NOTES

Adhesion of subsets of human blood mononuclear cells to porcine endothelial cells

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Abstract Cellular immune response is a major barrier to xenotransplantation, and cell adhesion is the first step in intercellular recognition. Flow-cytometric adhesion assay has been used to investigate the differential adhesions of monocyte (Mo), natural killer cell (NK) and T lymphocyte (T) present within human peripheral blood mononuclear cells (PBMC) to porcine aortic endothelial cells (PAEC), and to demonstrate the effect of human interferon-γ (hIFN- γ) or/and tumor necrosis factor-α (hTNF-α) pretreatment of PAEC on their adhesiveness for different PBMC subsets. The preferential sequence for PBMC subset binding to resting PAEC is Mo, NK and T cells, among which T cells show the slightest adherence; hTNF- α can act across the species, and augment Mo, NK and T cell adhesion ratios by 40%, 110% and 3 times, respectively. These results confirm at the cell level that host Mo and NK cells are major participants in the cellular xenograft rejection, thereby, providing a prerequisite for further studying the human Mo/NK-PAEC interactive mechanisms.

Keywords: human peripheral blood mononuclear cell (PBMC), porcine aortic endothelial cell (PAEC), human tumor necrosis factor-α (hTNF-α), cell adhesion, phenotypic analysis.

As an attractive research in the biomedical field, xenotransplantation may be the ultimate solution to the organ shortage problem. Pigs are suitable for choice because their organs have similar anatomical physiology to human organs. After transplantation, an untreated recipient rejects the immediately-vascularized xenograft, called hyperacute rejection (HAR), which results from the presence of the xenoreactive natural antibodies (XNAs) in recipients[1]. Although this humoral immunity could be averted by using transgenic pigs and removing XNAs in potential recipients, xenografts will be rejected in days by a cell-mediated process referred to as delayed xenograft rejection (DXR)[2]. Currently, the nature of the cellular immune response is known little.

Endothelium is a natural barrier between blood and tissue, and plays an important role in physiologic and pathologic processes. Adhesion of leukocytes to endothelial cells (EC) is vital to both afferent and efferent pathways in the cellular immune system. In the note, the adhesive interaction between human peripheral blood mononuclear cells (PBMC) and porcine aortic endothelial cells (PAEC) was measured using a flow cytometric assay, and the adhesive characteristics of monocyte (Mo), natural killer cell (NK) and T lymphocyte (T) within PBMC were compared. Furthermore, human proinflammatory cytokines, such as tumor necrosis factor- α (hTNF- α) and interferon-γ (hIFN-γ), were used to pretreat PAEC, and their effects on the adhesive interaction between the effector and target cells were investigated.

1 Materials and methods

(i) Reagent. PAEC medium: M199 (Gibco), 15% heat-inactivated fetal calf serum (FCS), 0.01 mmol/L thymidine and 30 μg/mL endothelial cell growth factor (ECGF, Boehringer Mannheim). PBMC medium: RPMI 1640 (Gibco) and 10% FCS, which was used for adhesion culture. Cytokines: recombinant human interferon-γ (rhIFN- γ, Boehringer Mannheim); recombinant human tumor necrosis factor-α (rhTNF-α, R&D systems). Fluorochrome-labeled monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-CD45 Mab (FITC-CD45, Pharmingen); phycoerythrin-conjugated anti-CD3/CD14/CD56 Mabs (PE-CD3, PE-CD14, PE-CD56, Pharmingen).

(ii) Endothelial cell isolation and culture. Porcine aortic endothelial cells were isolated after collagenase digestion according to the method described in ref. [3]. Briefly, sterile pig aorta was washed with phosphate buffer solution (PBS), and then treated with 0.1% collagenase (Gibco) at 37℃ for 10 min. The resulting PAECs were grown in 1% gelatin-coated tissue culture flasks, and used

between the fifth and ninth passages. The purity of PAEC assessed by Factor Ⅷ related antigen staining (ZYMED) was 85%—95%.

(iii) PBMC isolation. PBMCs were isolated from concentrated blood (purchased from Beijing Blood Center), which consists mainly of leukocytes. After proper dilution in PBS containing 5% FCS and 2 mmol/L ethylenediaminetetraacetic acid (EDTA), the blood was separated using Ficoll-Hypaque gradient centrifugation. The interfaces, containing mononuclear cells, were collected, washed twice with the above dilute solution, and finally resuspended in culture medium. Viability of the isolated PBMC always exceeded 95% as detected by trypan blue exclusion.

