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HUMAN GENE THERAPY 15:793–804 (August 2004)

Adenovirus-Mediated Fibroblast Growth Factor 1 Expression in the Lung Induces Epithelial Cell Proliferation: Consequences to Hyperoxic Lung Injury in Rats

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ABSTRACT

High concentrations of oxygen can induce pulmonary toxicity and cause injury to alveolar epithelial and endothelial cells. The present study was performed to determine whether the potent epithelial and endothelial fibroblast growth factor 1 (FGF-1) protected against hyperoxia-induced lung injury. Recombinant adenovirus carrying the gene encoding human secreted FGF-1 (Ad.FGF1) increased the proliferation of lung epithelial cells *in vitro*. Ad.FGF1 or control vector with an empty expression cassette (Ad.V152) was administered intratracheally to Wistar rats. With Ad.FGF1 (10^9, 5×10^9, 10^{10}, or 5×10^{10} viral particles [VP]), FGF-1 protein was found in bronchoalveolar lavage fluid 4 days postinfection at levels proportional to the viral dose and was detected in plasma after doses of 10^{10} VP or more were administered. Histological examination of the lungs showed intense proliferation and apoptosis of alveolar and bronchial epithelial cells, with few inflammatory cells. The alveolar architecture returned to normal within 17 days. Rats pretreated with Ad.FGF1 (10^9 or 5×10^9 VP) 2 days before exposure to hyperoxia (95% O_2) survived, whereas rats pretreated with Ad.V152 died within 3 days. In conclusion, adenovirus-mediated FGF-1 overexpression in the lungs causes epithelial cell proliferation and has beneficial effects in hyperoxic lung injury.

OVERVIEW SUMMARY

To test the effect of fibroblast growth factor 1 (FGF-1) overexpression on lung repair after hyperoxia-induced injury, recombinant adenovirus encoding the human secreted *FGF1* gene (Ad.FGF1) or control vector containing no gene (Ad.V152) was administered intratracheally to Wistar rats. With Ad.FGF1 (10^9 to 5×10^{10} viral particles [VP]), FGF-1 protein was found in bronchoalveolar lavage fluid 4 days postinfection at levels proportional to the viral dose. Histology showed proliferation and apoptosis of alveolar and bronchial epithelial cells, which were marked 4 days postinjection, without major inflammatory cell infiltration. Alveolar architecture returned to normal within 17 days. Rats pretreated with Ad.FGF1 (10^9 or 5×10^9 VP) 2 days before hyperoxia exposure (95% O_2) survived, whereas controls died within 3 days. In conclusion, adenovirus-mediated FGF-1 lung overexpression causes epithelial cell proliferation and has beneficial effects in hyperoxic lung injury.

INTRODUCTION

ALTHOUGH FIRST-GENERATION ADENOVIRUS VECTORS have been found efficient in transferring genes to the lungs, the short duration of gene expression has limited their use in the treatment of chronic diseases (Driskell and Engelhardt, 2003). In acute lung injury (ALI), in contrast, short-lived expression should be an advantage, and one study showed that intratracheally delivered adenoviral vectors were efficient in transfer-

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ring genes to severely injured, edematous lung tissue (Factor *et al*., 2002). Epithelial and endothelial proliferation may be critical to recovery from ALI, and the role of several growth factors in lung inflammation and repair has been extensively investigated. Transferring protective growth factors to the alveolar epithelium improves ALI in experimental models, but the potential effects of adenovirus-mediated transfer of growth factor genes in ALI have not been evaluated (Panos *et al*., 1995; Barazzone *et al*., 1999). Among growth factor families, fibroblast growth factors (FGFs) are expressed in specific spatial and temporal patterns and are involved in developmental and repair processes. FGF-1 has been isolated from a variety of tissues (Burgess *et al*., 1986), including adult rat lung, where it is found in the epithelial and interstitial cells of the alveolar septa, ciliated airway cells, nonciliated bronchiolar cells, smooth muscle cells, and type II pneumocytes (Sannes *et al*., 1992). In addition to being an angiogenic factor that stimulates the growth of endothelial cells, FGF-1 is mitogenic for a wide range of cell types including fibroblasts, smooth muscle cells, and epithelial cells. FGF-1 binds with high affinity to FGF receptors (FGFRs) 1 through 4 but does not seem to bind to FGFR-5 (Kim *et al*., 2001; Sleeman *et al*., 2001). Among FGFs, FGF-1 is the most potent mitogen *in vitro*, irrespective of the FGF receptor it activates. Moreover, FGF-1, together with other FGFs including FGF2 and keratinocyte growth factor (KGF; FGF-7), stimulates DNA synthesis in type II pneumocytes *in vitro* and *in vivo* (Leslie *et al*., 1985, 1990, 1997; Panos *et al*., 1993; Ulich *et al*., 1994).

FGF-1 overexpression may promote alveolar repair after ALI via its potent mitogenic effect on several cell types and its ability to stimulate the growth of both epithelial cells and microvascular endothelial cells. We therefore studied the effects of FGF-1 overexpression achieved by adenovirus-mediated gene transfer in a rat model of hyperoxic ALI with predominant damage to alveolar type I and endothelial cells.

MATERIALS AND METHODS

Construction and production of adenoviral vectors

A gene for human secreted FGF-1 (signal peptide FGF-1, sp-FGF-1) was engineered by fusion of the human fibroblast interferon signal peptide (FINsp) and the α-endothelial cell growth factor open reading frame (Jouanneau *et al*., 1991). The open reading frame was cloned under the control of the cytomegalovirus (CMV) promoter (positions -522 to +72) fused to the thymidine kinase 5′ untranslated region (UTR) (positions +51 to +101) and the simian virus 40 (SV40) late poly(A) signal with no intron (Fig. 1). This expression cassette was inserted into the E1 region of human adenovirus serotype 5 (Ad5) by recombination in *Escherichia coli*, as described in Crouzet *et al*. (1997). It is designated Ad.FGF1 in this article. We used the same method to construct the control virus (Ad.V152), which contained the CMV promoter and poly(A) sequences but no transgene. Adenoviruses were produced in the supernatant of 293 cells and then concentrated by tangential flow filtration (J.-M. Guillaume [Gencell, Vitry sur Seine, France], personal communication). Purification and viral titer determination (in viral particles [VP] per milliliter) were performed by high-performance liquid chromatography (Blanche *et al*., 2000). All viral stocks were subjected to restriction analysis to check the integrity of the virus. The ratio of VPs to plaque-forming units (PFU) was determined and found to be less than 1:100 for each virus. All viral stocks were tested for replication of competent adenoviruses and found to contain fewer than one replication-competent adenovirus per 10^{10} VP.

