Early growth response gene-1 promotes airway allograft rejection

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Chronic airway rejection, characterized by lymphocytic bronchitis, epithelial cell damage, and obliterative bronchiolitis (OB), limits long-term survival after lung transplantation. The transcription factor early growth response gene-1 (Egr-1) induces diverse inflammatory mediators, some involved in OB pathogenesis. An orthotopic mouse tracheal transplant model was used to determine whether Egr-1 promotes development of airway allograft rejection. Significantly higher Egr-1 mRNA levels were seen in allografts (3.2-fold increase vs. isografts, P = 0.012). Allografts revealed thickening of epithelial and subepithelial airway layers (51 ± 4% luminal encroachment for allografts vs. 20 ± 3% for isografts, P < 0.0001) marked by significant lymphocytic infiltration. Absence of the Egr-1 gene in donor (but not recipient) tissue resulted in significant reduction in inducible nitric oxide synthase (iNOS) expression. Egr-1 null allografts exhibited a striking reduction in inducible nitric oxide synthase (iNOS) expression. Effector cytokines previously implicated in OB pathogenesis with known Egr-1 promoter motifs (IL-1β and JE/monocyte chemotactant protein-1) were reduced in Egr-1 null allografts. These data suggest a paradigm wherein local induction of Egr-1 in tracheal allografts drives expression of inflammatory mediators responsible for lymphocyte recruitment and tissue destruction characteristic of airway rejection.

The majority of lung transplant patients develop obliterative bronchitis (OB) despite optimal immunosuppressive management. OB is characterized by progressive obliteration of the small airways with major pathological features including lymphocytic infiltration in early stages, inflammation, epithelial cell injury, and ultimately fibrosis (6). The clinical syndrome (bronchiolitis obliterans syndrome, BOS) that results is marked by progressive decline in pulmonary function (with obstruction to airflow), dyspnea, and ultimately death (7, 10). Once BOS is present, further immunosuppressive therapy is ineffective for preventing progressive airway obstruction and clinical deterioration. The failure to improve clinically despite augmentation of immunosuppression suggests that the genesis of OB is multifactorial, and it is therefore critical to recognize and study the reversible inflammatory process preceding irreversible OB (18).

Experiments here focus on one particular, ubiquitous initiator of diverse inflammatory processes, early growth response gene-1 (Egr-1). Egr-1 is a zinc finger transcription factor that drives expression of multiple gene families including those involved with leukocyte recruitment and adhesion, coagulation, and fibrosis. Downstream target genes of Egr-1 such as TNF-α, PDGF-A, PDGF-B, ICAM-1, FGF-2, IL-1β, JE/monocyte chemotactant protein-1 (MCP-1), and transforming growth factor-β1 have been implicated in OB pathogenesis (1, 3, 4, 11, 20, 31). Egr-1 induction contributes to tissue injury following ischemia through its actions as a master switch regulator of inflammatory, adhesion, and coagulation cascades. Conceptually, Egr-1 can be viewed as a main initiator of wound repair, as it is activated early, and the coordinated expression of Egr-1 responsive genes fits into a pattern of genes for which expression is likely important for wound repair. Under pathological conditions of tissue ischemia and reperfusion, Egr-1 is also induced, but some of its physiological effects, such as enhanced coagulation and leukocyte traffic, are injurious. Because lung transplantation is associated both with an initial ischemic insult (caused by severing the donor lung from its native blood supply) as well as ongoing immune attack (in the setting of allotransplantation), it is logical to consider whether a program of gene activation initiated by Egr-1 can in fact be pathological with respect to development of chronic airway rejection. In the case of transplanted small airways, wound “repair” with attendant leukocytic infiltration and fibrogenic response might be maladaptive. These experiments were therefore driven by the hypothesis that Egr-1 is induced by an airway transplant procedure and that this induction contributes to ultimate pathological luminal encroachment of the airway.

