Egr1: a novel target for ameliorating acute allograft rejection in an experimental lung transplant model

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Abstract

OBJECTIVES: Acute allograft rejection is one of the significant complications occurring in lung transplant recipients. Early growth response-1 (Egr-1), zinc-finger-type transcription factor, is known as a master switch regulator of diverse chemical mediators. We used an orthotopic mouse model of left lung transplant to elucidate the function of Egr-1 in acute pulmonary rejection.

METHODS: Left lung grafts retrieved from C57BL/6 wild mice or C57BL/6 Egr-1-null mice were orthotopically transplanted into BALB/c mice; the lungs were harvested at day 1, 3, 5 or 7 after lung transplantation. The grade of acute rejection was histopathologically evaluated. The intragraft gene expression levels of Egr-1 and downstream target mediators were quantitatively measured by real-time polymerase chain reaction. Immunohistochemical analysis was used to determine the location and distribution of the Egr-1 protein in the pulmonary graft.

RESULTS: Severe acute rejection was observed in allografts from wild-type mice at 5 days after transplantation. Only minimal rejection was seen in the lung graft from Egr-1-null donor mice at 5 days after transplantation. Strong upregulation of Egr-1 mRNA transcripts was observed at day 1, which then decreased during the next 5 days. The mRNA of Egr-1 target mediators [interleukin-1-beta (IL-1β), monocyte chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor-1] reached maximal levels at day 5. Egr-1-null allografts exhibited significantly lower expressions of IL-1β and MCP-1 mRNA (P < 0.05).

CONCLUSIONS: Our study showed that deletion of Egr-1 in lung allografts ameliorates severe acute rejection with the reduction of expression levels of chemical mediators, implying a new possible strategy for treating acute pulmonary allograft rejection.

Keywords: Lung transplantation • Acute rejection • Egr-1 • Inflammatory cytokines

INTRODUCTION

Lung transplantation has gained widespread acceptance as a treatment option for patients with end-stage pulmonary disease [1, 2]. However, acute rejection is one of the significant complications in lung transplant recipients; this form of rejection is also a risk factor for obliterative bronchiolitis (OB) [3–6]. OB is one of the primary causes of death in lung transplant recipients, and the outcomes for lung transplants remain far worse than those for transplants of other solid organs [1]. In patients with OB, irreversible distal airway obstruction gradually progresses because of the lack of an effective treatment and finally leads to death of the transplant recipient [7, 8]. OB is characterized by progressive damage and fibrosis and is thought to be a manifestation of chronic rejection [9].

Therefore, reduction of the rate and severity of acute allograft rejection is an important aspect of lung transplantation. In the cases of acute rejection of solid organ transplants, various chemical mediators, which are linked to cellular responses, may play pivotal roles. Therefore, these mediators may serve as therapeutic targets for the management of transplant recipients. Early growth response (Egr)-1 gene is a zinc-finger-type transcription factor [10] that is rapidly and transiently induced by several stresses, including mechanical trauma, hypoxia, high tidal volume, cytokines and ischaemia–reperfusion (I/R) [11–13]. Egr-1 is known as a master switch in the regulation of inflammatory cascades. Egr-1 may possibly function as an important mediator of the transcriptional response to rejection and serve as an attractive subject in studies on the therapeutic strategies for acute rejection. We hypothesized that attenuation of Egr-1 expression in the pulmonary graft reduces the severity of inflammatory responses and thus suppresses acute rejection. To date, a few reports have described the relationship between...
acute rejection and Egr-1. However, these studies have been hampered by the lack of a physiologically relevant mouse model of lung transplantation. In the current study, we used an orthotopic mouse left lung transplant model [14] to elucidate the function of Egr-1 in pulmonary acute rejection.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 and BALB/c [wild-type (WT)] mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Egr-1−/− mice were purchased from Taconic Farms Inc. (Hudson, NY, USA). Male mice, about 10 weeks old and weighing 25–30 g, were used in these experiments. This experimental protocol was approved by the Animal Care Committee at Okayama University.

**Transplant technique**

Left lung grafts retrieved from C57BL/6 (B6 WT) mice (WT group) or from B6 Egr-1-null mice (KO group) were orthotopically transplanted into BALB/c mice for allograft transplantation, and lung grafts from B6 mice were transplanted into syngeneic recipients for isograft transplantation (control group); the cuff technique, which has been previously described [14], was used for both transplantations (n=5 for each group). Recipient animals did not receive any immunosuppressive agents or antibiots. The lungs in the WT group were harvested at day 1, 3, 5 and 7 after transplantation. Harvested iso- or allografted lungs from WT donor mice. The lungs were fixed in 10% formaldehyde, sectioned and stained with haematoxylin and eosin (H&E). Grading for acute cellular rejection was performed using standard criteria developed by the Society for Heart and Lung Transplantation provisionally.