1HAEo$^-$ cell culture and proliferation studies

The 1HAEo$^-$ line is derived from human tracheal epithelial cells transfected with mutated SV40 T-antigen and has been extensively described (Cozens *et al*., 1992). 1HAEo$^-$ bronchial cells were grown in Dulbecco's modified Eagle's medium sup-

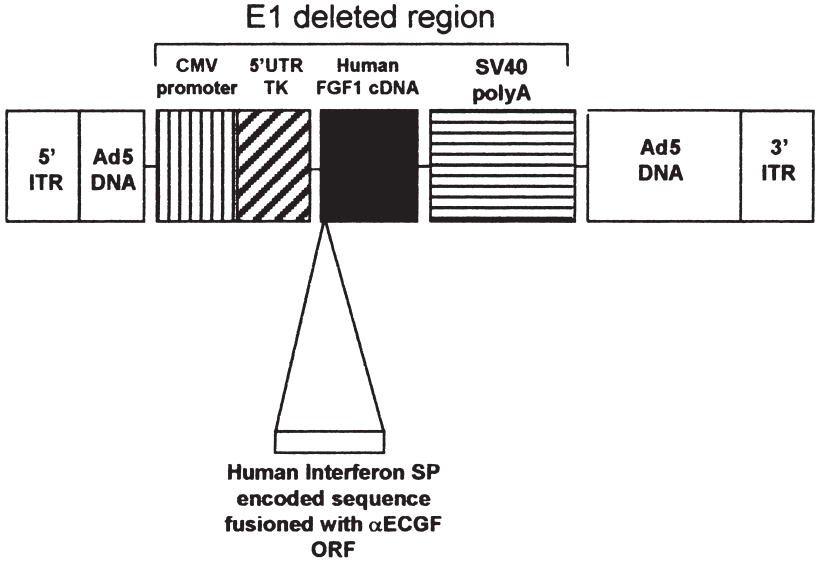

FIG. 1. Diagram representing the expression cassette inserted into the E1 region of the human Ad5 serotype. a-ECGF, acidic epithelial cell growth factor; CMV, cytomegalovirus; FGF-1, fibroblast growth factor type 1; ITR, inverted terminal repeat; ORF, open reading frame; SP, signal peptide; SV40, simian virus 40; TK, thymidine kinase; UTR, untranslated region.

plemented with serum substitute Ultroser G (2%; GIBCO-BRL, Gaithersburg, MD) on a collagen G (I+III) coating. Cells were grown to 80% confluence and incubated for 48 hr in fresh serum-free medium containing recombinant FGF-1 at various concentrations (10–200 ng/ml). For adenovirus infection, cells were incubated with Ad.FGF1 or Ad.V152 (1–100 multiplicities of infection [MOIs]) for 1 hr at 37°C, washed twice with new medium, and incubated for 47 hr in fresh serum-free medium. Cell counts were determined after 48 hr by harvesting cells in trypsin–EDTA and counting them in triplicate with a hemocytometer.

Animal experiments and delivery of adenovirus vectors to the lungs

Wistar rats (200–250 g body weight) were used for all studies. Animal care and procedures were in accordance with institutional guidelines. Ad.FGF1, or Ad.V152 as the control, was diluted before use in sterile saline, pH 7.4, in a final volume of 150 μl. The rats were anesthetized with intraperitoneal ketamine (7 mg/100 g) and xylazine (1 mg/100 g). Viral infection of the lungs was achieved by intratracheal instillation of diluted Ad.FGF1 or Ad.V152 (150 μl/rat).

After an intraperitoneal injection of pentobarbital (60 mg/kg), blood samples were drawn from the abdominal aorta. The plasma was separated after centrifugation of the clotted blood samples at 2000 rpm for 10 min and then stored at −20°C. For bronchoalveolar lavage (BAL), a median sternotomy was performed, the trachea was dissected free from the underlying soft tissues, and a 0.6-mm tube was inserted through a small incision in the trachea. BAL was performed by instilling 10 ml of 9‰ NaCl (one 5-ml and two 2.5-ml aliquots) *in situ* into the left lung and then recovering the fluid by gentle aspiration. The fluid was centrifuged at 300 × *g* for 7 min at 4°C. The cell-free BAL fluid was stored at −70°C until used for enzyme-linked immunosorbent assay (ELISA).

Immediately after BAL, the lungs were removed and the left lung was fixed by infusing neutral buffered formaldehyde into the trachea. After routine processing and paraffin embedding, multiple sections from each lobe were stained with hematoxylin and eosin.

For transmission electron microscopy, the lungs were fixed *in situ* by inflation with 10 ml of 2.5% glutaraldehyde in 0.045 *M* cacodylate buffer at pH 7.4, followed by immersion in fixative. Sections cut from blocks of tissue were examined under an EM 301 microscope (Philips, Eindhoven, The Netherlands).

Evaluation of gene transfer

FGF-1 protein detection in serum and bronchoalveolar lavage fluid. To evaluate the efficiency of gene transfer, serum and BAL levels of FGF-1 protein were measured in normoxic rats 4, 10, and 17 days after infection with various doses of Ad.FGF1 (10^9 to 5×10^{10} VP) or Ad.V152 (10^{10} VP) and in hyperoxic rats 4 days after infection with Ad.FGF1 (5×10^9 VP) or Ad.V152 (5×10^9 VP).

The ELISA kit used in this study was developed for Aventis (Strasbourg, France) by the custom development service of R&D Systems (Minneapolis, MN). The kit recognizes naturally occurring FGF-1 from both humans and rats.

Immunohistochemistry of FGF-1. To determine which cells were transduced with Ad.FGF1, we performed an immunochemistry study of lung sections in normoxic rats 4 days after Ad.FGF1 (5×10^9 VP) or Ad.V152 (5×10^9 VP) administration. Paraffin sections were dewaxed by passage through xylene and graded alcohols. Endogenous peroxidase activity was then blocked with 0.6% hydrogen peroxide in methanol, and nonspecific binding sites were blocked with normal goat serum. The slides were incubated overnight at 4°C with a polyclonal rabbit antibody against human FGF-1 (R&D Systems Europe, Abingdon, UK). The samples were then incubated for 30 min with a biotinylated goat anti-rabbit antibody and with avidin–biotin complex (DakoCytomation, Carpinteria, CA). Visualization was obtained after a few minutes of incubation with diaminobenzidine (DAB) as the chromatogen. Immunohistochemical controls also included slides of lungs from Ad.FGF1-treated rats incubated with normal dilute rabbit serum instead of the first antibody.

Evaluation of the inflammatory effects of Ad.FGF1

Cytological evaluation. For cytological evaluation of inflammation, BAL pellets were resuspended with the remaining supernatants and the cells were counted. Cytospin preparations were made by placing 0.2 ml (BAL aliquots) in each funnel of a Cytospin III (Shandon, Cergy-Pontoise, France). The cells were fixed and stained with Diff-Quick (Dade Behring, La Défense, France), and a differential leukocyte count was performed.

Histological evaluation. For histological evaluation, light microscopy was performed on samples from normoxic rats 4 days after administration of various doses of Ad.FGF1 (10^9, 5×10^9, 10^{10}, or 5×10^{10} VP) or Ad.V152 (10^9 to 5×10^{10} VP) and 10 and 17 days after administration of 10^9, 5×10^9, or 10^{10} VP. In hyperoxic rats, histological sections were also examined at various times after infection with Ad.FGF1 or Ad.V152 (10^9 or 5×10^9 VP). The inflammatory response was assessed on the basis of an empiric semiquantitative scale, as described previously (Brody *et al*., 1994).

Determination of lung wet-to-dry weight ratio. The medial lobe of the right lung was excised, dissected, immediately weighed, and then placed in a desiccating oven at 65°C for 72 hr, at which point the dry weight was measured. The wet-to-dry weight ratio was used to quantify lung water content (Staub, 1974). Hemoglobin concentration was measured in ultracentrifuged (14,000 × *g* for 10 min) lung homogenate.

Effect of Ad.FGF1 administration on animal survival

Two days after intratracheal Ad.FGF1 (10^9, 5×10^9 VP) or Ad.V152 (10^9, 5×10^9 VP) administration, the rats were exposed to 95% oxygen in a Plexiglas hyperoxia chamber. The flow of gas was kept constant at 8 liters/min. The inspired fraction of oxygen was checked with a gas analyzer at the end of each experiment. The rats had free access to food and water and were housed at room temperature. In a first experiment, the rats (n = 6–11 per group) were killed 48 hr after exposure to hyperoxia and used to evaluate pulmonary gene transfer, histology, edema, and apoptosis. In a second experiment, eight rats

per group were exposed to hyperoxia and their survival was evaluated.

