

C-reactive Protein and Complement Are Important Mediators of Tissue Damage in Acute Myocardial Infarction

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Summary

Myocardial infarction in humans provokes an acute phase response, and C-reactive protein (CRP), the classical acute phase plasma protein, is deposited together with complement within the infarct. The peak plasma CRP value is strongly associated with postinfarct morbidity and mortality. Human CRP binds to damaged cells and activates complement, but rat CRP does not activate complement. Here we show that injection of human CRP into rats after ligation of the coronary artery reproducibly enhanced infarct size by ~40%. In vivo complement depletion, produced by cobra venom factor, completely abrogated this effect. Complement depletion also markedly reduced infarct size, even when initiated up to 2 h after coronary ligation. These observations demonstrate that human CRP and complement activation are major mediators of ischemic myocardial injury and identify them as therapeutic targets in coronary heart disease.

Key words: heart • ischemia • necrosis • inflammation • acute phase response

Ischemia caused by coronary artery occlusion is the primary process underlying acute myocardial infarction, but inflammation around the zone of hypoxic necrosis also makes a major contribution to the final size of the lesion and the clinical outcome (1). Local activation of complement, which starts within 2 h of acute ischemic injury (2), and infiltration of neutrophils are universal features of both clinical and experimentally induced acute myocardial infarction (1). In experimental animals, inhibition of complement activation at the time of coronary artery occlusion both prevents neutrophil infiltration (3) and markedly reduces infarct size (4–8). The mechanisms that initiate complement activation are not known (9), although loss of CD59 (protectin) from cells compromised by anoxia may contribute to direct damage by the terminal complement complex (2).

The circulating concentration of human C-reactive protein (CRP),¹ the classical acute phase protein, is always increased after acute myocardial infarction, starting within 4–6 h of the onset of symptoms and reaching a peak after ~50 h (10, 11). This peak value is associated with outcome, both early and late. Ventricular rupture occurs only in patients with peak serum CRP levels >200 mg/liter (12), and high CRP levels predict mortality over the next 6 mo from all causes related to myocardial infarction (13). Fur-

thermore, at autopsy, as originally demonstrated in rabbits by Kushner et al. (14), CRP is found deposited on myocardial cells within the infarcted area, together with activated complement (15).

Human CRP, after aggregation or ligand binding, is a potent activator of the classical complement pathway (16–20), and, based on both experimental and clinical observations, we have previously discussed the possible proinflammatory role of CRP in exacerbating tissue injury during the acute phase response (21, 22). Hack et al. (23) and Beranek (24, 25) have lately returned to this concept, highlighting the potential pathogenetic importance of CRP binding to nonirreparably damaged cells leading to complement activation. This would opsonize the targeted cells and/or cause direct cytotoxicity, thereby increasing the amount of cell death as well as adding to the proinflammatory activity of complement activated by CRP or other mechanisms in the zone of direct ischemic necrosis (1, 2, 9).

We have now investigated this mechanism directly and show here, for the first time, that parenteral injection of human CRP does indeed markedly enhance tissue damage, via a complement-dependent mechanism, in experimental acute myocardial infarction produced by coronary artery ligation. In addition, we demonstrate that even initiation of complement depletion 2 h after coronary artery ligation still dramatically reduces infarct size. These findings have potentially major therapeutic implications.

¹Abbreviations used in this paper: CRP, C-reactive protein; NBT, nitroblue tetrazolium; SAP, serum amyloid P component.

Materials and Methods

Protein Reagents and Assays. Human CRP (26), human serum amyloid P component (SAP; reference 27), rat CRP (28), rat C3 (28), and cobra (*Naja naja*) venom factor (29) were isolated and purified and/or assayed precisely as previously described. The preparations of human CRP and SAP and of cobra venom factor used *in vivo* were all >99% pure.

