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Orthotopic and heterotopic tracheal transplantation model in studying obliterative bronchiolitis

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ABSTRACT

Background: Several animal models have been established to investigate the mechanisms of obliterative bronchiolitis after lung transplantation. In this study, we compared three prevalent murine models of obliterative bronchiolitis in terms of several basic pathologic changes in a relatively short span of time after transplantation.

Methods: Each of the recipient mice simultaneously received orthotopic, intra-omental and subcutaneous tracheal transplantation in both syngeneic and allogeneic settings. No immunosuppressive treatment was administered. Tracheal grafts were harvested on Day 14, 21 and 28 after transplantation for histological and immunohistochemical analyses.

Results: Syngeneic tracheal grafts from different transplant sites retained normal histologic structures, while their corresponding allografts demonstrated more occlusion of the airway lumen as well as more infiltration of $CD4^+/CD8^+$ mononuclear cells and myofibroblasts, but less regenerative epithelium and neovascularized vessels at indicated times ($P < 0.05$). Compared with two heterotopic allografts, orthotopic allografts had less occlusion of the tracheal lumen as well as less infiltration of $CD4^+/CD8^+$ mononuclear cells and myofibroblasts, but more regenerative epithelium and neovascularized vessels ($P < 0.05$).

Conclusions: Orthotopic tracheal transplantation in mice can be considered as a model to study early stages of obliterative bronchiolitis, and heterotopic tracheal transplantation can be a model for late stages of obliterative bronchiolitis.

1. Introduction

Lung transplantation becomes the only available therapeutic option for patients with selected end-stage pulmonary diseases. Despite that great improvements have been achieved, the long-term survival of patients with pulmonary allografts is still barely satisfactory, which continues to be hampered by obliterative bronchiolitis (OB) and its clinical correlate, bronchiolitis obliterans syndrome (BOS). BOS was reported in 49% of patients by 5 years after transplantation and in 75% by 10 years, on the basis of data including more than 13,000 recipients who survived at least 14 days [1]. OB is an inflammatory and fibroproliferative disorder affecting small airways of the transplanted lung and has been generally considered as a form of chronic rejection. Increasing clinical studies have indicated risk factors related to the development of OB [2]. However, the specific pathogenesis of OB remains unclear, and further research is necessary to elucidate the underlying pathogenic mechanisms.

Rodents, with the advantage of easy manipulation over a short-time frame, play an important role in OB research. As an experimental animal in transplantation models, the rat has been highly recommended in the past [3,4]. The mouse, however, would be a much more valuable tool owing to the widespread use of genetically defined inbred and engineered strains, and commercial availability of various reagents. The orthotopic lung transplant in mice might be best mimicking the clinical surgery, but has the drawbacks of technical difficulty and low level of reproducibility of OB lesions [5,6]. Therefore it has been generally used to study early postoperative problems, such as ischemia-reperfusion and acute rejection. In 1993, Hertz and colleagues implanted tracheal grafts into a subcutaneous pouch of the neck of recipient mice, and successfully induced typical OB lesions [7]. Afterwards, several transplantation models of a trachea in variable sites

Abbreviations: OB, obliterative bronchiolitis; BOS, bronchiolitis obliterans syndrome; α-SMA, actin, α-smooth muscle.

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such as intra-omental [8] and orthotopic sites [9,10], as well as various modifications and variants [11,12] were developed by the other investigators. Although the distributions of cartilage rings and submucosal glands in mice trachea are like those in human small airways [13], some may argue that differences may exist in the mechanisms that contribute to the tracheal obliteration in this model as compared to the bronchiole obliteration in human transplant lungs. Moreover, different groups were inclined to investigate diverse issues through their preferred models, but all the models failed to perfectly elucidate the mechanism of OB. So in this situation, investigators were confused to choose the appropriate model for their hypothesis or specific question.

In this study we combined orthotopic, intra-omental and subcutaneous tracheal transplantation, which have been well-established and reproducible OB models [9,10,14], to investigate several basic pathologic changes during the post-transplant period. Each donor trachea was divided into three segments and then respectively implanted into three sites of each corresponding recipient. Finally, the morphological changes of the grafts on various days after transplantation were analyzed and compared.

