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ORIGINAL ARTICLE

Cloning of pig serine proteinase inhibitor 9 and its use in protecting against apoptosis

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Summary

The activity of granzyme B, a main effector molecule of natural killer (NK) cells and cytotoxic T lymphocytes, is regulated by the intracellular serine proteinase inhibitor 9 (PI-9). Pig PI-9 was first cloned, and the sequences that encode pig PI-9, including the start codon and stop codon, were identified. The cDNA was inserted into the cloning site of pCXN2 (chicken beta actin promoter and cytomegalovirus enhancer), transfected into pig endothelial cells (PEC), and several stable PEC clones were established. An NK cell-mediated cytolysis test was next applied to the PEC clones, using YT cells (an NK-like cell line). The PEC transfectants with pig PI-9 had a significant inhibitory effect on NK cell-mediated PEC lysis. The overexpression of the anti-apoptotic molecule, pig PI-9, has the potential for use in protecting graft cells from human NK cells.

Introduction

When hyperacute rejection in pig to human xenotransplantation is prevented by the expression of a complement regulatory protein, such as decay-accelerating factor (DAF; CD55) [1,2], and the knocking out of the α1,3galactosyltransferase (α1,3GT) gene [3,4], the xenograft becomes susceptible to other types of rejection, cellular rejection, mediated by monocytes, macrophages, natural killer (NK) cells, and cytotoxic T lymphocytes (CTL) [5].

The cytolytic activity of human NK cells reflects a balance between signals delivered from both inhibitory and activating receptors, such as NKG2A and NKG2D [6]. Pig cells are highly susceptible to human NK cell-mediated xenogeneic cytotoxicity [3–5], probably because swine leukocyte antigen class I molecules are unable to deliver negative signals to human NK inhibitory receptors [6]. On the other hand, some molecules on pig cells, such as pig MHC class I related chain (MIC) [7,8], send signals to the activating receptors. Several strategies are available for inhibiting NK cell activity that involve the expression of the human class Ib molecules, such as HLA-G and HLA-E [9,10], the genetic remodeling of glycosyl antigens [11,12], and the expression of human DAF (CD55) [13]. Apart from specific strategies for the regulation of NK cells, some alternate methods for suppressing Killer cells, both NK cells and CTL, were considered.

Protease inhibitor 9 (PI-9) is a member of the large superfamily of serine protease inhibitors (serpins). Serpins are widely dispersed in nature and regulate the activity of proteases in diverse physiologic processes that include coagulation, inflammation, cell migration, and apoptosis. PI-9 belongs to a branch of the serpin superfamily exemplified by ovalbumin or intracellular serpins. These proteases are classified on the basis of amino acid sequence similarity. Compared with other members of the serpin

family, intracellular serpins lack a typical cleavable *N*-terminal signal sequence. As a result, they are mainly intracellular molecules [14–16]. In this study, the role of the pig anti-apoptotic molecule, pig PI-9, in protecting pig endothelial cells (PEC) from human NK cells against a cellular xenograft rejection was examined *in vitro*.

Materials and methods

Cell culture

The pig endothelial cell line, MYP30 [15] was cultured in Dulbecco's modified Eagle's medium. The human NK like cell line, YT cells, generously provided by Drs Junji Yodoi and Keisuke Teshigawara (University of Kyoto, Japan). Culture media were supplemented with 10% heat-inactivated fetal bovine serum and kanamycin/amphotericin B. Cultures were maintained in a 5% CO_2/95% air atmosphere at 37 °C.

Cloning of pig PI-9 cDNA

The sequence was queried in dbEST(NCBI) using the TBLASTN program to search for pig PI-9. Based on the expressed sequence tag (EST) cloning data and the homology to other animals, the first primer sets: 5′-CTCTCCTTCGCCAGCGCTGCAGAG-3′ and 5′-TCA CGGGGAGGAAAACCTGCCAC-3′ were identified. 5′ and 3′ RACEs were carried out by standard methods, using the primers; for 5′ RACE: 5′-TGGTCTGCTCAT CAATTGAGTTCCATG-3′ and for 3′ RACE: 5′-TGC TGATGGAGTGCTGCATGGATCC-3′.

