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Differential expression patterns of gangliosides in the tissues and cells of NIH-mini pig kidneys

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Gangliosides are a ubiquitous component of the membranes of mammalian cells that have been suggested to play important roles in various cell functions such as cell–cell interaction, adhesion, cell differentiation, growth control and signaling. However, the role that gangliosides play in the immune rejection response in xenotransplantation is not yet clearly understood. In this study, differential expression patterns of gangliosides in HEK293 (human embryonic kidney cells), PK15 (porcine kidney cells), NIH-kd (NIH-mini pig kidney cells, primary cultured) and the cortex, medulla and calyx of the NIH-mini pig kidney were investigated by high-performance thin-layer chromatography (HPTLC). The results revealed that HEK293, PK15 and NIH-kd contained GM3, GM2 and GD3 as major gangliosides. Moreover, GM3, which are the gangliosides of NIH-kd, were expressed at higher levels than HEK293 and PK15. Especially, GT1b were expressed in HEK293 and NIH-kd but not in PK15. Finally, GM1 and GD1a were expressed in NIH-kd, but not in HEK293 or PK15. These results suggest that differential expression patterns of gangliosides from HEK293, PK15 and NIH-kd are related to the immune rejection response in xenotransplantation.

Keywords: HEK293; PK15; NIH-kd; NIH-mini pig kidney; gangliosides xenotransplantation

Introduction

Gangliosides are glycosphingolipids that contain sialic acid and are ubiquitous membrane components in mammalian cells. Ganglioside GM3 is a common precursor of nearly all naturally occurring gangliosides, and it has been suggested that this GM3 plays an important role in a wide variety of cellular functions. Additionally, GM3 induces monocytic differentiation of the human leukemia cell lines HL-60 and U937 (Nojiri et al. 1986; Xia et al. 1989; Zeng 1995) and plays an important role in a wide variety of biological processes including cell–cell interaction, adhesion, cell differentiation, growth control and receptor function (Choo et al. 1995). GM3 is synthesized by the addition of sugar residues to the glycan side chain of a creamed backbone by a specific enzyme to yield developmentally regulated cell-type-specific structures (Van Echten and Sandhoff 1993; Duclos 2000). As a class of molecules, gangliosides are ubiquitous in mammalian cells, present in high concentrations in brain tissue and frequently altered in tumor cells, which are all reasons to probe their biological activity. However, it has been difficult to clearly delineate the impact of gangliosides on cell function until recently. One approach has been to alter the ganglioside content pharmacologically, either through inhibition of synthesis or the addition of exogenous gangliosides, and then assess cell function. However, the role that gangliosides play in immune rejection response by xenotransplantation is not yet clearly understood. In the present study, differential expression patterns of gangliosides in the kidneys of NIH-mini pigs were investigated.

Materials and methods

Monoclonal antibodies

Eight MAbs specific for one of the following gangliosides, GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b and GQ1b, were used in this study. HEK293, PK15, NIH-kd and various sections of the NIH-mini pig kidney were appreciably positive for MAbs against GM3, GM2, GM1, GD3, GD1b and GT1b, which correspond to GMR6, MK1-16, GMB16, GMR19, GGR12 and GMR5, respectively. Therefore, these six MAbs were used for further experiments. The production and characterization of these MAbs have previously

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been described (Ladisch and Gillard 1985; Ozawa et al. 1992; Kotani et al. 1993; Kanda and Tamaki 1998).

Glycosphingolipids

Rat brain and bovine brain gangliosides were used as a standard to categorize the individual ganglioside species.

Glycolipid extraction and purification

The methods used to extract and purify gangliosides have been previously described (Ladisch and Gillard 1985). Briefly, the cells were homogenized in distilled water at 4°C to extract the total lipids. The extracted total lipids in chloroform/MeOH (1:1, v/v) were then lyophilized using N_2 gas and subsequently dissolved in chloroform/MeOH/H_2O (15:30:4, v/v/v). The dissolved total lipid samples were then applied to a DEAE Sephadex A25 column (Sigma). The column was subsequently washed to remove the neural lipids with chloroform/MeOH/H_2O (15:30:4, v/v/v). Acidic lipids were eluted by adding chloroform/MeOH/sodium acetate (15:30:4, v/v/v). The eluted samples were then dried at 30°C under N_2 for 5 h, after which they were dissolved in chloroform/MeOH (1:1, v/v) and alkalized in 12 N ammonium hydroxide solution. Next, the acidic lipid samples dissolved in chloroform/MeOH (1:1, v/v) were applied to a Sep-Pak C18 cartridge column. The column was then washed to remove non-hydrophobic species lipids with H_2O. Finally, the gangliosides were eluted by adding MeOH, dried at 30°C under N_2 for 3 h and stored at −80°C until analysis.

