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Evaluation of Cytotoxicity for Immunity Rejection of US11, hDAF and FasL Transgene-Transfected Cells

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ABSTRACT

Xenotransplantation is proposed as a solution to the problem of organ shortage. However, transplantation of xenogeneic organs induces an antigen-antibody reaction in α-1,3-gal structure that are not present in humans and primates, and thus complement is also activated and organs die within minutes or hours. In this study, we used FasL gene, which is involved in the immune response of NK cell, and US11, which suppresses MHC Class I cell membrane surface expression, to inhibit cell mediated rejection in the interspecific immunity rejection, and also hDAF(CD55) was introduced to confirm the response to C3 complement. These genes were tranfeced into Korean native pig fetal fibroblasts using pCAGGS vector. And cytotoxicity of NK cell and human complement was confirmed in each cell line. The US11 inhibited the cytotoxicity of NK cell and, in addition, the simultaneous expression of US11 and Fas ligand showed excellent suppress to T-lymphocyte cytotoxicity, hDAF showed weak resistance to cytotoxicity of natural killer cell but not in $CD8^+$ CTLs. Cytotoxicity study with human complement showed that hDAF was effective for reducing complement reaction. In this studies have demonstrated that each gene is effective in reducing immune rejection.

(Key words : Cell-mediated immune rejection, Cytotoxicity, Xenotransplantation, Complemente)

INTRODUCTION

In the field of organ transplantation, beneficiaries who wish for allograft organs steadily increase annually, but due to the lack of donors, only a small number of recipients are receiving transplants. Animal organs for the solution to this shortage could be used as an organs supply strategy for life extension until a suitable organ donor appears (Cooper *et al*., 2003). And considering physiological similarity, organ size, and function of the organ must be considered for selecting the animals. For example, rodents have been widely used in the analysis of human disease, but the size of organ does not match the size of human organs (Shultz *et al*., 2007). Primates have a problem of not only a long generation gap but also a high cost for the xenotransplantation with possible ethical issues. However, livestock animals can overcome these problems for xenotransplantation due to organ size, similarity of function, and productivity, among which pigs are most similar in size and function to physiology (Rispat *et al*., 1993; Sachs *et al*., 2001; Cooper *et al*., 2002; Lunney *et al*., 2007).

The organs of pigs have been limited in their use for xenotransplantation because of the HAR (hyper acute rejection) that α-gal antibodies in the recipient blood that bind to α-1,3-gal complex present on the cell surface of pig organs (Good *et al*., 1992; Alwayn *et al*., 1999; Logan., 2000). However, in 2003, this problem was solved by producing knock-out (GTKO) pigs that the α-1,3-galactosyl transferase gene was removed through genetic engineering technology. Also. after implanting the heart of a GTKO pig into a baboon, the heart survived 8 months after transplantation (Mohiuddin *et al*., 2012). However, various immune rejections such as complement activation and thrombosis, acute vascular rejection and cell mediated immune rejection have to be remained for practical application with pig organs. Even if the hyper acute rejection and the acute

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rejection may be overcome, the cell-mediated rejection due to adaptive immunity appears has to be solved because the organ is continuously exposed to the human blood. This cell-mediated symptom could occur within 7 days after allograft or xenotransplantation and be mainly involved with NK cells and T-lymphocytes (Haddad *et al*., 1995). These immune cells recognize the Fas receptor which belongs to the tumor necrosis factor (TNF) or major histocompatibility complex class I (MHC Class I) on the surface of the target cell and induce apoptosis (Kagi *et al*., 1994; Natarajan *et al*., 2002; Russell *et al*., 2002). Studies have been conducted on the overexpression of human membrane bound Fas ligands or human decoy Fas antigens to eliminate such immune responses (Tanaku *et al*., 2002 Kawamoto *et al*., 2006). Transgenic pigs were produced by introducing genes such as CD46 (membrane-associated factor protein, hMCP), CD55 (human weakness-promoting factor, hDAF), and CD59 for long-term survival at transplantation than GTKO organs (Cozzi *et al*., 1995; Loveland *et al*., 2004; Ramirez *et al*., 2000 and 2005; Lee *et al*., 2006;). Those transgenic organs could be effective to overcome HAR.

In our studies to overcome cell-mediated rejection, we selected the US11 gene, which inhibits the expression of MHC class I on the cell surface, the Fas ligand (FasL) that blocks the Fas pathway, and hDAF, which affects mainly the complement system, Transgene cell lines were established using these genes and cytotoxicity experiment was performed.

