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Bronchial Epithelial Cell Matrix Production in Response to Silica and Basic Fibroblast Growth Factor

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Abstract

Background: Previous studies show that macrophages, lung fibroblasts, and their soluble mediators are responsible for the onset and development of pulmonary fibrosis. This study was conducted to determine whether airway epithelial cells are also directly involved in response to fibrogenic agents and consequently in the pathogenesis of lung fibrosis. To verify the hypothesis, we determined whether silica acts directly on human bronchial epithelial cells by stimulating cytokine and growth factor release and by modifying matrix production.

Materials and Methods: An SV40 large T antigen-transformed human airway epithelial cell line, 16HBE14o (16HBE), was used. The expression profile of some pro-inflammatory interleukins (ILs), such as IL-1α, IL-1β and IL-6 and their modulation by silica, were evaluated by polymerase chain reaction (PCR) analysis. Transforming growth factor beta (TGFβ) and basic fibroblast growth factor (bFGF) mRNA levels were tested by Northern blotting in the presence and in the absence of silica. The silica- and/or bFGF-induced effects on matrix components (total proteins, collagen, and fibronectin) were also evaluated using radiolabeled precursors.

Results: The results demonstrated 16HBE internalized silica particles. Silica induced a little IL-6 secretion, without affecting IL-1 and TGFβ isoform production and strongly stimulated bFGF mRNA level and bFGF protein secretion. Silica also induced changes in 16HBE production of total proteins, collagen, and fibronectin production. When added in combination with the growth factor, it strengthened bFGF stimulation of matrix component secretion.

Conclusions: These results support the hypothesis that the changes in matrix components are due to a direct effect of silica on bronchial epithelial cells. Silica-induced oversecretion of bFGF suggests that autocrine and paracrine differentiation loops for bFGF may also be operative and that these mechanisms may be involved in the pathogenesis of pulmonary fibrosis. In the future, cytokine-directed therapeutic strategies might find a place in clinical practice.

Introduction

Pulmonary fibrosis, resulting from chronic exposition to fibrogenic agents, is mainly interstitial. The sequence of events leading to lung fibrosis involves inflammation and disruption of the normal tissue architecture, followed by tissue repair with mesenchymal cell accumulation and excess extracellular matrix (ECM) production (1). In response to fibrotic growth factors or cytokines, released principally by macrophages, lung fibroblasts increased productions of fibrillar type I and II collagen (2,3). Interactions between macrophages and lung fibroblasts after fibrotic particle translocation across the airway epithelium play a pivotal role (4–6). In addition to alveolar macrophages and lung fibroblasts, epithelial cells and basal lamina may be considered not only as barriers preventing the egress of particles into the interstitial space (7), but also as effectors of mineral dusts.

When the epithelium is damaged, the wound site is immediately covered by ECM and basal lamina proteins such as laminin, type IV collagen, and fibronectin (8–13). Bronchial epithelium enhances pulmonary host defense mechanisms by releasing cytokines and growth factors that induce cell differentiation with changes in matrix production, chemotaxis, and activation (14).

Interleukin 1 (IL-1) and interleukin 6 (IL-6) commonly participate in local injury and inflammatory reactions (15,16). They have multifunctional roles, which are all central in the development of inflammation within the airways of individuals with respiratory disease. Transforming growth factor (TGFβ) and basic fibroblast growth factor (bFGF), major mediators in regulating cell growth, differentiation, signaling, and repair, are mainly associated

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with fibrotic changes involved in tissue repair and lung fibrosis (17,18).

To better understand the role of epithelial tissue in lung fibrosis, we conducted an electron and scanning microscopy study on a human bronchial epithelial cell line that was exposed to silica in vitro. The fluorescent antibody technique was used to evaluate laminin and collagen IV immunoreactivity. Using polymerase chain reaction (PCR) analysis, we tested the expression profile of some pro-inflammatory cytokines such as IL-1α, IL-1β, and IL-6, and evaluated TGFβ and bFGF mRNA levels by Northern blotting. Quantitative analysis of secreted cytokines and growth factors and the effects of silica on them were evaluated by specific enzyme-linked immunosorbent assay (ELISA) kits. To postulate bFGF autocrine mechanisms that could enhance the effects of silica on matrix production, we used radiolabeled precursors to study total protein, collagen, and fibronectin synthesis in bronchial epithelial cells maintained in vitro in presence of bFGF alone, silica, or bFGF and silica in combination. Finally, matrix production was related to cell proliferation.

Materials and Methods

Cell Cultures

An SV40 large T antigen-transformed human airway epithelial cell line, 16HBE14o (16HBE), was obtained from the Dr. D. Gruenert Laboratory (University of California, San Francisco, Calif) and kindly provided by A.M. Vignola and A. Merendino (University of Palermo, Italy). The transformed cells that retain differentiated morphology and function of normal human airway epithelia (19) were maintained in Eagle's minimum essential medium (Gibco, UK) with 10% fetal calf serum (FCS), nonessential amino acids (2 mM), L-glutamine (2 mM), 100 U/ml penicillin, and 100 μg/ml streptomycin to achieve subconfluence. For the experiments, 16HBE cells were utilized between 3 and 20 passages, seeded at 10^5 cells/cm^2 for 24 hr in MEM 1 0.5% FCS and treated according to the following experimental schemes.

