DNA Microarray-Based Gene Expression Profiling in Porcine Keratocytes and Corneal Endothelial Cells and Comparative Analysis Associated with Xeno-related Rejection

Porcine to rat corneal xenotransplantation resulted in severe inflammation and rejection of the corneal stroma, whereas an allograft showed mainly endothelial cell-associated rejection. We, therefore, investigated and compared the gene expression between porcine keratocytes and corneal endothelial cells. RNA was isolated from primary cultured porcine or human keratocytes and porcine corneal endothelial cells. Gene expression was comparatively analyzed after normalization with microarray method using Platinum pig 13 K oligo chip (GenoCheck Co., Ltd., Ansan, Korea). Real-time polymerase chain reaction (PCR) was performed for C1R, CCL2, CXCL6, and HLA-A in porcine keratocytes and corneal endothelial cells. As a result, up-regulated expression more than 2 folds was observed in 1,162 genes of porcine keratocytes versus porcine endothelial cells. Among the immune-regulatory genes, SEMA3C, CCL2, CXCL6, F3, HLA-A, CD97, IFI30, C1R, and G1P3 were highly expressed in porcine keratocytes, compared to porcine corneal endothelial cells or human keratocytes. When measured by real-time PCR, the expression of C1R, CCL2, and HLA-A was higher in porcine keratocytes compared to that in porcine corneal endothelial cells. In conclusion, the increased expression of C1R, CCL2, and HLA-A genes in porcine keratocytes might be responsible for the stromal rejection observed in a porcine to rat corneal xenotransplantation.

Key Words: Cornea; Microarray analysis; Porcine; Polymerase Chain Reaction; Transplantation, Heterologous

INTRODUCTION

The cornea is consisted of 3 different cell layers; epithelial cells, stromal fibroblasts (keratocytes), and endothelial cells. In the cornea, endothelial cells are the main target for the rejection in an allo-corneal transplantation, because fibroblasts are known to be less immunogenic than endothelial cells (1, 2).

In Asian countries, including Korea, cultural circumstances, including Confucianism, have caused a profound shortage of donor tissues for corneal transplantation. Therefore, the use of tissue from a xenogeneic source has been considered for a long-time by many Asian ophthalmologists. Recently, pig has been widely studied as a possible donor for xenotransplantation, because the pig’s organ size as well as its anatomy and physiology make it an ideal substitute as a xenograft (3-5). The feasibility of porcine cornea as a xenograft has currently been evaluated (6). However, we observed that the xeno-corneal graft in a porcine to rat model showed different features from the murine or human allograft; the stromal fibroblasts, which are keratocytes, presented with more severe rejection than endothelial cells did (7). Hence, we wondered if the gene expression of porcine keratocytes would be different from that of corneal endothelial cells when it comes to the xeno-related rejection.
Since the first description of high-density DNA microarrays in 1995 (8), DNA microarrays have been widely used in genomics research. The long oligonucleotide microarray, which is composed of gene-specific oligonucleotides of 40-70 nt in length, spotted on glass slides, has become a powerful tool for globally detecting differential gene expression. Since the first-generation porcine oligonucleotide set, representing 13,297 cDNAs and expressed sequence tags (ESTs), has been designed by Qiagen-Operon for transcriptional profiling (9), we are now able to hybridize genes efficiently, using such porcine oligonucleotide set, to detect different expression levels between keratocytes and corneal endothelial cells. Therefore, this study was undertaken to investigate the different gene expression of porcine keratocytes in xenotransplantation in comparison to corneal endothelial cells, using cDNA microarray with porcine oligonucleotide set.

**MATERIAL AND METHODS**

The Ethics Research Committee of the Seoul National University Hospital approved the protocol of the experiment.

**Pig-to-rat orthotopic corneal transplantation**

Twenty-one fresh porcine corneas, obtained within 6 hr of death, were used. To match the corneal thickness, the 500 μm anterior lamella was removed, using the Barron anterior chamber maintainer (Katena Products, Inc., Denville, NJ, U.S.A.) and crescent knife (Satin, Alcon surgical, Fort Worth, TX, U.S.A.). The remaining posterior lamella and endothelium was trephined from the inside with a 6 mm sized blade (Kai industries Co., ltd, Seki City, Japan), and the recipient rat cornea was also trephined carefully with a 4 mm sized blade (Kai industries Co., ltd), grasping the globe firmly with two fine tooth forceps until the immediate entrance into the anterior chamber. The graft was secured with 12 to 14 interrupted nylon sutures, and tarsorrhaphy was performed finaly at the lateral one-third of the lid margin. Antibiotics with 1.2 U/mL dipase I for 2 hr at 37°C, and 5 mL of type I collagenase was added and the tissues were shaken 3 times every 30 min. The harvested keratocytes were centrifuged at 1,200 rpm for 5 min, and the precipitate was inoculated into culture dish with DMEM.F12 (1:1) containing 10% FBS (fetal bovine serum; HyClone Laboratories, Utha, U.S.A.). The primary keratocytes were cultured at 37°C in a carbon dioxide incubator for 1 to 2 weeks. The cells were passaged at 7-9 days before RNA isolation.