MATERIALS & METHODS

Production of Expression Vector

The US11 gene was obtained by PCR with pig cell DNA as a template with a specific gene primer (Table 1). And hDAF and FasL genes were amplified from Hela cell DNA using target specific primers (Table 1) The reaction was maintained at 94℃ for 5 minutes and then 36 cycles of denaturation at 94℃ for 30 seconds. The parameters for both US11 and FasL in the annealing step were 30 seconds at 55℃ and 30 seconds at 65 ℃ in hDAF. Extend for 30 seconds at 72℃. The final extension was carried out at 72℃ for 10 minutes, and the whole process was performed using GeneAmp® PCR system 9700 (Applied Biosystems). These genes were inserted into pCAGGS expression vector and transformed into DH5a strain. After cloning, the vector DNA was linearized with Ssp I.

Establishment of Transgene Cell Lines

Korean pig fetal fibroblast (KNPFF) was grown in DMEM (Gibco, USA) containing 10% FBS (Gibco), 1% glutamine (Gibco) and 1% P/S (Gibco) at 100 mm dish and these cell was incubated at 38.5℃, 5% CO_2, 10% humidity for 2 days. Then, mononuclear cell was isolated in a 38.5℃ incubator by treating with 0.05% trypsin / EDTA (Gibco) for 3 minutes. The cell-DNA mixture which was consisted of 1×10^6 of cell, 800 μL of GenePulser® electroporation buffer (Bio-Rad, USA) and each 2 μg of plasmid vectors, pCAGGS-BSD-hDAF, pCAGGS-BSD-FasL and pCAGGS-BSD-US11. This mixture was loaded into the electroporation cuvette (Bio-Rad), and exposed under conditions of 300 V, 250 Ω and 4 cuvette using by GenePulser® Xcell (Bio-Rad). After that, the cell was immediately propagated in 100 mm dishes with fresh media. After 48 h of transfection, 6 μg/mL of Blastasticidin S (Sigma-Aldrich, USA) was administered for 2 days, followed by treatment with 3 μg/mL of Blasticidin S (Sigma-Aldrich, USA) for 14 days.

Analysis of NK Cell Cytotoxicity

NK cell (ATCC, USA) were cultured in α-MEM (Welgene, Korea) with 10% FBS (Gibco), 6.4 μL of 2-mercaptoehanol (Sigma-Aldrich), 6 mL of MEM vitamin solution (Gibco) at 25T flask in 37℃, 5% CO_2, 10% humidity. TG cell as a target cell were seeded in

Table 1. hDAF, FasL and US11 gene-specific primers used for PCR screening

Primer name	F/R	Primer sequence (5′→3′)
hDAF (GenBank: AB240570.1)	F	ATGACCGTCGCGCGGCCGAG
	R	CAGGTCGAGGGATCTCCATA
FasL (GenBank: AY225406.1)	F	ATGCAGCAGCCCTTCAATTAC
	R	TTTTGGCAGAGGGAAAAAGA
US11 (GenBank: AY072776.1)	F	CAGTGGTACCATGGATCTTGTAATGC
	R	TATTAGCCAGAAGTCAGATGCTCAAG

96-well plates at 2×10^4 cells with 100 μL medium/well, and incubated at 38.5℃, 5% CO_2, 10% humidity for overnight. And NK cell was added to target cell in same 96-well culture plates at 2×10^5 cells with 100 μL medium/target cell well, then incubated at 37℃ for 4 hours. After incubation, MTT assay was conducted in triplicate wells. 20 μL of MTT (Sigma-Aldrich) solution (5 mg/mL in PBS) was added to each well, then incubated at 37℃ for 4 hours. Medium was removed by pipetting, 200 μL of dimethyl sulphoxide (DMSO, Sigma-Aldrich) was added to each well to dissolve MTT crystal, and incubated at 37℃ for 5 min. Absorbance was measured in ELISA reader at 540 nm. The cytotoxicity of NK cell with the target cell was calculated by using the following formula:

$$\%\,Cytotoxocity = \left\{1 - \frac{(OD\,of\,experiment\,group - OD\,of\,NK\,group)}{OD\,of\,target\,group}\right\} \times 100$$

