Expression of Egr-1 in Late Stage Emphysema

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The transcription factor early growth response (Egr)-1 is an immediate-early gene product rapidly and transiently expressed after acute tissue injury. In contrast, in this report we demonstrate that lung tissue from patients undergoing lung reduction surgery for advanced emphysema, without clinical or anatomical evidence of acute infection, displays a selective and apparently sustained increase in Egr-1 transcripts and antigen, compared with a broad survey of other genes, including the transcription factor Sp1, whose levels were not significantly altered. Enhanced Egr-1 expression was especially evident in smooth muscle cells of bronchial and vascular walls, in alveolar macrophages, and some vascular endothelium. Gel shift analysis with ³²P-labeled Egr probe showed a band with nuclear extracts from emphysematous lung which was supershifted with antibody to Egr-1. Egr-1 has the capacity to regulate genes relevant to the pathophysiology of emphysema, namely those related to extracellular matrix formation and remodeling, thrombogenesis, and those encoding cytokines/chemokines and growth factors. Thus, we propose that further analysis of Egr-1, which appears to be up-regulated in a sustained fashion in patients with late stage emphysema, may provide insights into the pathogenesis of this destructive pulmonary disease, as well as a new facet in the biology of Egr-1. (*Am J Pathol* 2000, 157:1311–1320)

Early growth response (Egr)-1 is a zinc finger transcription factor^{1,2} associated with “immediate-early responses” because of the rapid and transitory nature of its expression after a spectrum of stimuli ranging from growth factors to physical injury.^{1,3–6} For example, denuding injury to the rat aorta caused rapid expression of Egr-1 and downstream target genes, such as platelet-derived growth factor-B chain, in endothelium at the wound edge,³ and, after angioplasty, up-regulation of Egr-1 in migrating/proliferating smooth muscle cells was observed in rat carotid arteries.⁷ In a study of the response to oxygen depriva-

tion, we found acute hypoxia to induce expression of Egr-1, reaching a maximum by 30 minutes.^{5,6} Similarly brisk Egr-1 expression has been observed in response to renal and cardiac ischemia.^{8–13} Although brief in duration, important physiological effects have been ascribed to such pulses of Egr-1 expression. In this context, Egr-1 was once considered to have a profound influence on macrophage differentiation.^{14,15} However, our understanding of Egr-1 biology has been redefined by the phenotype of homozygous Egr-1 null mice which, under laboratory housing conditions, display only infertility in females due to deficiency of luteinizing hormone- β .^{16,17} Otherwise, unstressed Egr-1-null mice appear normal.

The increasing frequency with which lung reduction surgery is being performed has resulted in the availability of fresh emphysematous tissue for molecular and other analyses. Although this tissue represents only a snapshot of the pathophysiological picture of evolving emphysema, necessarily slanted toward end-stage disease, it provides a useful resource. By hybridization studies with a cDNA expression array, we have identified Egr-1 as a gene product whose expression is selectively enhanced in emphysematous lung, compared with controls. Levels of Egr-1 antigen appear to be increased in a variety of cells, including bronchial epithelium, vascular and bronchial smooth muscle cells, endothelium, and alveolar macrophages. In the apparent absence of an intercurrent infection in the patients with lung disease in our study, the most likely inference is that sustained Egr-1 induction occurs in late stage emphysema. We propose that emphysema may provide a setting to analyze functions of Egr-1 relevant to tissue remodeling and chronic inflammation, new facets of the biology of this transcription factor.

Materials and Methods

Study Population

Emphysematous lung from 28 patients was obtained from the block of grossly diseased tissue excised at the time of lung reduction surgery from the sample directed to the Division of Surgical Pathology. The patients ranged in

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age from 45 to 80 years, all were smokers, and all had severe emphysema ($FEV_1 < 40\%$ of predicted). Six control samples were obtained from patients undergoing excisional procedures for lung nodules, either primary (malignant or benign) or metastatic (ages 45–70 years). The tissue designated as control was distant from the nodule and appeared grossly normal. Two control samples were obtained from brain-dead individuals from whom consent had earlier been obtained for organ harvesting and research. These patients, despite absence of an identifiable cause of death, had suboptimal arterial pO_2 for lung donation likely related to prolonged brain death and associated systemic decompensation.

