附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※　**異種器官供人體移植之可行性研究-使用多基因轉殖小鼠與豬器**　※

※　**：HO-1/Hdaf/HLA-DR 多基因轉植豬細胞體外之功能測試**　※

※　**In Vitro Test for the Multiple Transgenic Pig Cell**　※

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計畫主持人：李章銘

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計畫參與人員：黃淑靖

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

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計畫名稱:異種器官供人體移植之可行性研究-使用多基因轉殖小鼠與豬器官
：HO-1/Hdaf/HLA-DR 多基因轉植豬細胞體外之功能測試
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中文摘要:

關鍵詞：異種移植（Xemo transplantation）、HLA、豬（Pig）
以豬器官來源的異種器官，為目前臨床移植所面臨的器官短缺的問題，提供了解決之道。但是其可行性，目前仍受限於人類對豬隻的異種抗體所誘發的超急性，及亞急性異種器官排斥，及物種間組織抗原歧異所引起的 T 細胞排斥。先前在我們所產製的 HLA DPW0401 基因轉殖豬中，發現人類 HLA DP 的抗原性，可完整表達在豬細胞表面，而相較於其他非基因轉殖同胞豬，HLA DPW0401 基因轉殖豬所引起人類周邊單核球(具 HLA DPW0401 基因型者)的增生反應，有減少的趨勢。本研究的目的在觀察 HLA DQW0601 的基因轉植豬是否可產生類似的作用。我們以人類與豬細胞的直接混合淋巴球培養，觀察人類周邊單核球對豬隻細胞的細胞增生反應是否因 HLA DQW0601 的基因轉殖而得以減緩。此外我們也利用培養劑中 INFr 的產生，而測定人類周邊單核球對豬細胞的 TH1 反應。結果，與控制組非基因轉殖豬細胞相比 HLA DQW0601 基因轉殖豬可減緩人類周邊單核球對豬細胞的異種細胞增生反應。而異種的 TH1 反應，在 HLA DQW0601 也有相同減少的趨勢。這種差異存在於 HLA DQW0601（-）與 HLA DQW0601（+）的人類細胞中。我們的實驗進一步地支持了增加 MHC II 的相似性，可減少人類細胞對豬隻細胞的異種反應。

Abstract

Pig to human xenotransplatation is a promising strategy to overcome the organ shortage in clinical transplantation. However, the application is hampered by the xenoreactive antibody mediated hyperacute rejection, delayed xenograft rejection and MHC disparity associated T cell rejection. Previous, we have produced the HLA DPW0401 transgenic pig, which was shown equipped with the human HLA antigenecity and, as compared to the non-transgenic litermate pig, induced a minor cellular response to the human PBMCs that came from the HLA DPW 0401(+) human. In this study, we tried to evaluate whether there is similar effect on human to pig xenogenic cellular response for the cells from transgenic pig of HLA DQw 0601. We used direct xenogenic mixed lymphocyte culture to see whether the human –to-pig xenogenic cellular response is attenuated by the trasngenosis of HLA DQ0601 on the porcine cells. The human anti-porcine Th2 response was evaluated by the production INF γ in the culture media. **Results**：The cellular proliferation of human PBMC under the stimulation of porcine PBMC was reduced by the presence of HLA DQ molecules on the porcine cells as compared to that of the non-transgenic littermate control. The human to porcine xenogenic Th1 response, as represented by the production of INF γ, was also attenuated in the HLADQ transgenic pig cells. These phenomenons can be demonstrated in the human PBMCs with or without the HLADQw0601 allele. This study on the HLA DQw0601 transgenic pig further support the concept that increasing the similarity of MHCII between pig and human in the HLA transgenic pig can help to reduce the xenogenic cellular response between human and pig.

Text

Xenotransplantation with graft organ from pig is a promising strategy to overcome the shortage

of organ, which has been the main restriction for clinical transplantation (1,2). The xenoreactive antibody-mediated hyperacute rejection (HAR) and delayed xenograft rejection (DXR) (or acute vascular rejection) are the major obstacles to impede the survival of xenofgraft organ in human (3-7). Various strategies have been developed to suppress the HAR or DXR successfully, including using organs from transgenic pigs of human Decay Accelerating Factor (8,9), depletion, or suppression of production of xenoreactive antibodies (10-14). The cellular response induced by differences of MHC between disconcordant species can provide a continuous detrimental effect to graft organ, even though the xenograft has gone through the stages of HAR or acute vascular rejection. It has been demonstrated that the porcine MHC molecules can effectively induce a strong human T cell response, through direct or indirect antigen recognition (15-17). The human NK cell associated anti-porcine cytotoxicity was also maintained, which was T cell and antibody-independent (18). Previously, we has produced the HLA DPw0401 transgenic pig and found that the expression of the DPw0401 exogenes on pig can help attenuate the human-to-pig xenogenic cellular response (18). In this study, we tried to evaluate whether there is similar effect on human to pig xenogenic cellular response for the cells from transgenic pig of HLA DQw 0601.

