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Protective role of the complement regulatory protein human CD-55 in cardiac xenograft: a descriptive study and a revision of the literature

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Summary. The limited and inadequate availability of organs from human donors has resulted in the utilisation of xenografts as an alternative tool. Nevertheless, hyperacute rejection (HAR) following xenograft determines the loss of the transplanted organ. The "primum movens" is the activation of the complement pathway mediated by the binding of natural xenogenic antibodies to the endothelium of the graft, followed by the lysis of the endothelial cells with subsequent oedema, thrombosis and necrosis of the transplanted organ. In this work we describe morphological and biomolecular observations of isolated human-decay accelerating factor (h-DAF, CD55) transgenic pig hearts, after perfusion for four hours with human blood. H-DAF is a membrane glycoprotein inhibiting the complement activation in humans. We describe considerably reduced damages in transgenic hearts, compared to controls. The cardiac function resulted preserved. Our data are in agreement with what was already shown by other groups using different experimental models. In conclusion, we encourage the use of new sources of transgenic animals, pointing out the importance of morphological analysis in evaluation of xenograft.

Key words: Complement, Endothelial cells, Membrane glycoproteins, Xenotransplantation, Genetic engineering

Introduction

Recent advances in surgical techniques and modern immunosuppressive therapies have increased the demand for organ transplantation. The lack of donors has encouraged the utilisation of animals as a potential source of organs (Calne, 1970). The main obstacle for this procedure, called xenograft, is the hyperacute rejection (HAR) that arises in the transplanted organs; this is characterised by vascular endothelium destruction, massive interstitial oedema, haemorrhage and necrosis (Platt and Bach 1991). The transplanted organs do not show the same sensitivity for HAR. Heart and kidney undergo an early and serious damage, while liver and skin show a greater resistance to this phenomenon. The cause for this different behaviour is still not clear (Bach et al., 1995).

The histopathological features of HAR are the consequence of an immediate and aggressive immunological response, in which preformed circulating antibodies, present in the host, bind to specific graft endothelial antigens and activate the complement system (Hammond, 1989). Briefly, the events of the complement cascade consist of a specific sequence of proteolytic steps, in which inactive precursor proteins are cleaved to yield a large and a small fragment. The former binds to the host cell surface and contributes to the following proteolytic cleavage; the latter often mediates the inflammatory response.

A critical step in the complement sequence is the production of a protease, called C3 convertase, which determines the activation of the complement component 3 (C3). This protein is the first component shared by both the classical and alternative pathways and its cleavage generates the most important mediators of the complement system. In particular, its cleavage determines the production of the two mediators, C3a and C3b. The latter is subsequently cleaved in another two fragments, C3c and C3d. These peptides are deposited in large quantities on the host cell surface, representing an inflammatory stimulus and triggering the late events of the complement cascade (Janeway and Travers, 1997a).

Specific proteins regulate the complement pathway, such as CD46 (Membrane Cofactor Protein, MCP), CD55 (Decay Accelerating Factor, DAF) and CD59 (Homologous Restriction Factor, HRF); they act specifically, protecting the cells from accidental damage. In particular, human-DAF (h-DAF) lays on the surface of blood and endothelial cells, where it acts by

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dissociating specific complement catalytic subunits and preventing the progression of this pathway (Janeway and Travers, 1997a). Since these complement regulatory proteins show a high species specificity, their presence on animal endothelial cells can not protect transplanted organs from the attack of human complement (Hansch et al., 1981; van der Berg and Morgan, 1994). Therefore, organs from transgenic animals expressing human proteins like CD46, CD55 and CD59 undergo a limited damage caused by HAR in xenografts (McCurry 1995a; Schmoeckel et al., 1996, 1997).

For this reason, pigs are frequently used to create transgenic animals. Although they are not phylogenetically the closest animals to humans, they are rather similar in size, immune system and organ structures. In addition, their progeny is quite numerous and they have already had a successful clinical application in heart valve transplantation and insulin production (Cozzi and White, 1995).

A modern approach to create transgenic pigs is the sperm-mediated gene transfer (SMGT). The technique uses ejaculated sperm cells as vectors to transfer an exogenous DNA sequence into eggs during fertilisation (Lavitrano et al., 1997). The transgene was integrated and transmitted to the following generations; h-DAF resulted assembled correctly on pig cell membrane and it conferred resistance to the complement-mediated lysis of the pig cells (Lavitrano et al., 1997).

We have already demonstrated that this method produces a high percentage of first generation human-DAF (h-DAF) transgenic pigs (Cappello et al., 2000). Moreover, we have also shown that h-DAF was strongly expressed in specimens obtained from heart, lung, kidney, aorta and skeletal muscle, not only in the first generation but also in the following, with efficiency up to 40% (Lazzareschi et al., 2000). The function of h-DAF has now been assessed by an *ex vivo* experimental model of perfusion.