Induction and Measurement of Myocardial Infarction. Female Wistar rats bred at Imperial College School of Medicine, Hammersmith campus were used at age 9–10 wk and weighed 230–280 g. Within each experiment, the whole body and heart weights were the same in control and experimental groups. All animals were carefully examined and were healthy before fasting overnight before surgery. General anesthesia was induced with inhaled isoflurane, and supplemental oxygen was provided at 1.0 liter/min. The chest was opened through the sixth intercostal space, anesthesia was discontinued but supplemental oxygen continued at 0.5 liter/min, and the pericardium was opened to allow the heart to be lifted out of the thorax. The left anterior descending coronary artery was immediately ligated at a constant distance just below the atrium with a 5/0 silk suture, the heart was replaced, and the chest was closed while active ventilation with 100% oxygen was continued. The duration of pneumothorax was ~30 s. Resuscitation was completed by gentle chest massage with the rat lying supine, and there was usually rapid recovery of consciousness and the righting reflex. Buprenorphine (0.05 mg/kg) was given immediately for postoperative analgesia and repeated 12-hourly. Development of myocardial infarction was confirmed by early postoperative four lead electrocardiogram showing marked ST segment elevation in leads I and aVL as well as various arrhythmias.

Intraoperative and immediate postoperative mortality was up to 20%, but thereafter there were few deaths before day 5, when the rats were killed for measurement of infarct size. Deep anesthesia was induced with isoflurane, and the hearts were excised while still beating in sinus rhythm and immediately arrested in diastole by immersion in 10 ml of 30 mM KCl. They were then cleaned of any extraneous adherent tissue and weighed before being briefly chilled at –20°C to produce sufficient rigidity to facilitate cutting into defined sections. Each heart was cut into four slices of equal thickness perpendicular to the course of the left coronary artery, starting from the apex of the heart and ending at the position of the ligature around that artery. These slices were designated A, B, C, and D starting from the apex, and each was washed with pure water and then immersed in 0.02 M phosphate buffer, pH 7.4, containing 0.5 mg/ml of nitroblue tetrazolium (NBT; Sigma Chemical Co.) at 37°C for 30 min. NBT stains viable but not infarcted myocardium. After staining with NBT, the slices were washed briefly in cold water before fixation in 10% buffered formalin for 48 h, and the proximal cut surface of each slice was then imaged under standard conditions with a high resolution digital camera. The captured images were coded and analyzed 'blind,' without knowledge by the operators of the treatment received by the rat in question. Lines were drawn around the total area of the slice, the NBT-negative infarct zone, and any parts of the image to be excluded from the estimation, including valves, chordae tendinae, shadows, and edges, and the area of the infarct was determined as a percentage of the total area. The infarcts were confined to slices B, C, and D, and the mean percentage in these three was taken as the infarct size in each heart. Statistical significance of differences between infarct sizes in different treatment groups within each experiment was sought by one-way analysis of variance and by Bonferroni *t* tests.

In Vivo Treatments. Complement depletion was induced in

vivo by a single intraperitoneal injection of cobra venom factor at 250 U/kg. Human CRP or SAP was injected intraperitoneally at 40 mg/kg 1 h after coronary artery ligation and then at 24-h intervals until the rats were killed on day 5. The proteins were dissolved in 10 mM Tris and 140 mM NaCl, pH 8.0, and also 2 mM CaCl₂ in the case of human CRP, and the control rats received injections of the buffer alone at the same intervals. In one experiment, rats were killed 24, 48, and 72 h after coronary artery ligation. For analysis of the effect of cobra venom factor on plasma C3, the usual dose was given and normal, unoperated rats were bled from the tail at intervals up to 6 d. For measurement of plasma clearance of human CRP and SAP, normal unoperated rats received a single 40 mg/kg dose of each protein and were bled at intervals up to 72 h later. Plasma clearance of human CRP was also studied in the same protocol in normal unoperated rats that had received cobra venom factor 24 h previously. These experiments could not be performed in rats that had undergone coronary artery ligation because they do not survive the anesthesia required for bleeding, but there is no reason to suppose that the results in those rats would be significantly different.