To elucidate a potential mechanistic link between Egr-1 induction and airway rejection, an orthotopic trachea transplantation model was employed using tissue derived from mice lacking the Egr-1 gene. Experiments were done with special reference to epithelial cell injury and inflammation. These are the main pathological features of lymphocytic bronchitis (LB; Ref. 37), which is believed to be a harbinger of OB (38). The importance of LB is underscored by the fact that it is a reversible inflammatory process that leads to irreversible OB (5, 13, 33). Conceivably, understanding LB contributory mechanisms could lead to new therapeutic options to prevent OB. As data in this manuscript indicate that the Egr-1 is induced by the transplantation procedure and contributes substantially to epithelial injury, LB, and loss of the airway lumen, it could conceivably become a new target of therapeutic opportunity to prevent OB.

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MATERIALS AND METHODS

Mice. C57BL/6 (H-2b) mice and B10.A (H-2d) mice were purchased from Jackson Laboratories, and BALB (H-2d) mice were purchased from Charles River Laboratories. For allografts, C57BL/6J (H-2b) mice were used as donors, and B10.A (H-2d) or BALB (H-2d) mice were used as recipients. For isografts, C57BL/6J (H-2b) mice were used as both donors and recipients. For the experiments in which Egr-1 knock-out (Egr-1^{-/-}) mice were used, littermate control wild-type (WT; Egr-1^{+/+}) mice were used; both had the same complete H-2b genotype on a background strain of 129xC57BL/6. Male mice between 8 and 15 wk old were used in these experiments, and the genotype of each Egr-1^{-/-} or littermate control mouse was confirmed by genomic PCR. All experiments were performed according to the protocols approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Orthotopic trachea transplantation model. All experiments were performed using an orthotopic tracheal transplantation model for studying chronic airway rejection, which has been previously reported (23, 24). This model mimics LB and allows studies focusing on inflammation. As a brief methodological synopsis, donor mice were anesthetized, and whole tracheas were harvested under sterile conditions by transecting below the cricoid cartilage distal to the carinal bifurcation. Recipient mice were similarly anesthetized, and whole tracheas were harvested under sterile conditions for anastomosis in a base mold in liquid nitrogen and stored at −80°C until the time of analysis. Histochemical staining was performed for elastin (Accustain, Sigma-Aldrich) to help delimit subepithelial and subepithelial areas using a computer-assisted image analysis system (AxeoCamHR; Carl Zeiss Microimaging, Thornwood, NY). These methods have been previously reported (23, 24).

mRNA isolation and real-time PCR analysis. Total RNA was extracted from each frozen mouse trachea using TRIZol reagent (Life Technologies). The PCR primers and TaqMan probes were purchased from Applied Biosystems (Foster City, CA). The sequences of forward and backward primers and probes for target (mouse Egr-1) and housekeeping (β-actin) genes were as follows. For Egr-1, the forward and backward primer sequences were 5’-GCTCTGAGACGATGAC-CAAT-3’ and 5’-GCAGAGGGAAGACGATGAGCA-3’; those for β-actin were 5’-CTCTGGTCAGGCACTTCCGGCTGT-3’ and 5’-GCTGATGCCACATCTGCTGAAA-3’. The probes for Egr-1 and β-actin were 5’-CCTCGGATCCATTATCTCCGGGTG-3’ and 5’-CCGCGT-GCTCCATCTGGGTCTAC-3’, respectively. Real-time PCR was performed using one-step RT-PCR master mix reagents (Applied Biosystems) and an ABI PRISM 7000 Sequence Detection System. Data are calculated by the 2^{ΔΔCT} method (21) and are presented as fold induction of Egr-1 mRNA in WT allografts normalized to β-actin compared with WT isografts.

