Successful tracheal transplantation using cryopreserved allografts in a rat model

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Abstract

Objectives: The purpose of this study was to determine the appropriate cryopreservation period of tracheal allografts based on morphological and immunological findings and to test the possibility of tracheal transplantation in rats using cryopreserved allografts without immunosuppression. Methods: Morphological and immunological studies were performed to compare the differences between non-cryopreserved grafts and cryopreserved grafts. Orthotopic tracheal transplantation using cryopreserved allografts, non-cryopreserved allografts, and non-cryopreserved autografts was performed and the rejection score of each group was evaluated. Results: Epithelial cells were lost when the grafts were cryopreserved for more than 20 days. Immunohistochemical staining of the trachea revealed that the MHC class II antigen was expressed on normal epithelium. These findings suggest that cryopreservation for more than 20 days decreased the antigeneicity of allografts because of epithelial desquamation. All rats that received allografts cryopreserved for more than 20 days survived until the scheduled sacrifice day. Microscopically, cryopreserved allografts that had been preserved for more than 20 days had a significantly lower rejection score than that of non-cryopreserved allografts (P < 0.05). Conclusions: We conclude that the appropriate period for cryopreservation of allografts would be 20 days or more, because cryopreservation for more than 20 days depleted epithelium, which possessed the MHC class II antigen. Therefore, a longer period of cryopreservation decreases the antigeneicity of allografts. Rat tracheal transplantations using cryopreserved allografts is possible without immunosuppression when the grafts have been cryopreserved for more than 20 days. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Rat tracheal transplantation; MHC class II antigen; Appropriate cryopreservation period

1. Introduction

Recently, an excellent freezing technique [1] and freezing machines have been developed for the cryopreservation of tissue [2,3]. Successful clinical cardiac valve reconstructions with cryopreserved allograft have been reported [4,5]. The advantage of cryopreservation is as follows: (1) where a cryopreservation tissue bank is established, allografts are available whenever needed [2,6]; (2) Allogeneicity of allografts may be decreased by cryopreservation. Although several authors have reported results from studies of cryopreserved tracheal transplantations in animal models [7–11], few studies have shown that allogeneicity of allografts is decreased by cryopreservation. If it is possible to decrease allogeneicity, tracheal reconstruction using cryopreserved allografts is a promising surgical alternative to solve airway problems due to malignancy.

The purpose of this study was to determine the appropriate period of graft cryopreservation based on morphological and immunological status. An additional purpose of this study was to test the possibility of cryopreserved rat tracheal transplantation without immunosuppression.

2. Materials and methods

2.1. Experimental animals

Pathogen-free male ACI (RT1^a) and Lewis (RT1^b) rats (Charles River, Kanagawa, Japan) were used for this study. Lewis rats were used as recipients and ACI rats were used as tracheal donors. Recipient and donor pairs were fully mismatched at both major and minor histocompatibility loci. The rats were maintained in accordance with the guidelines for animal experimentation of Niigata University, and
the study was approved by The Niigata University Committee on Animal Research.

2.2. Cryopreservation of rat trachea

2.2.1. Freezing method and preservation

ACI rats received anesthesia by ether and ten rings of cervical trachea were removed using sterile techniques. The excised trachea was placed immediately into a preservation solution containing a balanced buffered salt solution with L-glutamine (RPMI 1640 Medium; Wako Pure Chemical Industries, Osaka, Japan), 10% dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Osaka, Japan), and 20% fetal calf serum. The graft was frozen in a computer-controlled unit (KRYO 10 SERIES III, PLANER, UK) at approximately 1°C/min to −80°C. After achieving the correct temperature, the grafts were stored in liquid nitrogen for 1 day in group A, 10 days in group B, 20 days in group C, and 30 days in group D, respectively. After the cryopreservation, the graft was thawed in a 37°C water bath and evaluated for morphological and immunological examination. They were compared with the normal rats (non-cryopreserved ACI rats) as controls.

2.2.2. Morphological examination

Four rings of trachea from each specimen was fixed in 10% buffered formalin. Two rings were stained with hematoxylin and eosin for light microscopic examination. Another two rings of trachea were put in an 2% aqueous solution of tannic acid for 3 h, dehydrated in ethanol, transferred to isoamyl acetate and critical point-dried using liquid carbon dioxide. The dried trachea were mounted on aluminium stubs with silver paste and evaporated with gold-palladium. The inside of the tracheal lumen was examined using a Hitachi S-2380N Scanning Electron Microscopy (Hitachi Seisakusyo, Hitachi, Japan).