Immunohistochemical Analysis. The formalin-fixed heart slices were embedded in wax and sectioned at 2- μ m thickness for routine hematoxylin and eosin staining and for immunohistochemical staining. Optimal dilutions of the IgG fractions of monospecific goat anti-human CRP, rabbit anti-rat CRP, and sheep anti-rat C3 antisera, raised by immunization with the respective isolated pure antigens, were used as primary antibodies after blocking the sections by incubation in 20% vol/vol normal serum of the species of origin of the secondary antibody. In all cases, endogenous peroxidase activity was blocked by incubation with H₂O₂ before testing. Secondary antibodies were optimal dilutions of goat anti-rabbit IgG or rabbit anti-goat IgG (both from DAKO Corp.), which cross-reacts with sheep IgG. Finally, rabbit or goat peroxidase-antiperoxidase complexes (DAKO Corp.) were used, followed by visualization with diaminobenzidine. All positive staining was completely abolished when the primary antibodies had been absorbed before use with an excess of the respective specific antigen. In the case of rat C3, which was not available in pure form, parallel absorptions of the sheep anti-rat C3 antibody were performed with normal rat serum and with C3-depleted serum from a cobra venom-treated rat. The latter did not affect anti-C3 staining, whereas C3-sufficient rat serum abolished it completely. To confirm that the antigens under investigation were not affected by formalin fixation and tissue processing, sample hearts were snap frozen without fixation, and 5- μ m cryostat sections were cut for immunoperoxidase staining. For all antigens, appearances were identical to those of the fixed sections, except for poorer preservation of histological detail as expected. Frozen sections of a heart from a human CRP-treated rat killed on day 3 were also stained for human CRP by indirect immunofluorescence and the nuclei counterstained with ethidium bromide to visualize binding of CRP to cell surfaces.

Results

Infarct Size Is Increased by Human CRP. We have previously shown that rat CRP does not activate rat complement, whereas human CRP does so very efficiently (28). The rat model therefore permits specific analysis of the complement-dependent effect of human CRP on infarct size *in vivo*. Rats that received 40 mg/kg of isolated pure

human CRP by intraperitoneal injection 1 h after coronary artery ligation rapidly became clinically less well than buffer-treated controls, and some died during the next 3 d while receiving further daily injections of the same dose of human CRP. In contrast, rats that had not been operated on but received the same doses of human CRP showed absolutely no ill effects. Injections of human SAP, the pentraxin protein very closely related to CRP (30), had no adverse clinical effects in either normal or coronary artery-ligated rats. When all surviving animals were killed on day 5 after coronary artery ligation, the infarcts in those receiving human CRP were ~40% larger than in control rats treated with either buffer or human SAP (Table I).

The plasma clearance of human CRP and SAP after single intraperitoneal injections in control, nonoperated rats and the clearance of human CRP in decompensated rats is shown in Fig. 1. The clearance of human CRP was not affected by complement depletion. The peak values for human CRP were comparable to massive acute phase responses in humans (31), and those for human SAP were much higher than ever seen in humans (32). Rats that have undergone coronary artery ligation do not survive the anesthesia required for bleeding and were therefore not bled before they were killed on day 5. However, when these animals were killed 24 h after the last of five daily injections, the serum concentrations of human CRP were still in the range of 13–53 mg/liter, typical of a moderate clinical acute phase response in humans. In animals that had received human SAP, the serum concentrations of this protein were between 45 and 175 mg/liter. Values for rat CRP in serum at the time of exsanguination 5 d after coronary artery ligation were 215–525 mg/liter, which is within the normal range for this species (28).

Complement Dependence of the Enhancement of Infarct Size by Human CRP. Administration of cobra venom factor in vivo rapidly produces profound and sustained depletion of C3 (29), with no active C3 remaining in the circulation at 6 h. Traces of C3 antigen detectable thereafter are inactive cleavage fragments (33). With the dose of cobra venom factor used here, 250 U/kg (29), active C3 starts to reappear

