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Distribution of Epidermal Growth Factor Receptor and Ligands during Bronchiolar Epithelial Repair from Naphthalene-Induced Clara Cell Injury in the Mouse

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Clara cells are primary targets for metabolically activated pulmonary toxicants because they contain an abundance of the cytochrome P450 monooxygenases required for generation of toxic metabolites. The factors that regulate bronchiolar regeneration after Clara cell injury are not known. Previous studies of naphthalene-induced bronchiolar injury and repair in the mouse have shown that epithelial cell proliferation is maximal 1 to 2 days after injury and complete 4 days after injury. Proliferation is followed by epithelial re-differentiation (4 to 14 days). In this study, mice were treated with the environmental pollutant naphthalene to induce massive Clara cell injury. The distribution and abundance of three growth-regulatory peptides (epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), and transforming growth factor (TGF)-α) was determined immunochemically during repair of this acute bronchiolar injury. EGFR and its ligands were detected at low levels in cells throughout the lung including peribronchiolar interstitial cells, blood vessels, and conducting airway epithelium. Immediately after naphthalene injury (1 to 2 days), EGFR, EGF, and TGF-α are expressed in increased abundance in squamous epithelial cells of the injury target zone, distal bronchioles. These immunopositive squamous cells are detected in clumps in the distal bronchioles at the time when cell proliferation is maximal. EGFR protein expression is decreased slightly 4 to 7 days after injury and continues to decrease below control levels of abundance 14 to 21 days after injury. This down-regulation of EGFR is not reflected in a corresponding decrease in EGF and TGF-α protein expression, indicating that control of cell proliferation is regulated at the receptor level. Co-localization of EGFR and bromodeoxyuridine-positive proliferating cells in the same bronchiole indicates that EGFR is up-regulated within the proliferative microenvironment as well as in specific proliferating cells within the injury target zone. The coincident localization within terminal bronchioles of EGFR, EGF, and TGF-α to groups of squamous epithelial cells 2 days after naphthalene injury suggests that these peptides are important in up-regulating cell proliferation after Clara cell injury in the mouse. *(Am J Pathol 1997, 151:443–459)*

The epithelium lining the distal conducting airways of the lung is a target for injury from many inhaled environmental pollutants and metabolically activated xenobiotics. The potential for repair of an acute injury to distal bronchioles, including the local production of factors that regulate epithelial cell proliferation and differentiation, may be key to the initiation or exacerbation of a number of chronic lung diseases, including asthma, chronic bronchitis, bronchopulmonary dysplasia, and some forms of distal lung cancer. Here we report the use of a recently characterized model of bronchiolar repair in which acute Clara cell injury is produced in mice by the bioactivated pulmonary toxicant naphthalene.[1] In this study we used this model to define the expression of potential regulators of epithelial proliferation and differentiation during resolution of acute bronchiolar injury.

Nonciliated bronchiolar epithelial (Clara) cells are the progenitor cells for repair of bronchiolar epithelium after injury to ciliated cells.[2–4] The Clara cell is also a primary injury target of both inhaled and systemically administered xenobiotics.[5–7] Parenteral administration of naphthalene, a component of tobacco smoke and fossil fuels, causes a dose-dependent, cell- and site-selective injury to distal airway Clara cells.[8,9] Clara cell-specific injury has been related to metabolism of naphthalene to a toxic intermediate by cytochrome P450 monooxygenases located within mouse distal airway Clara cells.[10,11] In this

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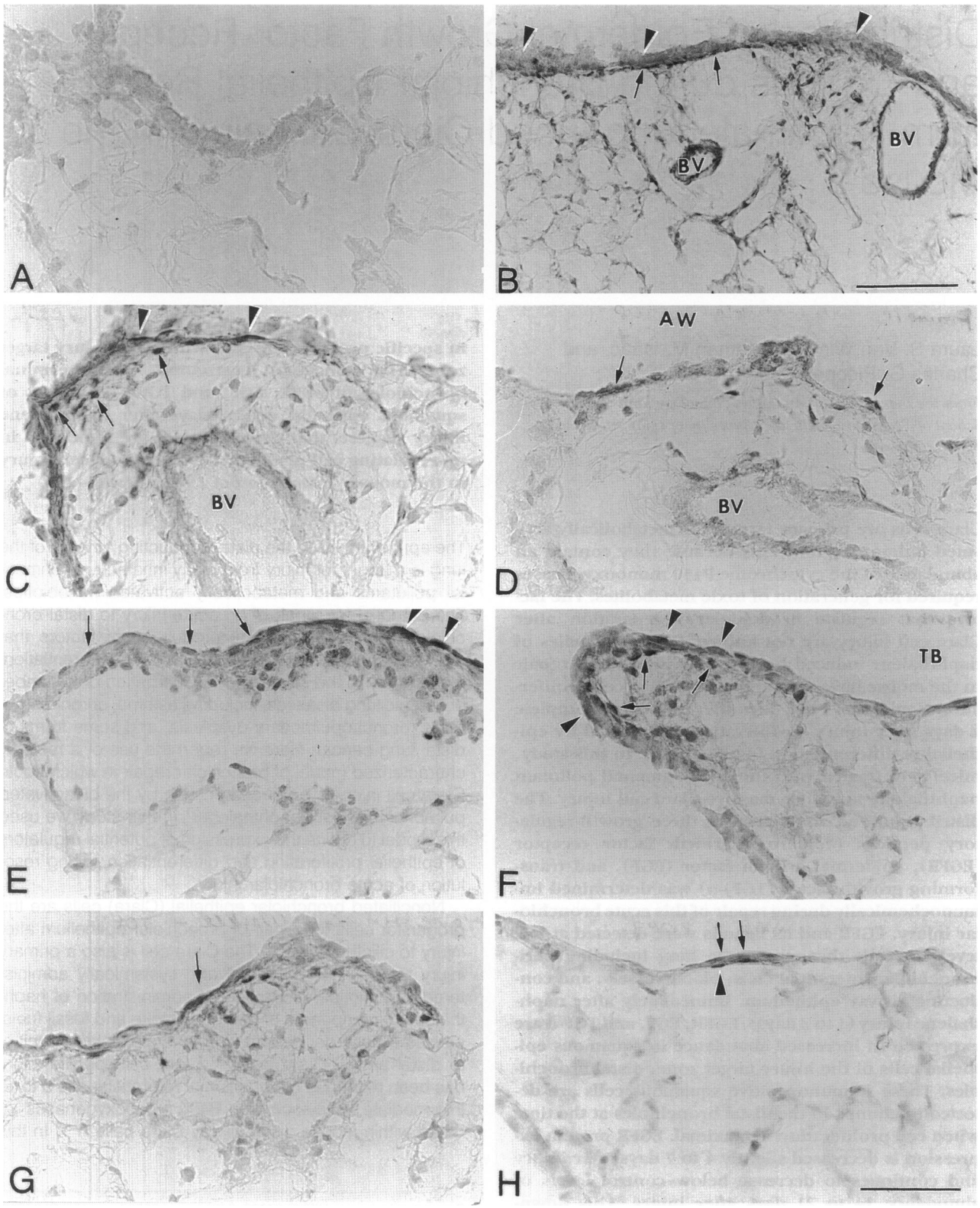
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study we induced massive Clara cell injury in mouse distal bronchioles by administration of a 200-mg/kg dose of naphthalene. This model has a defined pattern of repair that involves several overlapping stages: exfoliation of the injured Clara cells, squamation of the remaining ciliated cells, proliferation, migration, differentiation, and return of the epithelium to steady state (pre-injury appearance and composition).[1] The proliferative response peaks 1 to 2 days after injury and returns to near normal levels 4 days after injury and includes the injury, squamation, and proliferative stages. The differentiation phase follows the proliferative phase and is complete 14 days after injury.[1]

The factors that regulate distal airway epithelial proliferation and differentiation after injury are not known. However, it is likely that growth factors have a role in signaling bronchiolar epithelial cells to proliferate. Epidermal growth factor (EGF), transforming growth factor (TGF)-α, and EGF receptor (EGFR) are important epithelial cell growth regulatory peptides.[12,13] EGF and TGF-α bind to a common receptor, the EGFR, a member of the tyrosine kinase receptor family.[14] Expression of EGFR is crucial to mediating the action of EGF and TGF-α; only cells that express the receptor can respond to the growth factor(s). EGFR (and TGF-α) have been detected in macrophages, alveolar septal cells, and the conducting airway epithelium of rats.[15] The distribution of EGFR in human lung is also characterized by the localization of EGFR to many types of lung epithelial cells: nonciliated bronchial and bronchiolar cells, basal cells, and type II pneumocytes.[16] The role of EGFR in maintenance of normal lung cell function is still unclear, but Aida et al[16] have speculated, based on the distribution of EGFR in the lateral membranes of human Clara cells, that EGFR and EGF are involved in lung epithelial cell proliferation after injury. They hypothesize that the flattening of epithelial cells that occurs after injury may expose the membrane portions that contain EGFR and thus stimulate cell proliferation.[16] The regulatory role of EGFR and/or its ligands on cell proliferation after lung injury is supported by evidence of increased levels of these mediators after bleomycin-induced diffuse alveolar injury,[15] at sites of asbestos fiber deposition[17] and after exposure to silica particles[18] in rats and mice. Although it is clear that EGFR and its ligands are present in Clara cells, evidence of their possible role in bronchiolar epithelial repair has been lacking.

