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

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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” 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 N2). 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. Co23, 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) for 10 minutes with normal rabbit antibodies using L-C-biotin-Hydroxysuccimide ester (Pierce Chemical Co., Rockford, IL) was performed according to the manufacturer’s instructions.

**FACS ANALYSIS**

The human leukaemia T cell line Jurkat was maintained in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco, Grand Island, New York, USA) supplemented with 5% (vol/vol) fetal calf serum (FCS), 100 µg/ml streptomycin (Gibco), 50 µM 2-mercaptoethanol, and 20 µg/ml human transferrin (Sigma) (culture medium) at 37°C in a humid atmosphere and 5% CO₂.

Apoptosis was induced in the Jurkat cells by incubation with 25 µM etoposide (Sigma) overnight. The cells were then separated from the medium by centrifugation at 800 xg for six minutes, resuspended in IMDM (no FCS) in a 96 well round-bottom plate (100 µl/well, 200 000 cells/well), and washed once with IMDM. Thereafter, cells were incubated with serum (or purified apoH) diluted in IMDM (50 µl/well), for 30 minutes at 37°C. After washing four times with BB (1.19 g NaCl, 0.19 g KCl, 0.10 g CaCl₂, 0.1 g MgCl₂, in 500 ml, pH 7.4) and BA (0.5% (wt/vol) bovine serum albumin (BSA) (Boehringer Mannheim), 0.02% (wt/vol) azide), biotinylated anti-apoH antibodies (5 µg/ml in buffer BB-BA, 50 µl/well) or anti-C3 monoclonal antibody (C3-9) were added to the cells for 30 minutes at 4°C. After three washes with BB-BA buffer, a mixture of PE labelled streptavidin (Becton Dickinson, San Jose, California, USA) and FITC labelled annexin V (Boehringer Ingelheim), diluted 1/200 and 1/2000 in BB-BA, 50 µl/well) or anti-C3 monoclonal antibody (C3-9) were added to the cells for 30 minutes at 4°C. After three washes with BB-BA buffer, a mixture of PE labelled streptavidin (Becton Dickinson, San Jose, California, USA) and FITC labelled annexin V (Boehringer Ingelheim), diluted 1/200 and 1/2000 in BB-BA buffer, respectively, was added to the cells (final volume 50 µl/well). After 20 minutes of incubation at 4°C in the dark, the cells were washed three times with BB-BA buffer, respectively, was added to the cells (final volume 50 µl/well). After 20 minutes of incubation at 4°C in the dark, the cells were washed three times with BB-BA. Finally, cells were resuspended in 100 µl BB-BA in epi-tubes and analysed on a FACScan (Becton Dickinson, San Jose, California, USA).

**IMMUNOHISTOCHEMISTRY**

Frozen sections (4 µm thick) were mounted on to glass slides, dried for one hour by exposure to air, and fixed in acetone (“Baker analysed reagent”; Mallinkrodt Baker BV, Deventer, Holland). The slides were incubated at room temperature for 10 minutes with normal rabbit serum (Dakopatts A/S, Glostrup, Denmark) and diluted 1/50 in phosphate buffered saline (PBS) containing 1% (wt/vol) BSA (PBS-BSA) after rinsing in PBS. Incubation of the slides with specific antibody solutions was performed for 60 minutes at room temperature (C3-9 diluted 1/1000 in PBS-BSA; biotinylated antibody: IgG1 and mouse myeloma protein, MOPC (Cappel, Organon Teknika, Turnhout, Belgium).

The slides incubated with antibodies against complement were washed for 30 minutes with PBS and incubated with horseradish peroxidase conjugated rabbit antimouse immunoglobulins (RaM-HRP; Dakopatts A/S), diluted 1/25 in PBS-BSA. The slides incubated with biotinylated anti-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 myofibres, 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 (HWNN, 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 microscopy, was classified, as follows: (1) <12 hours, (2) >12 to <24 hours, (3) 1–3 days, (4) 3–5 days, (5) 5–9 days, and (6) >9–14 days.

**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>
scopical criteria, varied from less than 12 hours to more than 2 weeks.

