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Infiltration of CD4+, CD8+ T Cells and IgM+ Cells in Guinea Pig-to-Rat Cryopreserved Tracheal Xenografts

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Abstract : Objective : To assess the effect of cellular and humoral immune mechanisms in obliterative airway disease (OAD) in rodent tracheal xenografts, the infiltration of CD4+, CD8+, and IgM+ cells was analyzed in guinea pig-to-rat cryopreserved tracheal xenografts by histopathologic and immunohistochemical staining. Methodology : Guinea pig or Brown Norway (BN) rat tracheas were transplanted into F344 rat peritoneal cavities. Those were then divided into three groups ; consisting of a F344 rat syngenetic transplantation group (Group Ⅰ, n=8), a BN rat-to-F344 rat allotransplantation group (Group Ⅱ, n=12), and a guinea pig-to-F344 rat xenotransplantation group (Group Ⅲ, n=11). Results : In Group Ⅰ, the graft lumen showed almost a normal appearance. A mild proliferation of fibrous tissue was observed in Group Ⅱ, and luminal obliteration of the grafts was observed with a complete obstruction of the lumen by 2 weeks after transplantation in Group Ⅲ. There was no significant difference in the histopathologic scores between the 2-week cryopreserved grafts and, the 4-week cryopreserved grafts. CD4+ and IgM+ cell infiltrations were grade 0 in all of the grafts of Group Ⅰ. CD4+ and IgM+ cell infiltrations were grade 1 or 2 in 60% of the grafts of Group Ⅱ. All of the grafts of Group Ⅲ also showed a CD4+ and IgM+cell infiltration of grade 1 or 2. CD8+ cell infiltration of grade 1 or 2 was observed in 75% of the grafts of Group Ⅰ. In Group Ⅱ and Group Ⅲ, CD8+cell infiltration of grade 1 or 2 was found in all of the grafts. Conclusions : These findings suggest that the T-cell responses in grafts influence the occurrence of OAD and that humoral immune reactions may enhance the progression of occlusive lesions.

Key words : Tracheal transplantation, Obliterative airway disease, Xeno, Cryopreservation

Introduction

In Japan, in contrast to western countries, there has been no clinical case of tracheal transplantation, however, we can find many patients with benign or malignant tracheal obliteration in whom a surgical resection and/or tracheal stenting could not relieve their symptoms. Numerous reports on experimental tracheal allotransplantation have been so far published worldwide. One of the major problems, is that allografts are not always performed because of the shortage of donor grafts. In various organs, xenotransplantation has recently been investi-

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gated as a potential as therapeutic strategy.

The study of xenotracheal transplantation has been utilized for the analysis of obliterative bronchiolitis (OB) in human lung allograft. Rodent obliterative airway disease (OAD) is similar to OB in human lung allografts.[1)2)] In rodent concordant tracheal xenotransplantation, a luminal obstruction in grafts is characterized by luminal obliteration, a lack of bronchial epithelium, a degeneration of the tracheal cartilage and proliferation of fibrotic tissue in the graft lumen.[3)4)]

Several mechanisms contribute to the development of xenogenic rejection. There is general agreement that complement activation is an essential step in the development of hyperacute rejection (HAR). IgM and NK cell may play an important role in mediating HAR.[3)4)10)]

The purpose of this study was to investigate whether and to what much extent OAD might occur after discordant xenogenic tracheal transplantation and analyze how a cellular and humoral immuno reaction is associated with OAD experimentally. We suppose that cellular events also may be activated in the development of xenogenic rejection within 14 days as 'late' xenogenic rejection, and evaluated the infiltration of CD4+, CD8+ T cells and IgM+ cells in guinea pig-to-rat cryopreserved tracheal xenografts.

Materials and Methods

Animals. Guinea pigs weighing 150–200 g and Brown Norway (BN) rats were used as donors. F344 rats weighing 200–250 g were used as recipients. Animal care was provided according to the Fukuoka University Experimental Animal Care and Use Committee guidelines, and all animals were housed under specific pathogen-free conditions with free access to food and water.

Transplantation. The donor animals were given lethal doses of sodium pentobarbital (100 mg/kg i.p). The neck and upper chest were incised and dissected bluntly. The trachea was resected and placed in ice-cold phosphate-buffered saline (PBS) containing penicillin (1000 mg) and was cryopreserved at −80℃ for 2 to 4 weeks. Prior to transplantation, the cryopreserved tracheas were thawed in 35℃ water. The recipient rats were anesthetized with sodium pentobarbital (25 mg/kg i.p.). A midline laparotomy was performed, and the graft was wrapped with the recipient omentum and implanted in the recipient peritoneal cavity. The abdominal wall was closed with vicryl sutures, and the skin was closed with 4–0 nylon sutures.