IMMUNOFLUORESCENCE PROCEDURE. 16HBE cells were treated with or without crystalline silica (Sigma Chemical Company, St. Louis, Mo; 99% microcrystalline silica, containing particles between 1–5 μm) at the concentration of 50 μg/ml in MEM 1 0.5% FCS for 24 hr. Determination of the number of particles was carried out by counting particles with light microscopy. The silica suspension containing 4.4 3 10^4 particles per μg of silica (10 μg/cm^2, initial ratio of 16 particles per cell) was treated for 2 hr at 2008F to inactivate any endotoxin. After the incubation period, 16HBE cells were washed with phosphate-buffered saline (PBS) and immediately fixed in 2.5% parafolmaldeyde in PBS buffer for 10 min at room temperature (20). Coverslips were incubated for 1 hr with bovine serum albumin (BSA) in PBS buffer before overlaying for 1 hr with mouse monoclonal anti-laminin (1:1000, Sigma), and mouse monoclonal anticollagen IV (1:100, Sigma). After incubation for 1 hr, cells were washed three times and overlaid for 1 hr with anti-mouse IgG TRITC coniugate (1:50) antibody developed in goat (Sigma). Negative controls omitting the primary antibody were totally unstained (data not shown). The coverslips were mounted in 90% glycerol in PBS and examined under a Zeiss Dialux 20-EB epifluorescence microscope, equipped with automatic microphotography. Pictures were taken with Fujichrome HP 1600 film and developed by the Cibachrome method.

Electron Microscopic Procedure

16HBE were treated with or without silica at the concentration of 50 μg/ml in MEM 1 0.5% FCS for 24 hr. After prolonged washes, cells were dissociated in 0.25% phosphate-buffered trypsin (pH 7.2), and collected by centrifugation. Pellets were fixed in gluteraldehyde (1.7% in 0.1 M phosphate buffer, pH 7.2) for 30 min, washed in PBS, and postfixed in osmium tetroxide (1% in 0.1 M phosphate buffer) for 30 min. They were then dehydrated through a graded ethanol series and embedded in araldite resin (Durcapan-Fluka AG, Buchs SG, Switzerland). Thin sections were obtained on a Reichert ultramicrotome, transferred to a copper grid, stained with uranyl acetate and lead citrate, and examined through a Hitachi 800 electron microscope. For scanning electron microscopy (SEM) examination, cells were fixed in 1% glutaraldehyde in PBS for 15 min at room temperature, washed three times in PBS, and dehydrated stepwise in ethanol. After critical point drying by the Freon method, the samples were coated with gold-palladium (60:40) by vacuum evaporation on a moving stage and viewed under a 501 Philips SEM. The acceleration voltage was 15 KV.

Detection of IL-1α, IL-1β, and IL-6 by RT-PCR Analysis

Total RNA was isolated from 16HBE cells (21) treated or not with silica (50 μg/ml) for 24 hr in MEM 1 0.5 FCS and the RNA treated immediately with DNAse I (Gibco). The integrity of the treated RNA was examined by detection of ribosomal RNA bands (28S and 18S) in ethidium bromide stained agarose gels. The RNA was quantified by reading the optical density at 260 nm. One to two micrograms of total RNA were reverse transcribed for 1 hr at 378C. RT was accomplished in a final volume of 50 μl containing 50 mM Tris-HCl (pH 8.3 at 258C), 3 mM $MgCl_2$, 75 mM KCl, 10 mM DTT, 0.5 mM each deoxynucleotide triphosphate (dNTP), 40 units/tube RNAsin (Roche Molecular Biochemicals, Italy), 1 μg random exanucleotide primers (Roche), and 40 units/tube Moloney murine leukemia virus (M-MLV) reverse transcriptase

(Gibco). CR was performed using 2 μl of the cDNA prepared by the RT reaction in a final volume of 50 μl. The PCR reaction mixture contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4 at 258C), 1.5 mM $MgCl_2$, 2.5 units Taq polymerase (Platinum *Taq;* Gibco), 0.2 mM each dNTP, and 0.2 μM of each pair of primers. The sequence for each 39- and 59-cytokine primer pair used in this study is based on published oligonucleotide sequence data (Seid JM, et al., 1993) and is summarized in the table.

The predicted product sizes for the IL-1α, IL-1β, and IL-6 primers are 222, 249, and 240 bp, respectively. PCR was run in a thermal cycler (Hybaid) for 25 cycles, each cycle consisting of denaturation stage for 15 sec at 948C, annealing for 20 sec at 578C, and polymerization for 30 sec at 728C. At the end of amplification, the PCR products were separated on a 1.6% agarose gel, stained in ethidium bromide, and photographed under ultraviolet light. To confirm the sizes of the fragments, a 100-bp DNA ladder (Gibco)

PCR primer sequences

Gene	Primer Sequence	Product Size (bp)
IL-1α		
Primer 39	59-CGAGCCAATGATCAGTACCTC-39	222
Primer 59	59- GGTAGTGTCCATCACTCTGG -39	
IL-β		
Primer 39	59-GGCAGACTCAAATTCCAGCT -39	249
Primer 59	59-ACAACGAGGTATAGGACAGG-39	
IL-6		
Primer 39	59-CAGGAACTGGATCAGGACTT-39	240
Primer 59	59-GACGAAAGTGTGTACAATGA-39	
Actin		
Primer 39	59-ACGGGGTCACCCACACTGTG -39	660
Primer 59	59-CTAGAAGCATTGCGGTGGACGATG -39	

was used as molecular weight marker. Actin was included as the control gene, which is expressed in all cells. The presence of 660-bp actin bands in all the PCR products confirms the similar conversion of RNA to cDNA in both control and silica-treated samples.