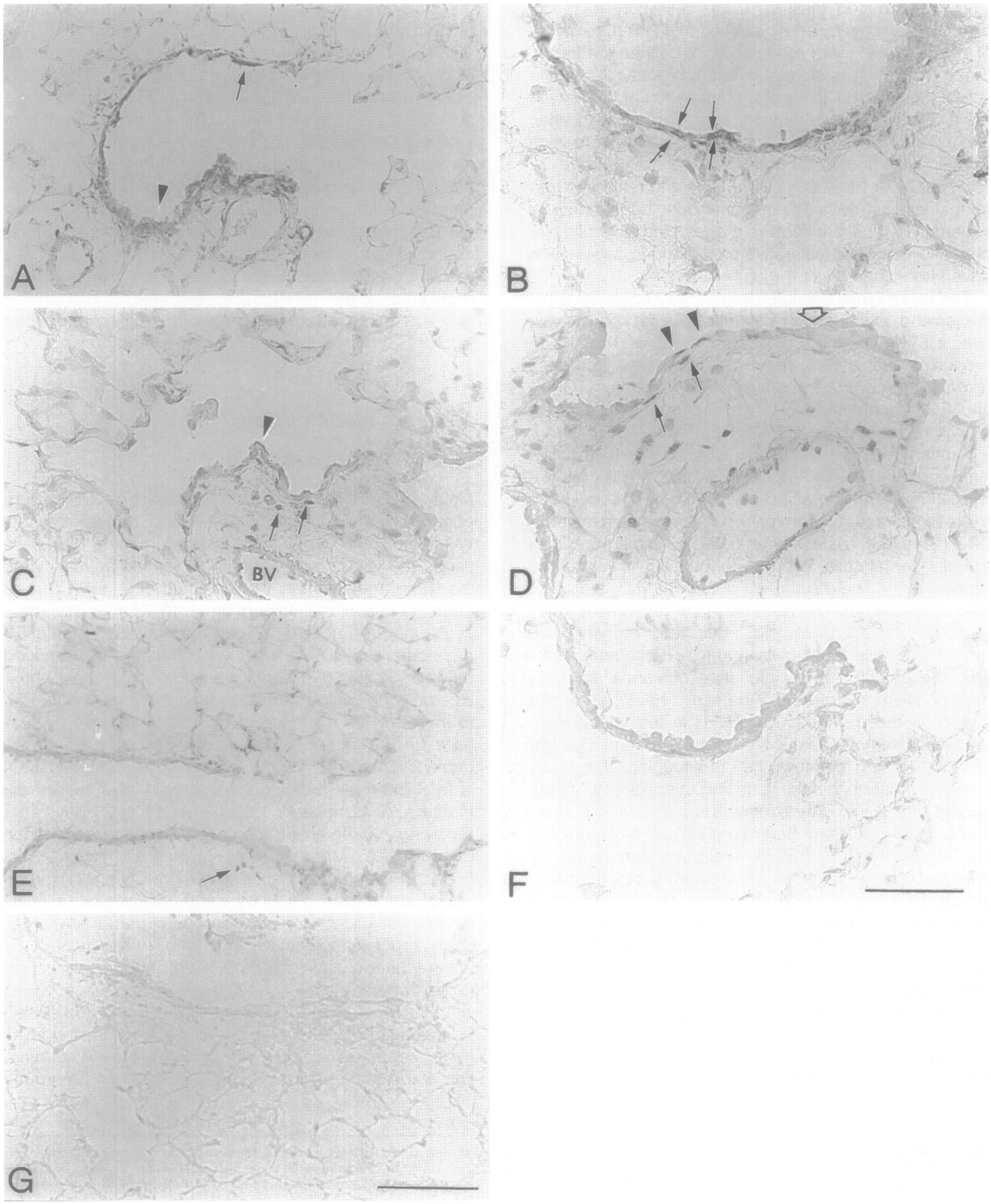
Lung response to injury has been shown to clearly involve increases in growth factor expression. The goal of this study was to define the distribution and abundance of both the receptor, EGFR, and its ligands, EGF and TGF-α, during the proliferation and differentiation phases of repair after bronchiolar Clara cell injury. This study demonstrates that EGFR, EGF, and TGF-α are present in normal mouse lung and that naphthalene injury results in increased expression of EGFR and its ligands within airway epithelium and peribronchiolar interstitial cells (fibroblasts and macrophages) adjacent to injured airway regions. In addition, peptide expression is maximal when cell proliferation is greatest, 2 days after injury, and EGFR co-localizes to sites of increased cell proliferation. These results suggest that EGF and TGF-α are important upregulators of the proliferative response to bronchiolar epithelial injury and that changes in the level of expression of EGFR modulate the proliferative response.

Materials and Methods

Experimental Protocol

Male viral antibody-free Swiss-Webster mice (CFW) were purchased from Charles River Breeding Laboratories, Wilmington, MA. Adult mice (2 to 3 months old) were used, and within each experiment, all the animals were the same age. Animals were housed in a HEPA filtered cage rack and maintained on a 12/12-hour light/dark cycle with free access to food and water for at least 7 days before use. All animals were dosed and sacrificed between 8 and 10 AM, with the exception of the 12-hour time point, for which sacrifice was between 8 and 10 PM, to minimize the influence of diurnal glutathione fluctuations on the extent of naphthalene injury. 5-Bromo-2′-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) in PBS (50 mg/kg) was administered 1 hour before euthanasia. At 12 hours or 1, 2, 4, 7, 9, 14, or 21 days after receiving 200 mg/kg intraperitoneal naphthalene (Aldrich Chemical Co., Milwaukee, WI) in corn oil (Mazola, Best Foods/CPC International, Englewood Cliffs, NJ) or an equivalent volume per body weight of corn oil, animals were anesthetized with an overdose of pentobarbital sodium and exsanguinated. The lungs were inflated by intratracheal infusion of 50% OCT embedding compound (Miles Laboratories, Elkhart, IN) in PBS and the left lobes were frozen in 100% OCT, immersed in freon, and cooled in liquid nitrogen. The remaining right half of the lung was frozen separately in liquid nitrogen for protein determination. Expression of growth factor proteins and morphol-

Figure 1. Immunohistochemical characterization of the distribution of EGFR within distal bronchioles of mice during the first 2 days of injury and repair after naphthalene treatment. **A:** Corn oil control lung has diffuse labeling in the epithelium and within the interstitium. **B:** In the proximal portion of a distal bronchiole 12 hours after naphthalene injury, EGFR protein is abundantly expressed in blood vessels (BV) and in the interstitium adjacent to the bronchiole. Very dark staining is detected underneath the basal lamina (**arrows**). Bronchiolar epithelial cells have diffuse labeling (**arrowheads**). **C:** In terminal bronchioles (TB) 12 hours after naphthalene injury, EGFR protein is abundantly expressed in the areas between the swollen epithelial cells and the basal lamina (**arrowheads**) as well as in cells located between adjacent blood vessels (BV) and the basal lamina (**arrows**). **D:** The terminal bronchioles of mice 1 day after treatment with naphthalene have fewer positive interstitial cells positioned between the airway (AW) and the blood vessel (BV). A few squamous epithelial cells contain abundant EGFR protein (**arrows**). **E:** In the proximal portion of a distal bronchiole 2 days after naphthalene injury, the surface of the squamous epithelial cells (**arrows**), and some entire cells (**arrowheads**), are intensely immunopositive for EGFR protein. **F:** At bifurcations just proximal to terminal bronchioles (TB), there are a number of EGFR-positive cells in the epithelium (**arrowheads**) and the interstitium immediately adjacent to the labeled epithelium (**arrows**). **G:** In terminal bronchioles 2 days after injury, clusters of squamous EGFR-positive cells are evident (**arrow**). **H:** Even in terminal bronchioles, where the basal lamina appears denuded 2 days after injury, clusters of squamous epithelial cells (**arrows**) and interstitial cells (**arrowhead**) that contain EGFR are evident. B Bar, 100 μm (**B**) and 50 μm (**A** and **C** to **H**).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ogy in corn oil control mouse lungs did not vary between time points. Control mice from different time points were grouped together for immunohistochemical analysis.

