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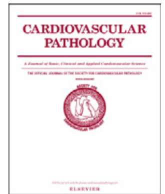

Original Article

Endogenous C1-inhibitor production and expression in the heart after acute myocardial infarction

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ABSTRACT

Background: Complement activation contributes significantly to inflammation-related damage in the heart after acute myocardial infarction. Knowledge on factors that regulate postinfraction complement activation is incomplete however. In this study, we investigated whether endogenous C1-inhibitor, a well-known inhibitor of complement activation, is expressed in the heart after acute myocardial infarction.
Materials and methods: C1-inhibitor and complement activation products C3d and C4d were analyzed immunohistochemically in the hearts of patients who died at different time intervals after acute myocardial infarction (n=28) and of control patients (n=8). To determine putative local C1-inhibitor production, cardiac transcript levels of the C1-inhibitor-encoding gene *serping1* were determined in rats after induction of acute myocardial infarction (microarray). Additionally, C1-inhibitor expression was analyzed (fluorescence microscopy) in human endothelial cells and rat cardiomyoblasts in vitro.
Results: C1-inhibitor was found predominantly in and on jeopardized cardiomyocytes in necrotic infarct cores between 12 h and 5 days old. C1-inhibitor protein expression coincided in time and colocalized with C3d and C4d. In the rat heart, *serping1* transcript levels were increased from 2 h up until 7 days after acute myocardial infarction. Both endothelial cells and cardiomyoblasts showed increased intracellular expression of C1-inhibitor in response to ischemia in vitro (n=4).
Conclusions: These observations suggest that endogenous C1-inhibitor is likely involved in the regulation of complement activity in the myocardium following acute myocardial infarction. Observations in rat and in vitro suggest that C1-inhibitor is produced locally in the heart after acute myocardial infarction.
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1. Introduction

Acute myocardial infarction (AMI) is the most prominent cause of cardiovascular-related mortality. Reperfusion of the infarct area after AMI activates a local inflammatory response. This inflammatory response clears the wound of necrotic cells and debris and provides signals to initiate reparative pathways. At the same time, however, this also results in additional cardiomyocyte loss and enlargement of the infarct area [1]. Complement activation is an important contributor to this inflammatory process in the infarcted myocardium [2] and postischemic necrosis and inflammation decreases when complement is inactivated [3]. However, the knowledge regarding complement regulation in the infarcted human myocardium remains incomplete. Such

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knowledge is vital for the development of complement-modulating therapies, which have previously been successful in different animal models of AMI [4] but had mixed success in AMI patients [5,6].

Complement regulation in the infarcted heart is complex and involves multiple factors that either activate or inhibit complement via interactions with different complement proteins. For instance, in the infarcted human myocardium, complement-activating properties have been described for C-reactive protein [7,8], IgM [9,10], and type 2 secretory phospholipase A_2 [11–13] and for complement-inhibitory properties for clusterin [14,15] and C4b-binding protein [16,17].

Another well-known inhibitor of complement activation is C1-inhibitor (C1-inh) [18,19]. Although exogenously administered, C1-inh has been shown to inhibit complement activation both in AMI patients and animal AMI models [4,20]; to the best of our knowledge, it has never been investigated in humans whether endogenous C1-inh is also involved in the regulation of complement activity in the post-AMI heart. In this study, therefore, we investigated the presence of endogenous C1-inh in time and in relation to complement activation postmortem in the hearts of AMI patients. In addition, we investigated whether ischemia/reperfusion can result in C1-inh production in the heart itself.

2. Materials and methods

2.1. Patients

Thirty-six patients, autopsied at the VU University Medical Center (VUMC), were selected retrospectively for this study (see Supplementary Table 1 for patient details). At the VUMC, either as part of the patient contract or when relatives have given explicit consent, residual autopsy material may be used for research after completion of the diagnostic process, in agreement with the Declaration of Helsinki. At autopsy, the infarct area was identified using a lactate dehydrogenase staining on heart sections. Cardiac tissue specimens were then taken from the infarcted area from 28 patients diagnosed with AMI affecting the anterior left ventricular wall and from a corresponding area of 8 patients who died of a cause unrelated to cardiac disease (controls).

Based on the infarction age, patients were subdivided into acute phase AMI (*phase 1*, $n=10$; infarctions 0–12 h old), polymorphonuclear leucocytes phase AMI (*phase 2*, $n=11$; infarctions 12 h to 5 days old), and chronic phase AMI (*phase 3*, $n=7$; infarctions 5–14 days old), according to histopathological criteria as described before [15]. The tissue was immediately frozen after autopsy and stored in liquid nitrogen.