2. Materials and methods

2.1. Animals

Specific pathogen-free, female C57BL/6 (H-2^b) mice and Balb/C (H-2^d) mice (Jackson Laboratory) weighing 20–24 g were used. All animals were housed in the specific pathogen-free facility (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) and had access to water and food ad libitum. All the studies were performed in compliance with the Principles of Laboratory animal care (NIH publication Vol 25, No. 28 revised 1996) and the Tongji Medical College Animal Care and Use Committee Guidelines.

Tracheas from Balb/c mice were implanted into Balb/c mice (syngeneic, n = 45) or C57BL/6 mice (allogeneic, n = 45). Each donor tracheal graft was evenly divided into three segments, and then simultaneously implanted into orthotopic, intra-omental and subcutaneous sites of each recipient. Grafts (15 syngeneic or 15 allogeneic grafts from each transplant site) were harvested on Days 14, 21 and 28 after transplantation for histologic and immunohistologic analyses.

2.2. Donor procedure

The donors were euthanized by intraperitoneally injecting pentobarbital (80 mg/kg). A midline cervical incision was performed to expose the entire trachea. The trachea below the cricoid cartilage distal to the bifurcation was dissected, harvested, and then it was flushed and preserved with cold sterile saline at 4 °C. Prior to implantation, the full-length trachea (approximately 12 cartilage rings) was divided into three segments of 4 cartilage rings, which were then randomly transplanted into various sites respectively.

2.3. Recipient procedure

The recipients were anesthetized by intraperitoneally injecting pentobarbital (50 mg/kg). Initially, a short midline cervical incision was performed to visualize the entire laryngotracheal complex. The recipient trachea was carefully dissected, and then transected at the third intercartilage below the epiglottis while spontaneous breathing was maintained. One of the 4-ring donor tracheal segments was implanted end-to-end, starting with the distal anastomosis using 9-0 Prolene suture (Ethicon). The cervical incision was closed in layers with continuous 7-0 Vicryl suture (Ethicon). Subsequently, the recipient mouse underwent a midline laparotomy followed by exposure of the greater omentum. The second tracheal segment was wrapped and fixed into the greater omentum using 9-0 Prolene, and then the abdominal wall was closed in layers with continuous 7-0 Vicryl suture. Finally, a small incision was made in the dorsal suprascapular area of the recipient mice. A subcutaneous pouch was made with blunt dissection, and then the third tracheal segment was placed into it. The skin was closed with interrupted 7-0 Vicryl suture. The operative procedures were performed with the assistance of a surgical microscope (×10 magnification) in a sterile fashion. All recipient animals received no immunosuppression.

2.4. Histology and immunohistochemistry

The grafts were harvested from CO_2 euthanized recipient mice on Day 14, 21, and 28 after transplantation for histologic and immunohistochemical analyses. Tracheal grafts were cut into longitudinal sections for hematoxylin and eosin (H&E) or immunohistochemical staining. Each graft segment for H&E staining was fixed in 4% formalin at room temperature for 24 h. The formalin-fixed tissues were embedded in paraffin, later cut into 4-μm sections and then stained with H&E.

For immunohistochemistry studies, 5-μm routine sections were used. CD4 and CD8 positive cells were respectively identified by mouse monoclonal anti-CD4 and anti-CD8 (BD Biosciences). Vessel endothelial cells were identified by mouse monoclonal anti-CD31 (BD Biosciences). For fibroproliferative tissue staining, mouse monoclonal anti-actin, α-smooth muscle (α-SMA, Sigma-Aldrich) was used. For each primary antibody, an appropriate irrelevant IgG was used as negative control to ensure that effects of nonspecific binding were recognized.

2.5. Morphologic studies

A microscope (BX51, Olympus) with camera (AxioCam MRc, Carl Zeiss) and Image-Pro Plus 6.0 for Windows (Media Cybernetics) analysis program were used for morphometric analysis, which were performed by two independent, blinded reviewers. All measurements were performed on six random sections from each graft. Lumenal occlusion was defined as the area containing tissue inside of the cartilage ring. The percentage of luminal occlusion was calculated as follows: (area within cartilage-area within residual lumen) / area within cartilage × 100%. Mucus, produced by airway epithelial cells, in the lumen was not calculated as obliteration. The histologic changes in respiratory epithelium were evaluated as percentage of lumenal circumference covered by ciliated epithelium. $CD4^+/CD8^+$ mononuclear cells were quantified as the total number of positively stained, mononuclear cells in the lamina propria of the graft in each selected section. $CD31^+$ blood vessels were counted in same fashion with $CD4^+/CD8^+$ cells. The percentage of α-SMA positive area inside of the cartilage ring was calculated in the same fashion as lumenal occlusion.

