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An *in vivo* model of hyperacute rejection: characterization and evaluation of the effect of transgenic human complement inhibitors

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Abstract

Hyperacute rejection (HAR) occurring after transplantation within phylogenetically distant species is a severe reaction triggered by preexisting xenoreactive antibodies and complement activation, leading to the destruction of the donor organ. Expression of human complement inhibitors in transgenic pig organs prolongs the survival of xenograft in experimental models. Moreover, the extent of protection from hyperacute rejection is dependent on the level and site of expression of the transgenic molecules and, probably, on the combination of different molecules. In this regard a small animal model to test the efficacy of expression vectors and different human molecules could be very advantageous. A murine model developed in our laboratory was characterized by measurement of several parameters characteristic of HAR in the livers of control and transgenic mice expressing transgenic human DAF (CD55) or MCP (CD46) at the end of 2 h of perfusion with human plasma and after 1 day. The parameters studied were heamatological values of hepatic functions (GOT and GPT), induction of pro-inflammatory molecules and histopathological evaluation. Cytokines (IL-1α, IL-1β, IL-6) induction and exposure of P-selectin on the endothelial cell surface, was only observed in control animals after 2 h of perfusion, as an early event. GOT and GPT values increase drammatically after 2 h perfusion and 1 day after the treatment according to the histopathological observation of liver damage. On the contrary, the livers of hDAF or hMCP transgenic mice, under the same treatment were significantly protected although the extent of this protection is dependent on the level of expression of transgenic human molecules.

Abbreviations: HAR – hyperacute rejection; hMCP – human membrane cofactor protein (CD46); hDAF – human decay accerating factor (CD55); GOT – aspartic aminotransferase; GPT – alanine aminotransferase; GAPDH – glyceraldehyde-3-phosphate dehydrogenase

Introduction

The present decade has witnessed an increasing interest in interspecies transplantation. Xenotransplantation appears to be an interesting solution to the

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urgent problem of shortage of human cadaveric organs suitable for allotransplantation. However, the success of this procedure is dependent on the ability to prevent the devastating humoral immune response named hyperacute rejection (HAR). Xenoreactive natural antibodies and Complement are seen as the major initiating factors leading to the infarction and destruction of discordant xenografts (Dalmasso et al., 1992; Samuelsson et al., 1994; Sandrin & McKenzie, 1994; Romanella et al., 1997; Carrington et al., 1997). Host complement activation leads to endothelial cell (EC) activation, loss of vascular integrity, interstitial hemorrhage and rapid xenograft destruction. Complement activation pathways are regulated by a number of species-specific regulatory proteins, the regulators of complement activation (RCAs) (Hourcade et al., 1989). Within the RCAs family, the decay accelerating factor (DAF or CD55) and the membrane cofactor protein (MCP or CD46) play a central role in the inhibition of the complement activation cascade. DAF is a glycoprotein anchored by a GPI tail to various types of human cell membrane. Its role is to accelerate C3 convertase dissociation in both the classical and alternative pathways (Lublin & Atkinson, 1989). MCP is also a glycoprotein with a transmembrane domain acting as a cofactor for factor I-mediated cleavage of C3b and C4b, thus interfering with the formation of C3 convertase (Lublin & Atkinson, 1989). Xenogeneic cells or transgenic mice expressing human complement inhibitors are protected from human complement-mediated damage (Mulder et al., 1995a,b; Diamond et al., 1995; Mora et al., 1996; van Denderen et al., 1996; Lazzeri et al., 1998). The use of genetically engineered animals as organ donors could, therefore, represent a good opportunity for clinical xenotransplantation.

Numerous reports have shown that the expression of complement inhibitors in organs derived from transgenic pigs prolongs the survival of xenografts in experimental models (Fodor et al., 1994; Cozzi & White, 1995; McCurry et al., 1995; Kroshus et al., 1996; Pierson et al., 1997; Bach, 1997; Byrne et al., 1997). However, the protection from HAR injury is affected by several factors, mainly by the level and site of expression of the transgenic molecules.

