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Increased Expression of Basic Fibroblast Growth Factor in Hyperoxic-Injured Mouse Lung

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Abstract Basic fibroblast growth factor (bFGF) is a mitogenic polypeptide for a wide variety of cell types and has been immunolocalized in the rodent and human lung. We investigated the mRNA and protein expression of bFGF in hyperoxic-injured adult mouse lungs using northern blot analysis and immunohistochemistry. Mice (6–8 weeks) were continuously exposed to 80% oxygen up to 4 days. Levels of bFGF mRNA were increased from room air control on days 3 and 4 of hyperoxia. mRNA levels of acidic fibroblast growth factor (aFGF), fibronectin, and transin/stromelysin were also examined in this injury model. Similar to bFGF, the fibronectin and transin/stromelysin mRNA levels were increased after 3 days of hyperoxia. In contrast, the aFGF mRNA levels were gradually reduced on each day of hyperoxia. A rabbit polyclonal anti-bFGF antibody was used to determine the distribution and levels of expression in the hyperoxic-injured lungs. The room air control and day 1 hyperoxic-exposed lungs exhibited staining for bFGF in the basement membranes of the blood vessels, airways, and alveoli. Patchy but intense alveolar staining was prominent on day 4 of hyperoxia. The bFGF immunoreactivity of blood vessels and airways was unaffected by the hyperoxia exposure. These results suggest that bFGF may play a role in the alveolar response to hyperoxic-induced injury by virtue of the altered mRNA levels and protein distribution in this injury model.

Key words: acute lung injury, basic fibroblast growth factor, fibronectin, transin, stromelysin

There is increasing evidence that polypeptide growth factors play a central role in lung injury and repair by regulating cellular proliferation, migration, function, and extracellular matrix production [Kelley, 1990]. Both platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) have been localized by immunohistologic and in situ hybridization techniques to fibrotic areas in lung from patients with idiopathic pulmonary fibrosis (IPF) [Antoniades et al., 1992; Broekelmann et al., 1991]. Growth factor expression in lung has also been shown to be altered during hyperoxic exposure of adult rats and premature baboons [Fabisiak et al., 1989; Jones et al., 1987]. Basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor (aFGF) are both members of the heparin-binding growth factor family. aFGF and bFGF have 55% amino acid homology, and both have been isolated from a variety of tissues [Burgess and Maciag, 1989]. Both peptides are angiogenic and are mitogens for a wide range of cell types including fibroblasts, smooth muscle cells, endothelial cells, and epithelial cells. Recently, using immunohistochemical methods, bFGF has been localized in the lung of fetal, neonatal, and adult rats, as well as the large airways and blood vessels in the adult human lung [Han et al., 1992; Sannes et al., 1992; Cordon-Cardo et al., 1990]. Increased levels of bFGF-like activity has been identified in lavage fluid from patients with acute lung injury [Henke et al., 1991]. The adult mouse lung develops acute endothelial and epithelial cell injury as a result of hyperoxic exposure [Frank et al., 1978; Smith, 1985]. We investigated the expression of bFGF in the mouse during hyperoxic-induced lung injury to determine if it has a potential role in the lung's response to acute lung injury.

The cellular response to a growth factor often will often depend on the ECM context [Nathan and Sporn, 1991]. bFGF is stored in the ECM and it is hypothesized that this peptide is released and biologically active during periods of tissue remodeling [Baird and Ling, 1987; Folkman et al., 1988]. Therefore we also investigated

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the mRNA expression of a matrix glycoprotein, fibronectin (FN), and the metalloproteinase, transin (the rat homologue to human stromelysin), in hyperoxic-injured adult mouse lungs. These two ECM components are altered during periods of tissue inflammation and ECM turnover [Crouch, 1990; Case et al., 1989].