Total RNA was collected from a pig liver, using the TRIZOL Reagent (Sigma, Saint Louis, MO, USA). Purified mRNA was obtained using an oligo-dT cellulose, and was then reverse-transcribed by means of an oligo-dT primer to cDNA for use as a template for PCR. The PCR products were subcloned into the *Eco*RV site of pBluescript. Sequence analyses were performed by means of an ABI 310 autosequencer (Perkin-Elmer Corporation, Norwalk, CT, USA). cDNA of pig PI-9 with a FLAG-tag was next inserted into the cloning site of pCXN (chicken beta actin and cytomegalovirus enhancer) [17].

Establishment of stable transfectants

The cDNA constructs (20 μg) were introduced into PEC by means of the electroporation method [18]. The transfected cells were maintained in a complete medium for several days in an atmosphere of humidified 5% CO_2 at 37 °C, and then transferred to the selection medium containing 0.7 mg/ml G418 (Nacalai tesque, Kyoto, Japan). The stable PEC transfectants were isolated by the limiting dilution method.

Quantitative real-time RT-PCR

Total RNA was collected from the stable clones and the mock control clone, using the TRIZOL LS Reagent (Sigma). The total RNA was used in a reverse transcriptase reaction. To evaluate the PI-9 mRNA, SYBR-green real-time PCR was performed with a Smart Cycler II System (Takara, Osaka, Japan) and the SYBR premix Taq (Takara). The pig PI-9 sequence was amplified using two primer pairs. RT was carried at 42 °C for 15 min, followed by 95 °C for 2 min, using random primers, followed by PCR at 45 cycles at 95 °C for 5 s and 60 °C for 20 s. The amount of mRNA in the transfectants was normalized with the level of GAPDH RNA.

Western blotting

The protein content of the transfectant and naive cell lysates was determined by the BCA method (Pierce, Rockford, IL, USA) and approximately 30 μg aliquots of the resulting proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions. The separated proteins were then electrophoretically transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membrane was blocked by treatment with 5% skim milk in tris-buffered saline/0.05% tween 20 (TBST) for 1 h at 25 °C and then incubated in 1% bovine serum albumin/0.5% skim milk/TBST with an anti-FLAG mAb (M2) (Sigma) for 1 h at 25 °C. After washing, the blots were incubated with horseradish peroxidase conjugated secondary antibody, porcine anti-rabbit Ig (DAKO, Carpinteria, CA, USA), and the signal was developed using an ECL detection system (Amersham, Princeton, NJ, USA).

Lactate dehydrogenase assay

Lactate dehydrogenase assay (LDH) was performed following a previously described method, using an MTX-LDH kit (Kyokuto, Tokyo, Japan). The test involved estimating the amelioration of NK cell-mediated lysis by the transfectant molecules using PEC. Target cells (1.5×10^4) were plated in each well of a microtiter plate and cultured for 15 h prior to assay. The plates were incubated with YT effector cells at an effector:target (E:T) ratio. Each assay was performed in triplicate. After a 4-h incubation at 37 °C, the released LDH was determined. The spontaneous release of LDH activity from the effector and target cells was less than 10% and 5%, respectively, compared with the maximal release obtained by sonication. The results are expressed as the percent of specific lysis.

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Statistical analysis

Data are presented as the mean ± SEM. The Student's *t*-test was used to determine the significance of the differences within groups. Differences were considered to be statistically significant when $P < 0.05$.

Results

Identification of the positive clones and analysis of homology

cDNA sequence isolated from pig liver cells was subcloned into the *Eco*RV site of pBluescript. The cDNA of these clones was amplified by PCR and the sequence was determined. The sequence that encodes pig PI-9 including the start codon and stop codon were defined (Fig. 1). The open reading frame for pig PI-9 consisted of 1125 bp (375aa). Compared with other animals, pig PI-9 has a 74.5% homology in human [HSU71364] [14], and 65.9% in the mouse [BCO29900] [19] amino acid sequence (Fig. 2).

Real-time PCR for mRNA of the transfectant in an additional experiment

Several new PECs with PI-9 clones were established. Real-time PCR was performed to detect differences in the mRNA of PI-9 in each clone. mRNA levels of the pig PI-9 were measured individually by means of a SYBR green system and normalized to GAPDH. The amount of pig PI-9 mRNA in the clones, PI-9#1, PI-9#2 and PI-9#3, was compared with that in the mock clones (Fig. 3a).

Western blot

Western blots were performed using an anti-FLAG mAb. Therefore, the Western blot of naive pig PI-9 was not detected as background. We selected three clones that showed a clear expression in Western blots and indicated good cell growth for the next assay (Fig. 3b).