(iv) Flow-cytometric adhesion assay. The adhesion of PBMC to PAEC was assayed by flow cytometry[4]. PAECs were seeded on 24-well tissue culture plates precoated with 1% gelatin at 1×10^5 per well, and grown to confluence. Some monolayers of PAEC were incubated with rhIFN-γ (500 U/mL), rhTNF-α (2.5—15 ng/mL) or a combination of both cytokines for 24 h.

5×10^5 of PBMCs were added to each well after the monolayer was washed twice with PBS. If not stated otherwise, the plates were incubated for 1 h at 37℃ to allow adherence. Monolayers of PAEC were then rinsed 3 times with warm PBS to remove non-adherent PBMC. The remaining cells were detached from plastic and dissociated to single-cell suspension using 0.25% trypsin-0.02% EDTA.

Aliquots of cell mixes, consisting of adherent PBMC as well as PAEC, were stained at 4℃ with FITC-CD45 for 20 min. To analyze the phenotype of the adherent PBMC subsets, cells were subjected to double staining with the respective antibodies listed in table 1. Moreover, preparations of the PBMC used in the experiments, but not incubated with PAEC, were also treated with the Mabs specified in table 1 for comparisons with the bound PBMC.

Table 1 Phenotype analysis of PBMC subsets

Preparation	1	2	3
Staining mode	PE-CD14,FITC-CD45	PE-CD56,FITC-CD45	PE-CD3,FITC-CD45
Phenotype	Mo	NK	T

At least 1×10^4 cells per reading were measured by FACS Calibur (Becton-Dickinson). Data analysis was performed by Cell Quest software (Becton-Dickinson). Adhesion assay data were expressed as the ratio of the number of adherent cells to that of PAEC. The student's t test was used for statistical analysis. P values above 0.05 were considered not significant.

2 Results

(i) Quantification of adhesion of PBMC subsets. Based on cellular granularity (sideward scatter, SSc) and relative fluorescence intensity, FITC-CD45-stained adherent PBMC could be easily distinguished from the unstained PAEC. Fig. 1(a) illustrates a dot plot for flow cytometric analysis of adherent PBMC and PAEC after coculturing for 1 h, the PBMC adhesion ratio to PAEC was 0.60±0.15 (n=4, in triplicate) derived from the ratio of events in region 2 (R2) to that in region 1 (R1). Phenotypic analysis of the adherent PBMC showed that within PBMC both Mo and NK could adhere to PAEC (fig. 1 (b), (c)); however, T cells exhibited a slightest binding to PAEC (fig. 1(d)). So their adhesion ratios to PAEC were (0.31±0.05) (Mo), (0.23±0.07) (NK) and (0.02±0.01) (T), respectively (n=4, in triplicate. P>0.05, Mo vs. NK; P<0.01, T vs. Mo/NK).

As the abundance of each subpopulation in separated PBMC was different from one another, their adhesion ratio could not indicate their binding preference. Therefore, a comparison was made between the proportion of each subset in the bound PBMC and that in the original PBMC, and a high ratio value denoted the preferential binding of a specific subset. Fig. 2 shows the differential binding of PBMC subsets to PAEC; thus their preferential adhesion sequence was Mo>NK >>T.

(ii) Time course of adhesion. In order to investigate the kinetics of adhesion of a specific PBMC subset to PAEC, PBMC and PAEC were co-incubated for different time course ranging from 15 to 120 min. Within PBMC, NK and T cell adhered to PAEC quickly, and their adhesion ratios arrived at maximal values after 30 min. In contrast, Mo required at least 60 min to reach the plateau. Once these subsets came to optimal attachment, they maintained maximal adhesion up to 120 min (table 2).