LOCALISATION OF ACTIVATED COMPLEMENT AND apoH IN INFARCTED MYOCARDIUM

As shown recently, complement was localised in infarcted sites of human myocardium. Apo-H, 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>Annexin V (-) Serum</th>
<th>Annexin V (+) Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
</tr>
<tr>
<td>Anti-apoH</td>
<td>13</td>
</tr>
<tr>
<td>Anti-Clq</td>
<td>10</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%. Results are mean fluorescent intensity of five experiments. Anti-apoH was biotinylated rabbit polyclonal 34.

(and thus were beyond the early phase of apoptosis as characterised by the flip flop phenomenon) bound apoH. This indicates that staining for apoH is not discriminative in detecting cells that only underwent flip flop of the membrane. However, similar to the immunohistochemical staining results in the heart, apoH positive cells also bound complement, suggesting a role of both mediators in the inflammatory mediated process of cell death.

Discussion
The precise mechanisms contributing to myocardial cell death in the human myocardium after infarction are still not fully understood. We have hypothesised that in jeopardised myocardium the membrane of cardiomyocytes might become perturbed, thereby becoming a target for acute phase proteins such as serum phospholipase A2 and CRP. This would result in fixation of CRP to the cells and the subsequent activation of complement. Indeed, colocalisation of complement and CRP in jeopardised human myocardium was recently demonstrated, supporting this hypothetical mechanism.

Perturbation of the cell membrane involves an exchange of phospholipids of the inner and outer leaflets, resulting in flip flop of the membrane. As a consequence of this process, cells expose PS in the outer leaflet. Flip flop of cell membranes in vivo is difficult to demonstrate. It has been shown that in vitro apoH binds to PS. Because of this property, apoH deposition in tissues may reflect flip flop of membranes in vivo. In our present study, we indeed found that apoH is localised to jeopardised human myocardium. Moreover, apoH in particular appeared to bind to the membranes of cardiomyocytes, supporting its supposed function as a marker for membrane flip flop. However, in vitro experiments in human leukemia T cell Jurkat cells indicate that flip flop itself is not sufficient for apoH binding.

In addition to binding to PS, apoH might also bind to cardiolipin. Cardiolipin is a constituent of the inner and outer mitochondrial membrane. Thus, cardiolipin might also serve as a binding site for apoH. This could explain the cytosolic localisation of apoH that we saw in a few of the infarctions. The identity of the ligands for apoH on (apoptotic) cells is currently under investigation.

In addition, another apolipoprotein, apolipoprotein J (clustrin) has been found to be colocalised in ischaemic human hearts together with complement membrane attack complex (MAC). It has been suggested that apolipoprotein J might be involved in the clearance of damaged and necrotic tissue, together with MAC. A similar role might be played by apoH, especially because the clearance of liposomes by the liver in mice is in part mediated by plasma derived apoH. Interestingly, after interaction with serum, liposomes not only become positive for apoH but also for complement. In the human myocardium and in vitro experiments with Jurkat cells, we have shown colocalisation of activated complement and apoH. This raises the possibility that apoH, bound to cells, might trigger or enhance complement activation, directly or indirectly via CRP-ligand complexes.

In conclusion, for the first time we have shown that apoH is localised in infarcted human myocardium to areas that also contain CRP and activated complement. We therefore hypothesise that apart from complement, CRP, and apolipoprotein J, apoH is a new player in the inflammatory changes ensuing in infarcted myocardium.

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Apolipoprotein H in jeopardised human myocardium

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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 IF staining, possibly related to a subpopulation of epitopes on MPO that do not redistribute with ethanol fixation. 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 and diagnostic 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 Combi-kit ELISA (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/permeability 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 ANCA 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 ELISA 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/precision 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 coeliac disease 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 esophagus as substrate (Binding Site, Birmingham, UK). Positive samples were confirmed using an enzyme linked immunosorbant assay (ELISA) for the detection of antityrosine transglutaminase antibodies (Menarini Diagnostics, Wokingham, UK).

