Transfection of lung cells in vitro and in vivo: effect of antioxidants and intraliposomal bFGF

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Luo, Xiaoping, Rosetta Belcastro, Judy Cabacungan, Vicky Hannam, Anna Negus, Yanxia Wen, Jonathan Plumb, Jim Hu, Brent Steer, David R. Koehler, Gregory P. Downey, and A. Keith Tanswell. Transfection of lung cells in vitro and in vivo: effect of antioxidants and intraliposomal bFGF. *Am J Physiol Lung Cell Mol Physiol* 284: L817–L825, 2003. First published January 3, 2003; 10.1152/ajplung.00479.2001.—We hypothesized that constitutive formation of reactive oxygen species by respiratory cells is a barrier to gene transfer when liposome-DNA complexes are used, by contributing to rapid degradation of plasmid DNA. When plasmid DNA is complexed to liposomes it is protected against H_2O_2-mediated degradation but not that mediated by the hydroxyl radical. Treatment of distal rat fetal lung epithelial cells ($RFL_{19}Ep$) with the vitamin E analog Trolox (50 μM) reduced intracellular plasmid degradation. Both Trolox (50 μM) and an iron chelator, phenanthroline (0.1 μM), significantly increased transgene expression in $RFL_{19}Ep$ approximately twofold, consistent with a hydroxyl radical-mediated inhibition of transgene expression. When basic fibroblast growth factor (bFGF; 20 ng/ml), a growth factor with antioxidant properties, was included within liposomes, we observed a significantly greater enhancement of $RFL_{19}Ep$ transgene expression (~2-fold) over that seen with Trolox or phenanthroline. Inclusion of bFGF within liposomes also significantly enhanced (~4-fold) transgene expression in mice following intratracheal instillation.

gene transfer; reactive oxygen species; growth factors; basic fibroblast growth factor

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THE INTRACELLULAR FORMATION of reactive oxygen species (ROS) under pathological conditions has been implicated in a wide variety of degenerative processes, diseases, and syndromes (12). However, it has more recently been appreciated that constitutively formed intracellular ROS are also important stimulators of signal transduction during a variety of cell processes (31), including DNA synthesis (9, 31). The hydrogen peroxide (H_2O_2) content of normal human breath condensate is ~0.26 μM (1, 18), with a range from 0.01 to 0.4 μM (21), presumably reflecting constitutive formation of ROS by lung cells. Consistent with this observation are the findings that both type II pneumocytes from adult animals (2) and distal rat fetal lung epithelial cells ($RFL_{19}Ep$) (24) generate and release into their culture medium significant quantities of H_2O_2. In conditions in which there is pulmonary inflammation, the H_2O_2 concentration of breath condensate may increase considerably to >1 μM (15).

We have previously reported that liposome-mediated transfection of primary cultures of distal $RFL_{19}Ep$ cells is, at least in part, limited by rapid intracellular degradation of the transfected DNA (34). That these cells generate and release into their culture medium significant quantities of H_2O_2, even when maintained in a fetal oxygen tension of 20 mmHg (24), suggested to us that constitutive formation of ROS by these cells may contribute to degradation of transfected DNA. H_2O_2 in the culture medium of distal $RFL_{19}Ep$ cells is derived from the reduction of cell-derived superoxide through spontaneous or enzymatic dismutation (14). In the studies reported here, we have examined whether constitutive ROS production by these cells is an impediment to transfection using liposome-DNA complexes, as assessed by antioxidant interventions. In addition, we have evaluated the use of intraliposomal basic fibroblast growth factor (bFGF), a growth factor with antioxidant properties (36) that has the potential to enhance transgene expression not only by its antioxidant properties (36) but also through enhanced cellular uptake of DNA (29) or, because bFGF produced within a cell is largely directed toward receptors on the nuclear membrane (5, 22), through facilitating delivery of DNA in the liposome-DNA complex to the nuclear membrane.

MATERIALS AND METHODS

Materials. 1,[2-14C]Dioleoyl-*sn*-glycero-3-phosphoethanolamine ([14C]DOPE), [methyl-3H]thymidine, and triethylam-

monium deoxycytidine $5'[\alpha-^{33}P]$triphosphate were from Amersham Canada (Oakville, Ontario, Canada). [8-^{14}C]Adenine, [5,6,8,9,11,12,14,15-^{3}H(N)]prostaglandin $F_{2\alpha}$, and ^{125}I-bFGF were from New England Nuclear (Boston, MA). Proteinase K was from Promega (Madison, WI). Recombinant human bFGF, recombinant human FGF receptor 1α (IIIc)/Fc chimera, and recombinant human nerve growth factor (NGF) receptor/Fc chimera were from R&D Systems (Minneapolis, MN). 1,10-Phenanthroline was from ICN Biomedicals (Costa Mesa, CA). Diphenyl-p-phenyldiamine was from Eastman Kodak (Rochester, NY). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was from Sigma-Aldrich (Oakville, Ontario, Canada). All other chemicals were from Sigma (St. Louis, MO). Vetbond tissue adhesive was from 3M (St. Paul, MN). Human erythrocyte catalase was from Calbiochem-Novabiochem (San Diego, CA). DEAE Sepharose FF and CM Sepharose FF were from Amersham (Baie d'Urfé, Québec, Canada). Porcine trypsin, heat-inactivated fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were from GIBCO Canada (Burlington, Ontario, Canada). Collagenase and type I DNase (2,367 U/mg) were from Worthington (Freehold, NJ). Restriction enzymes were from Pharmacia (Baie d'Urfé, Québec, Canada). 1,2-Dioleoyldimethylammonium chloride (DODAC) was from Inex Pharmaceuticals (Vancouver, British Columbia, Canada). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was from Avanti Polar Lipids (Alabaster, AL). GL67/dimyristylphosphatidyl-ethanolamine-polyethylene glycol 5000 (DOPE/DMPE-PEG5000) was from Genzyme (Framingham, MA). A 10-ml thermobarrel liposome extruder was from Lipex Biomembranes (Vancouver, British Columbia), and extrusion filters were from Nucleopore (Pleasanton, CA). The pCMV4-chloramphenicol acetyl transferase (CAT) construct was a generous gift from Drs. K. Brigham and J. Conary (Vanderbilt University School of Medicine, Nashville, TN). The secreted human placental alkaline phosphatase plasmid (pSEAP) construct was purchased from Tropix (Bedford, MA). An 8-isoprostane enzyme immunoassay kit was from Cayman Chemical (Ann Arbor, MI). A rhodamine nucleic acid labeling kit was from PanVera (Madison, WI), and the labeling procedure was according to the manufacturer's instructions. Fluorescent labeling of cell nuclei with 4',6-diamidino-2-phenylindole (DAPI) was performed when cells were mounted with a DAPI-containing mounting medium from Vector Laboratories (Burlingame, CA).