The animals were classified into 3 groups, including a F344 rat syngenetic transplantation group (Group Ⅰ, n=8), BN rat-to-F344 rat allotransplantation group (Group Ⅱ, n=12), and guinea pig-to-F344 rat xenotransplantation group (Group Ⅲ, n=11). Immunosuppressive therapy was not provided to any recipient animals. The animals were then killed 7 to 14 days after transplantation.

The grafts were harvested and snap-frozen at −60℃ in embedding medium (Tissue-Tek OCT compound Miles Inc., Elkhart, IN). Frozen sections (6 μm) were cut from tissue blocks onto gelatin-coated 3-inch glass slides at −19℃ on a Leica CM1800 cryostat (Leica -Nussloch City, Germany), fixed in acetone, and stored at -80℃ until they were used for staining. Separate sections from grafts were stained with hematoxylin and eosin.

Slides were warmed to room temperature, and sections were hydrated with Tris-HCL-buffered saline (TBS) for 15 minutes. Endogenous peroxidase activity was eliminated by incubation in 0.3% hydrogen peroxidase/methanol for 15 minutes, and slides were washed with TBS. Nonspecific protein binding was blocked by incubation in diluted goat serum for 20 minutes, followed by incubation with 2 μg/ml anti-rat T helper cell monoclonal antibody (Mouse IgG 1; Cederlane Laboratories Ltd., Hornby, Ontario, Canada), anti-rat T cytotoxic/suppressor cell monoclonal antibody (Mouse IgG 1; Cederlane Laboratories Ltd.) or Mouse IgG1 kappa against the mu heavy chain of rat IgG (for control; J. Vanhandenhoven). The sections were then stained with peroxidase-conjugated goat-anti mouse IgG (Sigma Biosciences, St. Louis, MO.) followed by 3,3′-diaminobenzidine tetrahy-

drochloride (WAKO Pure Chemicals, Ltd.) mixed with H_2O_2 and NaN_3.

Evaluation. Two reviewers examined coded microslides of grafts blindly. T-cell infiltration was graded on a scale of 0 to 2, based on the degree of CD4+, CD8+, and IgM+ mononuclear cell infiltrations. Hematoxylin and eosin-stained sections were also examined and were assigned pathologic scores (scale of 0 to 2) based on the degree of epitheli alization, luminal fibrosis, cell infiltration, and cartilage degeneration (Table 1).

Statistical analysis. Differences in the mean infiltration scores of the grafts were analyzed by the Mann-Whitney U test for nonparametric data. Differences in the mean pathologic scores were analyzed statistically by a factorial analysis of variance methods. P values of <0.05 were considered to indicate statistical significance.

Results

Histopathologic findings. In Group Ⅰ, the graft lumen showed a normal appearance. Partial defects of the epithelium and a mild proliferation of fibrous tissue were observed in grafts in Group Ⅱ, however, no luminal obliteration was found. In Group Ⅲ, the proliferation of fibrous tissues and luminal obliteration of grafts were observed 1 week after transplantation, and a complete obstruction of the graft lumen occurred 2 weeks after surgery (Fig. 1). The histopathologic total score of the grafts was higher in Group Ⅲ than in Group Ⅰ ($p<0.05$) (Table 2). There was no significant difference in the histopathologic score between Group Ⅱ and Group Ⅲ, and there was no significant difference in histopathologic score between the 2-week and 4-week-cryopreserved grafts.

Immunohistochemical findings. In Group Ⅰ, 100% (4/4) of the grafts showed grade 0 in CD4+ cell and IgM+ cell infiltrations (Fig. 2), but in Group Ⅱ, over 40% of the grafts (2/5<) showed grade 1 or 2 infiltration, and in Group Ⅲ, 100% (5/5) of the grafts showed grade 1 or 2 of CD4+ cell and IgM+ cell infiltration (Figs. 3 and 4, Table 3).