2.6. Statistical analysis

All data are presented as mean ± SEM. GraphPad Prism 5 for Windows (GraphPad Software, Inc.) was used for statistical analysis. One-way repeated measures analysis of variance (ANOVA) followed by Tukey's test or Friedman test followed by Dunn's test (nonparametric) was used within a group. Comparisons between syngeneic grafts and allografts were performed using *t*-test or Mann–Whitney test (nonparametric). $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Histological assessment

The syngeneic grafts basically retained normal tracheal architecture with lumenal patency and no aberrant granulation tissue found (Fig. 1A–C, G–I, M–O). Among the syngeneic grafts, their percentages of lumenal occlusion were around 20% which were close to the normal trachea (Fig. 2A), and significantly different among various transplant sites

($P = 0.002$): the airway lumen of intra-omental grafts demonstrated more patent than subcutaneous grafts ($P < 0.05$), which demonstrated more patent than orthotopic grafts ($P < 0.05$). Among allografts, the percentages of lumenal occlusion were significantly different ($P = 0.0067$. Fig. 2A): the tracheal lumen of orthotopic allografts progressively occluded (Fig. 1D–F), and the percentage of lumenal obliteration exceeded 40% on Day 28; heterotopic allografts exhibited typical histological changes of OB with complete occlusion occurred by Day 28 (Fig. 1J–L, P–R), and tracheal lumen of heterotopic allografts was more occlusive than orthotopic allografts ($P < 0.05$), while lumenal occlusion of two different heterotopic allografts was not significantly different ($P > 0.05$). Compared with the corresponding syngeneic grafts, airway lumen of allografts demonstrated to be more occlusive at various time points ($P < 0.05$ respectively).

Syngeneic grafts after transplantation maintained normal or nearly normal ciliated mucosa (Fig. 1A–C, G–I, M–O inset): pseudostratified ciliated epithelium with glands lined almost the entire tracheal lumen, and the secretory function was restored. Among syngeneic grafts, the levels of epithelization were significantly different ($P = 0.0022$) (Fig. 2B): orthotopic syngeneic grafts covered less ciliated epithelium than heterotopic syngeneic grafts ($P < 0.05$); the two heterotopic grafts were not significantly different ($P > 0.05$). Allografts progressively lost epithelium, and levels of remaining ciliated epithelium were significantly different ($P = 0.0025$): orthotopic allografts underwent squamous metaplasia and ulceration in varying degrees (Fig. 1D–F inset), and had higher level of epithelization than the heterotopic allografts ($P < 0.05$) (Fig. 2B); in heterotopic allografts, the tracheal mucosa underwent progressive degrees of denudation, and finally lost nearly all of the epithelium and basement membrane (Fig. 1J–L, P–R inset), and the level of epithelization of two heterotopic allografts was not significantly different ($P > 0.05$) (Fig. 2B). Compared with their corresponding syngeneic grafts, allografts regenerated lower level of epithelium at various times following transplantation ($P < 0.05$) (Fig. 2B).

3.2. Immunohistochemical assessment

There were mild infiltrations of $CD4^+/CD8^+$ mononuclear cells in syngeneic grafts, which were not significantly different among various transplant sites ($P = 0.1944$). Compared with syngeneic grafts, more severe infiltration of $CD4^+/CD8^+$ mononuclear cells was detected in allografts during the observation period ($P < 0.05$ respectively) (Fig. 3A, B). Infiltrations of $CD4^+/CD8^+$ mononuclear cells in allografts were significantly different ($P = 0.0003$): orthotopic allografts demonstrated a continual increase in cellular infiltration over time; heterotopic allografts demonstrated cellular infiltrate, peaked on Day 21 (intra-omental allografts, $CD4^+/CD8^+$: $160 \pm 13/184 \pm 24$; subcutaneous allografts, $CD4^+/CD8^+$: $164 \pm 11/175 \pm 17$) and sustained high level on Day 28 (intra-omental allografts, $CD4^+/CD8^+$: $154 \pm 15/177 \pm 14$; subcutaneous allografts, $CD4^+/CD8^+$: $160 \pm 14/161 \pm 15$), which were more than orthotopic allografts ($P < 0.05$) but were not significantly different between two heterotopic allografts ($P > 0.05$) (Fig. 3E).