**Histopathological evaluation**

In order to confirm the changes in the state of the transplanted lung with time, we performed histopathological evaluation of iso- or allografted lungs from WT donor mice. The lungs were harvested at days 1, 3, 5 and 7 after transplantation. Harvested lungs were fixed in 10% formaldehyde, sectioned and stained with haematoxylin and eosin (H–E). Grading for acute cellular rejection was performed using standard criteria developed by the Society for Heart and Lung Transplantation provisionally.

**Real-time polymerase chain reaction**

Intragraft gene expression levels of Egr-1 and downstream target mediators induced by Egr-1, including interleukin-1-beta (IL-1β), plasminogen activator inhibitor-1 (PAI-1), monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1), were quantitatively measured by real-time polymerase chain reaction (PCR). Total RNA was extracted from frozen lung tissue of each mouse by using the Isogen reagent (Nippon Gene, Tokyo, Japan). Reverse transcription was performed with the SuperScript™ III-RT Kit (Invitrogen, Tokyo, Japan), according to the manufacturer’s protocol. The reaction mixture contained 1:100 diluted template cDNA, 0.4 µl primers (Invitrogen), 0.2 µl probe (Roche Diagnostics, Indianapolis, IN, USA) and 4 µl Light Cycler TaqMan Master in a final volume of 20 µl. Real-time PCR was performed using a Light Cycler rapid thermal cycler system (Roche Applied Science, Indianapolis, IN, USA) with 45 amplification cycles (95°C, 10 s; 60°C, 30 s and 72°C, 1 s) and a final cooling step (4°C). The DNA melting curve was analysed using the Light Cycler software. All extracted RNA samples were examined in duplicate.

**Immunohistochemical analysis**

Lung tissue was cut into small pieces, washed with phosphate-buffered saline (PBS), fixed in formalin and embedded in paraffin. The sections were treated with hydrogen peroxide in methanol for 20 min to quench endogenous peroxidase activity. The primary antibody (rabbit anti-mouse Egr-1 polyclonal antibody sc-189 diluted to 1:200; Santa Cruz Biotech, Santa Cruz, CA, USA) was applied, and the sections were incubated overnight at 4°C. After the sections were washed with TBST [50 mM Tris–HCl buffer (pH 7.6) containing 0.01% Tween-20 and 0.15 M NaCl], they were incubated for 30 min with peroxidase-labelled polymer-horseradish peroxidase (HRP) conjugated with anti-rabbit immunoglobulins [EnVision™-HRP kit (K4003), Dako, Tokyo, Japan]. Staining was completed by incubation with 3,3-diaminobenzidine chromogen solution (DAB+, a part of the EnVision kit). Nuclei were counterstained with haematoxylin.

**Flow cytometric analysis**

Flow cytometry was performed to determine the severity of rejection grade. Harvested whole lungs were cut into minute pieces and incubated in RPMI medium containing 1 mg/ml
Intracellular collagenase (SERVA Electrophoresis, Heidelberg, Germany) and 5 U/ml RNase-free DNase set (Qiagen, Tokyo, Japan) at 37°C for 90 min. Single-cell suspensions were obtained by passage of digested tissue through a 70 µm tissue strainer (BD Biosciences, San Jose, CA, USA). Cells were centrifuged, washed twice and resuspended in 500 µl PBS containing bovine serum albumin. Cellular infiltration into lungs was assessed by staining with fluorochrome-labelled anti-CD90 (Acris Antibodies, San Diego, CA, USA), anti-CD4 (eBioscience, San Diego, CA, USA) and anti-CD8 (BD Biosciences) by using a FACS Caliber flow cytometer (BD Biosciences). The data analysis was performed using the CellQuest Pro software (BD Biosciences).

**Statistical analysis**

When the results were significant according to one-way analysis of variance (ANOVA), differences between series were determined using Tukey’s test with StatView for Windows (J-5.0 SAS Institute Inc.). Values were expressed as means±SD. A P-value of <0.05 was considered statistically significant.