Recently, we have reported an *in vivo* murine model of perfusion with human plasma which recreates the histopathological evidence of HAR in solid organs (Mulder et al., 1995b; Lazzeri et al., 1998). By this procedure it was possible to establish the effect of transgenic human complement inhibitors and compare it to the level of expression of transgenic molecules in different organs.

Endothelial cell activation is one of the first event of HAR and is characterized by the induction of several pro-inflammatory molecules such as IL-1α, IL-1β, IL-6 etc. whose upregulation leads to the loss of vascular integrity, an irreversible damage, essential for the HAR process to proceed (Coughlan et al., 1993; Bach et al., 1995; Saadi et al., 1995; Selvan et al., 1998).

The possibility to follow the HAR events induced by perfusion with human plasma for a longer time (24 h) strengthened our murine model. In this paper, we report the analysis of several parameters representative of the multitude of HAR reactions. The results of these experiments, carried out on the liver of control and transgenic mice, indicated an irreversible damage in the livers of perfused controls, while the inflammatory process observed in the livers of perfused transgenic mice was totally reversible.

Materials and methods

Construction of MT-hMCP and MT-hDAF vectors

The construction of the MT-hMCP expression vectors is described elsewhere [8]. Briefly, the inducible hepato-specific promoter of the mouse metallothionein gene has been used to direct the expression of the human DAF minigene, or the human MCP cDNA, to the liver.

Production of transgenic mice

Transgenic mice were produced according to the method described by Hogan et al., 1986. The MT-hDAF-27 and MT-hMCP-35 transgenic mice used in all experiments described in this paper were treated with 25 mM $ZnSO_4$ in their drinking water for 5 days prior to the experiments to induce the metallothionein promoter (Palmiter et al., 1993).

Cytofluorimetric analysis of isolated hepatocytes

Livers derived from control and transgenic (MT-MCP and MT-DAF) mice were perfused, via portal vein, with 50 ml of a salt solution (140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 15 mM glucose, 0.5 mM EGTA, pH 7.4) followed by a second perfusion with 100 ml of a collagenase solution (68 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 5 mM galactose, 4.7 mM $CaCl_2$

100 U/ml collagenase, pH 7.4). The perfused livers were pressed through a cell dissociation sieve (Sigma) and then through a 60-mesh screen. The released hepatocytes were purified by isodensity Percoll centrifugation.

To detect the expression of hMCP or hDAF on hepatocyte surface, isolated hepatocytes were treated with monoclonal antibodies anti-hMCP (J4-48, AMAC Inc, Westbrook, ME, USA) or anti-hDAF (WAKO, Osaka, Japan) followed by FITC rabbit anti-mouse IgG (Sera-Lab, Crawley Down, Sussex, England) and analyzed by flow cytometry (FACScan, Beckton-Dickinson, Mountainview, CA, USA). The data were analyzed using Lysys II software (Consort 30, HP 340 computer).

Perfusion with human plasma

Perfusion with human plasma has been described in details elsewhere (Mulder et al, 1995b; Lazzeri et al., 1998). Briefly, infusion with fresh human plasma by cannulation of the jugular vein allows the perfusion of the entire animal. Sets ($N = 5$) of control and transgenic mice were sacrified after 2 h of perfusion at a perfusion rate of 10 μl/min (1.2 ml total perfused human plasma; about 1:1 dilution of the murine blood volume), or let to survive and sacrified 1 day after treatment. Blood samples were collected at each time point for haematological evaluation, the major organs removed, one sample of each was snap-frozen in liquid nitrogen for imunohistochemistry or RNA analyses, and a sample was fixed with 10% buffered formaldehyde and processed for histopathological evaluation.