Detection of bFGF and TGFβ1 mRNA by Northern Blot Analysis

Total cellular RNA was isolated from 16HBE treated or not with silica (50 μg/ml) for 24 hr in MEM 1 0.5% FCS. For Northern blot analysis (22), equal amounts of total RNA (20 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde and transferred onto nylon filters (Hybond-N plus, Amersham, Little Chalfont, UK). Before blotting, the gels were rinsed in 203 SSC (SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) for 20 min. The blots were prehybridized (0.2 ml/cm^2) in a mixture containing 50% formamide, 63 SSC, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA, and 53 Denhardt's solution for 4 hr at 428C. The probes were labeled with [α^{32}P] dCTP (3000 Ci/mmol, Amersham) by random priming (multiprime DNA labeling system, Amersham). Hybridization (0.1 ml/cm^2) was performed at 428C overnight using a 10^6-cpm/ml probe in the same buffer used for prehybridization. After hybridization was completed, the nylon membranes were washed once in 23 SSC, 0.1% SDS at room temperature and then once in 0.13 SSC, 0.1% SDS at 658C for 30 min. Autoradiography was performed by exposing membranes to Kodak X-Omat film at 2 808C for 4 days. Differences in the intensity of the specific hybridization bands from the autoradiograms were evaluated by computerized scanning densitometry. The human cDNA probes used in the Northern blotting analyses were a 1.05-kb human TGFβ_1 cDNA (clone SP64βas, ATCC), a 0.8-Kb human bFGF cDNA (a generous gift of Judith Abraham, PhD, Scios Nova, Calif) and a 1.3-kb rat glyceraldehyde-3-phosphate dehydrogenase cDNA (pRGAPDH). To assess blot loading, the membranes were stripped and rehybridized with the GAPDH probe. For autoradiography, the filters were exposed to Kodak X-Omat film at 2 808C for 4 days.

Quantitative Analysis of Cytokines and Growth Factor by ELISA Kit

16HBE were maintained for 24 hr in MEM 1 0.5% FCS with or without silica (50 μg/ml). The cultures were then rinsed and maintained in serum-free

medium for an additional 24 hr. After incubation, conditioned medium (CM) from untreated (control-CM) or silica-treated (silica-CM) cells was collected and centrifuged for 10 min at 2000 g in the presence of protease inhibitors (1 mM phenyl-methyl-sulfonyl-fluoride) to remove cells and debris. ELISA analysis (Amersham Life Science Products) of lyophilized and stored at 2 708C CM was carried out according to the manufacturer's instructions. Standard curves were run to determine IL-1α, IL-6, TGFβ_1, and bFGF concentrations.

TGFb Bioassay

TGFβ activity was assayed using a bioassay based on the ability of TGFβ to inhibit proliferation of CCL-64 cells (ATCC, Mv-1-Lu, mink lung epithelial cells). For the assay (23), CCL-64 (10,000 cells/well) were seeded in 96-well microtitre plates and maintained in MEM plus 10% FCS and 1% nonessential amino acids. Parallel sets of CCL64 were incubated with aliquots of control-CM or silica-CM, dialyzed against water for 72 hr, lyophilized, and dissolved in the medium to a final protein concentration of 100 μg/well. TGFβ standard (0.2 ng/well) or silica standard (10 ug/well) was used as internal controls. All cultures were maintained in a final volume of 200 μl for 72 hr and pulsed with 2.5 μCi/ml [^{3}H]-thymidine for the last 6 hr of incubation. Identification of TGFβ isoforms were identified by preincubating CM with neutralizing antibodies against TGFβ_1 (2 μg/ml, mouse IgG, R&D System, Minneapolis, Minn), TGFβ_2 (0.06 μg/ml mouse IgG, R&D Systems), and TGFβ_3 (0.6 μg/ml mouse IgG, R&D Systems). CCL-64 were harvested with an automated cell harvester and the amount of [^{3}H]-thymidine incorporated was determined by the standard liquid scintillation method and reported as percent of inhibition of [^{3}H]-thymidine incorporation.

Analysis of Total Protein and Collagen Synthesis

Confluent 16HBE epithelial cells were incubated for 24 hr in MEM plus 0.5% FCS, L-glutamine, nonessential amino acids with and without silica (50 μg/ml), bFGF (20 ng/ml) silica, and bFGF together diluted with the medium to yield solution of 10^{26} M 50 μg/ml. Parallel cultures exposed to silica at doses of 25, 100, and 250 μg/ml did not show any dose dependent effects. Sodium ascorbate and 10 μCi/ml ^{3}S-methionine (AS 185 MBq in 5 mCi) were added in the last 3 hr. The chase media were dialyzed in the presence of proteolytic enzyme inhibitors against three changes of distilled water (24). Cell layers were washed three times with ice-cold tris buffered saline (TBS), pH 7.4, then scraped into 1.0 ml of 50 mM Tris/HCl, pH 7.4, containing protease inhibitors, and then sonicated. Equal aliquots of the culture media containing 1–5 3 10^4 dpm were freeze dried and reconstituted in 1.0 ml assay buffer (50 mM Tris/ HCl, 5 mM $CaCl_2$, pH 7.5). Samples of 0.5 ml were incubated with and without 25 μg highly purified bacterial collagenase (25) in the presence of 2.5 mM *N*-ethylmaleimide at 378C for 2 hr. The undigested noncollagenous protein was then precipitated by adding 50 μg BSA and ice-cold trichloroacetic acid (TCA), 7% (Wt/vol) final concentration. The supernatant was extracted with diethyl ether to remove the TCA. Radioactivity was determined by scintillation counting. The radioactivity incorporated into collagenous proteins was determined by subtracting the radioactivity in the control from the collagenase-digested sample. The radioactivity in the noncollagenous protein was determined after extracting any residual collagen from the pellet with hot TCA and dissolving the remaining pellet in 100 μl of 70% (vol/vol) formic acid before scintillation counting.