Analysis of Cytotoxicity with $CD8^+$ CTLs

Normal fetal fibroblasts were cultured for 48 hours with IFN-γ (10 ng / mL) as a stimulant, and 50 Gy of γ-rays were removed and cultured in T75 cell culture flask for 1 day. The $CD8^+$ CTLs was isolated from human blood using Dynabeads® CD8 Positive Isolation Kit (Invitrogen, USA). The extracted lymphocytes were incubated with feeder cell in T75 flask for 3 days. After incubation, medium changed with stimulation media, which 10% RPMI (with antibiotics) containing PMA(25 ng/mL) and IL-2 50 U/mL and incubated for 1 weeks. After that, it was culture with normal media for 1 weeks. Lymphocytes were disaggregated from feeder cell by pipetting. And it was immediately proceeded MTT assay. MTT process was same MTT assay of NK cell, but it was differ at some points that seeded target cell and CTLs number were 2×10^3 and 2×10^4. Also the cytotoxicity of CTL cell with the target cell was calculated by using the following formula:

$$\%\,Cytotoxocity = \left\{1 - \frac{(OD\,of\,experiment\,group - OD\,of\,CTLs\,group)}{OD\,of\,target\,group}\right\} \times 100$$

Analysis of Human Complement Serum

1×10^6 TG cells were transferred to a 96-well dish, which was performed in triplicate After incubation, medium was changed with human serum (Innovative)-containing media. And 25%, 50% and 75% human complement serum were added to treatment group, and control was used with medium contained 10% FBS. Then, cells were incubated for 1.5 h at 37.0℃, 5% CO_2 in humid environment. Following reaction, medium in 96-well dish was aspirated and 100 μL of fresh 10% FBS DMEM was added. CCK (10 uL) was added each well and incubated for 2 h at 37.0℃, 5% CO_2 in humid environment. ELISA (microplate reader, Epoch) was used to measure cytotoxicity at 450 nm, and cytotoxicity was calculated as follows:

$$\%\,Cytotoxicity = \left\{\frac{(OD\,of\,control\,group - OD\,of\,experiment\,group)}{OD\,of\,control\,group}\right\} \times 100$$

Statistical Analysis

At least three replicates were performed for each cytotoxicity experiment, and the results were presented as mean values (mean±SEM). For cytotoxicity analysis of hDAF, FasL and US11, KNPFF cells were set as a control group, and all data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test as the post-hoc test using the SPSS.

RESULTS

Vector Screening

The 4.8 kb pCAGGS-BSD vector was selected for strong expression in animal cellss. Transcription of transgenes occured through the CMV immediate early enhancer (CMV IE), the first intron of the chicken β-actin gene as promoter, and the CAG promoter recombined with the rabbit β-globin gene as a splice acceptor (Okabe *et al.*, 1997; Alexopoulou *et al.*, 2008; Niwa *et al.*, 1991). The 648 bp product of the US11 gene was amplified from pig cell DNA with specific primers by PCR, and analyzed by 1% agarose gel electrophoresis. And, this gene was confirmed by sequencing analysis (Fig. 1). Two other genes, hDAF and FsaL, were also produced by PCR using a specific primer designed to amplify the DNA fragment at sizes of 1800 bp (hDAF) and 800 bp (FasL) (Fig. 2).

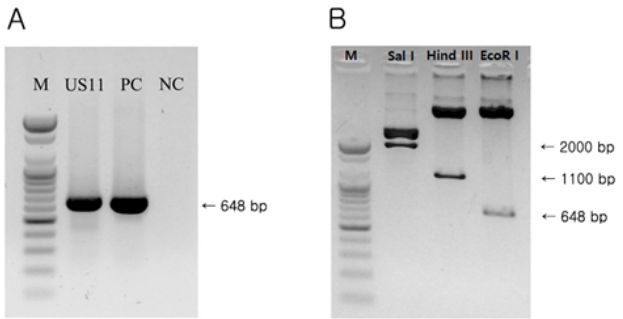

Fig. 1. Identification of pCAGGS-BSD-US11 vector construction. (A) US11 gene amplified by PCR using pig gDNA with specific primers. (B) Digestion of three restriction enzymes for vector screening. Sal I digestion produced 2,000 bp of fragment to confirm the insertion of US11. Hind Ⅲ digestion showed the size of the BSD, and EcoR Ⅰ digestion confirmed the insertion of the US11 gene. M, marker; PC, positive control; NC, negative control.

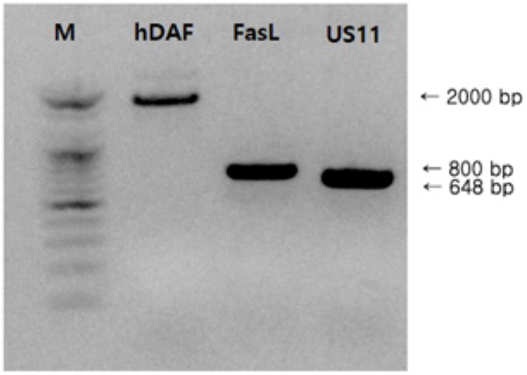

Fig. 2. PCR screening of pCAGGS-BSD-hDAF, pCAGGS-BSD-FasL and pCAGGS-BSD-US11 vectors. The size of hDAF, FasL and US11 gene are matched with 2,000 bp, 800 bp and 648 bp, respectively.