Immunohistochemistry

Specimens were sectioned at 5 μm on a Reichert-Jung 2800 Frigocut cryostat. The avidin-biotin peroxidase procedure and 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) substrate with nickel enhancement was used to identify antibody-binding sites for bright-field immunohistochemistry. After fixation in ice-cold methanol, sections were processed for immunohistochemistry using the procedure outlined by the supplier (Vector Laboratories, Burlingame, CA) with the exception that an extra avidin-biotin blocking step (Vector) was added before incubation with the primary antibody. The presence of the growth factor proteins was detected using the following specific antibodies: sheep anti-human EGFR (UBI, Lake Placid, NY), rabbit anti-mouse EGF (UBI), and rabbit anti-rat TGF-α (TGF-1, Peninsula Labs, Belmont, CA). To limit nonspecific binding of the primary antibody, sections were blocked with 5% bovine serum albumin. Controls included substitution of PBS for the primary antibody and preincubation of the antibody with ligand before tissue staining as a control for specificity. The optimal dilution at which there was positive staining with minimal background staining was determined separately for each antibody using a series of dilutions on sections from corn oil- and naphthalene-treated animals. A minimum of three mice were assayed for each treatment group. All treatment groups were included in each immunohistochemical assay.

A 10-fold excess by weight of EGF or TGF-α was used to preabsorb the anti-EGF and anti-TGF-α antibodies, respectively, for 4 hours at 4°C (human recombinant TGF-α from Collaborative Biomedical, Bedford, MA, or receptor-grade purified mouse EGF from UBI) before overnight incubation with tissue at 4°C. The anti-EGFR antibody was preincubated with a 40-fold excess of human A431 cell lysate (UBI) overnight and then incubated with tissue for 2 hours.

Fluorescent co-localization of CC10/B-tubulin IV and EGFR/BrdU was performed on adjacent serial sections of both control and 2-day post-naphthalene-treatment mouse lungs to demonstrate the phenotypes of the proliferating cells in the context of EGFR distribution.

CC10/B-Tubulin IV

Rabbit anti-rat CC10 (Clara cell 10-kd secretory protein, a marker of differentiated Clara cells) was a generous gift of Dr. Gurmukh Singh (Veterans Affairs Medical Center, Pittsburgh, PA). Mouse monoclonal anti-B-tubulin-IV (Biogenix, San Ramon CA) was used to identify ciliated cells.[19] Both the anti-CC10 and the anti-tubulin antibodies were applied to the tissue simultaneously, and primary antibody binding was detected after overnight incubation at 4°C using the fluorescent secondary antibodies (Jackson Immunoresearch, West Grove, PA) donkey anti-rabbit-Cy3 (red) and donkey anti-mouse-Cy2 (green) applied sequentially (with PBS washes in between).

EGFR/BrdU

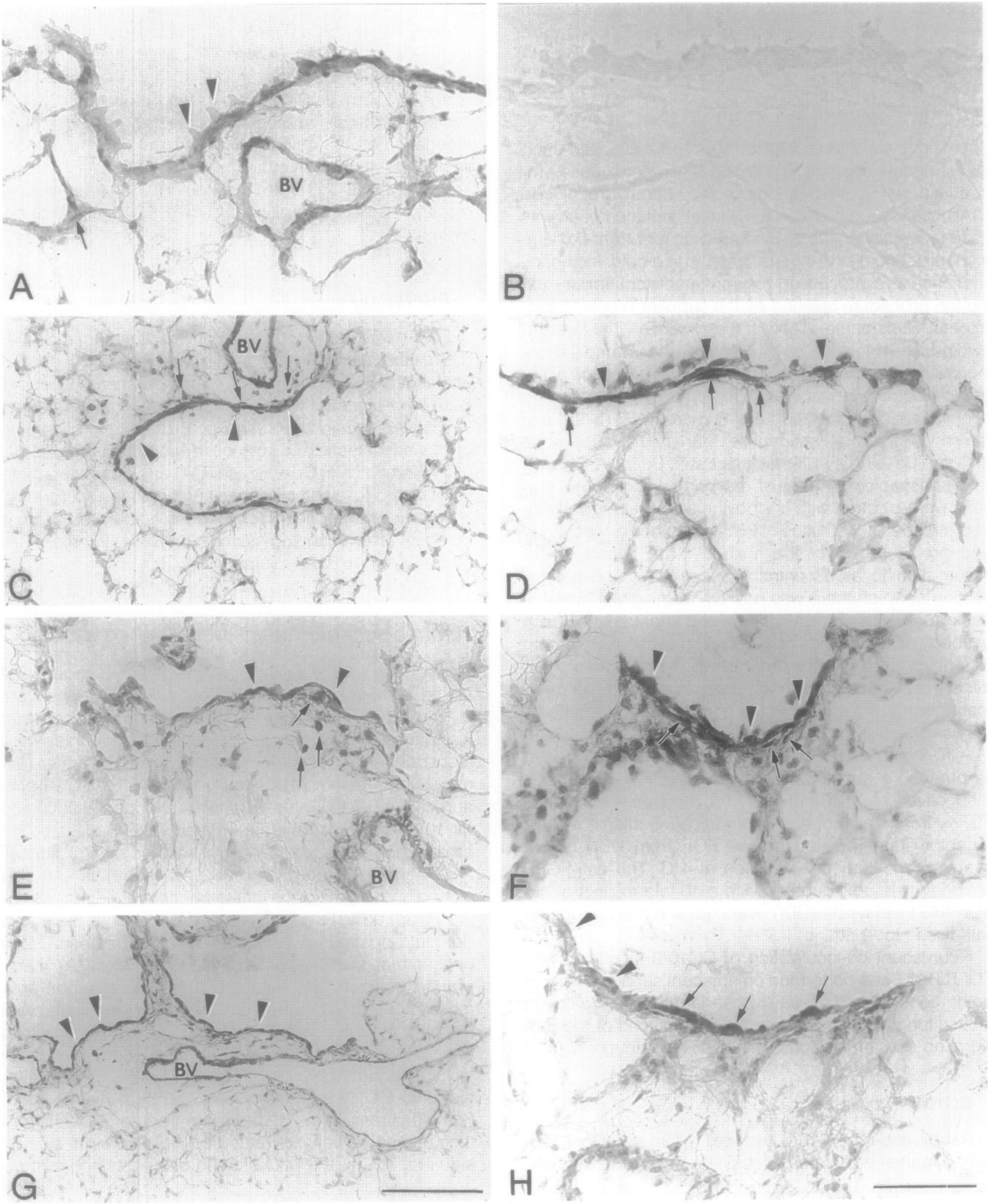
EGFR was detected using sheep anti-human EGFR (UBI) incubated overnight at 4°C followed by donkey anti-sheep-Cy3 (red; Jackson Immunoresearch). Tissues were then digested with 2 N HCl for 10 minutes to denature DNA, and incorporation of BrdU into proliferating cells was detected using mouse monoclonal anti-BrdU (ICN Biomedicals, Costa Mesa, CA) and incubated overnight at 4°C followed by donkey anti-mouse-Cy2 (green).

Fluorescently stained sections were analyzed using a confocal laser scanning system (BioRad, Hercules, CA) on an Olympus BH-2 microscope.

Immunoblots of Protein

Mouse lungs were flash frozen in liquid nitrogen and stored in a −80°C freezer until used. Frozen lungs were pulverized in a stainless steel beaker, and the powder was poured into a tissue homogenizer. The samples were resuspended in 1 vol of ice-cold suspension buffer (0.1 mol/L NaCl, 10 mmol/L Tris/HCl, 1 mmol/L EDTA, 1 μg/ml aprotinin, pH 7.6). Samples were centrifuged at 9000 × *g* for 10 minutes, and the supernatants were collected for protein determination using the method of Bradford.[20] Protein homogenates and purified reference proteins were diluted to the appropriate concentration ranges using PBS/0.05% Tween 20 and vacuum blotted using a dot-blot apparatus (Bethesda Research, Bethesda, MD) onto Immobilon-P (Milliipore, Marlborough, MA) membranes. Antigen was detected using either rabbit anti-mouse EGF (UBI) or rabbit anti-rat TGF-α (Peninsula) with a biotinylated secondary antibody and the avidin-biotin technique as recommended by the manufacturer (Vector). TMB membrane peroxidase substrate (KPL, Gaithersburg, MD) was used as the peroxidase substrate. Samples from three naphthalene-treated animals and their respective vehicle-treated controls were run side by side in a series of dilutions. Each blot contained a refer-

