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ORIGINAL ARTICLE

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Adenovirus-mediated gene transfer of triple human complement regulating proteins (DAF, MCP and CD59) in the xenogeneic porcine-to-human transplantation model

Part I: in vitro assays using porcine aortic endothelial cells

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Abstract We assessed whether the adenovirus-mediated gene transfer of triple human complement regulating proteins (hCRPs) to the porcine aortic endothelium (PAE), could possibly exert a synergistic effect to inhibit human complement activation. Adenovirus vectors, encoding *E.Coli* β-galactosidase (AxCALacZ), human membrane cofactor protein (MCP) (AxCAMCP), decay-accelerating factor (DAF) (AxCADAF), and CD59 (AxCACD59) were produced by the COS-TPC method. AxCALacZ was transfected to porcine aortic endothelium cells (PAECs) under various multiplicities of infection (MOI) to determine the efficiency of adenovirus-mediated gene transfer by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) staining. The mRNA expressions of transfected CRPs were examined by reverse transcriptase-polymerase chain reaction (RT-PCR). Cellular damage to the PAEC was assessed by an MTT assay. PAEC was most efficiently transfected with the LacZ gene at 10^3 MOI/60-min incubation time (89.1%). In all samples transfected with the CRP gene, the corresponding mRNAs were detected in the RT-PCR. In the MTT assay, PAECs co-cultured with 20% human serum, showed the highest cellular viability after gene transfer of triple CRPs (117.7%), when compared with those of marker LacZ, single or double CRPs. The adenovirus-mediated multiple gene transfer of CRPs may thus be an efficient method for suppressing complement activation in the porcine-to-human model of hyperacute rejection.

Keywords Hyperacute rejection · Porcine aortic endothelial cell · Adenovirus vector · MCP · DAF · CD59

Introduction

In xenogeneic transplantation or xenoperfusion, rapid destruction of the xenoperfused organs is caused by hyperacute rejection (HAR), in which the complement activation on the endothelial cells plays a fundamental role [15, 16]. Since the complement activation is caused by an inability of complement regulating protein (CRP) to act beyond species [17], transgenic pigs which express human CRPs (hCRPs) have thus been developed to cope with HAR. In these models, single to double CRPs such as decay-accelerating factor (DAF) and CD59 were reported to be effective in suppressing the cascade of human complement activation [3]. Human CRPs, however, can be divided into four major proteins, namely membrane cofactor protein (MCP), decay-accelerating factor (DAF), CD59, and complement receptor type 1 (CR1) [8, 11, 21], which inhibit complement activation at different levels. DAF inhibits converting enzymes of C3 and C5, CR1 and MCP accelerate I factor-mediated inactivation of C3b, and CD59 inhibits formation of the membrane attack complex (MAC). At least theoretically, the multiple and synchronous expression of CRPs may thus induce effective complement inactivation.

Transgenic animals reported to express double CRPs (DAF and CD59) by Byrne et al. [3] had a relatively high endothelial expression of CD59, but a low level of DAF. More recently, Cowan et al. reported transgenic pigs expressing CD55, CD59 and H-transferase, in which two different hCRPs were equally expressed in the heart, lung, liver and pancreas, but the third transgene (H-transferase) was expressed at low levels [6]. Simultaneous expression of CD55 and CD59 in these transgenic pigs resulted in prolonged survival of the kidney xenograft of up to 5 days, when compared with the relatively short survival time of single hCRP (CD55) expression [6]. Although these might be the first transgenic pigs to express two different CRPs at similar levels, multiple and equal expressions of more than two hCRPs have yet to be established. It thus appears important to find a different method for achieving a multiple and simultaneous expression of CRPs in xenogeneic pig organs, other than through transgenic technology.

We previously reported the adenovirus-mediated gene transfer to the porcine endothelium in vitro [14], and to the whole porcine liver in vivo [18]. In these reports, adenovirus-mediated gene transfer was found to efficiently transduce the marker LacZ gene into the porcine endothelium and hepatocytes, without damaging these cells. Our next objective was thus to establish the effective gene transfer of the multiple physiologically active proteins in the porcine liver, and thus obtain an interaction between these proteins. In the present study, we tried to establish the adenovirus-mediated multiple gene transfer of three different hCRPs in a HAR model. Since the target of HAR in xenogeneic organ transplantation is the endothelium, the porcine aortic endothelial cell (PAEC) was selected as the target. In the literature, there has so far been no report concerning whether multiple exogenous genes, such as hCRPs, transferred to the porcine endothelium by adenovirus-mediated gene transfer could exert their own physiological activities. Moreover, it is also unknown whether these multiple exogenous genes can exert their own physiological activities in an interactive manner, as expected for transfected hCRPs, and thereby act cooperatively in inhibiting human complement activation.

