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ORIGINAL ARTICLE

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# Human decay-accelerating factor expressed on rat hearts inhibits leukocyte adhesion

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**Abstract** In xenotransplantation the use of donors transgenic for recipient-type complement regulatory protein decay-accelerating factor (DAF/CD55) or membrane cofactor protein (MCP/CD46) protects grafts against hyperacute rejection (HAR), which is primarily mediated by xenoreactive natural antibodies and complement. In the Langendorff model, we previously demonstrated that rat hearts transgenic for human CD55 (hCD55), perfused with human serum, were protected against HAR. However, ex vivo, these hearts were found to be destroyed by a process occurring after the period of HAR. The question arose as to whether hearts transgenic for hCD55 are also protected against adhesion and infiltration by cells implicated in the early phases of xenograft rejection. The aim of the present study was to analyze this process in the ex vivo heart perfusion model. hCD55-transgenic rat hearts and their controls were perfused with either heat-inactivated or normal human blood solutions for 60 min. Although most of the hearts had stopped beating within the 60-min perfusion period, the perfusion was not stopped to enable adhesion of cells during a fixed period identical for all groups. Independent of the presence of complement, H&E-stained tissues of hCD55-transgenic hearts revealed fewer PMN leukocytes adhering to the endothelium than the controls (mean: 31% vs 60%). Standard histology and immunohistochemistry showed that hCD55-transgenic hearts exhibited less interstitial edema, hemorrhage, microthrombosis, fibrin deposition, and leukocyte infiltration than did the controls. All hearts showed mild to moderate levels of P-selectin and similar levels of ICAM-1, C3c, C9, IgA, IgG, and IgM deposition. hCD55 expressed on rat hearts not only inhibits complement activation, but also human leukocyte adhesion and apparently functions as an anti-adhesion molecule. hCD55 is an efficient factor in protecting grafts against HAR and protects the graft against adhesion of leukocytes as well.

**Keywords** Decay-accelerating factor · Human leukocytes · Rat · Heart · Xenotransplantation

## Introduction

Hyperacute rejection (HAR) is the first immunological barrier to be overcome before organs can be transplanted between discordant species [1, 6, 11]. HAR is primarily mediated by the binding of xenoreactive natural antibodies to the vascular endothelium followed by complement activation, resulting in activation or

damage of endothelial cells. The major features of HAR in vivo are platelet aggregation and adhesion, fibrin deposition, thrombosis, interstitial edema, hemorrhage, and vasoconstriction. HAR can be averted by the blocking of either xenoreactive natural antibodies or complement [2, 8]. The second immunological barrier in the discordant situation is acute vascular rejection (AVR), which can occur without the presence of T cells. AVR involves type-II endothelial cell activation, including up-regulation of proinflammatory molecules, infiltration of host monocytes and natural killer cells into the graft, and cytokine production, leading to chemotaxis of leukocytes [2]. The third immunological barrier to be overcome is the cellular immune response, in which mechanisms similar to those in allograft rejection are involved. The use of donors transgenic for a certain recipient-type complement regulatory protein protects grafts against HAR. We demonstrated earlier that rat hearts transgenic for human CD55 (hCD55), perfused with human serum, were protected against HAR [7]. Although the hearts were not rejected by HAR, they were finally rejected by a delayed form of HAR. Since CD55 prolongs the survival of hearts perfused with human serum, by regulating the activation of endothelial cells, we wondered whether CD55 might have an extra effect, i.e., inhibition of the adhesion of cells to the vascular endothelium. Others have found that CD55 can, indeed, function as an anti-adhesion molecule [9]. Therefore, the aim of our study was to analyze whether rat hearts transgenic for hCD55 are protected against cell-mediated immunity upon perfusion with human blood in the Langendorff device. We analyzed the contribution of complement in the adhesion process by using either normal or decomplemented (=heat-inactivated) blood.

## Materials and methods

### Animals

Male and female heterozygous and homozygous hCD55-transgenic rats were bred using a construct containing the hCD55 cDNA under the transcriptional control of the endothelial cell-specific human ICAM-2 promoter [7]. hCD55 expression was assessed by immunohistology, FACS analysis of purified endothelial cells, and Southern blot techniques as described by Charreau et al. [7]. Wistar rats were used as controls. When control hearts were perfused with 12% control blood solution, the mean beating time was 55 min, and if pure Krebs-Henseleit solution was used, hearts kept on beating for 190 min. Based on the results in the controls, we performed a 60-min perfusion of all hearts in the experimental groups. The animals were housed in a certified animal breeding facility under standard conditions and had free access to standard rat chow and water acidified to pH 2.2 ad libitum. The code of *Principles of Laboratory Animal Care* (NIH publication No. 86-23, revised 1985) was followed. The experimental protocol was approved by the Committee on Animal Research of Erasmus University.