Haematological analysis

Blood samples were analyzed in an automatic Spotchem Apparatus (Menarini Diagnostics, Florence, Italy). This apparatus allows the evaluation of several parameters by dry chemistry. Blood samples (0.3–0.5 ml) were collected in heparinized tubes (provided by the apparatus supplier) and centrifuged for 2 min at $6{,}500 \times g$ per min. Aspartic aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) were compared to the values of untreated controls present in each experiment and to control values measured on a set of 10 animals of the same age and species.

Histopathological analysis

Sample of the organs to be examined were fixed with 10% buffered formaldehyde, embedded in paraffin, sectioned and stained with hematoxilin/eosin or endothelium specific staining (Masson's trichromic) as specified in the figure legend.

Immunohistochemistry

Murine P-selectin was detected on cryostat liver sections by direct immunofluorescence standard technique using specific anti-mouse P-selectin rabbit antibody (PharMingen, San Diego, CA, USA) and FITC-anti rabbit antibody. Sections were counterstained with Blue Evans.

RNA analysis by RT-PCR

To analyze the induction of IL-1α, IL-1β and IL-6, the messenger RNA from control and transgenic mouse livers was isolated using Oligotex Direct mRNA Mini Kit (Qiagen, Hilden, Germany) and 0.5 μg of each sample were reverse-trascribed with Superscript RT (GIBCO-BRL, Life Technologies, Italy) and oligo(dT) (Pharmacia Biotech, Italy). PCR products were generated from 0.5 μg of the resulting cDNA using both cytokine specific primers and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, as internal control, in a final concentration of 1 and 0.1 μM, respectively. The PCR conditions were 30 cycles at 94°C for 20 s, 58°C for 20 s, 72°C for 75 s. The following primers were designed to anneal to sequences in different exons to differentiate between cDNA and contaminating genomic DNA.

IL-1α:	5′ATGTATGCCTACTCGTCGGG3′ and 5′CAGACTGTCAGCACTTCCCA3′
IL-1β:	5′CCACCTCAATGGACAAATATCA AC3′ and 5′CAGCCCATACTTTAGGAAGACA CAG3′
IL-6:	5′GGAGAGGAGACTTCACAGAGG ATAC3′ and 5′TTGTTCTTCATGTACTCCAGGT AGC3′
GAPDH:	5′ACCACCATGGAGAAGGCCGG3′ and 5′CTCAGTGTAGCCCAAGATGC3′.

The PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining and quantitative values were obtained by the Image-Master VDS (Pharmacia Biotech, Italy).

Results

Expression of hMCP and hDAF in transgenic mice

The expression of hMCP or hDAF was analyzed by RT-PCR of total RNA extracted from various organs. Although we have used a hepato-specific promoter to direct the expression of the human complement inhibitors into the liver, expression of hMCP or hDAF was also detected in other organs. High levels of expression of hMCP were detected in the liver, heart, testis. On the contrary, hDAF was expressed in all the organs examined with higher levels in the heart and kidney, but its expression in the liver was significantly lower than that of hMCP (data not shown). Immunohistochemical analysis of different tissues of transgenic mice revealed the presence of the proteins on the endothelium system in all the organs examined (data not shown), while in the liver their presence could be detected both on vascular endothelia and on the hepatocyte cell surface (Mulder et al., 1995a,b).

Cytofluorimetric analysis of isolated hepatocytes derived from MT-MCP or MT-DAF transgenic mice (Figure 1) showed that the number of hMCP molecules present on the MT-MCP transgenic hepatocyte surface (Figure 1C) was higher than that of h-DAF on the membrane of hepatocytes derived from MT-DAF transgenic mice (Figure 1B), thus confirming the expression data obtained by RT-PCR analysis.