Evaluation of lung apoptosis and proliferation after Ad.FGF1 administration

Proliferating cell nuclear antigen labeling. A PCNA (proliferating cell nuclear antigen) staining kit (Zymed, South San Francisco, CA) was used. The kit uses a biotinylated PCNA mouse monoclonal antibody (clone PC 10) that eliminates the need for a species-specific secondary antibody. Immunolocalization was performed on paraffin-embedded formalin-fixed rat lungs. Briefly, after paraffin removal in xylene, the sections were rehydrated and exposed for 10 min to H_2O_2 (3% in methanol) to quench endogenous peroxidase. The sections were exposed to PCNA monoclonal antibody for 50 min. To avoid background staining, the primary antibody was diluted (1:5) with bovine serum albumin (1% in 10 m*M* phosphate-buffered saline [PBS]). Immunodetection was then performed with peroxidase-conjugated streptavidin as the signal generator and DAB as the chromogen to stain PCNA-containing nuclei dark brown. Counterstaining was done with hematoxylin. Negative controls for nonspecific binding were performed by omitting the step with the primary antibody.

Image analysis of PCNA-labeled sections was done with a charge-coupled device Iris camera (CCD Iris; Sony, Tokyo, Japan) coupled with a light microscope (Laborlux; Leica Microsystems, Bensheim, Germany). PCNA staining was estimated with Perfect Image software (ClaraVision, Orsay, France), which discriminates between two colors on the basis of their proportions of red, green, and blue (dark brown nuclei for DAB staining and blue for counterstained areas) with ±10% variation. From binary images, at least 20 fields per section were analyzed. Results were obtained as the ratio of dark brown staining to blue staining, and expressed as a percentage (DAB nuclear staining relative to total lung parenchyma).

Terminal deoxyribonucleotidyltransferase-mediated dUTP nick end-labeling staining. The terminal deoxyribonucleotidyltransferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method is derived from the method described by Gavrieli *et al*. (1992), with digoxigenin being substituted for the hapten biotin. We used the ApopDETECT peroxidase kit from Qbiogene (Strasbourg, France). Briefly, after paraffin removal in xylene, the sections were rehydrated and exposed to proteinase K (Promega, Madison, WI) to improve histological marker penetration. Endogenous peroxidase was quenched with 3% H_2O_2. The enzyme TdT was added to attach UTP–digoxigenin nucleotides to the 3′-OH ends of single- or double-stranded DNA (located in apoptotic bodies). The reaction was stopped with specific buffer, and labeled DNA fragments were allowed to bind to an anti-digoxigenin antibody conjugated to a peroxidase reporter molecule. DAB was used to stain nuclei dark brown and hematoxylin was used for counterstaining.

Caspase 3-like activity assay. After isolation, right lungs were homogenized in a buffer containing 50 m*M* HEPES, 0.1% CHAPS, 5 m*M* dithiothreitol (DTT), 0.1 m*M* EDTA, and 0.1% Triton X-100, using an Ultra-Turrax homogenizer (IKA Werke, Staufen, Germany). Lung tissue homogenates were centrifuged at 10,000 × *g* for 15 min, and supernatants were stored at −70°C until use in the caspase 3-like activity assay. Direct protein quantitation was done by the Bradford method with IgG standardization purchased from Bio-Rad (Ivry sur Seine, France). The cytosolic extract was incubated at 37°C with a 200 μM concentration of the substrate Ac-DEVD-pNA in 96-well microtiter plates (CASPASE-3 cellular activity assay kit PLUS; BIOMOL Research Laboratories, Plymouth Meeting, PA). At various time points, hydrolytic activities were determined by measuring the absorbance of *p*-nitroaniline at 405 nm. Caspase activity was expressed as picomoles per minute per microgram of protein.

Statistical analysis

All results are reported as means ± SEM. Two-way analysis of variance (ANOVA) was performed to compare the effect of Ad.FGF1 and Ad.V152 pretreatment in normoxic and hyperoxic animals, followed by the Fisher test to compare Ad.FGF1 and Ad.V152 for each oxygenation condition.

Survival curves (Kaplan–Meier plots, $n = 8$ for each group) were compared by log rank test. p Values < 0.05 were considered statistically significant.

RESULTS

Effect of Ad.FGF1 gene transfer in vivo *and* in vitro

Effect of Ad.FGF1 on epithelial cell proliferation. We first confirmed the growth effect of recombinant FGF-1 on 1HAEo$^-$ cells and determined the efficacy of Ad.FGF1 *in vitro*. As shown in Fig. 2, Ad.FGF1 induced a dose-dependent increase in 1HAEo$^-$ cell counts that was similar in magnitude to that induced by recombinant FGF-1.

Detection of FGF-1 in lung tissue, BAL fluid, and plasma. FGF-1 immunostaining of adult rat lungs 4 days after Ad.FGF1 (5×10^9 VP) administration was localized in bronchiolar (Fig. 3B, panel b) and alveolar (Fig. 3B, panel a) epithelial cells, type II epithelial cells, macrophages, and fibroblasts. Immunostained cells were visible throughout the right and left lungs. They were located mainly in the bronchiolar and alveolar areas. Immunostaining occurred only in proliferating areas. There was no immunostaining in lungs from Ad.V152-pretreated controls.

In BAL fluid obtained 4 days after adenovirus administration to normoxic rats, FGF-1 levels increased in a dose-dependent manner up to 10^{10} VP (Fig. 3A). Only small amounts of FGF-1 were detected after Ad.V152 administration, with no differences across doses. After Ad.FGF1 (5×10^9 VP) or Ad.V152 (5×10^9 VP) pretreatment, FGF-1 levels were similar in BAL fluid from hyperoxic and normoxic rats (5.9 ± 1.4 and 1.0 ± 0.2 ng/ml BAL fluid versus 5.8 ± 1.3 and 1.3 ± 0.1 ng/ml BAL fluid, respectively) on day 4. FGF-1 levels on days 10 and 17 were not significantly higher than in the controls (data not shown) in normoxic or hyperoxic rats.

Plasma FGF-1 assays found FGF-1 at levels ranging from 0 to 150 pg/ml after administration of 10^{10} to 5×10^{10} VP of

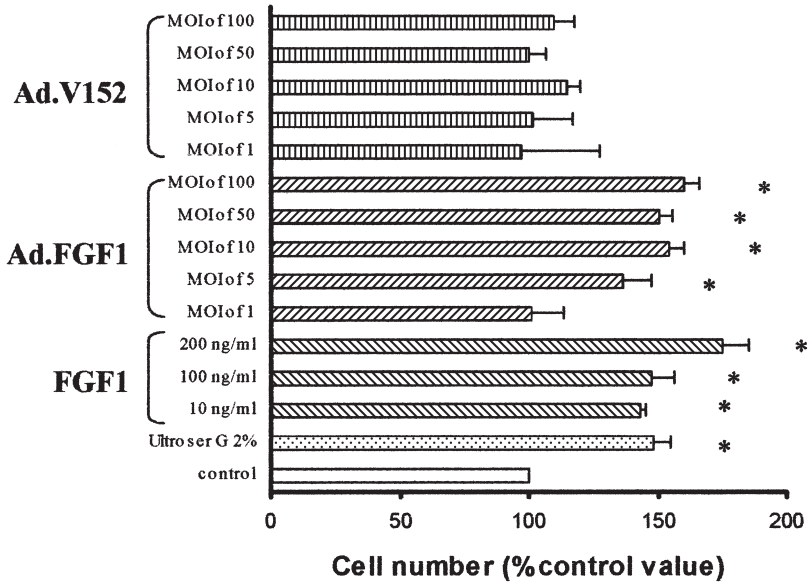

FIG. 2. Effects of recombinant FGF-1, Ad.FGF1, and Ad.V152 on the proliferation of human bronchial epithelial cells (1HAEo$^-$). At the end of incubation, cells were harvested and counted with a hemocytometer. Shown are the effects of serum substitute Ultroser G (2%), FGF-1 (10 to 200 ng/ml of medium), and Ad.FGF1 or Ad.V152 at the concentrations indicated. Values represent means ± SEM of six experiments. *Significant difference compared with control values, $p < 0.05$.