To clarify these unknown issues concerning adenovirus-mediated gene transfer, we thus investigated whether or not the multiple gene transfer of hCRPs to the PAEC was effective in suppressing the activation of human complement activities, in a HAR model in vitro.

Materials and methods

Cell isolation and culture of porcine aortic endothelial cells

Porcine aortic endothelial cells (PAECs) were harvested from the abdominal aorta of a female pig (20–25 kg; Ryukyu Biotec, Okinawa, Japan). The abdominal aorta was removed immediately after the pig had been killed, and all minor branch vessels were ligated. The aorta was flushed with phosphate buffer saline (PBS) and penicillin/streptomycin (GIBCO GRL, Tokyo, Japan) and then filled with 0.05% collagenase solution for 15 min at 37 °C. The endothelial cells were then harvested, centrifuged, and suspended in a culture medium (RPMI1640; GIBCO GRL) containing 10% fetal bovine serum (FBS; BIO Whittaker) with L-glutamine and penicillin/streptomycin (GIBCO GRL). The endothelial cells were then seeded in 3-cm collagen-coated culture dishes. The culture medium was changed every 2 days. Once the primary cultures had reached confluence, the cells were subcultured by trypsin-EDTA (GIBCO GRL) treatment until the eighth to 11th passages. The cells were identified as endothelial cells, both morphologically and based on their ability to take up acetylated low-density lipoprotein (Ac-LDL) [9]. Each assay was performed at least in triplicate using PAECs obtained from three pigs.

Construction of adenovirus vectors

All adenovirus vectors were constructed with an "Adenovirus Expression Vector Kit" (TaKaRa Biomedical, Japan). The full length cDNA of MCP was kindly provided by J.P. Atkinson, M.D. (Washington University Medical School), DAF by M.E. Medof, M.D. (Institute of Pathology, Case Western Reserve University School of Medicine, University Hospital of Cleveland), and CD59 by A. Davies, Ph.D. (Medical Research Council Centre, Molecular Immunopathology Unit). The cDNAs of MCP, DAF and CD59 were inserted at ECoRI, ECoRI and XbaI in the multi-cloning site of plasmid vector (pHβAPr1neo, pUC19 and CDM8). The full lengths of MCP, DAF and CD59 cDNA were 1,540 bp, 2,200 bp and 1,140 bp, respectively. These cDNAs were adjusted by the restriction enzymes and were sub-cloned into the SwaI site of the cosmid vector (pAxCAwt) which had a CAG promoter (cytomegalovirus enhancer, chicken β-actin promoter, rabbit β-globin polyA signal; "Adenovirus Expression Vector Kit"). Similarly, the cosmid vector coding *E.Coli* β-galactosidase (pAxCALacZ) was also prepared [10]. Eight micrograms of cosmid vectors and 1.5 μg of restriction enzyme (EcoT22I) which digest DNA-TPC were co-transfected into 1×10^5 293-kidney embryonal cells [12]. The supernatant containing the adenovirus vector was transfected four times to fresh 293-kidney embryonal cells, with increasing culture medium up to 15 ml. The construct of the adenoviral vector is schematically illustrated in Fig. 1. The viral titers were determined, based on the 50% tissue culture infectious dose ($TCID_{50}$), in which, the virus titer in the $TCID_{50}$ corresponds to the plaque-forming units (pfu) [20]. These virus vectors of AxCALacZ (6×10^9 pfu/ml), AxCAMCP (3×10^9 pfu/ml), AxCADAF (3×10^9 pfu/ml), and AxCACD59 (1×10^8 pfu/ml), were stored at –80 °C until required.

