C-Reactive Protein Activates Complement in Infarcted Human Myocardium

Remco Nijmeijer,^{*†‡§} Wim K. Lagrand,^{*‡}
Yvonne T. P. Lubbers,[§] Cees A. Visser,^{*‡}
Chris J. L. M. Meijer,[†] Hans W. M. Niessen,^{*†} and
C. Erik Hack^{*§¶}

From the Institute for Cardiovascular Research* and the Departments of Pathology,[†] Cardiology,[‡] and Clinical Chemistry,[¶] Vrije Universiteit Medical Center, Amsterdam; and the Department of Immunopathology,[§] Sanquin Research at the Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands

Circulating levels of C-reactive protein (CRP) constitute a cardiovascular risk marker. Immunohistochemical studies have revealed co-localization of CRP and activated complement in human infarcted myocardium suggesting CRP to enhance inflammation in ischemic myocardium by inducing local complement activation. The aim was to establish whether CRP activates complement in infarcted human myocardium and to assess the relationship between this activation and the duration of infarction. Myocardial tissue samples from 56 patients that had died from acute myocardial infarction were evaluated. Specimens were taken from infarcted as well as noninfarcted sites of the heart. CRP-mediated complement activation was assessed by immunohistochemistry and by measuring levels of complement, CRP, and CRP-complement complexes, specific markers for CRP-mediated activation, in homogenates of the heart. Infarctions of 12 hours to 5 days had significantly more extensive depositions of complement and CRP and contained significantly more CRP, activated complement, and CRP-complement complexes than infarctions that were less than 12 hours old. Levels of CRP complexes correlated significantly with CRP and complement concentrations in the infarctions, as well as with the extent of complement and CRP depositions as measured via immunohistochemistry. Specific activation products of CRP-mediated activation of complement are increased in infarcts of more than 12 hours in duration and correlate with the extent of complement depositions. Hence, CRP seems to enhance local inflammatory reactions ensuing in human myocardial infarcts of more than 12 hours duration. (*Am J Pathol* 2003, 163:269–275)

Circulating levels of the acute phase reactant C-reactive protein (CRP) constitute a cardiovascular risk factor: levels in healthy persons or patients with stable or unstable angina pectoris are associated with an increased incidence of cardiovascular events.^{1–3} In addition, the course of CRP after acute myocardial infarction (AMI) is associated with the development of complications and with outcome.^{4,5} These associations between CRP and cardiovascular events are considered to reflect that CRP, by virtue of its acute phase behavior, is an indirect parameter for the degree of inflammation ensuing in atherosclerotic lesions or in the infarcted myocardium. However, we have shown that CRP localizes in infarcted human myocardium, similarly as has been reported in a rabbit model for AMI.^{6,7} This raises the possibility that CRP participates in the inflammatory changes locally ensuing in the infarcted myocardium. As a matter of fact CRP is able to activate complement via the classical pathway, implying that CRP may enhance inflammation by activating complement.^{8–10} Indeed, Griselli and co-workers¹¹ have demonstrated in a rat model that human CRP enhances myocardial infarct size by activating complement. Immunohistochemical co-localization of CRP and activated complement in myocardial tissue of patients with AMI suggests a similar proinflammatory role for CRP in human myocardial infarction.⁷ However, co-localization of CRP and activated complement does not definitely prove that CRP indeed activates complement locally in infarcted myocardium.

In the present study we sought for evidence that CRP activates complement in human myocardial infarction, and determined the relationship between such activation and the duration of infarction. Myocardial tissue specimens were obtained from patients who had died from AMI. These specimens were analyzed for deposition of complement and CRP. In addition, we measured levels of CRP, complement, and CRP-complement complexes, complexes that are specific parameters for CRP-mediated complement activation, in homogenates prepared from the specimens.

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Address reprint requests to Dr. H. W. M. Niessen, VU-Medical Center, Department of Pathology, De Boelelaan 1117, 1007 MB Amsterdam, The Netherlands. E-mail: jwm.niessen@vumc.nl.