Immunohistochemistry. Immunohistochemical staining was performed for CD3 (hamster anti-mouse CD3, BD Biosciences), Egr-1 (rabbit anti-mouse Egr-1, Santa Cruz Biotechnology), and inducible nitric oxide synthase (polyclonal rabbit anti-mouse iNOS, Transduction Laboratories). Quenching of endogenous peroxidase was accomplished with 0.3% H2O2, and nonspecific binding was blocked with 10% horse serum. Sections were incubated with primary antibodies for 1 h at room temperature and then with biotinylated secondary antibodies for 1 h. Enzymatic activity was revealed with a peroxidase-conjugated avidin–biotin complex and diaminobenzidine. Stained sections were counterstained with 0.1% methyl green and mounted in DPX. Immunohistochemical sections were scored by two independent observers corresponding with airway wall thickening. Significant hypertrophy and thickening of the epithelial layer were associated with luminal encroachment with maximal occlusion seen 1 wk after transplantation. Magnification is ×400.

Histopathological evaluation. Frozen sections were cut 5 μm thick and placed on glass slides (Fisher Scientific), after which sections were air-dried, fixed for 15 min in acetone at 4°C, and stored at −80°C until the time of analysis. Histochemical staining was performed for elastin (Accustain, Sigma-Aldrich) to help delimit subepithelial–epithelial boundaries and facilitate morphometric measurements of graft luminal narrowing. Morphometric measurements of cross-sectional area were performed by blindly tracing both epithelial and subepithelial areas using a computer-assisted image analysis system (AxeoCamHR; Carl Zeiss Microimaging, Thornwood, NY). These methods have been previously reported (23, 24).

Graphs. Epithelial and subepithelial cross-sectional areas were determined using a computer-assisted image analysis system (AxeoCamHR; Carl Zeiss Microimaging, Thornwood, NY). Data are presented as fold induction of Egr-1 mRNA in WT allografts normalized to β-actin compared with WT isografts.

Fig. 1. Time course of graft luminal narrowing is displayed. Representative histology (Van Gieson stainings) and morphometric analysis for each of the indicated time points are shown. Significant mononuclear cell infiltration was seen in both the epithelial and subepithelial layers corresponding with airway wall thickening. Significant hypertrophy and thickening of the epithelial layer were associated with luminal encroachment with maximal occlusion seen 1 wk after transplantation. Magnification is ×400. #P < 0.05 vs. day 1. Data represent analysis of 4–6 transplantations per group.
10% normal serum derived from the host species in which the secondary antibody was prepared. Sections were then incubated with a suitable concentration of the primary antibody. Immunodetection was performed by biotinylated secondary antibody incubation followed by Vectastain ABC kit (Vector Laboratories). 3,3'-Diaminobenzidine (BD Biosciences) was used as the developing reagent followed by a hematoxylin counterstain or elastin staining. Quantitative analysis of T cell infiltration was performed by manually counting the number of CD3-positive cells under high-power magnified fields.

Cytokine assays. Serum levels of IL-1β and MCP-1/JE were measured using commercial ELISA kits according to the instructions of the manufacturers (Quantikine; R&D Systems, Minneapolis, MN).

Statistics. All statistical comparisons were performed using a commercially available statistical package for the Macintosh personal computer (Stat View J-5.0, SAS Institute). Student’s t-tests were used to determine P values when comparing two groups. ANOVA with a post hoc Bonferroni test was used to compare conditions between groups. Values are expressed as means ± SE with differences considered statistically significant at P < 0.05.

RESULTS

Time course of graft histopathology. Histopathological evaluation of tracheas transplanted orthotopically in an allogenic milieu (C57BL/6 trachea implanted into B10.A) harvested at days 1, 3, 7, and 10 after transplantation revealed the sequential change of airway allograft rejection. Maximal infiltration of mononuclear cells was demonstrated in both the epithelial and subepithelial layers at 1 wk after transplantation (Fig. 1). Hypertrophy and thickening of the epithelial layer leading to significant luminal encroachment was demonstrated by morphometric analysis in allografts compared with isografts.