**RESULTS**

**Time course of graft histopathology**

The rejection grade increased sequentially with time, and severe rejection was observed at 7 days after transplantation. Infiltrating mononuclear cells were mainly observed in the perivascular and peribronchial regions. Differences in the amount of infiltrating mononuclear cells were clearly observed to be associated with time, especially in perivascular regions. At day 3 after transplantation, mononuclear cells begin to infiltrate around vessels while peribronchial regions were almost intact, and a large number of mononuclear cells abruptly infiltrated not only into perivascular region but also into peribronchial region at day 5 after allotransplantation (Fig. 2). In contrast, lungs of isografts were almost normal in appearance even at day 5 after transplantation (Fig. 3A, control group). A few cells had infiltrated around vessels and small bronchi; as in the case of isografts, only minimal rejection was observed in the lung grafts from Egr-1-null donor mice at day 5 after transplantation (Fig. 3A, KO group).

**Egr-1 mRNA expression and downstream target mediators**

The intragraft gene expression levels of Egr-1 and of the downstream target mediators induced by Egr-1, IL-1β, PAI-1, MCP-1 and ICAM-1, were quantitatively measured by real-time PCR after allotransplantation. Strong upregulation of normalized Egr-1 mRNA transcripts was observed at day 1 after allotransplantation, which then gradually decreased in the following 7 days (Fig. 4). The mRNA of chemical mediators (IL-1β, PAI-1, MCP-1 and ICAM-1) reached maximal levels at day 5 after
allotransplantation (Fig. 4). Egr-1-null allografts exhibited a significantly lower expression of IL-1β and MCP-1 mRNA at day 5 ($P = 0.02$). The mRNA levels of the other downstream targets, ICAM-1 and PAI-1, in Egr-1 knockout mice tended to be lower than those of WT mice, but there were no significant differences between the mRNA levels in these two groups (Fig. 5).
**Expressions of the Egr-1 protein**

Immunohistochemical analysis was used to detect the location and the distribution of the Egr-1 protein in the pulmonary grafts. Egr-1 expression was enhanced with time (Fig. 2B), contrary to the expression of Egr-1 mRNA. Egr-1 expression was mainly localized in the perivascular region and bronchial endothelial cells (Fig. 3B). In the lungs retrieved from knockout donor mice and isografts from WT donor mice, Egr-1 protein expression was not observed in the perivascular regions (Fig. 3).

**Flow cytometric analysis**

Flow cytometric analysis was performed on graft tissue retrieved at day 5 after transplantation (Fig. 6). In the lungs from isografts, the number of CD8+ T-cells was less than that of CD4+ T-cells; the ratio of intragraft CD8+ to CD4+ was 0.64 ± 0.30. In contrast, in the lungs harvested at day 5 after allotransplantation, CD8+ T-cells outnumbered CD4+ T-cells. The ratio of intragraft CD8+ to CD4+ was 2.65 ± 0.51. In the lungs harvested from Egr-1 knockout donor mice, the ratio of CD8+ to CD4+ was 0.51 ± 0.11 (Fig. 6, n = 3 for each group). The ratio of CD8+ to CD4+ in Egr-1 knockout donor mice allografts and isografts was significantly lower than that of WT allografts [Egr-1 knockout B6 (KO day 5) vs. B6 BALB/c (allo day 5), P < 0.01; B6B6 (iso day 5) vs. allo day 5, P < 0.01; iso day 5 vs. KO day 5, P > 0.05].

**DISCUSSION**

In lung transplantation, the frequency and severity of acute rejection have been shown to influence the development of OB, which ultimately causes lung dysfunction [3-6]. However, the mechanisms that lead to both acute and chronic lung allograft rejection have still not been clarified.

Acute pulmonary rejection is likely to be associated with interactions between the immune cells in the grafted lungs and the cytokines produced as a part of the host immune response. In the current study, 5 days after transplantation, the lung allografts of WT mice showed obvious evidence of severe acute rejection along with increases in the transcript levels of IL-1β, MCP-1 and PAI-1. However, the coincident induction of these molecules is not consistent with the decrease in the mRNA levels of Egr-1. Egr-1 mRNA expression in vascular and immune cells is rapid, peaking within 30 min of stimulation, and transient, falling to undetectable levels by 2 h after stimulation [15]. This expression pattern is not consistent with the mechanism in which the expression of one mediator, such as IL-1β, causes downstream expression of the other mediators.