High-performance thin-layer chromatography (HPTLC)

HPTLC analysis of the gangliosides was conducted using a 10 × 10 or 10 × 20 cm thin-layer chromatography (TLC) 5651 plate (Merck, Darmstadt, Germany) described in a previous study (Ladisch and Gillard 1985). The purified gangliosides (2 mg protein/lane) were loaded onto TLC 5651 plates that were subsequently developed in chloroform/MeOH/0.25% $CaCl_2 \cdot H_2O$ (50:40:10, v/v/v). The gangliosides were then stained with resorcinol, after which the densities of the ganglioside bands were quantified by HPTLC densitometry (Beta 4.0.3 from Scion Image, Frederick, MD). Bovine brain gangliosides were used as a standard to categorize individual ganglioside species.

Tissue preparation

The cortex, medulla and calyx of female NIH-mini pig kidneys (3 years old; National Institute of Animal Science, Korea) were rinsed several times in phosphate-buffered saline (PBS) and then drained. The kidney tissues were then mixed with Tissue-Tek® O.C.T. compound (Miles Inc., USA), frozen in liquid nitrogen and stored at −80°C until use.

Tissue staining and immunofluorescence microscopy

The expression of gangliosides in the cortex, medulla and calyx of the NIH-mini pig kidneys was determined by indirect immunofluorescence microscopy of frozen sections. Serial sections (3 μm thick) cut with a cryostat microtome were thaw-mounted on albmin-coated glass slides. The mounted sections were then air dried for 2 h, after which they were fixed with acetone at −20°C for 5 min (Graus et al. 1984).

Sections were washed twice with PBS for 10 min and then incubated with 5% BSA in PBS for 15 min at room temperature. Next, the sections were incubated with mouse monoclonal antibody diluted in PBS containing 5% BSA overnight at 4°C. They were then washed with cold PBS four times, after which they were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibody diluted in PBS to 1:500 for 1 h. After washing with PBS five times, the sections were sealed with a coverslip. To identify the nuclei, 1 μl/ml of DNA-specific fluorescent dye (Hoechst 33342) was added. All fluorescent samples were observed under a fluorescence microscope (Model FV300, Olympus).

HEK293, PK15 cell culture and primary culture of NIH-mini pig kidneys

HEK293 and PK15 cells (KCLB®; Korean Cell Line Bank., Korea) were cultured in growth medium containing DMEM (Sigma) supplemented with 4500 mg/L glucose, L-glutamine and sodium pyruvate (Sigma), 3.7 g/L sodium bicarbonate (Sigma), 1% antibiotic-antimycotic (Gibco) and 10% fetal bovine serum (Gibco) at 37°C under 5% CO_2. NIH-kd primary cells were cultured from female NIH-mini pig kidneys. The inner parts of the NIH-mini pig kidneys were dissected and the cortex, medulla and calyx were removed. The kidney tissue was then washed with PBS and transferred to a 1.5 ml EP tube with 1 ml PBS. Next, the tissue was chopped and rotated on a 37°C heating stack for 30 min, after which 10 ml of DMEM media were added to the chopped tissue and the samples were filtered with a cell strainer (70 μm, BD Falcon™). To harvest the tissue cells, chopped tissues were centrifuged for 15 min at 1000 rpm and then washed three times. The cell pellets were then seeded in a T25 flask and incubated at 37°C under 5% CO_2. The medium was changed 5 days after incubation.

Cell staining and immunofluorescence microscopy

Cells were washed twice with PBS for 10 min and then permeabilized with 0.25% Triton X-100 for 10 min at 37°C, after which they were fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. The samples were then incubated with 5% BSA in PBS for 15 min at room temperature, washed twice with PBS and then incubated with mouse monoclonal antibody diluted in PBS containing 5% BSA overnight at 4°C. Next, the samples were washed with cold PBS four times and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibody diluted in PBS to 1:500 for 1 h. After washing with PBS five times, the sections were sealed with a coverslip. To identify the nuclei, 1 μl/ml of DNA-specific fluorescent dye (Hoechst 33342) was added. All fluorescent samples were observed under a confocal scanning laser fluorescence microscope (Model FV300, Olympus Co., Tokyo, Japan).