CD31, a vascular cell-specific cell–cell adhesion molecule, has been identified to play an important part in the process of angiogenesis. We stained CD31 to investigate the angiogenesis ability in different transplant sites (Fig. 3C). The quantities of $CD31^+$ blood vessels in various syngeneic grafts were significantly different ($P = 0.0002$): intra-omental syngeneic grafts had more $CD31^+$ blood vessels than subcutaneous syngeneic grafts ($P < 0.05$), which had more than orthotopic syngeneic grafts ($P < 0.05$). The quantities of $CD31^+$ blood vessels in various allografts were also significantly different ($P = 0.0093$): the quantity of $CD31^+$ blood vessels in orthotopic allografts was more than heterotopic allografts ($P < 0.05$), while the quantities were not significantly different between two heterotopic allografts ($P > 0.05$). Compared with the corresponding syngeneic grafts, all of the allografts had revascularization at lower level ($P < 0.05$) (Fig. 4A).

Myofibroblasts with capacity of collagen synthesis are involved in tissue remodeling. We used α-SMA as a marker for myofibroblasts to determine the fibrosis degrees in transplanted trachea (Fig. 3D). In syngeneic grafts, myofibroproliferation was nearly undetectable during the observation time, whereas allografts had more proliferation of myofibroblasts in lamina propria of transplanted trachea ($P < 0.05$). The percentages of α-SMA positive area were not significantly different in syngeneic grafts ($P = 0.5278$). The percentages were significantly different in allografts ($P = 0.0030$): The percentages of $\alpha\text{-SMA}^+$ area in two different heterotopic allografts were similar ($P > 0.05$), but significantly higher than orthotopic allografts ($P < 0.05$) (Fig. 4B).

4. Discussion

The optimal tool to study OB pathogenesis, no doubt, is human lung transplantation. However, drawbacks such as sparse OB samples, and difficulties of sampling at various times, in addition to complications after sampling like infections, hamper human lung transplantation to act as a "model". There is therefore a critical need for some animal models that could elucidate the pathogenesis of OB.

Of the different tracheal transplantation models employed in this study, each has obvious advantages and drawbacks [15], and previous investigators have not yet come to a consistent conclusion on which of the transplantation models is more qualified as a model for studying OB. Since evidence is mounting that epithelial damage [16,17], immune-mediated tissue injury [18], angiogenesis [19,20] and fibroproliferative remodeling [21] may be involved in the development of OB, we compared transplantation models in terms of these hotspot issues in this study. In addition, we combined transplantation