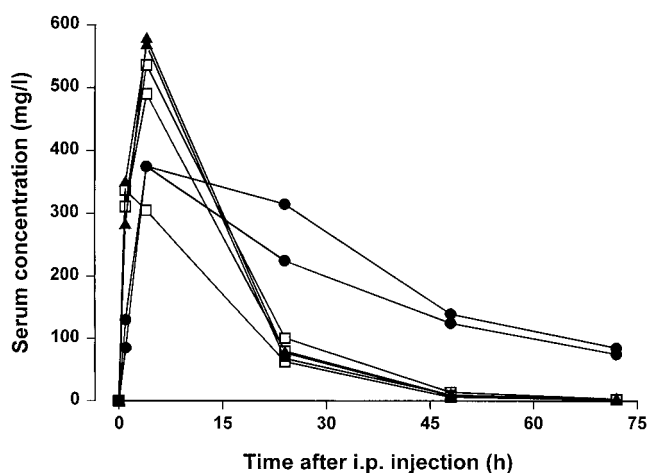


Figure 1. Plasma clearance of human CRP and SAP after single intraperitoneal injections of 40 mg/kg in normal adult female rats weighing 230–280 g. Each data set represents values in a single animal. □, human CRP in untreated rats; ▲, human CRP in rats decompensated by intraperitoneal injection of 250 U/kg of cobra venom factor 24 h beforehand; ●, human SAP in untreated rats.

in the circulation after ~4 d and is within the normal range by day 5 or 6. When rats had been decompensated by in vivo administration of cobra venom factor 24 h before coronary artery ligation, their infarcts at 5 d were ~60% smaller than those in control untreated animals (Table II). Complement-sufficient rats injected daily with human CRP developed, as before, infarcts ~40% larger than those in control untreated animals. However, injection of human CRP had no effect at all on the reduced infarct size in decompensated rats (Table II). The damaging effect of human CRP in this model is thus absolutely complement dependent.

Deposition of CRP and Complement in Rat Myocardial Infarcts. Human CRP and rat C3 (Fig. 2) and rat CRP (not shown) were all deposited in the infarcted myocardium. On day 5, human and rat CRP were present homogeneously throughout the infarcted muscle and also in a more intense, speckled pattern in multiple foci that occasionally coin-

Table I. Human CRP Increases Myocardial Infarct Size in Rats

Treatment	No. treated	Day 5 survivors	Infarct size mean (SD)	Bonferroni <i>t</i> test vs. controls
			%	
<i>Experiment 1</i>				
Buffer only	5	5	14.5 (1.1)	–
Human CRP	5	4	21.2 (2.0)	<i>P</i> = 0.0007
<i>Experiment 2</i>				
Buffer only	3	3	12.8 (0.9)	–
Human SAP	5	5	12.4 (1.9)	NS
Human CRP	5	3	17.6 (0.4)	<i>P</i> = 0.0022

Human CRP or SAP was injected intraperitoneally at 40 mg/kg.

Table II. Complement Dependence of the Enhancement of Infarct Size by Human CRP

Treatment	No. treated	Day 5 survivors	Infarct size mean (SD)	Bonferroni <i>t</i> test vs. controls
			%	
Buffer only	8	8	14.3 (1.2)	–
Human CRP	5	3	19.7 (1.6)	<i>P</i> = 0.0020
Complement depletion	5	5	6.5 (0.9)	<i>P</i> = 0.0000
Complement depletion + human CRP	6	6	6.9 (0.3)	<i>P</i> = 0.0000

Human CRP at 40 mg/kg was injected intraperitoneally 1 h after coronary artery ligation and then at 24-h intervals until all rats were killed 5 d later; controls received buffer alone. Parallel groups had been decompartmented by intraperitoneal injection of cobra venom factor at 250 U/kg 24 h before coronary artery ligation.

cided with hematoxyphil nuclear remnants. These foci may be nuclear ghosts from which chromatin has been cleared but that retain the small nuclear ribonucleoprotein particles to which human CRP binds avidly (34, 35) and possibly other CRP ligands. Rat C3 was present predominantly in the same speckled foci, with no diffuse immunoreactivity on the infarcted muscle cells, in contrast to the distribution of CRP. Immunofluorescence staining of sections of unfixed, snap-frozen myocardial tissue taken 72 h after coronary ligation clearly demonstrated the presence of CRP on the surfaces of damaged myocardial cells in and around the infarct, as well as the same distribution seen in fixed sections stained by the immunoperoxidase method. Staining for human CRP was always more intense than for rat CRP, although it is not clear whether this represents greater abundance of the human protein in the tissue sections or just greater sensitivity of the respective immunostaining procedure. Numerous

mononuclear cells in the dense periinfarct infiltrate also stained strongly for human CRP (Fig. 2) but not for rat CRP (not shown) or rat C3 (Fig. 2).