**Primary culture of porcine endothelial cells**

The Descemet’s membranes were mechanically peeled off from the corneas of 50 common adult pigs. 0.05% trypsin and 0.01% EDTA were added to the tissues and the whole mixture was shaken 4 times every 20 min. The harvested endothelial cells were centrifuged at 1,200 rpm for 5 min and the precipitates were inoculated onto culture plate coated with collagen type IV. The resulting precipitate was submerged in M-199 medium (Cambrex, Charles City, IA, U.S.A.) containing 10% FBS. The primary endothelial cells were cultured at 37°C in a carbon dioxide incubator for 1 to 2 weeks. The primary cells were passaged at 7-9 days before RNA isolation.

**RNA isolation**

Total RNA was extracted from the cells using Micro-to-Midi total RNA purification system (Life Technologies, Inc., Atlanta, GA, U.S.A.). Lysis buffer was added to the cells, and the cells were immediately homogenized using a Turrax homogenizer. The final product yielded 260/280 nm ratio of 1.8-2.0 and the purity was confirmed via gel electrophoresis. The concentration was determined based on 260 nm absorbance using a spectrophotometer.

**Hybridization in microarray**

A porcine platinum 13 K oligonucleotide microarray, which was developed in-house at GenoCheck Co., Ltd. (Ansan, Korea), was used for evaluation of the gene expression profiling. Control genes used in platinum 13 K oligonucleotide microarray chips were as follows: Arabidopsis thaliana photo...
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Ansan, Korea

The primers for real-time PCR (GenoCheck, Co. Ltd., Ansan, Korea) were designed according to Zhao SH’s updates of the genes (10). A total of 13,610 spots including control genes were spotted on platinum 13K oligonucleotide microarray chips which were composed of 24 blocks with 568 genes each. We spotted and analyzed four different kinds of spike control samples on each block to confirm the quality of the chips and normalization of data. Oligonucleotide probes were resuspended in spotting buffer kit (GenoCheck Co., Ltd.) and spotted onto CMT-GAPS II slides (Corning Inc., Corning, NY, U.S.A.).

For microarray hybridization, total RNA of corneal endothelial cells was pooled and used for hybridization, and cDNAs were made from RNA sample via reverse transcription and labeled to produce the fluorescent-labeled cDNAs. Each total RNA sample (30 μg) was labeled with Cyanine3 (Cy3) or Cyanine5 (Cy5)-conjugated dCTP (NEN Life Science Products Inc., Boston, MA, U.S.A.) by a reverse transcription reaction using reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). The fluorescent-labeled cDNAs were mixed and simultaneously hybridized to the oligonucleotide microarray chip.

The oligonucleotide chips were scanned with an Axon 4000B Scanner (Axon Instruments, Foster City, CA, U.S.A.) using laser excitation of 2 fluorescence at 532 nm and 635 nm in wavelength for the Cy3 and Cy5 labels, respectively. The scanned images were analyzed with the software program GenePix Pro 5.1 (Axon Instruments, Inc., San Francisco, CA, U.S.A.) and GeneSpring GX 7.3.1 (Silicon Genetics, Santa Clara, CA, U.S.A.). The fluorescent-labeled cDNAs were mixed and simultaneously hybridized to the oligonucleotide microarray chip.

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The raw intensity data were globally normalized by an intensity dependent on the LOESS (locally weighted regression scatter plot smoothing) method, and then was normalized by the with-print-tip group normalization method for each print-tip. The results were presented as M vs A scatter plot smoothing method, and then was normalized by the with-print-tip group normalization method for each print-tip. The results were presented as M vs A scatter plots. Twenty four tips were used for making 13K oligonucleotide microarray chips which were composed of 24 blocks with 568 genes each. We spotted and analyzed four different kinds of spike control samples on each block to confirm the quality of the chips and normalization of data. Oligonucleotide probes were resuspended in spotting buffer kit (GenoCheck Co., Ltd.) and spotted onto CMT-GAPS II slides (Corning Inc., Corning, NY, U.S.A.).