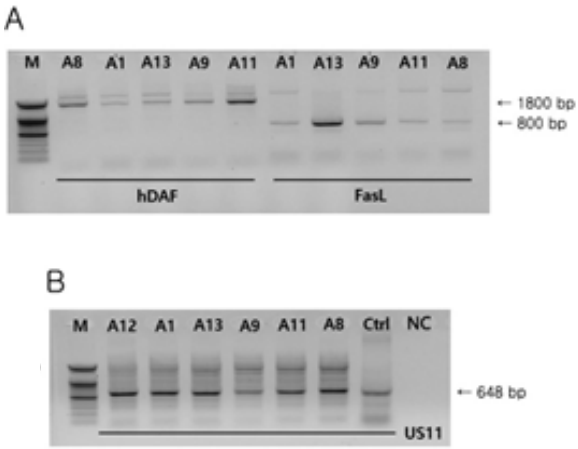

Fig. 3. PCR screening of transfected cells. (A) The colonies of A1, A8, A9, A11 and A13 with both hDAF and FasL genes. (B) US11 gene transfected clolnies. M, marker; Crtl, Control; NC, negative control.

Identification and Efficiency of Transfected Cells with Multiple Genes

The pCAGGS-BSD-hDAF, pCAGGS-BSD-FasL and pCAGGS-BSD-US11 vectors were co-transfected with KNPFF using electroporation. The colonies of surviving transformed cell in the selection with Blasticidin S for 16 days were determined by PCR using specific primers (Fig. 3). Cell colonies were introduced as triple for 7 of 80 (8.8%), as double for 21 of 80 (26.2%) and as single for 30 of 80 (37.5%) while 22 of 80 (27.5%) colonies were died out (Table 2).

Cytotoxicity Test of Transformed Cell Line Using NK Cell

To confirm the cytotoxicity by NK cell, normal swine fibroblasts were used as a control. In addition, cytotoxicity experiment was performed at a 1:1, 10:1 concentration ratio of target cell to human NK cell for 8 hours. FasL-transfected cell line (A13) significantly inhibited NK cell cytotoxicity than control cells. The US11-transfected cells showed about 73% cell protection against NK cells at a ratio of 10:1, and hDAF-trnasfected cells had about 64% inhibition of the NK cell response. And FasL-introduced cells showed 13% lower cytotoxicity compared to US11×hDAF cell and A13 cell (Fig. 4).

Cytotoxicity of Human Complement Serum

In the cytotoxicity of human complement serum, hDAF was effective for reducing complement reaction. Cytotoxicity of hDAF group was 2.7% (25% human serum), 7.0% (50% human serum) and 15.9% (75% human serum). Cell expressing US11/hDAF genes also showed reduced reaction with 6.1% (25% human serum), 11.9% (50% human serum) and 16.5% (75% human serum) cytotoxicity (Fig. 5). However, cells with only US11 gene only did not show a significant difference from control non-transgenic cell. Cell expressing

Table 2. Efficiency of DNA transfection into Korean native pig fibroblasts by electroporation

Genes	No. experiments	No. total colonies	No. transgene colonies (%)			No. death	No. at 21 days
			Single	Double	Triple		
Control (pCAGGS vector)	1	15	13(86.7)	N/A		2(13.3)	9
Transgene (hDAF+FasL+ US11)	1	16	6 (37.5)	4 (25.0)	1 (6.3)	5 (31.5)	0
	2	18	7 (38.9)	4 (22.2)	1 (5.5)	6 (33.3)	0
	3	21	9 (23.8)	5 (23.8)	2 (9.5)	5 (23.8)	1
	4	25	8 (32.0)	8 (32.0)	3 (8.0)	6 (24.0)	1
Total		80	30 (37.5)	21 (26.2)	7 (8.8)	22 (27.5)	2 (2.5)

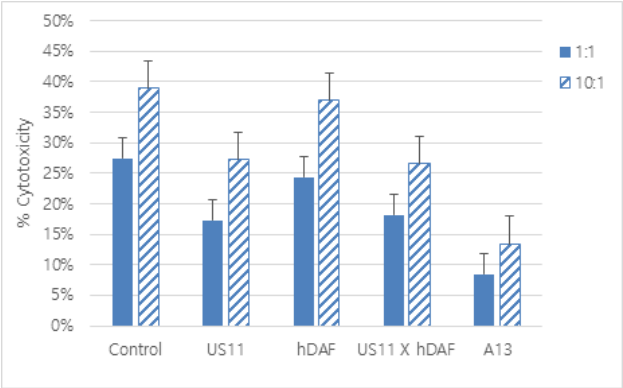

Fig. 4. Cytotoxicity of NK cell against cell lines [Control, US11, hDAF and US11XhDAF, FsaL(A13)]. The blue and stripe squares of right top indicate the effector : target ratio.