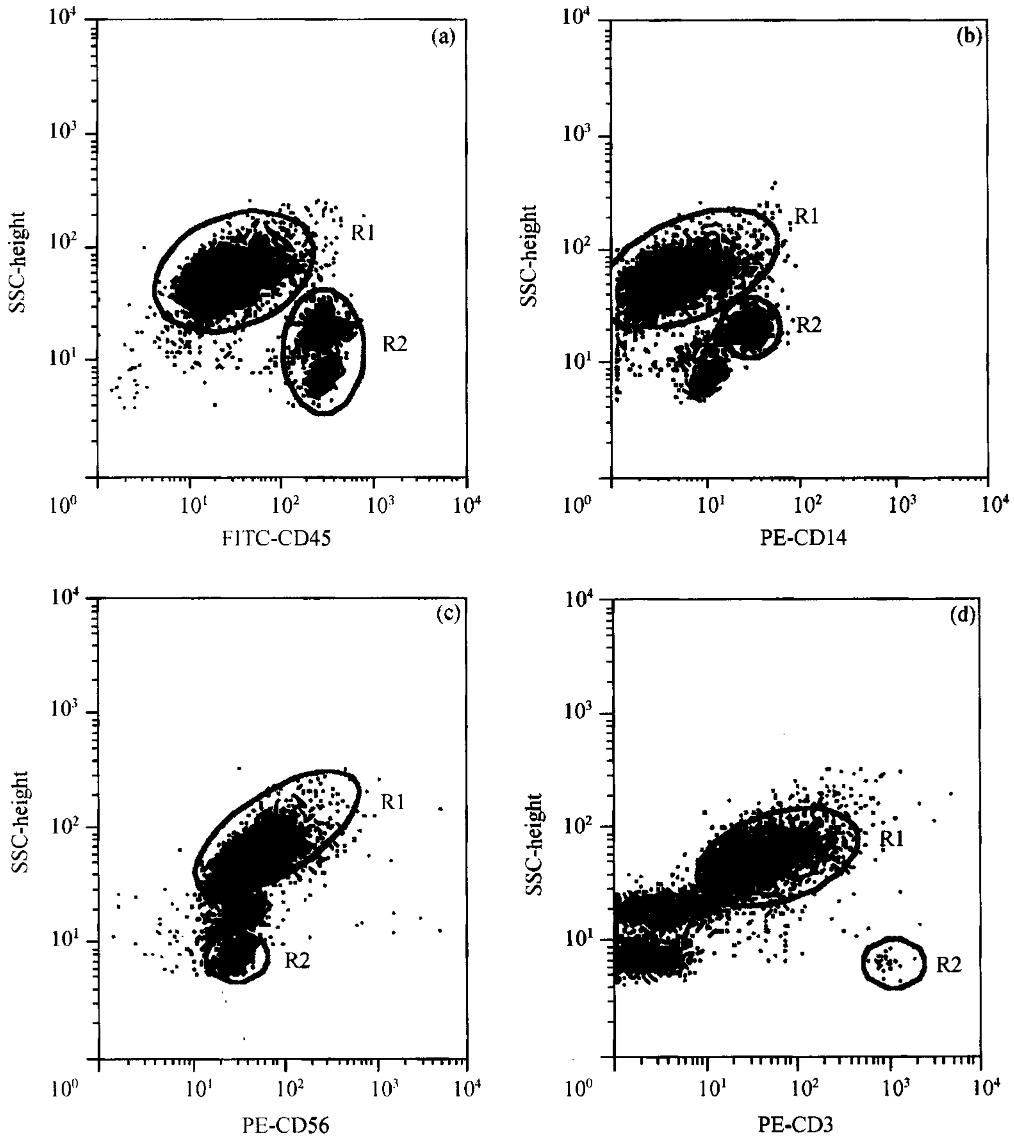

Fig. 1. Flow-cytometric adhesion assay dot plot. (a) PAEC and adherent PBMC distribution; (b) PAEC and adherent Mo distribution; (c) PAEC and adherent NK distribution; (d) PAEC and adherent T cell distribution, among which, (b), (c) or (d) was derived from gating PAEC and CD45$^+$ (adherent PBMC) populations in the corresponding SSc—FITC-CD45 dot plot. R1, Unstained PAEC; R2, FITC-CD45-stained PBMC(a), PE-CD14/FITC-CD45-stained Mo(b), PE-CD56/FITC-CD45-stained NK(c) and PE-CD3/FITC- CD45-stained T cell(d).

Table 2 Adhesion kinetics of PBMC subsets

Co-culture course/min	15	30	60	90	120
Mo adhesion ratio	0.15 ± 0.02	0.24 ± 0.04	0.34 ± 0.04	0.33 ± 0.06	0.36 ± 0.05
NK adhesion ratio	0.11 ± 0.03	0.21 ± 0.05	0.21 ± 0.04	0.20 ± 0.04	0.19 ± 0.05
T adhesion ratio	0	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.00

Representative result for 1 of 3 independent experiments done in triplicate. Values are given as means ± S.D.