METHODS

Animals and Exposures

Adult C57BL mice (8–10 weeks) (Simonsen Laboratories, Gilroy, CA) were housed in the Oregon Health Science University animal care facilities and treated in accordance with NIH guidelines. All animals were provided food and water ad libitum and were kept on a 12 h day/night schedule at an ambient temperature of 22°C. To examine the effects of hyperoxia, the mice were continuously exposed to humidified 80% oxygen. Animals were sacrificed after 0, 1, 2, 3, and 4 days of hyperoxia. The lungs were carefully dissected, removing the large vessels and bronchi at the hilum. The lungs were then snap-frozen in liquid nitrogen and stored in liquid nitrogen until the RNA was extracted for northern blot analysis. For the immunohistochemistry studies, the lungs and heart were removed en bloc, gently inflated with 10% neutral buffered formalin (NBF), and then placed in 10% NBF for 1–2 days. The left lung was then dissected and routinely processed for paraffin embedding.

RNA Extraction

Total RNA was extracted from whole lungs for each time point with a modified LiCl/urea isolation procedure based on methods of Auffray and Rougeon [Auffray and Rougeon, 1980]. Briefly, the frozen lungs were ground in liquid nitrogen with a mortar and pestle. The frozen tissue was then mixed with LiCl/urea lysis buffer (50.8 g LiCl, 144 g urea, 0.54 g Na acetate, and 0.08 g heparin, in diethyl pyrocarbonate (DEPC) treated water to a final volume of 400 ml; 10 ml buffer/gm tissue) and homogenized with a polytron (Brinkman, Luzern, Switzerland). The homogenate was incubated overnight at 4°C and then centrifuged at 12,000*g* for 20 min. The pellet fraction was resuspended in lysis buffer and centrifuged again at 12,000*g* for 20 min. The pellet was resuspended in a 10 mM Tris buffer, pH 7.5, heated to 65°C for 5 min, and then cooled quickly on ice. This suspension underwent a series of phenol/chloroform extractions, and then the RNA was ethanol precipitated and dissolved in DEPC-treated water. The amount of RNA recovered was determined by absorbance at 260 nm.

cDNA Plasmids/Probes

The human bFGF and human aFGF cDNA plasmids were a generous gift of Dr. Judith Abraham, California Biotechnology, Inc., San Francisco, CA. The rat fibronectin cDNA plasmid was generously provided by Dr. Richard Hynes, Massachusetts Institute of Technology, Cambridge, MA. The rat transin cDNA plasmid cDNA was a gift of Dr. Bruce Magun, Oregon Health Sciences University, Portland, OR. The rat cyclophilin cDNA plasmid (1B15) was a gift from Dr. James Douglass, Vollum Institute, Oregon Health Sciences University, Portland, OR. ^{32}P-cDNA probes for FN and transin were prepared with a random hexamer priming kit (Pharmacia, Piscataway, NJ). The ^{32}P-cRNA probes for aFGF, bFGF, and cyclophilin were prepared with a riboprobe transcription kit (Promega, Madison, WI).

Northern Blot Analysis

Total RNA was denatured and electrophoresed (20 μg per lane) through 1% agarose/formaldehyde denaturing gels with 0.25 μg/ml of ethidium bromide [Davis et al., 1986]. The RNA was transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) by capillary action in the presence of 10× SSC (1.5 M sodium chloride and 0.15 M sodium citrate). The Nytran blot was then baked at 80°C for 2 h. The blots were then prehybridized overnight in a buffer consisting of 50% formamide, 0.1% SDS, 250 μg/ml heat-denatured herring sperm DNA (U.S. Biochemical Corp., Cleveland, OH), 10× Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% BSA in DEPC-treated water), 50 μg/ml polyadenylic acid (Pharmacia, Piscataway, NJ), and 5× SSC at 43°C for cDNA probes or 60°C for cRNA probes. The prehybridization buffers were then removed and replaced with fresh buffer supplemented with the ^{32}P-labeled cDNA/cRNA probes. The blots were incubated for an additional 16–20 h at the appropriate temperature. Blots incubated with cDNA probes were washed in 2× SSC + 0.5% SDS (15 min, three times) at 60°C and 1× SSC + 0.5% SDS (15 min, three times) at 60°C. Blots incubated with cRNA probes were washed in 5× SSC

