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# Inhibition of Human CD8$^+$ Cytotoxic T Lymphocyte (CTL) -mediated Cytotoxicity in Porcine Fetal Fibroblast Cells by Overexpression of Human Cytomegalovirus Glycoprotein Unique Short (US) 2 Gene*

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**ABSTRACT :** Xenotransplantation of pig organs into humans is a potential solution for the shortage of donor organs for transplantation. However, multiple immune barriers preclude its clinical application. In particular, the initial type of rejection in xenotransplantation is an acute cellular rejection by host CD8$^+$ cytotoxic T lymphocyte (CTL) cells that react to donor major histocompatibility complex (MHC) class I. The human cytomegalovirus (HCMV) glycoprotein Unique Short (US) 2 specifically targets MHC class I heavy chains to relocate them from the endoplasmic reticulum (ER) membrane to the cytosol, where they are degraded by the proteasome. In this study we transfected the US2 gene into minipig fetal fibroblasts and established four US2 clonal cell lines. The integration of US2 into transgenic fetal cells was confirmed using PCR and Southern blot assay. The reduction of Swine Leukocyte Antigen (SLA)-I by US2 was also detected using Flow cytometry assay (FACS). The FACS analysis of the US2 clonal cell lines demonstrated a substantial reduction in SLA-I surface expression. The level (44% to 76%) of SLA-I expression in US2 clonal cell lines was decreased relative to the control. In cytotoxicity assay the rate of CD8$^+$ T cell-mediated cytotoxicity was significantly reduced to 23.8±15.1% compared to the control (59.8±8.4%, $p<0.05$). In conclusion, US2 can directly protect against CD8$^+$-mediated cell lysis. These results indicate that the expression of US2 in pig cells may provide a new approach to overcome the CTL-mediated immune rejection in xenotransplantation. (**Key Words :** Cytotoxic T Lymphocyte (CTL) cells, Swine Leukocyte Antigen (SLA)-I, Unique Short (US) 2, Xenotransplantation)

## INTRODUCTION

Pig has been considered as an ideal source of donor organs for xenotransplantation, primarily because of organ size, plentiful supply, physiological and anatomical comparabilities with human organs. However, vigorous humoral and cellular immune responses to xenografts severely limit the clinical applications of xenotransplantation (Morgan et al., 2001).

The major and most well-known immunologic barrier to the xenotransplantation of pig organs into humans is the natural human anti-Gal antibody that binds to α-gal epitopes, which are abundantly expressed on pig cells. This induces complement activation and results in hyperacute rejection of the xenograft (Good et al., 1992; Galili, 1993; Sandrin et al., 1993; Tanemura et al., 2000). However, hyperacute rejection can be prevented by using several tactics, such as by blocking complement activation. The recent generation of an α-1,3-galactosyltransferase (α-gal) gene knockout pigs may allow to be overcome the antibody-mediated hyperacute rejection (Lai et al., 2002; Phelps et al., 2003; Kuwaki et al., 2005; Yamada et al., 2005). Furthermore, the α-gal antigen expression is not ubiquitously present in all porcine cell types, and in particular it is not expressed on the beta cells of the islets of Langerhans (Vales-Gonzalez et al., 2005). Pancreatic islet xenotransplantation has been proposed as one of the initial model of xenotransplant to undergo clinical trials (O'connell, 2002). Therefore, to overcome the other immune rejection rather than hyperacute rejection may be more important for cellular xenograft such as islet cells.

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Once the hyperacute rejection is overcome, another immunological obstacle to long-term xenograft survival may be the human $CD8^+$ cytotoxic T lymphocytes (CTL)-mediated killing of xenograft cells in xenograft recipients. The human $CD8^+$ CTL directly recognizes MHC class I (Swine Leukocyte Antigen-I; SLA-I) on pig endothelial cells and has the ability to kill these xenograft cells in xenograft recipients (Tanemura et al., 2002). Therefore, the removal of the SLA-I expressed on the cell surface is needed to escape cellular rejection and to overcome the CTL-mediated killing in xenograft recipients.

Human cytomegalovirus (HCMV) has evolved a number of strategies to evade host immune response. These include the down-regulation of class I molecule on the surface of host cells to escape cytotoxic T cell recognition by utilizing the unique short region protein (US), US2, US3, US6, and US11. In particular, US2 have the ability to dislocate class I heavy chains back into the cytosol for subsequent degradation by proteasomes (Wiertz et al., 1996; Helgel et al., 1997).