Materials and Methods

Patients

Two overlapping groups of patients with myocardial infarction at autopsy were included in the study: 56 patients in the immunohistochemical study and 64 patients in the homogenate study. Forty-three patients participated both in the immunohistochemical as well as in the homogenate study. Nineteen participated in earlier studies on the involvement of CRP, complement, and ICAM-1 in infarcted human myocardium.^{7,12} At autopsy, performed within 24 hours after death, macroscopic signs of AMI were determined by decreased lactate dehydrogenase staining of the affected myocardium. Tissue samples were taken from the infarcted myocardium, as well as from noninfarcted parts of the heart, from each patient. All specimens were stored in liquid nitrogen (-196°C). The study was approved by the ethics committee of the VU Medical Center Amsterdam, and complied with the principles of the declaration of Helsinki. Use of leftover material after the pathological examination is part of the standard patient contract in our hospital.

Antibodies and Immunohistochemistry

Monoclonal antibodies 5G4 (IgG-2A subclass) against human CRP and C4-4 (IgG-1 subclass) against human C4d have been previously used for immunohistochemistry.^{7,12} Briefly, 4- μm -thick frozen sections were mounted onto glass slides (SuperfrostPlus; Menzel-Glaser, Braunschweig, Germany), dried for 1 hour by exposure to air, and fixed in acetone (Baker Analyzed Reagent; Mallinckrodt Baker BV, Deventer, Holland). The slides were incubated at room temperature for 10 minutes with normal rabbit serum (Dakopatts A/S, Glostrup, Denmark) 1 to 50 diluted in phosphate-buffered saline (PBS), pH 7.4, containing 1%, w/v, bovine serum albumin (PBS-BSA), after a rinse in PBS. The slides were incubated with specific antibody for 60 minutes at room temperature (monoclonal antibody C4-4 at a 1:300 dilution in PBS-BSA; monoclonal antibody 5G4 against CRP 1:500 in PBS-BSA). Thereafter, they were washed for 30 minutes with PBS and incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (RaM-HRP; Dakopatts A/S, Glostrup, Denmark), 1 to 25 diluted in PBS-BSA. The slides were washed again in PBS and incubated for 4 minutes in 3,3'-diamine-benzidine-tetrahydrochloride (Sigma) at 0.5 mg per ml in PBS containing 0.01% v/v H_2O_2 , washed again, counterstained with hematoxylin for 40 seconds, dehydrated, cleared, and finally mounted. In control experiments, specific antibodies were incubated in the presence of soluble antigen. In addition, similar incubations were performed with irrelevant monoclonal antibodies: IgG1, IgG2A, and mouse myeloma protein, MOPC (Cappel, Organon Teknika, Turnhout, Belgium).

Microscopic criteria were used to estimate infarct duration in all myocardial tissue specimens.^{13,14} Three phases of infarction were discriminated: the early phase, corresponding to an infarct duration of 0 to 12 hours after

the onset, and with no or minor polymorphonuclear leukocyte (PMN) infiltration; a PMN phase corresponding to a duration of 12 hours to 5 days and characterized by massive infiltration of PMN; and a chronic phase corresponding to infarct duration of 5 to 14 days and characterized by infiltration of lymphocytes and macrophages and proliferation of fibroblasts and vessels. The extent of complement and CRP depositions was determined as follows: tissue slides were subdivided into four parts at a magnification $\times 250$. The percentage of the microscopic field positive for complement or CRP was then assessed for each part. Subsequently the average positive area for the entire slide was calculated. Two investigators (RN and HWMN) independently judged and scored all slides for infarct duration and for anatomical localization of complement or CRP as visualized by immunohistochemical staining. For the final scoring results, consensus was achieved between the two investigators.

Measurement of Parameters in the Infarcted Myocardial Tissue

Myocardial tissue was homogenized using a Dismembrator and dissolved in Veronal buffer (10 mmol/L CaCl_2 , 20 mmol/L MgCl_2 , 10 mmol/L Na-diethyl-barbital, 0.65 mol/L NaCl, pH 7.4). The suspension was then centrifuged for 20 minutes at $10,000 \times g$. All procedures thus far were performed at 4°C to prevent *in vitro* activation of complement. In the supernatant CRP, CRP-complement complexes (CRP-C4d) and total amount of activated C4 (this is further referred to as "C4b/c" because the assay does not discriminate between C4b, C4bi, or C4c) were quantitatively measured with enzyme-linked immunosorbent assays as previously described.^{9,10} The concentration of parameters in homogenates was adjusted for protein content (Bio-Rad protein assay, catalog no. 500-0006).