In this study, we evaluated the HAR phenomena in isolated h-DAF transgenic pig hearts after perfusion with human blood. We analysed anatomo-clinical features, histological damage, ultrastructural alterations, h-DAF expression and complement deposition, in order to verify the protective role of h-DAF in this experimental model and to better codify the spectrum of morphological modifications following a heart xenograft simulation.

Materials and methods

Tissue sampling

Hearts were isolated from five adult h-DAF transgenic pigs and six controls. The transgenic pigs, obtained following the SMGT technique (Lavitrano et al., 1997), belonged to the third generation. In previous experiments we assessed the h-DAF expression by immunoperoxidase on frozen skeletal muscle biopsy (data not shown) to choose only strongly expressing h-DAF transgenic pigs. Pigs were pre-anaesthetised with pentothal, intubated, anaesthetised with isofluoran and nitric protoxide and curarised with pavulon. After thorax opening, the hearts were isolated and perfused with a cold cardioplegic solution containing heparin and subsequently jointed to an extra corporeal perfusion system by aorta. The hearts of transgenic pigs and of five controls were perfused with heparinized fresh human blood (heterologous blood) at an output of 2 litres/min for 4 hours. One control pig heart was perfused with its own blood (homologous blood). During perfusion, haematic pH, by regulation of pO_2 and pCO_2, and glycemia were maintained at physiological levels. A transmitralic balloon monitored the coronary perfusion pressure. The hearts were weighed before and after perfusion and the weight was registered. After perfusions, multiple biopsies from each ventricle and from interventricular septum for traditional histological examination were performed. Other biopsies, obtained from the left ventricle, were processed for electron microscopy analysis and frozen in liquid nitrogen for immunohistochemistry and molecular biology.

Morphological analysis

Sections of formalin-fixed, paraffin-embedded specimens were stained with hematoxylin and eosin to evaluate the morphological status of the myocardium. Oedema, necrosis, thrombosis, endothelial damage and lymphocytic and plasmacellular infiltration were semiquantitated, by two independent observers, according to the tissue percentage involved in the pathological process on 10 high power fields (HPF, x40) (absent: -; less than 33% of tissue: +; 34-66%: ++; more than 67%: +++).

Electron microscopy

Ultrathin sections of 2,5% glutharaldehyde-fixed, epon-embedded specimens were observed by a JEOL 1220 transmission electron microscope. Sarcomeral, sarcotubular and mitochondrial damage was estimated on the basis of damaged cell percentage in 5 meshes (absent: -; less than 33% of cells: +; 34-66%: ++; more than 67%: +++). This semi-quantitative score was obtained by two independent observers.

Immunohistochemistry

Immunostaining by the avidin-biotin complex method (LSAB2 kit peroxidase, DAKO Cat. No K0677) was performed, using primary antibodies against h-DAF (monoclonal mouse, 1:100, UBI, Cat. No 05-285), human C3c complement (polyclonal rabbit, 1:100, DAKO, Cat. No A0062) and isotype-matched control. Aminoethilcarbazole was used as chromogen. The results were semiquantitated by two expert observers, on cell percentage presenting the antigens on 10 HPF (absent: -; less than 33% of cell: +; 34-66%: ++; more than 67%: +++).

Preparation of tissue extracts

Fresh tissues from perfused pig hearts were homogenized in lysis buffer (LB: 50mM Tris HCl pH 7.5, 150mM NaCl, 1% NP-40, 1mM EGTA), containing protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem, Cat. No 539134), and centrifuged at 13,000 rpm for 10 minutes at 4 °C. The supernatant was collected and the protein concentration was determined using a colorimetric assay (DC Protein Assay, Biorad, Cat. No. 5000116).

Western blot analysis

20 μg of total cell extracts in each lane and a protein marker (Kaleidoscope prestained standard, Bio-Rad, Cat. No 1610324) were separated by electrophoresis on denaturing 10% polyacrylamide slab gel (SDS-PAGE) and transferred to a nitrocellulose membrane (Nitrocell Paper, Bio-Rad, Cat. No 1620115). After 1 hour at room temperature (RT) with a blocking buffer (5% low-fat dried milk in TBST: 50mM Tris-HCl pH 7.5, 150 mM NaCl, 0,1% Tween-20) under gentle shaking, the membrane was incubated with anti-h-DAF primary antibody (monoclonal mouse, 1:300, UBI, Cat. No 05-285) overnight at 4 °C.

