RESEARCH NOTES

Identification of the candidate genes associated with cellular rejection in pig-to-human xenotransplantation*

HU Weimin, CHENG Jingqiu, LI Youping**, LI Shengfu, LU Xiaofeng and WAN Lin

(Laboratory of Transplant Engineering and Immunology, West China Hospital, Sichuan University, Chengdu 610041, China)

Received March 4, 2002; revised April 8, 2002

Abstract To identify the genes associated with cellular rejection in pig-to-human xenotransplantation, the suppression subtractive hybridization (SSH) was used in screening the up-regulated genes from a co-culture of human peripheral blood mononuclear cells (PBMCs) and porcine vascular endothelial cell line PIEC. The up-regulated cDNAs were cloned into pGEM-T Easy vector and then sequenced. Nucleic acid homology searches were performed using the BLAST program. A subtracted cDNA library including about 300 clones with the expected up-regulated genes was obtained. Twenty-four of these clones were analyzed by sequencing and homology comparison was made. These clones represent the genes of human perforin (PRF1), proteasome, lymphocyte specific interferon regulatory factor/interferon regulatory factor 4 (LSIRF/IRF 4), muscleblind-like (MBNL) protein and a porcine expressed sequence tag (EST) which has 81% homology with human oxidative-stress responsive 1 (OSR 1). These genes might be the candidate genes which are associated with cellular rejection in pig-to-human xenotransplantation.

Keywords: xenotransplantation, rejection, endothelial cell, suppression subtractive hybridization.

Xenotransplantation is developed for overcoming the severe shortage of human organ donors. Most researches in the area have focused on the humoral xenogenetic response of hyperacute rejection, the most immediate impediment to successful pig-to-human xenotransplantation. However, even hyperacute rejection is controlled or prevented, the subsequent cell-mediated response to porcine antigens must still be combated. This cellular response is one component of discordant xenograft rejection that is receiving increased attention[1,2].

The endothelial cells (ECs) are antigen-presenting cells (APCs) that line on the inner side of blood vessels of solid organ grafts and are the first foreign cells to come into contact with circulating host immune cells. Thus, the endothelium is both a major stimulator and a major target of human immune responses to solid organ grafts. Endothelial cells are activated to express adhesion molecules and to secrete cytokines during acute and delayed xenograft rejection[3]. Specific cytokines, receptors, and adhesion molecules appear to cross the xenograft barrier and play a critical role in T cell-endothelial cell interactions. Such interactions are likely to affect EC activation and immune responses to porcine xenografts *in vivo*[1]. Therefore, it is important to further understanding of the interaction between porcine ECs and human peripheral blood mononuclear cells (PBMC).

The objective of this study is to use a human anti-porcine xenogeneic mixed lymphocyte endothelial cell culture (XMLEC) and the suppression subtractive hybridization (SSH) method to investigate up-regulated genes in lymphocytes and ECs after XMLEC, which should help us to reveal the molecular events related to xenograft rejection and survival.

1 Materials and methods

The porcine vascular endothelial cell line PIEC[4] was a gift of Dr. Zhou Guangyan at Shanghai Institute of Immunology, China. Human PBMC were isolated by the routine method. The 1×10^6 PIECs were co-cultured with 5×10^6 PBMCs in RPMI 1640 with 10% fetal calf serum (FCS) for 18 h and the numbers of PIEC and PBMC were counted respectively. The PIECs and PBMCs were mixed again at certain num-

* Supported by the Major Program of National Natural Science Foundation of China (Grant No. 39993430)

** To whom correspondence should be addressed. E-mail: yzmy@www.mcwcums.com

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

bers and total RNA was extracted as the tester of SSH. The driver of SSH was a mixture of total RNA from un-co-cultured PIECs and PBMCs with the same numbers.

Total RNA of tester and driver were extracted and double-stranded cDNA was synthesized. To obtain shorter blunt-ended molecules the cDNA was digested with *Rsa* I at 37℃ for 3 h. The product of co-culture was used as tester cDNA, and the un-co-cultured as driver cDNA.

SSH was performed according to the PCR-Select™ cDNA subtraction kit (Clontech). The PCR-amplified cDNAs obtained from SSH were cloned with pGEM-T Easy Vector System (Promega). Plasmids with inserts were sequenced by an ABI PRISM™ 377XL DNA sequencer. Nucleic acid homology searches were performed using the BLAST program.

2 Results and discussion

After XMLEC for 18 h, the cell number kept unchanged, PIEC was 1×10^6 and PBMC was 5.2×10^6. After two hybridizations and two PCR amplifications, the subtracted tester showed a number of distinct bands, and the unsubtracted tester control demonstrated a smear without distinct bands (Fig. 1). The distinct bands are differentially expressed sequences.

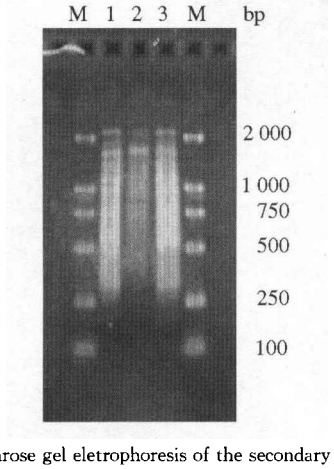

Fig. 1. Agarose gel eletrophoresis of the secondary PCR products of subtracted and unsubtracted tester. M, DNA size marker; 1, subtracted tester after purification; 2, unsubtracted tester; 3, subtracted tester before purification.

