Molecular mechanisms of complement activation by damaged cells

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IgM co-localizes with complement and C-reactive protein

in the infarcted human myocardium

IgM localizes in the infarcted human heart.

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Abstract

Reperfusion of ischemic myocardium after acute myocardial infarction (AMI) may induce ischemia/reperfusion (I/R)-injury, which amongst others results from local activation of the complement system. Previously, we provided evidence for involvement of C-reactive protein in this activation. In this study we analyzed a potential role of IgM in complement activation in the human heart subsequent to AMI.

We performed an immunochemical analysis of heart specimens from 59 patients who died from AMI. Serial slides of frozen tissue from the infarction site, were stained for IgM, complement factors C3d and C5b-9 (membrane attack complex) and CRP.

Depositions of IgM were found on the plasma membrane, cross-striations and in the cytoplasm of jeopardized cardiomyocytes in infarct of 1 to 5 days duration. IgM depositions were remarkably similar to those of CRP and both complement factors C3d and C5b-9. Relative staining intensities of IgM and CRP varied greatly among patients.

IgM just as CRP, targets complement locally to jeopardized cardiomyocytes in the human heart following AMI. An identical deposition of IgM with C5b-9 on jeopardized cardiomyocytes proves co-localization of IgM with the membrane attack complex of complement. Localization patterns and relative staining intensities suggest IgM and CRP recognize similar epitopes in the ischemic heart, but that the relative contribution of either protein to complement activation in the ischemic myocardium differs among patients.

Introduction

Reperfusion of the impaired myocardium after acute myocardial infarction (AMI) results in a local inflammatory response [1]. This inflammatory response damages the ischemic tissue, a phenomenon also designated as ischemia/reperfusion (I/R)-injury. Prevention of this I/R-induced inflammation has been shown to reduce the infarct size up to 50% in animal models [1], and may offer new therapeutic opportunities for patients with AMI. Hence, knowledge of the mechanisms of I/R-injury in humans is warranted.

An important mediator involved in experimental I/R-injury in animals is the complement system. In a rat model of reperfusion injury of ischemic myocardium, prevention of complement activation resulted in a marked reduction of I/R-related injury [2]. Also in humans, there is preliminary evidence that inhibition of complement by C1 inhibitor reduces infarction size by up to 57% [3]. These results point to complement as an attractive target to
limit I/R-injury. However, the molecular basis of I/R-induced complement activation is not completely elucidated as yet.

Various molecules have been claimed to target activated complement to the ischemic myocardium during I/R injury. One of these molecules is C-reactive protein (CRP), which activates complement via the classical pathway [4-6]. In rabbits, CRP was localized in the inflamed myocardium after AMI [7,8]. Administration of human CRP in rats challenged with coronary artery occlusion enhances infarction size in a complement-dependent fashion [9]. In myocardial tissue specimens from patients that died from acute myocardial infarction (AMI), CRP has been shown to co-localize with complement [10], suggesting that in humans this acute phase protein contributes to complement activation in the ischemic myocardium. This notion is supported by observations that during AMI the human heart contains increased amounts of activation products that specifically reflect complement activation induced by CRP [11].

In mice, another molecular mechanism for complement activation induced during I/R-injury was revealed. This mechanism involves immunoglobulin M (IgM) [12,13]. In I/R models of the intestine and skeletal muscle, IgM-deficient mice developed substantially less I/R-injury than their wild-type littermates; this injury was restored in the deficient mice by supplementation with normal murine IgM. The specificity of the IgM mediating I/R-injury in mice is not known. It is also not known whether a similar IgM-dependent mechanism occurs in humans during I/R. We hypothesized that in humans IgM might also contribute to ischemic injury in the heart following AMI. In this study we therefore analyzed tissue specimens from the heart of patients that had died from AMI. These specimens were analysed for the presence of IgM, CRP, complement factor C3d and complement factor C5b-9 of the membrane attack complex (MAC).

Materials and Methods

Patients

Patients referred to the Department of Pathology for autopsy were included in this study when autopsy was performed no later than within 24 hours after death, and when at autopsy they showed signs of a recently developed AMI: i.e. on histochemical examination they had decreased lactate dehydrogenase (LD) staining (de-coloration) of the affected myocardium. 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 left-over material after the pathological examination is part of the standard patient contract in our hospital.

*Processing of tissue specimens*

Myocardial tissue specimens were obtained from the infarcted zone as well as from remote sites of the healthy part of the heart. These remote sites showed normal LD staining patterns and were studied as internal non-infarcted controls. A control heart tissue sample from the left ventricle was obtained from a patient that died from a cause not related to heart disease. Before being prepared as cryo-sections, tissue specimens were stored at -196°C (liquid N2). Frozen sections were mounted onto SuperFrost®Plus glass slides (Menzel-Gläser, Braunschweig, Germany).