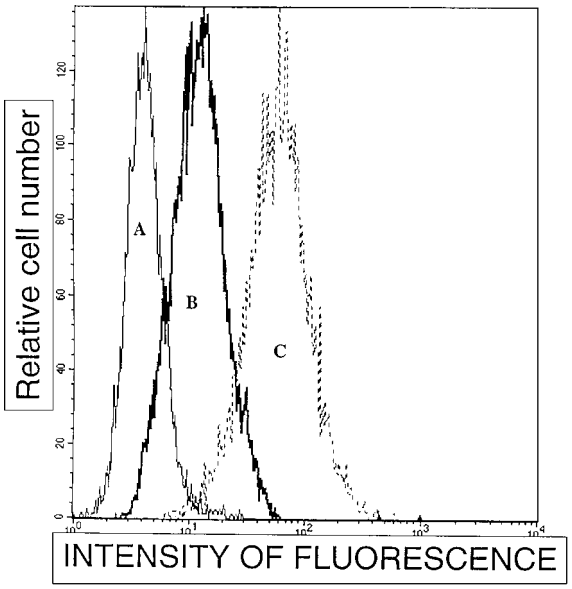

Figure 1. FACS analysis of hMCP and hDAF expression on isolated hepatocytes. Hepatocytes were isolated from control, MT-hCD55-27.4 and MT-hCD46-38.2 transgenic mice. Anti-hDAF or anti-hMCP mAb were used to detect hDAF or hMCP molecules on the cell surface. A, Hepatocytes derived from control mouse; B, hepatocytes derived from MT-hDAF transgenic mouse; C, hepatocytes derived from MT-hMCP transgenic mouse.

Human complement C3 deposition

Cryostat liver sections derived from control and $ZnCl_2$-induced or uninduced transgenic mice after 2 h perfusion with human plasma were examined by immunostaining with FITC anti-human C3 specific antibodies. As already described (Mulder et al., 1995a,b) a heavy deposition of human complement C3 was only observed on the livers of control animals and uninduced transgenic mice after 2 h of perfusion with human plasma. Deposition of human C3 was virtually absent on the liver derived from perfused hMCP or hDAF transgenic mice (data not shown).

Histopathological analysis

Livers derived from controls immediately after, or 1 day after 2 h of perfusion with human plasma were analyzed and compared to the livers derived from transgenic mice under the same treatment. As already described (Mulder et al., 1995b; Mora et al., 1996), a severe damage of the liver was observed immediately after perfusion, that is endothelium swelling, neutrophil infiltration, intralobular haemorrhage, fibrin deposition (Figure 2A,B). The livers of control animals, 1 day after perfusion showed a more extensive injury with large areas of haemorrhagic necrosis, mononuclear cell infiltration and cell death (Figure 2C). Livers derived from transgenic mice under the same treatment were significantly protected after 2 h of perfusion to an extent dependent on the level of expression of the complement regulators. In the case of livers from perfused MT-hMCP transgenic mice (Figure 2D) in which high levels of hMCP was observed, the protection seems to be almost complete. The livers derived from perfused MT-DAF transgenic mice, where lower levels of hDAF expression was detected, show small foci of inflammation (Figure 2E). However, one day after perfusion, the livers derived from either hMCP or hDAF transgenic mice are comparable to untreated control livers (Figure 2F). These results suggest that the inhibition of human C3 deposition due to the presence of the complement regulators also prevents the induction of those pro-inflammatory molecules that in perfused non-transgenic mice allows the hyperacute reaction to proceed to an irreversible damage, while