Statistical analysis

All data are expressed as the means ± S.E. Statistical differences were determined using a Student's unpaired *t*-test. Differences were considered significant at $p < 0.05$.

Results

Cryosection and GIEMSA staining

The vertical section revealed the location of the cortex, medulla and calyx in the kidney. GIEMSA staining of the NIH-mini pig kidney sections (3 μm thick) clearly revealed the cortex, medulla and calyx (Figure 1).

High-performance thin-layer chromatography of NIH-mini pig kidney sections

Figure 2A shows a profile of the gangliosides in the cortex, medulla and calyx of NIH-mini pig kidneys. The results suggest that GM3, GM1, GD3 and GT1b are the major gangliosides in the cortex, GM3, GM2, GM1, GD3, GD1b and GT1b are the major gangliosides in the medulla and GM2, GD3, GD1b and GT1b are the major gangliosides in the calyx. Additionally, GM2 and GD1b were not expressed in the cortex, while GM3 and GM1 were not expressed in the calyx. Quantitative analysis of gangliosides expression that are presents the quantitative values of expressed gangliosides in NIH-mini pig kidney (Figure 2B).

Immunofluorescence staining of NIH-mini pig kidney sections

Nuclear staining and confocal scanning laser fluorescence microscopy showed the cortex, medulla and calyx of the NIH-mini pig kidney. Gangliosides were expressed in the cytoplasm. The cortex samples were negative for anti-GM2 and GD1b MAb and the calyx samples were negative for anti-GM3 and GM1 MAb (Figure 3).

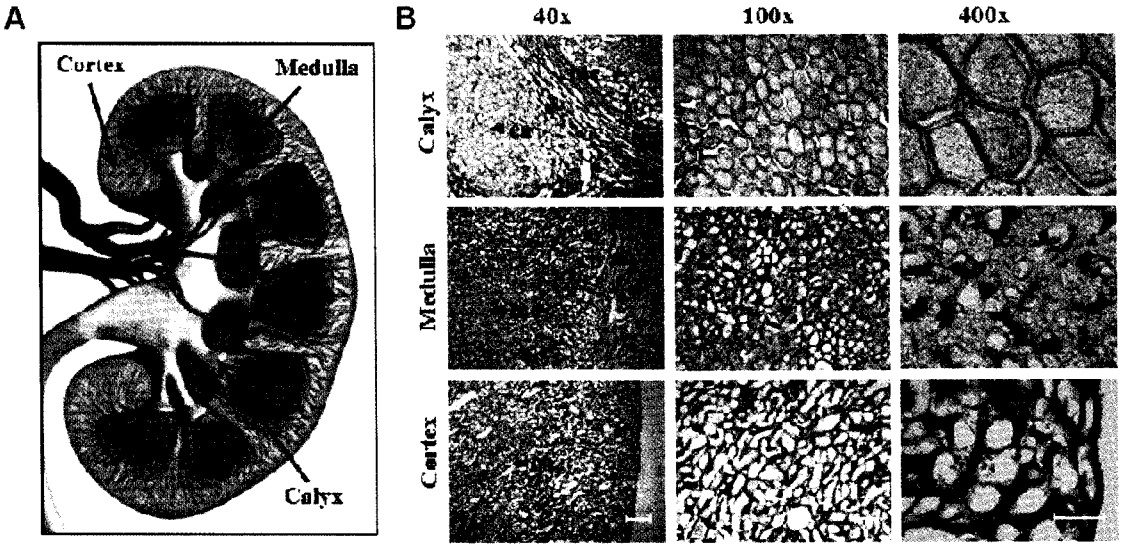


Figure 1. Vertical sections of kidney. A. Vertical section of kidneys. B. Cryosections and GIEMSA staining of NIH-mini pig kidneys. Serial sections of NIH-mini pig kidneys showing the vertical sections of the kidney cortex, medulla and calyx. ca, calyx; co, cortex; me, medulla. The bar represents 50 μm.