RNA Purification

Total RNA was purified from freshly excised lung tissue using TRIzol reagent according to the manufacturer's protocol (GIBCO/BRL). The tissue was cut into small pieces, placed in TRIzol, homogenized, and total RNA was purified. Poly A⁺ mRNA was isolated using an oligo-dT column (FastTrack 2.0 Kit from Invitrogen, Carlsbad, CA). In brief, 0.5–1 mg of total RNA resuspended in 10 mmol/L Tris/HCl (pH 7.5) buffer was added to FastTrack lysis buffer (10 ml) containing RNase/protein degrader. The solution was heated to 65°C for 15 minutes, oligo(dT) cellulose was added, and the mixture was incubated for 2 hours at room temperature. The resin was then washed and mRNA eluted. RNA was monitored by absorbance at 260/280 nm (the ratio was generally ≥ 1.8) and agarose gel electrophoresis.

cDNA Expression Array Study

³²P-labeled cDNA probes were prepared by transcribing 1 μ g of each RNA population (poly A⁺ RNA derived from emphysematous or control lung) using reagents purchased from Atlas cDNA Expression Arrays Kit (Clontech, Palo Alto, CA) and [α -³²P]dATP (Amersham, Piscataway, NJ, no. PB 10204; 10 μ Ci/ μ l; 3,000 Ci/mmol). Labeled cDNA probes were separated from unincorporated ³²P-labeled nucleotides and small (<0.1 kb) cDNA fragments, using Chroma Spin-200 DEPC-H₂O columns. Then, equal amounts of ³²P-labeled cDNA probes were separately hybridized for 4 hours at 68°C to duplicate Atlas Array membranes with immobilized cDNA arrays (Clontech, 588 genes). Membranes were subjected to high stringency washing conditions (0.1 $\times$ SSC with 0.5% sodium dodecyl sulfate) for 2 hours at 68°C, and autoradiography was performed at –70°C with Kodak XAR film. Hybridization signals for different poly A⁺ RNA samples were compared using NIH Image software. Four preparations from normal controls and four from emphysema patients were studied using the arrays. Levels of Egr-1, based on hybridization signals, were normalized according to the average levels of four housekeeping genes, ubiquitin, GAPDH, β -actin, and 23-kd highly basic protein, as suggested by the manufacturer.

Northern Blotting

Total cellular RNA (10–20 μ g/sample) was subjected to denaturing gel electrophoresis in 0.8% agarose-formaldehyde gels and transferred to Duralon UV membranes (Stratagene, La Jolla, CA). Membranes were hybridized for 1 hour at 68°C with ³²P-labeled partial cDNAs for human Egr-1 (271–1902CDS; GenBank NM 001964) and Sp1 (human cDNA, 2.1-kb fragment),⁶ and autoradiography was performed as above. Gel-purified DNA fragments were labeled using α -³²P-dCTP with a random primer labeling kit (Stratagene).

Immunohistochemistry

Lung tissue was collected, immediately cut into small pieces, and placed in buffered formalin (10%). Following fixation, tissue was paraffin-embedded and stained for routine histochemistry (H&E) or processed for immunohistochemistry to detect Egr-1 antigen. Lung tissue was washed with phosphate-buffered saline (pH 7.0), fixed in formalin, and embedded in paraffin. Sections were first stained with rabbit anti-Egr-1 IgG (8 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA), and then were incubated with secondary antibody, an affinity-purified peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO). Where indicated, an excess of Egr-1 peptide (Santa Cruz Biotechnology) used as immunogen to generate the anti-Egr-1 antibody was preincubated with tissue sections, and then the primary antibody was added for a further incubation period.