Analysis of Fibronectin Synthesis

Confluent 16HBE cells were incubated for 24 hr (see above). Ten μCi/ml ^{3}S-methionine (AS 185 MBq in 5 mCi) were added in the last 3 hours. Fibronectin was selectively and quantitatively bound to gelatin sepharose pre-equilibrated in TBS in individual minicolumns (24). Aliquots of media containing 1–4 3 10^4 dpm radioactivity were brought to 0.5% (vol/vol) Triton X-100 using a 20% (vol/vol) Triton stock solution in water, and allowed to percolate through minicolumns of 100 μl gelati-Sepharose resin (Pharmacia Inc., Piscataway, NJ) pre-equilibrated with TBS. The fibronectin was eluted with 100 μl of 2 3 SDS-PAGE sample buffer and analyzed by electrophoresis on 6% SDS-polyacrylamide slab gels. The fluorographs were analyzed for quantification via densitometric scanning using the Quanti-Scan software running on a 486 IBM desktop computer.

Cell Proliferation

The incorporation of [^{3}H]-thymidine (Radiochemical, Amersham) was used as index of DNA synthesis. 16HBE cells were treated for 24 hr in MEM 1 0.5% FCS with silica (50 μg/ml) or bFGF (20 ng/ml), or silica 1 bFGF. Untreated HBE cells were seeded for 24 hr in MEM 1 0.5% FCS (controls). One μCi/ml of [^{3}H]-thymidine (81.0 Ci/mmol SA, Radiochemical, Amersham) was added to all cultures. After in vitro maintenance, cells were solubilized in 0.5 M NaOH and an aliquot precipitated with 10% TCA (30 min at 48C) on glass fiber filters (Millipore, 0.45 μm). After washing with cold 5% TCA, the filters were dried and counted in 10 ml of scintillation fluid (Ultima Gold, Packard Chemical, Groningen, The Netherlands) in an LKB scintillation counter. Incorporation of the radioactive label into the TCA-insoluble fraction was calculated as cpm/well. After incubation, 16HBE cells were scraped with a rubber policeman, pelleted by centrifugation at low speed, and resuspended in a small volume (0.1–0.5 ml) of medium. To determine cell viability, Trypan blue was added to the cellsuspension to obtain a final concentration of 2 mg/ml. Cells were incubated for 5 min at room temperature, and the number of stained cells counted in a hemocytometer.

Statistical Analysis

Results reported in the table and figures are the mean ± SD of three separate experiments, each performed in quadruplicate. Statistical analysis was performed by analysis of variance (ANOVA) followed by Sheffe F-test (26).

Results

Immunofluorescence Staining of Collagen IV and Laminin Pattern

Indirect immunofluorescence studies showed that collagen IV is expressed in irregular amorphous patches in the perinuclear region and in a punctate distribution in the cell periphery in silica-treated and untreated 16HBE cells (Fig. 1AB).

Laminin protein stained as spots, which probably corresponded to adhesion points in the substratum in control and silica-treated 16HBE cells (Fig. 1CD).

Morphologic Analysis

Scanning electron microscopy of 16HBE cultured in medium alone (control) revealed numerous surface microvilli projecting from the plasma membranes (Fig. 2A). In silica-treated cells, silica particles were attached to the plasma membrane in and around the membranous structures (Fig 2B). Transmission electron microscopy revealed cytoplasmic internalization of silica particles within single-membrane vacuoles. There were no other ultrastructural changes compared with the controls (Fig. 3AB).

IL-1α, IL-1β, and IL-6 Expression by PCR

Total RNA was extracted from untreated and silica-treated cultures and reverse transcribed. The cDNA were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific oligonucleotide primers for IL-1α, IL-1β, and IL-6 (Fig. 4). IL-1 and IL-6 bands were expressed in control and silica-treated cultures; IL-1β was the less consistent isoform.

Northern Blotting Analysis for bFGF and TGFβ Transcripts

Basic FGF and TGFβ expression was evaluated by hybridizing Northern blots with radiolabeled human bFGF and TGFβ probes. Densitometric analysis of autoradiographs normalized with GADPH levels showed 16HBE cells expressed constitutively high levels of bFGF and TGFβ1 mRNA (Fig. 5). The human bFGF and TGFβ1 cDNA probes recognized a 2.5-Kb mRNA and a 7-kb RNA, respectively. Silica did not induce any differences in TGFβ1 expression, but it increased bFGF gene expression by 26%.

Quantitative Analysis of Secreted Cytokines and Growth Factors by ELISA Kit

Analysis by ELISA of the amounts of IL-1α, IL-6, bFGF, and TGFβ secreted by 16HBE cells treated or not with silica are reported in Table 1. There were no significant differences in IL1α and TGFβ secretion rates in control and silica-treated cells. Silica enhanced IL-6 (↑ 18%) and bFGF secretion (↑ 172%).