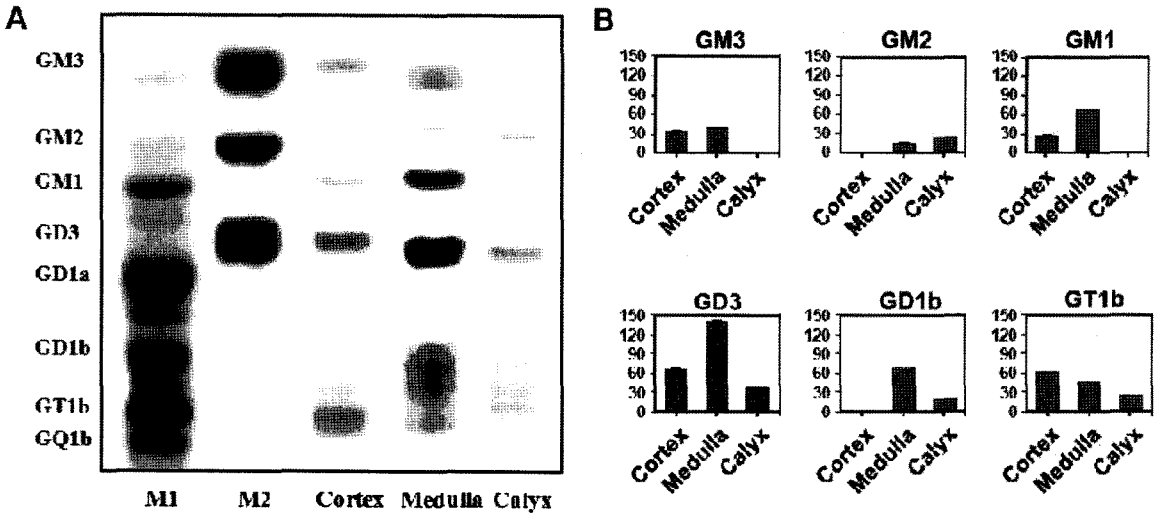
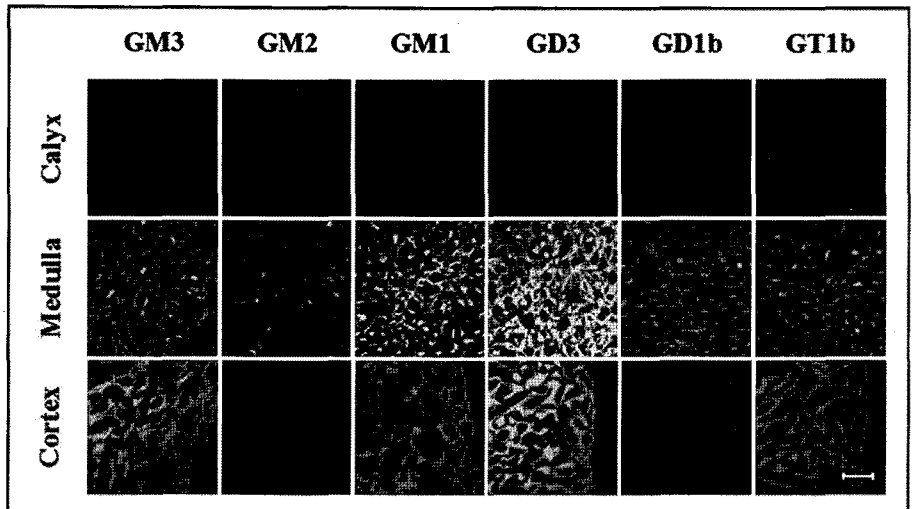


Figure 2. HPTLC analysis of ganglioside expression in NIH-mini pig kidneys. A. The ganglioside extracts were loaded onto a TLC silica gel plate with chloroform:methanol:0.22% $CaCl_2$ (55:45:10, v/v/v), and the gangliosides were visualized using resorcinol solution. B. Quantitative analysis of ganglioside expression in NIH-mini pig kidneys. Lanes 1 and 2, ganglioside standard markers, M1 and M2; lane 3, cortex (NIH-mini pig kidney); lane 4, medulla (NIH-mini pig kidney); lane 5, calyx (NIH-mini pig kidney).



	Degree of expression					
Tissue type	**GM3**	**GM2**	**GM1**	**GD3**	**GD1b**	**GT1b**
Calyx	-	+	-	+	+	+
Medulla	+	+	+	+	+	+
Cortex	+	-	+	+	-	+

Figure 3. Indirect immunofluorescence analysis of NIH-mini pig kidney sections. Sections were immunostained with GMR6 (anti-GM3 Mab), MK1-16 (anti-GM2 Mab), GMB16 (anti-GM1 Mab), GMR19 (anti-GD3 Mab), GGR12 (anti-GD1b Mab), GMR5 (anti-GT1b Mab) and FITC-labeled goat anti-mouse IgM antibody and then stained with Hoechst 33342 for DNA. The bar represents 50 μm.