Western Blotting

Small pieces of frozen lung were thawed in RIPA buffer (sodium dodecyl sulfate, 0.1%; sodium deoxycholate, 0.5%; Nonidet P-40, 1%; Tris-HCl, 20 mmol/L, pH 7.5, and NaCl, 150 mmol/L) with freshly added inhibitors (phenylmethylsulfonyl fluoride, 200 μ mol/L; leupeptin, 1 μ g/ml; pepstatin, 1 μ g/ml; and aprotinin, 1 μ g/ml) using 1 ml of ice-cold buffer per gram of tissue. Tissue was further disrupted by dounce homogenization; the temperature was maintained at 4°C throughout the procedure. The mixture was centrifuged at 10,000 $\times g$ for 10 minutes at 4°C, and the supernatant (total tissue lysate) was harvested for further study. Protein concentration was determined by the method of Bradford, lysate (50 μ g) was mixed with 2 $\times$ electrophoresis sample buffer, and electrophoresis was performed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% reduced). Proteins in the gel were transferred to nitrocellulose membranes, and the membranes were blocked by incubation with nonfat milk (10%) at 4°C overnight.¹⁸ Membranes were then exposed to anti-Egr-1-specific antibody (1:2000 dilution; no. 588, Santa Cruz Biotechnology) for 90 minutes at room temperature and washed 6 $\times$ for 10 minutes each using Tris-buffered saline containing Tween-20 (0.05%). Horseradish peroxidase-conjugated secondary antibody was used to detect sites of primary antibody binding, and the enhanced

chemiluminescence method was used. Blots were also incubated with antibody to β -actin purchased from Sigma.

Gel Shift Analysis

Nuclear extracts were prepared from lung tissues stored at -80°C by the procedure of Dignam et al.¹⁹ as modified by Schrieber et al.²⁰ The following double-stranded oligonucleotide probes, purchased from Santa Cruz, were used (only the 5'–3' sequence is shown): Egr consensus sequence (5'-GGATCCAGCGGGGCGAGCGGGGCGA-3'), mutationally inactivated Egr consensus sequence (5'-GGATCCAGCTAGGGCGAGCTAGGGGGA-3'), and Sp1 consensus sequence (5'-ATTCGATCGGGGCGGGGCGAG-3'). Oligonucleotides were radiolabeled using [γ - ^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Promega, Madison, WI). For electrophoretic mobility shift assay (EMSA), reaction mixtures (20 μl) containing nuclear extract (5 μg) and ^{32}P -labeled oligonucleotide probe (50,000 cpm) in gel shift reaction buffer (Promega) were incubated for 20 minutes at room temperature. Supershift experiments were performed by adding anti-Egr-1 antibody (no. 588, Santa Cruz Biotechnology) or the same concentration of nonimmune IgG to reaction mixtures (1 μl antibody per 20 μl of reaction mixture) for 1 hour at 4°C . DNA-protein complexes were resolved by nondenaturing gel electrophoresis on 4% polyacrylamide gels in $0.5\times$ TBE buffer at 120 V at 4°C . Gels were dried and subjected to autoradiography.

Cell Culture

Human lung fibroblasts (HLF; strain CCL-202, American Type Culture Collection, Manassas, VA) were grown in complete medium (minimal essential medium with non-essential amino acids, L-glutamine, and penicillin/streptomycin supplemented with fetal calf serum [10%; GIBCO/BRL]). Human bronchial smooth muscle cells (BSMC) and human pulmonary artery smooth muscle cells (PASMC) were obtained from Clonetics (San Diego, CA). BSMC and PASMC were grown in complete medium (Smooth Muscle Cell Basal Medium [Clonetics], human epidermal growth factor [0.5 ng/ml], insulin [10 $\mu\text{g}/\text{ml}$], human fibroblast growth factor [2 ng/ml], gentamicin [500 $\mu\text{g}/\text{ml}$] and amphotericin B [0.1 $\mu\text{g}/\text{ml}$]) supplemented with 5% fetal calf serum. Confluent cells were washed with phosphate-buffered saline and stimulated in medium containing cytokines, IL-1 α (R&D Systems, Minneapolis, MN), TNF- α (R&D), IFN- γ (Sigma), TGF- β 1 (R&D) or phorbol myristate acetate (PMA; Sigma) alone. Total cellular RNA was extracted from adherent cells and used for Northern blot analysis.