Analysis of Data

Data were analyzed with the statistical program SPSS for Windows, version 9.0 (SPSS Inc., Chicago, IL). The Kolmogorov-Smirnov test was used to evaluate the distribution of data. Median and 25th and 75th percentage values were used to describe data that were not normally distributed. These data were depicted as box and whisker plots. The Mann-Whitney *U*-test and the Wilcoxon signed rank test were used to evaluate the significance of differences. Spearman's correlation rank test was used to assess the correlation (*R*s) between parameters. A *P* value of <0.05 was considered to represent a significant difference.

Results

CRP and Complement Depositions in Infarcted Myocardium

In line with our previous study, CRP and complement depositions were found co-localized on jeopardized car-

Figure 1. Localization of CRP (A) and activated complement (C4d) (B) in infarcted human myocardium. J, jeopardized area; N, normal area. Original magnifications, $\times 250$.

diomyocytes, in particular on the plasma membrane and the cross striations and in the cytoplasm.⁷ At $\times 250$ magnification, we quantified the extent of these depositions as described in Materials and Methods. Examples of the observed immunohistochemical staining patterns of CRP and complement are shown in Figure 1. Figure 2 summarizes the quantitative data of the immunohistochemical

Figure 2. Course of CRP and complement depositions in infarcted myocardium as assessed with immunohistochemistry. The extent of the depositions is expressed as percentage of the microscopic tissue slide at $\times 250$ magnification. Early, infarct duration 0 to 12 hours; PMN, 12 hours to 5 days; and chronic, 5 to 14 days.

analysis. Both CRP and complement depositions in the infarcts were significantly more extensive during the PMN phase (12 hours to 5 days) than during the early phase (0 to 12 hours). The median of CRP deposition in the PMN phase was somewhat higher than that for complement, although this difference did not reach statistical significance. During the chronic phase (5 to 14 days), depositions of CRP and complement became less intense. This decline was significant for complement ($P < 0.05$) and borderline for CRP ($P < 0.09$). However, as compared to the findings in the early phase, the depositions of CRP and C4d in the chronic phase were significantly more intense (CRP, $P < 0.03$; and C4d, $P < 0.01$). In the control specimens of noninfarcted sites of the heart, no or only focal depositions of complement were observed, whereas CRP was not found on cardiomyocytes in these areas at all (not shown). In control hearts of patients that had died from other causes than AMI, neither complement nor CRP depositions were found. Thus, the localization of complement and CRP in the myocardium appeared to be a phenomenon specifically occurring in infarcts, in particular in those with duration of 12 hours to 5 days.

CRP and Complement Levels in Infarcted Myocardial Tissue

To confirm the immunohistochemical data, we attempted to quantify intrainfarct levels of CRP and complement by measuring levels of these proteins in homogenates of the tissue specimens using sensitive enzyme-linked immunosorbent assays. A significant increase of the intrainfarct amount of activated complement, ie, C4b/c, and CRP during the PMN phase was found as compared to levels in the specimens from the early phase (Table 1). In the chronic phase, levels of either parameter were lower, although this decrease was not significant as compared to the PMN phase. An increase of tissue levels of C4b/c or CRP during the PMN phase was not observed for the control specimens from the noninfarcted sites (Table 1), although these levels tended to increase during the chronic phase. As no or only focal CRP or complement depositions were found in the noninfarcted control areas, the amount of mediators measured in the homogenates of the control areas likely represented the amount of mediators present in the intravascular compartment. Hence, subtracting the concentration of mediators in the control areas from that in the infarcted area, likely better reflects the amount of mediator present in the extravascular compartment of the infarcts. After this correction, a significant increase of the intrainfarct amount of C4b/c and CRP was found during the PMN phase as compared to levels during the early phase (Figure 3, a and b). In contrast, corrected intrainfarct levels in the chronic phase were comparable to those in the early phase.

