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Studies of innate immune systems against human cells☆,☆☆

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ABSTRACT

Background: Pigs are frequently used as animal models for experiments in the surgical field, including allo- and xeno-transplantation. Regeneration studies, including studies dealing with human- and monkey-induced pluripotent stem cells (iPSC), have gradually progressed, with pigs sometimes being used as the scaffold. However, the immunological response of pigs against humans, especially innate immunities, remain unclear. This study reports on a comprehensive study of pig innate immunity against humans.
Methods: Hemolytic complement activity of pig serum was measured using a microtitration technique. The pig natural anti-human antibody (Ab) was examined using human peripheral blood mononuclear cells (PBMC). The reaction of pig natural killer (NK) cells and monocytes/macrophages against human cells was also assessed.
Results: Most of the pig complement titers were measured based on methods used in human complement assays. The alternative pathway for pig complement reacts with human cells, indicating that pig complement can react with human cells. Pig serum contains relatively high levels of natural antibodies, IgM and IgG, to human PBMC. Furthermore, the killing of NK cells- and monocyte/macrophage-mediated human cells was clearly confirmed.
Conclusion: The collective findings indicate that the pig innate immunological systems, not only serum but also cellular factors, are able to recognize and injure human cells.

1. Introduction

Pigs have recently become popular experimental animals that are used in the surgical field, including allo- and xeno-transplantation. Xenotransplantation using pig organs, tissues and cells is expected to alleviate the worldwide shortage of human transplantation donors. Studies of discordant xenografts have progressed dramatically, due developments in immunological analyses, including the alternative pathway of complement [1,2], and biological analyses for carbohydrate-antigens [3,4], the α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R) [5], the Hanganutziu-Deicher (H-D) antigen [6,7], etc., together with the rapid progress being made in gene technology and animal science [8,9].

On the other hand, regeneration studies, using not only mouse induced pluripotent stem cells (iPSC) but human and monkey iPSC as well, have also progressed, sometimes with pigs being used as the scaffold [10–12]. However, the immunological response of pigs against humans, especially the pig complement system and the other innate immunity systems, is unclear.

In this study, we report on an investigation of the possibility of using pigs in immunological experiments, including the surgical field, from the point of view of the pig innate immune system. Based on our previous study for pig complement titers [13], the complement system in pigs was further studied, and the reaction of natural antibodies and the alternative pathway of complement to humans was further investigated. As the next step, the responses of cellular factors of innate immunity, such as natural killer (NK) cells and monocytes/macrophages against humans, were also examined.

2. Materials and methods

2.1. Cell culture

HAOEC (Human Aortic Endothelial Cells) was cultured using CSC Medium (Cell systems Corporation, Kirkland, WA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Nichirei

Abbreviations: the α-Gal epitope, Galα1-3Galβ1-4GlcNAc-R; H-D, Hanganutziu-Deicher; iPSC, induced pluripotent stem cells; HAOEC, human aortic endothelial cells; EDTA, ethylenediamine tetra-acetic acid; EGTA, ethylene glycol tetra-acetic acid; Ab, antibody; PBMC, peripheral blood mononuclear cells.

☆ Complement components are identified according to World Health Organization recommendation (1981).

☆☆ RS performed research and participated in writing the article. EK, AM, PL and MW participated in performing research. HE, HN and HO participated in analysing the data, and SM participated in making the research design, and writing the article.

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Biosciences INC., Tokyo, Japan) and 1% Penicillin-Streptomycin (Meiji Seika Pharma, Tokyo, Japan). Human fibroblast cell lines, NTI-4 and NTI-5, were from Health Science Resources Bank (HSRRB) (Osaka, Japan), and cultured using Dulbecco's modified Eagle's medium (D-MEM) (Sigma-Aldrich Co., MO, USA) supplemented with 10% heat-inactivated FBS, L-glutamine and Penicillin-Streptomycin. Cultures were maintained in a 5% CO_2/95% air atmosphere at 37 °C.

2.2. Blood

Samples of pig blood were obtained from several young adult or newborn pigs (Large White/Landrace x Duroc, female, at almost 6-months, 45 days or 2–3 weeks old, respectively). Human blood was obtained from healthy adult volunteers.

2.3. Assay of hemolytic complement activity

The hemolytic complement activity of pig serum was measured using a microtitration technique. CH50 was determined according to a method reported by Mayer [14]. ACH50 was assayed according to methods reported by Platts-Milles and Ishizaka [15]. The hemolytic activities of C4, C2, C3, C5, C8 and C9 were estimated using intermediate cells and reagents, as described previously. C1 activity was typically estimated using sensitized erythrocytes (E) bearing human C4 (EAC4hu), guinea-pig C2 (C2gp) and normal human serum (NHS) diluted in an ethylenediamine tetra-acetic acid (EDTA) buffer containing C3–C9 (Chu-EDTA). C4 activity was estimated using sensitized erythrocytes (E) bearing guinea-pig C1 (EAC1gp), C2gp and NHS diluted with EDTA buffer containing C3–C9 (Chu-EDTA). C2 activity was determined using EA-bearing human C1 and C4 (EAC14hu), prepared from EA and human serum by the TTHA method, and from Chu-EDTA. The hemolytic activity of C3 was detected using EA-bearing human C1, C4, C2 (EAC142hu) and C5hu, and incubation with C6-9hu-EDTA. The hemolytic activity of C5 was detected using EA-bearing human C1, C4, C2, and C3 (EAC1423hu), and incubation with C6-9hu-EDTA. The hemolytic activity of C8 and C9 were detected using EA-bearing human C1 to C7 (EAC1-7hu), and incubation with C9gp or C8gp in KKB^{2+} buffer, respectively [16].