Endothelial enzyme-linked immunosorbent assay for xenogeneic IgG and IgM

To determine the IgG and IgM deposition on the PAE co-cultured with human serum (HS), we performed endothelial enzyme-linked immunosorbent assay (ELISA) [2]. PAECs were seeded on microtiter plates (tissue culture grade, 96-well, flat bottomed; Becton Dickinson Labware, USA) at a concentration of 1×10^4 cells/well in 100 μl RPMI1640 containing 10% FBS, and were incubated for 16 h with 5% CO_2 at 37 °C. The PAECs were then immediately fixed with 100 μl of 4% paraformaldehyde for 5 min at room temperature and then were washed with PBS. The plates were stored at –70 °C until required. The detection of alkaline phosphatase was performed using AmpliQ (DAKO, UK). To inhibit a non-specific protein reaction, a blocking buffer (0.1% gelatin, 1% non-fat dry milk in PBS) was incubated for 1 h at room temperature. After removing the supernatant, we incubated target PAECs in 100% HS

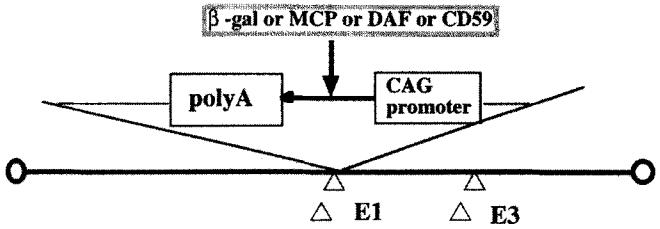

Fig. 1. Construction of adenovirus vector. The adenovirus vector carried the expression cassette of β-galactosidase, MCP, DAF or CD59, under control of the CAG promoter and upstream of a polyadenylation signal. The expression cassette replaced the viral E1 region (early viral protein) and E3 region. β-gal *E.Coli* β-galactosidase, *MCP* membrane co-factor protein, *DAF* decay-accelerating factor, *CAG promoter* cytomegalovirus enhancer, chicken β-actin promoter, rabbit β-globin polyA signal

at 37 °C for 1 h in 96-well microplates. PAECs were washed once by PBS (100 μl), and were reacted with alkaline phosphatase-conjugated rabbit anti-human IgM (μ-chain specific) and anti-human IgG (γ-chain specific) (DAKO, Japan), which were diluted 10^3 while being maintained for 60 min in PBS. After incubation with antibodies, the wells were washed four times with wash buffer (250 μl), and 200 μl of substrate for the alkaline phosphatase was added to each well. The detection of the absorbance was measured at 490 nm using an ELISA plate reader. As a negative control, porcine serum was used instead of HS. The HS was kindly provided by three healthy volunteers who all had different blood types, and an equal amount of HS was added to each well.

Gene transfer of the marker LacZ gene to the PAECs

Each 1×10^4 portion of purified PAECs was distributed and cultured in 96-well plates for 24 h, and then the AxCALacZ were added to each well in consecutive dilutions of 1, 10, 10^2, 10^3 and 10^4 MOI for different exposure times (5, 30, and 60 min). Next, each well was washed once with 100 μl of PBS, and then incubated at 37 °C for 24 h with RPMI1640 medium containing 10% FBS. Cell monolayers of transfected PAECs were fixed for 15 min in 0.5% glutaraldehyde and rinsed twice in PBS. Samples were then incubated at 37 °C for 2 h in 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution containing $K_3Fe(CN)_6$ (41 mg/ml), $K_4Fe(CN)_63H_2O$ (52.5 mg/ml), and 1 mM $MgCl_2$. After completion of the reaction, the X-gal solution was removed and the samples was washed thoroughly with PBS. Any cells that stained blue were considered to express *E.Coli* β-galactosidase, and the averages of the positive cell percentages per field were computed using the densitograph software program (ATTO, Tokyo, Japan).

Experimental design in gene transfer of the hCRP genes to the PAECs

The PAEC was transfected with AxCALacZ in group1 (column 6), AxCAMCP in group 2 (column 1), AxCADAF in group 3 (column 2), AxCACD59 in group 4 (column 3), AxCADAF + AxCACD59 in group 5 (column 4), and AxCAMCP + AxCADAF + AxCACD59 in group 6 (column 5). Columns 7 and 8 (group 7) showed the viability of the untreated PAECs (without adenovirus vector) with (column 8) or without (column 7) 20% HS.