Cardioprotective Effect of Complement Depletion. Marked reductions in infarct size and in ischemia/reperfusion injury have previously been demonstrated in animals in which complement activation had been blocked by treatment, either with cobra venom factor or with recombinant soluble complement receptor type 1, before or at the time of induction of ischemia (4–8). The therapeutic implications of this observation are obvious, but for clinical implementation, it is critical to know whether complement depletion initiated after the onset of coronary occlusion can still have a protective effect. We therefore administered cobra venom factor at various times between 24 h before and 24 h after coronary artery ligation. There was, as shown before, a marked, ~60% reduction in infarct size in rats that had re-

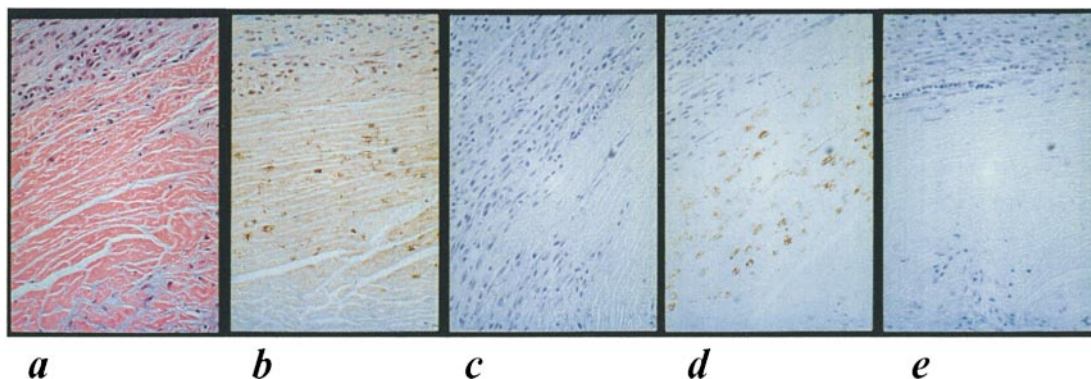


Figure 2. Immunohistochemical staining of rat myocardial infarcts on day 5. (a) Hematoxylin and eosin stain showing infarcted myocardial cells and adjacent dense mononuclear cell infiltrate. (b) Immunostain with anti-human CRP with uptake localized to the infarcted area and both diffuse and focal patterns of immunoreactivity; many of the adjacent infiltrating macrophages are also immunoreactive. (c) Immunostain with anti-human CRP preabsorbed with isolated pure human CRP, showing complete absence of any staining and confirming the immunospecificity for human CRP of the pattern observed in b. (d) Immunostain with anti-rat C3 with uptake confined, in contrast to the anti-human CRP (b), to focal structures, possibly nuclear ghosts, within the infarct. (e) Immunostain with anti-rat C3 preabsorbed with whole rat serum, showing complete absence of any staining. Absorption of the anti-rat C3 antibody with C3-depleted rat serum did not affect the staining pattern, confirming its immunospecificity for rat C3. Original magnifications, 40.

ceived treatment 24 h beforehand and that were maximally complement depleted at the time of operation (Table III). Although injection of cobra venom factor 6 or 24 h after ligation had no effect, the infarcts in animals that received cobra venom factor either 0.5 or 2 h after ligation were almost 50% smaller (Table III). Maximal complement depletion is achieved only from 6 h after intraperitoneal injection of cobra venom factor, and in this rat model there is therefore a window of therapeutic opportunity of perhaps up to 8 h after acute coronary artery occlusion during which immediate inhibition of complement activation could potentially reduce infarct size.

Discussion

CRP has been very stably conserved in evolution (30, 36, 37), and no structural polymorphism or deficiency of CRP has yet been reported in humans, suggesting that this protein has important normal functions that contribute to survival. In experimental models, CRP is protective against pneumococcal infection (38, 39) and may contribute to innate immunity to other microorganisms to which it binds (40). By analogy with this role of SAP in relation to chromatin that we have recently demonstrated (41), CRP also probably plays an important role in scavenging autologous ligands and preventing development of autoimmunity. This does not mean, however, that CRP may not also contribute to pathogenesis of disease, especially conditions developing in postreproductive later life. Natural selection is 'blind' to phenomena occurring after reproduction, provided they do not affect viability of the species as a whole (42).