Table 1. The primers for real-time PCR (GenoCheck, Co. Ltd., Ansan, Korea)

| Gene  | Primer | Sequence | Size |
|-------|--------|----------|------|
| GAPDH | Sense  | CTA CTG CCA ACC TCT CGG TT | 128 bp |
|       | Antisense | CTC AGT GTA GCC CAG GAT GC | |
| CCL2  | Sense  | GCT TGA ATC CTC ATC TCT CA | 194 bp |
|       | Antisense | TGC TGC TGG TGA TCT TCG | |
| CXCL6 | Sense  | CCC TCC TCC TCC ACT CCT CT | 158 bp |
|       | Antisense | GAT AGG ACT AGC GCT GGC AA | |
| HLA-A | Sense  | CAG TGG TTT TCT GGA TGC TA | 195 bp |
|       | Antisense | CAG GGA TGC TCT GCT CTG | |
| C1R   | Sense  | GCA GCC TCA GTA CGA GTT CC | 87 bp |
|       | Antisense | GAC AGT AGC ACC TGC TTC CC | |

to reduce variations of local background intensity caused by multiple pins (9). Over two-fold changed genes were selected for further analysis (M ≥ 1 and M ≥ -1; M=log₂ [red Cy5 intensity/green Cy3 intensity] ratio).

Real-time quantitative PCR

In order to validate the results from the microarray analysis, we selected four genes, which are C1R, CCL2, CXCL6 and HLA-A, and compared their gene expression in porcine keratocytes and corneal endothelial cells as measured by real-time PCR. Real-time quantitative PCR was performed in triplicate in 384-well plates. A 384-well high-throughput analysis was performed by using the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Weiterstadt, Germany) and white colored 384-well plates (ABgene, Hamburg, Germany) for intensification of the fluorescent signals by a factor of three. The system operates using a thermal cycler and a laser that is directed via fiber optics to each of 384 sample wells. The fluorescence emission from each sample is collected by a charge-coupled device-camera and the quantitative data were analyzed using the Sequence Detection System software (SDS version 2.0, PE Applied Biosystems). Reaction mixtures contained 10 pM/μL of each primer and 2X SYBR Green PCR Master Mix (PE Applied Biosystems), which includes the HotStarTaq DNA-Polymerase in an optimized buffer, the dNTP mix (with dUTP additive), the SYBRs Green I fluorescent dye, and ROX dye as a passive reference. Each of the 384-well real-time quantitative PCR plates included serial dilutions (1, 1/2 1/4, 1/8, and 1/16) of cDNA, which were used to generate relative standard curves for genes. All primers (Table 1) were amplified using the same conditions. Thermal cycling conditions 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec, and 72°C for 30 sec.

In order to exclude the presence of unspecific products, a melting curve analysis of products was performed routinely after finishing amplification by a high-resolution data collection during an incremental temperature increase from 60°C to 95°C with a ramp rate of 0.21°C/sec. We then converted real-time PCR cycle numbers to gene amounts (ng) on the basis of the equation. The real-time PCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System (PE Applied Biosystems).

RESULTS

Histology of rejected corneal xenografts in a pig-to-rat transplantation

The hematoxylin-eosin staining of cornea with time after porcine to rat corneal transplantation showed massive infiltration of neutrophils and monocytes into the stroma at the
early stage (Fig. 1). Severe inflammation and rejection against stromal fibroblasts were observed.

Different gene expression in porcine keratocytes and corneal endothelial cells on microarray

After we confirmed that cultured keratocytes and endothelial cells did not cross-contaminate with each other using the reverse transcriptase PCR (RT-PCR) (Fig. 2), we used cDNA microarray to detect different expression levels between keratocytes and corneal endothelial cells. Hybridization image of the genes in keratocytes versus those in endothelial cells using a platinum pig 13 K biochip is presented in Fig. 3. Data intensity was normalized via M vs A scatter plot. Fig. 4 shows a log intensity signal ratio of the gene expression in porcine keratocytes versus that in porcine endothelial cells.

Up-regulated expression more than 2 folds was found in 1,162 genes of porcine keratocytes. Table 2 shows the genes involved in inflammation or immune reaction among the ones upregulated in porcine keratocytes than in porcine corneal endothelial cells. Of those immune-associated genes, whose signal intensity in porcine keratocytes was higher than that in porcine endothelial cells, the genes expressed more highly in porcine keratocytes than in human keratocytes were presented in Table 3; SEMA3C, CCL2, CXCL6, F3, HLA-A, CD97, IFI30, C1R, G1P3.