After washings, the membrane was incubated with HRP-conjugated secondary antibody (anti-mouse, Pierce, 1:8000, Cat. No 31432) for 1 hour at RT with shaking and the specific binding was detected using a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Cat. No 34080) for autoradiography.

The same membrane was stripped with a stripping buffer (Restore TM Western Blot Stripping Buffer, Pierce, Cat. No 21059) and incubated with anti-human C3c complement primary antibody (polyclonal rabbit, 1:500, DAKO, Cat. No A0062), following the procedures described above (secondary antibody: anti-rabbit, Pierce, 1:10000 Cat. No 31462).

Statistical analysis

Standard statistic analyses were employed to calculate the means and the standard deviation (SD). One-way analysis of variance was used to determine the presence of significant differences within the data. Differences between the means were regarded as significant when a value of $p<0.05$ was obtained.

Results

Anatomo-clinical results

After perfusion, the increase of coronary perfusion pressure was 137±10% in heterologous blood-perfused control hearts (HEBPCH), 54±16% in heterologous blood-perfused transgenic hearts (HEBPTH), and 25% in homologous blood-perfused control heart (HOBPCH) ($p<0.001$) (Fig. 1). Moreover, heart weight increased 39.7±3.5% in HEBPCH, 21±3.6% in HEBPTH and 17% in HOBPCH ($p<0.005$) (Fig. 1). Statistical analysis revealed that the difference between HEBPCH and HEBPTH was significant ($p<0,05$).

Histological data

All observed HEBPCH samples showed a moderate interstitial oedema. In contrast, oedema in HEBPTH and HOBPCH was negligible (Tab1e 1; Fig. 2, H,E). Moreover, HEBPCH showed a severe endothelial damage; the endothelial lamina was discontinuous, compared to HEBPTH, in which the endothelium resulted scarcely altered in an irrelevant number of small vessels (Fig. 2, H,E inset). In addition, small vessels in HEBPCH resulted abnormally dilated whereas HOBPCH presented a normal endothelium.
Furthermore, a low-moderate thrombosis was observed in HEBPCH, often associated with capillary congestion. These features were inconspicuous in HEBPTH and absent in HOBPCH (Table 1). In addition, necrosis, hypereosinophilia of myocytes, contraction band formation and intercalated disc disruption were uncommon features. Indeed, we noted a negligible presence of these phenomena in three out of five HEBPCH (Table 1).

Finally, no lymphatic or plasma cellular infiltration was present in any examined specimens (Table 1).

Electron microscopy

The myocardial fibres did not show any sarcomeral or sarcotubular alteration in any observed specimens. In contrast, mitochondrial damage was present in only two

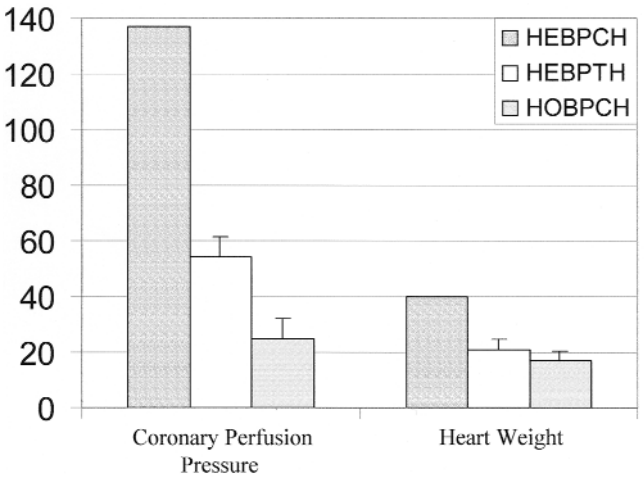

Fig. 1. Histograms showing the increment of coronary perfusion pressure and heart weight in control and transgenic pig hearts. HEBPCH: heterologous blood perfused control heart; HEBPTH: heterologous blood perfused transgenic heart; HOBPCH: homologous blood perfused control heart.

