The In Vitro Protection of Human Decay Accelerating Factor and hDAF/Heme Oxygenase-1 Transgenes in Porcine Aortic Endothelial Cells Against Sera of Formosan Macaques

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ABSTRACT

To mitigate hyperacute rejection, pigs have been generated with α-Gal transferase gene knockout and transgenic expression of human decay accelerating factor (hDAF), MCP, and CD59. Additionally, heme-oxygenase-1 (HO-1) has been suggested to defend endothelial cells. Sera (MS) (0%, 1%, 5%, 10%, and 15%) from Formosan macaques (*Macaca cyclopis,* MC), an Old World monkey wildly populated in Taiwan, was used to test the protective in vitro, effects of hDAF or hDAF/hHO-1 on porcine aortic endothelial cells (pAEC) derived from $hDAF^+$, $hDAF^+/hHO\text{-}1^+$, and $hDAF^+/hHO\text{-}1^-$ and 1 nontransgenic pAEC. Ten percent human serum (HS) served as a positive control. When MS addition increased to 10% or 15%, all transgenic pAEC exhibited a greater survival than nontransgenic pAEC. Noticeably, 15% MS reduced survived to $<10\%$ versus $>40\%$ in nontransgenic and transgenic pAEC, respectively. These results revealed that hDAF exerted protective effects against MC complement activation. However, comparing with 10% MS and HS in pAEC of nontransgenic pigs, the survivability was higher in HS, suggesting that complement activation by MS was more toxic than that by HS. Furthermore, $hDAF^+/hHO\text{-}1^+$ showed no further protection against effects of MS on transgenic pAEC.

THE PIG IS considered to be a good xeno-donor, but the grafts inevitably encounter rejection. Genetic modifications of pig α-Gal transferase gene knockouts and transgenic hosts with human decay accelerating factor (hDAF), MCP, and CD59 have proven somewhat effective to overcome hyperacute rejection.[1,2] Additionally, heme oxygenase-1 (HO-1) has been suggested to defend endothelial cells.[3] We generated hDAF[4] and hDAF/hHO-1[5] transgenic pigs for xenotransplantation studies. Using ex vivo perfusion of transgenic pig kidneys with human blood, we confirmed that an high expression of hDAF overcomes hyperacute xenograft rejection; the expression of HO-1 provided little advantage to prolong xenoperfusion.[5] Before using a nonhuman primate model to study the function of hDAF and hDAF/hHO-1 transgenes, we have established aortic endothelial cells from these transgenic pigs as an in vitro model to test the function of transgenes. The Formosan macaque (*Macaca cyclopis* [MC]) which is an Old World monkey wildly populated in Taiwan, is closely related to the Cynomolgus monkey (*M fascicularis*),[6] which is widely used in xeno-kidney transplantation.[7] Serum samples, from MCs that had been well quarantined and maintained in a specific pathogen-free (SPF) animal house, were used to examine in vitro protective effects of hDAF or hDAF/hHO-1 on porcine aortic endothelial cells (pAEC).

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MATERIALS AND METHODS

Animals and Cells

MC kept in a SPF animal house were used in accordance with the Wildlife Conservation Act of ROC. Care and usage of animals accorded with the Guide for the Use and Care of Laboratory Animals prepared by the International Animal Care and Use Committee. Sera were obtained from 3 MC. We established pAEC from aortic blood vessel of 3 transgenic lines (hDAF$^+$, hDAF$^+$/hHO-1$^+$, and hDAF$^+$/hHO-1$^-$) and 1 nontransgenic pig.

Reverse Transcriptase Polymerase Chain Reaction Analysis

The mRNA expressions of hDAF and hHO-1 transgenes were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA extracted from pAEC with the Trizol reagent (Invitrogen, Carlsbad, Calif) was reverse transcribed to cDNA by M-MLV reverse transcriptase (Promega, Madison Wisc). The PCR analysis was performed using the One Step RT-PCR System with Taq DNA Polymerase (Takara, Japan) and an HO-1 primer pair: forward, 5′-TCT GCT AAC CAT GTT CAT GC-3′ and reverse, 5′-TGG GCA ATC TTT TTG AGC ACC-3′. The hDAF primer pair, was forward, 5′-TCT GCT AAC CAT GTT CAT GC-3′ and reverse, 5′-AGA ACT CTT CAA TAT CTG ACC-3′.