By means of SSH and cloning, we obtained a subtracted cDNA library including about 300 clones. Twenty-four clones were sequenced and analyzed for homology in the GenBank. These clones represent 24 genes, and a summary of these data is shown in Table 1. Four out of them are known human genes, eight are known porcine genes (ESTs), nine have homology with known human genes, and three have no match in the database. Several known genes are related to the activation of lymphocyte and EC and graft rejection, such as perforin (PRF1)[5], proteasome (26S subunit)[6], lymphocyte specific interferon regulatory factor/interferon regulatory factor 4 (LSIRF/IRF 4)[7] and porcine MHC class I[1]. We also found a porcine EST which had 81% homology with human oxidative-stress responsive 1 (OSR1), and

Table 1. Up-regulated candidate genes in XMLEC

Clone	Length (bp)	Homogenecity (%)	Homogeneous sequence
X1	482	85	Human DNA sequence from clone RP11-449I6 on chromosome 6
X2	189		No homology with known genes
X3	354	100	MARC 1PIG Sus scrofa cDNA 5′ end
		81	Homo sapiens, oxidative-stress responsive 1
X4	567		No homology with known genes
X5	507	99	32027 MARC 2 PIG Sus scrofa cDNA 5′ end
X6	429	94	Human DNA sequence from clone RP3-434P1 on chromosome 22, contains KCNJ4, KDELR3 genes
X7	478	98	Homo sapiens mRNA for muscleblind-like (MBNL) protein
X8	435	100	UNL-P-FN-cf-g-04-0-UNL. s1 UNL-P-FN Sus scrofa cDNA clone
		91	Homo sapiens mRNA; cDNA DKFZp586N012
X9	370	95	Homo sapiens muscleblind-like (*Drosophila*) (MBNL)
		97	Sus scrofa cDNA clone
X10	593		No homology with known genes
X11	643	99	MI-P-AY1-nqz-b-11-0-UI. s1 MI-P-AY1 Sus scrofa cDNA clone
X12	486	98	Homo sapiens perforin (PRF1) mRNA 3′ end
X13	846	92	Homo sapiens muscleblind-like (*Drosophila*) (MBNL)
X14	1448	86	Human DNA sequence from clone CTD-2530H13 on chromosome X
		99	373112 MARC 2PIG Sus scrofa cDNA 5′ end
X15	547	97	Homo sapiens chromosome 19 complete sequence
X16	451	86	Homo sapiens mRNA KIAA1707 protein
X17	338	97	Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase
X18	1002	83	Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 4 (putative function) (ENPP4)
X19	554	88	Human DNA sequence from clone 593C16 on chromosome 1q24.1-25.2, contains a gene for ras GTPase activating protein, and a CpG island
X20	682	99	54567 MARC 1PIG Sus scrofa cDNA 5′ end
X21	911	84	Sus scrofa MHC class I SLA genomic region, haplotype H01
X22	592	97	Human lymphocyte specific interferon regulatory factor/interferon regulatory factor 4 (LSIRF/IRF4) mRNA
X23	662	89	Homo sapiens KIAA0970 protein (KIAA0970)
X24	1438	85	Human DNA sequence from clone CTD-2530H13 on chromosome X
		99	373112 MARC 2PIG Sus scrofa cDNA 5′ end

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net

oxidative stress is being considered to be associated with the graft rejection and ischemia / reperfusion (I/R)[3]. Another interesting finding is muscleblind-like (MBNL) gene, which is related to myotonic dystrophy and has also a high expression in myeloma cells and hematopoietic stem cells[8]. It may play an important role in xenograft rejection.

In summary, our data reveal that SSH is a powerful technique for the detection of differential gene expression in xenograft rejection. The up-regulated genes may play a critical role in xenograft rejection. They may become the target genes for inhibiting or preventing xenograft rejection. The further studies are undergoing, which includes: (1) using semi-quantitative RT-PCR analysis, Northern blot and *in situ* hybridization (ISH) to further identify these up-regulated genes; (2) obtaining full cDNAs with 5′-RACE and 3′-RACE to investigate the role of these molecules in xenograft rejection; and (3) sequencing and identifying other clones. All of these will help us to understand more about xenograft rejection.

References

1 Coleman, T. S. et al. Human T-cell-porcine endothelial cell interactions induce human Th1 cytokines and porcine activation markers. J. Surg. Res., 2001, 97(2): 184.
2 Dorling, A. et al. Xenotransplantation: Immune barriers beyond hyperacute rejection. Clin. Sci., 1997, 93(6): 493.
3 Tsuyuki, S. et al. Effect of redox modulation on xenogeneic target cells: The combination of nitric oxide and thiol deprivation protects porcine endothelial cells from lysis by IL-2-activated human NK cells. J. Immunol., 2001, 166(6): 4106.
4 Yun, S. et al. The induction of major histocompatibility complex class II expression is sufficient for the direct activation of human CD4+ T cells by porcine vascular endothelial cells. Transplantation, 2000, 69(5): 940.
5 Shulzhenko, N. et al. Intragraft activation of genes encoding cytotoxic T lymphocyte effector molecules precedes the histological evidence of rejection in human cardiac transplantation. Transplantation, 2001, 72(10): 1705.
6 Luo, H. et al. A proteasome inhibitor effectively prevents mouse heart allograft rejection. Transplantation, 2001, 72(2): 196.
7 Grumont, R. J. et al. Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: Modulation of interferon-regulated gene expression by Rel/nuclear factor B. J. Exp. Med., 2000, 191(8): 1281.
8 Phillips, R. L. et al. The genetic program of hematopoietic stem cells. Science, 2000, 288(5471): 1635.

?1994-2018 China Academic Journal Electronic Publishing House. All rights reserved. http://www.cnki.net