### Surgical procedure and ex vivo heart perfusion (Langendorff)

Prior to the surgical procedure the rats were anesthetized with isoflurane inhalation (PCH Pharmachemie, Haarlem, The Netherlands), and 1.0 ml heparin (50 IU/ml) (Leo Pharmaceutical Products, Weesp, The Netherlands) was injected intravenously. Via two lateral incisions the thorax was opened and the heart was removed. A 14-gauge in-dwelling cannula (Vasocan, Braun, Melsungen, Germany) was inserted into the aorta and secured with a 4–0 suture (NC-Silk, Braun). The heart was directly linked to the Langendorff circuit and perfused with oxygenated Krebs-Henseleit (KH) solution, as previously described by Verbakel et al. [12]. All hearts were perfused with KH solution (pure KH solution, without Haemaccel) for 10 min and were then switched to perfusion for 60 min with 12% human blood or 12% heat-inactivated human blood. During this 60-min perfusion, hearts were monitored constantly for apex frequency and flow by multichannel registration. Most of the hearts perfused with blood stopped beating within the 60-min perfusion period. The perfusion was not stopped in order to enable adhesion of cells during a fixed period identical for all groups. The time measured until they stopped beating was also noted.

### Perfusion solutions

Frozen, pooled, human O-type plasma was obtained from the blood bank of University Hospital Dijkzigt (Rotterdam, The Netherlands). For each experiment fresh human serum was prepared from thawed human plasma after the induction of clotting with thrombin (Dade Behring, Newark, N.J.; 0.5 ml thrombin/200 ml human plasma). Heat inactivation, i.e., complement inactivation, was realized by the heating of serum at 56 °C for 30 min. O-type blood was collected in 9-ml EDTA test tubes and immediately processed as follows. After centrifugation (2,000 rpm, 10 min), the plasma was removed and replaced by a similar volume of serum. To prepare the 12% (heat-inactivated) solution for perfusion, we diluted the blood with KH stock solution, prepared as described earlier. Erythrocytes, platelets, and white blood cells (WBCs) were present in the blood solution. A sample of the 12% (heat-inactivated) human blood mixture was taken from each freshly prepared solution, and the WBCs counted in a blood cell counter (Sysmex F800, Japan). The mean WBC concentration of this sample was $0.5\times10^9$/l. In all perfusion solutions we finally added 0.1–0.2 ml heparin (50 IU/ml) to prevent coagulation.

### Immunohistochemistry

After 60-min perfusion each heart was cut in two. One sample was fixed in 3.6% formaldehyde solution. Following dehydration and paraffin embedding, 4-$\mu$m-thick sections were cut and stained with hematoxylin and eosin (H&E). By means of standard histology (H&E), all hearts were analyzed for differences in the number of leukocytes adhering to the endothelium. In these stained tissues, we examined 20 medium-sized vessels to assess the presence of leukocytes. In each vessel the number of leukocytes adhering to the endothelium and the number of leukocytes present in the lumen were counted. Finally, the percentage of adherence was calculated. The other part of the sample was snap-frozen and stored at −80 °C until sectioned for immunohistochemistry. Frozen tissue sections (6 $\mu$m thick) were prepared in a Microm HM 560 cryostat and stained for anti-human C3c and C9 antibodies, mouse anti-rat CD54 (=ICAM-1), rabbit anti-human CD62P (=P-selectin), and rabbit anti-human IgA, IgG, and IgM. Rabbit anti-human C3c complement fluorescein isothiocyanate (FITC), rabbit anti-human C9, and rabbit anti-human IgA, IgG, and IgM, were obtained from DAKO, ITK, and Clindia Benelux, Leusden, The Netherlands. Rabbit anti-human CD62P, which cross-reacts with rat P-selectin, was obtained from Pharmingen, Becton Dickinson, Woerden, The