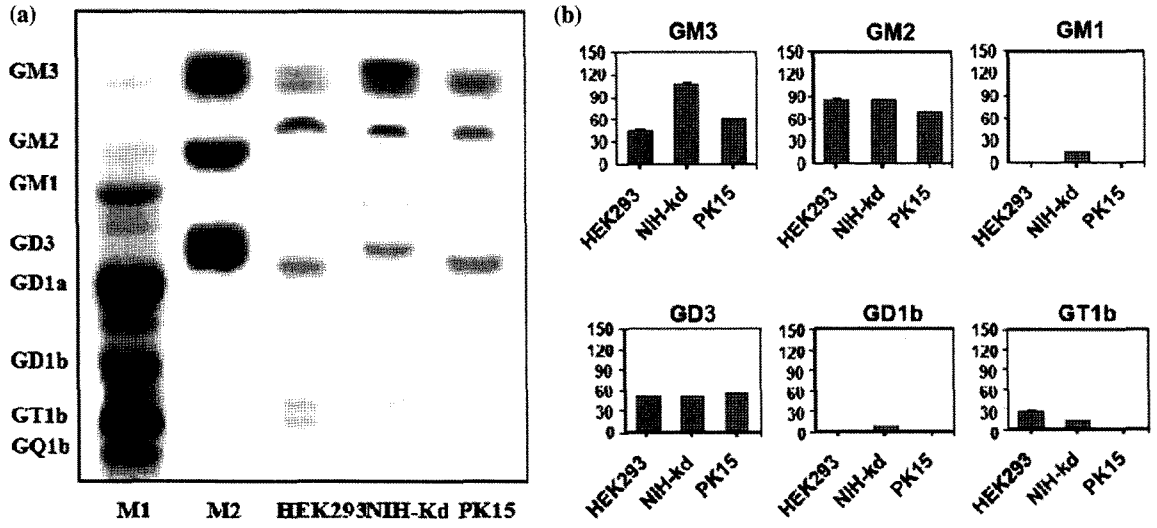


Figure 4. HPTLC analysis of ganglioside expression in human and pig kidney cells. A. The ganglioside extracts were loaded onto a TLC silica gel plate with chloroform:methanol:0.22% $CaCl_2$ (55:45:10, v/v/v), and gangliosides were visualized with resorcinol solution. Lanes 1 and 2, ganglioside standard markers, M1 and M2; lane 3, HEK293 (human embryonic kidney cells); lane 4, NIH-kd (NIH-mini pig kidney cells, primary cultured); lane 5, PK15 (porcine kidney cells). B. Quantitative analysis of ganglioside expression in human and pig kidney cells.

High-performance thin-layer chromatography of HEK293, NIH-kd and PK15 cells

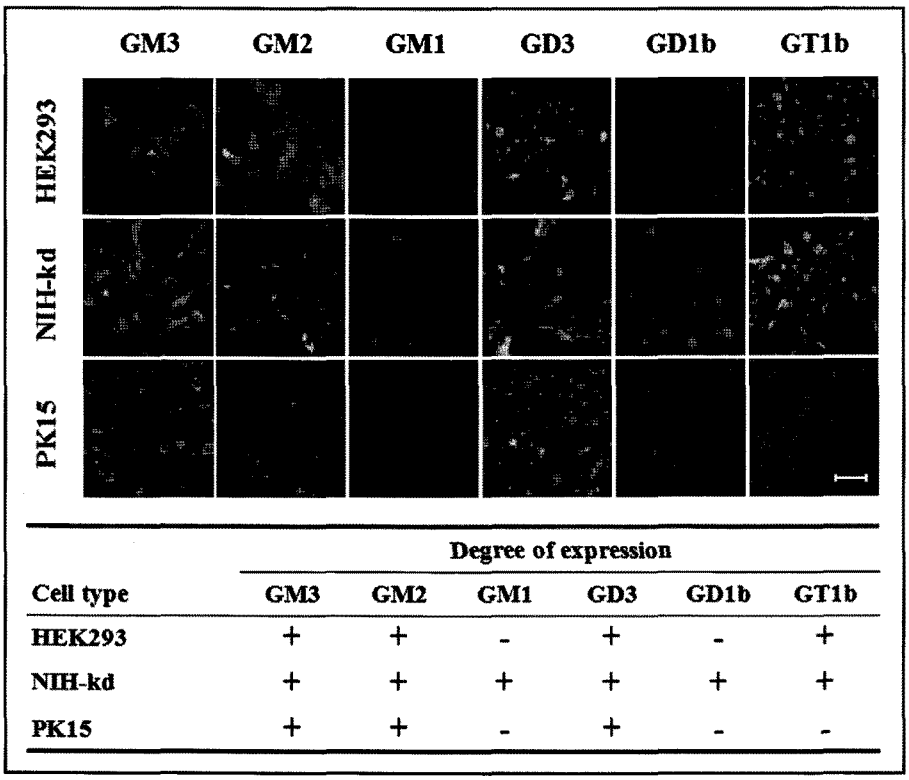
Figure 4A shows a profile of gangliosides in HEK293, NIH-kd and PK15 cells. The results suggest that GM3, GM2, GD3 and GT1b are the major gangliosides in HEK293 cells, that GM3, GM2, GM1, GD3, GD1b and GT1b are the major gangliosides in NIH-kd cells and that GM3, GM2 and GD3 are the major gangliosides in PK15 cells. Additionally, GM1 and GD1b were not expressed in the HEK293 cells, while GM1, GD1b and GT1b were not expressed in PK15. Quantitative analysis of the expression of gangliosides is presented as the quantitative values of the gangliosides expressed in the NIH-mini pig kidney (Figure 4B). Moreover, the expression patterns of the gangliosides in the medulla of the NIH-mini pig kidney were similar to those of NIH-kd (Figures 2A and 4A).