In the present study, we investigated whether insertion of US2 gene into pig cells could inhibit the human CTL-mediated cytotoxicity by down-regulating the surface expression of MHC class I molecules (Swine Leukocyte Antigen-I; SLA-I).

## MATERIALS AND METHODS

### US2 cDNA cloning and transfection

All the experiments using animals were conducted under protocol approved by MGEN, Inc. institutional animal care and use committees as specified by guideline.

US2 cDNA was prepared from the total RNA extracted from accurun 350 serum CMV DNA positive control (BBI Diagnostics) using a QIAamp DNA mini kit (QIAGEN). The US2 gene was amplified by PCR using the CMV cDNA as the template. Forward (5' CAG TGG AAC CAT GAA CAA TCT CTG GAA AGC CTG GGT) and reverse (5' CAA CTC TAG ATT AGC ACA CGA AAA ACC GCA TCC ACA) primers were used. Thirty cycles of PCR were performed as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were isolated from the gel and cloned into the plasmid (pCX-US2) using CMV promoter as a controller. The sequence of the cloned gene was analyzed and it was matched with the US2 gene sequence (NCBI No. AY446894). The constructed US2 vector was transfected into minipig fetal fibroblast cells by Lipofectamine 2000 (Invitrogen). The transfected cells were screened by using Blasticidine S (Fluka) (6 μg/ml) for 2 weeks. The integration of US2 gene into minipig fetal fibroblasts was confirmed by PCR.

### Southern blot analysis

Clonal cell lines were lysed overnight at 55°C with 1 ml TES buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K). Genomic DNA was isolated to phenol/chloroform extraction and precipitated absolute alcohol. Resolubilized DNA was resolved into TE buffer and incubated at 55°C for 2 h. The US2 gene was amplified by PCR using the isolated gDNA as the template. Forward (5'CAG TGG AAC CAT GAA CAA TCT CTG GAA AGC CTG GGT) and reverse (5'CAA CTC TAG ATT AGC ACA CGA AAA ACC GCA TCC ACA) primers were used. PCR products were diluted with TE buffer and separated on 0.7% agarose gel. Following the electrophoresis, the DNA was transffered to nylon membrane and treated probes labeled with anti-fluorescein-AP (Amersham). Bands were detected using a chemiliminescent substrate system (Amersham).

### Flow cytometry analysis

Anti-SLA I-FITC (VMRD) and FITC-conjugated anti-mouse IgG (Abcam) was used to detect the down-regulation of MHC class I by US2. Isotype control was stained with anti-mouse IgG2b (Abcam). The cells ($1\times10^6$) were washed twice with ice cold PBS and then incubated for 30 min in anti-SLA-I-FITC diluted with PBS. The cells were washed twice with ice cold PBS and then analyzed as 10,000 gated events using a FACSCalibur cytometer (BD Biosciences). Some cells were washed with ice cold PBS, stained with anti-CD3-FITC (BD Biosciences) and anti-CD8-PE (BD Biosciences) for 30 min. To verify the phenotype of IL-2 activated lymphocytes population, anti-CD3-FITC and anti-CD8-PE were used. Finally, the cells were washed twice in ice cold PBS and then fixed with 3% formaldehyde (Sigma) in PBS. The samples were analyzed as 10,000 gated events using a FACSCalibur cytometer.

### Lymphocyte isolation, activation and expansion for cytotoxicity assay

Minipig's fetal fibroblast cells were 50Gy gamma-irradiated to sensitize the lymphocytes. The human lymphocytes for effector cells were obtained from the heparinized blood of healthy volunteers. Isolation was carried out using density centrifugation of 1:1 DMEM diluted blood on Ficoll (Amersham). The lymphocyte fraction was recovered using a sterilized pastier pipette and washed with PBS containing 1% BSA by centrifugation in DMEM at 600×g for 10 min at 4°C. Those lymphocytes were isolated magnetic beads. In order to isolate $CD8^+$ lymphocytes, cells were incubated $CD8^+$ DynaBeads (Invitrogen) for 20 min at 4°C. To eliminate the beads from the $CD8^+$ lymphocytes, DETACHaBEAD (Invitrogen) were added to the incubated lymphocytes using RPMI 1640 containing 1% FBS. After isolation, the cells transferred to normal gamma-irradiated minipig's fetal fibroblast cells and