Netherlands, and mouse anti-rat CD54 (=ICAM-1) from Serotec, Breda, The Netherlands. C3c and C9 staining was done as previously described by Verbakel et al. [12]. P-selectin, (1:200), IgA (1:400), IgG (1:400), and IgM (1:400) staining was performed in the same way as C9 staining [10]. ICAM-1 staining was performed in the following way: the frozen sections were thawed at room temperature for 1 h, fixed with acetone for 10 min and air-dried for 15 min. With a peroxidase anti-peroxidase (PAP) pen, circles were drawn around the tissue sections which were then washed with PBS three times. Sections were incubated for 10 min with 0.03–0.1% $H_2O_2$ (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in a humidified atmosphere and rinsed three times with PBS. After preincubation for 15 min with 0.1% normal rabbit serum, diluted (1:200) mouse anti-rat ICAM-1 antibody was added, and the sections were incubated for 60 min in humidified surroundings. Again the sections were washed three times with PBS. Sections were incubated with secondary rabbit anti-mouse antibody for 30 min and washed three times with PBS. Diaminobenzidine (DAB; DAKO, Copenhagen, Denmark) was added and sections were incubated for 5–15 min. The DAB reaction was stopped by flushing with water for 5 min. Sections were put in Mayer's hematoxylin for 40 s, washed, and were then put into an ammonia solution. Dehydration was followed by a xylene step. The slides were covered with a coverslip.

Anti-human C3c-stained sections were examined under a fluorescence microscope, and anti-human C9-, P-selectin-, ICAM-1-, IgA-, IgG-, and IgM-stained sections under a light microscope. The intensity of C3c and C9 depositions and P-selectin and ICAM-1-expression on the heart tissues was scored blind via semi-quantitative analysis: 0 = no staining, 0.5 = mild staining, and 1 = dense staining. IgA, IgG, and IgM depositions were scored semi-quantitatively as well: 0 = no staining, 0.5 = mild staining, 1 = moderate staining, and 2 = dense staining.

### The experimental groups

The experimental groups were composed as follows:

Group 1: control hearts perfused with 12% human blood ($n=6$).

Group 2: hCD55 hearts perfused with 12% human blood ($n=11$).

Group 3: control hearts perfused with 12% heat-inactivated human blood ($n=8$).

Group 4: hCD55 hearts perfused with 12% heat-inactivated human blood ($n=8$).

### Statistical analysis

We performed the non-parametric Kruskall-Wallis test to determine which test had to be used. The non-parametric Mann-Whitney test was performed on (1) the difference in cell adhesion in H&E-stained tissues and (2) P-selectin expression. We used Student's *t*-test to evaluate (1) xenograft survival, (2) C3c deposition, (3) C9 deposition, (4) ICAM-1 expression, and (5) IgA, IgG, and IgM deposition. All tests were considered significant when *P*-values were below 0.05.

## Results

### Langendorff perfusions

The mean beating times (MBTs ± SD) for all groups are shown in Table 1. Control hearts perfused with human blood (group 1) beat for a significantly shorter time than did hearts in group 2, i.e., hCD55 hearts perfused with human blood ($P=0.008$), and group 4, i.e., hCD55 perfused with heat-inactivated human blood ($P=0.045$). Similar beating times were found in control hearts and hearts transgenic for hCD55 perfused with heat-inactivated human blood (groups 3 and 4). During the 10-min perfusion with KH, the flow through transgenic and non-transgenic hearts remained constant at a mean level of 27 ml/min. After switching to (heat-inactivated) human blood the flow decreased to a mean level of 10 ml/min, and when the hearts stopped beating the mean flow further decreased to 3 ml/min.

**Table 1** MBTs of hearts from hCD55-transgenic rats andcontrols perfused with either human blood (*HB*) or heat-inactivated human blood (*HIHB*)

| Group | MBT ± SD (min) |
|---|---|
| 1. Controls + HB | 19 ± 11 |
| 2. hCD55 + HB | 43 ± 19 |
| 3. Controls + HIHB | 34 ± 15 |
| 4. hCD55 + HIHB | 38 ± 19 |

$P<0.05$: group 1 vs group 2

### Histology

Standard histology showed interstitial edema, hemorrhage, fibrin depositions, microthrombi, and cellular infiltration. The cellular infiltrates contained leukocytes, being predominantly polymorphonuclear cells (PMNs) (Fig. 1).

### Human leukocyte adhesion to endothelial cells

The percentage of leukocytes adhering to the vessel wall in rat hearts transgenic for hCD55 and control hearts, perfused with human blood, is summarized in Table 2. In H&E-stained hCD55 hearts perfused with human blood an adherence of only 34% of the leukocytes was found, whereas in control hearts perfused with human blood 66% of the leukocytes adhered ($P=0.003$). H&E-stained hCD55 hearts perfused with heat-inactivated human blood (group 4) showed significantly fewer adhering leukocytes than did control hearts perfused with heat-inactivated human blood (group 3; $P=0.002$).