Cell culture. Primary cultures of 19-day-gestation distal RFL$_{19}$Ep were prepared as previously described (20, 33, 34). Briefly, the lungs of 19-day-gestation fetal rats were isolated and separated from major vessels and airways. The lungs were minced and gently vortexed to remove erythrocytes. This step was repeated until the supernatant was clear. The minced lung tissue was then subjected to proteolytic digestion with trypsin and DNase, a process that was arrested by the addition of FBS. The resultant cell suspension was eluted through 100-μm-mesh nylon bolting cloth. The eluted cells were next subjected to a collagenase digestion, which also arrested by the addition of FBS, and fibroblasts were removed by differential adherence. Epithelial cells of >95% purity, as assessed by staining for cell cytoskeleton components, were obtained using this technique (20). Cells were seeded in DMEM with 10% (vol/vol) FBS at a sufficient cell density to allow near-confluence 24–48 h after seeding, at which time the cells were changed to serum-free medium for the individual experiments. Cells were maintained in an humidified gas mixture of 3% O_2, 5% CO_2, and 92% N_2 to maintain the cells at a normal fetal arterial oxygen tension of ~20 mmHg. Concentrations of catalase-inhibitable H_2O_2 re-

leased into serum-free culture medium collected over 24 h were confirmed (data not shown) to be similar to those previously reported (24), when measured using an alternate methodology (16).

Cationic liposome preparation. An equimolar solution of DODAC/DOPE in chloroform was dried under vacuum in a rotary evaporator, then maintained under vacuum for 24–48 h. The resulting lipid film was rehydrated at 1 μg total lipid/μl phosphate-buffered saline (PBS) and sonicated for 2× 30 s. For calculations of lipid recovery [^{14}C]DOPE was included in the initial lipid mixture. Liposomes were initially extruded through sequentially smaller gauge syringe needles from 18- to 27-Fr and were then subjected to five freeze-thaw cycles with liquid N_2 and extrusion through a 400-nm filter (25). Liposomes were recovered by centrifugation at 165,000 g for 1 h. For DODAC/DOPE liposomes, plasmid DNA was added at a 1:10 (wt/wt) DNA-to-lipid ratio. The charge ratio that allows optimal uptake of DODAC/DOPE liposome/DNA complexes by RFL$_{19}$Ep occurs in a narrow band between DNA/lipid molar ratios of 1:10–1:15 (34). Preparations were diluted with culture medium as necessary. GL67/DOPE/DMPE-PEG5000 was provided as a lyophilized powder, which was vortexed in ultrapure water ± 20 ng/ml bFGF. For in vivo studies with GL67/DOPE/DMPE-PEG5000 liposomes, DNA was added at a 3.6:0.6 (mol/mol) DNA-to-lipid ratio. The mixture was allowed to stand at 30°C for 15 min, without mixing, to allow the formation of lipid-DNA complexes.

When bFGF was included within liposomes, it was added to the rehydration buffer at a concentration of 20 ng/ml, which we have previously defined to be the optimal concentration for stimulation of RFL$_{19}$Ep DNA synthesis when added to the culture medium (32). In preliminary experiments (data not shown), it was observed that bFGF added to preformed liposomes modestly reduced transfection efficiency, presumably by altering the charge ratio of the liposome-DNA complex. Liposomes were, therefore, washed in 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA to degrade extraliposomal bFGF. In preliminary experiments, liposomes were incubated with ^{125}I-bFGF to allow ^{125}I-bFGF/liposome complexes to form. These were washed free of unassociated ^{125}I-bFGF, then washed in 0.05% (wt/vol) trypsin and 0.02% (wt/vol) EDTA for 1 h, which resulted in a 71 ± 2% ($n = 3$; means ± SE) removal of ^{125}I-bFGF from ^{125}I-bFGF/liposome complexes.

For in vitro transfection experiments, liposome-DNA complexes were added to cell cultures for 24 h at a lipid concentration of 2.5 nmol/cm^2, as previously described (34). Uptake of DODAC/DOPE liposome-DNA complexes by RFL$_{19}$Ep is linear up to 24 h, and exposure to a lipid concentration of 2.5 nmol/cm^2 is not associated with cytotoxicity (34).

Cytotoxicity index. Cytotoxicity was assessed by the release of [8-^{14}C]adenine from cells preincubated with 0.2 μCi/ml [8-^{14}C]adenine (27). As reported elsewhere, this assay has an ability to detect cell injury equivalent to measurements of lactate dehydrogenase release (33) or trypan blue exclusion (11). The medium containing [8-^{14}C]adenine was added for 2 h before the monolayer was washed 2× with fresh medium. The percentage of preincorporated [8-^{14}C]adenine released into the culture medium was assessed after 48 h and expressed relative to the basal release by cells not exposed to additives, which was given an arbitrary index value of 1.

DNA synthesis. This was assessed by incorporation of [^{3}H]thymidine (1 μCi/ml) into cell DNA (17) under serum-free conditions. The isotope was added 48 h after plating, and the duration of the incubation was 24 h, as previously described (18).
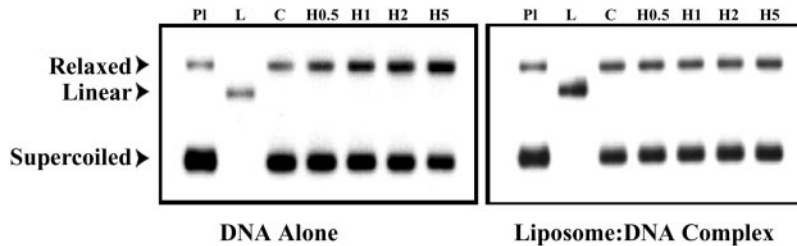
Fig. 1. Plasmid DNA was separated on 1% agarose gels before (Pl) and after (L) linearization as standards. Compared with control plasmid DNA (C), pCMV4-chloramphenicol acetyl transferase (CAT) DNA that had been exposed to 0.5, 1, 2, or 5 μM H_2O_2 (H0.5, H1, H2, H5) for 24 h showed a concentration-dependent loss of the supercoiled form with a reciprocal increase in the damaged relaxed form. However, when plasmid DNA was complexed with liposomes, the DNA was protected against injury at these concentrations of H_2O_2.