(10 min, three times) at 62°C, 0.1× SSC + 5 mM EDTA + 0.1% SDS at 62°C (120 min), and 0.1× SSC for 10 min at 62°C. Blots were then autoradiographed at −70°C using Kodak XAR-5 film against an intensifying screen for 1–5 days. The size of RNA transcripts was determined according to the position relative to ribosomal RNA and RNA standards (Promega, Madison, WI). The results were quantified with a GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). The probes were removed from the blots by washing in 50% formamide/6× SSPE (1× SSPE = 0.18 M NaCl, 10 mM $NaPO_4$, pH 7.7, and 1 mM EDTA) at 65°C for 30 min and rinsing in 2× SSPE. To verify that approximately equal quantities of RNA were in each lane blots were then reprobed for the constitutively expressed cyclophilin mRNA [Danielson et al., 1988]. New blots were used to probe for bFGF, aFGF, transin/stromelysin, and fibronectin to optimize hybridization efficiency.

Immunohistochemistry

Five micron sections from the paraffin-embedded lungs were cut and mounted on poly-1-lysine-coated slides and stored at room temperature until stained. Sections were deparaffinized with xylene, hydrated in graded concentrations of ethanol and Tris-buffered saline (50 mM Tris, 0.15 M NaCl, pH 7.5; TBS). The sections were digested with 2 mg/ml hyaluronidase (H-6254, Sigma Chemical Co., St. Louis, MO) in 0.1 M acetate buffer, pH 5.2, for 30 min at room temperature. The sections were rinsed with deionized water and then washed three times with TBS. Non-specific binding sites were blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA.), 0.1% BSA, and 0.3% Triton X-100 in TBS for 30 min. The sections were incubated overnight at 4°C in a humidified chamber with a 1:500 dilution (1 mg IgG/ml) of a rabbit polyclonal anti-bFGF antibody (BT-583, Biomedical Technologies, Inc., Stoughton, MA). According to the manufacturer, the antibody is specific for bFGF and does not cross react with aFGF. Normal rabbit serum IgG, 1:1,000 dilution (2 mg/ml, Vector Laboratories, Burlingame, CA), was used for a negative control antibody. Subsequent steps were carried out at room temperature. The tissue sections were washed three times in TBS and incubated with a biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA). The sections were then washed three times in TBS and incubated with an avidin-biotin-alkaline phosphatase complex for 1 h (Vectastain ABC-AP Kit, Vector Laboratories, Burlingame, CA). The sections were washed three times in TBS and developed using Fast Red (BioGenex Laboratories, San Ramon, CA) with levamisole (Vector Laboratories, Burlingame, CA). The levamisole was included to block endogenous alkaline phosphatase. The tissue sections were counterstained with hematoxylin and covered with Crystal/Mount (Biomeda, Foster City, CA).

RESULTS

Northern Blot Analysis

The adult mouse develops both endothelial and epithelial cell injury in response to hyperoxia [Smith, 1985]. To determine if acute injury elicits an alteration in bFGF expression, northern blot analysis was performed on total RNA isolated from normal and hyperoxic-injured adult mouse lungs (Fig. 1). The normal adult mouse lung had unmeasurable levels of bFGF mRNA but the expression of bFGF mRNA gradually increased on each day of hyperoxia, approximately a 4-fold increase from day 1 to day 4, as measured by scanning densitometry. The major bFGF transcript was approximately 7.0 kb, and upon much longer exposures of the autoradiograph a minor 3.7 kb transcript was visble in lane 4. These transcripts for bFGF are similar in size to those obtained in our laboratory from rabbit retinal tissue and human synoviocytes [Planck et al., 1992; Melnyk et al., 1990]. For comparison with bFGF, we also examined the hyperoxic-injured mouse lungs for aFGF mRNA expression. Unlike bFGF mRNA, the aFGF mRNA was readably detectible in normal mouse lung and its levels are gradually reduced on each day of hyperoxia, with day 4 levels being reduced approximately 10-fold from basal levels. Transcripts were visualized at approximately 4.4 and 3.3 kb for aFGF (Fig. 2).