*Assessment of infarct phase*

Microscopic criteria [14,15] were used to estimate infarct duration and viability of cardiomyocytes in all myocardial tissue specimens. As morphological judgment is more reliable with paraffin slides, corresponding paraffin slides were also made. Jeopardized myocardium was characterized by the intensity of eosinophilic staining of involved myofibers, condensation, loss of nuclei and cross striation. We characterized jeopardized myocardium without microscopic changes but with macroscopic LD-decolorization as an early phase infarct (phase 1), infiltration of polymorphonuclear leukocytes (PMNs) as a PMN-phase (phase 2), lymphocytes and macrophages and fibrosis as a chronic phase (phase 3). Furthermore, patients showing typical changes of phase 3 morphology together with those of phase 1 morphology were classified as reinfarct early phase (phase 4). Patients with phase 3 morphology and phase 2 morphology were classified as reinfarct PMN phase (phase 5). Two investigators (P.A.J.K. and H.W.M.N.) each judged and scored independently all slides for infarct phase. In case of discrepancy, slides were re-evaluated by both investigators until consensus was reached. The distribution of the various infarct phases among the patients is given in Table 1. In all cases, infarct age as assessed with histology corresponded with the clinical course.

*Antibodies*

Horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibody against human IgM (American Qualex, San Clemente, CA, USA) was used for immunohistochemical detection of IgM. Monoclonal antibodies (mAbs) against the complement factor C3d (mAb C3-15,
subtype: IgG-1) and against CRP (mAb 5G4, subtype: IgG-2a) have been used previously for immunohistochemical studies [10]. mAb aE11 (subtype: IgG-2a, Dako, Carpinteria, CA, USA) was used for detection of complement factor C5b-9. The mAbs were stored at 1 mg/ml in phosphate-buffered saline, pH 7.4 (PBS). Irrelevant MAbs (2 IgG-1, 1 IgG-2a and 1 IgM) were used as negative controls, and tested at concentrations similar to those used for the anti-complement and anti-CRP mAbs. These controls yielded negative results.

Immunohistochemistry

Frozen sections (5 μm thick) were mounted onto glass slides, dried for 1 h by exposure to air and fixed in acetone (‘Baker analyzed reagent’, Mallinckrodt Baker, Deventer, Netherlands). After a rinse in PBS, the slides were incubated at room temperature for 10 minutes with normal swine serum (NSS; for IgM), normal rabbit serum (NRS; for complement and CRP) (both Dakopatts; Glostrup, Denmark) or 5% (w/v) bovine serum albumin (BSA) in PBS (for C5b-9), diluted 1:10 (NSS) or diluted 1 to 50 (NRS) in 1% (w/v) BSA in PBS (PBS-BSA), (BSA from Boehringer, Mannheim, Germany). Incubation of the slides with specific antibody solutions (Abs diluted in PBS-BSA) was performed for 60 min except for mAb aE11, which was incubated overnight at 4°C (pAb against IgM was diluted 1:400; mAb C3-15 was diluted 1:1500; mAb 5G4 was diluted 1:300; mAb aE11 was diluted 1:50). The slides were washed for 30 min with PBS and slides stained with mAbs were incubated with HRP-conjugated rabbit-anti-mouse immunoglobulins (RaM-HRP; Dakopatts), diluted 1 to 25 in PBS-BSA except for mAb aE11, which was detected using EnVision (Dako, Carpinteria, CA, USA). Thereafter the slides were washed again in PBS and incubated for 3 min in 0.5 mg/ml 3,3’-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO, USA) in PBS, pH 7.4, containing 0.01% (v/v) H₂O₂, washed again, counterstained with hematoxylin for 1 min, dehydrated, cleared and finally mounted.

Co-localization of IgM, C3d, C5b-9 and CRP as well as relative staining intensities of IgM and CRP were evaluated in each patient. Furthermore, the percentage of positive surface area was determined, by subdividing the total area of the slide into four equal parts and then estimating the percentage of positive areas per visual field. Finally, an average percentage of positive surface area was calculated from these subdivisions. The slides stained with pAb against IgM, or with mAb C3-15, mAb 5G4 or mAb aE11 were serial slides.