Figure 2. Immunohistochemical characterization of the distribution of EGFR within distal bronchioles of mice during injury and repair 4 to 21 days after naphthalene treatment. **A:** In a terminal bronchiole 4 days after injury, the majority of the epithelium is low cuboidal and is diffusely immunopositive (**arrowhead**). In contrast, squamous epithelial cells contain abundant EGFR protein (**arrow**). **B:** A higher magnification of the bronchiole shown in **A** illustrates that the EGFR protein is localized to the basolateral and luminal regions of squamous epithelial cells (**arrows**). **C:** Seven days after naphthalene injury, the squamous cells remaining in terminal bronchioles have dense reaction product (**arrowhead**). A few cells located between the blood vessel (BV) and the basal lamina also contain dark staining (**arrows**). **D:** Higher magnification of a bronchiole 7 days after injury illustrates that the EGFR protein is localized primarily to the apical portions of the few squamous epithelial cells (**arrowheads**) and to cells on the opposite side of the basal lamina (**arrows**). Cuboidal cells contain light staining for EGFR (**open arrow**). **E:** Fourteen days after injury, the EGFR is localized diffusely within terminal bronchiolar epithelium and a few, rare cells in the interstitium contain dense reaction product (**arrow**). **F:** Twenty-one days after injury, there is virtually no labeling. **G:** When PBS was substituted for the primary antibody on a 2-day-treated mouse lung section, there was no labeling. Bar, 100 μm (**A**, **C**, **E**, and **G**) and 50 μm (**B**, **D**, and **F**).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ence standard of purified protein (human recombinant TGF-α from Collaborative Biomedical or receptor-grade purified mouse EGF from UBI) also in a series of dilutions. Blots containing both purified EGF and TGF-α were immunoblotted with either rabbit anti-mouse EGF or rabbit anti-rat TGF-α to demonstrate antibody specificity.

Immunoblot Quantification

Immunoblots were allowed to air dry after antigen detection and were scanned on a Hewlett Packard ScanJet IIcx flatbed scanner using DeskScan II software. The images were opened in NIH Image, version 1.57, on a PowerMac 7100/80 computer, and image contrast was optimized for the entire blot to allow better visualization of the dots. An analysis circle with a diameter of 0.12 inches was made and then placed over each dot, and an average intensity for that dot was read. The purified protein readings were used to develop a standard curve for each blot. The protein concentration in the samples was determined from the standard curve of the purified protein on that blot.

Statistics

EGF and TGF-α protein concentrations were determined for a lung homogenate from each mouse (n = 3 per time point) by comparison to a reference standard of purified protein that was run with each blot. Levels measured in each treatment group are normalized to their respective controls run on the same blot, and data are expressed as the mean percentage of the control value ± SD. Differences between groups of values were determined by analysis of variance and one-way regression analysis and determination of significance, based on Scheffe's F-test, as $P < 0.05$.[21]

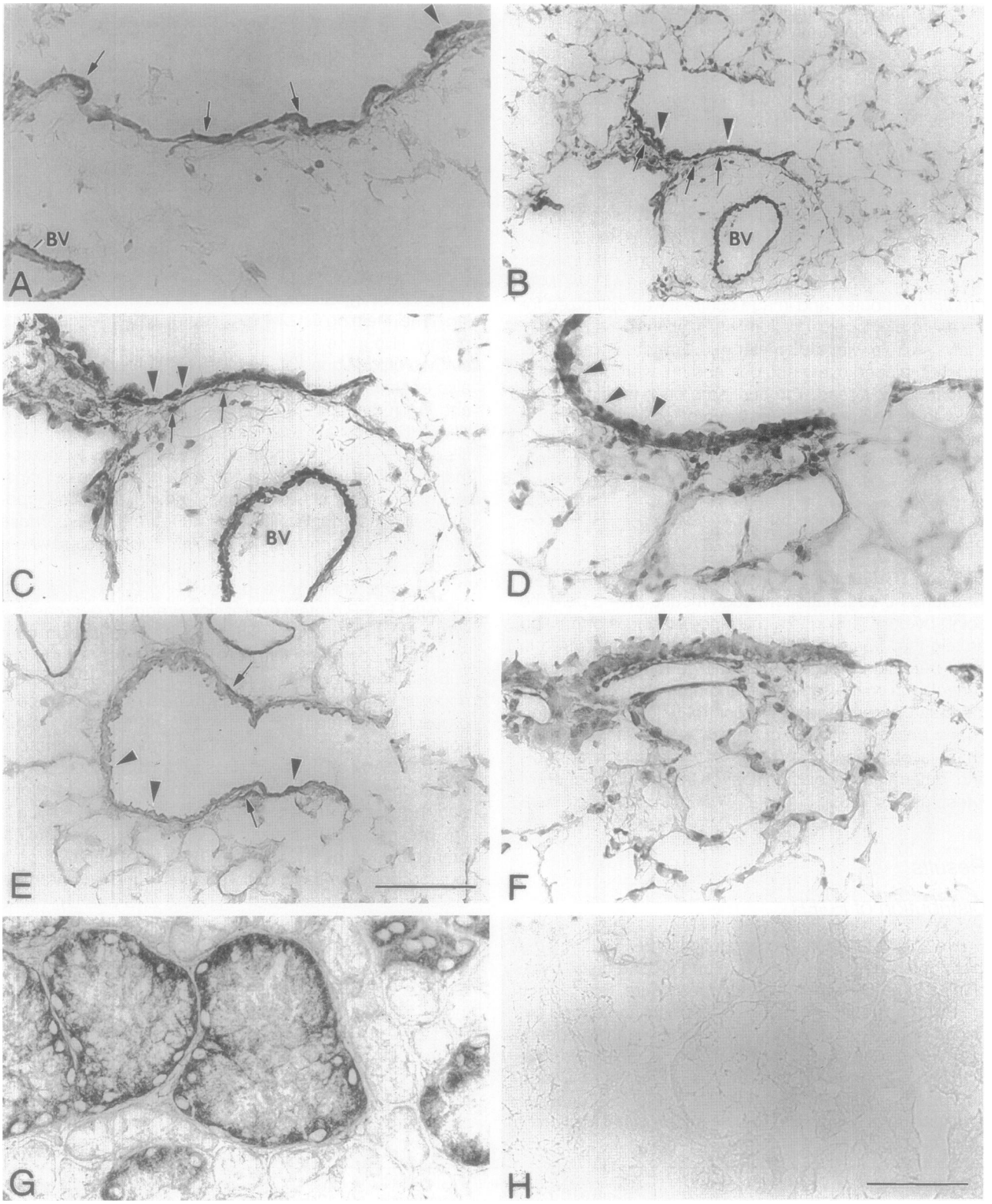
Results

Distribution of EGFR

Figures 1 and 2 define the distribution of EGFR during the injury and regeneration events in and around distal bronchioles in naphthalene-treated mice. Figure 1 describes the distribution of EGFR in control animals and during the period of maximal cell proliferation after naphthalene injury (1 to 2 days). In corn oil-treated mice (Figure 1A), EGFR is present in low abundance throughout the lung (in macrophages and alveolar type II cells) and diffusely in distal bronchiolar epithelium. In the proximal section of the distal bronchiole in Figure 1B, 12 hours after naphthalene treatment, EGFR protein is detected in increased abundance throughout the lung but is especially abundant in blood vessels and peribronchiolar interstitial cells. EGFR protein is abundantly expressed on both sides of the basal lamina: on the basal side of swollen, injured epithelial cells and in attentuated peribronchiolar interstitial cells (Figure 1C). In terminal bronchioles 1 day after injury, there are fewer positive interstitial cells located between the airway and the blood vessel. Squamous epithelial cells in injured distal airways have abundant EGFR protein that fills the entire cell (Figure 1D). In proximal portions of distal bronchioles 2 days after injury (Figure 1E), squamous cells have intense immunostaining. The staining is of two general types: either completely filling the cell or localized only to the luminal surface of the squamated cells. This duality of staining is also observed in squamous cells in terminal bronchioles and at bifurcations 2 days after injury (Figure 1, G and F). Within the distal bronchioles, squamous cells completely filled with reaction product appear in clusters in the epithelium. Directly opposite these immunopositive cell clusters, on the other side of the basal lamina, are immunopositive attenuated interstitial cells (Figure 1, F–H). These are especially common in terminal bronchioles where severe injury has occurred (Figure 1H).