Validation of genes by real-time PCR

Real-time PCR showed that the expression of C1R, CCL2,
and HLA-A was higher in porcine keratocytes, compared to that in porcine corneal endothelial cells (Fig. 5).

**DISCUSSION**

Allograft rejection in the corneal transplantation is mainly an endothelial rejection, and the stromal rejection against fibroblasts is very rare in human or murine allograft (2). Surprisingly, however, we observed severe inflammation and rejection against stromal fibroblasts in an orthotopic porcine to rat corneal transplantation (Fig. 5). In addition, in a mixed reaction with human sera or human PBMC (7), we found a higher susceptibility of porcine keratocytes to an immune-mediated damage than corneal endothelial cells. This is the reason why we analyzed and compared the gene expression of porcine keratocytes and porcine endothelial cells in this study. We tried to elucidate major factors involved in the

**Table 2.** Signal intensity and map of immune-associated genes of porcine keratocytes versus porcine corneal endothelial cells

| Gene title                                                                 | Intensity | Map    | Gene title                                                                 | Intensity | Map    |
|---------------------------------------------------------------------------|-----------|--------|---------------------------------------------------------------------------|-----------|--------|
| Mannan-binding lectin serine peptidase 1                                  | 19.4      | 3q27-q28 | Fc fragment of IgG, low affinity IIb, receptor (CD16b)                    | 4.5       | 1q23   |
| (C4/C2 activating component of Ra-reactive factor)                         |           |        | Coagulation factor III (thromboplastin, tissue factor)                     | 3.7       | 6p21.3 |
| Tumor necrosis factor, alpha-induced protein 6                            | 18.9      | 1p22-p21| Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)               |           |        |
| Cathepsin C                                                               | 12.5      | 11q14.1-q14.3 | Interferon, gamma-inducible protein 30                                  | 3.5       | 19p13.1|
| Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)      | 8.3       | 4q21   | Major histocompatibility complex, class I, A                             | 3.4       | 6p21.3 |
| Peroxidasin homolog (Drosophila)                                          | 7.0       | 2p25   | CD97 antigen                                                              | 3.3       | 19p13  |
| Chemokine (C-X-C motif) ligand 14                                          | 6.8       | 5q31   | Complement component 1, r subcomponent                                   | 3.2       | 12p13  |
| Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C | 5.2 | 7q21-q31 | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4 | 2.8       | 14q11.2|
| Interleukin 6 (interferon, beta 2)                                         | 5.1       | 7p21   | Interferon, alpha-inducible protein (clone IFI-6-16)                      | 2.7       | 1p35   |
| Chemokine (C-C motif) ligand 2                                             | 4.8       | 17q11.2-q12 | Major histocompatibility complex, class II, DR alpha                   | 2.7       | 6p21.3 |
|                                                                             |           |        | Nuclear factor, interleukin 3 regulated                                   | 2.5       | 9q22   |

**Fig. 3.** Hybridization image of the genes of keratocytes (A) versus those of endothelial cells (B) using platinum pig 13 K biochip.

**Table 3.** Signal intensity of immune-associated genes of porcine keratocytes/human keratocytes, which expression was higher than in porcine endothelial cells

| Gene title                                                                 | Gene symbol | Intensity |
|---------------------------------------------------------------------------|-------------|-----------|
| Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C | SEMA3C      | 46.8      |
| Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)      | CCL2        | 25.5      |
| Chemokine (C-C motif) ligand 14                                            | CXCL6       | 16.8      |
| Coagulation factor III (thromboplastin, tissue factor)                    | F3          | 12.2      |
| Major histocompatibility complex, class I, A                              | HLA-A       | 12.1      |
| CD97 antigen                                                              | CD97        | 8.5       |
| Interferon, gamma-inducible protein 30                                    | IFI30       | 8.4       |
| Complement component 1, r subcomponent                                   | C1R         | 6.1       |
| Interferon, alpha-inducible protein (clone IFI-6-16)                      | G1P3        | 4.6       |
| Tumor necrosis factor, alpha-induced protein 6                            | TNFAIP6     | 3.5       |
| Cathepsin B                                                               | CTSB        | 3.1       |
| Transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)               | TAP1        | 2.8       |
| Peroxidasin homolog (Drosophila)                                          | PXDN        | 2.6       |
First, we found 20 genes having high signal intensity ratios of porcine keratocytes/endothelial cells among the immune-related genes. Next, we compared the expression of those candidate genes with their expression in human keratocytes and sorted out highly expressed genes, because they might be involved in the development of porcine stromal inflammation or rejection, which was not observed in stromal keratocytes of human allograft. As a result, target genes turned out to be \textit{SEMA3C}, \textit{CCL2}, \textit{CXCL6}, \textit{F3}, \textit{HLA-A}, \textit{CD97}, \textit{IFI30}, \textit{C1R}, and \textit{G1P3}. When we compared the gene expression of \textit{C1R}, \textit{CCL2}, \textit{CXCL6}, and \textit{HLA-A} between porcine keratocytes and porcine endothelial cells using the real-time PCR, \textit{C1R}, \textit{CCL2}, and \textit{HLA-A} were highly expressed in porcine keratocytes. Notably, the level of \textit{C1R} gene in porcine keratocytes was as almost 16 times as that in porcine endothelial cells.