Oxidants and antioxidants. For studies of intracellular plasmid DNA stability, Trolox (50 μM) was added to the culture medium at the time of liposome addition. After 24 h, when the medium was changed, fresh medium containing 50 μM Trolox was added for a further 24 h. For studies of transgene expression, Trolox (50 μM), diphenyl-phenyldiamine (0.2 μM), and phenanthroline (100 nM) were introduced into the culture medium at the time of liposome addition and maintained in fresh culture medium for 48 h after the 24-h transfection period. Cell counts were performed on an automated cell counter (Coulter Electronics, Hialeah, FL). Accuracy of cell counts was regularly validated against hemocytometer counts. Measurement of 8-isoprostane by immunoassay was used as an index of lipid peroxidation (26). We have previously validated this measurement against aldehyde production by RFL$_{19}$Ep as assessed by gas chromatography-mass spectrometry (34). Results have been presented as picograms per milligrams of protein, with protein measurements made according to the method of Bradford (6).

DNA degradation. Plasmid DNA alone or an equivalent amount of DNA in a liposome-DNA complex was exposed to 0.5, 1, 2, or 5 μM H_2O_2 for 24 h or to 20 mM H_2O_2 $\pm$ 0.1 or 1 μM cupric (Cu^{2+}) sulphate for 10 min, to generate the hydroxyl radical (3). In preliminary studies, H_2O_2-mediated degradation of plasmid DNA was shown to be inhibitable by preincubation of the H_2O_2 solution with either erythrocyte catalase (100 U/ml) or Trolox (2 mM). Catalase was removed from the solution, prior to further treatment of the plasmid, by stirring with sequential additions of DEAE Sepharose FF and CM Sepharose FF, followed by centrifugation. The plasmid was linearized using 100 units of the restriction enzyme *Sma*I with 100 μg of pCMV4-CAT in 100 μl of buffer (containing in mM: 100 Tris acetate, 100 Mg acetate, and 500 K acetate, pH 7.5) at 30°C overnight. After a 24-h exposure to DNA with cationic liposomes, cells were washed twice with culture medium to remove any free liposome-DNA complexes. Cells were harvested immediately after washing or,

after a further 24 h in culture, by treatment with 0.1% (wt/vol) trypsin and 0.001% (wt/vol) DNase and centrifugation, to remove cell surface-associated liposomes and DNA (8). After resuspension in PBS, a DNA extract was prepared from the cells with proteinase K (13). DNA extracts were subjected to electrophoresis in 1% (wt/vol) agarose gels. Blots were probed with probes specific to the CAT nucleotide sequence.

DNA uptake. For studies of DNA uptake, pCMV4-CAT plasmids were nick translated with triethylammonium deoxycytidine 5′[α-^{33}P]triphosphate. We removed unincorporated nucleotides by passing the reaction mixture through a small column of Sephadex G-50. For uptake experiments, [^{33}P]plasmid DNA was mixed with unlabeled plasmid DNA to give a final specific activity of ~1.6 × 10^8 dpm/μg DNA. Integrity of the labeled DNA was confirmed by electrophoresis on a 1% (wt/vol) agarose gel with transfer onto a nylon membrane and autoradiography. For studies of liposome-associated plasmid uptake, the cells were harvested at the end of the incubation period by treatment with 0.1% (wt/vol) trypsin and 0.001% (wt/vol) DNase and centrifugation. Trypsin removes cell surface-associated liposomes (8), and DNase was included to degrade any surface-associated DNA not associated with liposomes. Total DNA uptake was calculated from the ^{33}P-content of the cell pellet.

Reporter gene activity. The bacterial CAT gene, which is not present in eukaryotic cells, was used as one reporter gene to measure transgene expression. The gene product is an enzyme that catalyses the transfer of acetyl groups to the substrate chloramphenicol from acetyl 1-CoA. Catalytic activity was determined by a radiometric assay, as described by Stribling et al. (30). In other studies, a mammalian expression vector containing SEAP was used as a reporter gene (4) with chemiluminescent detection. Reporter gene activities were measured 48 h after the end of the exposure of cultured cells to liposome-DNA complexes or 48 h after intratracheal instillation of liposome-DNA complexes.

Fluorescent DNA localization. Digital images of fluorescent DNA and cell nuclei were superimposed for calculations
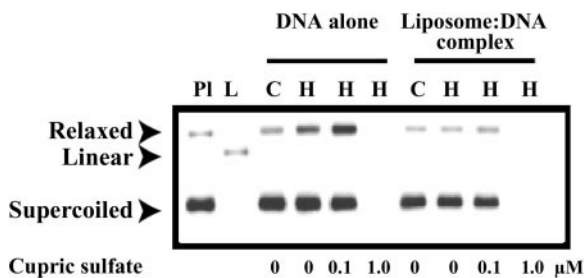

Fig. 2. pCMV4-CAT DNA was separated on 1% agarose gels before (Pl) and after (L) linearization as standards. The hydroxyl radical was generated by the addition of cupric (Cu^{2+}) sulfate to 20 mM H_2O_2 (H) for 10 min and compared with unexposed control samples (C). In the presence of 0.1 μM Cu^{2+} sulfate, a small increase in the relaxed form of plasmid DNA was evident. In the presence of 1 μM Cu^{2+} sulfate, all DNA forms were completely degraded, and no protection was evident from complexing DNA with liposomes.