(iii) Adhesion under cytokine-stimulated conditions. After preincubation of PAEC with rhIFN-γ (500 U/mL), rhTNF-α (10 ng/mL) or a combination of both cytokines for 24 h, the adhesion ratios of PBMC to each differently treated PAEC were (0.56 ± 0.11) (control, n=3, in triplicate), (1.13 ± 0.16) (rhTNF-α-stimulated PAEC, P<0.01 as compared with control, n=3, in triplicate), (0.61± 0.14)

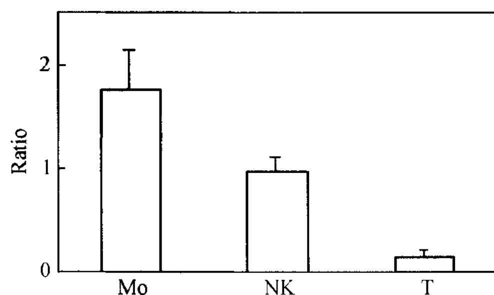

Fig. 2. Differential binding of PBMC subsets to PAEC. Ratio denotes the proportion of a subset in the adherent PBMC divided by the proportion of the corresponding subset in the original PBMC. Given are means ± S.D. of 4 independent experiments done in triplicate. $P<0.01$ when compared with one another.

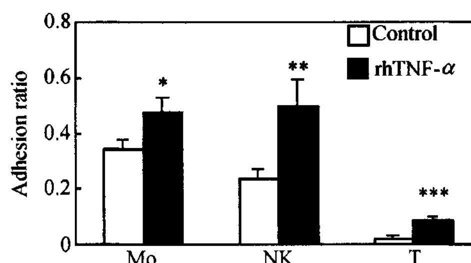

Fig. 3. Effect of rhTNF-α on adhesion of PBMC subsets. PAEC had been preincubated with rhTNF-α (10 ng/mL) for 24 h, and then the adherence of PBMC subsets to PAEC was assessed. Given are means ± S.D. of 3 separate experiments carried out in triplicate. * $P<0.05$ when compared with control; ** $P<0.02$ when compared with control; *** $P<0.01$ when compared with control.

(rhIFN-γ-stimulated PAEC, $P>0.05$ as compared with control, $n=3$, in triplicate) and (1.19± 0.21) (a combination of both cytokines-stimulated PAEC, $P>0.05$ as compared with the rhTNF-α-stimulated PAEC, $n=3$, in triplicate). Therefore, pretreatment of the target cell with rhIFN-γ for 24 h could not affect PBMC binding to PAEC; however, stimulation of PAEC with rhTNF-α resulted in a significant enhancement of PBMC adhesion. Furthermore, phenotypic analysis indicated that rhTNF-α pretreatment of PAEC augmented differently the adhesion ratios of PBMC subsets. That is to say, the bindings of Mo, NK and T cells to such treated PAEC were up-regulated by 40%, 110% and 3 times (fig. 3). When PAECs were preincubated with various concentrations of rhTNF-α (2.5—15.0 ng/mL) for 24 h, accordingly, the adhesion of PBMC subsets increased dose dependently (table 3), reaching their maximums at 10 ng/mL rhTNF-α.

Table 3 rhTNF-α stimulation-dependent adhesion of PBMC subsets

rhTNF-α/ng • mL^{-1}	0	2.5	5	10	15
Mo	0.29 ± 0.02	0.33 ± 0.02	0.36 ± 0.03*	0.41 ± 0.05*	0.38 ± 0.04*
NK	0.21 ± 0.04	0.28 ± 0.03	0.37 ± 0.02**	0.50 ± 0.04**	0.47 ± 0.05**
T	0.02 ± 0.00	0.03 ± 0.01	0.04 ± 0.01*	0.07 ± 0.01**	0.08 ± 0.01**

Values are given as means ± S.D. Representative result for 1 of 3 separated experiments carried out in triplicate. * $P<0.05$ when compared with control; ** $P<0.01$ when compared with control.

3 Discussion

At present, an important obstacle to xenotransplantation is the delayed xenograft rejection (DXR). In guinea pig-to-rat cardiac[5] and porcine-to-baboon cardiac or lung[6,7] xenotransplantation models, which were undergoing delayed rejection, immunohistochemical studies showed an extensive cellular infiltrate, composed mainly of host monocytes/macrophages (Mϕ) and NK cells with very few T cells, in the xenografts obtained by biopsy, suggesting that Mo/Mϕ and NK cell could be involved in the delayed rejection. Additionally, *in vitro* studies on the interactions between human PBMC and PAEC indicated the production of interleukin-1(IL-1) and interleukin-2 (IL-2)[6], and the lytic activity of NK cells[6,8]. However, the investigation of attachment of PBMC to PAEC, which is the first step in their interactions, has not been reported in detail. In the note, an *in vitro* co-culture model of human PBMC and PAEC was used to investigate differential adhesion of different PBMC subsets to PAEC. Flow cytometric adhesion assay indicated that within PBMC, Mo and NK, but not T cell, could adhere to PAEC vigorously, and therefore confirmed at the cell level that host Mo and NK cell could contribute to the xenograft rejection.