TGFβ Activity Assay

TGFβ bioassay was based on the capacity of active TGFβ to inhibit CCL-64 growth as expressed by [^{3}H]-thymidine incorporation. Preincubation of CM with TGFβ1, -β2, and -β3 neutralizing antibodies distinguished the different isoforms of secreted TGFβ. CM obtained from untreated 16HBE cells (CM-control) inhibited the proliferative response of CCL-64 by approximately 84%, suggesting high release of active TGFβ. TGFβ2 and -β3 antibodies inhibited CCL-64 growth by 74% and 80%, respectively, revealing that TGFβ1 is the most secreted TGFβ isoform. The effects of silica CM on CCL64 thymidine incorporation overlapped with the control CM, showing that silica treatment did not affect 16HBE release of any TGFβ isoform (data not shown).

Collagen and Total Protein Synthesis

Cell-associated collagen was enhanced by bFGF treatment (↑ 30%), whereas silica had a reducing effect (↓ 25%) (Fig. 6). When silica and bFGF were added together, the stimulatory effect of bFGF is conserved (↑ 15%). Collagenous protein secretion into the medium was stimulated either by bFGF or silica (increases of 25% and 19%, respectively) and the combination of bFGF and silica resulted in additive effects (increase of 48%). Total protein synthesis had a similar trend (data not shown). Basic FGF treatment enhanced cell-associated protein synthesis (↑ 20%), and silica had an inhibitory effect (↓ 14%). With the bFGF–silica combination, total protein synthesis increased by 30%. An increase induced either by bFGF or silica on total protein secretion into the medium was observed (increases of 16% and 11%, respectively). The simultaneous administration of silica and bFGF enhanced the increase of total secreted proteins to 42%.

Fibronectin Synthesis

Intracellular and secreted fibronectin was isolated by affinity chromatography on gelatin sepharose and analyzed by SDS-PAGE (Fig. 7). Fluorograph of cell-associated fibronectin were increased only after bFGF treatment (↑ 20%). Silica alone or in combination with bFGF decreased cellular fibronectin (↓ 20% and ↓ 40%). No significant differences were seen in the amount of radiolabeled fibronectin secreted when 16HBE were treated or not with bFGF or with silica added individually. On the contrary, a stimulatory effect on fibronectin secretion was induced by bFGF and silica association (↑ 30%).

Cell Proliferation

16HBE cultures treated with or without bFGF and silica, alone or together, were labeled with

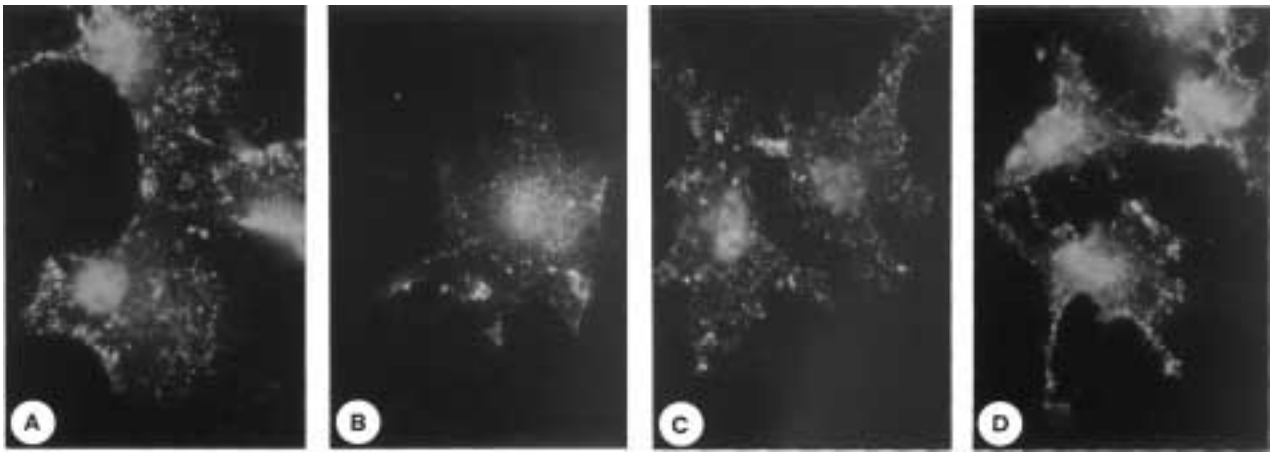


Fig. 1. Immunofluorescence staining of collagen IV and laminin in 16HBE cells. Immunofluorescence staining of collagen IV in control (A) and in silica-treated 16HBE cells (B). Laminin patterns in untreated (C) or silica-treated (D) 16HBE cells. Original magnification: 3 1200.