2.4. The alternative pathway of pig complement

The susceptibility of human erythrocytes and fibroblast cell lines to pig complement, the whole complement pathway and the alternative pathway were assessed, using Mg^{2+} ethylene glycol tetra-acetic acid (EGTA) (for the Alternative Pathway) [2]. Human blood was centrifuged at 3,000 rpm for 10 min at room temperature and washed twice with PBS. The pellet containing erythrocytes was diluted to 1.25% with saline. The diluted human erythrocyte suspension was then incubated with 10% or 20% pig serum with or without 10 mM Mg^{2+} EGTA for 1 h at 37 °C and the hemolytic titer was measured (OD = 415 nm) by means of a microplate reader SH9000 (CORONA, Tokyo, Japan). As a control for complete hemolysis, erythrocytes were diluted with water.

Concerning NTI-4 and NTI-5, the percent cytotoxicity for these cells by 20% pig serum with or without 10 mM Mg^{2+} EGTA for 4 h at 37 °C was calculated by the WST-8 assay. Briefly, after the incubation, 10 μl of a WST-8 solution (Nacalai tesque, Kyoto, Japan) was added to each well, followed by incubation at 37 °C for 45 min. Each sample was then measured using a microplate reader SH9000 (OD = 450 nm).

2.5. Natural antibody titer

The pig natural anti-human antibody (Ab) was examined using human peripheral blood mononuclear cells (PBMC). Human blood was diluted 1:1 with PBS, overlaid on a Lymphoprep system (Axis-Shield, Oslo, Norway) and then centrifuged at 1,800 rpm for 20 min. PBMC were sampled from the monolayer and washed twice with PBS. The PBMC were incubated with 10% pooled pig serum for 1 h on ice, followed by staining with a fluorescein isothiocyanate (FITC)-labeled Goat anti-pig IgM (BETHYL Laboratories, Montgomery, AL, USA) and IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min on ice. The resulting stained cells were quantified using a FACS Calibur system (BD Bioscience, Franklin Lakes, NJ, USA) [2].

2.6. NK cell-mediated cytotoxicity assay

Whole blood, obtained from pigs, was acquired following the same protocol as that for human PBMC, as previously reported [16].

The HAOEC were plated at 2×10^4 cells per well in a flat-bottomed gelatin-coated 96-well plate. Fifteen hours after plating the cells, the plates were incubated with effector cells, PBMC, at various effector: target (E:T) ratios = 20:1, 50:1 or 100:1. Each assay was performed in triplicate. After a 4 h incubation at 37 °C, the lactate dehydrogenase (LDH) that was released was measured using a MTX "LDH" kit (Kyokuto Pharmaceutial Industrial, Tokyo, Japan) according to the manufacturer's recommended protocol. Briefly, a 25 μl aliquot of LDH reagent was added to 25 μl of the supernatant from each well, and the resulting suspension incubated for 15 min at 37 °C, followed by adding 50 μl of the stop reaction solution. Each sample was then measured by means of a microplate reader SH9000; OD 560 nm. The spontaneous release of LDH from effector cells and target cells was less than 10% and 5%, respectively, compared to the maximal release obtained by sonication. The results are expressed as the percent of specific lyses [16].

2.7. Monocytes/macrophages-mediated cytotoxicity assay

Pig PBMC were stained with an anti-monocyte/granulocyte-FITC Ab (Abcam) for 30 min on ice, followed by treatment with anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min on ice. The stained monocytes/macrophages were finally isolated by positive selection using a SuperMACS separator and an LS column (Miltenyi Biotec).

The WST-8 assay was used as a cytotoxicity assay. Briefly, 1×10^4 HAOEC were co-cultured overnight with pig monocytes/macrophages and LPS (100 ng/ml) in a 96 well plate, at various effector: target (E:T) ratios = 20:1 or 50:1. Thereafter, 10 μl of a WST-8 solution (Nacalai tesque) was added to each well, followed by incubation at 37 °C for 45 min. Each sample was then measured using microplate reader SH9000 (OD = 450 nm).

3. Results

3.1. Hemolytic titers of pig complement

Hemolytic complement activity of pig sera, #1–4: adult pig serum, #5–7: newborn pig serum and # 8: serum from a 45 days-old pig, was measured using a microtitration technique identical for that for human serum, as described previously.

Both the CH50 and ACH50 titers for the pig and the components, except for C3, were measured using the same procedures that were used for measuring human complement components and indicated in the Table 1.

3.2. The reaction of the pig complement system to human cells

The lysis of human erythrocytes and fibroblasts by pig serum with/without EGTA was also assessed. Ten percent serum with/without EGTA was found to lyse 35.2 ± 6.9% or 13.5 ± 5.2% of human erythrocytes, and 20% serum with/without EGTA resulted in the killing of 79.2 ± 2.9% or 49.5 ± 14.5% of human erythrocytes, 85.7 ± 7.5% or 58.9 ± 10.7% of NTI-4, and 72.6 ± 20.2% or 62.1 ± 6.3% of NTI-5, respectively. Human erythrocytes were not affected by 10 mM EGTA, whereas, in the case of NTI-4 and NTI-5 35.8 ± 2.8% and 43.8 ± 2.5% lysis was detected. The