Discussion

A previous report described a modified model of obliterative bronchiolitis (OB) in which a rat trachea was heterotopically transplanted in the greater omentum of a recipient animal, to study the pathogenesis of grafts after allotransplantation.[5] OB is characterized by inflammatory cell infiltra-

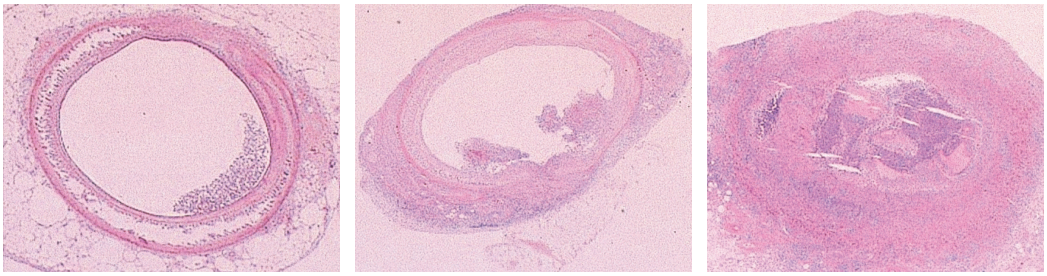
Table 1. Scoring and Grading

Findings	Score/Grade
Histopathologic staining	**Score**
Epithelialization	
Normal epithelialization	0
Limited defect and/or squamous metaplasia	1
No epithelialization and luminal fiblosing	2
Inflammatory Cell Infiltration	
No infiltration	0
Limited	1
Dense	2
Cartilage	
Normal	0
Atrophy	1
Destruction	2
Immunohistochemical staining	**Grade**
0～5 positive cells per field	0
6～30 positive cells per field	1
>30 positive cells per field	2

Table 2. Histopathologic Scores per Group

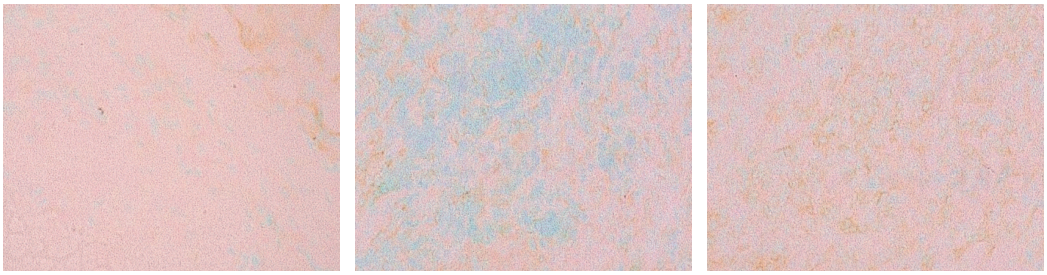
Group	Epithelialization	Cell infiltration	Cartilage	Total destruction
Group Ⅰ : syngenetic grafts				
2-week cryopreservation (n=4)	0.3±0.5	1.3±0.5	1.0±0.0	0.8±0.6
4-week cryopreservation (n=4)	1.0±0.0	1.5±0.6	0.0±0.0	0.8±0.7
Total (n=8)	0.6±0.5[a]	1.4±0.5	0.5±0.5	0.8±0.6[i]
Group Ⅱ : allogenetic grafts				
2-week cryopreservation (n=6)	1.1±0.3	0.0±0.5	0.7±0.7	1.6±0.4
4-week cryopreservation (n=6)	1.5±0.5	1.7±0.5	0.8±0.8	1.3±0.7
Total (n=12)	1.3±0.5[b]	1.6±0.5	0.7±0.7	1.2±0.7[ii]
Group Ⅲ : xenogenetic grafts				
2-week cryopreservation (n=6)	1.8±0.4	2.0±0.0	0.8±0.9	1.6±0.8
4-week cryopreservation (n=5)	1.6±0.5	1.6±0.5	0.4±0.9	1.2±0.8
Total (n=11)	1.7±0.5[c]	1.8±0.4	0.6±0.9	1.4±0.8[iii]

Scores are shown as the mean±SD values
a vs. b, a vs. c, ⅰ vs. ⅱ, i vs. ⅲ : $p<0.05$
b vs. c, ⅱ vs. ⅲ : NS



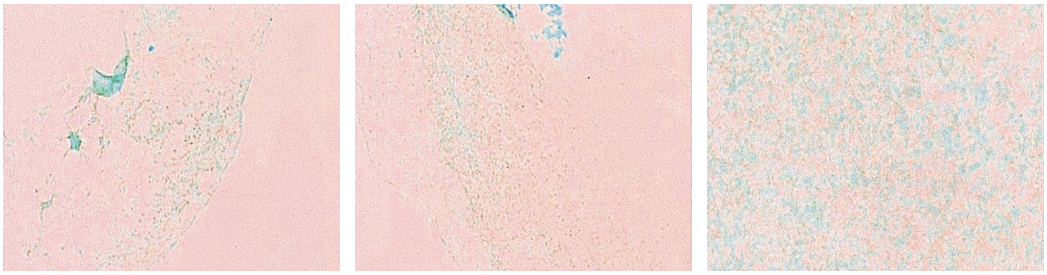
(A) (B) (C)

Fig. 1. Photomicrographs of the tracheas from F344 rat syngenetic transplantation, BN rat-to-F344 rat allotransplantation, and guinea pig-to-F344 rat xenotransplantation. (A) Complete epithelialization and mild mononuclear cell infiltration are found in syngenetic grafts. (B) A partial denudation of the tracheal epithelium and squamous metaplasia are found in the allografts. Mononuclear cell infiltration and fibroproliferation in the peritracheal tissue and a mild narrowing of the tracheal lumen are observed. (C) Severe monocyte infiltration, fibrosis, and luminal obstructions are found in the xenografts.