Ad.FGF1. No plasma FGF-1 was detected with lower doses of Ad.FGF1 or with any dose of Ad.V152.

Histological findings after intratracheal adenovirus administration. Sections taken 4 days after administration of 10^9, 5×10^9, 10^{10}, or 5×10^{10} VP of Ad.FGF1 showed intense proliferation of alveolar epithelial cells and bronchial cells located only in the terminal bronchoalveolar areas. This strong effect is illustrated in Fig. 4A, which compares 5×10^9 VP of Ad.FGF1 and Ad.V152. Alveolar filling and bronchial budding were seen in areas showing intense cell hyperplasia (Fig. 4A, panel a). Bronchiolar hyperplasia was visible as invagination and pseudostratification of the epithelial lining of the small distal bronchi, which normally are lined by a single layer of epithelium (Fig. 4A, panel d). The large airways did not show epithelial hyperplasia. Pulmonary type II epithelial cells were identified by electron microscopy as proliferating cells in hyperplastic alveolar areas (Fig. 5). Mitotic figures were seen in pneumocytes (Fig. 5) of hyperplastic areas, indicating cellular proliferation. These changes occurred in response to each of the Ad.FGF1 doses tested, although the number of areas involved was greatest with the highest doses of Ad.FGF1. However, the major alterations in the alveolar region precluded histological evaluation of the endothelial cells. No epithelial cell hyperplasia or proliferation was visible in the lungs from control rats injected with Ad.V152 (Fig. 4A, panels b, e, and h).

Over time, the histological alterations noted on day 4 resolved gradually. On day 10, the slides showed persistent thickening of the alveolar septa due to an increase in the number of cells. On day 17, the alveoli seemed almost normal; however, a detailed examination of the septa revealed focal increases in the number of nuclei (Fig. 4A, panel c), bronchial budding (Fig. 4A, panel f), and mesenchymal cell proliferation.

In the rats exposed to hyperoxia, the histological findings were similar to those in the normoxic rats 4 days after Ad.FGF1 administration (2 days after the beginning of exposure to 95% O_2) (Fig. 4B, panels j and m), except for marked inflammation (see below). In the surviving hyperoxic rats 17 days after Ad.FGF1 administration (15 days after the beginning of exposure to 95% O_2), cell proliferation was less marked, although the decrease occurred at a slower rate than in the normoxic rats. The histological profile on day 17 in the hyperoxic rats was comparable to that on day 10 in the normoxic rats (Fig. 4B, panels l and o).

Lung inflammation after adenovirus administration

Histological and cytological evaluation of inflammation after gene transfer. First, the location and extent of the inflammatory response were assessed. Both characteristics showed dose-dependent changes after adenovirus administration: 4 days after Ad.FGF1 or Ad.V152 administration at doses of 10^9 to 10^{10} VP, no inflammation was detectable by this method, and with doses greater than 5×10^{10} VP only mild inflammation was seen. In the rats exposed to 95% O_2, on day 4 after Ad.FGF1 or Ad.V152 (10^9 and 5×10^9 VP) administration, we found patchy mononuclear and mixed cell infiltrates composed primarily of macrophages and neutrophils, as well as sparse foci of mild damage to alveolar and bronchiolar epithelial cells.

Second, a Cytospin BAL fluid preparation was used to compare BAL cell recruitment after adenovirus administration in normoxic and hyperoxic rats. On day 4 after adenovirus administration, total cell counts were similar under the two oxygenation conditions but were significantly affected by adenovirus administration ($p < 0.05$), with no interaction. In both normoxic and hyperoxic rats, total cell counts were similar after administration of Ad.V152 or Ad.FGF1 at 10^9 VP but were significantly increased after Ad.FGF1 administration at 5×10^9 VP (Table 1). The BAL fluid neutrophil count was significantly