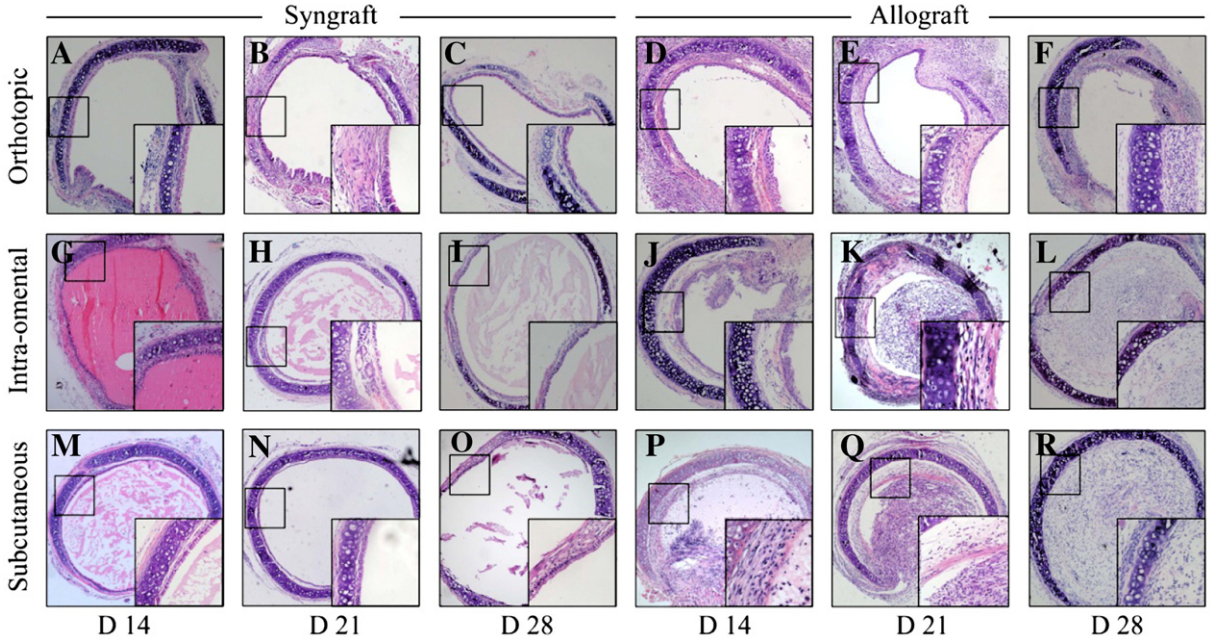

Fig. 1. Histological features of syngeneic grafts and allografts on D 14, 21 and 28 after transplantation. Orthotopic syngeneic grafts on D 14 (A), 21 (B), and 28 (C) post-transplant. Syngeneic grafts wrapped in greater omentum on D 14 (G), 21 (H) and 28 (I) post-transplant. Subcutaneous syngeneic grafts on D 14 (M), 21 (N) and 28 (O) post-transplant. Orthotopic allografts on D 14 (D), 21 (E), and 28 (F) post-transplant. Allografts wrapped in greater omentum on D 14 (J), 21 (K), and 28 (L) post-transplant. Subcutaneous allografts on D 14 (P), 21 (Q), and 28 (R) after transplantation (H&E; original magnification: ×100; inset magnification: ×400).

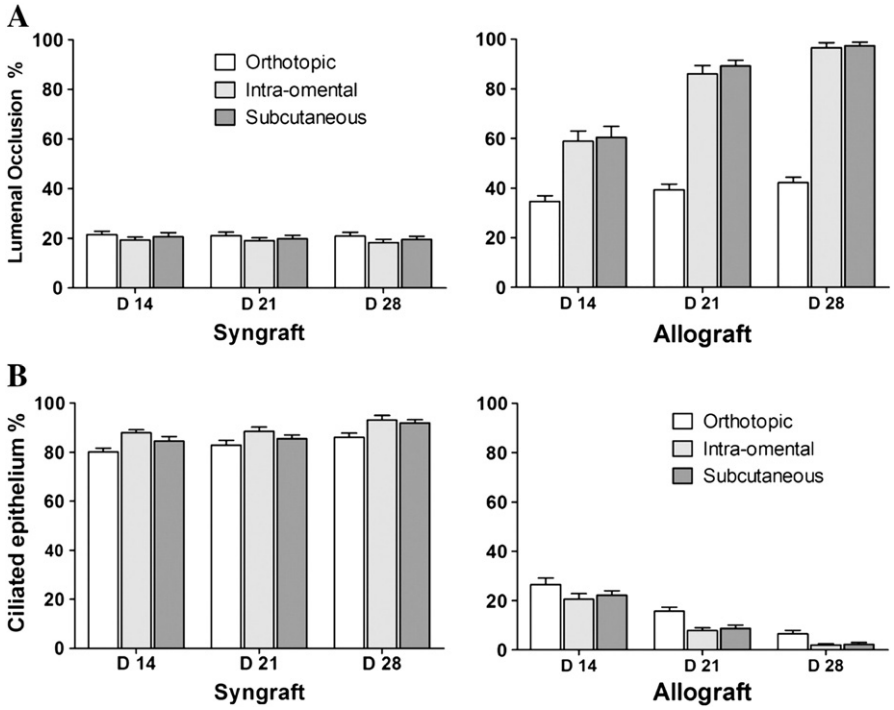

Fig. 2. A, Quantitive analysis of lumenal obliteration of tracheal grafts. B, Quantitive analysis of percentage of tracheal lumen covered by ciliated epithelium. Data are expressed as mean ± SEM.

models to decrease the consumption of the animals as well as improve individual error and the experimental efficiency.