### Adhesion molecule expression

An overview of the mean adhesion molecule expression per experimental group (± SD) is given in Table 3. hCD55-transgenic hearts perfused with human blood (group 2) expressed no P-selectin on their vascular endothelium. This differed significantly from all the other

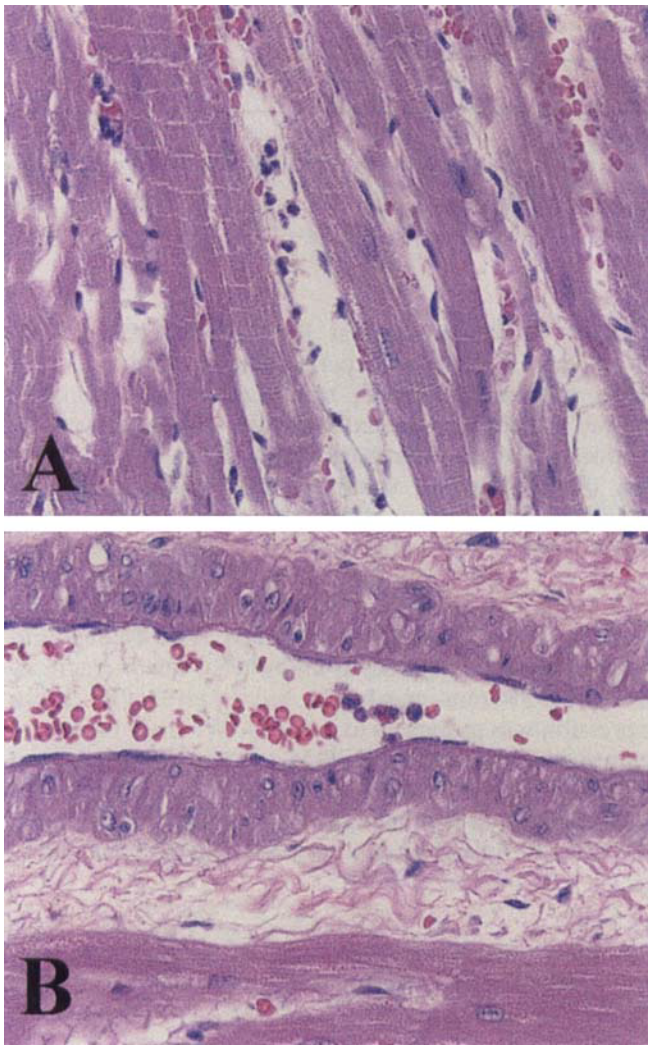


**Fig. 1** Standard histology showed interstitial edema, hemorrhage, fibrin depositions, and microthrombi. These features were more prominent in control hearts (**a**) than in hCD55-transgenic hearts (**b**). Cellular infiltrates contained leukocytes, being predominantly PMNs. In controls (**a**) the percentage of PMN adhesion is significantly higher than in hCD55 hearts (**b**)

groups ($P < 0.003$). Controls perfused with human blood (group 1), controls perfused with heat-inactivated human blood (group 3), and hCD55 hearts perfused with heat-inactivated human blood (group 4) expressed similar levels of P-selectin. The ICAM-1 expression was found to be similar in all experimental groups (data not shown).

### Anti-human C3c and C9 deposition

An overview of the mean anti-human C3c and C9 depositions per experimental group (± SD) is given in Table 3. C3c and C9 were mostly diffusely present along the cardiomyocytes and on some vessels. Both control groups expressed similar levels of C3c and C9 compared to the hCD55 transgenes. A trend is seen in the pattern of C3c and C9 deposition: control hearts perfused with human blood (group 1) expressed higher levels of C3c and C9 than did hearts in the other experimental groups, although these differences were not significant.