Results

cDNA Expression Array Study of Lung Tissue

cDNA made from poly A⁺ RNA derived from four emphysema patients and four controls was analyzed using the

A

B

C

Figure 1. cDNA expression array analysis of lung from control individuals and emphysema patients. **A–B:** Total RNA was isolated from lung of control or emphysema patients, poly A⁺ RNA was prepared, cDNA was made, labeled with ^{32}P -ATP, and hybridized with a cDNA expression array. **A** and **B** demonstrate representative results of autoradiograms from one control individual and one emphysema patient, respectively. The arrow in **B** indicates the duplicate spots corresponding to Etr103/Egr-1 (each sample was present as a duplicate). A total of four control and four emphysema patients were studied in this manner. **C:** Autoradiograms from the four control and four emphysema patients were analyzed by NIH Image, and results are shown. *, $P = 0.036$; TGF- β 1, transforming growth factor- β 1; Mch4, apoptotic cysteine proteinase; TRAF-2, TNF receptor-associated factor 2; CD11A, integrin α L; IL-13, interleukin-13.

cDNA expression array system. Figure 1 is a representative autoradiogram of one emphysema (Figure 1B) and one control patient (Figure 1A). Visual inspection suggested a strong increase in a cDNA corresponding to

Figure 2. Northern blot analysis of mRNA from emphysematous and control lung for Egr-1 transcripts. Total RNA harvested (20 μ g total RNA/lane) from emphysematous (E; lanes 6–15) or control (C; lanes 1–5) lungs were subjected to Northern blot analysis with 32 P-labeled cDNA for Egr-1 or β -actin.

Etr103 (note arrow in Figure 1A), a gene encoding a zinc finger protein induced during phorbol ester-stimulated differentiation of HL60 cells identical to Egr-1.²¹ This impression was confirmed by image analysis, based on data from all eight individuals (Figure 1C). The relative expression of Egr-1 cDNA (the Egr-1 hybridization signal was normalized based on levels of housekeeping genes; see Methods section) in emphysema patients appeared to be increased ≈ 3.3 -fold compared with controls ($P = 0.036$). In contrast, multiple housekeeping genes, including ubiquitin, phospholipase A2, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), β -actin, and 23-kd highly basic protein, displayed similar levels in the cDNA expression array assay comparing control and emphysema tissue (not shown). Expression of several other potentially relevant genes was also assessed: transforming growth factor (TGF)- β 1 because of its role in fibrotic disorders,²² two apoptosis-related genes, Mch4 (apoptotic cysteine proteinase) and TRAF-2 (TNF receptor-associated factor-2),^{23,24} and interleukin (IL)-13, a cytokine implicated in allergic disorders such as asthma.^{25,26} Although there appeared to be small and variable increases in the hybridization signal for TGF- β 1, Mch4, TRAF-2, and IL-13 in cDNA prepared from poly A⁺ RNA from some samples of emphysematous lung, they did not reach statistical significance (Figure 1C). Note that levels of Sp1 transcripts assessed by this method also did not appear different in emphysema and control patients. Taken together, these results focused our attention on analyzing Egr-1 expression in lung tissue from patients with emphysema.

Northern Blot Analysis of Egr-1 mRNA in Emphysematous Tissue

Total RNA from 28 patients with emphysema and 8 non-emphysematous controls was analyzed by Northern blotting using a labeled cDNA for Egr-1. Figure 2 shows the results with 15 samples (10 emphysema and 5 controls); a strong increase in Egr-1 transcripts in the patients with emphysema (lanes 6–15) is observed compared with the controls (lanes 1–5). When these data were pooled with those obtained from Northern blot analysis of the other samples (18 emphysema and 3 controls), 21/28 samples from emphysema patients showed a strong increase in Egr-1 (Figure 3, lanes 6–15), whereas the others (7/28) showed a lesser or no increase in Egr-1 transcripts. The