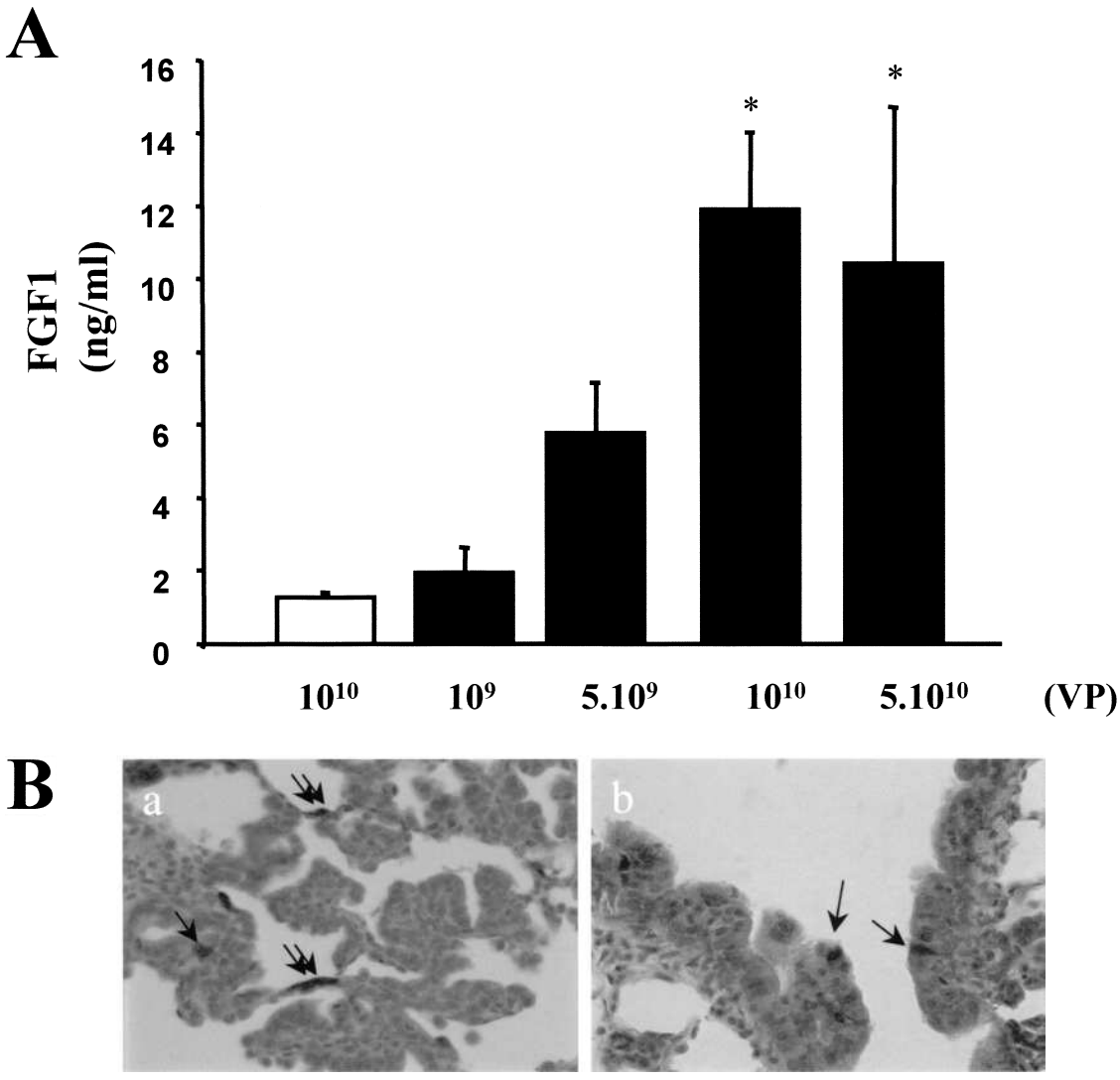

FIG. 3. Evaluation of *in vivo* gene transfer. (**A**) FGF-1 levels in bronchoalveolar lavage (BAL) fluid from normoxic rats 4 days after treatment with Ad.V152 (open column) at a dose of 10^{10} VP ($n = 5$) or with Ad.FGF1 (solid columns) at doses of 10^9 VP ($n = 5$), 5×10^9 VP ($n = 6$), 10^{10} VP ($n = 5$), and 5×10^{10} VP ($n = 4$). BAL was performed with saline, and immunoreactive FGF-1 was measured in a final volume of 10 ml for each rat. Values represent means ± SEM of four to six experiments. *Significant difference compared with Ad.V152 values, $p < 0.05$. (**B**) Light micrograph of adult normoxic rat lung sections stained with anti-FGF-1 antibody 4 days after pretreatment with Ad.FGF1 (5×10^9 VP). (a) Positive staining (brown) of epithelial cells in the alveolar septum (double arrows) and of macrophages (single arrows). Original magnification: ×40. (b) Positive staining (brown) of bronchiolar epithelial cells (single arrows). Original magnification: ×40.

FIG. 4. (**A**) Histological lung sections of animals treated with adenovirus in normoxia. (a, d, and g) Four days after intratracheal instillation of Ad.FGF1 (5×10^9 VP) in normoxic rats, abnormal hyperplastic areas are visible at the terminal bronchioalveolar junctions (a); higher power views show epithelial hyperplasia with bronchial budding (d) and alveolar filling resulting from typical alveolar epithelial cell hyperplasia (g). (b, e, and h) Four days after intratracheal instillation of the control Ad.V152 (5×10^9 VP) in normoxic rats, the lung architecture is normal and slight inflammation is visible in some alveolar spaces (e). (c, f, and i) On day 17 after intratracheal instillation of Ad.FGF1 (5×10^9 VP) in normoxic rats, the hyperplastic areas are no longer visible but bronchial budding is still present (f) and numerous nuclei of alveolar epithelial cells are seen (f). (**B**) Histological lung sections of animals treated with adenovirus in hyperoxia. (j and m) Four days after intratracheal instillation of Ad.FGF1 (5×10^9 VP) and 2 days after 95% O_2 exposure, hyperplastic areas are visible, as well as an inflammatory cell infiltrate. (k and n) Four days after intratracheal instillation of Ad.V152 (5×10^9 VP) and 2 days after 95% O_2 in rats, mild damage to alveolar and bronchiolar spaces is visible with areas of edema and inflammatory cell infiltrate. (l and o) Seventeen days after intratracheal instillation of Ad.FGF1 (5×10^9 VP) in rats exposed to 95% O_2 for 15 days, the hyperplastic areas are decreased, but numerous alveolar epithelial cells are visible, as well as inflammatory cells. Light micrographs; hematoxylin–eosin staining; original magnification: ×5 (**A** and **B**, top row), ×20 (**A**, middle row; **B**, bottom row), and ×40 (**A**, bottom row).

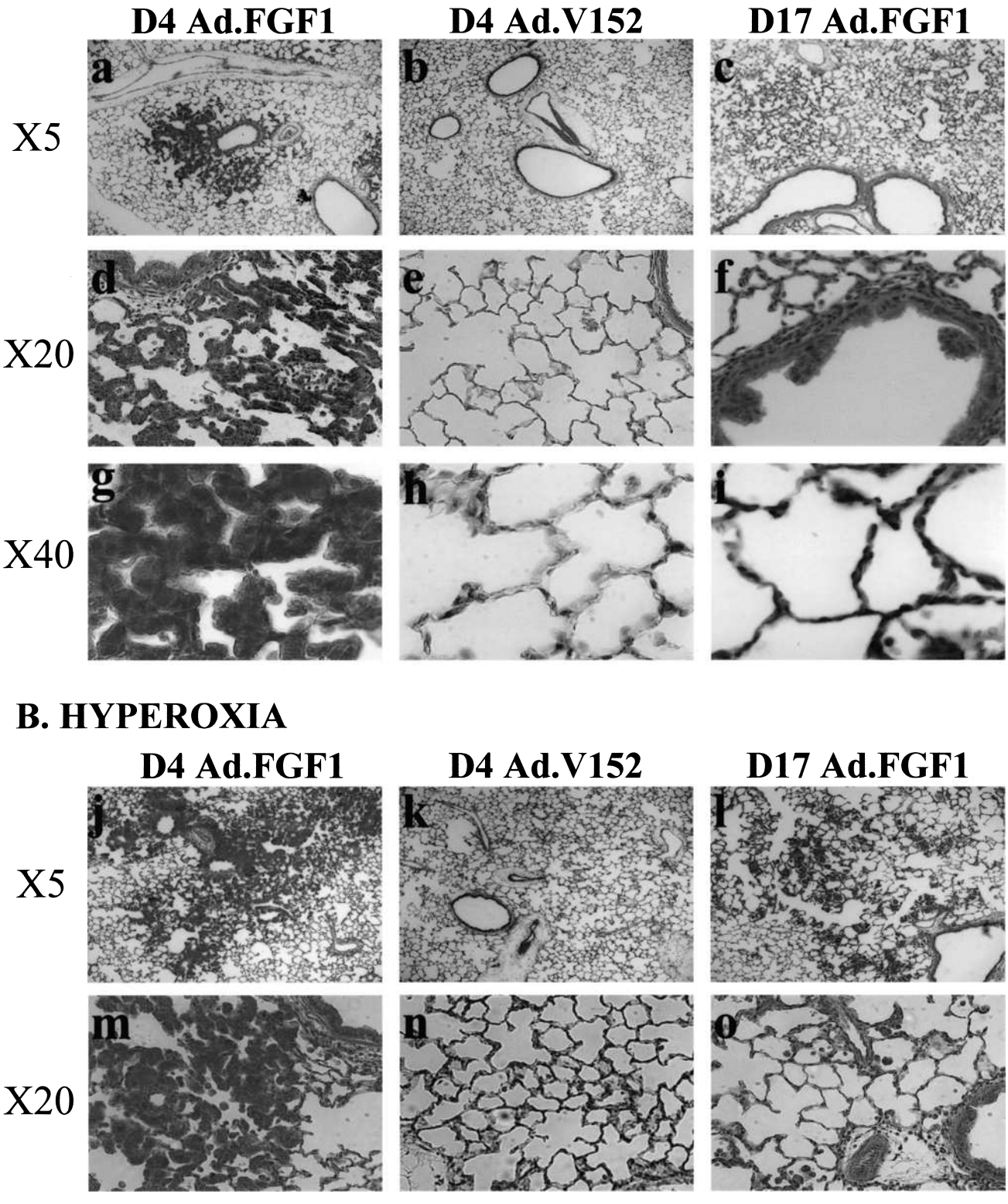
A. NORMOXIA
D4 Ad.FGF1
D4 Ad.V152
D17 Ad.FGF1
X5
X20
X40
a
b
c
d
e
f
g
h
i
B. HYPEROXIA
D4 Ad.FGF1
D4 Ad.V152
D17 Ad.FGF1
X5
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FIG. 4.