As a measurement of tissue injury and remodeling in this model, we then examined both FN and transin mRNA levels. We chose to examine FN because of its correlation with lung injury in humans and animals [Crouch, 1990]. We examined the expression of transin/stromelysin because its broad pH optima and substrate specificity makes it a potentially important proteinase in the lung [Woessner, 1991; Murphy and Docherty, 1992]. This proteinase has been shown to be altered during the inflammation and ECM

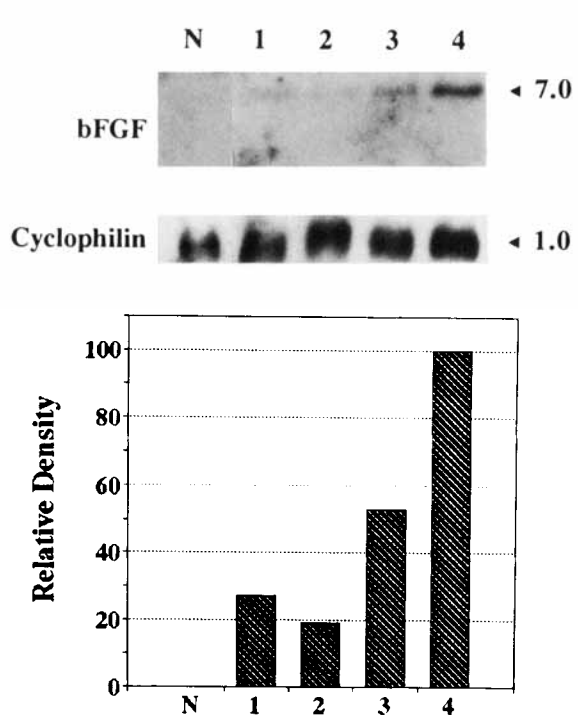

Fig. 1. Upper panel: Autoradiograph of a representative northern blot hybridized with ^{32}P-labeled human cRNA for bFGF. Total RNA was isolated from whole mouse lungs after continuous exposure to 80% oxygen. Lane N is normal room air control lung mRNA and lanes 1 through 4 have mRNA from the indicated days of hyperoxic exposure. The major transcript for bFGF mRNA was identified at 7.0 kb. The autoradiograph was exposed for 4 days. The same blot was stripped and reprobed with ^{32}P-labeled rat cDNA probe for the constitutively expressed cyclophillin mRNA at 1.0 kb. **Lower panel:** Relative abundance of bFGF mRNA after hyperoxic exposure. Bands in the autoradiographs from the upper panel were quantified with a GS 300 scanning densitometer. The bFGF results were normalized to the cyclophilin levels and plotted as the percent of maximum. Experimental n = 3.