A. Western

B. Northern

Figure 3. Comparison of Egr-1 transcripts and antigen in emphysematous lung. **A:** Western blot analysis of lung protein extracted from six different patients with emphysema (50 μ g protein/lane), two demonstrating low levels of Egr-1 antigen (lanes 1–2) and four showing relatively higher levels of Egr-1 (lanes 3–6). Immunoblotting used antibodies to Egr-1 or β -actin. **B:** Northern blotting of total RNA extracted from the same six patients with emphysema (20 μ g RNA/lane). Blots were hybridized with 32 P-labeled cDNAs for Egr-1 or β -actin.

control samples displayed low levels of Egr-1 in 7/8 samples, though one showed higher levels of Egr-1.

It is important to note that the controls for our experiments were not normal (and therefore are not designated "normal controls"). Control tissue was obtained from patients with lung nodules, and the samples used for our analyses were from a portion of lung at the resection margin which appeared to be uninvolved in the pathological process. However, it is difficult to be certain that such lung samples were completely normal. Two samples were obtained from transplant donor candidates whose lungs were rejected due to poor blood gases immediately before organ harvest, although the tissue appeared grossly normal. We initially considered lung tissue obtained at autopsy as an additional source of control material, but the conditions under which it could be procured are markedly different from that for the emphysema samples. Variable and unclearly defined post-mortem times for the autopsy samples introduced a major uncertainty in interpretation of the results. Thus, apparently normal portions of lung from patients with nodules and suboptimal donor candidates seemed to represent a reasonable, though not necessarily ideal, control group.

Increased levels of Egr-1 transcripts in emphysematous lung did not reflect a general enhancement in expression of transcription factors. For example, levels of

Sp1 were virtually the same in total RNA harvested from emphysema and control groups study by Northern blotting (not shown). This is especially relevant since GC-rich DNA binding motifs for Sp1 and Egr-1 can be distributed in an overlapping topology, and it has been shown that increased expression of Egr-1 can displace Sp1 from such sites, thereby modulating gene expression.^{3,27}

Expression of Egr-1 Antigen in Emphysematous Lung

Levels of Egr-1 antigen in lungs of patients with emphysema appeared to be directly proportional to the amount of Egr-1 mRNA. Figure 3, A and B, displays results of Western (Figure 3A) and Northern (Figure 3B) blot analysis in representative emphysema patients with low (lanes 1 and 2) and high (lanes 3–6) levels of Egr-1 expression. It is evident that patients with low levels of Egr-1 transcripts display low levels of Egr-1 antigen (lanes 1 and 2). The latter does not reflect RNA or protein degradation in the samples, as β -actin transcripts and antigen were represented by discrete bands and β -actin was highly expressed in these two individuals (lanes 1 and 2). Emphysema patients whose lung samples displayed high levels of Egr-1 transcripts showed, in parallel, elevated Egr-1 antigen (lanes 3–6).

Sites of Egr-1 expression in emphysematous lung were visualized immunohistologically in freshly procured lung samples fixed in formalin. Six samples from emphysema patients and four controls were analyzed in this fashion, and results in one representative emphysema patient and one control are shown in Figure 4. All patients had severe emphysema; evidence of greatly dilated alveolar airspaces and typical clubbing is observed by H&E staining (Figure 4, A and B). Egr-1 antigen was observed in bronchial epithelium and smooth muscle, and in many peribronchial cells, presumed to be monocyte-macrophages (Figure 4, C and D). Immunoreactive Egr-1 was also seen in alveolar macrophages (Figure 4E). In each case, Egr-1 immunoreactivity appears to be principally cytoplasmic. Similar strong staining for Egr-1 was seen in the other five emphysema patients whose lungs were subjected to immunohistochemical analysis (not shown). When samples were preincubated with the Egr-1 peptide used as immunogen, and then anti-Egr-1 IgG was added, the specific staining pattern was prevented (Figure 4, F–H). In addition, replacement of anti-Egr-1 IgG with nonimmune IgG, also resulted in no staining (Figure 4, I–K). In contrast to these results in lung from emphysema patients, lungs from controls (Figure 4M) displayed virtually no detectable Egr-1 under the same conditions in a sample from a control individual (Figure 4L). Similar results were obtained when tissue from three other control individuals was analyzed (not shown).