Materials and Methods

Prodcuction of HLA DQ0601 transgenic pig

Using the Yorkshire inbred strain, the HLA DP transgenic pig was produced with the technique of microinjection (with PAKQ 056 for DQB1 0601 gene and 4116 for DQA1 0103 gene).

Mixed lymphocyte culture;

1. Dilution the heparinized human blood with double volume of HBSS
2. Overlayer the above mentioned mixture with 10ml Ficoll-paque
3. Centrifuge with 1500 rpm for 30 minutes
4. Harvest the mononuclear cell layer in the interface
5. Wash with HBSS for three times (PBMC)
6. Incubate the PBMC for 4 hours at 37^{o} C in 75-cm^{2} plastic flask (Falcon #3023, Becton Dickinson Labware, Lincoln Park, NJ) to deplete the adherent cells.
7. Recover the adherent cells with rubber policemen and analysis with flowcytometry
8. Incubate non-adherent cells for 60 min at 37o C on nylon wool (Fenwal Laboratories, Deerfield, IL) and then gently elute enrich the T cells
9. Treat the T cells with 1:100 dilution of the ascitic fluid of a murine anti-porcine macrophage- and granulocyte-specific antibody for at 4^{o}C for 30 min.
10. Add rabbit complement 9HLA-ABC, Pel Freeze, Roger, AR) at 1:8 dilution and incubate for 45 min at 37^{o}C
11. Wash the cells with culture media
12. Prepare the responder and stimulator cells
    a) responder cells adjust the concentration to $1x10^{6}$ cells/ml
    b) stimulator cell:(PBMC) adjust the concentration to $2x10^{6}$ cells/ml
    c) Irradiate the stimulator cells with 4500 cGy and incubate in 37^{o} C, 5% CO_2 for 20 minutes.
    d)Wash the stimulator cells with HBSS for three times

e)Adjust the concentration of stimulator cells to $1x10^6$ cells/ml

13. Add into each well with 100 μl of stimulator and responder cells respectively and done in triplicate
14. Incubate in 37^o C and 5% CO_2 for 6 days
15. Add 20 μl H^3 thymidine incubating for 6 hours.
16. Harvest with glass fiber and dry in air
17. Add scintillating cocktail
18. Count the cpm with β-counter.

Xenogenic MLC

Direct xenogenic MLC (mixed lymphocyte culture) was performed with responder-PBMCs from HLA DQw0601 (+) and HLA DQw0601 (-) humans respectively, which was stimulated with mitomycin (0.25 mg/mL) treated PBMCs of transgenic pig (TG) and non-transgenic littermate pig (NTG). The PBMCs from human responder himself and humans of HLA DQw0601 (+), HLA DQw0601 (-) genotypes, were also used as stimulators.

Analysis of TH1 response

The Th1 response in the xenogenic MLC was evaluated by the production of INF γ in the culture media. The media was harvested after 3 days of co-culture of stimulators and responders in the xeogenic MLC. INF γ was measured in duplicate with commercially available ELISA kits (Qantikine; R&D systems, Minneapolis , MN, USA).

Data analysis and statistics

The SI (stimulating index) was used with the following definitions:
the cpm of (^{3}H) thymidine uptake with various stimulators/ cpm of (^{3}H) thymidine uptake with stimulator of TG
Mann-Whitney test was used to test the significance of difference between independent groups. The data were presented with mean + standard error

Results and discussion

After 7 days of stimulation, the human responders without the HLA DQw0601 allele, have SIs of 1.37 (+0.53), 1.85 (+0.19) and 1.76 (+0.14) under stimulated by PBMCs from non-transgenic littermate control pig (NTG), normal pig (NP) and third party human control respectively (H) (HLADQ0601+). ($p<0.05$ for TG vs. NP and TG vs. H). (Fig 1) With the human PBMCs responders with the HLA DQw0601 allele, the SIs were 1.35 (+0.12), 1.42 (+0.09) and 1.10 (+0.16) under stimulated by PBMCs from non-transgenic littermate control pig (NTG), normal pig (NP) and third party human control respectively(HLADQ0601-) (H). ($p<0.05$ for TG vs. NP and TG vs. NTG). (Fig 2). Under three days of stimulation, the human responder PBMCs without the human DQw0601 allele produced a higher level of INF γ when the stimulators came from the PBMCs of normal control pig, as compared to that of the transgenic pigs (NP vs. TG: 55 +3.75 vs. 24 + 7.92 pg/ml).(Fig 3) Similar trend was found when the responders PBMCs came from the human DQw0601+ genotype (NP vs. TG : 153 + 21.2 vs. 69 + 0 pg/ml) . (Fig 4)

The individual difference in the HLA phenotypes is the major triggering factor for T-cell mediated rejection in tissue transplantation. The HLA II include three subsets of polymorphic molecules, DP, DQ, and DR, which are mainly present in the antigen presenting cells and interact with CD4+ T-cell receptors (19). In this T-cell associated rejection, the Th1 cells in the CD4+ T population can secret cytokines of IL2, INF γ or GM-CSF etc, and promote allograft rejection (20).