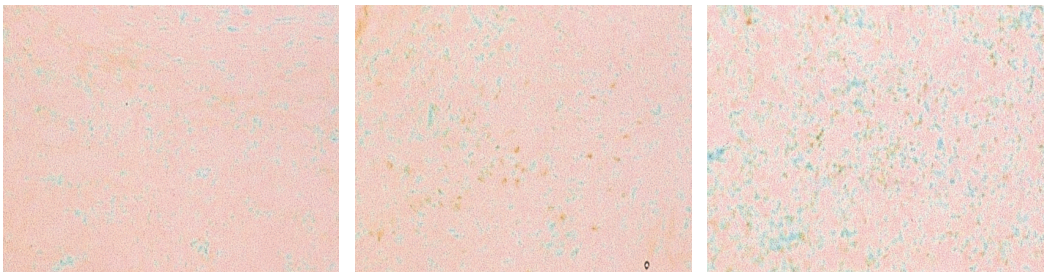
(A) (B) (C)

Fig 2. Immunohistochemical staining of CD4+ cells. (A) CD4+ cell infiltration is not observed in syngenetic grafts (grade 0). (B) Mild CD4+ cell infiltration in allografts (grade 1) and (C) a severe infiltration of CD4+ cells in the xenografts is shown (grade 2).



(A) (B) (C)

Fig. 3. Immunohistochemical staining of CD8+ cells. (A) CD8+ cell infiltration in the syngenetic grafts (grade 1), and moderate or severe infiltration in (B) allo- and (C) xenografts are shown (grade 2).



(A) (B) (C)

Fig. 4. Immunohistochemical staining of IgM+ cells. (A) No IgM+ cell infiltration is found in the syngenetic grafts (grade 0). (B) Mild IgM+ cell infiltration in the allografts (grade 1) and (C) the severe infiltration in the xenografts (grade 2) is shown.

Table 3. CD4+, CD8+, and IgM+ Cell Infiltrations per Group

		Number of Grafts showing positive immunohistochemical staining (%)		
		Group Ⅰ : syngenetic (n=4)	Group Ⅱ : allogenetic (n=5)	Group Ⅲ : xenogenetic (n=5)
CD4	Grade 0	4 (100)	2 (40)	0 (0)
	Grade 1	0 (0)	2 (40)	3 (60)
	Grade 2	0 (0)	1 (20)	2 (40)
CD8	Grade 0	1 (25)	0 (0)	0 (0)
	Grade 1	2 (50)	0 (0)	2 (40)
	Grade 2	1 (25)	5 (100)	3 (60)
IgM	Grade 0	4 (100)	3 (60)	0 (0)
	Grade 1	0 (0)	1 (20)	3 (60)
	Grade 2	0 (0)	1 (20)	2 (40)

Grade 0 : 0～5 positive cells per field
Grade 1 : 6～30 positive cells per field
Grade 2 : >30 positive cells per field

tion of peribronchiolar tissue by the migration of fibroblasts, proliferation of interstitial tissues, and fibrosis of bronchioles. The mechanism underlying OB in human lung allografts still unknown. It is considered to be a different phenomenon from acute allograft rejection characterized by perivascular mononuclear cell infiltration.[2] Histologically, OB in human lung allografts is also similar to Obliterative airway disease (OAD) in rodent tracheal xenotransplantation grafts.[3)4)] OAD of the grafts is also characterized by mononuclear cell infiltration, a denudation of the airway epithelium, fibroproliferation, and obliteration of the airway lumen. In addition, the mechanism underlying OAD is still not known.

Reichenspurner H and colleagues reported that OAD occurs in rat allografts and hamster-to-rat xenografts after heterotopic tracheal transplantation.[3)-5)] In their xenotransplantation, OAD after transplantation progressed more severely and rapidly than after tracheal allotransplantation. For the above, we used a heterotopic model to study the pathogenesis, humoral and cellular events of OAD after xenotransplantation.