T-cell activation might be partially stimulated in response to cytokines and adhesion molecules, and this stimulation can result in accelerated alloimmune responses and consequent rejection processes. Immunohistochemical analysis indicated that Egr-1 expression in allotransplanted grafts from WT mice was localized to alveolar epithelial cells, bronchial epithelial cells, vascular endothelial cells and infiltrating immune cells. These cells might contribute to the activation of T-cells and progression of allograft rejection. Autieri et al. [16] performed biopsies of cardiac allografts and concluded that the Egr-1 expression in vascular endothelial cells is likely to be caused by low levels of host-derived inflammatory cytokines present in the grafted vessels, regardless of the rejection status. Conversely, Harada et al. [17] reported that Egr-1 is induced in mouse allogeneic tracheal transplantation and contributes substantially to epithelial injury, and in animals that received allografts lacking the Egr-1 expression.
gene, reduction in Egr-1 expression also suppressed the serum levels of the inflammatory cytokines that are downstream targets of Egr-1. Consistent with their results, our study showed that blockade of the Egr-1 pathways attenuates acute lung allograft rejection at the early phase of lung transplantation. Moreover, the transcript levels of IL-1β and MCP-1 were remarkably lower in the transplanted allografts lacking Egr-1. These results imply that Egr-1 functions as a molecular master switch that transactivates a number of genes, including genes encoding proinflammatory cytokines and chemokines, and it may represent an attractive therapeutic target to prevent acute pulmonary rejection. However, the severity of rejection continued to show a gradual progression, and complete rejection was observed within 14 days (data not shown). The possible explanation for this finding is that the reduction in inflammatory responses by Egr-1 pathway blockade and the consequent delay in T-cell activation occur only in the early period of transplantation.

The inflammatory responses occurring immediately after transplantation have been considered to play a role in accelerating rejection processes. Oishi et al. [18] showed that transfer of hIL-10 into transplanted lung appears to cause a deviation of cytokine immune responses from a predominant Th1 to a Th2 profile, which leads to a reduction in the gene expression of IL-2, INF-gamma and TNF-alpha and a slight improvement in pulmonary acute rejection. Moreover, ischaemia followed by reperfusion causes severe stresses, resulting in strong upregulation of Egr-1 transcripts and inflammatory mediators. Okada et al. [19] reported that the reduction in Egr-1 levels attenuates I/R injury in the animal lung transplant model. I/R injury has been known to be a risk factor for rejection episodes. I/R injury impairs both acute and long-term graft function and is associated with an increased number of acute-rejection episodes that affect long-term graft outcome [20]. Another possible explanation for the interaction of Egr-1 and graft rejection is that the minimization of I/R injury with deletion of Egr-1 might attenuate the severity of acute pulmonary rejection during the early period after transplantation. Thus, Egr-1 may have an important role in cases showing a rapid onset of acute lung rejection.

The successful treatment for acute rejection in this study, which was performed by deletion of Egr-1, was associated with reduced transcript levels of inflammatory cytokines and a reduction in the CD8+ T-cell proportion. In mouse lung allografts that show acute rejection, graft-infiltrating CD8+ T-cells predominate over CD4+ T-cells [21–24]. Similar results were observed in our study. Furthermore, elimination of Egr-1 caused a decrease in the number of graft-infiltrating CD8+ T-cells and a significant reduction in the grade of acute pulmonary rejection. The detailed mechanisms of the correlation between acute pulmonary rejection and Egr-1 pathway should be studied by analysing alloimmune cascades and T-cell activation and proliferation, including the proportion of CD4(+)CD25(+)Foxp3(+) T-cells.

In conclusion, although further studies will be required to elucidate these findings and to validate our hypotheses, our results provide new mechanistic insights into the acute rejection of lung allografts and reveal the prominent role of Egr-1 in the severity of acute rejection. Moreover, it is conceivable that the use of Egr-1 blockade to achieve broad-spectrum inhibition of the expression of cytokines, adhesion molecules, other genes pertinent to inflammation and other proliferative pathologies may become a new therapeutic strategy to prevent acute pulmonary rejection.

Figure 6: Flow cytometric analysis. Flow cytometric analysis was performed on graft tissue retrieved at day 5 after transplantation. In the lung tissue from isografts, the number of CD8+ T-cells was less than that of CD4+ T-cells; the ratio of intragraft CD8+ to CD4+ was 0.65 ± 0.51 (n = 3). On the contrary, in the lungs harvested at day 5 after allotransplantation, CD8+ T-cells outnumbered CD4+ T-cells. The ratio of intragraft CD8+ to CD4+ was 2.65 ± 0.51 (n = 3). In the lungs harvested from Egr-1 knockout donor mice, the ratio of CD8+ to CD4+ was 0.51 ± 0.11 (n = 3). These support the histopathological differences of the severity of the rejection among three groups.
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