LDH assay of the stable transfectants with PI-9 gene

The suppressive function of the PI-9 molecule on cytolysis mediated by YT cells was next examined using an LDH assay. PI-9 molecules significantly suppressed NK cell-mediated PEC lyses in transfectants with PI-9. All three transfectants showed an inhibitory effect on NK cell-mediated PEC lyses. Compared with the mock transfectant, the PEC transfectants with pig PI-9 show a significant decrease [(E:T = 2:1 and 5:1) ($n = 6$)] (Fig. 4a,b).

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   1 GAGTGGGACCTGCTGCGGCCGGATCTGCGCCTGGGCTCGGCGGAGCTGCGGCGGCAGAGC   60
  61 CAGCGTGATGGACGCTCTCTCTGAAGCAAACGGCACCTTCGCCCTCCGCCTTTTAAAGAT  120
           M  D  A  L  S  E  A  N  G  T  F  A  L  R  L  L  K  I

 121 ACTGTGCCAAGATGACCCGTCACACAACGTGTTTTATTCTCCTGTGAGCATCTCCTCTGC  180
       L  C  Q  D  D  P  S  H  N  V  F  Y  S  P  V  S  I  S  S  A

 181 CCTGGCCATGGTCCTCCTGGGGGCAAAAGGAGACACCGCTGCCCAGCTGGCCCAGGTGCT  240
       L  A  M  V  L  L  G  A  K  G  D  T  A  A  Q  L  A  Q  V  L

 241 TTCTTTAAACACAGAGAAAGACATTCACCAGGATTTCCAGGCACTTCTTGCCGAGCTGAA  300
       S  L  N  T  E  K  D  I  H  Q  D  F  Q  A  L  L  A  E  L  N

 301 CAAACCCAGCACTCGGTACTTGCTCAGAACGGCCAACAAGCTCTTTGGGGAGAAGTCTCG  360
       K  P  S  T  R  Y  L  L  R  T  A  N  K  L  F  G  E  K  S  R

 361 TGAATTTCTCTCTACCTTCAAGGAATCCTGTCTTCGGTTCTACGATGCGGAGCTGGAGCA  420
       E  F  L  S  T  F  K  E  S  C  L  R  F  Y  D  A  E  L  E  Q

 421 GCTCTCCTTCGCCAGCGCTGCAGAGGCATCCAGAAAGCAGATAAACGCTTGGGTCTCTAA  480
       L  S  F  A  S  A  A  E  A  S  R  K  Q  I  N  A  W  V  S  K

 481 AAAGACAGAAGGTAAAATTCCAGAGGTGCTGCCATGGAACTCAATTGATGAGCAGACCAG  540
       K  T  E  G  K  I  P  E  V  L  P  W  N  S  I  D  E  Q  T  R

 541 GCTGGTTCTTGTCAATGCCGTCTACTTCAAAGGAAGGTGGGACCAGCAATTTGACAAAAA  600
       L  V  L  V  N  A  V  Y  F  K  G  R  W  D  Q  Q  F  D  K  K

 601 GTACACGAGGGAGATGCCTTTTAGGGTAAACCAGAAGGAGCAGAGGCCGGTGCAGATGAT  660
       Y  T  R  E  M  P  F  R  V  N  Q  K  E  Q  R  P  V  Q  M  M

 661 GTTTCAGGAGGCAACGTTCAGACTCGGGCGCGTGGAGGAGGTGCCGGCCCAGGTCCTGGA  720
       F  Q  E  A  T  F  R  L  G  R  V  E  E  V  P  A  Q  V  L  E

 721 GCTGCCCTACGAGGACCGGGAGCTGAGCATGGTCGTCCTGCTCCCCGACGACCACGTGGC  780
       L  P  Y  E  D  R  E  L  S  M  V  V  L  L  P  D  D  H  V  A

 781 TCTGAGCGAGGTGGAGAGACAGCTCACCTTTGAGAAACTCCTGGCCTGGACGAAGCCGGA  840
       L  S  E  V  E  R  Q  L  T  F  E  K  L  L  A  W  T  K  P  E

 841 ACGCATGCAGAGCCTCGACGTGGAAGTTTTCCTCCCGAGGTTTAAGCTGGACGCGAGTTA  900
       R  M  Q  S  L  D  V  E  V  F  L  P  R  F  K  L  D  A  S  Y

 901 CGACCTGGAGTTGCTGCTCGGGCATTTGGGGGTGGTGGACGCCTTCCAGCAGGGCAAGGC  960
       D  L  E  L  L  L  G  H  L  G  V  V  D  A  F  Q  Q  G  K  A