In human, the development of BOS is rare within the first year after lung transplant, but the cumulative incidence ranges from 43 to 80% within the first five years of transplantation [22]. Histologically, early lesions of BOS demonstrate submucosal lymphocytic inflammation and disruption of the epithelium of small airways, followed by a buildup of granulation tissue in the airway lumen, resulting in partial obstruction. Subsequently, granulation tissue organizes in a cicatricial pattern with resultant fibrosis and eventually completely obliterates the airway lumen [23]. It is difficult to define the distinct stages of OB development, but each stage has different main pathological features.

Our results demonstrate that orthotopic tracheal allografts were partially obstructed, in which the mucosa underwent denudation and squamous metaplasia as well as re-epithelization to various degrees, while the submucosa had few myofibroblasts but rising number of inflammatory cells. On the other hand, heterotopic allografts were

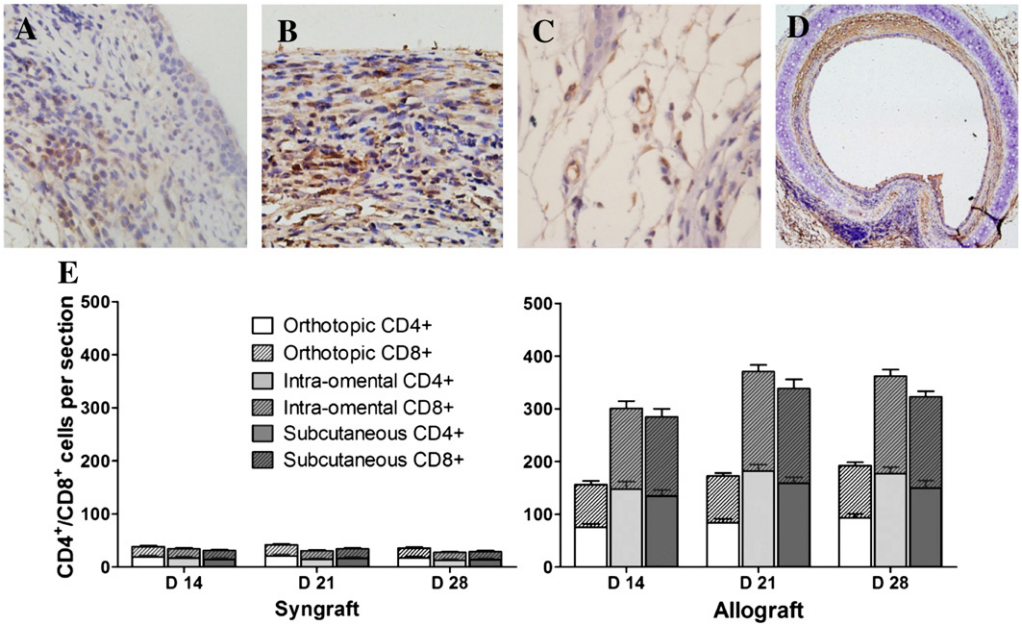

Fig. 3. A, Immunohistochemical staining of $CD4^+$ cells from orthotopic allograft on D 21 post transplantation. B, Immunohistochemical staining of $CD8^+$ cells from subcutaneous allograft on D 21 post transplantation. C, Immunohistochemical staining of $CD31^+$ blood vessels from intra-omental allograft on D 21 post transplantation. D, Immunohistochemical staining of α-SMA^+ from intra-omental allograft on D 21 post transplantation. Original magnification: ×400 (A–C); ×100 (D). E, Quantitive analysis of $CD4^+/CD8^+$ mononuclear cell infiltration in tracheal grafts. Data are expressed as mean ± SEM.