Immunohistochemical Staining and Western Blotting

Fresh pig ear sections were placed into plastic cryomolds, covered with OCT media (Leica Microsystems, Nussloch, Germany) for storage at −80 °C. Frozen-sections were immunohistochemically (IHC) stained with an hDAF primary antibody (BRIC 216; Serotec, Oxford UK), biotinylated horse anti-mouse immunoglobulin (Ig)G (Vector, Burlingame, Calif), and avidin-biotin peroxidase complex (ABC-HRP; Vector). The first primary antibody was revealed by a brown reaction with diaminobenzidine (Vector). Western blotting was performed to confirm the expression of the hHO-1 transgene. Briefly, protein extracts from pAEC separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto a PVDF membrane (Perkin Elmer, Norwalk, Conn), which was then incubated with appropriate primary HO-1 antibodies (1:3000; Stressgen, Ann Arbor, Mich). Following detection by incubation with horseradish peroxidase-conjugated antibodies, the immunoblotting signals were visualized by treatment with the ECL reagent (Millipore, Billerica, Mass).

MTS Assay and Statistic

The pAECs cultivated in M199 (Sigma, St Louis, Mo), supplemented with 10% fetal bovine serum (Gibco, Grand Iste, NY), penicillin (50 U/mL), and streptomycin (0.05 mg/mL; Sigma), were incubated in a humidified, 5% CO_2 incubator at 37 °C. Each well contained 1×10^4 seeded pAEC cells. When it reached 90% confluence, the culture medium was changed for 24 hours to 1%, 5%, 10%, or 15% MS or 10% human serum (HS) as a positive control. After MTS (Promega) solution was added for 3 hours, we recorded absorbance at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. The survival of transgenic and nontransgenic pAECs was analyzed by paired Student's *t*-tests.

RESULTS

The expressions of both transgenes were confirmed by RT-PCR, Western blots and IHC staining (Fig 1). The transgene function tested by addition of MS was evaluated by MTS. The addition of 1% MS caused little difference in survival of pAEC among transgenic and nontransgenic lines. However, when MS reached 5% in the pAEC culture medium, hDAF$^+$/hHO-1$^-$ transgenic pigs (3-04) showed greater survival rates than nontransgenic pigs ($P < .05$). Moreover, if MS was increased to 10% or 15%, all transgenic pAEC exhibited greater survival than nontransgenic pAECs (Fig 2). When 15% MS was added, the survival rate

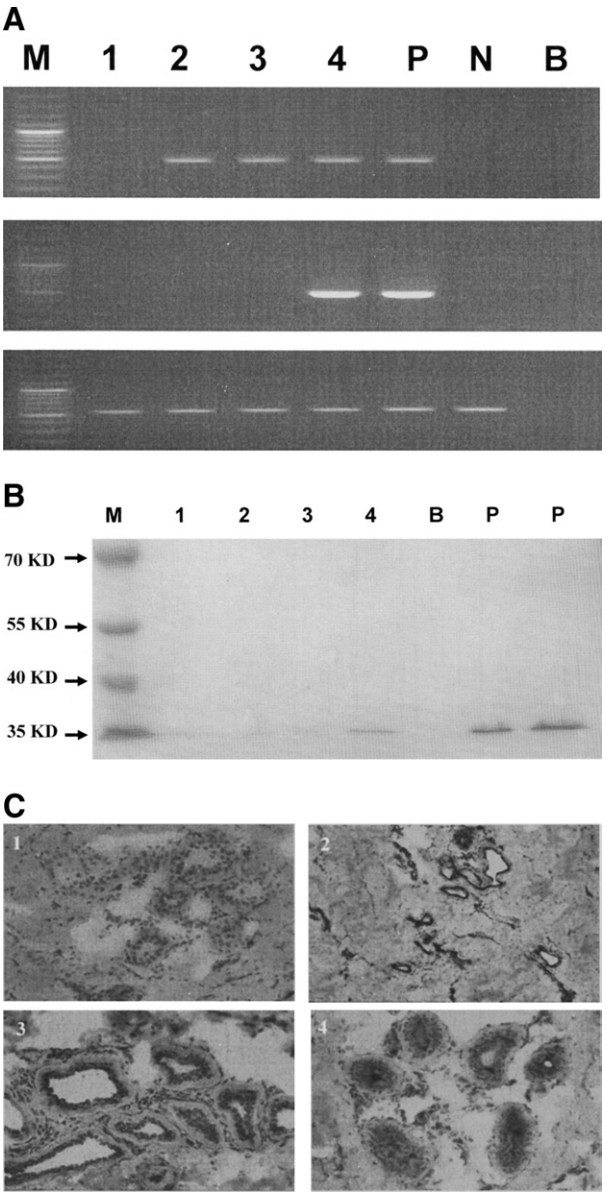

Fig 1. **(A)** RT-PCR. **(B)** hHO-1 Western blot. **(C)** hDAF IHC analysis of hDAF antigen. In A and B, lanes 1–4 are the pAEC of pigs 68-10, 3-04, 88-05, and 99-04, respectively. P, positive control; N, negative control; M, marker; B, blank. **(C)** Panels 1–4 are ear tissue of pigs 68-10, 3-04, 88-05, and 99-04, respectively.