Relation of CRP to Complement in Infarcted Myocardium

In an earlier study CRP was found to be co-localized with C4d in infarcted myocardium,⁷ suggesting that CRP con-

Table 1. Concentrations of Activated Complement and CRP in Tissue Homogenates from Infarcted and Control Sites in AMI Patients in Relation to Infarct Stage

	Infarct duration			P value*
	Early phase 0 to 12 hours (n = 42)	PMN phase 12 hours to 5 days (n = 14)	Chronic phase 5 to 14 days (n = 8)	
C4 b/c (nmol/L/mg) Median (25th to 75th %)				
Infarct	4.0 (2.9–5.8)	16.5 (5.0–23.0)	8.5 (4.9–11)	0.01
Control	4.4 (3–7.7)	4.6 (1.1–13)	9.5 (3.7–14.8)	–
CRP (μ g/mg) Median (25th to 75th %)				
Infarct	0.5 (0.1–1.6)	5.1 [†] (0.6–9.9)	2.4 (0.7–8.3)	0.01
Control	0.3 (0.1–1.7)	1.1 (0.3–6.0)	2.6 (1.0–4.8)	–

*0 to 12 hours versus 12 hours to 5 days.

[†]P value < 0.05 comparing infarcted versus control areas.

C4b/c (nmol/L/mg of tissue) and CRP (μ g/mg of tissue) were measured in homogenates of myocardial tissue specimens from the infarcted and control sites, and are expressed as median and 25th and 75th percentile. Differences between groups were analyzed with Mann-Whitney U and Wilcoxon signed rank tests.

tributed to complement activation in the ischemic myocardium. This co-localization was also found in the present study. Availability of the quantitative immunohistochemical data allowed the analysis of a putative correlation between either parameter. As expected from the immunohistochemical data, CRP and C4d depositions were found to be correlated (R_s , 0.886; P < 0.001; Figure 4). In a next step, we analyzed the correlation between the amount of CRP and C4d depositions, as found in immunohistochemistry, with the ininfarct concentrations of the mediators, as measured in the homogenates (Table 2). The immunohistochemical deposition of CRP correlated well with the amount of activated complement (C4b/c; R_s , 0.880, P < 0.005) and amount of CRP (R_s , 0.574; P < 0.005) in the homogenates. Furthermore, the deposition of C4d correlated well with the ininfarct concentration of C4b/c (R_s , 0.535; P < 0.005), as assessed in the homogenates. The immunohistochemical deposition of complement correlated only marginally with CRP (R_s , 0.41; P < 0.06). Together these data not only showed that immunohistochemical depositions of complement and CRP significantly correlated with ininfarct complement activation and CRP levels, respectively, but also that complement deposition and activation in the infarcts were significantly linked to CRP localization.

CRP-Mediated Complement Activation in Infarcted Myocardium

Thus far the data indicated a close correlation between CRP deposition and complement activation in the myocardial infarcts. This suggests, but does not prove, that CRP contributes to complement activation in the ischemic myocardium. Wolbink and colleagues⁹ have shown that complexes between activated complement and CRP are exclusively generated during complement activation by CRP, and not during other activation processes. Hence we measured CRP-C4d complexes in the homogenates of the tissue samples from the infarcts using a sensitive enzyme-linked immunosorbent assay.¹⁰ Significant increases of these complexes were observed in

Figure 3. Extracellular concentrations of C4b/c (A) and CRP (B) in the infarcts. Levels of C4b/c (A) or CRP (B) were measured in homogenates of tissue samples from the infarct and control areas. Extravascular levels in the infarcted area were calculated by subtracting values in the homogenates of control areas from those in infarct areas. Early, infarct duration 0 to 12 hours; PMN, 12 hours to 5 days; chronic, 5 to 14 days.

Figure 4. Relation of the deposition of CRP to that of complement. Immunohistochemical deposition of CRP and C4d were quantified as described in Materials and Methods, and expressed as a percentage of the microscopic tissue slide at $\times 250$ magnification. R_s , 0.886; $P < 0.001$.

samples taken during the PMN phase (Table 3). Similarly as for C4b/c and CRP (see above), extravascular intramyocardial levels were calculated by subtracting levels in the control sites from those in the infarct site. Corrected

Table 2. Correlations between Corrected Amounts of Inflammatory Mediators as Measured in Homogenates and Percent Immunohistochemical Depositions in Infarcted Myocardium

Immunohistochemistry versus homogenates Variable	R_s^*	$P <$
% CRP versus C4b/c	0.880	0.005
CRP	0.574	0.005
% Complement (C4d) versus C4b/c	0.535	0.005
CRP	0.410	0.06

* R_s , Spearman's coefficient of correlation.

Immunohistochemical deposition of complement and CRP were quantified as described in Materials and Methods, and correlated with intramyocardial concentrations of C4b/c and CRP. The latter were calculated by subtracting the concentration of the parameter measured in the homogenate of the control tissue specimens from that measured in the specimen from the infarct site.