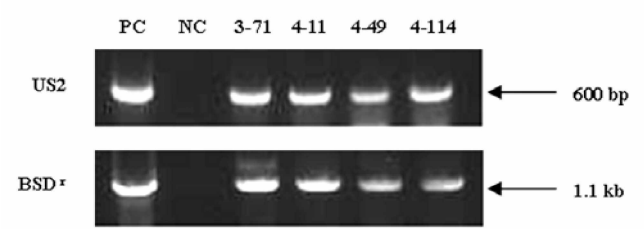


**Figure 1.** PCR analysis of US2 gene from the clonal cell lines. Genomic DNA was extracted from clonal cell lines transfected with US2 gene and analyzed them by PCR. As a control, pCX-US2 vector (positive control, PC) and normal minipig's fetal fibroblast cells genomic DNA (negative control, NC) were used. Other lanes are US2 clonal cell lines (3-71, 4-11, 4-49, 4-114).

incubated for 3 days in DMEM (10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin). The incubation medium was changed to new DMEM containing IL-2 and was treated with every 2 days. The duration of the IL-2 treatment was 14 days.

**Cytotoxicity assay**

The CTL assay was measured according to the MTT assay method. The target cells were prepared at a concentration of $2\times10^4$ in a 96-well bottom plate. After 24 h, the effector cells (isolated lymphocytes) were treated on the clonal cell lines. The effector/target ratio was 10:1 and 15:1. The effector cells and target cells were co-incubated, and then 20 μl MTT solution was added to the cells and they were then incubated for 4 h. After dissolving the crystals, the absorbance was measured at 570 nm. The percent cytotoxicity was calculated using the following formula: (1-(the mean value of experiment group-the mean value of CTL group)/the mean value of target group)×100.

## RESULTS AND DISCUSSION

Porcine xenograft destruction as a consequence of direct recognition of pig SLA by human T cells is likely to be a significant barrier to successful pig-to-primate xenotransplantation. To overcome this barrier the most straightforward solution is to eliminate the cell-surface expression of donor MHC. According to the previous reports, human cytomegalovirus (HCMV) down-regulates the surface expression of MHC class I molecules (Del et al., 1992). Therefore, we investigated whether this mechanism could be used to overcome the cellular rejection in the porcine cells. We transfected US2 into the minipig fetal fibroblast cells, which were selected by Blasticidine (BSD, 6 μg/ml) for 2 weeks. The integration of US2 was confirmed in four clonal cell lines (3-71, 4-11, 4-49, 4-114) by using PCR (Figure 1). To determine whether US2 gene has been truly integrated into clonal cell lines, Southern blot was carried out. Genomic DNA was isolated from clonal cell lines and US2 gene was amplified from the genomic DNA. The amplified PCR products were hybridized with a labeled US2 gene as a probe. All of four clonal cell lines showed the band with expected size clearly (Figure 2). This result indicates that US2 gene was successfully transfected into each cells.

US2 is known to induce the translocation of both MHC class I and II from the endoplasmic reticulum (ER) membrane to the cytosol, and to degrade these products (Hegde et al., 2003). A normal porcine cell line and four US2 transfected clonal cell lines were analyzed by FACS with porcine anti-SLA-I to determine the surface expression profiles of SLA-I. The level (44% to 76%) of SLA-I expression in US2 clonal cell lines was decreased relative to the control. In particular, the clonal cell line (3-71) showed 73% reduction in SLA-I surface expression (Figure 3). We found each clonal cell lines had different reducing level of SLA-I by FACS analysis. The different SLA-I expression in the cell lines might be due to different integration site of US2 gene in pig genome. Because we transfected US2 gene into pig cells using lipofectamine, the insertion site of US2 gene would be different in four cell lines respectively. The difference of gene insertion site could bring different gene expression and the levels of SLA-I expression were different from each cell lines. Similar to our results, HLA-E gene expression level was different from each porcine cells transfected with HLA-E gene (Forte et al., 2005). These results clearly indicate that the expression of viral protein US2 decreased the SLA-I expression level on the cell surface of the clonal cell lines.