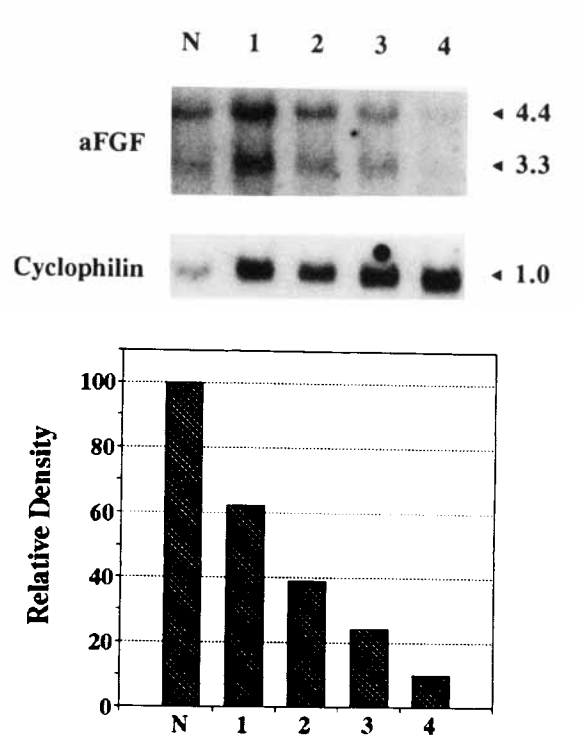

Fig. 2. Upper panel: Autoradiograph of a representative northern blot hybridized with ^{32}P-labeled human cRNA for aFGF. The experimental design was as in Figure 1. Lane N is normal room air control lung mRNA and lanes 1 through 4 have mRNA from the indicated days of hyperoxic exposure. The major transcripts for aFGF were identified at approximately 4.4 kb and 3.3 kb. The autoradiograph was exposed for 3 days. The same blot was reprobed for cylophillin mRNA. **Lower panel:** The relative abundance of aFGF mRNA after hyperoxic exposure. Densitometric readings of aFGF bands in the autoradiographs from the upper panel were normalized to the cyclophilin levels and plotted as the percent of maximum. Experimental n = 3.

turnover accompanying erosive arthritis in rats [Case et al., 1989]. Low levels of the FN transcript were noted in normal mouse lung and day 1 and 2 hyperoxia-exposed lungs (Fig. 3). FN expression significantly increased after 3 and 4 days of hyperoxic exposure. The transin/stromelysin mRNA was barely detectable in normal lung and day 1 and 2 hyperoxia-exposed lungs. Similar to the FN, the transin/stromelysin mRNA levels dramatically increased on days 3 and 4 of hyperoxia (10-fold) (Fig. 4).

Immunohistochemistry

We used a rabbit polyclonal anti-bFGF antibody to immunolocalize bFGF in normal and hyperoxic-injured mouse lungs. In the normal adult mouse lung, we found light to moderate immunoreactivity for bFGF in the basement membranes of the bronchial airways and alveolar septae and strong staining for bFGF in basement membranes of blood vessels (Fig. 5a). Light immunoreactivity was exhibited in the smooth muscle layer of large arteries and airways. The pattern of immunostaining of day 1 hyperoxic-injured lungs was similar to normal lungs. After 2 days of hyperoxia exposure the alveolar immunoreactivity for bFGF became more intense, but this enhanced staining was much more patchy in distribution (Fig. 5b). This pattern of patchy, but strong, immunoreactivity for bFGF in the alveolar regions of the lung continued on days 3 and 4 of hyperoxia. By day 4, the staining for bFGF appeared not only in the alveolar basement membranes but also in cellular regions of the alveolar septae (Fig. 5c,d). Some bFGF immunoreactivity is seen over the tips of septae in the alveolar ducts. The bFGF immunoreactivity of blood vessels and airways was unaffected by

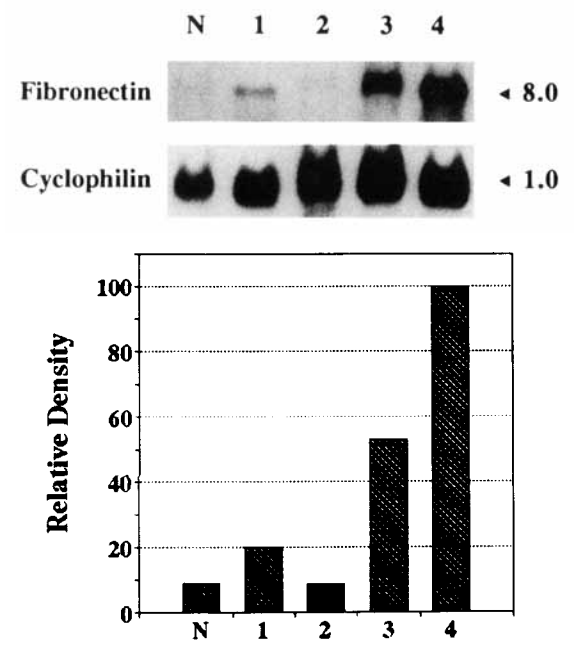

Fig. 3. **Upper panel:** Autoradiograph of a representative northern blot hybridized with ^{32}P-labeled rat cDNA probe for fibronectin. The experimental design was as in Figure 1. Lane N is normal room air control lung mRNA and lanes 1 through 4 have mRNA from the indicated days of hyperoxic exposure. The major transcript of was identified at approximately 7.5–8.0 kb. The autoradiograph was exposed for 1 day. The same blot was reprobed for cyclophilin. **Lower panel:** The relative abundance of fibronectin mRNA after hyperoxic exposure. Densitometric readings of fibronectin bands in the autoradiographs from the upper panel were normalized to the cyclophilin levels and plotted as the percent of maximum. Experimental n = 3.