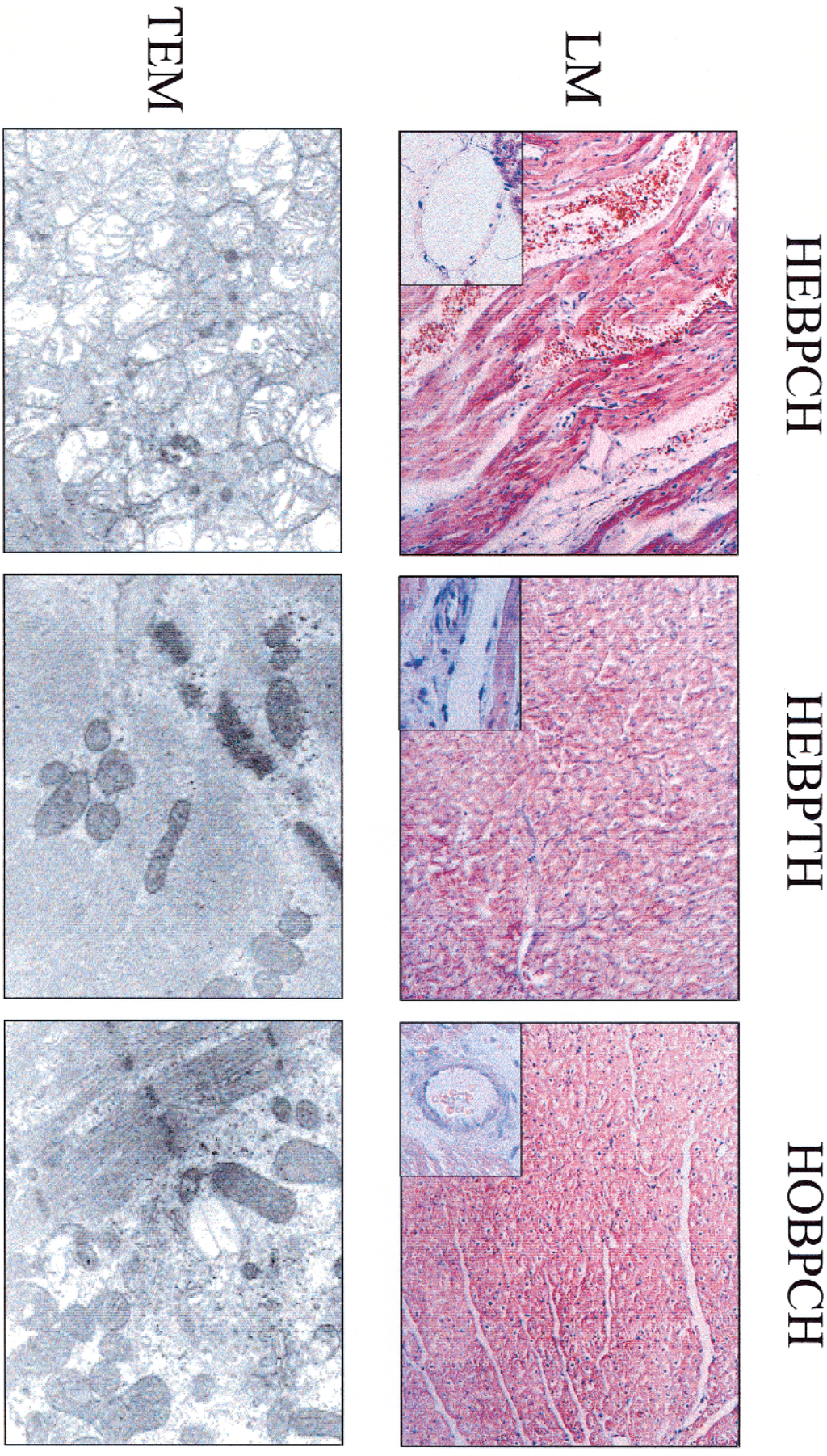

Fig. 2. Light Microscopy (LM). Oedema and necrosis are evident in HEBPCH (4X); the inset shows damage of endothelium (x 40). HEBPTH presents only a low pericellular oedema (x 4); the inset shows an unaltered endothelium in arteriolae (x 40). A negligible oedema is presents in HOBPCH (4X); the inset shows a normal endothelium (x 40). Trasmission Electron Microscopy (TEM). Damaged mitochondria in HEBPCH (x 5000). HEBPTH and HOBPCH show normal sarcomeral and mitochondrial status (x 5000). HEBPCH: heterologous blood perfused control heart; HEBPTH: heterologous blood perfused transgenic heart; HOBPCH: homologous blood perfused control heart

of the HEBPCH (Table 2; Fig. 2, TEM). In particular, mitochondria were swollen and showed disruption of the crests .

Immunohistochemistry

H-DAF was uniformly distributed in HEBPTH myocardiocytes from subepicardium to subendocardium. Particularly, h-DAF was mostly localized on the plasmalemma of myocardiocytes, compared to the scarce positivity found in the cytoplasm (Fig. 3, h-DAF). In addition, h-DAF was strongly expressed in endothelium of both endocardium and intraparietal vessels. In contrast, the h-DAF expression was negative in both myocardiocytes and endothelial cells of HEBPCH and HOBPCH (Fig. 3, h-DAF). Moreover, human C3c was abundantly deposited on plasmalemma of HEBPCH endothelial and myocardial cells, while it was scarcely present in HEBPTH and absent in HOBPCH (Fig. 3, C3c). C3c absence in HEBPTH can be strongly related to the presence of h-DAF.