The results presented here unequivocally demonstrate, in a robust experimental model, that human CRP markedly enhances the extent of myocardial damage produced by ischemic injury. Although the time-concentration profile of human CRP produced by daily CRP injections was not the same as the monophasic acute phase response that follows uncomplicated naturally occurring myocardial infarction in humans, it was comparable to the persistent, high, and fluctuating CRP pattern typically found in patients with postin-

farct complications (11) and was therefore not 'unphysiological.' Endogenous rat CRP, as well as the injected human CRP, was deposited in the infarct in vivo, but rat CRP does not activate rat complement (28). In contrast, human CRP is a potent activator of rat complement (28), and the enhancement of infarct size caused by administration of human CRP is completely abrogated by in vivo complement depletion. Human CRP production is always greatly increased after acute myocardial infarction, CRP is always deposited in human myocardial infarcts, and early and late clinical outcomes are significantly associated with peak postinfarction plasma levels of CRP. It is therefore very likely that CRP contributes significantly to the extent of damage in human acute myocardial infarction, and, based on our results here, it probably does so via complement activation. Although complement activation by CRP is not efficient in generating the terminal lytic complement complex, it very effectively cleaves C3, the critical step for opsonization by C3b and liberation of the C3a anaphylatoxin.

These findings have important therapeutic implications, suggesting that a drug capable of inhibiting the binding of human CRP to its target ligands in vivo and thereby preventing it from activating complement should reduce infarct size, with corresponding clinical benefit. Furthermore, increased CRP production is a feature of the nonspecific acute phase response to a very wide range of traumatic, infectious, inflammatory, and neoplastic tissue-damaging conditions (31). In all of these, including disorders as diverse as burns, surgical trauma, rheumatoid arthritis, sepsis, and invasive neoplasia, there are nonirremediably damaged cells that, by analogy with myocardial infarction, are likely to be targeted by the opsonic and proinflammatory actions of CRP and complement. Specific inhibition of CRP binding in vivo might thus be expected to be of wide clinical benefit, and suitable compounds are currently being sought, supported by our recent description of the high resolution 3-dimensional structure of the physiological CRP-ligand complex (43).

The cardioprotective effect of total complement depletion at the time of experimental coronary artery occlusion is well established (4-8). However, in the clinical context

Table III. *Cardioprotective Effect of Complement Depletion in Myocardial Infarction*

Cobra venom factor treatment	No. of rats	Infarct size on day 5 mean (SD)	Bonferroni <i>t</i> test vs. controls
		%	
None, buffer only	4	17.7 (1.9)	
24 h before coronary ligation	4	7.0 (1.2)	<i>P</i> = 0.0002
0.5 h after coronary ligation	4	9.7 (1.1)	<i>P</i> = 0.0007
2 h after coronary ligation	4	9.5 (1.6)	<i>P</i> = 0.0011
6 h after coronary ligation	4	16.9 (2.2)	NS
24 h after coronary ligation	4	16.9 (1.2)	NS

Cobra venom factor at 250 U/kg was injected intraperitoneally at the times shown in relation to coronary artery ligation; controls received buffer only. All rats were killed after 5 d for estimation of infarct size. One-way analysis of variance, total DF = 23, *F* = 36.45, *P* = 0.0000.

of patients presenting with acute myocardial infarction, complement depletion could only be initiated after the onset of symptoms. We show here that injection of cobra venom factor up to 2 h after ligation of the coronary artery significantly reduces infarct size, despite the fact that maximal depletion of plasma C3 is only achieved 6 h after cobra factor administration. This encouragingly suggests that, af-

ter acute ischemia, the major pathogenic action of complement occurs after some hours, and there is therefore a window of therapeutic opportunity during which inhibition of complement activation could still reduce infarct size, with corresponding reduction in morbidity and mortality. Although no anticomplement drugs are yet in clinical use, our results support their development and testing.

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