Expression of Egr-1 mRNA and protein. To quantify the levels of mRNA for Egr-1 in airway grafts, real-time PCR analyses were performed on graft tissue extracted at day 7, the peak of inflammation and airway luminal narrowing. Normalized Egr-1 levels for mRNA were significantly increased in WT allografts compared with isografts (3.2-fold, P = 0.012; Fig. 2A). Immunohistochemical staining for Egr-1 demonstrated that Egr-1 expression was principally localized in the graft epithelial cells, although there was some increase in fusiform and monocytoïd cells in the subepithelium (Fig. 2B).

Effects of Egr-1 on graft luminal narrowing. To determine the mediating role of Egr-1 on graft luminal narrowing, several sets of experiments were performed. In the first set, Egr-1 gene null allografts were implanted into WT recipient mice with the hypothesis that lack of the Egr-1 gene would mitigate luminal narrowing. Morphometric analyses were performed on isografts (C57BL/6 into C57BL/6), WT allografts (Egr-1+/+ mice into B10.A), and Egr-1 gene null allografts (Egr-1−/− mice into B10.A). These analyses were performed at day 7, the peak of inflammation and airway luminal narrowing. Isograft exhibited no significant cellular infiltration, with epithelial/subepithelium occupying 20 ± 3% of the graft lumen, similar to the volume of this tissue observed in native tracheas. In contrast, WT allografts revealed significant thickening of these airway layers, leading to luminal encroachment more than doubling that of the native tissue (51 ± 4% for allograft, P < 0.0001; Fig. 3A). Concordant with the hypothesis that Egr-1 promotes alloeffector/inflammatory responses, tracheas obtained from Egr-1−/− donors implanted into an allograft milieu demonstrated significant protection from graft luminal narrowing (34 ± 4%) compared with WT allografts (P = 0.0001; Fig. 3A).

To determine whether the site of Egr-1 expression (graft tissue vs. infiltrating cells) is most critical to the observed protection against graft luminal narrowing in Egr-1-null allografts, another set of experiments was performed. This question is particularly relevant with our previous data (24) demonstrating that iNOS expressed in airway graft tissue has a no significant effect on airway rejection, whereas recipient leukocyte-derived iNOS plays a critical exacerbating role. In new experiments, gene-competent (Egr-1+/+) grafts were placed into Egr-1 gene null recipients, or alternatively, Egr-1 null grafts were placed in Egr-1 gene-competent hosts. Contrary to the observation that iNOS expression in the recipient is a prime mover for OB development, whereas graft iNOS status has little effect, Egr-1 status of the donor graft tissue is most important. Allografts taken from Egr-1−/− donor mice exhibited less airway luminal narrowing; however, no significant mitigation of airway rejection was observed when only the recipient was Egr-1 null (Fig. 3B). These differences in pro-
tection conferred by the site of Egr-1 expression could not be ascribed to the reversal of donor and recipient strains (as has been observed in other transplant settings; Ref. 32) because graft luminal narrowing for Egr-1+/H1101/H1101 donors (129xC57BL/6 background) transplanted into B10.A recipients was similar when the reverse background strain combination (B10.A transplanted into Egr-1+/H1101/H1101 recipients) was used. These data indicate that donor Egr-1 status is more critical than recipient Egr-1 status in the pathogenesis of airway rejection.

Quantification of graft mononuclear cell infiltration.

To study the effect of Egr-1 on T cell infiltration, pan-T cell-marked CD3-positive cells were quantified using immunohistochemically stained frozen sections in Egr-1−/− allografts compared with WT allograft. Total CD3-positive cell counts were obtained for an entire section taken from the middle one-third of the tracheal grafts. There were significant differences in the number of infiltrating T cells between WT and Egr-1−/− allografts (number of cells per slice, 1,263 ± 206 vs. 709 ± 136; \( P = 0.0018 \); Fig. 4). These quantitative data demonstrate that numbers of CD3-positive cells directly correlate with the exacerbation of airway luminal narrowing.