Western blot analysis

Anti-h-DAF antibody recognised specifically the transgenic h-DAF protein (63 KDa) in HEBPTH and did not cross-react with any other protein present in HEBPCH and HOBPCH (Fig. 4a). Moreover, the human C3c (70 KDa) (Fig. 4b) accumulated only in HEBPCH, confirming that h-DAF inhibited the complement activation in HEBPTH. Therefore, these data confirmed the immunohistochemical observations.

Table 1. Histologic evaluation of damages in HOBPCH, HEBPCH and HEBPTH. Damages are semiquantitated on four levels: -: absent, +: low; ++: moderate; +++: severe, as described in Materials and Methods.

	PERICELLULAR EDEMA	ENDOTHELIAL DAMAGE	THROMBOSIS	NECROSIS	MYOCYTES HYPEREOSINOPHILIA	CONTRACTION BANDS	LYMPHOCYTIC AND PLASMACELLULAR INFILTRATION
HOBPCH	+	-	-	-	-	-	-
HEBPCH-1	++	++	+	-	-	-	-
HEBPCH-2	++	+++	++	+	+	+	-
HEBPCH-3	+	++	++	-	-	-	-
HEBPCH-4	++	+++	++	+	+	+	-
HEBPCH-5	++	++	+	+	+	-	-
HEBPTH-1	+	+	+	-	-	-	-
HEBPTH-2	+	+	-	-	-	-	-
HEBPTH-3	+	-	-	-	-	-	-
HEBPTH-4	+	+	+	-	-	-	-
HEBPTH-5	+	+	+	-	-	-	-

-: absent; +: low; ++: moderate; +++: strong. HEBPCH: heterologous blood perfused control heart; HEBPTH: heterologous blood perfused transgenic heart; HOBPCH: homologous blood perfused control heart.

Table 2. Immunohistochemistry results and TEM evaluation of damages in HOBPCH, HEBPCH and HEBPTH. Antibody expression and damage evaluation are semiquantitated on four levels: -: absent, +: low; ++: moderate; +++: severe as described in Materials and Methods.

	DAF EXPRESSION	C3 DEPOSITION	SARCOMERAL DAMAGES	SARCOTUBULAR DAMAGES	MITOCHONDRIAL DAMAGES
HOBPCH	-	-	-	-	-
HEBPCH-1	-	++	-	-	-
HEBPCH-2	-	+++	-	-	
HEBPCH-3	-	+++	-	-	-
HEBPCH-4	-	+++	-		++
HEBPCH-5	-	++	-	-	+
HEBPTH-1	++	++	-	-	-
HEBPTH-2	+++	+	-	-	-
HEBPTH-3	+++	+	-	-	-
HEBPTH-4	+++	+	-	-	-
HEBPTH-5	++	+	-	-	-

-: absent; +: low; ++: moderate; +++: strong. HEBPCH: heterologous blood perfused control heart; HEBPTH: heterologous blood perfused transgenic heart; HOBPCH: homologous blood perfused control heart.