Two investigators (P.A.J.K. and H.W.M.N.) assessed anatomical localization of the specific antibodies, as visualized by immunohistochemical staining. For the final scoring results, consensus was achieved by the two investigators.
Statistics

Statistics were performed with the SPSS statistics program (windows version 9.0). To evaluate whether observed differences were significant, paired or non-paired T-tests were used when appropriate. A $P$-value (two-sided) of less than 0.05 was considered to represent a significant difference.

Results

**IgM depositions in the human heart**

Immunohistochemical depositions of IgM were found on cardiomyocytes that were morphologically characterized as jeopardized as described in Methods (Fig. 1). Depositions of IgM found on the plasma membrane, were strikingly intensive in different areas of the macroscopic infarction zone (Fig. 1a, arrow I; figures 1b, 1c, arrows IV, V, VI), on cross-striations (Fig. 1a, arrow II) and in the cytoplasm (Fig. 1a, arrow III) of cardiomyocytes. As a negative control, an IgM-subtype antibody against Leu7 was used; this antigen is not present in cardiomyocytes of the left ventricle of the adult heart. Staining of the slides with this antibody was negative (Fig. 1g).

Focal depositions of IgM were found inconsistently on the endothelium of blood vessels in the heart of patients who died of AMI (not shown). This endothelial IgM staining was independent of the phase of infarction, since within each phase a subgroup of patients had no IgM staining of the endothelium, whereas the rest of the patients of that group had a varying amount of IgM positive vessels. Moreover, endothelium staining for IgM was not limited to the infarction area, but also occurred in adjacent sites and remote sites of the healthy part of the heart. IgM depositions on cardiomyocytes were found in the infarcted myocardium of patients with PMN-phase infarcts and PMN-phase reinfarcts. No deposition of IgM was found in the infarcted myocardium of patients with early- or chronic phase infarcts or early reinfarcts. IgM deposition was never found in the healthy, remote myocardium. Also, in the heart of a patient who died from a disorder not related to heart disease no IgM was found on cardiomyocytes. As discussed in Materials and Methods, no staining with IgG-1 or IgG-2a was found on cardiomyocytes. Replacement of specific antibody with IgG-1 or IgG-2a isotype controls yielded negative results on the cardiomyocytes.
Co-localization of IgM, complement and CRP

To test a putative co-localization of IgM, complement and CRP in the infarcted myocardium, we stained serial slides of the tissue specimens. IgM (Fig. 1d and 1h) was found to co-localize with complement factor C3d (Fig. 1e), complement factor C5b-9 (Fig. 1f) and CRP (Fig. 1f).

Figure 1: Localization of IgM on cardiomyocytes in myocardial infarct and co-localization of IgM, C3d, C5b-9 and CRP in the human heart. Localization of IgM on cardiomyocytes in the heart of a patient who had died from AMI. a: IgM deposited on the plasma membrane (arrow I), on cross-striations (arrow II) and in the cytoplasm (arrow III) of jeopardized cardiomyocytes (Magnification x630). b+c: Close up of IgM staining of the plasma membrane of jeopardized cardiomyocytes (arrows IV, V, VI) (magnification x1000). d and h: Localization of IgM, e: localization of complement factor C3d, f: localization of CRP and i: localization of complement factor C5b-9 in the heart of a patient who died after AMI (Magnification x100). As a negative control an IgM-subtype mAb against Leu7 was used (g).
As a matter of fact staining patterns of IgM, CRP and complement were strikingly similar. As was found for IgM, no deposition of complement and CRP occurred at sites remote from the infarction area. We also determined the extent of the depositions by estimating the mean surface area occupied by cardiomyocytes that were positive for IgM, complement or CRP as a percentage of the total surface area of the slides in the infarcted region (Fig. 2). The percentage of IgM-/complement-/CRP-positive surface area in patients with PMN phase infarcts or PMN phase reinfarcts was significantly higher than that of patients with early phase infarcts ($P=0.001$) or early phase reinfarcts ($P<0.021$), respectively. Moreover, this IgM-/complement-/CRP-positive area tended to be higher in PMN phase reinfarctions as compared with PMN phase infarctions ($P=0.539$ for IgM, $P=0.578$ for complement, and $P=0.698$ for CRP). To analyse the correlation between depositions of IgM, CRP and complement, scatter plots were made in which the percentages of IgM-/complement-/CRP-positive surface area of each patient was plotted, irrespective of infarct phase. This analysis revealed a linear relationship between the percentages of IgM and complement depositions (Fig. 3a; $R=0.999$, $P=0.000$), those of IgM and CRP (Fig. 3b; $R=0.994$, $P=0.000$), and those of complement and CRP (Fig. 3c; $R=0.996$, $P=0.000$).