Table 3. Concentration of CRP-Complement Complexes in Tissue Homogenates from Infarcted and Control Areas in AMI Patients in Relation to Infarct Stage

	Infarct duration			P value*
	Early phase 0 to 12 hours ($n = 42$)	PMN phase 12 hours to 5 days ($n = 14$)	Chronic phase 5 to 14 days ($n = 8$)	
CRP-C4d (pmol/L/mg)				
Median (25th to 75th %)				
Infarct	1.0 (0.8–4.3)	23.0 [†] (10.0–37.0)	4.8 (1.8–10.3)	0.002
Control	1.3 (0.8–5.3)	5.8 (1.0–18.0)	3.9 (1.4–6.1)	–

*0 to 12 hours versus 12 hours to 5 days.

[†] P value < 0.05 comparing infarcted versus control areas.

CRP-C4d complexes (pmol/L/mg tissue) were measured in homogenates of myocardial tissue specimens from the infarcted and control sites, and are expressed as median and 25th and 75th percentile. Differences between groups were analyzed with Mann-Whitney U and Wilcoxon signed rank tests.

Figure 5. Extravascular concentration of CRP-complement complexes in infarcted myocardium in relation to infarct stage. Complexes were measured in the tissue homogenates from infarcted and control sites, and expressed as nmol/L/mg. Extravascular concentration in the infarcts was calculated by subtracting levels in control sites from those in the infarct sites. Early, infarct duration 0 to 12 hours; PMN, 12 hours to 5 days; and chronic, 5 to 14 days.

intramyocardial levels of CRP-C4d were significantly increased during the PMN phase as compared to those in the early stage (Figure 5). Furthermore, the corrected intramyocardial concentration of these complexes correlated significantly with the extent of immunohistochemical depositions of C4d and CRP in the infarcted area (R_s , 0.692, $P < 0.005$, for the relation of CRP-C4d versus CRP; and 0.486, $P < 0.005$, for that of CRP-C4d versus C4d, respectively). Also when CRP, C4b/c, and CRP-C4d in the homogenates (corrected values) were correlated with each other, significant correlations were found (Table 4). Notably, the amount of complexed CRP in the homogenates was up to 50% of the total amount of CRP.

Discussion

CRP constitutes a risk factor for cardiovascular events,^{1,2,15–18} although the molecular link between circulating CRP and cardiovascular disease is still not completely understood. CRP was found to become deposited in the infarcted myocardium in experimental models as

Table 4. Correlations of Extravascular CRP, Activated C4, and CRP-C4d Complexes in Infarcted Human Myocardium

Correlations between inflammatory parameters			
Variable 1	Variable 2	Rs*	P<
CRP-C4d	C4b/c	0.708	0.005
CRP-C4d	CRP	0.600	0.005
CRP	C4b/c	0.482	0.005

*Rs Spearman's coefficient of correlation

CRP, C4b/c, and CRP-C4d complexes were measured in homogenates of tissue specimens from infarcted and control sites. Levels in the homogenates were normalized for protein content. Extravascular levels in infarcts were calculated by subtracting levels in the control sites from those in the infarcts. Spearman's rank coefficient of correlation was assessed to determine relation between mediators.

well as in patients with AMI.^{6,7,11} Moreover, deposition of CRP coincided with that of activated complement, suggesting but not proving, a role for CRP in complement activation occurring locally in infarcted myocardium.⁷ Indeed, *in vitro* and *in vivo* studies have shown that CRP is able to activate complement via the classical pathway.⁸⁻¹⁰ We now report in a large group of patients that 1) C4d and CRP are co-localized in infarcted human myocardium; 2) localization of either protein is maximal during the PMN phase (infarcts of 12 hours to 5 days); 3) the extent of the depositions and the concentrations of C4d and CRP in tissue homogenates from the infarct sites are correlated; and 4) CRP-complement complexes, which are specific parameters for CRP-mediated complement activation, accumulate in infarcts of 12 hours to 5 days. These data together point to a role for CRP in activating complement in the ischemic human myocardium and provide a time frame for this activation process.