Reverse transcriptase-polymerase chain reaction

The expressions of mRNA from transfected CRPs were detected by reverse transcriptase-polymerase chain reaction (RT-PCR). PAECs (1×10^5) were incubated with adenovirus vector in 6-cm dishes, at 37 °C under 5% CO_2 for 1 h, under consecutive dilutions (10, 10^2 and 10^3 MOI) of each adenovirus vector. Each dish was washed once with 3 ml of PBS, and then was incubated at 37 °C for 1, 2 and 3 days in RPMI1640 medium with 10% FBS. The cells were then collected with a cell scraper. Total RNA extraction was performed by the acid guanidinium phenol–chloroform (AGPC) method [5] on these cells, and the RNA concentration was checked by a spectrophotometer. The RT-PCR was performed with the Geneamp EZ rT*th* RNA PCR kit (Perkin Elmer) [13]. The sequences of the specific oligonucleotide primers were as follows: CCCTACTATGAGATTGGTGAACG and TTCGACTACTGGAAATCGACATT for MCP, TATTATTTGGTGCAACCATCTCC and TTTGTGGTGGTTTTCTGAGAAGT for DAF, and CATTACCAAAGCTGGGTTACAAG and GAAGCTCTCCTGGTG TTGACTTA for CD59. The mRNA was amplified by an RT-PCR in a 50-μl reaction-mixture volume. The contents of the mixture consisted of 5× EZ buffer, 10 μl; dNTP, 10 mM; 5 units of rT*th* DNA polymerase; 25 mM $Mn(OAc)_2$ solution, 5 μl; 200 pmol each of primer; and 1 μg of sample, which were reverse transcripted at 60 °C for 30 min. The protocol of DNA amplification for CRPs consisted of a single cycle at 94 °C for 2 min, and 30 cycles at 94 °C for 1 min and 60 °C for 1 min, and a final extension step was done at 60 °C for 7 min. Positive controls were mRNA of 293 cells post-transfected with AxCAMCP, AxCADAF or AxCACD59. The negative control was mRNA of 293 cells post-transfected with AxCALacZ.

MTT assay

An MTT assay was used to determine the cellular damage of the PAE caused by human complement activation [19]. PAECs were seeded on microtiter plates at a concentration of 1×10^4 cells/well in 100 μl RPMI1640 containing 10% FBS, and were incubated for 16 h with 5% CO_2 at 37 °C. The target PAECs were incubated in 20% or 40% HS at 37 °C for 1, 3, and 5 h in 96-well flat-bottomed microplates, and then the supernatant was removed. PAECs were washed once in 100 μl PBS. Cell viability was analyzed by a colorimetric assay (MTT assay). As a negative control, porcine serum was used instead of HS. We also used the same assay in the HAR inhibition model in vitro. PAECs (1×10^4) were incubated with adenovirus vector (groups 1 to 6), under 10^3 MOI at 37 °C, 5% CO_2, for 1 h, in 96-well flat-bottomed microplates. Immediately after transfection, the supernatant was removed and the PAECs were washed once in 100 μl PBS. Next, 100 μl of RPMI1640, containing 10% FBS, were added to the PAECs, which were then incubated at 37 °C under 5% CO_2 for another 1, 2 and 3 days. PAECs were further incubated with 20% HS for 1 h at 37 °C, under 5% CO_2. The supernatant was removed and the PAECs were washed again in 100 μl PBS. Finally, cellular viability was checked by the MTT assay, in which the complete solubilization of the purple formazan crystals was measured, based on the spectrophotometric absorbance of the samples, using a microtiter plate (ELISA) reader. Cellular viability was determined by the following formula: percent viability = (absorbance in the sample/absorbance in the negative control)×100. The wavelength used to measure the absorbance of the formazan product was 570 nm, and the reference wavelength was 655 nm.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD) of the mean value. Group comparisons were performed with the one-factor analysis of variance (ANOVA) and a paired *t*-test when appropriate, and differences were considered to be statistically significant at $P < 0.05$.

Results

Endothelial ELISA

As a model of HAR in vitro, the deposition of xenoreactive antibody on the PAEC was confirmed, and the appropriate concentration of HS was determined. Xenoreactive IgM and IgG antibodies deposited on the PAEC were detected by endothelial ELISA. In those samples incubated with 100% HS, IgM and IgG depositions were positively detected at 10^3 dilution of primary antibody. The IgM and IgG deposition levels (absorbance: 1.140 ± 0.132 and 0.764 ± 0.091, respectively) were found to be higher than those incubated with porcine serum (0.264 ± 0.010 and 0.109 ± 0.008, respectively), and the differences were significant ($P < 0.001$ and $P < 0.001$) (Fig. 2).