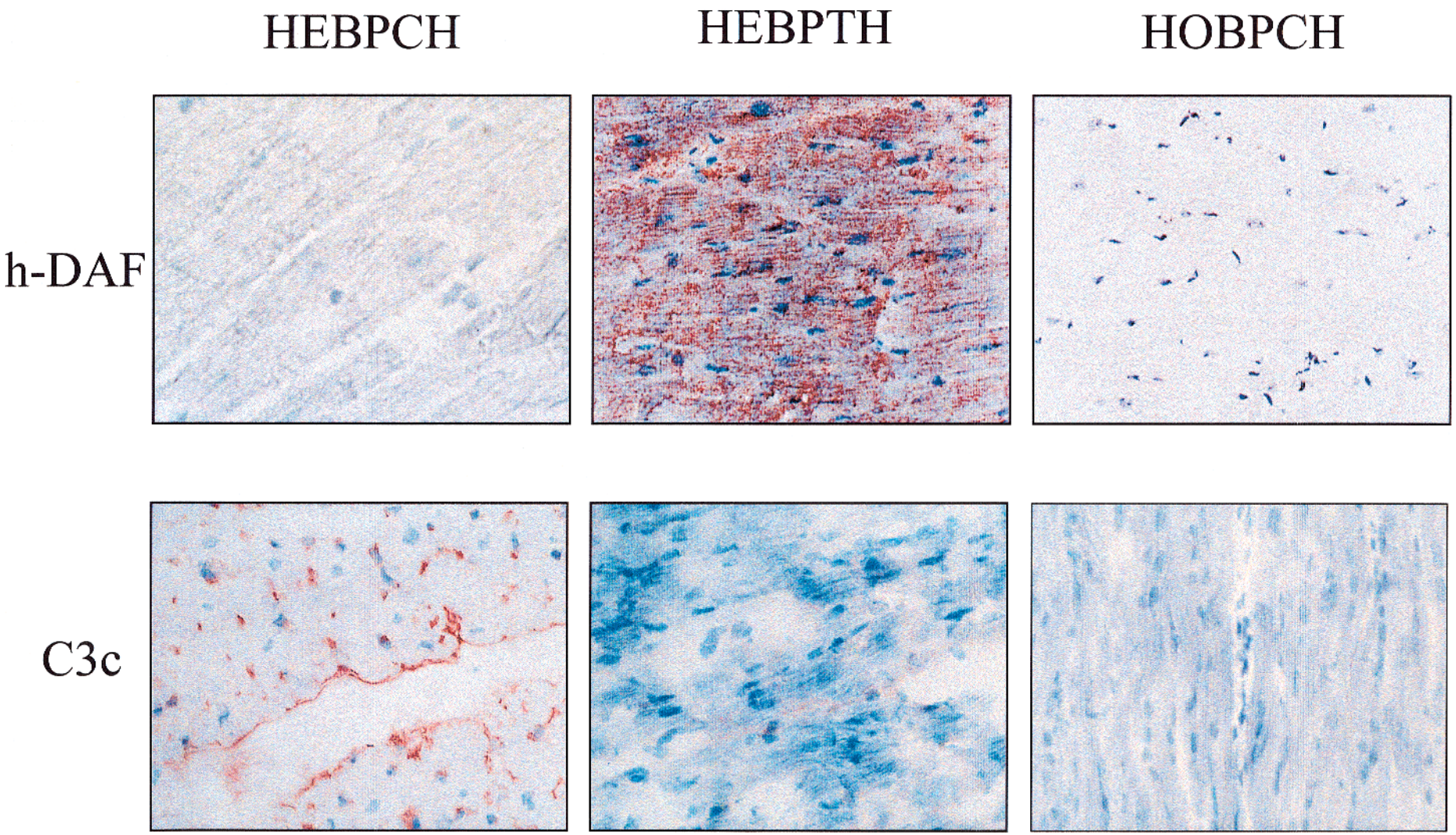

Fig. 3. Immunohistochemistry. H-DAF: Photomicrographs showing strong presence of immunostaining for h-DAF in HEBPTH and absence in HEBPCH and HOBPCH (x 10). C3c: immunostained sections showing C3c presence in HEBPCH and absence in HEBPTH and HOBPCH (x 10). (EBPCH: heterologous blood perfused control heart; HEBPTH: heterologous blood perfused transgenic heart; HOBPCH: homologous blood perfused control heart.

Discussion

Until a few years ago, HAR was considered as an absolute barrier to xenografts between phylogenetically distant species. Indeed, Lexer et al. (1996) hemoperfused eight freshly excised pig hearts in baboons *in vivo*, finding that six of them ceased their function after a mean period of 90 minutes, while only two continued functioning for the 4-hour study period. On microscopic examination, seven hearts including one that continued beating, presented histopathological features of HAR, with a strong deposition of C3 on the myocardium. HAR was normally detected within a few hours from the xenograft and was considered as the inevitable consequence of it. Moreover, in another experiment, pig livers perfused for 9 hours with human blood showed that, despite biochemically and histologically confirmed tissue injury, graft viability was well-maintained in xenoperfusion even without immunological manipulations (Terajima et al., 1996).

The initiating factor of pig organ HAR by human or non-human primates is the presence of preformed antibodies in the host (Cooper et al., 1994). These xenoantibodies recognise the terminal alpha-1,3-galactose disaccharide present on pig endothelium. Moreover, the species incompatibility between human