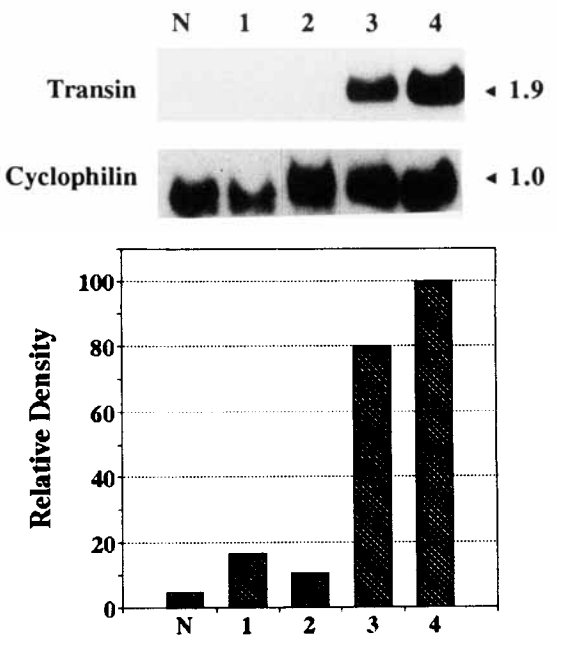

Fig. 4. **Upper panel:** Autoradiograph of a representative northern blot hybridized with ^{32}P-labeled rat cDNA probe for transin/stromelysin. The experimental design was as in Figure 1. Lane N is normal room air control lung mRNA and lanes 1 through 4 have mRNA from the indicated days of hyperoxic exposure. The transcript was visualized at 1.9 kb. The autoradiograph was exposed for 1 day. The same blot was reprobed for cyclophilin. **Lower panel:** The relative abundance of transin/stromelysin mRNA after hyperoxic exposure. Densitometric readings of transin/stromelysin bands in the autoradiographs from the upper panel were normalized to the cyclophilin levels and plotted as the percent of maximum. Experimental n = 3.

the hyperoxic exposure. Control sections which were incubated with nonimmune rabbit IgG showed no background staining (Fig. 5e).

DISCUSSION

The hyperoxic-injured adult mouse lung develops entothelial cell and type 1 epithelial cell damage, similar to human lungs [Smith, 1985; Kang et al., 1993]. We have examined the expression of bFGF mRNA and protein in normal and hyperoxic-injured mouse lungs to determine if this growth factor might have a role in the lung's response to hyperoxic-induced injury. The bFGF mRNA expression was undetectable in the normal mouse lung, while bFGF immunoreactivity was found in the basement membranes of the blood vessels, airways, and alveolar septae. This pattern of widely distributed bFGF protein, but difficult to detect mRNA, has been seen in other tissues [Burgess and Maciag, 1989]. Northern blot analysis measures steady-state mRNA levels and is not sufficiently sensitive to detect low levels of mRNA expression. The pattern of bFGF immunoreactivity in normal mouse lung is similar to that seen in normal rat lung in our laboratory using the rabbit polyclonal anti-bFGF (Biomedical Technologies, Inc., Stoughton, MA) and a mouse monoclonal anti-bFGF (148.6.1.1.1., ZymoGenetics, Seattle, WA) [Picou et al., 1993]. The basement membrane distribution of bFGF in the normal rat lung has been demonstrated by Sannes et al. using a commercially available monoclonal antibody to bFGF [Sannes et al., 1992]. Therefore, the pattern of basement membrane immunoreactivity in normal lung has been produced in two species with various antibodies to bFGF. Similar to Sannes et al. [1992] our localization of bFGF required hyaluronidase digestion, suggesting storage in the ECM and a possible source of bFGF which could be enzymatically released during lung injury. Levels of both bFGF mRNA and immunoreactive protein are altered in the mouse lung as a result of hyperoxic-induced injury. bFGF devel-