X-gal staining of PAECs

Since all four adenovirus vectors used in the present study, encoding either a marker LacZ gene or three different hCRP genes, had the same structure, we thus expected to find similar levels of protein expression from each transduced gene. The protein expression was thus enzymatically assessed using the PAECs transfected with maker LacZ genes (Table 1). The positive cell percentages of the PAECs exposed for 30 and 60 min further increased, when compared with those exposed for 5 min. The peak percentage of positive PAECs was $89.1 \pm 3.1\%$ at a titer of 10^3 MOI for 60 min. Based on these results, the PAECs were found to be the most efficiently gene transferred by the adenovirus vector after 60 min and 10^3 MOI of incubation.

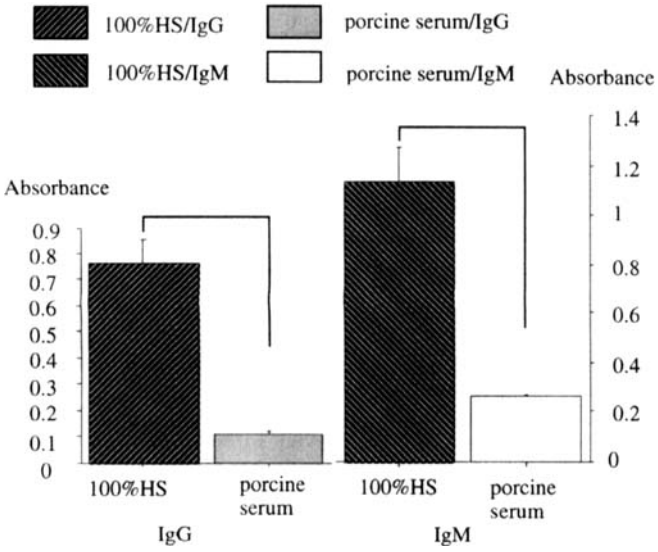

Fig. 2. Natural antibody levels deposited on the porcine endothelial cells (endothelial ELISA). Xenoreactive human IgM and IgG antibodies deposited on the PAECs were detected by endothelial ELISA. The IgM and IgG depositions were found to be higher (1.140 ± 0.132 and 0.764 ± 0.091, respectively) than those incubated with porcine serum (0.264 ± 0.010 and 0.109 ± 0.008, respectively), with significant differences ($P < 0.001$ and $P < 0.001$, respectively). *HS* human serum

Table 1. Positive cell percentage of the PAECs expressing marker LacZ genes (X-gal staining). Positive PAECs expressing β-galactosidase activities from the transfected marker LacZ genes were enzymatically assessed in X-gal staining. Percentages of the positive cells were calculated per field by a computer-driven densitograph software program. All data are expressed as the mean ± SD (%)

Incubation time (min)	MOI				
	1	10	10^2	10^3	10^4
5	2.2 ± 1.8	10.9 ± 1.1	10.2 ± 2.4	21.8 ± 1.6	16.4 ± 1.8
30	13.4 ± 1.7	16.5 ± 1.8	32.2 ± 2.4	58.9 ± 2.8	40.3 ± 2.6
60	10.7 ± 1.3	14.3 ± 1.6	20.1 ± 1.8	89.1 ± 3.1	42.5 ± 1.8

RT-PCR of CRPs (MCP, DAF and CD59)

The expression of mRNA from each transfected hCRP gene was also evaluated semiquantitatively in the PAEC. All RT-PCR products were detected on agarose gel. The expression of DAF mRNA increased along with an increase of MOI (10 to 10^3). At 10^3 MOI DAF mRNA expression peaked at 1 post-transfection day (905.8 ng at 416 bp) (Fig. 3b). On the other hand, the expression of MCP and CD59 mRNA gradually increased with the passage time. The amount of RT-PCR products of MCP and CD59 peaked at 3 post-transfection days at 10^3 MOI (MCP: 403.2 ng at 548 bp; CD59: 391.3 ng at 252 bp) (Fig. 3a and Fig. 3c). When the PAEC was tranfected with double or triple vectors encoding CRPs, there was no reduction in its mRNA expression for each CRP, when compared with the single transfection under compatible MOI (data not shown).