Figure 2: Extent of IgM, complement and CRP depositions in the infarcted myocardium.
Boxplot presentation of the percentage of IgM (grey bars)-/complement (white bars)-/CRP (shaded bars)-positive myocardium. For each patient the percentage of positive surface area for the particular antibody in relation to the total area of the examined tissue was calculated as mentioned in Materials and Methods. The error bars represent minimum and maximum values, while the boxes represent the lower- and upper quartiles. The black lines within the boxes represent the medians. $n =$ the number of patients examined.
IgM localizes in infarcted human hearts

Scatter plots of the extent of IgM- /complement-/CRP-positive areas of individual patients

Scatter plots in which the extent of depositions, expressed as % of surface, of IgM, complement and CRP per patient are plotted against each other. a: IgM- versus complement; b: IgM- versus CRP; c: CRP versus complement. For each plot, the corresponding correlation coefficient (R) and the two-tailed significance (P value) are given.

Staining intensity of IgM versus CRP

As both IgM and CRP can activate complement, we attempted to assess the contribution of either adaptor molecule in complement activation by comparing relative intensities of staining (Fig. 4). For this, only PMN phase infarcts and PMN reinfarcts were analyzed because only these episodes had IgM and CRP depositions. The staining intensity of IgM and CRP clearly varied between different patients (not shown). In the 11 examined patients of the PMN phase, 4 patients (36%) had more or less identical IgM and CRP staining intensity (IgM=CRP). In 4 patients (36%) staining of IgM was less intense than that of CRP (IgM<CRP), while in 3 patients (27%) IgM staining was more intense than that of CRP (IgM>CRP). In 3 of the 7
examined patients (42%) with PMN phase reinfarctions, IgM and CRP staining intensities were comparable, in 1 patient (14%) IgM staining was less intense than that of CRP, and in 3 patients (42%) IgM stained more intensely than CRP.

Table 1: Distribution of various phases of AMI in the patients

<table>
<thead>
<tr>
<th>AMI phase</th>
<th>infarct age</th>
<th>number of patients</th>
<th>male/female</th>
<th>age range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early phase</td>
<td>0 – 12 h</td>
<td>27</td>
<td>17 / 10</td>
<td>23 - 98</td>
</tr>
<tr>
<td>PMN phase</td>
<td>12 h – 5 days</td>
<td>11</td>
<td>6 / 5</td>
<td>42 – 85</td>
</tr>
<tr>
<td>Chronic phase</td>
<td>5 days – 14 days</td>
<td>5</td>
<td>3 / 2</td>
<td>58 – 83</td>
</tr>
<tr>
<td>Reinfarction Early phase</td>
<td>chronic phase + 0 – 12 h</td>
<td>5</td>
<td>3 / 2</td>
<td>63 – 85</td>
</tr>
<tr>
<td>Reinfarction PMN phase</td>
<td>chronic phase + 12 h – 5 days</td>
<td>7</td>
<td>3 / 4</td>
<td>30 – 89</td>
</tr>
</tbody>
</table>

h = hours

Discussion

The observation that complement is locally activated by infarcted myocardium dates back over 30 years [16]. Since then it has become apparent that the complement system is an important mediator of I/R injury in the heart during AMI, as well as in other organs. Studies on the molecular mechanisms of I/R-induced activation of complement may offer new clues for therapy. Recent evidence points to CRP as an activator of complement in human myocardial infarcts [11]. Here we provide evidence for involvement of IgM in this activation as well. IgM is a known activator of complement [17-21], and appeared to be deposited in the infarcted human myocardium. Indeed, co-localization of IgM with activated C3d and C5b-9 is indicative for MAC formation. Irrelevant antibody controls indicated specificity of the observed staining for IgM. In addition, IgG staining yielded negative results. To our knowledge this is the first study showing involvement of IgM in ischemic injury in humans. The deposition of IgM showed a remarkable co-localisation with that of complement and CRP, strongly suggesting that the IgM deposited had bound to the same ligands in the ischemic heart as CRP. Apparently, these ligands are only exposed during the PMN phase of
(re-)infarction, since depositions of CRP and IgM during the other phases were virtually absent. At this moment we can only speculate about the nature of these ligands.