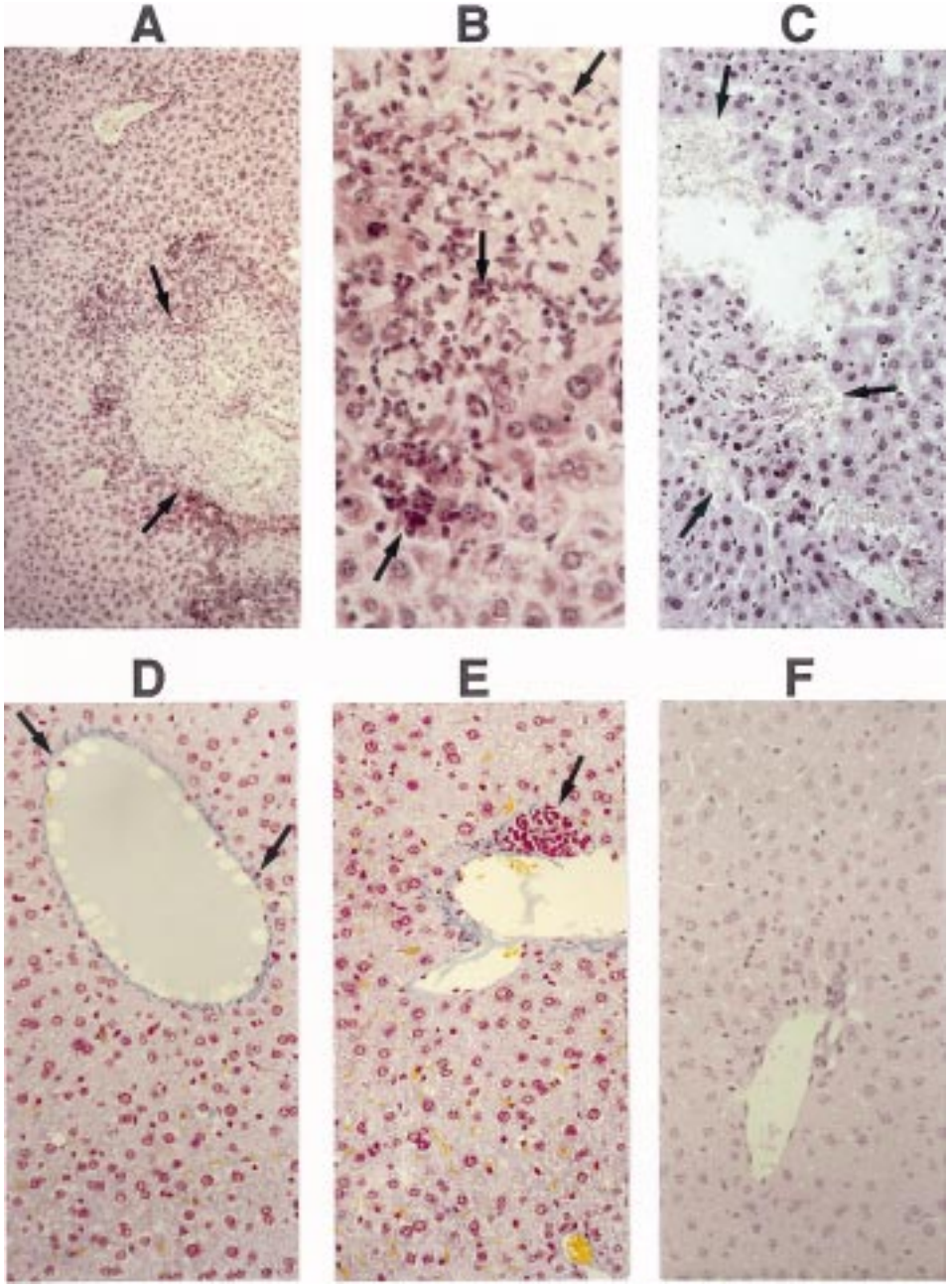

Figure 2. Histopathological analysis of perfused control and transgenic mice livers. Light micrographs of livers derived from control mice perfused for 2 h with human plasma (A, 150 ×; and B, 400 × original magnification) showing a massive intralobular neutrophil infiltration (B, arrows) and fibrin deposition (A, arrows). The liver damage is more severe 1 day after the 2 h of perfusion (C, 250 ×) presenting large areas of haemorrhagic necrosis (arrows). The livers derived from hMCP transgenic mice after 2 h of perfusion with human plasma (D, 250 ×) were significantly protected, arrows indicate a conserved structure of vascular endothelia. The livers derived from perfused hDAF transgenic mice occasionally shows small mononuclear cell foci (E, 250 ×, arrow). One day after the 2 h of perfusion, livers derived from transgenic mice were undistinguishable from the liver of untreated control mice (F, 250 ×). A, B, C, and F – sections stained with hematoxilin/eosin; D and E – stained with Masson's trichromic solution.