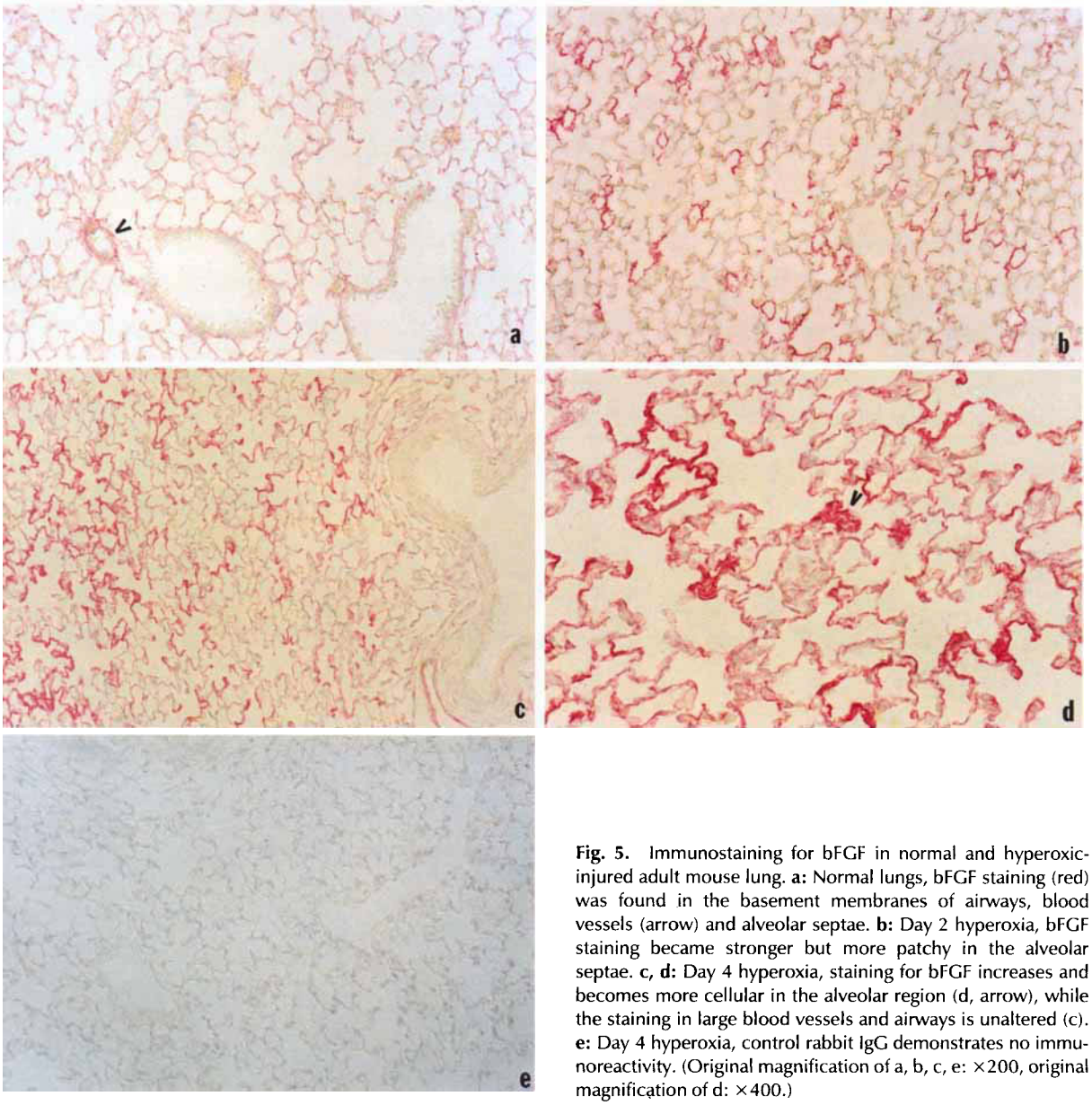

Fig. 5. Immunostaining for bFGF in normal and hyperoxic-injured adult mouse lung. **a:** Normal lungs, bFGF staining (red) was found in the basement membranes of airways, blood vessels (arrow) and alveolar septae. **b:** Day 2 hyperoxia, bFGF staining became stronger but more patchy in the alveolar septae. **c, d:** Day 4 hyperoxia, staining for bFGF increases and becomes more cellular in the alveolar region (d, arrow), while the staining in large blood vessels and airways is unaltered (c). **e:** Day 4 hyperoxia, control rabbit IgG demonstrates no immunoreactivity. (Original magnification of a, b, c, e: ×200, original magnification of d: ×400.)