2.2.3. Immunological examination

Two rings of trachea from each specimen were embedded in an OCT compound (Miles, Elkhart, USA) and rapidly frozen in liquid nitrogen. Cryostat sections were cut at 4 μm in thickness. After fixation in acetone for 5 min, the

![Fig. 1. Scanning microscopic and immunologic appearance of rat trachea before (a), (c) and after (b), (d) retrieval from 30 days in a cryopreserved state. (a) Ciliated epithelium is observed. (b) Epithelium was lost. (c) Epithelial cells contained classII positive cells. (d) ClassII positive cells were not detected within the epithelium.](image-url)
sections were allowed to react with a mouse anti-rat classII antigen (Serotec, Oxford, UK), biotinylated anti-mouse Ig (Amersham, Amersham, GB), and horseradish peroxidase (HRP)-labeled anti-mouse Ig (Serotec, Oxford, UK). HRP binding sites were detected in 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Sections were then counterstained with eosin.

2.3. Tracheal transplantation

2.3.1. Orthotopic tracheal transplantation in rats
Lewis rats were anesthetized with intraperitoneal administration of sodium pentobarbital and sodium atropine. Under spontaneous respiration with a transnasal endotracheal tube, five rings of cervical trachea was resected and a five rings graft was transplanted with a continuous 8-0 polypropylene suture. As controls, non-cryopreserved five-ring autografts were transplanted into Lewis rats and non-cryopreserved five-ring allografts from ACI rats were transplanted into Lewis rats. After transplantation, neither immunosuppressive agents nor antibiotics were administered.

2.3.2. Examination of transplanted grafts
Recipients rats were sacrificed at postoperative days 5, 7, 10, 20 and 30. The grafts were retrieved and stained with hematoxylin and eosin. Histological findings were graded as non: 0, slight: 1, mild: 2, severe: 3, in each three different parameters: bleeding, monocyct infiltration and fibrosis. Rejection was scored as sum of above points. Assessment was performed by two blinded observers.

Statistical analysis for the rejection scores was done using a Student t-test and a P value of less than 0.05 was considered significant.

3. Results

3.1. Morphological changes after different periods of cryopreservation
In the cryopreserved grafts, both membranous and cartilaginous portions appeared normal macroscopically in each group but lumen of grafts appeared more edematous in groups A and B than that in groups C and D. Light microscopic examination revealed that the epithelial cells were lost in groups C and D (Fig. 1a,b). For quantitative analysis, the residual ratio of the epithelium was calculated by the following formula: (the length of residual epithelium/the length of tracheal circumference)×100. The residual ratio was significantly less in groups C and D than in groups A and B by a Student t-test (Table 1). Microscopically, all portions of cartilage showed normal appearance in each group.

Scanning electron microscopic examination of normal rat trachea showed ciliated areas and non-ciliated areas in the luminal surface. Epithelial desquamation was evident in the groups that had been cryopreserved for longer period (groups C and D), and the expansion of the residual epithelium was evident in the groups that had been cryopreserved for less than 10 days (groups A and B).

3.2. Immunological change after cryopreservation

Examination of normal rat trachea showed that classII positive cells could be detected in the epithelium of all tested animals. In contrast, examination of cryopreserved rat trachea showed that classII positive cells could be detected in the residual epithelium. The number of classII positive cells in groups C and D decreased remarkably compared to that in groups A and B (Fig. 1c,d).

These findings suggest that the MHC classII antigen is expressed in the epithelium of normal trachea, and that cryopreservation for more than 20 days decreases antigenicity because of epithelial desquamation.

3.3. Orthotopic rat tracheal transplantation

All rats with non-cryopreserved autografts survived until the scheduled sacrifice day. Twenty of 40 rats that received allografts cryopreserved for less than 10 days (groups A and B) died before the scheduled sacrifice day. In contrast, rats survived until the scheduled sacrifice day when they received tracheal allografts that had been cryopreserved for more than 20 days (groups C and D). Based on these findings, allografts cryopreserved for 30 days were used for following experiments. Forty-four rats underwent successful orthotopic tracheal implantation and were allocated into

| Table 1 |
| Residual ratio of the epithelium  |
| Group (number) | Mean ± SD for A and B; and C and D | Combined P value (A,B,C,D) A and B vs. C and D |
| A (5) | 20 ± 7.9 | 28.5 ± 12.2 |
| B (5) | 37 ± 9.7 | |
| C (5) | 5.6 ± 4.3 | 6.2 ± 3.5 |
| D (5) | 6.8 ± 2.8 | P < 0.05 |

Data are shown as the mean ± SD. Residual ratio(%) = (length of residual epithelium/length of tracheal circumference) × 100.

| Table 2 |
| Experimental animals |
| Group (number) | Postoperative sacrificed days |
| | 5 | 7 | 10 | 20 | 30 |
| Non-cryopreserved autograft (13) | 2 | 2 | 3 | 3 | 3 |
| Cryopreserved allograft (15) | 3 | 3 | 3 | 3 | 3 |
| Non-cryopreserved allograft (16) | 3 | 3 | 4 | 3 | 3 |
experimental groups, as shown in Table 2. At the time of scheduled death, all wounds had grossly healed over.