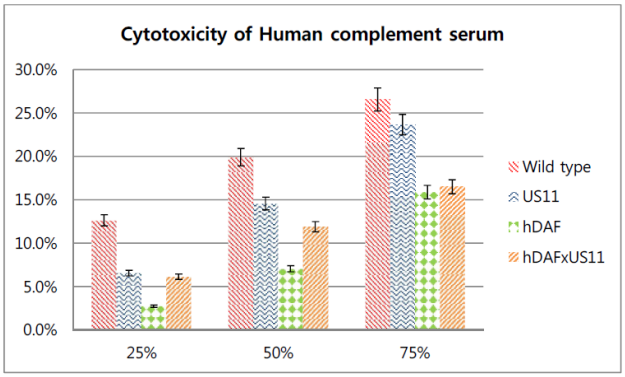

Fig. 5. Cytotoxicity of human complements serum in transfected cell lines. Complement density was 25% 50% and 75% for each transfected cell line (Wild type, US11, hDAF and US11XhDAF).

US11 gene appeared 6.5% (25% human serum), 14.6% (50% human serum) and 23.6% (75% human serum), while control cell showed 12.6% (25% human serum), 19.9% (50% human serum) and 26.6% (75% human serum) cytotoxicity.

DISCUSSION

Pig organs have been suggested as one of the ways to overcome the donor shortage problem of human organs. However, the pig organs could be transferred to the recipient's body, the survival rate was low due to exposure to the immune system (Bach *et al*., 1995; Platt *et al*., 1996). Also, even if organs function properly, rejection can occur both in xenotransplantation as in allograft (Galili *et al*., 1988). These immune problems are classified into three types of acute rejection, acute rejection and cell-mediated immune rejection. Hyper acute rejection is observed within a few minutes after transplantation and generally occurs when the anti-α-gal antibody binds to the vascular endothelial cell membrane of the donor organ by α-gal epitope α-1,3-gal structure, and therefore it brings producing xeno-reactive natural antibodies (XNAs) (Cooper *et al*., 1988; Platt *et al*., 1991). Acute vascular rejection occurs within days to weeks due to ischemia, diffuse intravascular coagulation and a deficiency of cellular infiltration (Platt *et al*., 1998; Bach., 1996). By overcoming above rejections, cell-mediated immune rejection occurs consistently even if organs survive. To improve the viability of the organs, first the active cellular immune response such as cytotoxicity of $CD8^+$ CTLs and NK cell must be reduced. In this study, it was confirmed that transgenic cell expressing hDAF, US11, and FasL genes can effectively avoid the reaction of NK cells. The cell with the US11 gene showed 27.2% and 28.56% cytotoxicity at a ratio of 10:1 for both NK cell and $CD8^+$ CTL. The US11 gene of HCMV produces glycoprotein 11 (gp-US11) in the cell membrane and prevents MHC class I expression in the endoplasmic reticulum from being expressed in the cell membrane expression (Jones *et al*., 1995; Noriega *et al*., 2009 and 2012; Wiertz *et al*., 1996; Besold *et al*., 2009). Therefore, $CD8^+$ CTLs and NK cell could show low cytotoxicity due to the difficulty of recognizing MHC class I in the cells in which the US11 gene was expressed. FasL showed the highest inhibition of cytotoxicity by NK cell at a 10:1 ratio of target and effector cell. This suggested that over-expressed FasL on the cell surface inhibited apoptosis by forming FasL / FasR-trimer complexes of $CD8^+$ CTL and NK cell (Tanemura *et al*., 2002; Kawamoto *et al*., 2006). In addition, hDAF (CD55) is generally known as a complement control protein that prevents conversion formation of C3bBb and C4b2a by dissociation between C2a and Bb proteins from C3b and C4b proteins (DaGe Liu *et al*., 2007; John S Logan, 2000). However, it also inhibited NK cell induced cytotoxicity, which may control cytotoxicity by modulating C3 released by NK cell.

Our results show that the US11, FasL, and hDAF genes effectively inhibit the cytotoxicity of NK cell that are involved in cell mediated immune rejection. Our study confirms that the US11, hDAF, and FasL genes could be used to produce transgenic pigs to effectively inhibit NK cell and $CD8^+$ CTLs that are involved in-cell-mediated immune rejection.

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