Table 1 MPO-ANCA and PR3-ANCA ELISA, ANCA Combi-kit® ELISA, and ANCA IIF results

<table>
<thead>
<tr>
<th>Sera</th>
<th>MPO-ANCA</th>
<th>Proteinase-3-ANCA</th>
<th>Inova Diagnostics</th>
<th>MBL</th>
<th>In house cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MPO positive (58)</td>
<td>MPO (6.4)</td>
<td>PR3 negative</td>
<td>P (1/160)</td>
<td>P (1/160)</td>
</tr>
<tr>
<td>2</td>
<td>MPO positive (61)</td>
<td>MPO (6.9)</td>
<td>PR3 negative</td>
<td>P (1/160)</td>
<td>P (1/160)</td>
</tr>
<tr>
<td>3</td>
<td>MPO positive (8)</td>
<td>MPO (1.6)</td>
<td>PR3 negative</td>
<td>P (1/160)</td>
<td>P (1/160)</td>
</tr>
<tr>
<td>4</td>
<td>MPO negative</td>
<td>MPO (1.3)</td>
<td>PR3 negative</td>
<td>P (1/160)</td>
<td>P (1/160)</td>
</tr>
<tr>
<td>5</td>
<td>MPO positive (&gt;100)</td>
<td>MPO (9.1)</td>
<td>PR3 negative</td>
<td>P (1/160)</td>
<td>P (1/160)</td>
</tr>
<tr>
<td>6</td>
<td>MPO positive (36)</td>
<td>MPO (5.2)</td>
<td>PR3 negative</td>
<td>P (1/160)</td>
<td>P (1/160)</td>
</tr>
<tr>
<td>7</td>
<td>MPO negative</td>
<td>PR3 (6)</td>
<td>PR3 negative</td>
<td>P (1/160)</td>
<td>P (1/160)</td>
</tr>
</tbody>
</table>

ORGenTec MPO-ANCA and PR3-ANCA IgG ELISA: positive, >5 U/ml; negative, <5 U/ml.

ORGenTec ANCA Combi-kit® IgG ELISA OD ratio: positive, >1; negative, <1 (only positive results shown).

IIF staining pattern: C, classic granular cytoplasmic IIF staining with central/interlobular accentuation; P, perinuclear.
UK), tissue transglutaminase being the auto-
antigen responsible for the IF pattern of
EMA. To exclude selective IgA deficiency,
sperm IgA concentrations were measured
by laser nephelometry using specific antisera
according to the manufacturer’s instructions
(Behring Laser Nephelometer II; Dade Be-
hring, Dortmund, Germany).

Two of the 100 CFS samples were positive
for EMA using IF, and this was confirmed by
ELISA, but none of the 100 control samples
was positive. None of the subjects had selec-
tive IgA deficiency. Mean (SD) sperm IgA
concentrations among patients with CFS
were 2.1 g/litre (0.98). Neither of the positive
cases, both women aged 27 and 54, had
reported symptoms typical of CD, although
one had a history of constipation. Routine
blood tests including serum proteins and full
blood count were normal, and both had been
seen by consultant physicians before eval-
uation. Both had histories of hypothyroidism,
were taking long term thyroxine, and were cur-
cently euthyroid. Before the diagnosis of CD
was made retrospectively, both had received
cognitive behaviour therapy (CBT), a stand-
dard treatment for CFS. In both cases, CBT
led to a substantial improvement in the qual-
ity of life and physical activity, but neither
case was considered to be a remission.

However, there is now evidence from
primary care of a surprisingly high frequency
of unsuspected positive EMA tests in people
with non-specific symptoms and a suggestion
that a higher index of suspicion is needed
when assessing such patients. We now
extend that observation to our CFS clinic.
Indeed, given our prevalence of 2%, and the
idea that there is treatment for CD, we now
suggest that screening for CD should be
added to the relatively short list of mandatory
investigations in suspected cases of CD.

Diagnostic Histopathology of breast
Disease
23–27 April 2001, Hammersmith Hospital
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BSSC London Spring Tutorial: Lung
and Pleural Cavity Fluid Cytology
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International Consultation on the
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Infectious Hazards of Donated Organs
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Recent Advances in Genetics
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