In the rats transplanted with non-cryopreserved autografts, the histological examinations at day 5 showed infiltration of red blood cells. After day 5, no further pathological changes were observed. At day 30, ciliated epithelial cells could be observed.

In rats transplanted with non-cryopreserved allografts, severe infiltration of mononuclear cells and red blood cells was observed in all layers at day 7. Infiltration of mononuclear cells remained until day 30 and fibrosis occurred.

In the rats transplanted with cryopreserved allografts, mild mononuclear cell infiltration was observed at day 5. This infiltration gradually decreased; at day 30 the grafts were lined with non-ciliated epithelium.

Microscopic rejection scores in each group are presented in Table 3. Compared with non-cryopreserved allografts, cryopreserved allografts had a significantly lower rejection score.

### 4. Discussion

Several reports have demonstrated that tracheal transplantation using cryopreserved allografts is feasible without immunosuppression [7–11]. However, these studies relied on different freezing techniques and preservation methods. In addition, the duration of cryopreservation ranged widely from 7 days [9] to 9 months [8]. Optimizing the parameters of preservation would be the key to successful tracheal transplantation using cryopreserved allografts. Therefore, we evaluated the effects of cryopreservation, especially its duration and changes of allogeneicity, on trachea.

Based on morphological and immunological studies, we demonstrated that the MHC classII positive cells disappeared in the trachea after cryopreservation for more than 20 days. The trachea is continuously exposed to organisms and particles in inhaled air, and high networks of resident dendritic cells develop in the trachea [12]. These cells stain positive with MHC classII antigens and function as antigen-presenting cells in the airway. Because MHC classII cells are essential for the recognition of alloantigen and for the initiation of allograft rejection [13], loss of these immunologically important cells would be effective for the minimization of allograft rejection. In our current study, we demonstrate that the epithelium of normal trachea stained positive with the MHC classII antigen and tracheal epithelium was depleted after cryopreservation when grafts had been cryopreserved for more than 20 days. For this reason, Yokomise et al. administered high-dose irradiation to canine trachea. They were thus able to deplete the epithelium and reported better results [14].

As regards the freezing methods, we used a computer-controlled unit based on the report by Lange et al. [15]. They suggested that the best freezing rate for cryopreservation using a computer-controlled unit would be −1°C/min, because at this rate, the latent heat of crystalization that will occur during the freezing process can be avoided. In addition, preservation in liquid nitrogen inhibits chemical and physical changes almost completely [16]. However, intracellular and intercellular ice formation may occur in the epithelium and therefore, the loss of epithelium can not be avoided when grafts are preserved for a longer period [17]. Similar results are reported by Lupineti et al. on studies of human valves [18] and by Bambang et al. on saphenous veins [19].

We next examined the effects of cryopreservation on allograft rejection. The scoring system for the evaluation of rejection used in the present study was based on the histological changes of tracheal allografts in incompatible rat strains [20]. In our study, impact of ischemia on five-ring grafts may be ignored because the light microscopic examination of the non-cryopreserved five-ring autografts showed no ischemic change. All rats that received non-cryopreserved allografts survived, although microscopic examination of these grafts showed rejection that Sesterhenn and Rose have studied in incompatible rat strains [20]. In our study, impact of ischemia on five-ring autografts may be ignored because the light microscopic examination of these grafts showed rejection that Sesterhenn and Rose have studied in incompatible rat strains [20]. On the other hand, 20 of 40 rats that received allografts cryopreserved for less than 10 days died, the reason of this different survival is not clear. As can be seen in Table 3, non-cryopreserved autografts had the lowest rejection score. Compared with non-cryopreserved allografts, allografts cryopreserved for more than 20 days had a significantly lower rejection score. However, histological examination of cryopreserved allografts showed mild rejection compared with that of non-cryopreserved autografts. One possible reason of this mild rejection may be immunological reaction against the cartilage that may have a tissue-specific antigen [21]. However, in our current study, the immunological
examination was not performed to compare the differences between non-cryopreserved tracheal cartilage and cryopreserved tracheal cartilage. With this model, administration of minimum immunosuppressive agents may prevent allograft rejection completely. Yokomise et al. [8] have reported that cryopreserved canine tracheal allotransplantation was successful due to depletion of the epithelium and preservation of the cartilage, which is in line with our present results.

We conclude that the appropriate period of allograft cryopreservation would be 20 days or more, because cryopreservation for more than 20 days depleted the epithelium that expressed the MHC classII antigen, and therefore it would decrease the antigenicity of allografts in a rat model. In cases where grafts have been cryopreserved for more than 20 days, tracheal transplantation using cryopreserved allografts would be possible without the use of immunosuppressive agents.

References