To ascertain whether Egr-1 antigen in emphysematous lung had sequence specific DNA binding activity, EMSA was performed (Figure 5). Nuclear extracts from a patient with emphysema and a high level of Egr-1 transcripts demonstrated a strong gel shift band (lane 1) with consensus ³²P-labeled Egr oligonucleotide probe, which

was not observed with mutationally inactivated Egr probe (lane 2). Supershift experiments were performed to determine whether the gel shift band observed with consensus Egr oligonucleotide probe and nuclear extracts from the patient with emphysema represented interaction with Egr-1: addition of anti-Egr-1 IgG to the reaction mixture resulted in a supershift (lane 3), whereas the same concentration of nonimmune IgG was without effect (lane 4). Further evidence for the specificity of protein-DNA interaction was shown by competition experiments in which 100-fold excess of unlabeled Egr probe blocked appearance of the gel shift band (lane 5) whereas similar experiments with unlabeled Sp1 were without effect (lane 6). Although these results provide strong support for the concept that Egr-1 might direct transcription in emphysematous lung, when nuclear extracts from five patients were studied, only two samples provided strong gel shift bands (as those shown in Figure 5), whereas the other three produced small to no increases in the gel shift band (though high levels of Egr-1 antigen were present). This apparent discrepancy between the presence of Egr-1 antigen and its activity in EMSA might be explained by the tissue from which the nuclear extracts were prepared. The latter extracts were prepared from frozen lung, and we have subsequently found that storage of lung at -80°C for intervals of weeks to months (in this case, up to 2 months) often results in loss of activity in the gel shift assay, although antigenicity by immunoblotting is maintained.

Expression of Egr-1 mRNA in Cultured Cells

In view of the expression of Egr-1 in a range of cells in emphysematous lung, experiments were performed to determine the potential of some of these cells to produce this transcription factor under basal conditions and in response to stimulation in cell culture. Human lung fibroblasts (HLF), human bronchial smooth muscle cells (BSMC), and human pulmonary artery smooth muscle cells (PASM) displayed low levels of Egr-1 transcripts under quiescent conditions in cell culture. After exposure of these cultures to a generic stimulus, PMA (50 ng/ml), there was a striking, time-dependent increase in the level of Egr-1 transcripts in each of these cell types (Figure 6A). Egr-1 mRNA was strongly up-regulated in HLF, PASM, and BSMC after 60 minutes of incubation with phorbol ester. In HLF and PASM, the level of transcripts was reduced by 120 minutes, whereas Egr-1 transcripts were more sustained in BSMC. Cultured cells were also stimulated with cytokines likely to be present in the inflammatory environment of emphysematous lung at various stages of the disease, including IL-1 α , tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and transforming growth factor (TGF)- β 1. Addition of IL-1 α , TNF- α , and TGF β 1, individually and together (combinations of IL-1 α + TNF α , IL-1 α + TGF β 1, and TNF α + TGF β 1) to BSMC (Figure 6C) resulted in robust induction of Egr-1 transcripts that was clearly sustained at the 2-hour time point, especially compared with results on PASM and HLF (see Figure 6, D and E). Cultured PASM (Figure 6D) and HLF (Figure 6E) exposed to the same combinations of

Figure 4. Immunohistological localization of Egr-1 in emphysematous (A–K) and control lung (L–M). Formalin-fixed, paraffin-embedded lung tissue was subjected to H&E staining (A and M) or immunostaining with anti-Egr-1 IgG alone (C–E, L), anti-Egr-1 IgG with blocking Egr-1 blocking peptide (F–H) or nonimmune IgG (I–K). Original magnifications, $\times 100$ (A–B, M), $\times 200$ (C–D, F–G, I–J, L), and $\times 400$ (E, H, K).

