuli in mice, whereas in rats, it is constitutively expressed at high levels, with only a severalfold increase after stimulation (Kushner and Rzewnicki, 1999). Other positive acute phase proteins include members of the complement system, including factor B, C1 inhibitor, C4b-binding protein, and mannose-binding lectin (Kushner and Rzewnicki, 1999). Ligand-bound or aggregated CRP binds to C1q by which it initiates activation of the classical complement pathway (du Clos., 2000) by means of an antibody-independent mechanism. After removal of an acute insult, plasma concentrations of the acute phase reactants return to their normal values, with CRP having a short half-life of 5 to 7 h. CRP plasma concentra-
tions may remain increased indefinitely in the presence of chronic inflammatory conditions [e.g., atherosclerosis (Carr et al., 1997)]. CRP in the blood of rabbits during inflammation (Anderson and McCarty, 1951) is analogous and immunologically related to CRP in humans (Gotschlich and Stetson, 1960).

CRP is a sensitive, but nonspecific, marker of an inflammatory response that has clinical use in patients suspected of disturbances of the immune system. Plasma concentrations of CRP are increased in infective, inflammatory, and ischemic diseases (Gabay and Kushner, 1999). Increased plasma CRP is associated with increased mortality due to cardiovascular events (Liuzzo et al., 1994); however, the mechanism linking the two is not clear.

Hepatic synthesis of CRP occurs upon stimulation by inflammatory cytokines produced in response to tissue injury (Gabay and Kushner, 1999). CRP has multiple biological actions including calcium-dependent binding to phosphocholine, fibronectin, chromatin, histones, and ribonucleoprotein, induction of the expression of adhesion molecules on endothelial cells (Pasceri et al., 2000), binding to specific receptors on leukocytes resulting in modulation of function (Mortensen and Zhong, 2000), and activation of the classical complement pathway (Volanakis, 1982) by binding to C1q (Williams et al., 1978). Unlike IgG, which specifically recognizes distinct epitopes, CRP appears to recognize altered self and foreign molecules (du Clos, 2000). Thus, CRP may recognize the ischemic/reperfused myocardium as an altered self (Gabay and Kushner, 1999), thereby leading to activation of complement localized to the region of tissue injury.

Coronary artery occlusion results in myocardial ischemia, and if blood flow is not restored within a reasonable time, infarction results. Restoration of blood flow, however, can contribute to injury. Tissue damage as a result of reflow is termed reperfusion injury (Jolly et al., 1984). Experimental (Friedrichs et al., 1994; Mathey et al., 1994; Black et al., 1995) and clinical studies (Ross, 1999) suggest that ischemia/reperfusion injury is mediated, in part, by complement-dependent mechanisms. Although there is increasing evidence that complement participates in ischemia/reperfusion injury, it is not clear how complement is activated. Local activation of the classical pathway in infarcted myocardium occurs in animal models of acute myocardial infarction (Pinckard et al., 1975; Kilgore et al., 1994; Yasojima et al., 1998a). Complement activation occurs in ischemia with or without reperfusion. Experimental studies in rabbits show the presence of the membrane attack complex on cardiomyocytes and endothelial cells in ischemic areas (Kilgore et al., 1994; Mathey et al., 1994; Yasojima et al., 1998a). Myocardial ischemia without reperfusion resulted in a late deposition of C5b-9 in the infarcted areas (Mathey et al., 1994) in contrast with the rapid appearance of the macromolecular complex as soon as 30 min after onset of reperfusion (Kilgore et al., 1994; Mathey et al., 1994; Yasojima et al., 1998a). Thus, reperfusion of the ischemic myocardium in the presence of a pre-existing and sustained increase in plasma CRP concentration may provide a setting for an exaggerated increase in tissue injury when compared with reperfusion in the presence of a physiological plasma CRP concentration.