Once the proliferative phase (1 to 2 days) is past, elevated EGFR protein expression gradually decreases 4 to 21 days after injury (Figure 2) compared with corn oil-treated controls (Figure 1A). The majority of the low cuboidal distal bronchiolar epithelium is diffusely immunopositive 4 days after injury (Figure 2A). Squamous cells present in the terminal bronchioles are strongly immunopositive on both their basolateral and luminal surfaces (Figure 2, A and B). Seven days after naphthalene injury, squamous cells with dense immunostaining and accompanying heavily labeled peribronchiolar interstitial cells are present only in the most distal portions of terminal bronchioles (Figure 2, C and D). A few interstitial cells located between the blood vessel and the basal lamina also contain abundant EGFR (Figure 2, C and D). These interstitial cells are generally of two types: either large round cells or attenuated cells directly adjacent to the basal lamina. In contrast, the cuboidal cells within the terminal bronchiolar epithelium have diffuse, light staining for EGFR protein (Figure 2D). The EGFR is distributed diffusely within terminal bronchiolar epithelium and a few, rare cells in the interstitium contain dense reaction product 14 days after injury (Figure 2E). There is virtually no labeling in the epithelium and interstitium 21 days after injury. At this time, the intensity of immunostaining is less

Figure 3. Immunohistochemical characterization of the distribution of EGF within distal bronchioles of mice during the first 2 days of injury and repair after naphthalene treatment. **A:** In a corn oil control animal, bronchiolar epithelial cells (**arrowheads**), type II alveolar cells (**arrow**), and blood vessels (BV) are immunopositive for EGF. **B:** When PBS was substituted for the primary antibody, there was no labeling. **C:** Twelve hours after naphthalene treatment, the terminal bronchiolar epithelium (**arrowheads**), the adjacent blood vessel (BV), and cells within the interstitium (**arrows**) contain abundant EGF protein. **D:** A higher magnification of the bronchiole shown in **C** illustrates that EGF protein is localized throughout squamous epithelial cells (**arrowheads**) and in adjacent interstitial cells (**arrows**). **E:** One day after naphthalene injury, squamous epithelial cells in distal bronchioles continue to have abundant immunoreactive protein (**arrowheads**). A few interstitial cells located between the blood vessel (BV) and the epithelium are also densely labeled (**arrows**). **F:** Intense labeling of terminal bronchiolar epithelium (**arrowheads**) and adjacent interstitial cells (**arrows**) in a lung section from another mouse 1 day after naphthalene treatment. **G:** Two days after naphthalene injury, the blood vessel (BV) adjacent to the injured terminal bronchiole and the squamous cells lining terminal bronchioles (**arrowheads**) contain intense EGF reaction product. **H:** Some squamous epithelial cells contain reaction product throughout the cell (**arrows**) and some have it localized to the cell surface (**arrowheads**). Bar, 50 μm (**A**, **B**, **D**, **F**, and **H**) and 100 μm (**C**, **E**, and **G**).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than or equal to corn oil-treated control animals (Figure 2F). When PBS was substituted for the primary antibody on a 2-day naphthalene-treated mouse lung section, there was no staining (Figure 2G).

Distribution of EGF

Figures 3 and 4 define the distribution of EGF during the injury and regeneration events in and around distal bronchioles in naphthalene-treated mice. Figure 3 illustrates the distribution of EGF in control animals and during the period of maximal cell proliferation after naphthalene injury (1 to 2 days). In vehicle-treated control mice (Figure 3A), EGF is expressed in bronchiolar epithelial cells (including Clara cells), type II alveolar cells, and blood vessels. When PBS is substituted for the primary antibody, no reaction product was detected in lung tissue from a control animal (Figure 3B). Twelve hours after treatment with naphthalene, labeling for immunoreactive EGF protein is higher in bronchiolar epithelial cells, blood vessels, and cells within the interstitium (Figure 3C) than in vehicle-treated control animals (Figure 3A). Within the most distal portion of terminal bronchioles, EGF immunoreactive protein is present in high abundance in squamous epithelial cells and in interstitial cells adjacent to the squamous epithelial cells (Figure 3D). There is some variability in staining for EGF protein within terminal bronchiolar epithelium of different mice 1 day after naphthalene treatment (compare Figure 3, E and F). Squamous epithelial cells contain abundant EGF protein as do adjacent peribronchiolar interstitial cells (Figure 3, G and H). Areas where the epithelium is more squamated (Figure 3E) have fewer associated immunopositive interstitial cells than areas where the epithelium contains low cuboidal cells (Figure 3F). Two days after naphthalene treatment, there is less interstitial cell labeling compared with 1 day after injury (compare Figure 3, G and H, with Figure 3, E and F). Most of the squamous epithelial cells in distal airways have dense deposits of immunoreactive protein filling the entire cell (Figure 3G). A few other squamous epithelial cells, located in more proximal portions of terminal bronchioles (Figure 3H) have EGF protein immunolocalized to the luminal surfaces of the cells. The regions of the blood vessels closest to the squamous bronchiolar epithelium have denser staining than other regions of the same blood vessel (Figure 3G).

After the phase of active cell proliferation (1 to 2 days), EGF protein expression remains at increased levels in the bronchiolar epithelium 4 to 7 days after injury and then decreases 14 to 21 days after injury (Figure 4). Four to seven days after naphthalene injury, squamous and cuboidal bronchiolar epithelial cells, peribronchiolar interstitial cells, and blood vessels are strongly immunopositive for EGF (Figure 4, A–C). EGF protein is present diffusely throughout the cytoplasm and more densely in the apex of some bronchiolar epithelial cells 14 days after injury (Figure 4D). The remainder of the bronchiolar epithelial cells continue to have abundant EGF reaction product that fills the entire cell (Figure 4D). This decrease in epithelial cell labeling is present throughout the terminal bronchioles in animals 21 days after naphthalene treatment (Figure 4E). The terminal bronchiolar epithelium is faintly immunopositive. Denser reaction product is located in epithelial cell apices, a few peribronchiolar interstitial cells, and the portions of blood vessels closest to the airways (Figure 4, E and F). At 21 days after injury, the distribution and abundance of EGF protein is similar to control animals (compare Figure 3A with Figure 4F). Normal male mouse salivary gland, a major site of EGF production, has abundant expression of EGF immunoreactive protein (Figure 4G). As a negative control, when PBS was substituted for primary antibody on mouse salivary gland tissue, no staining was detected (Figure 4H).

Distribution of TGF-α

Immunohistochemical characterization of the distribution of TGF-α within distal bronchioles of mice during the first 2 days of injury and repair (proliferative phase) after naphthalene treatment is illustrated in Figure 5. In vehicle-treated control animals, bronchiolar epithelial cells are labeled with varying intensities throughout the cell and on the luminal surface (Figure 5A). Twelve hours after naphthalene injury, squamous epithelial cells are densely stained (Figure 5, B and C). Interstitial cells positioned between the blood vessel and the bronchiolar basal lamina are densely labeled (Figure 5C). TGF-α protein is present in high abundance in attenuated peribronchiolar interstitial cells immediately adjacent to the basal lamina of injured bronchioles (Figure 5C). One day after naphthalene injury, TGF-α protein is detected in increased abundance in bronchiolar epithelial and in interstitial cells (Figure 5D) as compared with vehicle-treated control mice (Figure 5A). Two days after naphthalene injury, groups of squamous epithelial cells within the terminal bronchioles have increased levels of TGF-α protein (Figure 5, G and H). Interstitial cells and blood vessels are also immunopositive. There are fewer immunopositive interstitial cells present 2 days after injury than at 1 day after injury (compare Figure 5, E and F with D). TGF-α

Figure 4. Immunohistochemical characterization of the distribution of EGF within distal bronchioles of mice during injury and repair 4 to 21 days after naphthalene treatment. **A:** Four days after naphthalene injury, the squamous (**arrows**) and cuboidal (**arrowhead**) as well as adjacent blood vessels (BV) are strongly immunopositive for EGF. **B:** Seven days after injury, cuboidal bronchiolar epithelial cells (**arrowheads**), adjacent interstitial cells (**arrows**), and blood vessels (BV) contain abundant EGF protein. **C:** Higher magnification of the bronchiole shown in **B** illustrates the distribution of EGF protein within airway epithelial cells. The protein is abundantly expressed in the few squamous cells (**arrowheads**). Adjacent attenuated interstitial cells (**arrows**) and blood vessels (BV) also contain abundant immunoreactive protein. **D:** Fourteen days after naphthalene injury, EGF protein is present diffusely throughout the cytoplasm and more densely in the apex of bronchiolar epithelial cells (**arrowheads**). **E:** Twenty-one days after naphthalene injury, the terminal bronchiolar epithelium is faintly immunopositive. Denser reaction product deposition is located in the epithelial cell apices (**arrowheads**), in a few peribronchiolar interstitial cells (**arrows**), and in the sections of the blood vessel closest to the airways (BV). **F:** Higher magnification of a terminal bronchiole from a mouse 21 days after naphthalene treatment illustrates the distribution of EGF on the apices of the bronchiolar epithelial cells (**arrowheads**). **G:** Mouse salivary gland has dense deposition of EGF-immunoreactive protein. **H:** As an additional negative control, when PBS was substituted for primary antibody on mouse salivary gland tissue, there was no labeling. Bar, 50 μm (**A**, **C**, **D**, **F**, **G**, and **H**) and 100 μm (**B** and **E**).