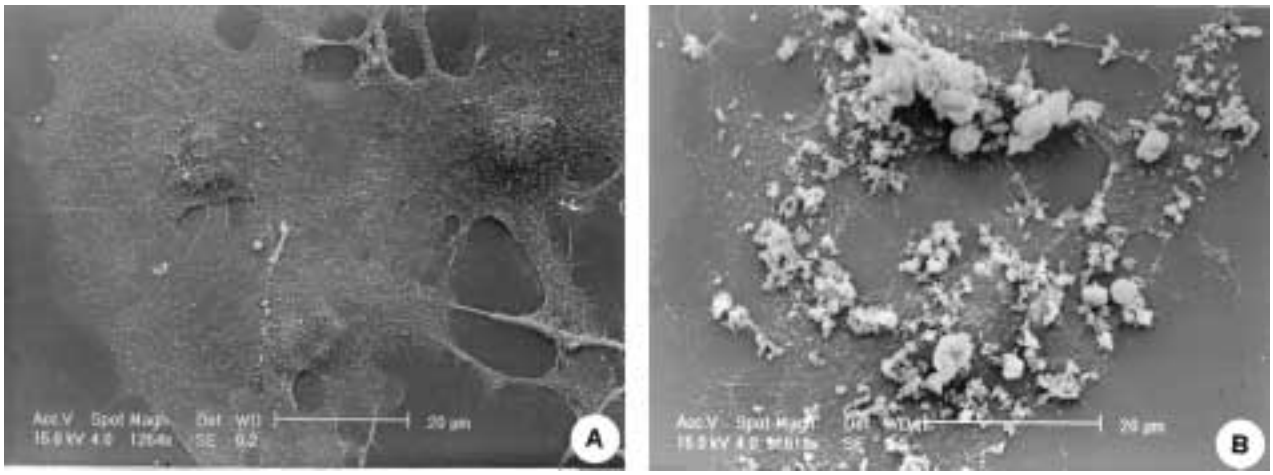


Fig. 2. Adherence of silica to surface of 16HBE cells. (A) Scanning electron micrograph of a control monolayer not exposed to silica. (B) Scanning electron micrograph of a monolayer exposed to silica (50 μg/ml) for 24 hr and then washed to remove unbound silica.

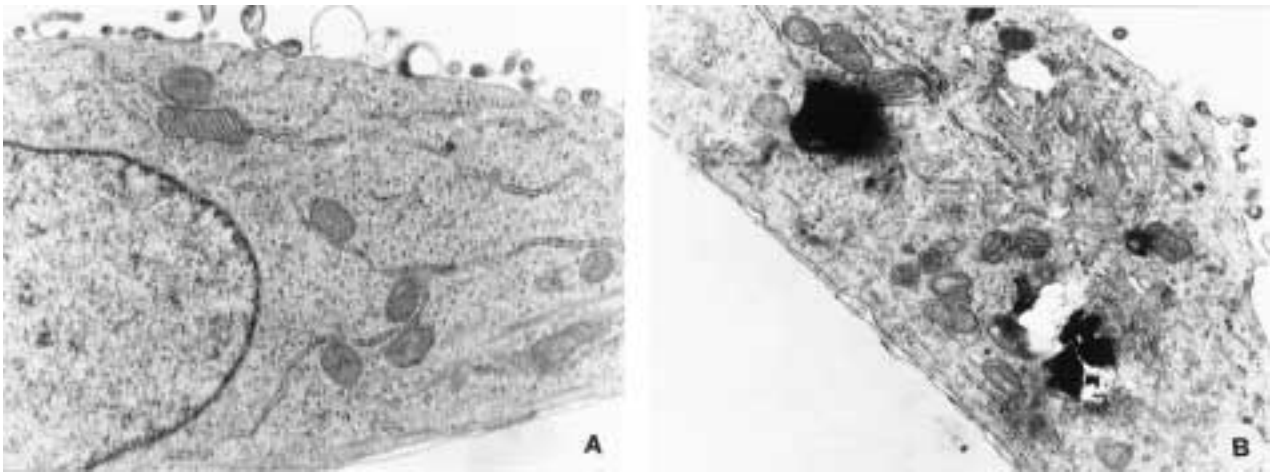


Fig. 3. Silica internalization into 16HBE cells. Electron microscope micrographs of 16HBE cells untreated (A) and treated with silica (B) (50 μg/ml) for 24 hr. Uranyl acetate and lead citrate staining. Micrograph B shows silica particle internalized by lung epithelial cells. Original magnification 3 21,000.

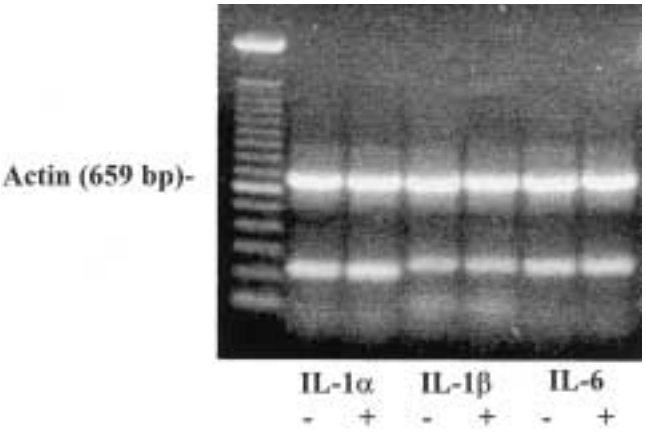
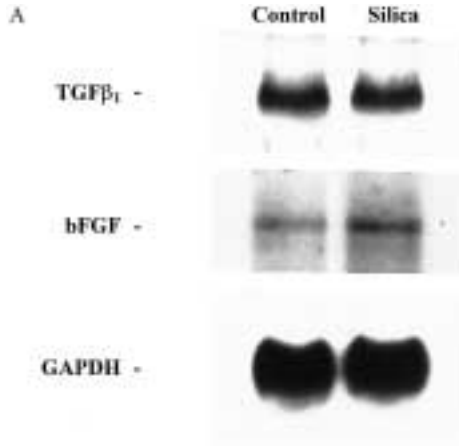


Fig. 4. Cytokine expression in human 16HBE cells cultured in the absence (−) and presence (+) of bFGF. Oligonucleotide primers complementary to human IL-1α, IL-1β, IL-6, and actin were used. Total RNA obtained from confluent cultures was reverse-transcribed and amplified by PCR for 25 cycles using the primers specific for each cytokine and for the actin. Ethidium bromide–stained agarose gel of PCR products is shown. A 100-bp DNA marker ladder is shown on the left side of the gel.