It was considered that interactions between cytokines and endothelial cells were of crucial importance for amplifying the immune response in allograft rejection. In the case of xenotrans-

plantation, it is necessary to investigate whether human cytokines could have cross-species effects on porcine endothelial cells. As is well known, IFN-γ and TNF-α, which could be produced by NK and Mo, respectively, possess multifunctions in initiating immune response. This report showed that although rhIFN-γ could not influence PBMC adhesion to PAEC, rhTNF-α could indeed double the PBMC adhesion. Thereby, it was inferred that in contrast to rhIFN-γ, rhTNF-α could cause considerable change in PAEC adherent property. Other investigators also demonstrated similar results for the cross-reactivity of human TNF-α. Batten et al.[9] indicated that rhTNF-α, but not human interleukin-1β (hIl-1β), or hIFN-γ, upregulated swine leukocyte antigens (SLA) class I and class II, and induced vascular cell adhesion molecule (VCAM) and E-selectin expression. Kwiatkowski et al.[10] also reported that porcine VCAM-1 expression was increased by 75%—85% after stimulation with rhTNF-α. From these results referred to here, it was extremely likely that human TNF-α would act as a proinflammatory factor in xenograft rejection.

It was notable that in spite of the significant increase in PBMC adhesion to PAEC preincubated with rhTNF-α, the magnitudes of the augmented adhesions of PBMC subsets exhibited great discrepancy, implying that the adhesion mechanism by which PBMC subsets bind to PAEC differs for Mo, NK and T cells; in other words, the extent to which cell adhesion molecules participate in adhesion to PAEC varies largely among Mo, NK and T cells. Therefore, further study is needed to address the adhesive mechanisms, so that in the future useful intervention could be provided to overcome the delayed xenograft rejection.

References

1. Millan, M. T., Ferran, C., Winkler, H. et al., Xenotransplantation: problems and approaches, Transplant Proc., 1994, 26: 3593.
2. Bach, F. H., Winkler, H., Ferran, C. et al., Delayed xenograft rejection, Immunol. Today, 1996, 17: 379.
3. Goodman, D. J., Albertini, M. V., Willson, A. et al., Direct activation of porcine endothelial cells by human natural killer cells, Transplantation, 1996, 61: 763.
4. Prasad Korlipara, L. V., Leon, M. P., Rix, D. A. et al., Development of a flow cytometric assay to quantify lymphocyte adhesion to cytokine-stimulated human endothelial and biliary epithelial cells, J. Immunol. Methods, 1996, 191: 121.
5. Blakely, M. L., Van der Werf, W. J., Berndt, M. C. et al., Activation of intragraft endothelial and mononuclear cells during discordant xenograft rejection, Transplantation, 1994, 58: 1059.
6. Itescu, S., Kwiatkowski, P., Wang, S. F. et al., Circulating human mononuclear cells exhibit augmented lysis of pig endothelium after activation with interleukin 2, Transplantation, 1996, 62: 1927.
7. Michler, R. E., Xu, H., O'Hair, D. P. et al., Newborn discordant cardiac xenotransplantation in primates: a model of natural antibody deletion, Transplant Proc., 1996, 28: 651.
8. Itescu, S., Kwiatkowski, P., Artrip, J. H. et al., Role of natural killer cells, macrophages, and accessory molecule interactions in the rejection of pig-to-primate xenografts beyond the hyperacute period, Human Immunol., 1998, 59: 275.
9. Batten, P., Yacoub, M. H., Rose, M. L., Effect of human cytokines (IFN-γ, TNF-α, Il-1β, Il-4) on porcine endothelial cells: induction of MHC and adhesion molecules and functional significance of these changes, Immunology, 1996, 87: 127.
10. Kwiatkowski, P., Artrip, J. H., Ankersmit, J. et al., Importance of CD49d-VCAM interactions in human monocyte adhesion to porcine endothelium, Xenotransplantation, 1998, 5: 67.

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