 961 CGACTTCTCGGCCATGGCCCCCGAGCGGGACCTGAGCCTGTCCACCTTCGTGCACAAGAG 1020
       D  F  S  A  M  A  P  E  R  D  L  S  L  S  T  F  V  H  K  S

1021 TGTGGTGGAGGTGAACGAGGAAGGCTCGGAGGCGGCGGCGGCCTCGGCCCTGGTGCTGAT 1080
       V  V  E  V  N  E  E  G  S  E  A  A  A  A  S  A  L  V  L  M

1081 GGAGTGCTGCATGGAGTCCGGGCCCAGGTTCTGCGCCGACCACCCCTTCCTCTTCTTCAT 1140
       E  C  C  M  E  S  G  P  R  F  C  A  D  H  P  F  L  F  F  I

1141 CAGGCACAACAAGGCCAAGAGCATCCTCTTCTGTGGCAGGTTTTCCTCCCCGTGAGCGGT 1200
       R  H  N  K  A  K  S  I  L  F  C  G  R  F  S  S  P  *

1201 GCACCCGCCTCGCGCGGGAGCCTGTCCCCTTCTGCGGCCCTGAGTCCCCCCACCAAGCCC 1260
1261 CAAGGGTGGCCAGGCAGACGCCCACCCGCTCCACGCGCCTTCCCCAGGCGGACCACTCAT 1320
1321 CCACGCCCCTCAGTCCAGACCGCAGGCCTGCAGGCTCTAAGGATGCCCTGGGGCATTTAA 1380
1381 GACGCAGCCCGGATCCCACAGGACGGCGGGCAGCGTGTCCCCACATGACCCCAGACTTCC 1440
1441 TCGCTGCCCTTGTCCGCGCTCACTGACATCTCAACTCTGGTCACACCCGTGCCTCAGCCT 1500
1501 CTTGCGATAGTAAATAGATGCCACTAGCCGTCGCTACTGTTTTATAAAAAAAAAAAAAAA 1560
1561 AAAAAAAAAA
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Figure 1 Sequence of pig proteinase inhibitor 9 (PI-9). The cDNA of pig PI-9 is shown. The pig PI-9 consists of 1545 bp. The open reading frame for pig PI-9 consists of 1125 bp (375aa).

Discussion

When a Killer cell attacks a target cell, two major lytic pathways are involved. One is a so-called 'contact-dependent pathway' which includes the Fas (CD95)–Fas ligand (FasL) system [20] and the TNF–TNF receptor (TNFR) system [21], and the other is a granule exocytosis pathway, which involves the release of soluble perforin and granzyme B [22]. The cytotoxic granules are released onto the surface of the bound target cell, and the pore-forming protein, perforin penetrates the cell membrane. The serine proteinase, granzyme B then promotes DNA degradation and apoptosis in the intra cellular spaces between the target cells. On the whole, killing is likely to

