Apolipoprotein H, a new mediator in the inflammatory changes ensuing in jeopardised human myocardium

H W M Niessen, W K Lagrand, H J A M Rensink, Ch J L M Meijer, L Aarden, C E Hack

Abstract
Aim—To investigate the presence of membrane “flip flop” in ischaemic human myocardium, we assessed depositions of apolipoprotein H (apoH; β2-glycoprotein 1) in ischaemic myocardium. Serum protein apoH can bind to negatively charged phospholipids and can also inhibit blood coagulation in vitro. We hypothesised that, because of its affinity for phosphatidyl serine, apoH might bind to “flip flopped” cells and would therefore be useful as a marker for membrane flip flop in vivo.

Methods—Myocardial tissue specimens were obtained from patients who had died within 14 days of acute myocardial infarction.

Results—Immunohistochemical analysis of these specimens revealed that apoH was selectively deposited in infarcted areas of human myocardium of at least one day’s duration. Depositions of apoH were not found in non-ischaemic myocardial tissue samples obtained from patients who died from other (extracardial) causes. In vitro experiments with the human leukaemia T cell line Jurkat, subjected to apoptosis by etoposide, showed that apoH was bound to the membrane of apoptotic cells. However, these experiments also indicated that flip flop itself is not sufficient for apoH binding. In addition, Jurkat cells that bound apoH were positive for activated complement complexes, as was also found in the human heart.

Conclusions—These results suggest that apoH is involved in the inflammatory processes that occur in ischaemic myocardium.

Keywords: myocardium; apolipoprotein H; inflammation; complement

Recently, we have shown co-deposition of complement with the acute phase protein C reactive protein (CRP) in infarcted sites of human myocardium. The ligand for CRP in infarcted myocardium, however, remains to be established. One possibility is that binding sites for CRP are generated in cells that have undergone so called “flip flop”. In normal cells, various phospholipids are asymmetrically distributed between the inner and outer leaflet of the membrane, PS being mainly located in the inner leaflet. In damaged cells (ischaemic, apoptotic, or necrotic cells) phospholipids of the inner and outer leaflet exchange, a phenomenon known as flip flop, leading to the exposure of PS in the outer leaflet. We hypothesised that because of its affinity for PS, apoH could be used as an in vivo marker for these “flip flopped” cells. In our present study we tested this hypothesis and searched for apoH depositions in the infarcted myocardium, in relation to those of activated complement.

Patients, materials, and methods

Patients
Patients, referred to the department of pathology for necropsy, were included in this study when at necropsy they showed signs of a recently developed acute myocardial infarction; that is, decreased lactate dehydrogenase (LD) staining (decolouration) of the affected myocardium. Most of the patients had participated in earlier studies on the involvement of CRP and complement in infarcted myocardium. Our study was approved by the ethics committee of the Free University Hospital Amsterdam.

Processing of tissue specimens
Myocardial tissue specimens were obtained from the infarcted as well as from adjacent sites. These latter sites showed normal LD staining patterns and were studied as internal controls. Before being prepared as cryosections, the tissue specimens were stored at –196°C (liquid N\textsubscript{2}). The glass slides used for microscopy were pretreated with 0.1% poly-L-lysine (Sigma Chemical Company, St Louis, Missouri, USA) to enhance the adherence of the frozen tissue sections.

Antibodies
We used a monoclonal antibody (C3-9; IgG-1 subclass) against activated complement factor C3 that has been used previously for immunohistochemical studies. Co2F3, directed against apoH, was a gift of Dr Koike (Department of
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after rinsing in PBS. Incubation of the slides (PBS) containing 1% (wt/vol) BSA (PBS-BSA) and diluted 1/50 in phosphate buffered saline (PBS-BSA) for one hour. Thereafter, the slides were incubated with antibodies against apoH monoclonal or polyclonal antibodies were washed for 30 minutes and incubated with streptavidin horseradish peroxidase conjugates (Dakopatts), diluted 1/500 in PBS-BSA for one hour. Thereafter, the slides were washed again in PBS and incubated for four minutes in 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in PBS, pH 7.4, containing 0.01% (vol/vol) H2O2. The slides were then washed again, counterstained with haematoxylin for 40 seconds, dehydrated, cleared, and finally mounted.