MTT assay

Since the complement activation itself could not be assayed in the present in-vitro system, cellular damage caused by an antibody deposition followed by complement activation was evaluated with an MTT assay. As a result, cellular damage of the PAE was somewhat stronger in those samples incubated with 20% HS than those incubated with 40% HS, but without any significant difference. PAECs incubated for 5 h with 20% HS showed stronger cell injury ($38.9 \pm 5.1\%$) than those incubated for 1 and 3 h ($41.0 \pm 2.4\%$ and $47.4 \pm 4.7\%$, respectively), with no significant difference (Fig. 4).

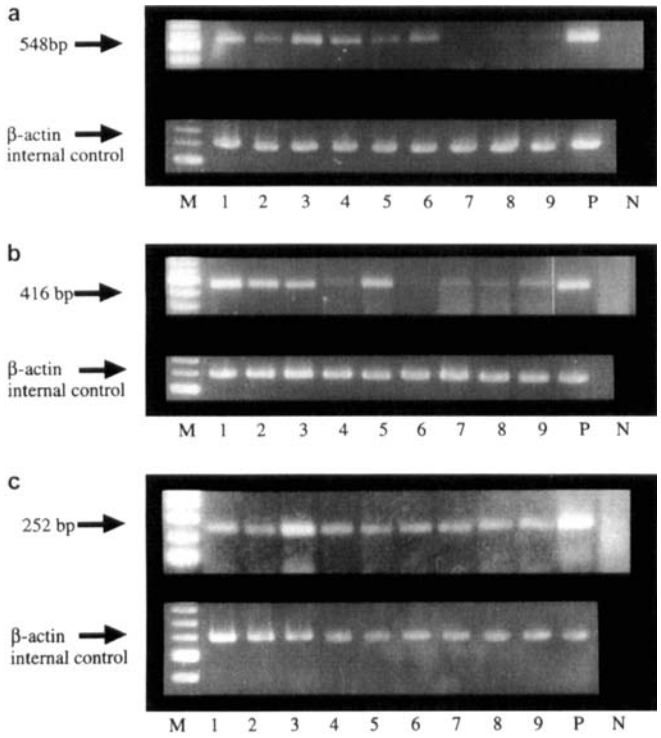

Fig. 3a–c. mRNA expression of hCRPs (MCP, DAF and CD59)(RT-PCR). The expression of mRNA from transfected hCRP each gene (MCP, DAF and CD59) was also evaluated semiquantitatively in the PAECs. All RT-PCR products were detected on agarose gel. The amounts of RT-PCR products were calculated by NIH image. RT-PCR product of **a** MCP (403.2 ng at 548 bp) and **c** CD59 (391.3 ng at 252 bp) peaked at 3 post-transfection days and 10^3 MOI. **b** The maximum dose of RT-PCR product DAF (905.8 ng at 416 bp) was at 1 post-transfection day and 10^3 MOI *M* marker/100 bp DNA ladder, *1* 1 post-transfection day and 10^3 MOI, *2* 2 post-transfection days and 10^3 MOI, *3* 3 post-transfection days and 10^3 MOI, *4* 1 post-transfection day and 10^2 MOI, *5* 2 post-transfection days and 10^2 MOI, *6* 3 post-transfection days and 10^2 MOI, *7* 1 post-transfection day and 10 MOI, *8*: 2 post-transfection days and 10 MOI, *9* 3 post-transfection days and 10 MOI, *P* positive control, *N* negative control