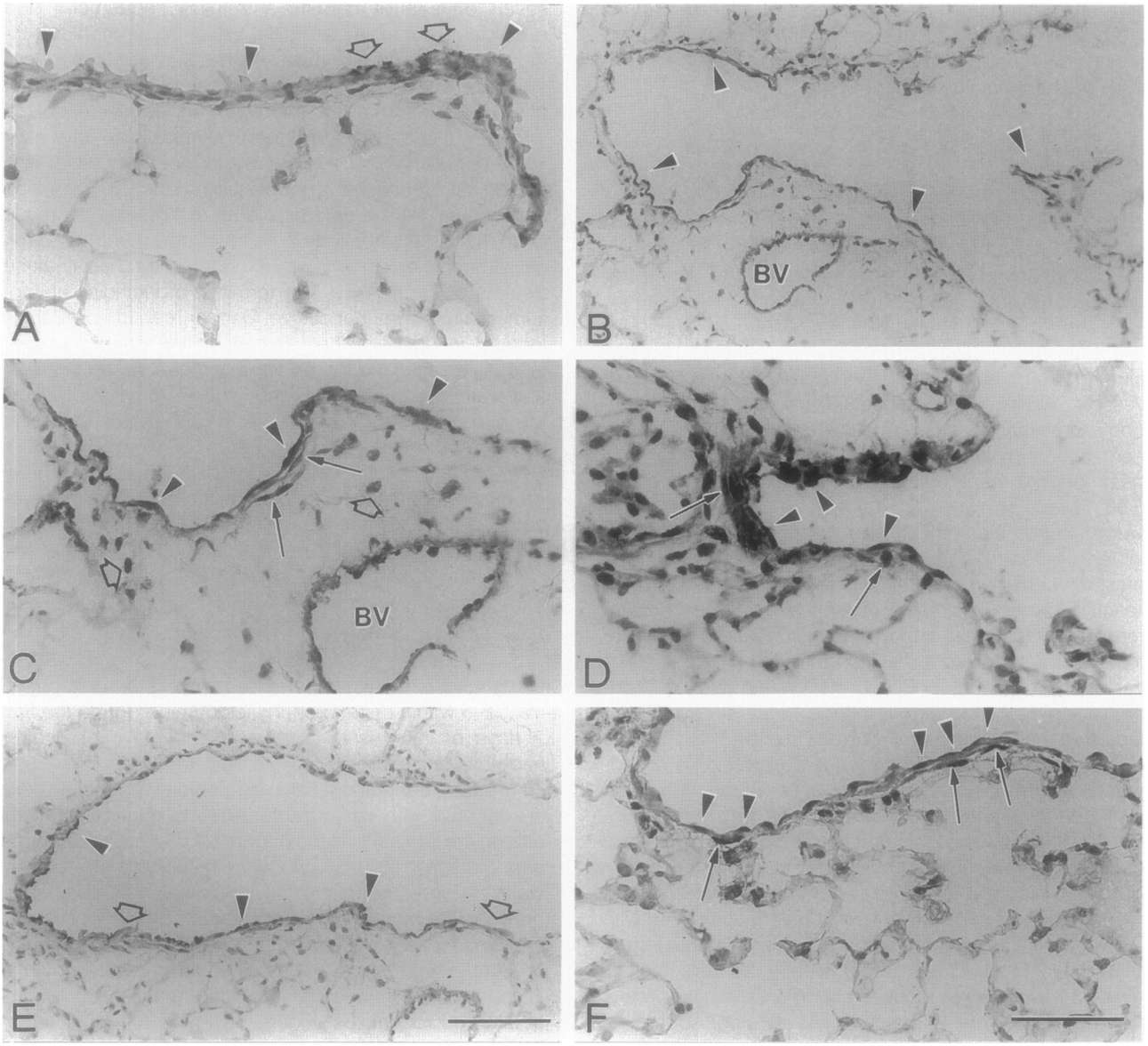

Figure 5. Immunohistochemical characterization of the distribution of TGF-α within distal bronchioles of mice during the first 2 days of injury and repair after naphthalene treatment. **A:** In a corn oil control animal, bronchiolar epithelial cells (**arrowheads**) are lightly labeled throughout the cell and more strongly on a few epithelial cell surfaces (**open arrows**). Some peribronchiolar interstitial cells and type II cells are also immunopositive. **B:** Twelve hours after naphthalene injury, the epithelium lining this injured terminal bronchiole is intensely labeled (**arrowheads**) for TGF-α protein. **C:** A higher magnification of the bronchiole shown in **C** illustrates that TGF-α protein is localized throughout squamous epithelial cells (**arrowheads**), in attenuated peribronchiolar interstitial cells (**arrows**), and in interstitial cells (**open arrows**) positioned between the blood vessel (BV) and the bronchiolar basal lamina. **D:** One day after naphthalene injury, TGF-α protein is detected in greatest abundance in bronchiolar epithelial cells (**arrowheads**) and in adjacent interstitial cells (**arrows**). **E:** Two days after naphthalene injury, groups of epithelial cells within the terminal bronchioles continue to express high levels of TGF-α protein (**arrowheads**) whereas other groups of epithelial cells have only diffuse staining (**open arrows**). **F:** A higher magnification of the bronchiole shown in **E** illustrates that TGF-α protein is intensely localized throughout clusters of squamous epithelial cells (**arrowheads**) that are juxtaposed with intensely labeled peribronchiolar interstitial cells (**arrows**). Bar, 50 μm (**A**, **C**, **D**, and **F**) and 100 μm (**B** and **E**).

protein is intensely localized throughout clusters of squamous epithelial cells that are juxtaposed with intensely labeled peribronchiolar interstitial cells 2 days after injury (Figure 5F).

TGF-α protein continues to be detected in high abundance in repairing bronchiolar epithelial cells 4 to 21 days after naphthalene injury (Figure 6). TGF-α protein is detected in the nucleus and apical membranes of distal airway epithelial cells 4 days after injury. Adjacent to the basal lamina, peribronchiolar interstitial cells and interstitial cells with circular profiles are also immunopositive (Figure 6A). Intense nuclear staining and diffuse cytoplasmic staining is present in cells throughout the bronchiolar epithelium and the interstitium 4 days after injury (Figure 6B). Cells with diffuse cytoplasmic and nuclear labeling were present throughout the epithelium and the interstitium at 7 (Figure 6C) and 14 (Figure 6D) days after naphthalene injury. Round and attenuated peribronchiolar interstitial cells are strongly immunopositive (Figure 6, C and D). Twenty-one days after injury, the abundance of