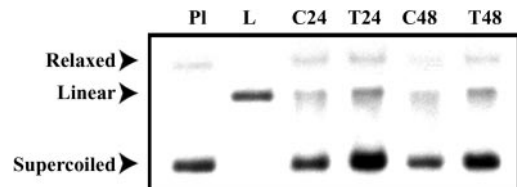

Fig. 3. pCMV4-CAT DNA was separated on 1% agarose gels before (Pl) and after (L) linearization as standards. Separated simultaneously was 20 μg of DNA that had been extracted from rat fetal lung epithelial cells (RFL$_{19}$Ep) immediately after transfection (C24, T24) or 24 h later (C48, T48). Cells were cultured in the presence (T24, T48) or absence (C24, C48) of 50 μM Trolox. An increased abundance of the supercoiled form of plasmid DNA was evident at 24 h (2.56-fold) and 48 h (2.05-fold) in cells that had been cultured in the presence of Trolox.

Fig. 4. *A*: the addition of 50 µM Trolox (T) , 0.2 µM diphenyl-phenyldiamine (D), and 0.1 µM phenanthroline (P) as antioxidant interventions before and during transfection of RFL$_{19}$Ep significantly enhanced pCMV4-CAT reporter gene expression compared with additive-free control (C) cells. *B*: RFL$_{19}$Ep transfected with liposome-DNA complexes in which the liposomes contained 20 ng/ml of basic fibroblast growth factor (bFGF, B) had a significantly increased expression of the pCMV4-CAT reporter gene relative to cells transfected with the liposome-DNA complex in the absence of intraliposomal bFGF (C). This effect was significantly greater than the enhanced reporter gene expression observed with cells transfected in the presence of 50 µM Trolox (T) or 0.1 µM phenanthroline (P) in the culture medium (n = 4; means ± SE; *P < 0.05 vs. control cells; #P < 0.05 vs. all other groups).
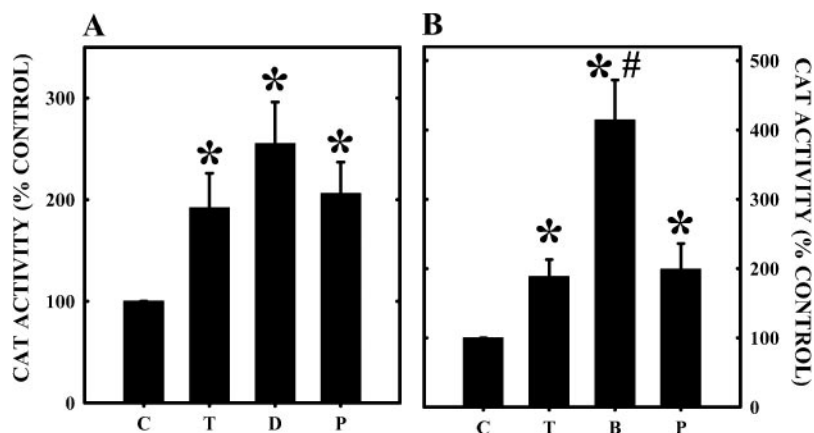
of the number of nuclear membranes in direct contact with fluorescent DNA. For these studies, the cells were cultured on chambered glass slides from Nalge Nunc International (Naperville, IL). Two to three random fields were used from each of four separate chambers, and mean values from each chamber used for calculations of means ± SE.

Instillation of liposomes into mouse lungs. Female CD1 mice at 10–12 wk of age were used. Inhalational anesthesia was established with 2% ethrane in 60% nitrous oxide with 40% oxygen in a chamber. Anesthesia was maintained with a nose cone. A small skin incision was made over the trachea for insertion of a 30-Fr needle to inject the liposome-DNA suspension into the trachea. The skin incision was closed with veterinary tissue adhesive. Liposome-DNA suspensions were in a final volume of 100 µl of ultrapure water. This contained 33.9 µg of GL67/DOPE/DMPE-PEG5000 with 118.8 µg of plasmid DNA. For comparison, some animals received plasmid DNA alone. Animals were killed 48 h after instillation of the DNA or liposome-DNA suspensions to allow removal of lung tissue for assay of reporter gene activity.

Statistical analysis. All values are shown as means ± SE of each group. Where error bars are not evident in the figures they fall within the plot point. Where figures show data from representative experiments, these experiments have been replicated on two to four occasions. Statistical significance (P < 0.05) was generally determined by analysis of variance followed by assessment of differences using Duncan's multiple range test (28).
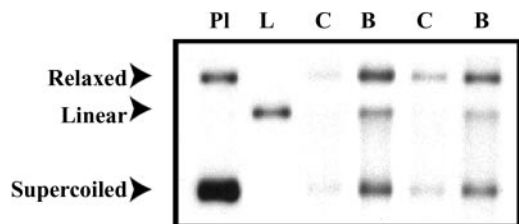
RESULTS

To determine whether plasmid DNA was susceptible to H_2O_2-mediated injury, we exposed plasmid DNA to 0.5–5 µM H_2O_2 for 24 h before and after being complexed with liposomes. As shown in Fig. 1, plasmid DNA exposed to 0.5–5 µM H_2O_2 showed a concentration-dependent loss of supercoiled DNA, with an increase in the relaxed form, consistent with oxidant injury. However, when the plasmid DNA was complexed to cationic liposomes, the DNA was protected against injury. In contrast, complexing DNA with liposomes did not protect it against a 10-min exposure to hydroxyl radicals (Fig. 2). When hydroxyl radical generation was initiated with low concentrations of Cu^{2+} sulfate (0.1 µM), an increase in relaxed DNA was evident with or without conjugation with liposomes. At an increased concentration of Cu^{2+} sulfate (1 µM), DNA was totally degraded with a complete loss of DNA bands, and no protection was seen when DNA was complexed with liposomes. In control experiments
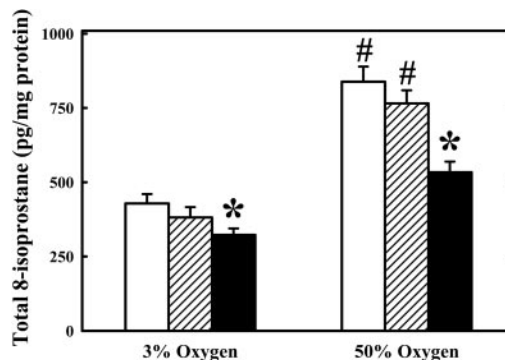
Fig. 5. pCMV4-CAT DNA was separated on 1% agarose gels before (Pl) and after (L) linearization as standards. Separated simultaneously was 20 µg of DNA that had been extracted from replicate RFL$_{19}$Ep immediately after a 24-h transfection with the liposome-DNA complex in the presence (B) or absence (C) of intraliposomal bFGF. An increased abundance of intracellular plasmid DNA was evident at 24 h in cells that had been transfected with the liposome-DNA complex containing intraliposomal bFGF.