Microscopic criteria were used to estimate infarct duration in all myocardial tissue specimens. Jeopardised fibres were characterised by the intensity of eosinophilic staining of involved myocardium, loss of nuclei and cross striation, polymorphonuclear neutrophil and lymphocyte infiltration, and fibrosis. However, because morphological judgement is more reliable in paraffin wax embedded slides, corresponding paraffin wax embedded tissue slides were also made, to confirm the determination of jeopardised versus non-jeopardised tissue. Two independent investigators (HWMN, WKL) each judged and scored all slides for infarct duration and anatomical localisation of specific antibody as visualised by immunohistochemical staining. Anatomical localisations examined were myofibre (membrane, cytoplasm, cross striations) and bloodvessel elements. For the final scoring results, a consensus was achieved by the two investigators.

Results

Patients

Myocardial tissue specimens were obtained from 17 patients who had died after acute myocardial infarction as confirmed by necropsy performed within 24 hours after death (table 1). Specimens were obtained from the infarcted as well as from the unaffected myocardial tissue. The infarct age, assessed by microscopic analysis of infarct duration in all myocardial tissue specimens. Jeopardised fibres were characterised by the intensity of eosinophilic staining of involved myocardium, loss of nuclei and cross striation, polymorphonuclear neutrophil and lymphocyte infiltration, and fibrosis. However, because morphological judgement is more reliable in paraffin wax embedded slides, corresponding paraffin wax embedded tissue slides were also made, to confirm the determination of jeopardised versus non-jeopardised tissue. Two independent investigators (HWMN, WKL) each judged and scored all slides for infarct duration and anatomical localisation of specific antibody as visualised by immunohistochemical staining. Anatomical localisations examined were myofibre (membrane, cytoplasm, cross striations) and bloodvessel elements. For the final scoring results, a consensus was achieved by the two investigators.

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Table 1  Duration of infarctions in the patients included

<table>
<thead>
<tr>
<th>Infarction age</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 12 hours</td>
<td>3</td>
</tr>
<tr>
<td>&gt; 12 to &lt; 24 hours</td>
<td>1</td>
</tr>
<tr>
<td>1–3 days</td>
<td>4</td>
</tr>
<tr>
<td>3–5 days</td>
<td>2</td>
</tr>
<tr>
<td>5–9 days</td>
<td>3</td>
</tr>
<tr>
<td>9–14 days</td>
<td>4</td>
</tr>
</tbody>
</table>
As shown recently, complement was localised in infarcted sites of human myocardium. ApoH, as detected by the biotinylated monoclonal antibody, was found in the same parts that stained positive for complement (fig 1). Notably, staining for apoH was most intense at the plasma membrane of cardiomyocytes. Cytoplasmatic localisation of apoH was also found, although staining for apoH in the cytoplasm was clearly less than that of the plasma membrane. Some staining of cross striations was also found (fig 2). In larger infarcts, the border zone appeared to stain more intensively for apoH than the centre of the infarcted region.

Similar to complement, apoH was not detected in infarctions of less than 24 hours duration. Furthermore, apoH was not found on endothelium, in contrast to complement. Similar staining results were obtained when a biotinylated rabbit polyclonal antibody was used against apoH (fig 3).

Staining of the myocardial tissue specimens with irrelevant control antibodies yielded negative results. In addition, internal controls—specimens taken from non-infarcted sites of the myocardium of the same patient—did not stain for C3 or apoH. Furthermore, myocardial tissue specimens from an immature child who died in utero at an amenorrhoea duration of 22 weeks (these specimens were taken to represent a pure, non-ischaemic myocardial control) did not stain for C3 or apoH, and neither did an old infarction (> 1 year old).

Unfortunately, immunohistochemical studies have the limitation that the well known marker of membrane flip flop, annexin V, cannot be used reliably in tissue slides of the heart. For this reason, we performed in vitro studies using Jurkat cells. These cells were incubated with etoposide overnight, which causes them to die as a result of apoptosis. Subsequently, the cells were incubated with anti-apoH or anti-C3. The flip flop phenomenon was assessed by staining for annexin V. The cells that were annexin V negative did not bind apoH (table 2), whereas annexin V positive cells did. Recently Manfredi et al have shown that Jurkat cells, positive for annexin V, stained for apoH. However, when we characterised these cells in more detail, it appeared that only cells that also stained with propidium iodide...
Table 2 Binding of annexin V, activated complement, and apolipoprotein H to apoptotic cells

<table>
<thead>
<tr>
<th>Serum</th>
<th>Annexin V (-)</th>
<th>Annexin V (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>(-)</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>(+)</td>
<td>47</td>
<td>55</td>
</tr>
</tbody>
</table>

Jurkat cells were incubated with 25 µM etoposide overnight. Cells were then analysed for binding of annexin V, activated complement, or apolipoprotein H via fluorescent activated cell sorter (FACS) analysis.

Serum concentration was 2.5%.