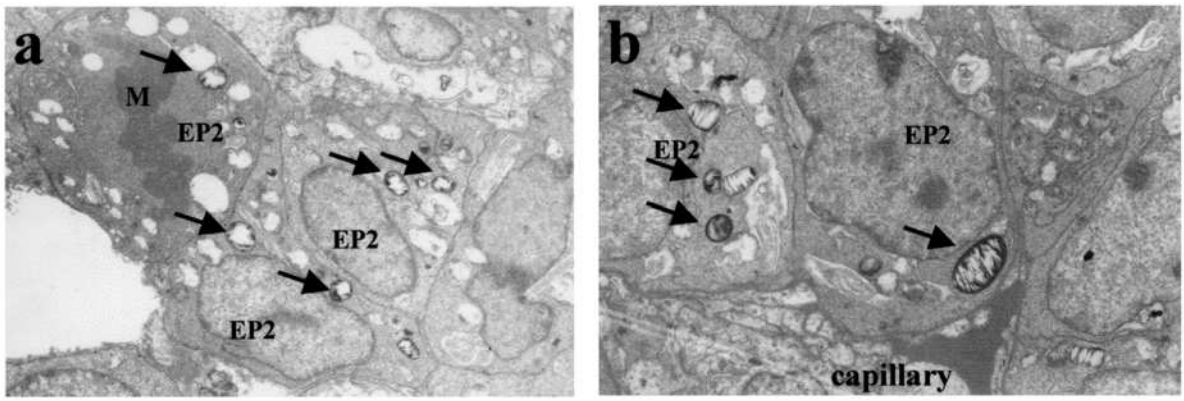

FIG. 5. (**a** and **b**) Electron micrographs of areas of alveolar hyperplasia in lungs from normoxic rats 4 days after intratracheal Ad.FGF1 administration (5×10^9 VP): alveolar type II epithelial cells (EP2) containing cytoplasmic lamellar inclusions (arrows) and undergoing division (M, mitosis). Original magnification: (**a**) ×2800; (**b**) ×3600.

influenced by the oxygenation condition ($p < 0.01$) and by adenovirus treatment ($p < 0.05$), with no interaction. BAL neutrophil counts were higher after hyperoxia than after normoxia ($p < 0.01$). Ad.FGF1 administration at 5×10^9 VP significantly increased the BAL neutrophil counts in normoxic but not hyperoxic rats, as compared with Ad.V152 or Ad.FGF1 at 10^9 VP ($p < 0.05$; see Table 1).

Wet-to-dry lung weight ratio. The respiratory rate was increased in normoxic rats given Ad.FGF1 at 5×10^9 VP. The gross appearance of the heart was normal, whereas the lungs were enlarged and edematous. The wet-to-dry lung weight ratio was increased in rats given Ad.FGF1 at 5×10^9 VP as compared with Ad.V152 or Ad.FGF1 at 10^9 VP (5.36 ± 0.16, 4.7 ± 0.06, and 4.91 ± 0.15, respectively, $n = 5$–10; $p < 0.05$) in normoxic but not hyperoxic rats (5.77 ± 0.23, 5.78 ± 0.19, and 5.83 ± 0.1, respectively, $n = 5$–8). To explore the hypothesis that neovascularization responsible for an increase in pulmonary blood volume contributed to increase the wet-to-dry lung weight ratio after Ad.FGF1, we measured the lung hemoglobin content (Saria and Lundberg, 1983) in ultracentrifuged lung homogenates; no increases were found at any time point, ruling out this hypothesis (data not shown).

Effect of Ad.FGF1 on rat survival during hyperoxia

To determine whether Ad.FGF1 limited O_2 toxicity, in rats exposed to 95% O_2 we compared survival between the groups ($n = 8$) pretreated with Ad.FGF1 (10^9 or 5×10^9 VP) versus the groups pretreated with Ad.V152 (10^9 or 5×10^9 VP) (Fig. 6). All the Ad.V152 control rats died within 72 hr of initiation of 95% O_2 exposure. In contrast, the Ad.FGF1-pretreated rats (Fig. 6) survived for at least 70 hr in 95% O_2; more than 60% of those given the 5×10^9 VP dose and 50% of those given 10^9 VP survived 10 days or longer ($p < 0.001$ for Ad.FGF1 versus Ad.V152 with the two adenovirus doses).

TABLE 1. INFLAMMATORY CELLS IN BRONCHOALVEOLAR LAVAGE FLUID FROM RAT LUNGS 4 DAYS AFTER INTRATRACHEAL Ad.FGF1 ADMINISTRATION[a]

Condition	*Parameter measured*	*Ad.V152*[b] *($10^9/5 \times 10^9$ VP)*	*Ad.FGF1*[c] *(10^9 VP)*	*Ad.FGF1*[c] *(5×10^9 VP)*
Normoxia	Total cell count ($\times 10^5$/ml BAL fluid)	1.6 ± 0.3	1.2 ± 0.1	2.2 ± 0.3[d]
	Macrophages (%)	96.7 ± 0.9	88.6 ± 3.0	72.5 ± 3.6[d]
	Neutrophils (%)	2.8 ± 0.6	9.4 ± 2.8	22 ± 0.5[d]
Hyperoxia	Total cell count ($\times 10^5$/ml BAL fluid)	1.6 ± 0.3	1.0 ± 0.3	2.3 ± 0.5[d]
	Macrophages (%)	85.3 ± 7.4	82.8 ± 4.0	79.2 ± 2.8
	Neutrophils (%)	13.5 ± 6.9	15.5 ± 4.3	18.8 ± 2.5

Abbreviations: BAL, bronchoalveolar lavage; VP, viral particles.

[a]Bronchoalveolar lavage was performed 4 days after intratracheal instillation of adenovirus (Ad.FGF1 or Ad.V152) in animals exposed to normoxia or hyperoxia for 2 days. Ad.V152 vector served as the control. Inflammatory cells in bronchoalveolar lavage fluid were recovered by Cytospin centrifugation and identified by differential staining. Polymorphonuclear neutrophils and macrophages are represented as percentages of total inflammatory cells. Values represent means ± SEM.

[b]$n = 11$.

[c]$n = 6$.

[d]$p < 0.05$ compared with Ad.V152 values.

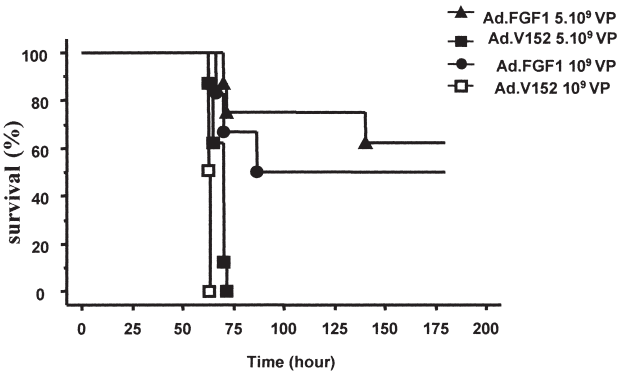

FIG. 6. Survival of adenovirus-pretreated rats exposed to 95% O_2. Rats were pretreated with adenovirus 2 days before the onset of hyperoxia exposure. Shown is a comparison of Ad.FGF1 administered at 5×10^9 VP (solid triangles) or 10^9 VP (solid circles) with Ad.V152 administered at 5×10^9 VP (solid squares) or 10^9 VP (open squares). Each value is the percentage of surviving rats in each group of eight animals. Kaplan–Meier survival curves were compared by log rank test. p Values less than 0.05 were considered statistically significant between animals treated with Ad.V152 or Ad.FGF1 at the same dose.