Fig. 6. RFL$_{19}$Ep were exposed to 3 or 50% O_2, and lipid peroxidation was assessed by measurement of 8-isoprostane. Exposure to 50% O_2 increased 8-isoprostane formation. Cells treated with liposomes containing 20 ng/ml of bFGF (solid bars) had a significantly reduced 8-isoprostane formation in both 3 and 50% O_2-exposed cells, relative to untreated cells (open bars). Cells treated with liposomes containing buffer only (hatched bars) had no significant change in 8-isoprostane formation (n = 4; means ± SE; *P < 0.05 vs. additive-free control cells at the same O_2 concentration; #P < 0.05 vs. additive-free control cells at 3% O_2).
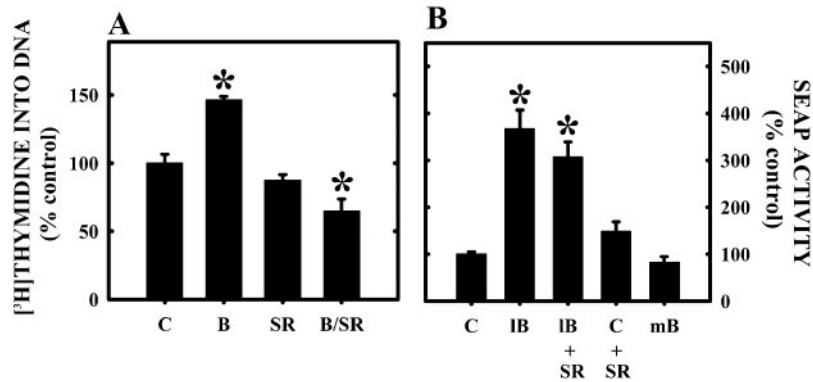
Fig. 7. *A*: exposure of RFL$_{19}$Ep to 20 ng/ml of bFGF (B) in the culture medium significantly increased cell DNA synthesis over that observed under additive-free control conditions (C). Addition of 5 ng/ml of chimeric truncated soluble FGF receptor to the culture medium had no significant effect on DNA synthesis under control conditions (SR) but did attenuate the increase in DNA synthesis observed in response to bFGF (B/SR). *B*: expression of the secreted human placental alkaline phosphatase (SEAP) transgene was enhanced by the presence of intraliposomal bFGF (lB) compared with transfection in the absence of intraliposomal bFGF (C). Neither the addition of 5 ng/ml of truncated soluble FGF receptor (lB + SR and C + SR) nor the presence of 20 ng/ml of bFGF in the medium (mB) had significant effects on transgene expression (*n* = 4; means ± SE; **P* < 0.05 vs. control cells).

(data not shown), 0.1 and 1 μM Cu^{2+} sulfate alone had no effect on the DNA bands.

That cell-derived generation of ROS contributes to degradation of transfected DNA intracellularly was suggested by an experiment in which plasmid DNA was extracted from RFL$_{19}$Ep after transfection in the presence or absence of the antioxidant Trolox, which we have previously shown to scavenge H$_2$O$_2$, arrest lipid peroxidation, and attenuate hydroxyl radical formation in oxidant-stressed RFL$_{19}$Ep (24). The loss of supercoiled DNA with time in culture was slowed by the presence of Trolox, with an ~2–2.5-fold difference in supercoiled DNA content (Fig. 3). In contrast, cells transfected and maintained in the presence of Trolox had clearly detectable bands of supercoiled DNA, consistent with reduced oxidant-mediated DNA injury. That antioxidants can not only preserve cell content of transfected DNA with time in culture but also enhance transgene expression is shown in Fig. 4*A*. The presence of Trolox (50 μM), diphenyl-phenyldiamine (0.2 μM), or phenanthroline (0.1 μM) in the culture medium resulted in a 2–2.5-fold increase (*P* < 0.05) in transgene expression. When liposomes containing bFGF (20 ng/ml) were used for transfection, transgene expression was significantly enhanced (*P* < 0.05) over and above that seen with either Trolox or phenanthroline in the culture medium (Fig. 4*B*). No additional transgene expression was observed by the combined addition of Trolox or phenanthroline with bFGF (data not shown), suggesting that the effect of bFGF was not simply due to an antioxidant effect alone. An increased intracellular content of plasmid DNA following transfection with liposomes containing bFGF was confirmed, as shown in Fig. 5.

That bFGF did have antioxidant activity when RFL$_{19}$Ep were treated with liposomes (2.5 nmol/cm^2) containing 20 ng/ml bFGF for 24 h is shown in Fig. 6. Control cells or cells treated with liposomes containing buffer alone exposed to 50% O$_2$ had a significant (*P* <

0.05) increase in 8-isoprostane release, as previously described (24). Cells treated with liposomes containing bFGF had both a significantly (*P* < 0.05) reduced basal level of 8-isoprostane release in 3% O$_2$ and a significant (*P* < 0.05) inhibition of the 50% O$_2$-mediated increase in 8-isoprostane production. There was no liposome- or bFGF-mediated effect on protein recovered from cells exposed to 50% O$_2$. Another potential contributor to the enhanced transgene expression observed with intraliposomal bFGF was enhanced binding to the cell surface through an FGF receptor. Should some bFGF escape the liposome but remain liposome-DNA complex associated, bFGF that had not been completely removed by washing liposomes in a trypsin and EDTA solution after preparation could act as a ligand for this receptor. To test this, accessible bFGF was exposed to a truncated soluble receptor to reduce its availability
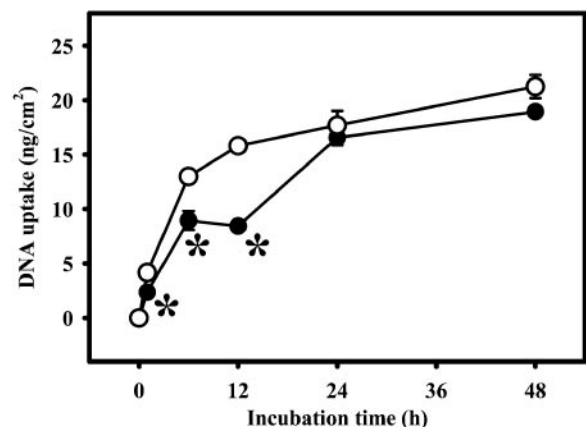