The cellular proliferation of human PBMC under the stimulation of porcine PBMC was reduced by the presence of HLA DQ molecules on the porcine cells as compared to that of the non-transgenic littermate control. The human to porcine xenogenic Th1 response, as represented by the production of INF γ, was also attenuated in the HLADQ transgenic pig cells. These phenomenons can be demonstrated in the human PBMCs with or without the HLADQw0601 allele. Compatible to our previous findings on HLADPw0401 transgenic pig (3), this study on the HLA DQw0601 transgenic pig further support the concept that increasing the similarity of MHCII between pig and human in the HLA transgenic pig can help to reduce the xenogenic cellular response between human and pig. Expressing the HLA II on the porcine cells might provide a promising strategy to make human better tolerate the porcine tissue in xenotransplantation. This preliminary result requires to be confirmed further by larger series of study.

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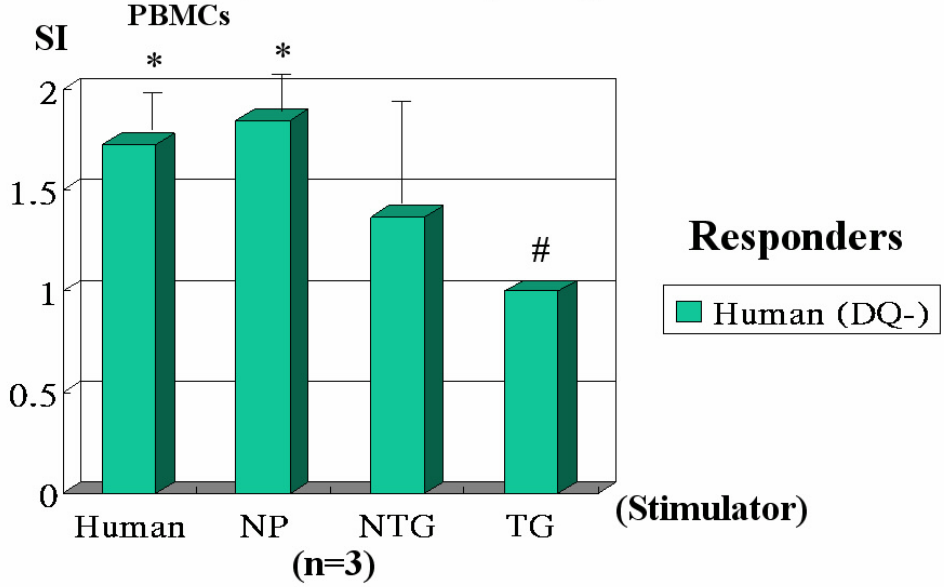
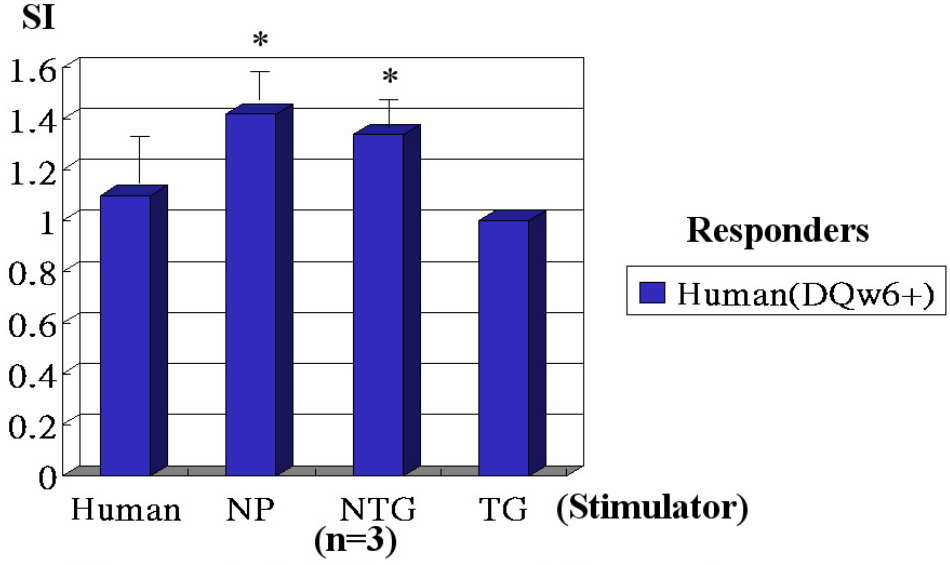
Figure 1: Stimulating indices of direct xenogenic MLC with responder of HLA DQw0601(-) human PBMCs