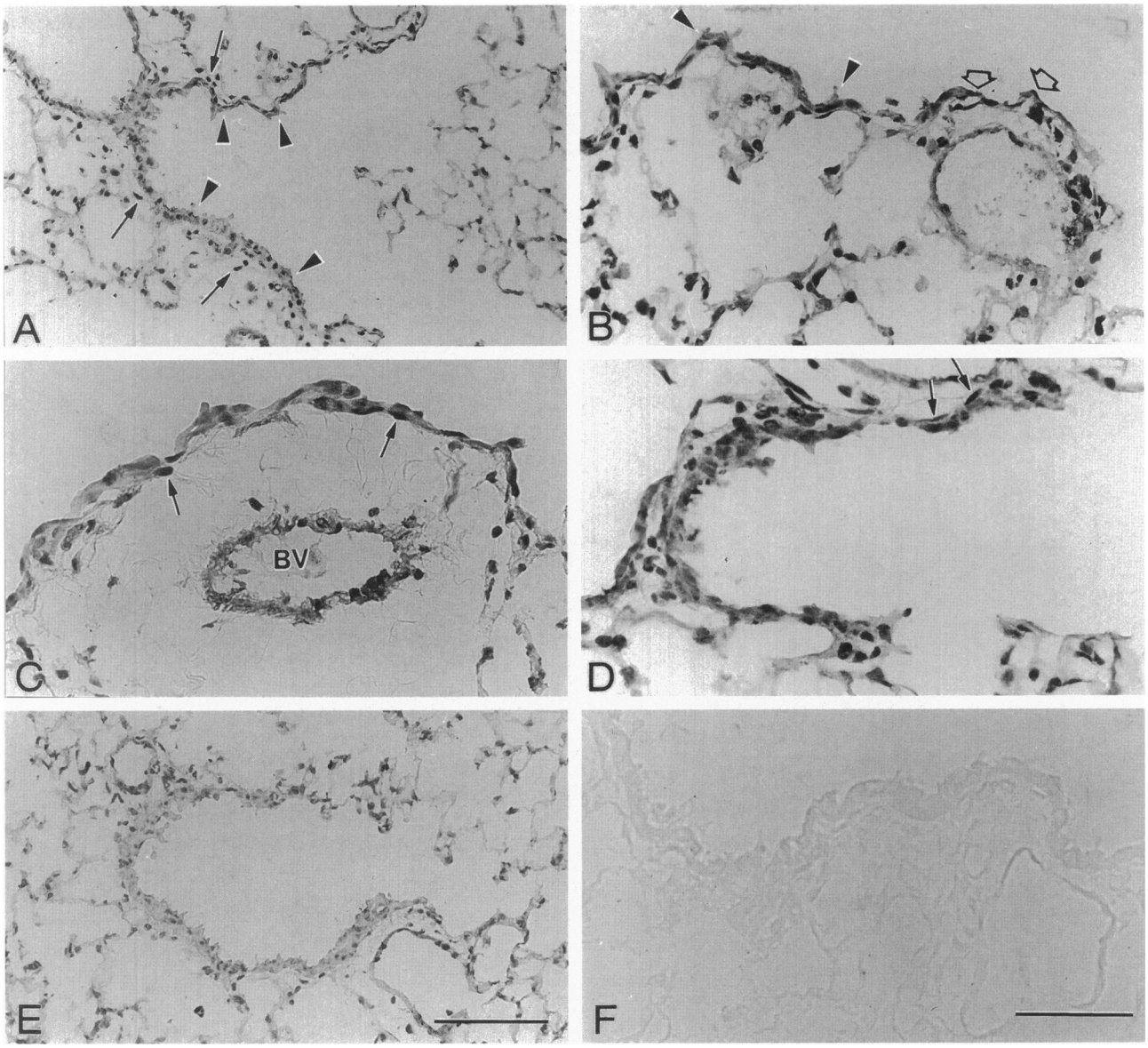

Figure 6. Immunohistochemical characterization of the distribution of TGF-α within distal bronchioles of mice during injury and repair 4 to 21 days after naphthalene treatment. **A:** TGF-α protein is primarily and uniformly localized to the nucleus of distal airway epithelial cells (**arrowheads**) and adjacent interstitial cells (**arrows**) 4 days after injury. **B:** A higher magnification of the bronchiole shown in **A** demonstrates the predominant nuclear labeling of low cuboidal epithelial cells (**arrowheads**). Squamous epithelial cells remain strongly immunopositive throughout the cell cytoplasm (**open arrows**). **C** and **D:** Seven (**C**) and fourteen (**D**) days after injury, cells with diffuse cytoplasmic and nuclear labeling were present throughout the epithelium and the interstitium. Some peribronchiolar interstitial cells still express high levels of TGF-α. **E:** Twenty-one days after injury, bronchiolar epithelial cells and adjacent interstitial cells express decreased TGF-α protein that is similar, or slightly less than, the control pattern of TGF-α abundance. **F:** When PBS was substituted for primary antibody, there was no labeling. Bar, 50 μm (**B**, **C**, **D**, and **F**) and 100 μm (**A** and **E**).

TGF-α protein in bronchiolar epithelial cells is slightly less than that seen in vehicle-treated control animals (compare Figure 6E with Figure 5A). When PBS was substituted for primary antibody on normal mouse lung tissue, no staining was detected (Figure 6F).

Antibody Specificity

Specificity of the antibody used in immunolocalization was confirmed using antisera preincubated with ligand (Figure 7). Preadsorption with ligand reduced staining for EGFR (compare Figure 7, A and B), EGF (compare Figure 7, C and D), and TGF-α (compare Figure 7, E and F).

Double Immunofluorescence

As shown in Figure 8, serial sections of mouse lung were double immunostained for CC10/B-tubulin IV (Figure 8, A and C) and BrdU/EGFR (Figure 8, B and D). In lungs of corn oil-treated control mice, CC10-expressing Clara cells (red) are detected in great abundance in terminal bronchioles (Figure 8A). A few B-tubulin-IV-positive cili-

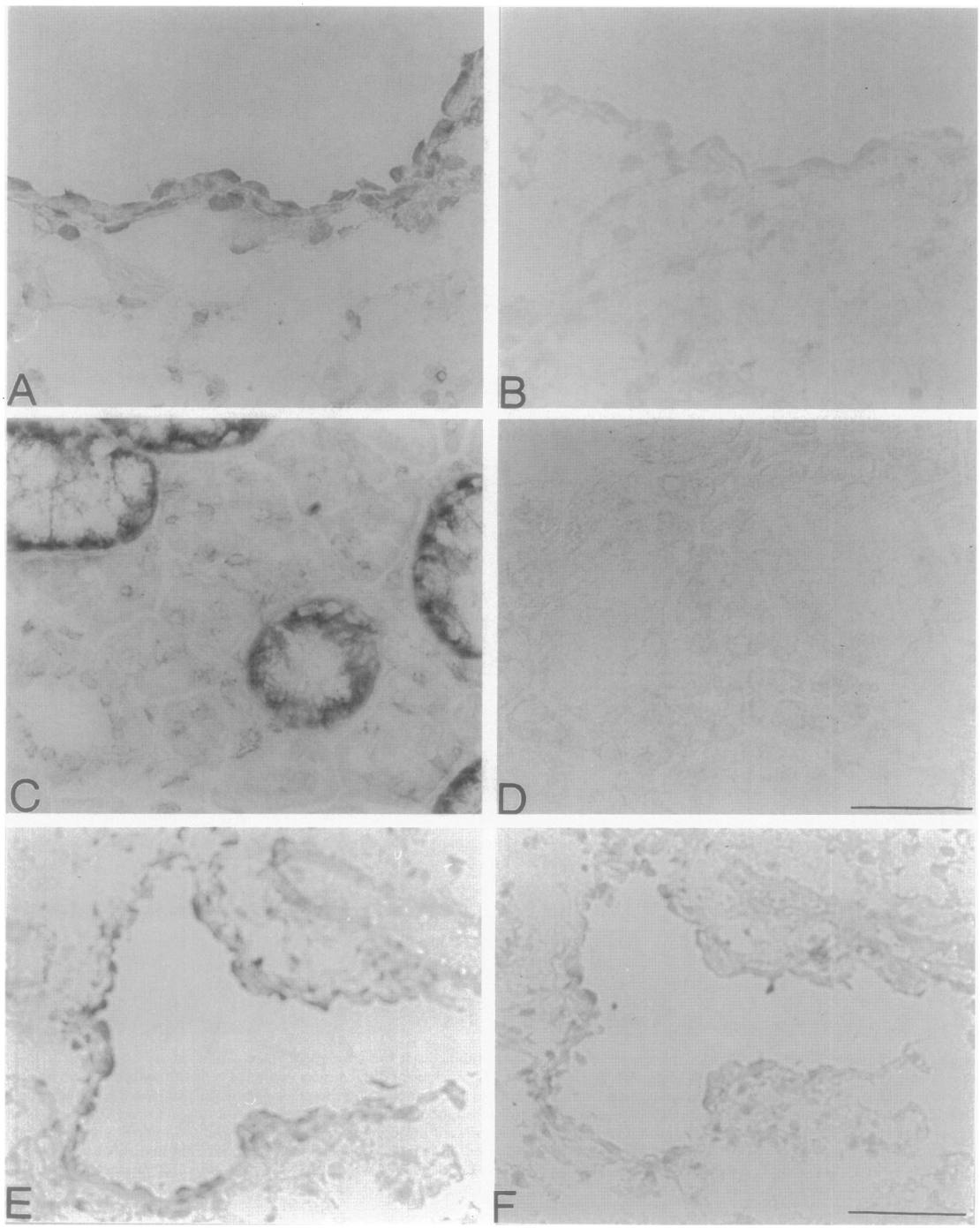

Figure 7. Additional positive and negative controls for immunohistochemistry. **A:** Positive EGFR staining is detected in the bronchiolar epithelium of an injured mouse lung 2 days after naphthalene treatment. **B:** After preincubation of the EGFR antibody with A431 cell lysate (EGFR rich), no staining was detected. **C:** Positive EGF staining was detected in normal male mouse salivary gland. **D:** Preincubation of the EGF antibody with mouse EGF resulted in a loss of staining. **E:** TGF-α protein was detected in bronchiolar epithelium of mouse lung 2 days after naphthalene treatment. **F:** After preincubation with recombinant TGF-α, staining was decreased. Bar, 50 μm (**A** to **D**) and 100 μm (**E** and **F**).

ated cells (green) are also detected in the epithelium. Serial sections of these lung specimens do not contain BrdU-positive epithelial cells (green) within or around the terminal bronchiole (Figure 8B). However, EGFR (red) is detected weakly in the bronchiolar epithelium and adjacent interstitium. Two days after injury, the bronchiolar epithelium in the naphthalene-treated mouse contains few positive Clara cells (red) and ciliated cells (green) and is predominantly lined by a squamous epithelium that expresses neither differentiated cell marker (Figure 8C). CC10 expression is remarkably decreased in the naphthalene-injured bronchiole compared with controls (compare Figure 8, C and A). Serial sections of naphthalene-injured lung contain numerous proliferating cells