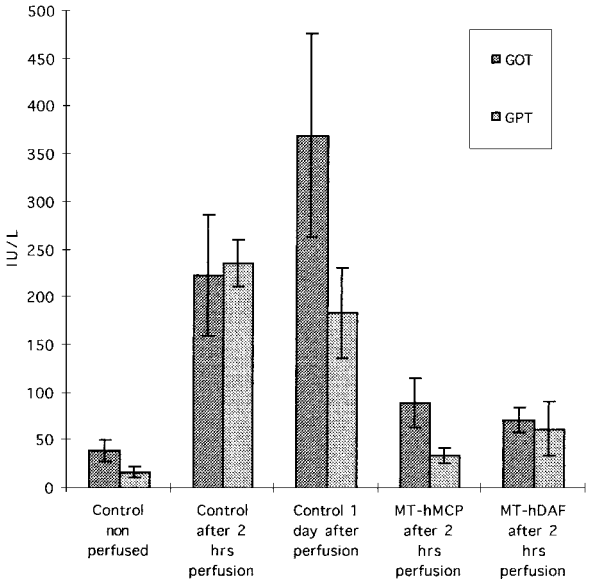

Figure 3. Haematological values of GOT and GPT of perfused control and transgenic mice. Dark gray columns represent the hematic values of GOT expressed in IU/L, and light gray columns represent the hematic values of GPT expressed in IU/L (ordinate). In the abscissa are indicated the animals from which the blood samples were derived and the treatments they received.

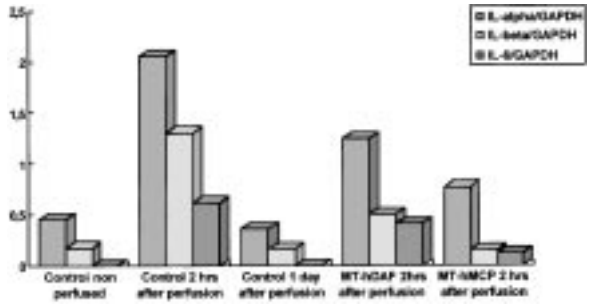

Figure 4. RT-PCR analysis of pro-inflammatory molecules of perfused control and transgenic mice livers. RT-PCR analysis of IL-1α, IL-1β and IL-6. The values were normalized to GAPDH internal control and are representative of three independent experiments. In the abscissa are indicated the treatment and the animal from which RNA was extracted. Dark gray columns indicate the fold increase (ordinate) of IL-6, medium gray columns IL-1α, and light gray columns IL-1β.

in the perfused transgenic livers the inflammatory process is completely reversible.

Haematological analysis

Hepatic functions in perfused control and transgenic mice at the indicated times after perfusion were determined by measuring the serum levels of GPT and GOT (Figure 3). The damage induced in the liver of perfused control animals was confirmed by the elevated values of GOT and GPT at the end of the 2 h of perfusion. These values continue to be elevated also 1 day after treatment, in agreement with the extensive necrosis observed by histopathological examination. The values of GOT and GPT in the serum of perfused transgenic mice were significantly lower and consistent with the histological evaluation which indicates a slight inflammatory process, inversely correlated with the expression of hMCP or hDAF.

Induction of pro-inflammatory molecules

To further characterize our *in vivo* perfusion model, we have carried out experiments of RT-PCR to analyze the induction of some pro-inflammatory molecules known to be involved in the endothelial cell (EC) activation, such as IL-1α, IL-1β, and IL-6. This analysis was carried out on livers derived from untreated controls, control and transgenic mice after 2 h perfusion and 1 day after perfusion. An internal control (GAPDH) allowed a rather accurate quantitation of the specific retrotranscripts. Figure 4 shows the results of this analysis. Two hours of perfusion with human plasma induces in control animals the synthesis of IL-1α, IL-1β, and IL-6. This induction is significantly lower in the livers of transgenic mice which presents some differences according to the level of expression of either hMCP or hDAF. One day after perfusion the level of induction in all groups examined is equally lower and comparable to the level observed in the untreated controls.