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Correspondence

MPO-ANCA may produce a combination of P-ANCA and atypical cytoplasmic ANCA indirect immunofluorescent patterns on certain ethanol fixed neutrophil substrates

The P-ANCA pattern is defined as perinuclear indirect immunofluorescent (IF) staining on ethanol fixed normal human neutrophils. This pattern is an artefact of ethanol fixation, dependent on the redistribution of certain cationic neutrophil granule proteins (such as myeloperoxidase (MPO), lactoferrin, and lysozyme) around the negatively charged nuclear membrane. However, certain MPO-ANCA can produce cytoplasmic rather than perinuclear IIF staining, possibly related to a subpopulation of epitopes on MPO that are not detected by the ORGenTec ANCA kit. This pattern is an artefact of ethanol fixation, dependent on the redistribution of certain cationic neutrophil granule proteins (such as myeloperoxidase (MPO), lactoferrin, and lysozyme) around the negatively charged nuclear membrane. We now report that MPO-ANCA positive sera may produce a combination of P-ANCA and atypical cytoplasmic ANCA IIF patterns on certain ethanol fixed neutrophil substrates, potentially leading to interpretative difficulties.

Sera from six patients with biopsy confirmed microscopic polyangiitis (at different stages of disease activity) were selected because of initial difficulties in the interpretation of their IIF patterns on ethanol fixed neutrophil slides from Inova Diagnostics (San Diego, California, USA). All six sera were MPO-ANCA positive and proteinase 3-ANCA (PR3-ANCA) negative by the corresponding ORGenTec (Mainz, Germany) enzyme linked immunosorbent assay (ELISA). PR3-ANCA positive serum from a patient with biopsy confirmed Wegener’s granulomatosis was also tested. To establish whether other ANCA antigen specificities were present, all sera were tested on the ORGenTec ANCA Combi-kit ELISA containing proteinase-3, MPO, lactoferrin, elastase, cathepsin G, lysozyme, and bacterial/peptidase increasing protein (BPI). IIF was then repeated on all sera on two separate occasions using in house (kindly supplied by the Division of Immunology, Royal Brisbane Hospital) and two commercial (Inova Diagnostics (different batch) and Medical and Biological Laboratories (MBL, Nagoya, Japan)) ethanol fixed neutrophil slides. The IIF staining patterns and end point titres were determined by consensus.

Table 1 summarised the results. In four of the six sera, no reactivity other than MPO-ANCA was detected using the ANCA Combi-kit ELISA. Of the other two sera, one also contained lactoferrin-ANCA and the other lysozyme-ANCA. Nevertheless, in addition to P-ANCA staining, atypical cytoplasmic staining was consistently produced by all six MPO-ANCA sera on the Inova slides, but not on the MBL or in house slides. These findings were reproducible on two different batches of neutrophil slides from the former manufacturer. Our small study demonstrates that sera containing MPO-ANCA may produce a combination of P-ANCA and atypical cytoplasmic IIF patterns on certain ethanol fixed neutrophil substrates. The recent International Consensus Statement recommends that such combined patterns be reported as “atypical ANCA”. Because atypical ANCA are not strongly associated with microscopic polyangiitis or Wegener’s granulomatosis, an atypical ANCA IIF report on these sera could potentially erroneously lead the requesting clinician away from the correct diagnosis. However, in all six sera, the positive MPO-ANCA result would hopefully redirect attention towards a possible diagnosis of systemic necrotising vasculitis. We have subsequently found that these combined IIF patterns do not occur with all MPO-ANCA positive sera on the Inova slides, and thus speculate that the phenomenon might be caused by factors in the ethanol fixation conditions of these slides resulting in the differential redistribution of different MPO epitopes. Therefore, we recommend that laboratories using this brand (and possibly other commercial brands) of ethanol fixed neutrophil slides be aware of this phenomenon, and consider repeating any sera producing such combined “atypical ANCA” IIF patterns on alternative ethanol fixed neutrophil substrates to clarify their true IIF pattern. Furthermore, antigen specific ELISA testing for MPO-ANCA and PR3-ANCA should also be performed on all such sera because combining IIF and ELISA in ANCA testing improves overall diagnostic specificity/predictive value compared with using either test alone.

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High prevalence of serum markers of coeliac disease in patients with chronic fatigue syndrome

There has been recent interest in the possibility that undiagnosed coeliac disease (CD) might be the cause of diverse clinical symptoms, most particularly “tired all the time”. A recent study reported a prevalence of three in 100 cases in a primary care environment in which samples were taken from patients with a range of symptoms and signs. The second most frequent symptom reported by the endomyosal antibody (EMA) positive patients was “being tired all the time”. We decided to examine the prevalence of EMA in patients attending our tertiary referral centre with the diagnosis of chronic fatigue syndrome (CFS).