In the present study, the croton oil-induced inflammatory response increased the plasma CRP concentration and was associated with an increase in myocardial infarct size. Taken together with the observation that CRP can activate the complement cascade (Volanakis, 1982) provides support for the notion that the degree of complement-mediated injury is influenced by the presence of a circulating concentration of endogenous CRP. The results of this study cannot exclude the possibility that one or more acute phase reactants mediates the observed effect. Evidence for the involvement of CRP in this phenomenon was reported in a rat model involving permanent coronary artery occlusion (Griselli et al., 1999). The latter study demonstrated that the exogenous administration of human CRP to rats after coronary artery ligation resulted in CRP deposition in the myocardium and the subsequent increase in infarct size determined 5 days later. Involvement of complement was demonstrated by showing...
that cobra venom factor abrogated the effect. The present results agree with the previous findings. They differ, however, in that the increased plasma CRP was the result of a sustained inflammatory lesion due to the subcutaneous administration of croton oil. CRP is expressed constitutively in the rat, with only a severalfold increase after stimulation (Kushner and Rzewnicki, 1999), thus differing from humans and rabbits in which CRP is expressed in response to an appropriate stimulus (Kushner et al., 1978). In our study, the rabbit heart was subjected to regional ischemia for 30 min followed by reperfusion for 3 h. In a rabbit model of coronary artery occlusion (without reperfusion), CRP was first detected in the heart in the zone distal to the ligation at 4.5 to 5.0 h after ligation and was demonstrated only in the zone of infarcted tissue (Kushner et al., 1963). This led to the conclusion that CRP was derived either as a result of inflammatory or necrotic changes in cardiac myofibers or from secondary deposition from blood into myofibers with altered permeability. As reported previously (Mathey et al., 1994; Yasojima et al., 1998a), reperfusion results in the early activation of the complement cascade. Thus, in the presence of an increased plasma concentration of CRP, there is an exaggerated extension of tissue injury beyond that which would result from the ischemic insult and the subsequent restoration of blood flow. Attention must be given to the significance of tissue-derived CRP and complement proteins that lead to formation of the membrane attack complex as contributing to the extension of tissue injury upon reperfusion. Activation of complement in ischemic myocardium may occur via the alternative pathway (Gralinski et al., 1996), as well as the involvement of C1q and C4, and consequently, the classical pathway (Volanakis, 1982). CRP might mediate increased infarct size after ischemia/reperfusion of the myocardium by providing a proinflammatory stimulus that results in local activation of complement. CRP binds to various negatively charged molecules including phosphocholine (Pepys, 1981). Normally, phosphocholine is not exposed to the extracellular milieu, but may be exposed by ischemia/reperfusion injury (Van der Vusse et al., 1994). Binding of CRP to phosphocholine and subsequent binding of C1q to CRP would result in activation of the classical complement pathway (Volanakis, 1982). CRP binding would ultimately lead to activation of complement within the region of the myocardium at risk and result in increased damage after reperfusion (Yasojima et al., 1998a). The observation that the increase in plasma CRP correlated with an increase in infarct size supports this hypothesis.

Previous studies demonstrated that glycosaminoglycans reduce infarct size in experimental models of myocardial ischemia/reperfusion (Friedrichs et al., 1994; Black et al., 1995). We now show that heparin and the nonanticoagulant derivative N-acetylheparin reduced infarct size despite the presence of an acute phase inflammatory response. This, plus the observation that croton oil-treated rabbits genetically deficient in C6 had smaller infarcts than similarly treated C6-competent controls, is consistent with the concept that activation of complement mediates injury to the myocardium after ischemia/reperfusion. Thus, preventing complement activation in the presence of increased plasma CRP is beneficial in terms of reducing tissue injury after ischemia/reperfusion. It can be inferred from this study, as well as from others (Griselli et al., 1999), that agents that prevent CRP from binding to its ligands and activating complement offer an opportunity to protect the myocardium after an ischemic insult.

Myocardial infarction in humans provokes an acute phase response, and CRP is deposited together with complement within the infarct (Yasojima et al., 1998b). The peak plasma CRP concentration is associated with postinfarct morbidity and mortality. Human CRP binds to damaged cells and activates complement. The present results show that coronary artery occlusion/reperfusion resulted in an increase in infarct size in the presence of a sustained inflammatory insult and its associated increase in plasma CRP. Infarct size was reduced in C6-deficient rabbits despite the continued presence of an increased plasma CRP when compared with C6-competent rabbits, suggesting a role for the complement system in mediating myocardial reperfusion injury. In the presence of an increased plasma CRP concentration, pretreatment with heparin or N-acetylheparin annulled the detrimental actions associated with increased endogenous CRP plasma values. The observations are consistent with the notion that the presence of an increased plasma CRP, derived in response to a remote inflammatory lesion, and activation of the complement cascade contribute to myocardial ischemia/reperfusion injury.

The results of the present study suggest that the long held concept of CRP as simply a marker for underlying inflammation requires modification to include the possibility that CRP is a mediator and amplifier of injury secondary to ischemia/reperfusion. Pharmacological modulation of the plasma CRP concentration and/or temporary repression of complement activation may be appropriate therapeutic targets for the management of patients with unstable acute coronary syndromes.

Despite compelling evidence supporting the concept that CRP is involved in the enhancement of infarct size, additional factors may directly or indirectly contribute to the development of myocardial injury. Cytokines and plasma proteins are altered during the acute phase response. Thus, many potential candidates may contribute to the increased infarct size during the acute phase response. Although the evidence presented in the present study suggests a role for CRP, caution should be exercised against over interpretation of the data. For example, it is not known whether rabbit CRP can activate rabbit complement. As indicated previously, rat CRP does not activate rat complement (de Beer et al., 1982), and human CRP does not activate rabbit complement in the absence of human serum. Further studies are necessary to resolve these important issues.

References


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