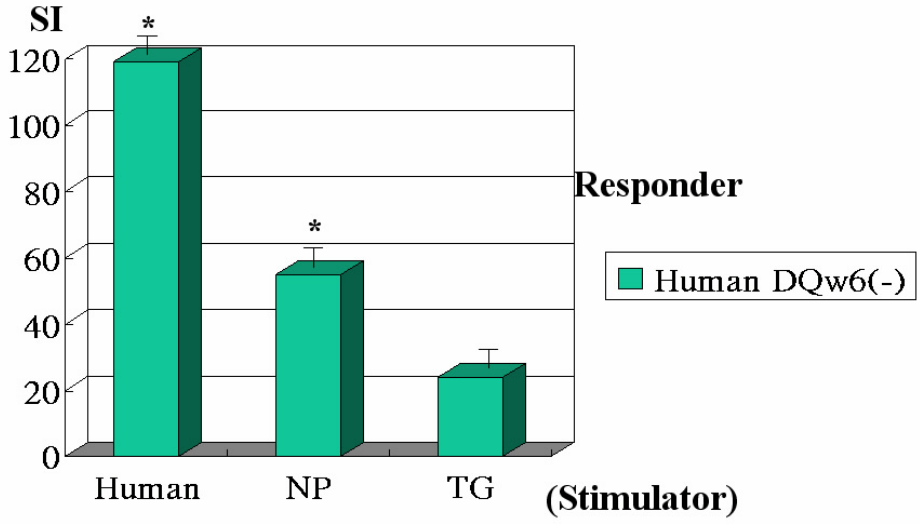
TG: transgenic pig; NTG: non-transgenic littermate pig
NP: normal pig *: p<.05 as compared to TG (SI=1)
#: using the cpm of TG as referent (SI=1)

Figure 2: Stimulating indices of direct xenogenic MLC with responder of HLA DQw0601(+) human PBMCs



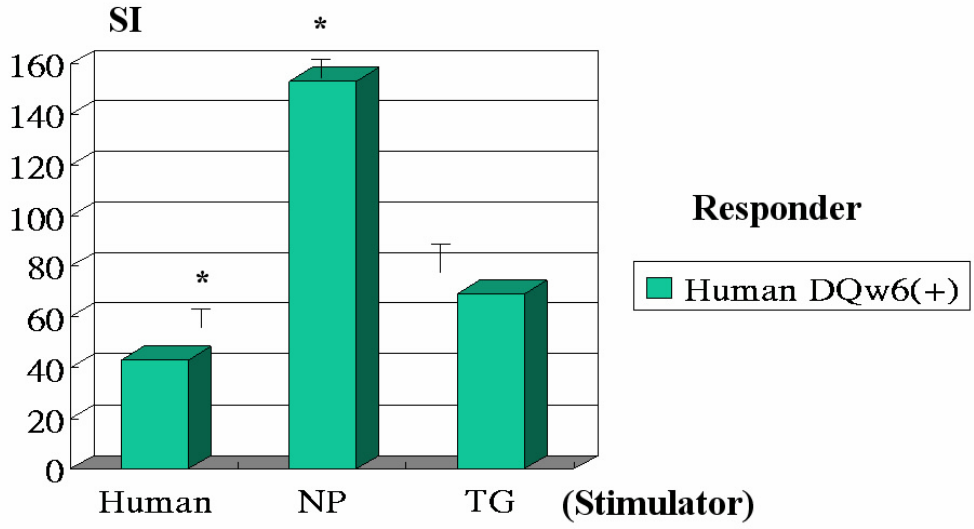
TG: transgenic pig; NTG: non-transgenic littermate pig
NP: normal pig *: p<.05 as compared to TG (SI=1)
#: using the value of TG as referent (SI=1)

Figure 3: TH1 response (INF γ production) in direct xenogenic MLC with responder of HLA DQw0601(-) human PBMCs after 3 days of stimulation



TG: transgenic pig; NP: normal control pig *: p<.05 as compared to TG

Figure 4: TH1 response (INF γ production) in direct xenogenic MLC with responder of HLA DQw0601(+) human PBMCs after 3 days of stimulation



TG: transgenic pig; NP: normal control pig *: p<.05 as compared to TG