Murine P-selectin localization

We have also studied the behaviour, in our *in vivo* model, of another molecule involved during hyperacute rejection, that is P-selectin, an adhesive glycoprotein normally included in the cytoplasmic vesicles of endothelial cells and platelets. During HAR, as a sign of endothelial cell activation, P-selectin is exposed on the EC surface and thus can be detected by the use of specific antibodies. As shown in Figure 5, in our *in vivo* model of HAR, P-selectin is mobilized and could be detected only in the livers derived from control animals after 2 h of perfusion with human plasma (Figure 5A). One day after perfusion of control mice (Figure 5B), as well after 2 h of perfusion of mice transgenic either for hMCP (Figure 5C) or hDAF (Figure 5D), P-selectin is not exposed and cannot be detected on the hepatic endothelium system, thus confirming the protective effect of the transgenic molecules on this early HAR event.

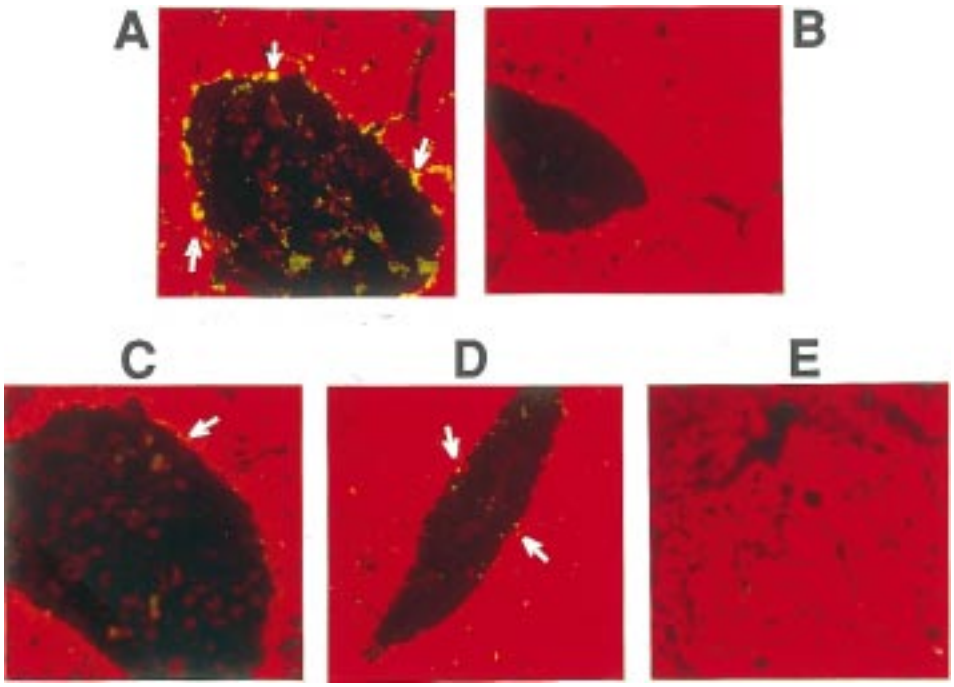

Figure 5. Immunohistochemical analysis of murine P-selectin in the liver of perfused control and transgenic mice. Fluorescent light micrographs of livers derived from control mice perfused for 2 h with human plsma (A), from control mice 1 day after the 2 h of perfusion (B), from hMCP transgenic mice (C), from hDAF transgenic mice (D) or from untreated control mice (E). Liver cryostat sections were immunostained with anti-mouse P-selectin rabbit antibody and FITC-anti-rabbit antibody. Sections were counterstained with Blue Evans. Arrows indicate P-selectin exposed on the EC surface. Original magnification 250 ×.