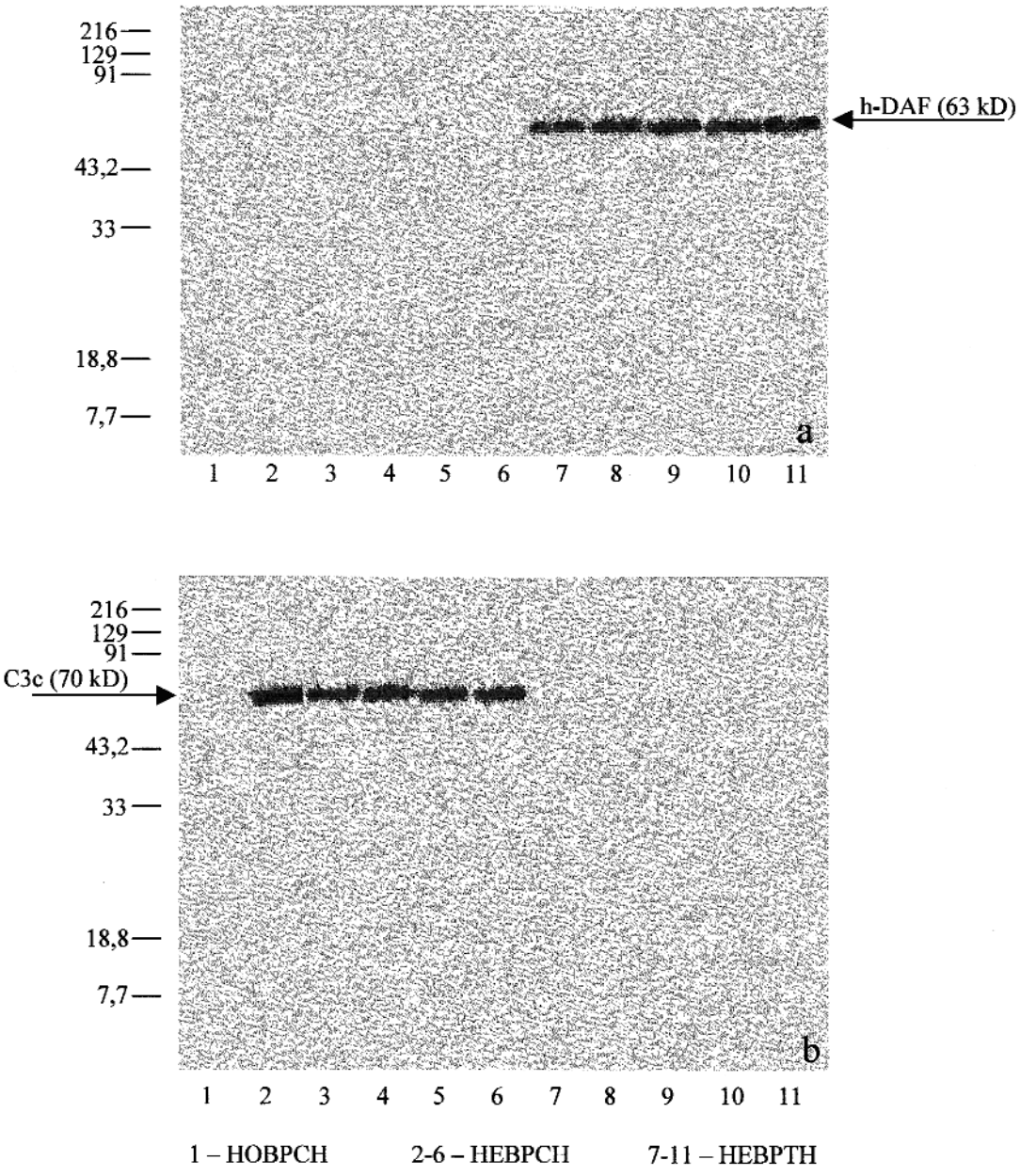

Fig. 4. Western blotting of extracted proteins from HOBPCH; HEBPCH and HEBPTH. **(a)** 10% SDS-PAGE and immunoblotting of h-DAF (63 KDa, lane 7-11). H-DAF is present only in HEBPTH. **(b)** 10% SDS-PAGE and immunoblotting of C3c (70 KDa, lane 2-6). C3c is present only in HEBPCH. HEBPCH: heterologous blood perfused control heart; HEBPTH: heterologous blood perfused transgenic heart; HOBPCH: homologous blood perfused control heart.

and porcine complement regulatory proteins is also a further element for HAR (van Denderen et al., 1997).

The employment of immunological techniques and the recent development of genetic engineering have permitted the extension of xenograft survival for a longer period after transplantation. For example, immunoabsorption techniques for anti-pig antibody using immunoaffinity columns permitted the prolongation of the survival of xenografted kidneys without HAR up to 13 days (Sachs, 1994). Moreover, the efficacy of highly selective immunoadsorption columns to deplete xenoreactive human anti-porcine antibodies, before ex vivo liver perfusion, resulted in an effective approach for delaying antibody-mediated xenogeneic HAR (Kroshus et al., 1995; Pascher et al., 1997). In addition, h-DAF expression combined with alpha-1,3-galactosyltransferase in transgenic transplanted splenocytes was a protection from complement-mediated injury in a model of HAR in mice (van Denderen et al., 1997).