Alveolar cell proliferation and apoptosis in hyperoxic rats

Alveolar cell proliferation in nonhyperplastic areas of lungs from rats exposed to 95% O_2 for 48 hr (Fig. 7A, panels a and b), as assessed by quantification of PCNA cell staining, was similar between the groups pretreated with Ad.V152 and Ad.FGF1 (2.67 ± 0.29 and 2.65 ± 0.74%, respectively). However, in hyperplastic areas of lungs from Ad.FGF1-pretreated rats, proliferation was increased (29.73 ± 1.3% in hyperplastic areas versus 2.65 ± 0.74% in normal areas).

The TUNEL technique was used to detect apoptosis 4 days after Ad.FGF1 or Ad.V152 administration followed by exposure to hyperoxia (95% O_2 for 48 hr). DNA strand breaks were detected mainly in bronchiolar cells and alveolar epithelial cells, either in normal or in hyperplastic areas. Some apoptotic cells appeared to be endothelial cells based on their location. Staining was more pronounced in hyperplastic areas than in normal areas in rats pretreated with Ad.FGF1 at any dose (Fig. 7A, panels c and d).

Evidence that caspase activation may be critical for many forms of apoptotic cell death prompted us to look for an increase in caspase activity in normoxic and hyperoxic rats (95% O_2 for 48 hr) 4 days after Ad.FGF1 administration. Figure 7B shows the changes in caspase 3 activity in lung tissue from six animals per group. No differences were found between rats pretreated with Ad.V152 or Ad.FGF1 (10^9 or 5×10^9 VP), and exposed to normoxia or to hyperoxia.

DISCUSSION

In this study, intratracheal administration of Ad.FGF1 resulted in expression of FGF-1 by bronchiolar and alveolar epithelial cells, as well as alveolar macrophages. The effect was dose dependent, with increasing doses of Ad.FGF1 producing patchy to diffuse lung transduction. In our model, a low dose of adenovirus induced strong expression of FGF-1 in BAL fluid but only minimal lung inflammation. Adenovirus-mediated lung FGF-1 overexpression increased the survival of rats exposed to hyperoxia. This effect was associated with a burst of alveolar type II cell proliferation and apoptosis.

Intratracheal Ad.FGF1 administration was efficient in ensuring local gene transfer with secretion of a functional protein by transduced cells. FGF-1 levels in BAL fluid recovered 4 days after Ad.FGF1 administration showed a dose-dependent increase. No increase in plasma FGF-1 was detectable with the Ad.FGF1 doses used in our study, suggesting that FGF-1 diffusion and expression in other organs were minimal. Histological examination revealed marked proliferation of bronchial and alveolar epithelial cells. This proliferation was confined to the terminal bronchoalveolar areas, indicating that adenovirus transduction and the resulting biological effects were heterogeneously distributed in the lungs after intratracheal adenovirus administration. The extent of lung involvement after Ad.FGF1 administration did not depend on the adenovirus injection technique but increased with the dose. Thus, after administration of 5×10^{10} or 10^{11} VP, epithelial cell proliferation was visible in most of the lung. Because the VP-to-PFU ratio for Ad.FGF1 was 100, 5×10^{10} and 10^{11} VP corresponded, respectively, to 5×10^8 and 10^9 PFU, which is the dose range used for most *in vivo* adenovirus transduction studies in rats. In our study, we used 10^9 and 5×10^9 VP, that is, 10^7 and 5×10^7 PFU, which are comparable to the doses defined as intermediate or low (10^9–10^{11} VP) in humans and well tolerated after local administration (Harvey *et al*., 2002). Although bronchial administration of adenovirus carrying the gene encoding the cystic fibrosis transmembrane regulator (Ad.CFTR) in humans was associated with local pneumonitis in one patient, adenovirus effect has not been tested at the onset of ALI in humans.

Over time, the histological changes seen on day 4 resolved gradually. Moreover, FGF-1 levels in BAL fluid peaked on day 4 and then decreased rapidly to values that were not significantly different from those in controls on days 10 and 17 after administration of 10^9 or 5×10^9 VP of Ad.FGF1. This finding may explain the gradual reversal of the lung epithelial proliferation induced by Ad.FGF1 administration. The short duration of FGF-1 expression is often a limitation to the use of adenoviruses as vectors for gene therapy in chronic diseases, but may be an advantage in our model of ALI. After exposure to hyperoxia, the rats died within 72 to 96 hr. Consequently, to improve survival, FGF-1 expression must reach a high level rapidly. However, prolonged expression is not needed: whereas alveolar type II cell proliferation during the early phase of ALI may restore injured epithelial cells, persistence of this effect with an inability to initiate spreading and differentiation to alveolar type I cells may produce a deleterious persistent hyperplastic response (Uhal *et al*., 1998). This persistent response may alter the extracellular matrix, thereby impairing recovery from ALI.

Hyperoxia causes extensive destruction of alveolar epithelial cells in monkeys (Kapanci *et al*., 1969) and humans (Gould *et al*., 1972). Although some studies found no significant damage to alveolar epithelial cells in rats exposed to hyperoxia (Crapo, 1986), ultrastructural studies showed disruption of the alveolar epithelium (Kistler *et al*., 1967; Harris *et al*., 1991). In