cytokines displayed prominent up-regulation of Egr-1 transcripts, though the level was clearly reduced by the 2-hour time point. Experiments with IFN- γ demonstrated that this cytokine also induced Egr-1 transcripts, with maximal levels of Egr-1 mRNA by 60 minutes for each of the three cell types (Figure 6B). Although levels of steady-state Egr-1 mRNA had clearly declined by 2 hours, the most persistent elevation was again seen in BSMC. These data indicate the responsiveness of cultured cells derived from the lung for expression of Egr-1 after exposure to cytokines. In the case of BSMC, up-regulation of Egr-1 appeared to have a somewhat longer time course, in that message levels were still high after 120 minutes.

Discussion

Our data, based on the analysis of lung tissue freshly obtained at the time of lung reduction surgery, indicate

that late stage emphysema provides a sustained stimulus in a majority of patients ($\approx 75\%$) for induction of Egr-1 in a broad range of lung cells. Compared with the rapid induction and decay of Egr-1 in response to acute injuries or environmental perturbations,^{1,3–6,8–12} it seems that Egr-1 expression is much more prolonged in emphysema. Consistent with these *in vivo* data, treatment of cultured lung-derived human fibroblasts and bronchial and pulmonary artery smooth muscle cells with a cocktail of cytokines resulted in enhanced Egr-1 transcripts. These data suggest the relevance of examining the contribution of Egr-1 to the biology of lung remodeling, including the active airway destruction and fibrosis characteristic of the earlier course of emphysema.

Because our studies involved analysis of only 28 patients with late stage emphysema, the relevance of these data to earlier stages of disease pathogenesis remains uncertain. This may be why it was difficult to identify increased expression of genes thought to be regulated

Figure 5. EMSA on nuclear extract from emphysematous lung. Nuclear extracts were obtained from emphysematous lung, incubated with wild-type/consensus 32 P-labeled Egr probe (lane 1) or mutationally-inactivated (MtEgr; lane 2) 32 P-labeled Egr probe, and subjected to nondenaturing PAGE. Where indicated, either anti-Egr-1 IgG (lane 3) or nonimmune (NI) IgG (lane 4; concentration of anti-Egr-1 IgG and nonimmune IgG were the same), or an 100-fold molar excess of Egr (cold Egr; lane 5) or Sp1 (cold Sp1; lane 6) was added to nuclear extracts and consensus 32 P-labeled Egr probe. The lower arrow indicates migration of the band corresponding to Egr-1-DNA complex. The upper arrow indicates migration of the supershift band in the presence of anti-Egr-1 IgG.

by Egr-1 in previous *in vitro* studies.⁴ Using cDNA expression arrays and the patient samples, enhanced expression of several genes thought (based on *in vitro* evidence) to be regulated by Egr-1 was not found. For example, evaluation of cDNA derived from lung RNA did not reveal increases in transcripts for TGF- β 1,²⁸ CD44,²⁹ TNF- α ,³⁰ macrophage-colony stimulating factor-1 (M-CSF),³¹ PDGF-A or -B,^{32,33} ICAM-1,³⁴ and NF- κ B p105³⁵ in emphysematous lung. There are several possible explanations for these results: 1) more sensitive methods are required to accurately detect possibly subtle changes in

Figure 6. Expression of Egr-1 in cultured human lung fibroblasts (HLF), human bronchial smooth muscle cells (BSMC), and human pulmonary artery smooth muscle cells (PASMC). Cultures were washed in phosphate-buffered saline, and fresh medium only or with either PMA (50 ng/ml) alone (A), IFN- γ [50 ng/ml] alone (B), or the indicated combination of cytokines (IL-1 α [5 ng/ml], TNF- α [5 ng/ml], TGF- β 1 [5 ng/ml]) (C-E) was incubated with cultures for up to 2 hours at 37°C. Total RNA (10 μ g/lane) was harvested for Northern blot analysis with 32 P-labeled cDNAs for Egr-1 and β -actin. This experiment is representative of two repetitions.

low abundance transcripts, such as those indicated above; 2) Egr-1-regulated genes are not expressed at this late stage of emphysema, despite high levels of the transcription factor; 3) other, yet to be identified, genes under control of Egr-1 are involved; 4) corepressors of Egr-1, especially NAB2, are also induced at high levels in emphysematous lung;³⁶ 5) Egr-1 has principally a re-

pressive effect on gene expression under these conditions; and 6) Egr-1 in emphysematous lung is not transcriptionally active.