The depletion of complement using a highly purified form of cobra-venom factor also significantly extended the cardiac xenograft survival in both guinea pig-to-rat and pig-to-baboon animal models (Leventhal, 1993). Finally, endogenously-expressed complement-regulatory molecules, like CD59 and CD46 on pig endothelium resulted in playing an important role in the protection against HAR in *in vitro* (van der Berg et al., 2000) and *ex vivo* (Perez de la Lastra et al., 1999) models.

In addition, transgenic organs expressing high levels of human complement regulatory proteins alleviated the humoral immunological barriers currently blocking xenograft, being able to contribute to the control of complement activation in many organs (McCurry et al., 1995b; Byrne et al., 1997)

Numerous works showed that h-DAF transgene combined with maintenance of immunosuppression is able to support life for a prolonged period in porcine cardiac xenografts in non-human primates (Waterworth et al., 1998; Vial et al., 2000). Moreover, h-DAF expression increased heart transplant survival in rat- (or mouse-) to-primate models of xenograft (Cowan et al., 1998; Charreau et al., 1999). In addition, h-DAF transgenic pig livers transplanted in five baboons resulted protected from HAR for 4-8 days (Ramirez et al., 2000).

The utilisation of pig organs in xenograft has prompted the purification and characterisation of some porcine complement inhibitors (van den Berg 1995, 1997). Recently, the cloning of cDNAs encoding multiple isoforms of the pig analogue to h-DAF was reported (Perez de la Lastra et al., 2000). Pig DAF was expressed in a wide range of tissues and on all circulating cells and it was transmembrane-anchored on erythrocytes, but its regulatory activity on the complement pathway is actually the object of further studies.

The extra-corporeal perfusion model employed in this study resulted highly efficient to simulate a xenograft, as demonstrated by the analysed controls (HOBPCH and HEBPCH).

We utilised heparinized fresh human blood of O hemogroup. The choice of which human blood group to use for xenoperfusions is not important. In fact, despite human type-A and -H blood antigens being present in both porcine exocrine secretions (Watkins, 1972) and erythrocytes (Sako et al., 1990), they are not detectable on vascular endothelium; the latter suggests that they are not involved in the HAR, although the pig A antigen can induce an immune response in O or B blood group recipients (Oriol et al., 1994).

Endothelial cell disruption is one of the most indicative features of HAR phenomena and its presence is considered strictly specific of this pathology (Rose et al., 1991). Indeed, immediately after the revascularisation of xenotransplanted organs, complement activation triggers the endothelial cell lysis, with the following thrombosis and tissue necrosis (Dalmasso et al., 1991); normally, HAR is observed within a few hours.

We found a conspicuous number of damaged endothelial cells only in HEBPCH. We considered this endothelial alteration due to the high titres of natural antibodies reacting with the carbohydrate residues present in porcine vascular endothelial cells and activating the complement cascade (Neethling et al., 1994). Moreover, we considered the endothelial damage as the triggering factor of oedema, which is responsible for both coronary perfusion pressure and heart weight increase. In addition, our results showed that the presence of h-DAF on both endothelial and myocardial cell surface significantly reduced the C3c deposition in transgenic hearts, protecting them from HAR in all five cases for the observed period of four hours. In contrast, HEBPCH showed C3c deposition and HAR phenomena. This evidence had already been demonstrated in human plasma-perfused CD55/CD59 transgenic mouse hearts, in which immunopathological analysis of serial biopsies revealed the inhibition of complement activation (Byrne et al., 1995).

Therefore, the anatomo-clinical features reported in HEBPCH permitted us to diagnose the HAR (Aretz 1999), even if they did not present a significant lymphoplasmacellular infiltration. The latter might concurrently occur, although remaining focal and mild. In our cases, its absence could be due to the fact that hearts were perfused for only four hours, a too short time to activate the biological events necessary for the leukocyte migration (Janeway and Travers, 1997b). Moreover, the absence of cellular lymphoid infiltrate had already been reported in 551 sequential endomyocardial biopsies from 36 consecutive cardiac allografts in the early period (3 weeks) after transplantation (Hammond. 1989).

We concluded that the h-DAF expression in HEBPTH protected them from HAR. These results are in agreement with what has already been shown in the literature, using different approaches. Moreover, these

data suggest that in a combined approach with immunoabsorption, immunosuppression and genetic engineering techniques, the presence of complement-regulating proteins (as h-DAF) in transgenic hearts should be considered as a useful means to alleviate the complement barrier upon graft of this highly vascularised organ into human, as well as to considerably extend the survival of transplanted organs. A detailed knowledge of morphological modifications following transgenic xenograft will permit the exclusion of HAR phoenomena in bioptic specimens after hours or days from transplantation.

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