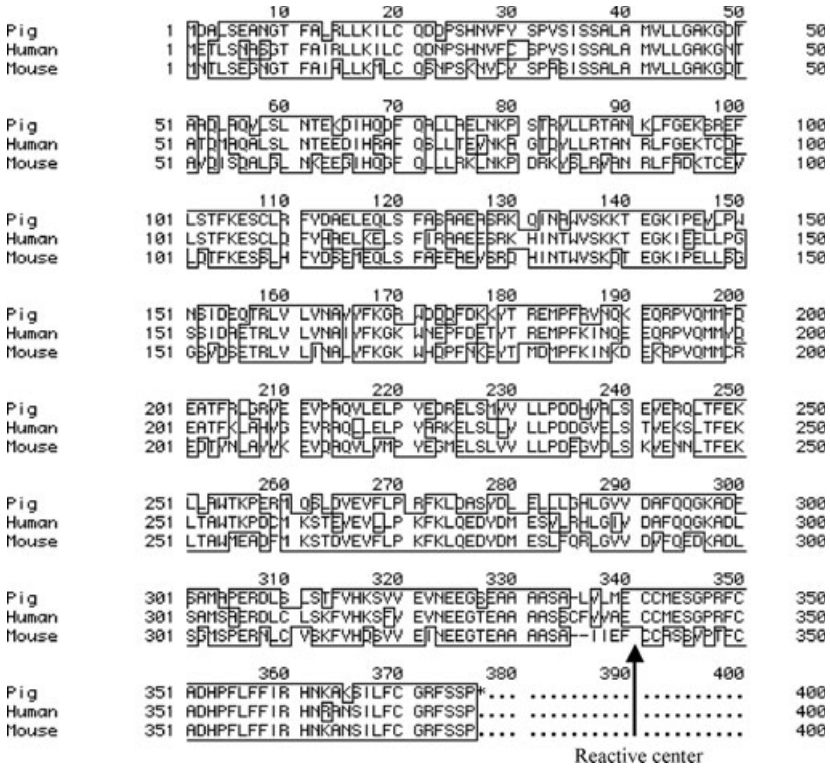

Figure 2 Comparison of amino acid sequences among pig proteinase inhibitor 9 (PI-9), human PI-9 [HSU71364] and mouse PI-9 [BCO29900]. Identical residues are boxed and the vertical arrow indicates the reactive center.

(a)

	Mock	Pig PI-9 #1	Pig PI-9 #2	Pig PI-9 #3
PI-9/GAPDH	0.005	0.156	0.888	1.403

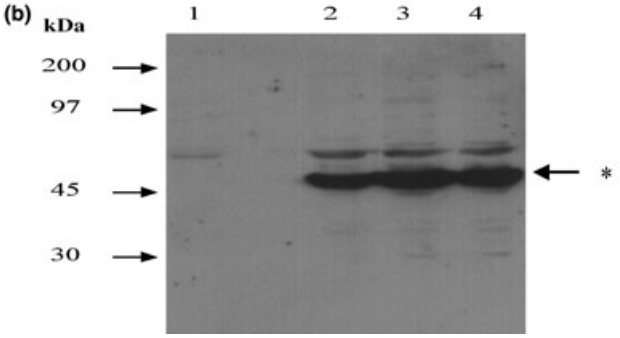

Figure 3 (a) Real time PCR values of the pig endothelial cells (PEC) transfectants with pig proteinase inhibitor 9 (PI-9). Real-time PCR was performed to detect differences in the mRNA of PI-9 in each clone. The amount of mRNA in the transfectants was normalized with the level of GAPDH mRNA. (b) Western blot analysis of transfectants. The cell lysate from transfectants with PI-9 was harvested and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with anti-FLAG mAb. (Non-reduced) Lane 1; cell lysate of mock transfectant. Lane 2; cell lysate of PEC transfectant with PI-9 #1. Lane 3; cell lysate of PEC transfectant with PI-9 #2. Lane 4; cell lysate of PEC transfectant with PI-9 #3. Specific band for pig PI-9 are indicated (*).

involve intracellular proteolytic pathways that require both serine and cysteine proteinases. However, target cells sometimes can be induced to resist killing if they contain synthetic serine or cysteine proteinase inhibitors [23,24].

On the other hand, it is well known that Killer cells are resistant to the effects of their own cytotoxins. During degranulation and target cell killing, intracellular PI-9 protects the killer cell itself from being misdirected into