Discussion

The success of xenotransplantation is limited owing to the immediate immune reaction named hyperacute rejection (HAR). This process is caused by preexisting host xenoreactive antibodies (XNAs) binding to the graft and by the activation of the complement system. The experimental approaches to overcome HAR have mostly exploited the genetic engineering of the donor (transgenesis) with the purpose of blocking either complement activation or XNAs (Cozzi & White, 1995; Sharma et al., 1996; Bach, 1997; McKenzie et al., 1998). Although, there is much evidence that transgenic animals expressing human complement inhibitors are protected from HAR (Fodor et al., 1994; Cozzi & White, 1995; McCurry et al., 1995; Kroshus et al., 1996; Carrington et al., 1997), it is also evident that the protection is affected by the level and site of expression of the human complement regulators in the donor organs (Cozzi & White, 1995; Byrne et al., 1997). In addition, to date it is not yet clear if one or more of the numerous inhibitors or even other molecules are required for a full protection (Sharma et al., 1996; van Denderen et al., 1997; Bach et al., 1997a,b; Soares et al., 1998; Tran et al., 1998; McKenzie et al., 1998; Badrichani et al., 1999). Obviously, an experimental model of HAR could be useful to test different expression vectors and molecules. We have described a mouse model of *in vivo* perfusion with human plasma, by which it is possible to recreate the initial events of HAR (Mulder et al., 1995a,b; Lazzeri et al., 1998). By this model we have also shown that the expression of transgenic human complement inhibitors (hDAF, hMCP, or both) protects the organs from complement-mediated damage (Mulder et al., 1995a,b; Lazzeri et al., 1998). This report describes a more detailed characterization of our HAR model. The analysis of different parameters representative of the HAR reactions induced by human complement, carried out at the end of 2 h of perfusion with human plasma and 1 day after the treatment, enable us to reach the following conclusions.

First, the human complement present in the perfused plasma induces, in control mice, EC activation (Coughlan et al., 1993; Bach et al., 1995; Saadi et al.,

1995; Selvan et al., 1998), that is the synthesis of pro-inflammatory molecules (IL-1α, IL-1β, IL-6) and the exposure of P-selectin on the EC surface, which in turn starts the process of HAR. The haematological values of the hepatic functions (GOT and GPT) of perfused control animals, confirmed the histopathological observation of a severe liver damage, that persists and increases 1 day after the 2 h of perfusion, showing extensive areas of necrosis.

Second, the analysis of the livers of perfused transgenic mice expressing human complement inhibitors, indicated that the protection from complement-mediated damage is dependent on the level of expression of the human molecules, as shown by the different extent of the inflammatory reactions. In fact, as shown in the figures, the livers derived from perfused mice transgenic for hDAF, whose expression in this organ is lower than that of hMCP, present evidences of an inflammatory process. In particular, small foci of mononuclear cells were occasionally observed in the liver sections. Transgenic RCA's expression-dependent protection was also observed in kidneys derived from perfused transgenic mice. In this organ the expression hDAF is higher than hMCP and accordingly, the kidneys derived from perfused MT-DAF transgenic mice are more protected than that derived from perfused MT-MCP transgenic mice (Lazzeri et al., 1998).

Cytokines induction, although significantly reduced in comparison to that observed in perfused control mice, was more relevant in the hDAF transgenic livers than in the livers derived from perfused hMCP transgenic mice.

Third, we demonstrated that in the presence of hDAF or hMCP, vascular integrity was preserved and the inflammatory process induced by perfusion was totally reversible.

In summary, our experimental model, reproducing the events characteristic of HAR described in humans, can be of help in the identification of appropriate expression vectors and to test single or different combinations of molecules capable of blocking hyperacute and acute rejection. It can, therefore, be a prelude for the appropriate genetic engineerization of pigs suitable for xenotransplantation.

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