However, microscopic evaluation revealed that especially the plasma membrane of the ischemic cardiomyocytes harbored the ligands for IgM and CRP, since in different areas in the infarcted zone either protein was found to bind particularly to the plasma membrane of cardiomyocytes. In a former study we have shown that apolipoprotein H (ApoH), which binds to phosphatidylserine in flip-flopped membranes, co-localizes with CRP in ischemic myocardium [22]. A loss of plasma membrane integrity, also designated as membrane flip-flop [23], is a feature of jeopardized cells. Together these data suggest that structures exposed in the flip-flopped membrane of jeopardized cardiomyocytes serve as ligands for CRP and IgM. For several reasons phosphorylcholine may constitute such a structure. CRP is well known to bind to phosphatidylcholine and particularly to lyso-phosphatidylcholine, via this chemical group [4,6,24]. Indeed, a significant amount of lyso-phospholipids is generated in
the infarcted myocardium [25]. In agreement with this, we have recently revealed that type-II secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}), which generates lyso-phospholipids, enhances the binding of CRP to the plasma membrane of ischemic challenged rat cardiomyoblasts [26]. Furthermore, we have found that sPLA\textsubscript{2} co-localizes in the infarcted human myocardium with complement and CRP [27] and thus also with IgM, according to the data described in this study. A similar specificity, i.e. preferential binding to lyso-phosphatidylcholine, has recently been described for anti-phosphorylcholine IgM [28]. This IgM failed to recognize phosphatidyl lipids but did bind to lyso-phosphatidylcholine on murine apoptotic T cells, and their binding was dependent on intracellular PLA\textsubscript{2} activity [28]. It was further revealed that increased IgM binding to the apoptotic cells was accompanied by complement activation [28]. In addition to lyso-phospholipids also oxidized phospholipids in membranes may expose phosphorylcholine in a way that allows binding of IgM as well as CRP [29,30]. Increased amount of oxygen radicals is generated in myocardial ischemia [31]. Its supposed specificity for phosphorylcholine is consistent with the idea that this IgM is natural IgM. Put together, a mechanism emerges in which increased production of oxygen radicals together with enhanced PLA\textsubscript{2} activity generates binding sites in the membrane of cardiomyocytes in the ischemic myocardium, which promotes the binding of both natural IgM and CRP, and which ultimately leads to activation of complement and subsequent irreversible injury to the tissue.

We observed differences in staining intensities of CRP and IgM implying that in some patients IgM, recognizing epitopes on the membranes of ischemic cardiomyocytes, mediates complement activation, whereas in others CRP, presumably binding to the same epitopes, activates complement. Serum levels of natural IgM vary between people [32]. In addition, there are variations in the magnitude of the CRP response between patients suffering from AMI [10]. Unfortunately, we did not have the opportunity to analyze the blood samples of the patients included in this study. Hence the relationship between circulating IgM and CRP levels and the relative contribution of either protein to complement activation in the ischemic myocardium remains to be established in further studies.

Autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and mixed connective tissue disease (MCTD) are accompanied by elevated IgM levels in the circulation [33,34] and an increased risk for cardiovascular events [35,36]. In particular, increased levels of natural auto-IgM against cardiolipin (CL) are supposed to be the link between cardiovascular events and IgM in these diseases, the more since patients with ischemic heart disease may also have elevated levels of these antibodies [37,38]. Our findings would imply that during AMI, higher IgM serum levels might result in more extensive IgM
deposition in the ischemic myocardium, thereby contributing to more extensive injury and a higher mortality in patients with autoimmune diseases. We included only one patient who was suffering from sclerodermia, with autoimmune disease in this study. Though this patient indeed had very intense IgM deposition in the infarcted myocardium, the causal link between increased levels of auto-phospholipid IgM and cardiovascular events in autoimmune disorders needs to be further studied.

A number of studies have shown surprising similarities between CRP and IgM in cardiovascular disease. CRP has been shown to localize in ischemic myocardium and to enhance ischemia-reperfusion injury in the heart in a complement-dependent fashion [9,10]. IgM, as we show here, also localizes in ischemic myocardium and in mouse models enhances ischemia-reperfusion injury [12,13]. CRP and IgM both enhance foam cell formation by interacting with lipoprotein particles [39,40]. Furthermore, anticardiolipin IgM levels constitute a risk factor for atherosclerotic vascular disease [41], similarly as CRP.

The role of natural IgM in vascular disease is not clear [42], and might be considered as two sides of a coin: on the one hand natural IgM antibodies against oxidized LDL protect against atherosclerosis, as was found in mice [29], rabbits [43] and in humans [44], supporting the recent view that induction of a humoral immune response to oxidized neoepitopes may be beneficial [45]. On the other hand, the data of this study suggest that IgM antibodies against epitopes in the membranes of ischemic cardiomyocytes may enhance cardiac injury during infarction and constitute an increased risk for cardiovascular disease. Future studies should reveal the relative contribution of each side of this IgM coin to cardiovascular disease in humans.

References