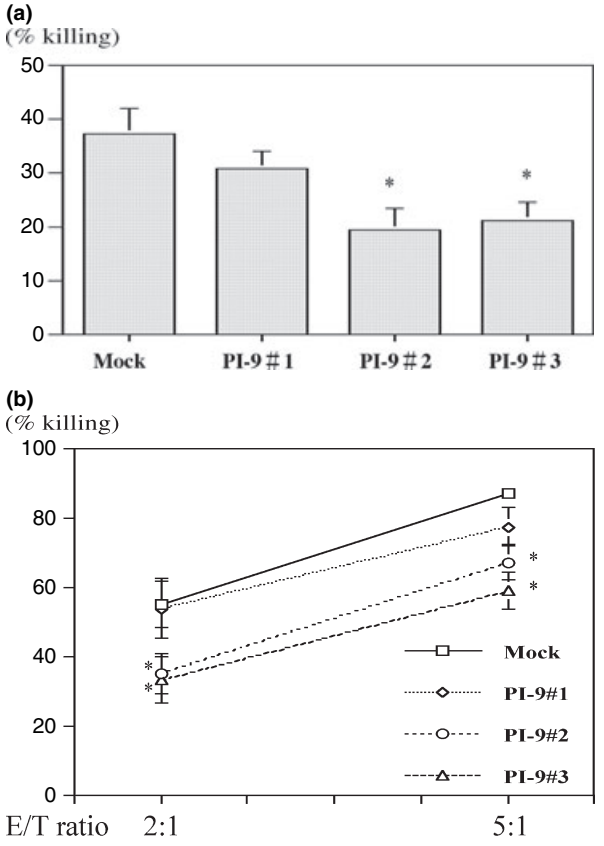

Figure 4 Natural killer (NK) cell-mediated cytotoxicity assay. (a) The cytotoxicity assays of YT cells against the newly cloned pig endothelial cells (PEC) transfectants with a relatively high proteinase inhibitor 9 (PI-9) expression in E:T = 5:1 ($n = 6$) were performed by an lactate dehydrogenase (LDH) assay. (b) The cytotoxicity assays of YT cells with high killing against the PEC transfectants with PI-9 in E/T = 2:1 and 5:1 (n = 7) were performed by an LDH assay. The % Killing of mock and the PEC transfectants is indicated. *indicates a significant difference ($P < 0.05$) compared with the mock transfectant.

the killer cell cytoplasm by inactivating granzyme B. That is, this inhibitor is expressed at high levels when it serves to protect these cells against endogenous and locally released granzyme B [25].

In the present study, protection against granzyme B-induced apoptosis by transfected-PI-9 was assessed using NK cell-dependent PEC lysis as an *in vitro* cellular xenograft rejection model. Pig PI-9 was first cloned. As in human PI-9, pig PI-9 contains an unusual reactive center P1(Glu)-P'(Cys), suggesting that it inhibits serine proteases that cleave peptide chains after acidic residues. Therefore, pig PI-9 can show inhibitory specificity for human granzyme B-like activity [14,15]. The possibility that the volume of pig PI-9 molecules expressed may not be sufficient to protect PECs from the released granzyme B cannot be excluded. However, the rate of inhibition as the result of expressed pig PI-9 was quite significant. Both of the high expression clones, #2 and #3, indicated the approximately 48% and 43% inhibition, respectively. Concerning *in vivo* situation, the data, such as the inhibition rate in this study would not directly reflect the graft survival. However, during the rejection of graft, the first target must be the endothelial cells. In addition, as a first clinical xenotransplantation, we are planning to use pig islet cell, which could closely reflect the *in vitro* data in this study.

In our previous study, transfection with the four glycosiltransferases genes, such as for β1,4-*N*-acetylglucosaminyltransferase III (GnT-III), α2,3-sialyltransferase (α2,3ST), α2,6-sialyltransferase (α2,6ST) and α1,2-fucosyltransferase (α1,2FT), led to a drastic reduction in the YT cell-mediated PEC lysis, approximately 30–70% [11]. On the other hand, the inhibitor effect of the class Ib molecule, HLA-G1 on the YT cell-mediated PEC lysis was approximately 20–30% (E:T ratio = 5 to 6:1) [9,26]. In addition, a complement regulatory protein, DAF (CD55) indicated an approximately 50% reduction of YT cell-mediated PEC lysis (E:T ratio = 5:1) [13]. In this study, compared with these molecules, PI-9 indicated a sufficient reduction on NK cell-mediated PEC lysis.

On the other hand, the Fas–FasL pathway might have a strong effect on NK cell-dependent PEC lysis. However, in general, an apoptotic reaction in the target cell requires a relatively long time for completion. Therefore, the 4-h cytotoxicity assay used in this study may represent the effect more on the perforin-granzyme B pathway than on the Fas–FasL or TNF–TNFR pathways. It is also possible that another possibility, the functional compatibility of pig PI-9 to human Granzyme B might have some relation to the inhibition rate of cytotoxicity. The 74.5% homology in the amino acid sequence between human PI-9 and pig PI-9, especially the similarity of the reactive center, indicates that the species specificity of these two combinations of molecules combination is of lesser importance.

Finally, to produce a transgenic pig producing this anti-apoptotic molecule, several obstacles need to be overcome, one of which is carcinogenesis. For example, an anti-apoptotic molecule, plasminogen activator inhibitor type 2 is related to papilloma formation in transgenic mice [27]. However, the findings herein do not indicate tumor formation in the transgenic pig with PI-9. Additional studies of the combined effects of PI-9 with class Ib molecules, glycosiltransferases [4,12] and DAF [2] will be needed for the clinical use of this technique in pig grafts.

In conclusion, the anti-apoptotic molecule, pig PI-9 was cloned, and the sequences were identified. The overexpression of pig PI-9 has the potential for use in protecting graft cells against human NK cells in preventing xenograft rejection. This paper proposes a new approach toward the inhibition of Killer cells, involving NK cells and CTL, in the xenotransplantation field.

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