B Relative levels of TGFβ1 and bFGF mRNA

	Control	Silica
TGFβ1	1	0.97
bFGF	1	1.26

Fig. 5. Expression of TGFβ_1, bFGF, and GAPDH genes in 16HBE cells treated or not with silica (50 μg/ml) for 4 hr in MEM + 0.5% FCS. (A) Total RNA was extracted by 16HBE and analyzed by Northern hybridization technique. Briefly, 20 μg total RNA/lane were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde, transferred onto nylon membranes, and sequentially hybridized with [^{32}P]-labeled cDNA probes for TGFβ_1 and bFGF. The same filters were stripped and rehybridized with a GAPDH cDNA. (B) Densitometric analysis and relative quantification for TGFβ_1 and bFGF messages were made on 2.5-kb bands and 7-kb bands, respectively, of the autoradiogram, normalized to the control GAPDH probe. Similar results were seen in three separate experiments.

[^{3}H]-thymidine and harvested for acid precipitable radioactivity. Neither bFGF nor silica affected the amount of acid-precipitable label in the cultures (Fig. 8), suggesting they did not affect DNA synthesis. Parallel experiments assessing cell density showed a similar trend; cell number did not differ in controls and bFGF or silica-treated 16HBE cells.

Discussion

Silica exposure results in an initial inflammatory response in the lung followed by a chronic fibrotic reaction (27). Lung macrophages and fibroblasts are usually considered target cells in fibrogenesis (28,29), but the mechanism of silica-induced fibrosis is not yet understood. Silica has been implicated in a direct fibrogenic role in an immortalized pulmonary lung fibroblast cell line (WI1003), as we observed in a previous study (30,31). Recent reports suggest that human bronchial epithelial cells serve not only as a physical barrier against exogenous insults, but also play a role in the maintenance of airway structures and actively control lung tissue restoration (32,33).

In this paper, we provide evidence for the first time that pulmonary epithelial cells behave as direct effectors of silica particles. Silica directly modifies cytokine and growth factor release, and induces changes in matrix components. As the use of primary bronchial epithelial cell cultures was not feasible because of loss of viability upon passage, we opted for the transformed human bronchial epithelial cell line (16HBE), which retains differentiated epithelial morphology and maintains normal epithelium functions (19,34). The immunofluorescence antibody technique showed 16HBE cells expressed laminin and collagen IV, which are proteins typically produced by epithelial cells (35).

Using scanning and transmission microscopy, we demonstrated that 16HBE cells were able to ingest silica particles without undergoing any ultrastructural changes. Silica internalization across the epithelium has been demonstrated (36–38); in this report, we showed that transport of silica particles into the epithelium modifies the bronchial cell phenotype. In fact, 16HBE cells constitutively expressed and released IL-1, IL-6, bFGF, and TGFβ. The secretion of inflammatory cytokines and fibrogenic factors is evidence that the bronchial epithelium regulates local and immune responses. Of the two IL-1 isoforms, IL-1α showed a more consistent band. Silica stimulated IL-6 secretion only slightly, but significantly, increased bFGF mRNA levels and bFGF protein release without affecting IL-1 and TGFβ isoform secretion. In silicosis and other fibrotic diseases, local IL-6 or bFGF release may contribute to abnormal connective tissue deposition by stimulating lung fibroblast proliferation and differentiation. Local IL-6 overproduction in asthma (34) and inasbestos-induced lung disorder (39), and

Table 1. Relative amounts of TGFβ_1, basic FGF, IL-6, and IL-1α in conditioned media obtained from 16HBE cells cultured in presence or in absence of silica (50 μg/mL) for 24 hr

	TGFβ_1 ng/10^6	bFGF ng/10^6	IL-6 U/10^6	IL-1α ng/10^6
Control	0.104 ± 0.008	3.2 ± 0.23	915 ± 48	0.494 ± 0.028
Silica	0.099 ± 0.005 (NS)	8.7 ± 0.46[a]	1125 ± 35[b]	0.512 ± 0.032 (NS)

TGFβ_1, bFGF, IL-6, and IL-1α were assayed by ELISA kit. Values (mean ± SD) were derived from three separate experiments, each in quadruplicate. Data were analyzed by ANOVA.
Differences versus control: [a]F-test significant at 99%; [b]F-test significant at 95%; NS not significant.

bFGF blockage by antibodies inhibiting lung fibroblast proliferation (34) have in fact been demonstrated.

The finding that silica selectively induced greater 16HBE cell secretion of bFGF prompted us to investigate bFGF action on matrix production to determine whether bFGF positive feedback mechanisms amplify the effect of silica. We observed that bFGF enhanced total protein and collagen production in cellular and extracellular compartments while silica stimulated secretion prevalently in the medium. Silica and bFGF together promoted functional changes in 16HBE cells and strengthened their effects. Neither silica nor bFGF affected cell proliferation; consequently, their effects on the matrix were not due to changes in DNA synthesis or cell density. Because matrix impairments alter lung structure, the silica-and/or bFGF-induced modifications may be involved in lung silicosis in vivo.