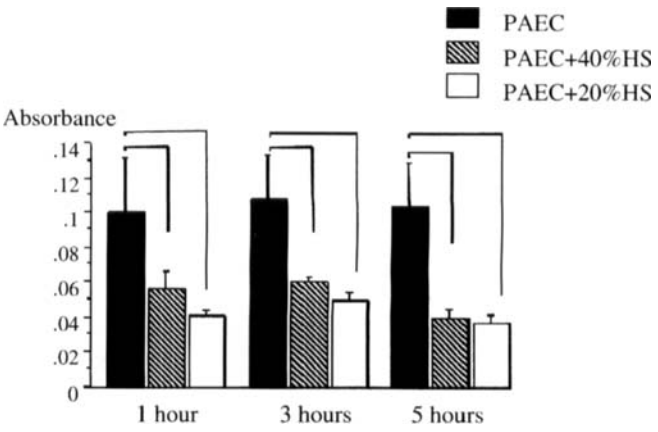

Fig. 4. Analysis of the cellular damage of the porcine aortic endothelial cell (*PAEC*) with human serum (*HS*) in MTT assay. The MTT assay was used to assess the cellular damage of the PAE caused by human complement activation. The complete solubility of the purple formazan crystals from PAECs was measured, based on the spectrophotometric absorbance of the samples, using a microtiter plate (ELISA) reader. Cellular damage of the PAE was somewhat stronger in the samples incubated with 20% HS than in those incubated with 40% HS, without significant difference. PAECs incubated for 5 h with 20% HS showed stronger cell injury ($38.9 \pm 5.1\%$) than those incubated for 1 and 3 h ($41.0 \pm 2.4\%$ and $47.4 \pm 4.7\%$, respectively), with no significant difference

From these results, 20% HS was used for further experiments. PAEC transfected with single CRP, in groups 1 (LacZ), 2 (MCP), 3 (DAF), and 4 (CD59), showed peak cellular viabilities at 10^3 MOI and 2 post-transfection days ($71.6 \pm 7.5\%$), ($87.4 \pm 4.0\%$), and ($92.8 \pm 2.7\%$), respectively, each showing a significant difference when compared with group 7 ($P = 0.01$, $P < 0.001$, and $P < 0.001$, respectively) (Fig. 5). PAEC transfected with double CRPs, in group 5 (DAF + CD59), showed a peak cellular viability at 10^3 MOI and post-transfection day 2 ($100.0 \pm 12.5\%$), with a significant difference ($P < 0.001$) when compared with group 7 (Fig. 5). In addition, the cellular viability was significantly higher in group 6 (MCP + DAF + CD59: $117.7 \pm 1.8\%$), than in group 7 ($P < 0.001$), under the same conditions (Fig. 5).

Discussion

The aim of the present study was first to investigate whether the adenovirus-mediated gene transfer of hCRPs could actually exert a complement inhibitory function against human complement activation, on the porcine endothelial cells. Secondly, we investigated whether or not these three different hCRPs transfected on the PAECs could actually interact with each other to exert their own complement inhibitory functions. Since porcine endothelial cells are a major target in HAR, the production of functional protein, such as hCRPs, after adenovirus-mediated gene transfer could thus be used to prevent HAR. Recently, there have been many reports concerning adenovirus-mediated gene transfer to the endothelial cells. In a transplant setting, however, adenovirus-mediated gene transfer to the endothelial cells has not yet been used to treat rejection, neither in allogeneic nor xenogeneic transplantation.

In the present in-vitro study, only the mRNA expression, not the protein expression, of hCRP was confirmed. The protein expression from the transfected marker LacZ genes, however, was confirmed by an enzymatic assay, and we thought that a similar protein expression could thus also be obtained for the hCRPs, since the same adenovirus vector with the same promoter was used for the adenovirus vector carrying hCRPs. In another line of experiments from our laboratory, we actually detected the protein expression of

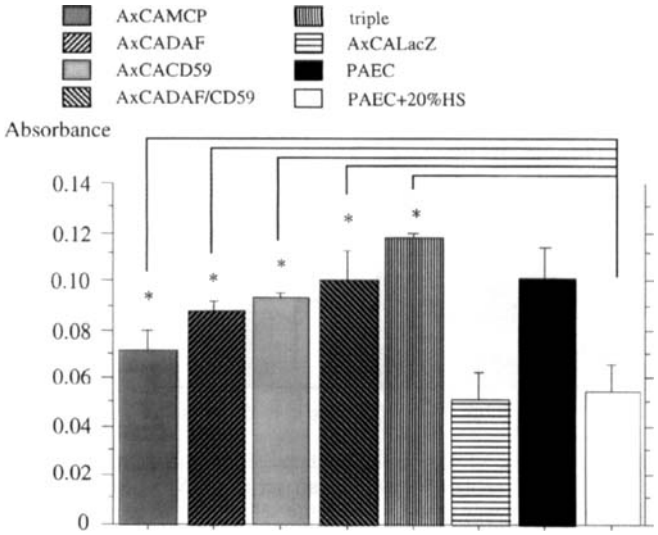

Fig. 5. Complement inhibitory effects of hCRP gene transfer on the porcine aortic endothelial cell (*PAEC*) treated with fresh human serum (*HS*) (MTT assay). Cellular damage of the PAEC was assessed in PAECs transfected with adenovirus vector, using an MTT assay. In five groups (AxCAMCP, AxCADAF, AxCACD59, AxCADAF + CD59 and triple adenovirus-vector transfected PAECs), the peak of viability on PAECs existed in 10^3 MOI at 2 days post-transfection, and the difference was significant when compared with group 7 ($P = 0.01$, $P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.001$)