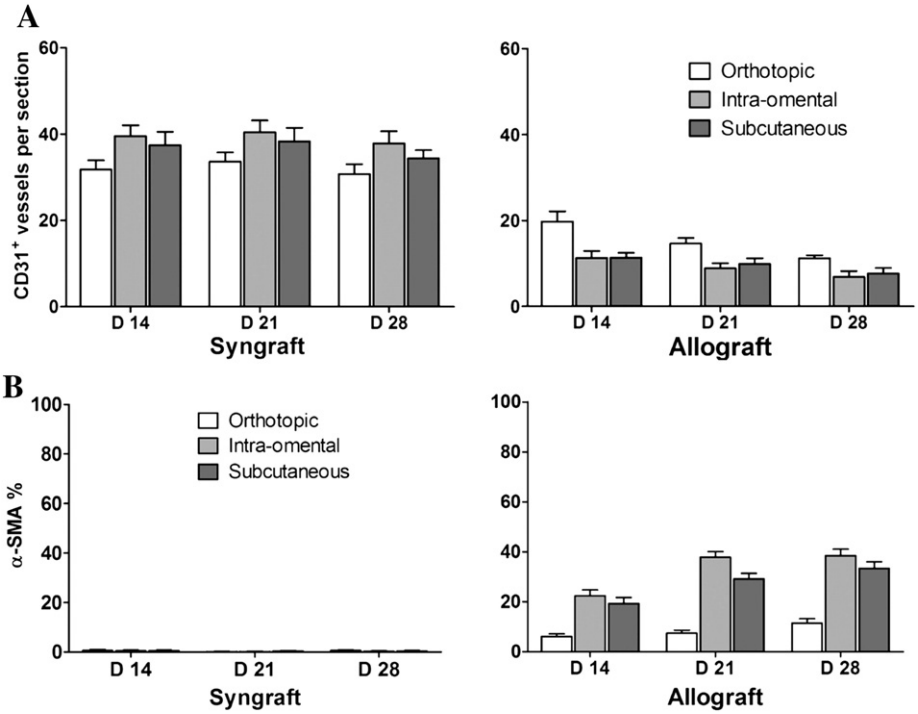

Fig. 4. A, Quantitive analysis of $CD31^+$ vessels in tracheal grafts. B, Quantitive analysis of α-SMA positive area in tracheal grafts. Data are expressed as mean ± SEM.

completely occluded within 4 weeks after transplant, in which the trachea had barely epithelium but abundant inflammatory cells and myofibroblasts. Therefore, pathological changes found in orthotopic and heterotopic allografts are respectively similar to those in different stages of BOS development in patients who received lung transplant.

Both orthotopic and heterotopic tracheal grafts are nonvascularized grafts, and there is no supply of blood to the grafts other than from angiogenesis, which is passively derived from surrounding tissue during the course of wound healing after transplantation. Although our study confirmed that the angiogenesis ability among various transplant sites was different, all the orthotopic syngeneic grafts basically retained normal histological structures. We speculate that transplant site would not be a major factor affecting the development of OB. In lung transplanted patients, OB is preceded by a decrease in microvascular supply to the small airways. This ischemic event may lead to airway damage or increase the tendency of scar tissue formation as a repair mechanism. The small airways then appear to respond to this insult by angiogenesis [24,25]. Compared with orthotopic allografts, heterotopic allografts formed lesions with less neovascularized vessels but more fibrous tissues like those in the more mature stage of scar formation. Hence, pathological changes in orthotopic and heterotopic allografts may represent the different stages of OB development: those of orthotopic allografts exhibit the early stage of OB development while heterotopic allografts exhibit the advanced stage, but the general trend of lesion development was identical.

20 years after the implementation of the first OB research model [7], the question is "what is the ideal model of OB." First, this model is time and cost saving: it is not practical to spend over months waiting for the development of OB lesions, while some models are limited in their high cost and availability. Second, the results of the model are reliable and highly reproducible: OB lesions and other pathological changes concerned should be easily detected and measured. Third, the model is physiological and clinically relevant: the grafts should be communicated with ambient air and with adequate blood supply which closely mimics environment of small airways in human. Fourth, the model has less technical difficulties: Among all the animal models of OB established now, the model of orthotopic mouse lung transplantation performed by Fan et al. [6] does not only reflect the full physiology of a transplanted graft, but also allows for the investigation other factors most affecting the evolution of OB. This model holds great promise for boosting clinically relevant research, but complicated operations and need of special mice strain combinations prevent its widespread adoption. Last but not least, the animals in models are easy to receive therapeutic intervention, in other words, animals with distinct immunological background have easy access to genetic or drug manipulation. Moreover, we should notice that this "ideal" model should be carefully employed based on the specific hypothesis or question. Under certain conditions, orthotopic or heterotopic tracheal transplantation, "the less-than-ideal models", can also be employed to explain some hypothesis, or provide useful evidences for further exploration of the question.

In conclusion, orthotopic tracheal transplantation in mice can be considered as a model to study early stages of OB, and heterotopic tracheal transplantation can be a model for late stages of OB. In addition, our results implicate that the development of OB in intra-omental and subcutaneous allografts followed a similar time course, we presume that the two different heterotopic transplantation models can substitute for each other.

Acknowledgments

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