**Table 2** Percentage of human leukocytes adhering to the vessel wall ± SD in hCD55-transgenic rat hearts and controls perfused with 12% human blood (*HB*) or heat-inactivated human blood (*HIHB*) in the Langendorff system

| Group | Percentage of adhering leukocytes (H&E) ± SD |
|---|---|
| 1. Controls + HB | 66 ± 14 |
| 2. hCD55 + HB | 34 ± 12 |
| 3. Controls + HIHB | 53 ± 14 |
| 4. hCD55 + HIHB | 28 ± 9 |

$P < 0.05$: group 1 vs group 2, group 3 vs group 4

### IgA, IgG, and IgM deposition

An overview of the mean anti-human IgA, IgG, and IgM deposition per experimental group (± SD) is given in Table 3. Control and hCD55-transgenic hearts perfused with human blood exhibited IgA, IgG, and IgM not only on the endothelium, but diffusely on the cardiomyocytes as well. In group 1, i.e., control hearts perfused with human blood, IgA and IgM reached high levels, whereas IgG did not, but these differences were not significant. In hCD55 transgenes perfused with human blood (group 2) all immunoglobulin levels were found to be similar. Control and hCD55 hearts perfused with heat-inactivated human blood (groups 3 and 4) expressed the same levels of IgA, IgG, and IgM.

## Discussion

In discordant xenotransplantation, both natural antibodies and complement are crucial factors in inducing HAR. Since the problem of HAR has almost been solved by the use of donors transgenic for recipient-type complement regulatory proteins, it is the second immunological barrier that has to be overcome: the AVR process. During AVR, type-II endothelial cell activation plays a central role via up-regulation of proinflammatory genes in endothelial cells and cytokine production [2, 3, 4, 5, 11], thus promoting the adherence of leukocytes.

The aim of our study was to analyze the intermediate phase between HAR and AVR, the so-called delayed HAR phase, in ex vivo perfused rat hearts. hCD55-transgenic rat hearts and controls were perfused with different blood solutions, and adhering leukocytes were

**Table 3** Mean score ± SD of P-selectin, C3c, C9, IgA, IgG, and IgM deposition in rat hearts perfused with human blood (*HB*) or heat-inactivated human blood (*HIHB*)

| Group | P-selectin | C3c | C9 | IgA | IgG | IgM |
|---|---|---|---|---|---|---|
| 1. Controls + HB | 1.2±1.0 | 0.8±0.4 | 0.5±0.4 | 1.5±0.7 | 0.7±0.3 | 1.5±0.7 |
| 2. hCD55 + HB | 0 | 0.3±0.5 | 0.2±0.3 | 0.7±0.4 | 0.8±0.4 | 0.8±0.5 |
| 3. Controls + HIHB | 1.1±0.6 | 0.4±0.4 | 0.4±0.4 | 0.8±0.3 | 0.8±0.5 | 1.1±0.6 |
| 4. hCD55 + HIHB | 1.5±0.1 | 0.4±0.4 | 0.4±0.4 | 0.9±0.5 | 0.9±0.2 | 1.0±0.7 |

$P < 0.05$: P-selectin: group 1 vs group 2, group 2 vs group 3, group 2 vs group 4

counted. If we consider the control group perfused with human blood, 66% leukocyte adherence was observed, which correlated with a moderate and mild expression of P-selectin and ICAM-1, respectively. Anti-human C3c and C9 depositions were present on the vascular endothelium and along the cardiomyocytes. The most important finding of the present study was that hCD55 hearts perfused with human blood showed low levels of C3c and C9 deposition, a moderate expression of ICAM-1 and no P-selectin, and fewer leukocytes adhering to the endothelial cells (34%) than in controls. The control-group hearts perfused with heat-inactivated human blood showed a similar leukocyte adhesion percentage as the hearts perfused with normal blood, indicating that not the absence of complement, but hCD55, is responsible for the inhibition of adherence. hCD55 hearts perfused with heat-inactivated blood showed low levels of C3c and C9 deposition and, as expected, low numbers of adhering leukocytes (28%). The adhering cells were predominantly PMNs. Adhesion of human PMNs to porcine aortic endothelial cells and inhibition of adhesion by hCD55 have been demonstrated earlier [10]. Our findings suggest that hearts expressing hCD55 might be protected against PMN-mediated rejection. Moreover, in previous experiments we found that rat hearts expressing hCD55, transplanted into primates, showed a PMN-dominated infiltrate upon rejection [7].

Our hypothesis was that when a delay in the activation of endothelium occurred, this would lead to a decline in the number of leukocyte-endothelium interactions and a low expression of P-selectin. This was found to be true only for the hCD55 hearts perfused with human blood (group 2). In hearts perfused with heat-inactivated human blood (groups 3 and 4), we found significantly fewer leukocyte–endothelial cell interactions in the hCD55-transgenic hearts, but the level of P-selectin expression was as high as in controls. We may, therefore, tentatively conclude that hCD55 acts as an anti-adhesion molecule, independent of the presence of complement. The beneficial effect of a high expression of hCD55 in the donor is not only restricted to HAR, but also extends to the ensuing process of AVR.

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