Effect of Egr-1 on iNOS expression in tracheal allografts.

Our previous study (24) indicated that iNOS expression in recruited cytoeffector lymphocytes affected the severity of graft rejection. To elucidate the potential link between Egr-1 induction and iNOS expression, we assessed iNOS expression in WT allografts and Egr-1−/− allografts by immunohistochemical staining. Allografts in Egr-1−/− donors exhibited...
Fig. 4. Quantification of graft CD3+ T cell infiltration. A: representative immunohistochemical staining for the pan-T cell marker CD3 (brown; individual T cell demonstrated by arrow). B: quantitative analysis of T (CD3 staining) cell infiltration. Total CD3-positive cell counts were obtained for an entire section taken from the middle one-third of the tracheal graft. Egr-1−/− allografts demonstrated significantly reduced infiltration of T cells compared with WT allografts (number of cell per slice, 709 ± 136 vs. 1,263 ± 206; *P = 0.0018). Each bar represents the means of 5 experiments ± SE. *P < 0.05 vs. isografts; #P < 0.05 vs. WT allografts. All data represent analysis at 1 wk post-transplant. Magnification is ×400.

Reduced iNOS expression (Fig. 5A), restricted to the luminal epithelium. In sharp contrast, strong iNOS immunoreactivity was identified in WT allografts encompassing cells in both the epithelial as well as subepithelial layers (Fig. 5B).

Cytokine expression. Egr-1 is a transcription factor for which promoter motifs are widely distributed in the regulatory elements of a number of inflammatory genes. To determine the effects of Egr-1 absence on the expression of representative known Egr-1 target genes, serum taken from tracheal transplant recipients was examined by ELISA for the presence of cytokines (IL-1β and JE/MCP-1). Data demonstrated significantly lower expression of IL-1β and JE/MCP-1 in serum from WT recipients receiving either isografts or allografts from Egr-1−/− donors compared with allografts from WT (Egr-1+/+) donors (Fig. 6, A and B). Although allotransplantation increased soluble ICAM-1 levels, the presence or absence of Egr-1 did not seem to make a difference for this soluble molecule (data not shown).

DISCUSSION

Lung transplantation is an effective treatment for many lung diseases unresponsive to other therapy. However, long-term survival of recipients is often limited by the development of OB, a fibroproliferative condition affecting small airways that ultimately causes lung dysfunction (2, 9, 17, 35). Histologically, OB develops after an initial period that is marked by an influx of inflammatory cells within airway epithelium (16), recognized as LB (37). LB is thought to be a reversible airway inflammatory process leading to subsequent irreversible airway damage and fibrosis, which are characteristic of OB. These studies used an orthotopic tracheal transplantation model (23, 24) that is characterized by intense mononuclear cell infiltration concomitant with both epithelial cell and subepithelial cell layer thickening, the main pathological features of LB.

The dense lymphocytic infiltrate characteristic of LB has led investigators to consider multiple individual cytokines and chemokines as potential pathogenic effector mechanisms. The approach taken in this manuscript was to examine one master control point that regulates deployment of multiple effector limbs involved in leukocyte trafficking. Egr-1 is an 80- to 82-kDa protein, which is the prototypic member of the early growth response gene family (25). It is a zinc finger transcription factor for which binding domains are present in the promoter regions of multiple inflammatory cytokines, chemokines, and adhesion receptors (36). The Egr-1-driven induction of downstream genes is responsible for the upregulation of numerous inflammatory cascades. To date, most studies have focused on the effect of Egr-1 induction on the pathogenesis of ischemic-reperfusion injury, including in the lungs (29, 36). Although several recent studies suggest an effect of Egr-1 induction on chronic obstructive pulmonary disease (27), chronic atherosclerosis (22), and coronary allograft vasculopathy (28), the role of Egr-1 in chronic airway allograft rejection still remains to be elucidated.