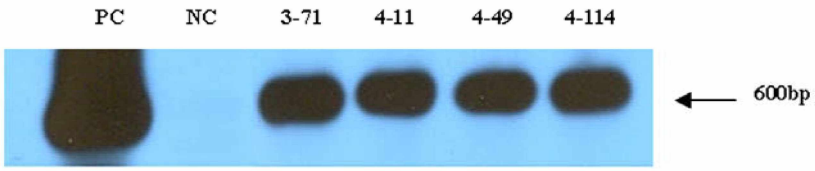


**Figure 2.** Southern blot assay of US2 clonal cell lines. US2 gene was amplified from US2 clonal cell lines by PCR. The diluted PCR product was analyzed by southern blot using US2 gene as probe. US2 gene from vector (positive control, PC) and PCR product from normal minipig's fetal fibroblast cells genomic DNA (negative control, NC) were used as the control. Other lanes are US2 clonal cell lines (3-71, 4-11, 4-49, 4-114).

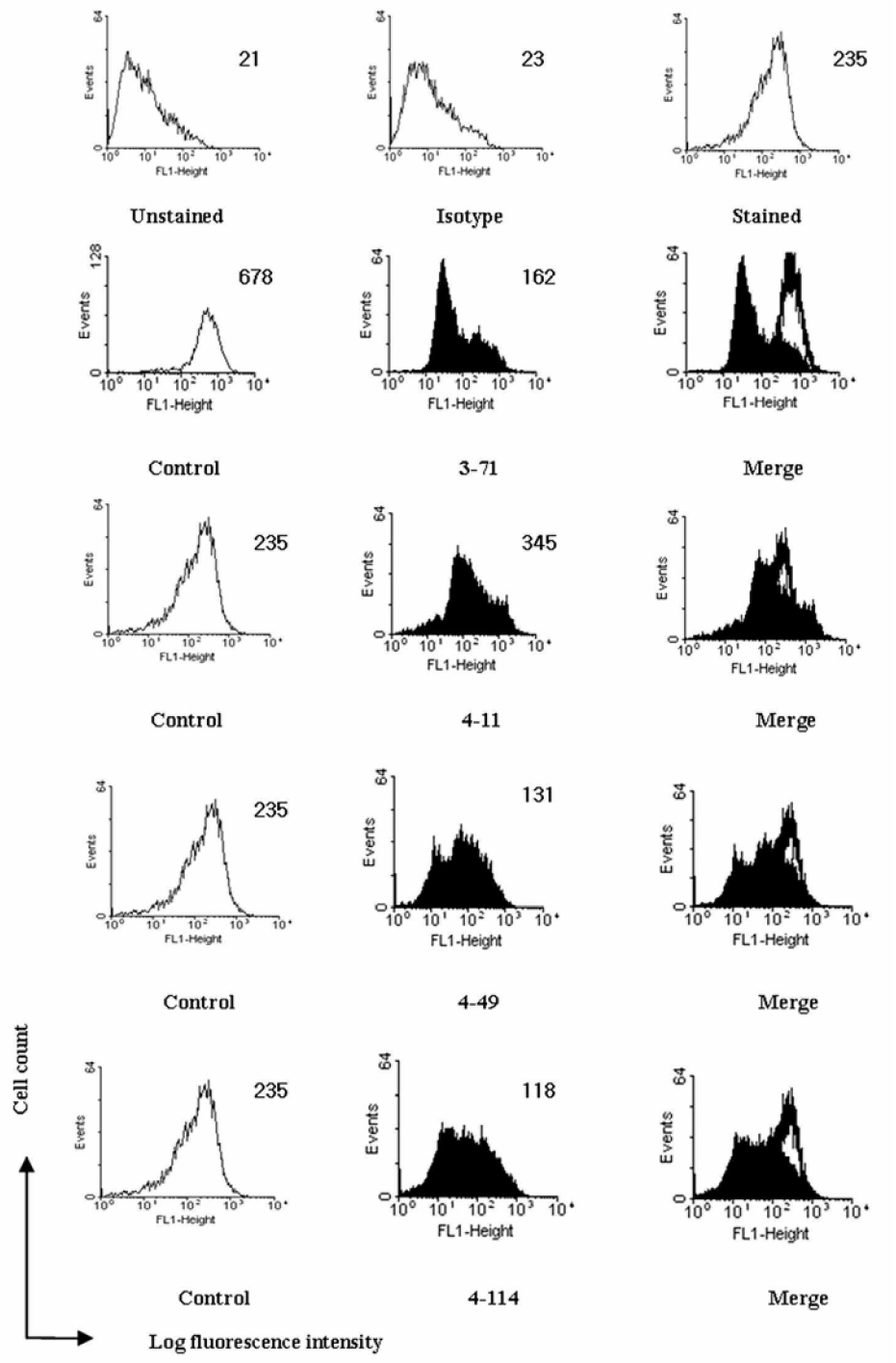


**Figure 3.** Dramatic reduction on cell surface expression of SLA-I in minipig's fetal fibroblast cells transfected with US2. Flow cytometry assay (FACS) was examined using each US2 clonal cell lines (3-71, 4-11, 4-49, 4-114) and normal minipig's fetal fibroblast cells. Normal minipig's fetal fibroblast cell lines were used as control group stained with anti-SLA I. US2 clonal cell lines were stained with anti-SLA-I and anti-mouse IgG2b was used as an isotype control.

Human $CD8^+$ CTL directly recognizes SLA-I on pig endothelial cells and has the ability to directly kill the xenograft cells in xenograft recipients (Murray et al., 1994; Shishido et al., 1997; Yi et al., 2000; Zhan et al., 2001; Tanemura et al., 2002). These reports suggest that human $CD8^+$ CTL-mediated cytotoxicity in xenograft recipients is one of the important immunological barriers to pig-to-human xenotransplantation. US2 has been reported to inhibit antigen presentation to $CD8^+$ T lymphocytes (Hegde et al., 2003); therefore, we attempted to determine whether

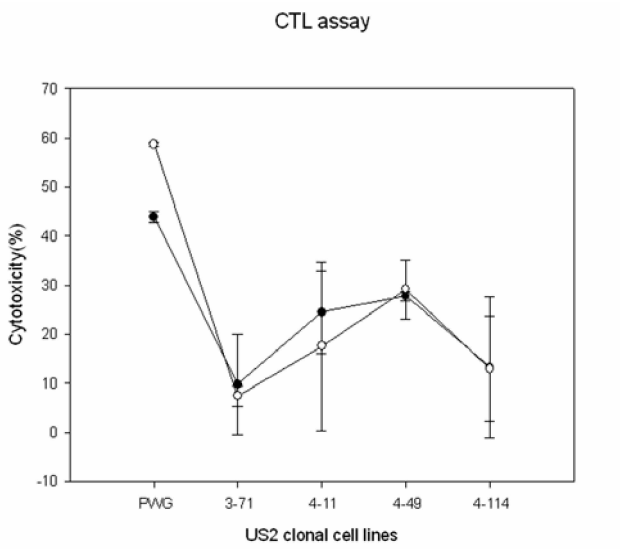