In general, disparate species combinations result in discordant acute reactions which occur within minutes or hours,[6)7)] and species that are concordant reject the grafts within 3-4 days after implantation due to vigorous humoral and cellular immune mechanisms.[8)9)] In concordant xenotransplantation, grafts can keep the intra-lumen open during HAR within minutes-to-hours post transplants. Few data in literature address the occurrence of OAD in discordant xeno-combination. We chose the guinea pig-to-rat model as a discordant xenograft model in this study.

In our previous study in pig-to-dog tracheal xenotransplantation using large animal model, orthotopic tracheal xenograft advanced rapidly to blockade of the lumen with fibrous tissue proliferation. The intra-lumen of pig grafts were maintained for a few days and reached obliteration by 4-5 days post transplantation. Similarly, xenografts showed a high degree of stricture by day 7 in our rodent model. Luminal obliteration further developed at a high rate by day 14 after implantation and was more remarkable in xenografts than in allografts. Reichenspurner H and colleagues described that the presence of OAD in concordant xenografts increased the number of T-cell subpopulations and macrophages in the peritracheal tissue suggested immune injury to be a major pathogenetic event and the deposition of rat Ig suggested evidence of a humoral response to the xenografts.[4)] However, the isotype and function of rat immunoglobulin remains unclear.

The major immunoglobulin isotype mediating complement activation is almost always IgM during HAR. If HAR is avoided by the inhibition of complement or depleting xenogenic natural antibody, another type of rejection may occur over the following days. Some authors may refer to this type of rejection as delayed xenograft rejection (DXR). DXR in discordant xenograft rejection generally appears to occur due to a progressive infiltration of mononuclear cells in the grafts.

Sato K, et al demonstrated in a hamster-to-rat heart transplantation model undergoing DXR,[10)] that the early xenoantibody formation, which was resistant to Cyclosporin A (CyA) and consisted mainly of IgM, was mainly T cell-independent and the late xenoantibody formation, which was suppressed by the addition of CyA, might have been T cell-dependent. T-deficient nude rats developed anti-hamster IgM xenoantibodies at least from day 2 to day 14 of transplantation with a determination by flowcytometry using hamster peripheral blood mononuclear cells as targets. Immunofluorescence staining of untreated grafts has been observed at day 3 post transplantation, however, it has not been observed continuously. We stained IgM+ cell in the grafts to confirm any humoral immuno reaction related with OAD using a discordant model. In our study, IgM deposition into xenografts increased over a 14 day post transplantation period. Further more, the degree of infiltration of T-cells, including CD4+ and IgM+ cell infiltration and luminal obliteration was more remarkable in xenografts than in allografts. The progression of OAD with above findings indicates that specific T lymphocytes are activated by

the continuous stimulus of the graft and helps to reinforce local inflammation with IgM deposition and macrophage infiltration.

The IgM isotype is possibly generated by reciprocal action with T cell-independent xenoantibodies formation and cell-mediated immunity during DXR in tracheal xenotransplantation. In the future, it is necessary to examine and distinguish the populations of T cell-independent IgM and-dependent IgM during xenogenic rejection.

Our finding, which showed the grade of infiltration of the CD8+ cells to the xenografts to be lower than that to the allografts is considered to be extremely interesting. This may contain many unsolved factors, which participated in the T cell responses to a different-species antigen. Although our results suggest the possibility that cellular immunity may have contributed to the destruction of the xenografts, the mechanism of such action remains to be elucidated. Further study is called for regarding cellular immunity in xenotransplantation.

In addition, we performed graft preservation in view of reducible graft antigenicity. Previously some reports have suggested that cryopreservation techniques were applied and were fairly effective in maintaining viability and structural integrity and reducing the immunologic response.[11)12)] Their studies of OAD also indicated that the tracheal epithelium may play an important role in graft rejection. We attempted to compare the 2-week cryopreserved graft group and the 4-week cryopreserved graft group, though differences in the cryopreservation time did not affect the degeneration of graft by day 14 post implant according to our results. Our investigation identified two main findings, one is that our model is heterotopic transplantation in which the host tracheal epithelium is not able to regenerate in the donor tracheal lumen, and another one is that the observation period after transplantation and cryopreservation time was too short in this study to sufficiently analyze the infiltration of T-cells and xeno-reactive antibody in xenografts after transplantation.

In conclusion, we believe that an analysis of OAD using the heterotopic xenotransplantation model will be useful for investigating the humoral and cellular immunity associated OB. Our study suggests that: i) the T-cell response may influence the development of OAD, and humoral immune reactions may enhance the progression of OAD: ii) the suppression of the humoral immune reaction in DXR may be required as well as the administration of cyclosporin or tacrolimus as suppressants for the T-cell immune response.

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