ops patches of increased immunoreactivity in alveolar regions by 2 days of hyperoxia. Increased mRNA levels of bFGF become apparent by 3 days of hyperoxia. After 4 days, bFGF immunoreactivity becomes more prominent and includes cellular localization in the alveoli and mRNA levels are increased further. The areas of strong immunostaining could be a result of locally increased bFGF synthesis following the rise in mRNA levels. Alternatively bFGF could be relocated from basement membranes which are damaged during the injury process. Nevertheless, our results demonstrate that both bFGF mRNA and immunoreactivity are concurrently altered in this injury model and suggest a role for bFGF in the wound repair response to hyperoxic-induced alveolar injury. In contrast to the bFGF, our results demonstrate that normal mouse lung contains measurable quantities of aFGF mRNA, but these levels are gradually reduced in response to hyperoxic-induced injury. This may indicate a greater biological potential for aFGF in the normal homeostasis of the lung than in lung injury.

The expression of FN and transin transcripts were significantly increased in hyperoxic-injured mouse lungs on days 3 and 4 of hyperoxia. These changes in FN mRNA levels are

consistent with previous studies of lung injury which demonstrated increased expression of FN. FN mRNA has been localized to areas of lung fibrosis in patients with IPF [Broekelmann et al., 1991]. Because of FN's adhesion properties, it may help stabilize disrupted alveolar basement membranes or be a substrate for alveolar and inflammatory cell migration in the injured adult lung [Ruoslahti, 1988]. FN may also enhance the cellular response to growth factors including bFGF [Feige et al., 1989].

We believe this is the first report to demonstrate the presence of transin mRNA in a lung injury model. Our work appears to parallel studies which have demonstrated increased levels of tissue inhibitor of metalloproteinases (TIMP) mRNA in the lungs of adult rabbits exposed to hyperoxia for 96 h, though these investigators were unable to detect the expression of transin mRNA in their studies [Veness-Meehan et al., 1991]. Transin/stromelysin expression may play a role in lung injury and repair because of its ability to digest many ECM components, including type IV collagen, FN, and laminin [Woessner, 1991]. Metalloproteinase activities in conditioned medium have been shown to parallel endogenous bFGF levels from cultured bovine capillary endothelial cells [Tsuboi et al., 1990]. The destruction of the basement membrane components may release bFGF from it ECM storage sites. Stromelysin is secreted from activated alveolar macrophages in vitro and thus a potential source in the injured lung [Welgus et al., 1990]. The bFGF, FN, and transin mRNA levels are simultaneously elevated in the injured adult mouse lung, suggesting a possible relationship between bFGF and these matrix proteins.

bFGF has been isolated from bronchoalveolar lavage fluid from adult humans with acute lung injury and is produced by alveolar macrophages [Henke et al., 1991, 1993]. These investigators hypothesized that bFGF may be responsible for neovascularization found in granulation tissue that is synthesized in the air space of patients who develop fibrosis after ARDS. Expression of FGF receptor-1 mRNA has been observed in the fetal rat and adult human lung [Wanaka et al., 1991; Luqmani et al., 1992]. These data together with our results imply that bFGF has the biological potential to play an important role in the lungs response to acute lung injury, in particular the repair of damaged alveoli.

Further studies to localize bFGF mRNA by in situ hybridization are needed to define sites of synthesis during lung injury and repair (during periods of room air recovery). Eventually, selective inhibition studies will be required to define the biological role of bFGF in normal lung processes as well as the response to injury.

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