We tested serum from 100 consecutive patients (47 men, 53 women; median age, 40 years; range, 18–75) referred to our specialist clinic and satisfying the standard CDC criteria for a diagnosis of CFS, and from 100 healthy control subjects (45 men, 55 women; median age, 40 years; range, 18–68) who were blood donors at the South East Thames Blood Transfusion Service. The CFS samples had been stored as part of other studies, and were analysed retrospectively. EMA of the IgA class were detected by indirect immunofluorescence (IF) using cryostat sections of distal primate oesophagus as substrate (Binding Site, Birmingham, UK). Positive samples were confirmed using an enzyme linked immunosorbant assay (ELISA) for the detection of antieti tissue transferase antibodies (Menarini Diagnostics, Wokingham, England).
of CD being misdiagnosed as CFS.

Only been two reports concerning three cases of unsuspected positive EMA tests in people primary care of a surprisingly high frequency of undiagnosed celiac disease with atypical features using antiretulcin and antigliadin antibodies. Q J Med 1995;88:717–19.


Correction


G Visser (Department of Cardiology, Free University Hospital, 1007 MB Amsterdam, The Netherlands) was mistakenly omitted from the list of authors of this paper. The journal apologises for any inconvenience that this may have caused.

Calendar of events

Diagnostic Histopathology of breast Disease
23–27 April 2001, Hammersmith Hospital (Imperial School of Medicine), London, UK
Further details: Wolfson Conference Centre, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK. (Tel +44 020 8383 3117/3227/3245; fax +44 020 8383 2428; email wcc@ic.ac.uk)

Gynecologic and Obstetric Pathology
26–29 April 2001, Fairmont Copley Plaza, Boston, Massachusetts, USA
Further details: Department of Continuing Education, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, USA. (Tel +1 617 432 1525; fax +1 617 432 1562; email hms-cme@hms.harvard.edu)

BSCC London Spring Tutorial: Lung and Pleural Cavity Fluid Cytology
27 April 2001, Guy's Hospital, London, UK
Further details: BSCC Office, PO Box 352, Uxbridge UB10 9TX, UK. (Tel +44 01895 274 020; fax +44 01895 274 080; email lesley.couch@psilink.co.uk)

International Consultation on the Diagnosis of Noninvasive Urothelial Neoplasms
11–12 May 2001, University of Ancona School of Medicine, Torrette, Ancona, Italy
Further details: R Montironi, Ancona Italy (email r.montironi@popsci.unian.it), DG Bostwick, Richmond, VA, USA (email bostwick@bostwicklaboratories.com), P-F Bassi, Padua, Italy (email bassip@ux1.unipd.it), M Droller, New York, USA (email michael.droller@smplink.mssm.edu), or D Waters, Seattle, WA, USA (email waters@vet.vet.purdue.edu)

Human Adverse Drug Reactions
30 May 2001, Royal College of Pathologists, London, UK
Further details: Michelle Casey, Academic Activities Coordinator, 2 Carlton House Terrace, London SW1Y 5AF, UK. (Tel +44 020 7451 6700; fax +44 020 7451 6701; www.rcpath.org)

Professional Standards of Pathologists in a Modern NHS Pathology Service
7 June 2001, Royal College of Pathologists, London, UK
Further details: Michelle Casey, Academic Activities Coordinator, 2 Carlton House Terrace, London SW1Y 5AF, UK. (Tel +44 020 7451 6700; fax +44 020 7451 6701; www.rcpath.org)

Infectious Hazards of Donated Organs
28 June 2001, Royal College of Pathologists, London, UK
Further details: Michelle Casey, Academic Activities Coordinator, 2 Carlton House Terrace, London SW1Y 5AF, UK. (Tel +44 020 7451 6700; fax +44 020 7451 6701; www.rcpath.org)

Recent Advances in Genetics
5 July 2001, Royal College of Pathologists, London, UK
Further details: Michelle Casey, Academic Activities Coordinator, 2 Carlton House Terrace, London SW1Y 5AF, UK. (Tel +44 020 7451 6700; fax +44 020 7451 6701; www.rcpath.org)

BSCC Annual Scientific Meeting
9–11 September 2001, Majestic Hotel, Harrogate, UK
Further details: BSCC Office, PO Box 352, Uxbridge UB10 9TX, UK. (Tel +44 01895 274020; fax +44 01895 274080; email lesley.couch@psilink.co.uk)