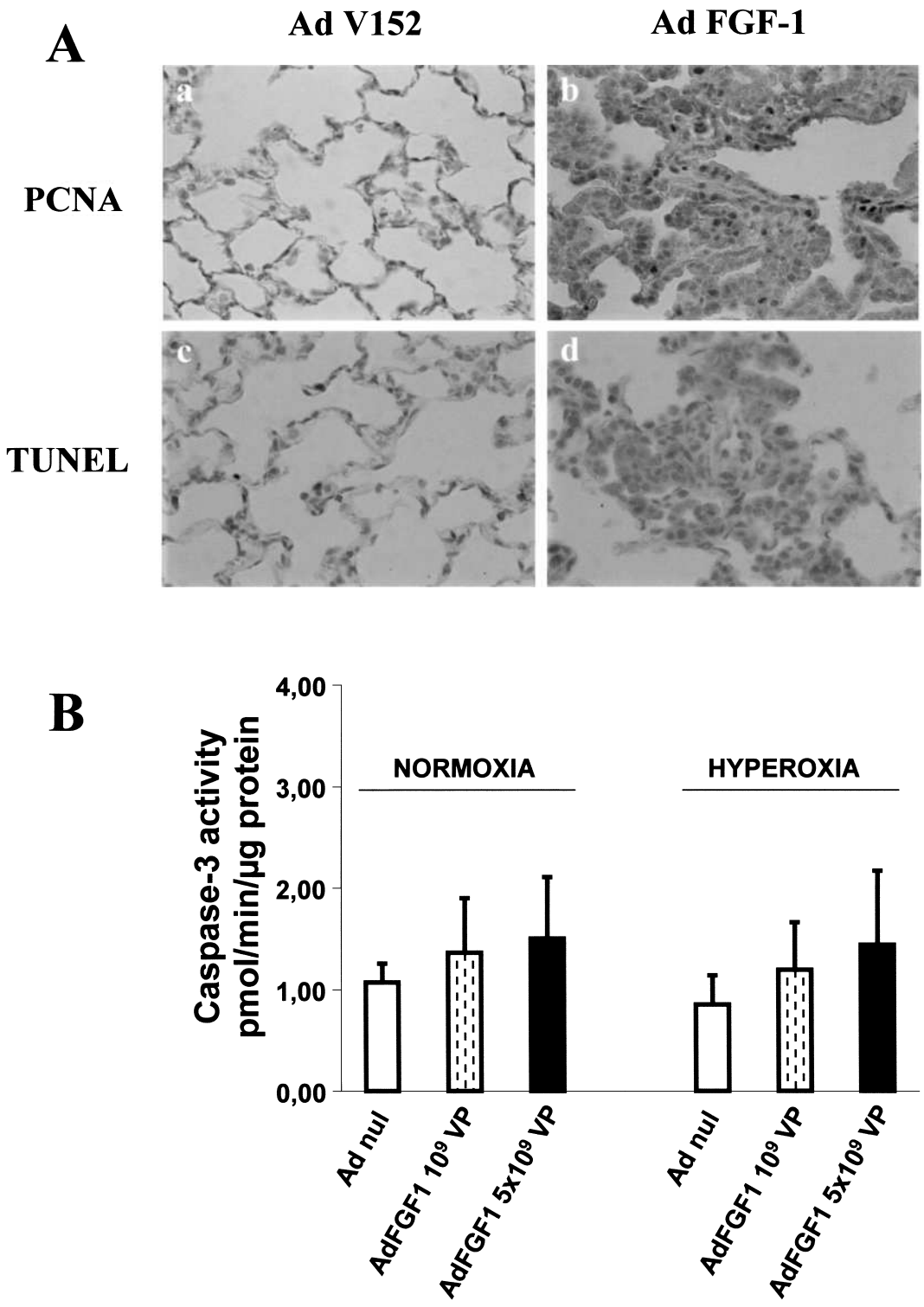

FIG. 7. Effect of Ad.FGF1 on cell proliferation and apoptosis. (**A**) PCNA (a and b) and TUNEL staining (c and d). Adenovirus (Ad.FGF1 or Ad.V152, 5×10^9 VP) was instilled intratracheally 2 days before a 48-hr period of exposure to 95% O_2. Immunohistochemistry for PCNA counterstained with hematoxylin shows positive staining (brown) for proliferating cells in the alveolar septa. (a) Ad.V152 (5×10^9 VP)-treated rat lungs (same findings as in nonhyperplastic areas of Ad.FGF1-treated rat lungs); (b) Ad.FGF1 (5×10^9 VP)-treated rat lungs (areas of hyperplasia). Original magnification: (a and b) ×25. DNA fragmentation was evaluated by TUNEL assay as described in Materials and Methods. (c) Representative photomicrograph illustrating the levels of nuclear staining in Ad.V152-pretreated animals (identical to those in nonhyperplastic areas of lungs from Ad.FGF1-pretreated animals). (d) Representative photomicrograph illustrating the levels of nuclear staining in hyperplastic areas of Ad.FGF1-pretreated animals. Original magnification: (c and d) ×25. (**B**) Comparison of caspase 3-like activity in the lungs 4 days after administration of Ad.V152 or Ad.FGF1 to rats exposed to normoxia or hyperoxia (95% O_2 for 48 hr). Caspase 3-like activity showed no differences between Ad.V152 and Ad.FGF1 (10^9 or 5×10^9 VP) in normoxic or hyperoxic rats. Values represent means ± SEM. $n = 6$ in each experiment.

rats briefly exposed to hyperoxia and then allowed to recover in room air, morphometric analysis showed a 2.5-fold increase in the number of alveolar type II cells (Thet *et al*., 1986). Moreover, intratracheal administration of KGF (FGF-7), which induces proliferation of alveolar type II cells and cell hyperplasia, protected rats against hyperoxic lung injury (Panos *et al*., 1995; Guo *et al*., 1998). These findings are consistent with a reparative response of alveolar type II cells to alveolar epithelium injury and justify our choice of the rat hyperoxia model for investigating potential effects of FGF-1 on ALI. We showed that Ad.FGF1 instillation induced massive type II cell proliferation in normoxic and hyperoxic animals. PCNA staining established that lung epithelial proliferation was far more marked in Ad.FGF1-treated animals than in the controls. If, as suggested by Witschi (1990), healing of the alveolar epithelium requires a proliferative response of alveolar type II cells, then intratracheal Ad.FGF1 administration may afford protection by accelerating this response. Moreover, replicating alveolar cells may be more resistant to the deleterious effects of oxygen, as suggested by findings in neonatal rats (Frank *et al*., 1978; Canada *et al*., 1995). Surprisingly, intravenous administration of KGF to mice also protected against the effects of hyperoxia but did not induce alveolar proliferation, suggesting a paracrine effect of KGF on the endothelium (Barazzone *et al*., 1999). However, using a tetracycline-inducible lung-specific KGF transgenic system, Ray *et al*. showed that KGF inhibited lung epithelial cell death induced by hyperoxia but did not increase survival of the animals (Ray *et al*., 2002). Their ultrastructural studies showed preservation of the epithelial lining contrasting with apoptosis in the endothelium and accessory cells lining the capillaries (Ray *et al*., 2002). This disagreement between the effects of exogenous KGF and transgene-produced KGF may be ascribable to the difference in alveolar KGF doses (1–5 mg/kg and 0.2–0.4 μg/kg, respectively). The protection afforded by exogenous KGF may be due to effects of KGF unrelated to binding of FGF receptor 2-IIIb (FGFR2-IIIb), such as binding to other FGF receptors or secondary effects. This is additional evidence that proper repair requires a protective effect on both the epithelial and the endothelial side of the airspace. We used the less specific growth factor FGF-1, the angiogenic properties of which might conceivably result in cytoprotective effects on the endothelium. However, we were unable to study endothelial integrity on the histological sections because the burst of epithelial cell proliferation profoundly modified the alveolar septa. The improvement in survival after exposure to hyperoxia found in our study may be ascribable in part to FGF-1-induced type II cell proliferation and in part to protective effects of FGF-1 on endothelial cells. After adenoviral gene transfer, FGF-1 may act on type II cell proliferation through intracrine, autocrine, and paracrine mechanisms and on endothelial cells through paracrine mechanisms.

Additional modes of protection have been hypothesized, such as an increase in surfactant protein, stimulation of fibrinolytic activity, and decreased apoptosis (Barazzone *et al*., 1999). Work performed with a generated transgenic mouse strain characterized by conditional overexpression of a soluble FGF receptor showed that the FGF receptor pathway was critical for maintaining surfactant homeostasis and lung function after hyperoxia (Hokuto *et al*., 2004). KGF attenuates programmed cell death in some cell lines (Buckley *et al*., 1998; Wu *et al*., 1998), but we found no reduction in apoptosis after Ad.FGF1 administration to hyperoxic rats. Apoptosis is an important mechanism for removing unwanted cells during injury repair, and extensive apoptosis has been documented during the resolution phase of ALI (Bardales *et al*., 1996). Persistent proliferation of alveolar type II cells may occur when the signals needed to end cell division are lacking and differentiation into type I cells cannot occur (Uhal *et al*., 1998). Our findings suggest that the hyperplasia-inducing stimulus caused by Ad.FGF1 administration was present only as long as FGF-1 was overexpressed. Nevertheless, Fehrenbach *et al*. (2000) emphasized the importance of alveolar type II cell apoptosis for the resolution of KGF-induced type II cell hyperplasia in rat lungs *in vivo*. They reported that KGF instillation induced terminal differentiation and apoptosis of hyperplastic type II cells *in vivo*, resulting in restoration of normal alveolar epithelium. Moreover, whether apoptosis plays a role in hyperoxia-induced lung injury remains debated. Work on A549 cells indicates that hyperoxia inhibits H_2O_2-induced apoptosis via an effect mediated by NF-κB (Franek *et al*., 2001). In our model, the slight increase in apoptosis may be related to resolution of the type II cell hyperplasia induced by FGF-1 rather than to a direct effect on oxygen toxicity.

ACKNOWLEDGMENTS

We thank E. Escudier and F. Bernaudin for processing the samples for transmission electron microscopy and for interpreting the results, and D. Gruenert for the gift of the 1HAEo$^-$ cell line. This work was supported in part by the nonprofit organization Association Française des Myopathies and by the Academy of Paris (Legs Poix).

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Received for publication November 29, 2003; accepted after revision June 29, 2004.

Published online: July 29, 2004.