2.2. Immunohistochemistry

Frozen heart tissue was sliced into 4-μm-thick sections, mounted on glass slides, dried, and fixed in acetone. The slides were subsequently treated with normal serum, primary antibody, and secondary antibody for 10, 60, and 30 min, respectively, at room temperature (see Supplementary Table 2 for details). Secondary antibodies were visualized by incubating the slides in 3,3-dianobenzidine (Dako, Glostrup, Denmark). The slides were counterstained with hematoxylin and covered.

Of each patient, consecutively sliced sections were used for the stainings. The necrotic cores of the infarcted cardiac tissues were located by the presence of jeopardized cardiomyocytes. Jeopardized cardiomyocytes were morphologically distinguished from nonjeopardized cardiomyocytes by a decrease in cell size and loss of nuclei and cross-striations [21]. The percentage of the surface area that stained positive for each marker relative to the total surface area of the analyzed tissue was measured using a Leica DM/LM microscope, with Qprodit v3.2 (Leica Microsystems, Rijswijk, The Netherlands) analytical software.

2.3. AMI rat model

All animal procedures were concordant to national guidelines and with permission of local Animal Ethical Committee of the VUMC.

Ten-week-old male Wistar rats were used ($n=21$; Harlan, Horst, The Netherlands). After 2 weeks of acclimatization, AMI was induced in 18 rats. The rats were anesthetized using a single subcutaneous hypnorm/dormicum (fentanyl/fluanisone 0.5 ml/kg and midazolam 5 mg/kg) injection and were ventilated at 75 breaths/min, 10–0.4 mbar (Zoovent ventilator; Instruvet, Amerongen, The Netherlands). The heart rate was monitored using Einthoven I ECG. A left thoracotomy in the fourth intercostal space was made, and the left anterior descending coronary artery was ligated subproximally using a 6.0 prolene suture (Ethicon, Somerville, NJ). Ischemia was maintained for 40 min, followed by reperfusion. The rats in the sham group ($n=3$) received the same surgical procedure without coronary artery ligation. After 30 min, 60 min, 90 min, 120 min, 4 days or 7 days of reperfusion ($n=3$ per group), the rats were terminated and heart tissue was stored at −80°C.

2.4. serping1 mRNA levels

Per rat, 100-mg heart tissue containing the infarction area was homogenized with a Polytron homogenizer (Thermo Scientific, Waltham, MA) and TriPure (Roche Applied Science, Penzberg, Germany) was used to isolate mRNA (see supplementary methods for details). From the three rats of each group, the mRNA samples were pooled prior to microarray analysis. RNA was hybridized to rat Agilent microarrays (Agilent Technologies, Santa Clara, CA) following manufacturer's protocol ("two-color microarray-based gene expression analysis"). Using Scanarray software, the image data were converted into numerical data and *serping1*, *pabpn1*, *hmbs*, and *ywhaz* mRNA levels of each time point after AMI was calculated relative to the sham-operated rats.

2.5. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated as described before [22], in agreement with all relevant guidelines. H9c2 cells (neonatal rat cardiomyoblast cell line) were purchased from ATCC (LGC Standards, Wesel, Germany). HUVECs and H9c2 cells were maintained in Medium 199 and Dulbecco's modified Eagle's medium, respectively (both from Lonza, Basel, Switzerland), enriched with serum and antibiotics (see supplementary methods for more details). Both HUVECs and H9c2 cells were cultured in an incubator under 5% CO_2/95% air atmosphere at 37°C. Cells were seeded in CC2 glass Lab-Tek chamberslides (Nunc, Roskilde, Denmark) for immunofluorescence analysis. For HUVECs, chamberslides were coated with 1% gelatine for 60 min at 37°C before seeding the cells.

Ischemia was mimicked by placing the cells in metabolic inhibition (MI) buffer, which disables cellular ATP production and uptake (see supplementary methods for more details).

Ischemia and reperfusion experiments were started when the H9c2 cells and HUVECs had grown to 70–80% and >80% confluence, respectively. For H9c2 cells, reperfusion was simulated by culturing the cells again in normal culture medium for 4 h after ischemia. Two chambers containing untreated cells were included in each experiment as controls: one stained for C1-inh and the other one stained with an isotype control antibody.

2.6. Fluorescence microscopy

After ischemia and reperfusion, the cells were fixed in 4% formaldehyde for 10 min and then permeabilized with methanol/acetone (70/30%) at −20°C for 10 min. Afterwards, the cells were blocked in 3% BSA (in PBS) for 10 min and incubated overnight at 4°C in 2% BSA (in PBS) containing primary antibodies against C1-inh or an isotype control. The next day, the cells were incubated with immunofluorescent secondary antibodies for 60 min in the dark (see Supplementary Table 2

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