Fig. 8. Uptake of radiolabeled liposome-DNA complexes by RFLE$_{19}$Ep in the presence (closed circles) or absence (open circles) of intraliposomal bFGF. The presence of intraliposomal bFGF significantly inhibited uptake of liposome-DNA complexes after 1, 6, and 12 h of incubation, but not after 24 or 48 h (*n* = 4; means ± SE; **P* < 0.05 vs. control cells at the same time point).
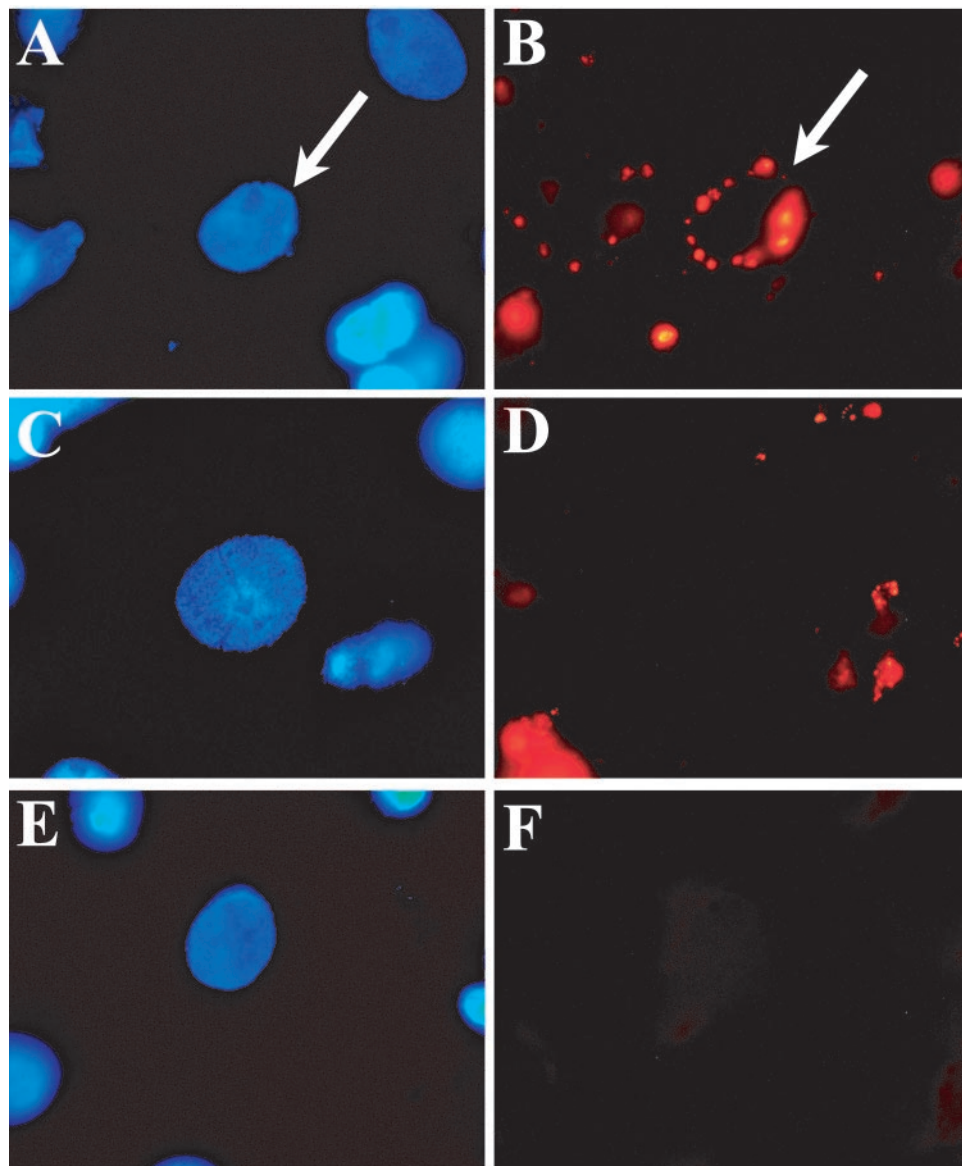
Fig. 9. Photomicrographs to show localization of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei (*A*, *C*, *E*) or rhodamine-labeled DNA (*B*, *D*). RFL$_{19}$Ep were transfected with fluorescent (*A–D*) or control (*E*, *F*) liposome-DNA complexes for 24 h. Fluorescent DNA (arrow, *B*) encircling nuclei (arrow, *A*) was observed in some cells treated with the liposome-DNA complex containing bFGF (*B*), but not in cells treated with the bFGF-free liposome-DNA complex (*D*).

for binding to cell surface receptors. That the selected concentration of truncated receptor (5 ng/ml) could block bFGF activity was confirmed with DNA synthesis as an index of bFGF activity secondary to binding to FGF receptors at the cell surface. As shown in Fig. 7*A*, the truncated soluble receptor attenuated the significant ($P < 0.05$) bFGF-mediated increase in cell DNA synthesis and also significantly ($P < 0.05$) reduced basal levels of DNA synthesis. The specificity of this effect was studied in separate experiments using a similarly constructed truncated soluble receptor to NGF. The truncated soluble receptor to NGF had no effect on cell DNA synthesis, and neither soluble receptor was cytotoxic at a concentration of 5 ng/ml (data not shown). Having confirmed that the truncated soluble FGF receptor was active at a concentration of 5 ng/ml, we added this concentration to the culture medium during transfections in the presence or absence of intraliposomal bFGF. The soluble truncated FGF recep-

tor had no significant ($P > 0.05$) inhibitory effect on the enhanced ($P < 0.05$) transgene expression brought about by the presence of intraliposomal bFGF, and there was no independent effect ($P > 0.05$) of extraliposomal bFGF (Fig. 7*B*).