Immunofluorescence staining of HEK293, NIH-kd and PK15

Images of HEK293, NIH-kd and PK15 were obtained by nuclear staining and confocal scanning laser fluorescence microscopy. Gangliosides were expressed in the cytoplasm. HEK293 were negative for the anti-GM1 and GD1b MAb, while PK15 were negative for the anti-GM1, GD1b and GT1b MAb (Figure 5).

Discussion

Transplantation of solid organs (heart, lung, liver, and kidney) from swine to humans would solve the current critical shortage of cadaver organs needed by patients with end-stage disease of these organs. However, currant xenografts are rejected within minutes to hours after transplantation. One of the major issues in contemporary kidney transplantation is the rapid diagnosis and prevention of acute allograft rejection episodes (AREs), which occur in approximately 20% of patients and significantly shorten the allograft survival (Tejani 2000). Cytokines are crucial mediators of immune reactions that lead to AREs in transplantations (Dallman 1995). Gangliosides are generally considered to play an important role in the cellular and humoral immune response. Previous studies have reported that gangliosides regulate cellular and humoral immune responses, and that gangliosides GM1, GD1a and GD1b inhibited LPS-induced proliferation of murine splenocytes (Esselman and Miller 1997). Additionally, GQ1b was found to enhance Ig production of human PBMC (peripheral blood mononuclear cells) (Kanda and Tamaki 1998), while GM2 and GM3 inhibited TNF-α and/or IL-10 production in cocultures of *Staphylococcus aureus* Cowan strain I and IL-2-activated human B cells (Kimata and Yoshida 1994, 1996). It is plausible that some of these immunomodulatory effects by gangliosides may be manifested by regulating signal transduction pathways, including those that are related to kidney transplants. Therefore, understanding the role of gangliosides in the ARE process may improve kidney transplant survival. To the best of our knowledge, the present study is the first immunohistochemical report that clearly demonstrates the expression pattern of gangliosides in the cortex,



	Degree of expression					
Cell type	**GM3**	**GM2**	**GM1**	**GD3**	**GD1b**	**GT1b**
HEK293	+	+	-	+	-	+
NIH-kd	+	+	+	+	+	+
PK15	+	+	-	+	-	-

Figure 5. Indirect immunofluorescence analysis of human and pig kidney cells. Cells were immunostained with GMR6 (anti-GM3 Mab), MK1-16 (anti-GM2 Mab), GMB16 (anti-GM1 Mab), GMR19 (anti-GD3 Mab), GGR12 (anti-GD1b Mab), GMR5 (anti-GT1b Mab) and FITC-labeled goat anti-mouse IgM antibody, after which they were stained with Hoechst 33342 for DNA. The bar represents 50 μm.

medulla and calyx of the kidneys of NIH-mini pigs. Our results revealed the differential expression patterns of gangliosides in HEK293, PK15 and NIH-kd. Specifically, ganglioside GM1 and GD1a expressed in NIH-kd tat are the cells which are primary cultured from the medulla of NIH-mini pig kidney. However, the ganglioside profiles in NIH-kd were similar to the medulla of NIH-mini pig kidney. Thus, our current findings suggest that the NIH-kd cells may originate from medulla of the NIH-mini pig kidney.

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