The present study demonstrates that Egr-1 is induced in an allogeneic airway transplantation milieu and contributes substantially to epithelial injury, LB, and loss of the airway lumen. T cell infiltration into the grafts was quantified by counting the number of CD3-positive cells, as both CD4+ and CD8-positive T cells have been demonstrated to play a critical role in the development of chronic airway rejection (14, 15). Data revealed that the lack of graft Egr-1 expression mitigates the infiltration of T cells into the airway graft tissue. Additionally, the levels of inflammatory cytokines that are downstream gene targets of Egr-1 were also suppressed in serum from recipients of allografts lacking the Egr-1 gene.

To ascertain the relative contribution of airway epithelial Egr-1 expression vs. Egr-1 expression in recipient graft-infiltrating leukocytes, histological difference between Egr-1−/− donor tissue transplanted into WT recipients and tissue transplanting in the reciprocal combination (WT airway grafts into allogeneic Egr-1−/− recipients) were histologically evaluated. Results demonstrated significantly decreased (P < 0.05) allograft luminal narrowing when the grafts themselves lacked the Egr-1 gene, implicating a critical contribution of epithelium-derived Egr-1, rather than infiltrating leukocyte-derived Egr-1, in exacerbating of airway rejection. Furthermore, epithelium has been demonstrated to be the primary source of inflammatory cytokines (IL-1β and JE/MCP-1; Refs. 24 and 4), which were found to be suppressed in the serum of Egr-1−/− donor graft recipients.
Previously, it was noted that during the process of airway rejection, upregulation of iNOS occurs in epithelial tissue and graft-infiltrating leukocytes. Transplantation of iNOS null donor or recipient tissue into the reciprocal host indicated that it was iNOS derived primarily from leukocytes that promoted massive lymphocyte influx and apoptosis coinciding with the release of chemotactic mediators and exacerbation of airway luminal narrowing (24). The pathophysiological effects of Egr-1 expression on developing airway rejection appear to contrast with this iNOS data in that it is the Egr-1 status in the graft rather than the Egr-1 status of the recipient that dominates the course of rejection.

The seminal role of Egr-1 in graft tissue as a driver of LB is not entirely unexpected, as Egr-1 induction in airway epithelial cells has been shown to contribute to airway inflammation in other airway remodeling diseases such as asthma (30). Immunohistochemical staining for Egr-1 in our study similarly demonstrated that the main source of Egr-1 expression was the airway epithelial cell. Additionally, we showed that allografts implanted into Egr-1−/− donors exhibited reduced iNOS expression in contrast to strong iNOS immunoreactivity identified in WT allografts. This could be due to two reasons. First, as iNOS has two putative Egr promoter motifs, there could be a direct causal link between upregulated expressions of the two (8). The second possibility is that several studies have suggested that iNOS is at least partly induced by several chemokines that are downstream target genes of Egr-1 (26).

Recently, it was reported that endogenous heme oxygenase-1 (Hmox-1) expression/CO production provides reciprocal protection against OB development by suppressing upregulation of iNOS expression in airway allografts (23). As CO protects ischemic lungs at least in part by suppressing ischemic induction of Egr-1 and its downstream target genes (25), it is possible that the effects of CO to limit OB might be due to its ability to suppress Egr-1 induction in an allograft milieu. Although further studies would be necessary to elucidate the specific mechanistic link between Egr-1 and iNOS induction in the pathogenesis of chronic airway rejection, the current data suggest a model wherein Egr-1 expression is upregulated in allograft tissue, which drives chemokine/cytokine expression, leading to an influx of iNOS-bearing inflammatory cells into the graft and development of chronic airway rejection. It is conceivable that broad-spectrum inhibition of inflammatory gene expression by a strategy of Egr-1 blockade, a transcriptional checkpoint in the regulation of multiple genes, might be more effective than a single anti-
cytokine strategy at preventing or limiting the development of airway rejection.

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