For genes traditionally associated with Egr-1, based on studies *in vitro*,⁴ these data must be confirmed *in vivo*. To the best of our knowledge, only three genes have been shown to be regulated by Egr-1 *in vivo* by study of the Egr-1 knockouts: luteinizing hormone- β in females,¹⁷ apo A-1,³⁷ and tissue factor.⁶ Thus, although the Egr-1 response element is present in a multitude of genes and its contribution to gene expression can be dissected by manipulation of environmental conditions in culture, it is possible that other factors predominate *in vivo*. In this context, it is likely that genes may prove to be regulated by Egr-1 *in vivo* that have been overlooked by *in vitro* analyses. Egr-1-null animals subject to a range of stresses clearly provide a rational experimental system in which to perform such studies. Finally, it is important to note that assessment of Egr-1-regulated genes by analysis of total RNA extracted from lung, or cDNA produced from total lung mRNA, principally detects striking changes in the expression of transcripts of relatively greater abundance. In view of the likelihood that focal changes in gene expression will occur in emphysema, especially earlier in the disease, careful *in situ* hybridization and immunohistochemical studies will be necessary for sensitive identification of such induced transcripts and the transcribed proteins. However, at this point, candidate Egr-1-regulated genes for this more in-depth analysis remain to be identified.

Two important issues should be clarified with respect to our data pertaining to the potential transcriptional competency of Egr-1 extracted from the lung tissue and concerning the control group for our study. In other work performed after the current study was completed, we have found that to reproducibly maintain sequence-specific DNA binding activity of Egr-1 extracted from emphysematous lung tissue, it is necessary to prepare the nuclear extracts immediately and to minimize storage time at -80°C . We believe this is the reason that the DNA binding activity detected in our samples with increased amounts of Egr-1 antigen was variable. However, it remains possible that the Egr-1 produced in emphysematous lung is not fully competent in terms of its transcriptional activity, possibly reflecting events at the level of protein phosphorylation.³⁸ This would also explain the principally cytoplasmic, rather than nuclear, distribution of Egr-1, and the lack of expression of Egr-1-regulated genes. Our next approach to this question will be to perform studies on optimally prepared nuclear extracts from lung samples. This cannot be decided with the current samples because of their extended time in storage and the limited amounts of remaining material. In terms of our control group, apparently uninvolved lung at the margins of resected nodules or samples from donors which proved unacceptable for clinical lung transplantation represent nonideal normal tissue to serve as the basis for comparison. However, these samples are remarkably similar to those harvested from emphysema patients in that they are freshly obtained from a living patient. Clearly, procuring the best normal control tissue

for this type of study will always have to be somewhat of a compromise, as it is not possible to obtain tissue from healthy, normal volunteers.

Despite these reservations, our study is the first to report increased expression of Egr-1 in patients with late stage emphysema. Our observations should serve as a stimulus for additional studies using a range of lung samples to determine whether Egr-1 is expressed earlier in the disease, if genes presumably regulated by Egr-1 are responsive, and, ultimately, to analyze whether Egr-1 contributes to the pathogenesis or progression of this destructive pulmonary disease. Induction of Egr-1 in lung-derived cultured lung fibroblasts and smooth muscle cells by a range of cytokines, including IL-1, TNF- α , IFN- γ , and TGF- β 1, which could potentially contribute to airway damage and fibrosis at different stages of chronic lung disease, suggests that induction of Egr-1 may be relevant to the pathology of this disorder.

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