**Figure 4.** Cytotoxicity assay of human cytotoxic T Lymphocytes against minipig's fetal fibroblast cells transfected with US2. *In vitro* cytotoxicity assay cultured $CD8^+$ T lymphocyte from the healthy volunteers. Effector cell:target cell = 15:1 (open circles), effector cell:target cell = 10:1 (closed circles).

the introduction of US2 into porcine cells could protect them from human CTL cytotoxicity. $CD8^+$ T lymphocytes were isolated from peripheral blood using magnetic beads. Phenotyping of lymphocyte population by flow cytometer showed that the population of CD8 positive cells is more than 80% in anti-CD3/CD8 double positive staining (data not shown). The down-regulation of CTL-mediated directed cell lysis by US2 was observed in four clonal cell lines. US2 significantly suppressed the lysis of clonal cell lines by human CTL. The rate of CTL cytotoxicity was reduced 16.8±9% compared to the control in the 15:1 of E/T ratio (59.7±0.3%, $p<0.01$) and reduced 18.85±8.5% compared to the control in the 10:1 of E/T ratio (43.9±1.1%, $p<0.02$) (Figure 4). The clonal cell line 3-71 showed the more ability for escaping the CTL-mediated killing than other cell lines. These results suggest that viral US2 can reduce the human CTL-mediated cell lysis of pig cells.

Taken together, this is the first report that US2 can regulate pig SLA expression for modulating the immune response. US2 has been considered an excellent candidate for the inhibition of CTL cell-mediated cytolysis after xenotransplantation. The production of the porcine somatic cells transfected with the US2 gene can be used the donor source of nuclear transfer to produce transgenic pigs for xenotransplantation.

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