Recognizing that liposome-DNA complexes prepared with intraliposomal bFGF might also affect DNA uptake by a variety of other mechanisms, we examined radiolabeled DNA uptake in the presence or absence of intraliposomal bFGF (Fig. 8). Liposome-DNA complexes prepared with intraliposomal bFGF had a significantly ($P < 0.05$) reduced uptake after 1, 6, and 12 h of incubation but not after 24 or 48 h.

Next, we sought to determine whether intraliposomal bFGF enhanced perinuclear localization of transfected DNA, using plasmid DNA that had been tagged with a fluorescent marker. Cells transfected with bFGF-containing fluorescent DNA-liposome complexes for 24 h had very different patterns of fluorescence
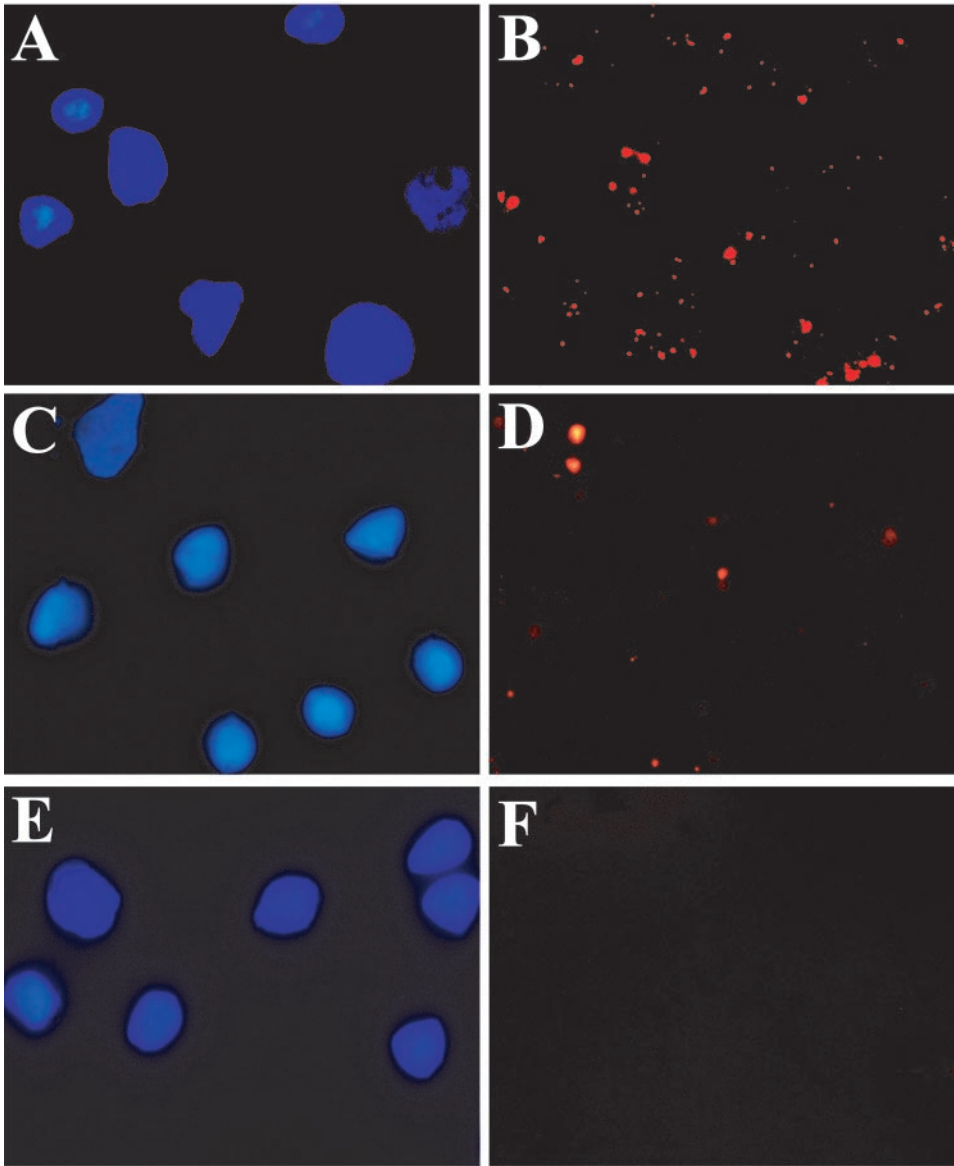
Fig. 10. Photomicrographs to show localization of DAPI-stained nuclei (*A*, *C*, *E*) or rhodamine-labeled DNA (*B*, *D*). RFL$_{19}$Ep were transfected with fluorescent (*A–D*) or control (*E*, *F*) liposome-DNA complexes for 24 h, followed by a further 24 h in culture. Loss of fluorescent DNA during time in culture was significantly greater in cells treated with the bFGF-free liposome-DNA complex (*D*) than in cells treated with the liposome-DNA complex containing bFGF (*B*).

when compared with cells transfected with fluorescent DNA-liposome complexes not containing bFGF. Fluorescent DNA appeared randomly distributed over the slide when bFGF-free liposomes were used (Fig. 9*D*), whereas cells that had been transfected with bFGF-containing liposomes had a proportion of cells with a distinctive perinuclear localization of fluorescent plasmid DNA (Fig. 9*B*). At 48 h, more fluorescent DNA was evident in cells that had been transfected with liposomes containing bFGF (Fig. 10*B*) than cells treated with control liposomes (Fig. 10*D*). Fluorescent DNA was observed in contact with the nuclear membrane of $9.7 \pm 1.1\%$ of cells transfected with control liposomes, but this was significantly increased ($P < 0.05$; $n = 4$; means $\pm$ SE) to $37.3 \pm 3.4\%$ for cells transfected with liposomes containing bFGF.