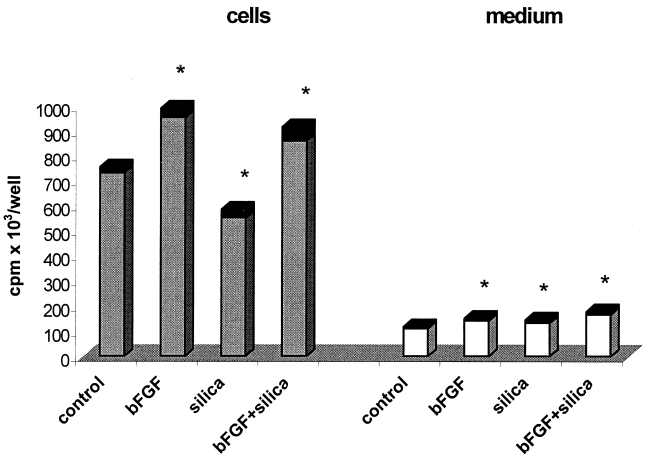
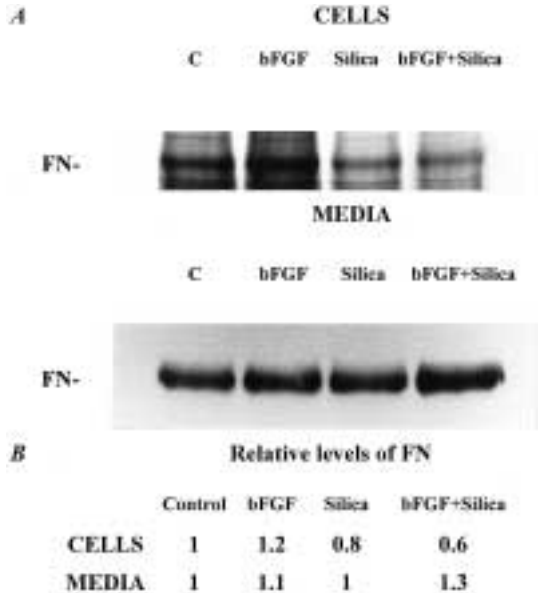


Fig. 6. 16HBE cells exposure in vitro to silica (50 μg/ml), bFGF (20 ng/ml), or silica + bFGF for 24 hr: Effects on collagen synthesis. All cultures were added with ^{3}H-methionine (25 μCi/ml). Values (mean ± SD) were derived from three separate experiments, each in quadruplicate. Data were analyzed by ANOVA. Differences versus control: *F-test significant at 99%; **F-test significant at 95%.

Fibronectin and collagen IV distribution in the lung have received considerable attention, and their role in the pathogenesis of fibrotic lung disease has been postulated. Increased collagen and laminin content, for example, determines changes in the viscoelastic behavior of the pulmonary parenchyma and alters dynamic parenchymal mechanisms (40). Patients with pulmonary fibrosis show increased fibronectin levels in pulmonary lavage fluid (41,42). Because fibronectin is involved in chemotaxis, fibroblast and bronchial epithelial cell proliferation, and migrating cell attachment, we can hypothesize



	Control	bFGF	Silica	bFGF+Silica
CELLS	1	1.2	0.8	0.6
MEDIA	1	1.1	1	1.3

Fig. 7. Effects of bFGF, silica, and silica + bFGF on fibronectin (FN) synthesized by 16HBE cells. Aliquots of cell extracts and media were subjected to gelatin-sepharose affinity chromatography and the isolated fibronectin was electrophoresed on 6% SDS-polyacrilamide gels and then fluorographed. (A) Film autoradiogram of the gels. (B) The fluorographs shown in (A) were analyzed by computerized scanning densitometry. The values are obtained assuming as 1 the untreated level for control cells. Similar results were seen in three separate experiments.

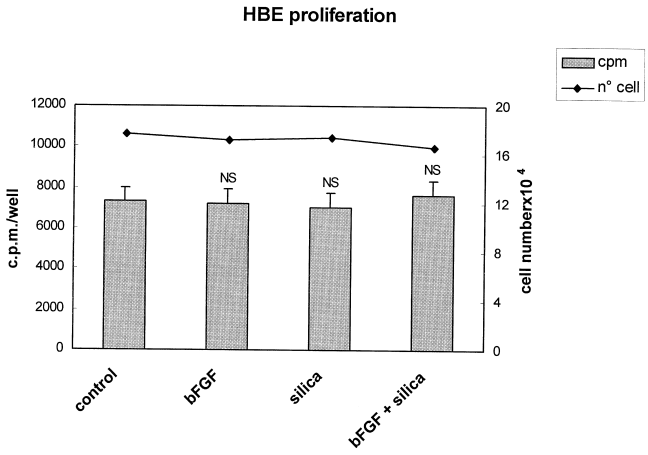


Fig. 8. [^{3}H]-thymidine incorporation and cell number in 16HBE cells treated with silica (50 μg/ml), bFGF (20 ng/ml), or silica + bFGF for 24 hr. Values (mean ± SD) were derived from three separate experiments, each in quadruplicate. Data were analyzed by ANOVA. Differences versus control were not significant (NS).

the silica and/or bFGF-induced change in fibronectin production might recruit more cells at the site of bronchial epithelial injury and modify fibroblast proliferation (43–45). After silica treatment, macrophage over-release of fibronectin has already been demonstrated (11). Our finding that silica combined with bFGF up-regulated fibronectin and collagen secretion even in 16HBE cells suggests that bronchial epithelial cells are directly involved in the pathogenesis of lung fibrosis.

In conclusion silica translocation into epithelial cells promotes direct effects on epithelial matrix components and silica-induced bFGF oversecretion may be another autocrine mechanism triggering pulmonary fibrosis. The data illustrate additional facets of cytokine–extracellular matrix interplay that still seed to be investigated to understand the molecular basis of lung fibrosis. The use of specific matrix components or cytokines to modify fibrotic processes and promote normal healing is an intriguing possibility requiring further evaluation.

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