\textit{C1R} is a highly specific serine protease that initiates the classical pathway of complement activation. A report presents elevated levels of \textit{C1rs-C1inh} complex in tears after the corneal transplantation, suggesting that the classical pathway of the complements may be activated in the early postoperative period of the corneal transplantation (11). Especially, complement-associated hyperacute- or acute-rejection is regarded as a very important rejection mechanism in xenograft of vascular organs such as kidney or heart. However, the effect of complements on rejection has been overlooked in corneal allograft because the concentration of complements in aqueous humor is lower than that in serum. Moreover, complements cannot reach the corneal stroma due to avascularity of corneal stroma especially in normal quiet cornea. Usually, complement proteins are mainly synthesized by hepatocytes but are also secreted by tissue macrophages and blood monocytes (12). The complements are also known to be secreted by keratocytes (corneal fibroblasts) (13). High secretion of complements by keratocytes is likely to be crucial in corneal xenograft, as well as the rapid infiltration of innate immune cells, that synthesize complements, may also contribute to increase the concentration of complements in the stroma. There is a report showing keratocyte apoptosis in an orthotopic human to cat corneal xeno-transplantation (14). The percentage of TUNEL-positive cells was higher in the stromal keratocytes than in the endothelial cells, which is consistent with our results. Taken all together, increased
expression of C1R gene in porcine keratocytes provides a plausible evidence on the possible complement-mediated stromal rejection of the porcine corneal xenograft.

CCL2 (MCP-1) displays a chemotactic activity for monocytes and basophils, but not for neutrophils or eosinophils. Many studies demonstrate the increase in MCP-1 and subsequent macrophage infiltration to the pig islet xenograft (15-17) and pig heart xenograft (18). Likewise, our in-vivo porcine to rat corneal transplantation showed early neutrophil migration and subsequent monocytes/macrophages infiltration (Fig. 5).

HLA-A or MHC Class I molecule is a major antigen to the immune system, and is easily recognized by CD8+ T-lymphocytes. Our porcine xenograft demonstrated high CD8+ infiltration as early as 7 days after transplantation (unpublished data), and this finding seems to be associated with the increase of HLA-A gene expression in this study.

Recruitment of neutrophil to inflammatory sites is mediated by two related receptors: CXCR1 and CXCR2. Both receptors share two ligands, interleukin-8 (CXCL8) and GCP-2 (CXCL6). The role of CXCL6 in inflammation to facilitate neutrophil infiltration is well established (19), while its involvement in xenorejection is uncertain. The porcine islet graft presents mostly monocytes/macrophages and T cells migration, but not neutrophils (15, 16). We found relative increasing expression of CXCL6 gene in porcine keratocytes, which is 16 and 8 folds higher than in human keratocytes and porcine endothelial cells, respectively, whereas real time PCR revealed expression of RNA did not. We do not know exact roles of CXCL6 right now, because we could not perform the real time PCR between porcine keratocytes and human keratocytes, due to technical difficulties that the cells can be transformed during cultivation and subculture. It would be more valuable if we use cells collected from rejected corneal xenografts rather than cells cultivated and collected from fresh cornea, because cells can be transformed during cultivation procedure, and it might not precisely reflect the pattern of gene expression during the early rejection period. Third, we analyzed different gene expression between human and porcine corneal xenografts and cell lines based on the observation in a pig-to-rat xenotransplantation model. Nevertheless, we still believe that the data provided in this study are worthy of understanding the rejection mechanism in porcine to rat xenotransplantation and further predicting the immune reaction in porcine to human xenotransplantation.

In conclusion, based on the known evidence-based function, the increased expression of C1R, CCL2, and HLA-A appears to be responsible for the stromal rejection of keratocytes in xeno-related corneal rejection. It can be mediated...
by the recruitment of acute inflammatory cells or possibly due to the complement-associated killing. The roles of other genes, which showed increased ratio, in the stromal rejection remain to be further investigated.

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