Last, we used mice to determine whether our observations of enhanced reporter gene expression with bFGF in vitro would also be evident in vivo. As shown in Fig. 11, when GL67/DOPE/DMPE-PEG5000 liposomes, prepared containing either control buffer or 20 ng/ml of bFGF, were instilled via the trachea into mice, a significant increase in pulmonary transgene expression was observed with the inclusion of bFGF in the liposomes ($n = 5$). There were no grossly obvious differences in lung histology following instillation of the two types of liposomes.

DISCUSSION

The prototypic lung disease for which gene therapy has been proposed is cystic fibrosis. Adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator gene has been less successful than originally anticipated, in that it is associated with dose-limiting toxicity, low efficiency, and brief efficacy, in part due to induction of an adenovirus-specific cell-mediated immunity (38). Liposome-medi-
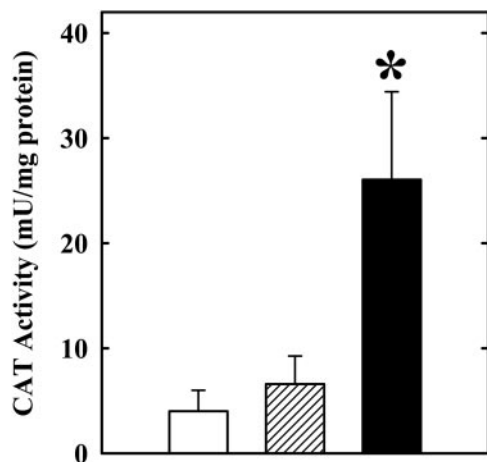
Fig. 11. GL67/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine/ dimyristylphosphatidyl-ethanolamine/polyethylene glycol 5000 (DOPE/DMPE-PEG5000) liposomes were prepared containing either control buffer (hatched bar) or 20 ng/ml of bFGF (solid bar) for in vivo instillation in mice. Naked DNA alone was also instilled for comparison (open bar). A significant increase in pulmonary transgene (pCMV4-CAT) expression was observed with the inclusion of bFGF in the liposomes ($n = 5$; means $\pm$ SE; $*P < 0.05$ vs. control liposome expression or naked DNA alone).

ated gene transfer has certain advantages over viral gene transfer, in that this approach is both nonimmunogenic and nononcogenic, but it suffers from being less efficient than the viral approach (37). Several barriers to efficient gene transfer have been identified, including inactivation by cystic fibrosis sputum (23) and the presence of preexisting inflammation (35). We hypothesized that intracellular and extracellular ROS may contribute to these barrier effects not only in cystic fibrosis but also in the various acute lung injuries that may be amenable to a gene therapy approach (7).

The feasibility of cationic lipid-mediated gene transfer to the developing human and rodent lung has been established (19). Our interest has been in using this approach for studies of neonatal lung injury, for which reason we conducted our experiments with distal lung epithelial cells from preterm rat fetuses. We assume that the majority of H_2O_2 found in the aqueous hypophase at the surface of the lung epithelium will be derived from lung epithelial cells and macrophages. Consistent with this, fetal lung epithelial cells in culture have a medium concentration of H_2O_2 similar to that reported for normal breath condensate (24). Under normoxic conditions, 1–2% of mitochondrial oxygen consumption can be accounted for in the production of superoxide and, upon dismutation, H_2O_2 (10). It has been recently recognized that ROS are important signal transduction molecules for various cell processes, including growth, under physiological conditions (31). In the absence of phagocyte myeloperoxidase, to form hypochlorous acid, the amount of H_2O_2 present in normal breath condensate (1, 18) and cell culture medium from $RFL_{19}Ep$ (24) would not be sufficient to significantly damage plasmid DNA. However, moderate damage would be evident at concentrations obtained from inflamed lungs (15). Plasmid DNA was protected from supraphysiological concentrations of H_2O_2 when complexed with liposomes, although not from hydroxyl radical-mediated injury.

Trolox, at a concentration that attenuates hydroxyl radical formation, arrests lipid peroxidation and scavenges H_2O_2 (24), and limits intracellular degradation of transfected supercoiled DNA, consistent with this degradation being mediated by ROS. Transfection in the presence of either of two antioxidants, Trolox and diphenyl-phenyldiamine, increased transgene expression ~2–2.5-fold. That these agents act through limiting hydroxyl radical-mediated DNA injury is suggested by an equivalent effect on transgene expression of phenanthroline, a cell-permeant iron chelator, at a concentration that we have previously shown to prevent increased hydroxyl radical production when $RFL_{19}Ep$ were exposed to hyperoxic conditions (24).

Of particular interest are the findings using intraliposomal bFGF, which resulted in an approximately fourfold increase in transgene expression. This growth factor has been reported to have antioxidant properties (36), which we have been able to confirm. It is, therefore, likely that an antioxidant effect contributes to its capacity to enhance transgene expression. The mechanism by which the remainder of this effect is mediated is not clear. Treatments of bFGF-containing liposomes with soluble FGF receptor and trypsin suggest that enhanced attachment of the liposome-DNA complex to the cell through binding of monovalent bFGF to FGF receptors on the cell is not the mechanism. Such treatments do not exclude such an effect through multimeric multivalent bFGF complexes masked in the liposome-DNA complex, which become unmasked on attachment of the liposome-DNA complex to the cell. However, because inclusion of bFGF in liposomes did not enhance their uptake, the bFGF-mediated enhancement of transgene expression cannot be attributed to an effect of enhanced binding to an FGF receptor, a conformational change, or a modification of surface charge on uptake of the liposome-DNA complex. Our finding of enhanced localization of fluorescent DNA to the nuclear membrane should be interpreted with caution. It is possible that enhanced nuclear targeting, through a chaperone effect of bFGF directed at FGF receptors on the nuclear membrane (5, 22), may contribute to this finding. However, this observation may simply reflect a reduction in DNA degradation with enhanced retention of DNA either at the nuclear membrane or within endosomes.

In summary, intraliposomal bFGF significantly enhances transgene expression both in vitro and in vivo. This effect is in part due to the antioxidant properties of bFGF, but other mechanisms also appear to be involved. Intraliposomal bFGF may be suitable for use as an adjuvant for studies in humans, in that a recombinant human protein, which would be nonimmunogenic, is available.

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