Notably, the pathological processes ensuing in infarcted myocardium of humans only can be studied in material obtained during autopsy. This approach has obvious limitations, including the influence of postmortem changes. We consider this influence minimal in our study, as tissue samples of the unaffected, noninfarcted areas showed no morphological signs of ischemia and had no complement or CRP depositions. This therefore excluded the possibility that CRP or complement had bound non-specifically to the myocardium, even despite the elevated plasma levels of this acute phase protein during the course of AMI. A second limitation of our study is that our observations were not serial. Yet insight into the time elapsing between the various processes and the onset of symptoms is essential to assess a time window for interventions. By alignment of the results according to the duration of infarction, we were able to define a time window for CRP-mediated activation of complement. Our results indicate that in general this activation starts at 12 to 24 hours after the onset, to wane off after 5 days.

Homogenates of tissue samples contain to some extent blood. Individual variation in plasma CRP and complement levels occurs in patients with myocardial infarcts.¹⁹ Hence, measurement of CRP and complement parameters in homogenates of specimens from infarcted myocardium may be flawed by a contribution of varying plasma levels.²⁰ We reduced this flaw by measuring the

parameters in homogenates from specimens of the unaffected myocardium of each patient, and by subtracting levels in these control samples from those in the infarcted myocardium. In case of hypoperfusion of the infarcted area this method may result in underestimation of the extravascular infarct levels. However, as the levels calculated in this way correlated well with the extent of CRP and complement depositions in the infarcted area as observed in immunohistochemistry, we considered this flaw acceptable.

The mechanisms by which the complement system is activated during AMI are still unclear, although the release of mitochondrial constituents, reperfusion, and thrombolytic agents have been proposed.²¹⁻²³ Previously, we have observed co-localization of CRP and complement in infarcted myocardium,⁷ suggesting that CRP contributes to complement activation in this condition. Application of antibodies against neo-epitopes exposed on activation products specific for CRP-mediated activation of complement in immunohistochemistry could definitely prove whether or not CRP indeed contributes to complement activation in AMI. However, to our knowledge such antibodies do not exist, and hence we decided to use another approach. Previously we have described that CRP-complement complexes constitute a specific marker for CRP-mediated complement activation, because they are only generated during activation by CRP and not during that by other activators.^{9,10} Hence, we measured CRP-C4d complexes in homogenates from infarcted myocardium. Both CRP and C4b/c levels in these homogenates correlated well with the extent of immunohistochemical depositions of CRP and complement, indicating that levels in the homogenates indeed reflected the processes identified in immunohistochemistry. Significant levels of CRP-C4d complexes were measured in the homogenates of the infarcted myocardium even when corrected for levels observed in those of the unaffected myocardium. In particular during the PMN phase a marked increase of CRP-C4d complexes was observed. Notably, the amount of complexed CRP appeared to be up to 50% of the total amount of CRP present in the homogenates. This percentage is much higher than that found in plasma (less than 1%). These data strongly suggest that CRP contributes to complement activation in locally infarcted human myocardium.

Our study does not allow conclusions regarding the ligand for CRP in infarcted myocardium. Co-localization of CRP with β -2-glycoprotein-I, a protein that specifically binds to the phospholipid phosphatidylserine,²⁴ however, suggests flip-flopped membranes of ischemic cells to expose these ligands. In addition, our study does not allow definite conclusions regarding the relative contribution of CRP in comparison to other activators of complement to the activation process in the ischemic myocardium. Yet, we found a pretty strong correlation ($R_s > 0.7$) between CRP-C4d complexes and C4b/c in the homogenates (see Table 4), which suggests that CRP is a main activator of complement in ischemic human myocardium.

Complement activation can enhance infarct size, as has been shown in animal experiments.^{25,26} Increases of C4d, CRP, and CRP-C4d complexes in the infarcted

areas of the heart from 12 hours on up to 5 days after onset of infarction, therefore has important clinical implications because they point to a window for therapeutic interventions. We suggest that inhibitors of complement and/or CRP in humans with AMI should be administered from 12 hours after the onset of AMI up to several days. Indeed in a preliminary study in patients with AMI the administration of the complement inhibitor C1-inhibitor from 6 hours after the onset for up to 2 days resulted in smaller infarcts.²⁷

In conclusion, our study demonstrates accumulation of CRP as well as CRP-complement complexes in infarcted human myocardium. These findings support the notion that CRP may function as a proinflammatory mediator during AMI by activating complement.

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