these hCRPs in the porcine liver in-vivo, using the same adenovirus vectors and in-situ perfusion of the liver (Part II of our paper). Regarding the ideal conditions for gene transfer, PAECs were increasingly transduced along with an increase in the titers between 1 MOI and 10^3 MOI, but inversely decreased at more than 10^4 MOI. Under the same conditions, PAECs were also increasingly transduced along with an increased culture time to within 60 min. Based on these data, PAECs were thought to be most effectively transduced at 10^3 MOI for 60 min. Since the adenovirus vector is known to have viral toxicity at extremely high titers, due to viral-coat proteins such as hexon or penton [7], a decreased efficiency of gene transfer at more than 10^4 MOI might thus be attributed to this cellular toxicity on PAEC. In fact, cellular damage observed in the MTT assay findings revealed increased PAEC damage at MOI of more than 10^4. These facts indicated that adenovirus-mediated gene transfer should thus be performed with appropriate titers and contact periods over the target cells, to obtain an effective transduction and the lowest cellular toxicity at the same time [1].

Based on the results obtained from the marker-gene transfer, we further investigated whether or not the adenovirus-mediated gene transfer is capable of expressing multiple hCRPs on the PAEC. As was expected, the PAEC transfected with adenovirus vectors encoding different CRPs, expressed corresponding mRNA of each hCRP. Even when the double or triple CRPs were simultaneously transfected, there was no reduction or enhancement in the expression of each CRP mRNA when compared with those in single transduction of each CRP. These findings thus indicated that adenovirus-mediated gene transfer might be useful for the simultaneous and uniform expression of different exogenous genes on the target cells. Recently, Cowan et al. reported that the simultaneous expression of CD55 and CD59 in the transgenic pigs resulted in prolonged survival of the kidney xenograft of up to 5 days, compared with the relatively short survival of single hCRP (CD55) expression [6]. Although these might be the first transgenic pigs to express two different CRPs at similar levels, the multiple and equal expressions of more than two hCRPs have yet to be established. These features of transgenic animals might be derived from the lethal effect of multiple and uniform gene expression, either in their birth or growth. When these features of transgenic technology are considered, adenovirus-mediated gene transfer might thus offer some advantages over transducing multiple genes.

Since the complement activation itself cannot be evaluated in very small amounts of supernatant in our in-vitro settings, the inhibitory effects of transduced hCRPs on PAECs were evaluated by a cytotoxic assay. Using the cytotoxic assay, we thus investigated whether or not the PAECs transduced with multiple hCRP genes may have any advantage in suppressing human complement activation, when compared with those transfected with single CRP or marker LacZ. As a result, PAECs transfected with triple CRPs (MCP + DAF + CD59) had the highest cellular viability, with a significant difference in comparison with those with single (MCP, DAF, or CD59) or double (DAF + CD59) CRPs. PAECs transfected with double CRPs (DAF + CD59) also had a significantly higher cellular viability than those transfected with single CRP. PAECs transfected with double CRPs (MCP + DAF), however, did not improve cellular viability when compared with PAECs transfected with single CRP (data not shown). These three CRPs act at two different levels within the complement pathway. DAF reacts on the level of the C3 and C5 convertases, and MCP resolves C3b into iC3b as a co-factor for factor I acting complementary toward DAF [4]. The third regulator, CD59 directly regulates the formation and function of the terminal cytolytic membrane attack complex (MAC) by specifically interacting with complement components C8 and C9 [8]. In this context, it is reasonable that combined use of CD59 yields a synergistic effect in suppressing complement activation. Based on these mechanisms in the regulation of the complement cascade and the results obtained in the present study, the expression of the multiple CRPs should thus be required to effectively inhibit complement activation.

The present study indicated that the adenovirus-mediated gene transfer of multiple hCRP genes could induce the complement inhibitory functions of these genes in an interacting manner on porcine endothelial cells. These methods might further increase the chance of successfully modulating the porcine liver in order to perform successful xenotransplantations in the future.

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