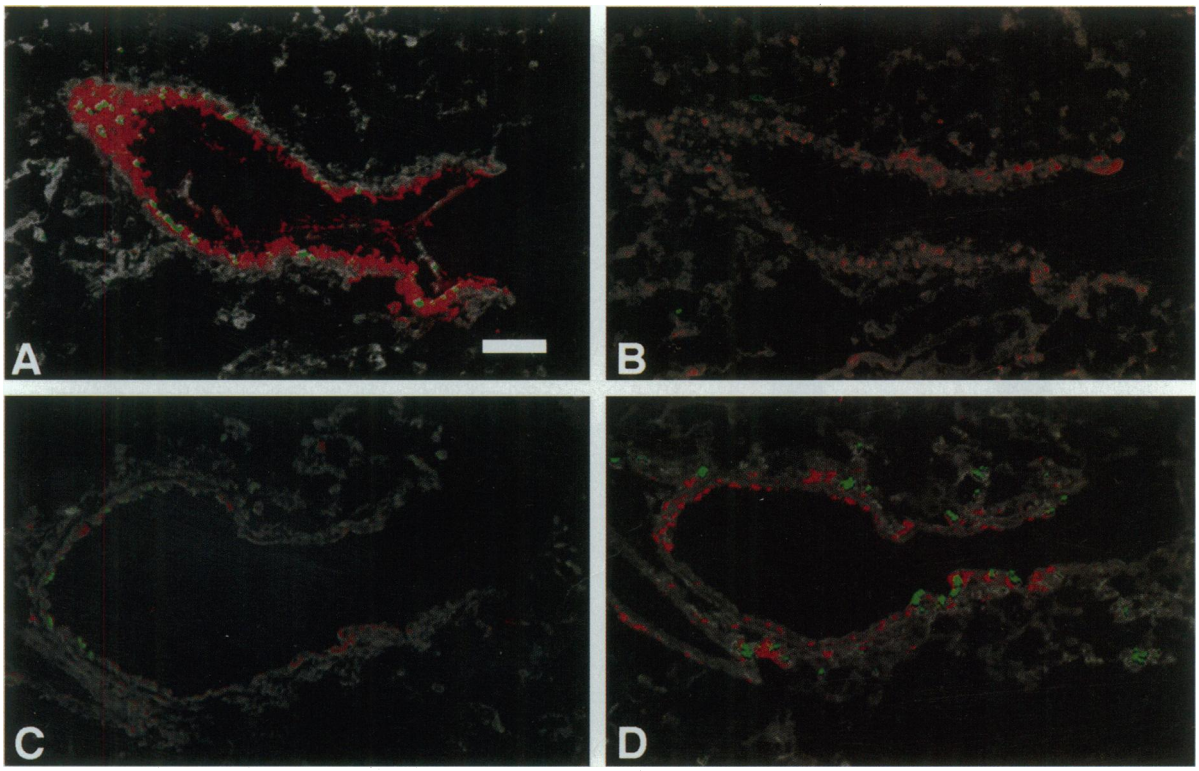

Figure 8. Double staining of either CC10 and B-tubulin IV or EGFR and BrdU in corn oil control and naphthalene-injured mouse lung. **A:** CC10-expressing Clara cells (red) are detected in great abundance in terminal bronchioles of control mice. A few B-tubulin-IV-positive ciliated cells (green) are also detected in the epithelium. **B:** A serial section of the control mouse lung shown in **A** does not contain BrdU-positive epithelial cells (green). However, EGFR is detected weakly in the bronchiolar epithelium and adjacent interstitium. **C:** Two days after injury, the bronchiolar epithelium in the naphthalene-treated mouse contains few positive Clara cells (red) and ciliated cells (green) and is predominantly lined by a squamous epithelium that expresses neither marker. **D:** In a section serial to that shown in **C**, the naphthalene-injured mouse lung has a pronounced increase in BrdU-positive proliferating cells (green) within both the epithelium and the interstitium. Some, but not all, of the proliferating cells are EGFR positive (red). Bar, 50 μm.

(green) that have incorporated BrdU for 1 hour (Figure 8D). The proliferating cells are found within both the epithelium and the interstitium surrounding the terminal bronchiole. Some, but not all, of the proliferating cells are EGFR positive (red and green). Some EGFR-positive cells do not contain BrdU-positive nuclei (red only).

Measurement of EGF and TGF-α Protein Levels

As shown in Figure 9A, immunoblots were sufficiently sensitive to detect a range of standard reference proteins. The linear range of detection for EGF (shown, 120 ng to 192 pg) was greater than for TGF-α (not shown, 3 μg to 24 ng). There was no cross-reactivity between the TGF-α antisera with EGF protein and *vice versa* (Figure 9B). Whole lung homogenates of treated and control mice did not contain significantly different amounts of immunodetectable EGF and TGF-α protein (Figure 9, C and D, respectively).

Discussion

The purpose of this study was to characterize the distribution of three growth regulatory peptides (EGFR, EGF, and TGF-α) during naphthalene-induced injury and repair in mouse bronchiolar epithelium. As has been previously established and is summarized in Figure 10, epithelial regeneration after naphthalene injury occurs in two distinct phases that include the overlapping stages of injury, squamation, proliferation, migration, differentiation, and steady state.[1] The first phase occurs 1 to 2 days after injury and includes concurrent vacuolization and exfoliation of injured Clara cells and squamation of remaining ciliated cells to cover the basal lamina as well as cell proliferation in the interstitium and the epithelium.[1] The second phase of repair takes place 4 to 21 days after naphthalene injury and includes migration and re-differentiation of the epithelial cells to return the epithelium to steady state. The distribution and abundance of EGFR, EGF, and TGF-α in normal mouse lungs is similar to the pattern that has been described previously in rats; peribronchiolar interstitial cells, blood vessels, and epithelial cells are immunopositive.[22,23] Increased expression of EGFR and its ligands TGF-α and EGF occurs concomitantly with cell proliferation in the epithelium and the interstitium 1 to 2 days after naphthalene injury. Although EGFR is down-regulated beginning with the second phase of repair (4 to 21 days after injury), its ligands TGF-α and EGF are slower to return to steady-state levels of abundance. Once elevated at 1 to 2 days after injury, TGF-α and EGF do not decrease and return to steady state until 21 days after injury.

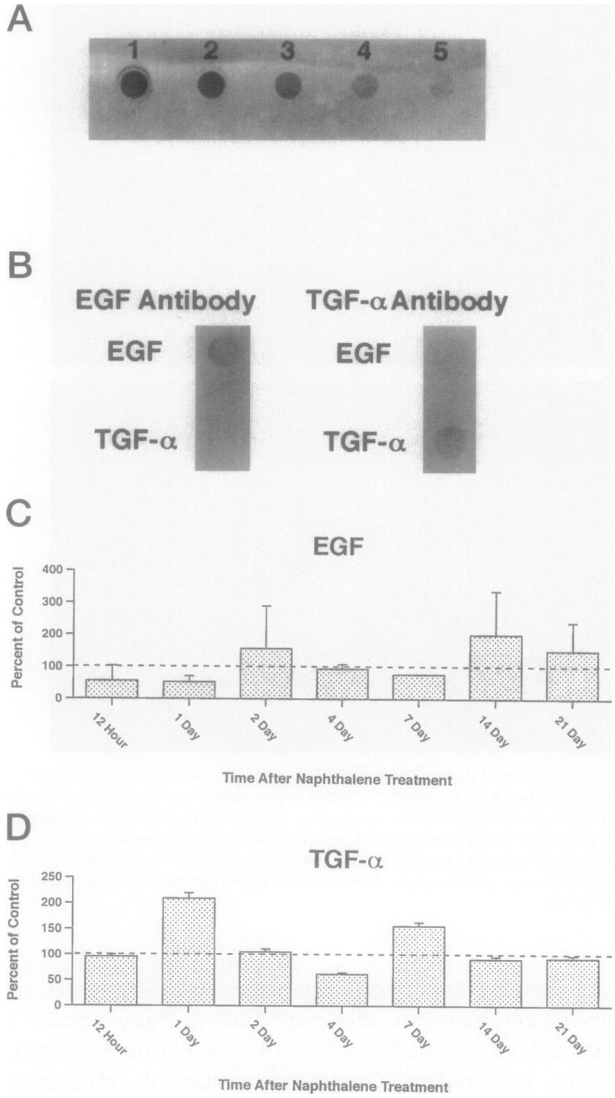

Figure 9. EGF and TGF-α protein expression was quantified by immunoblotting. A reference standard of purified protein was run with each blot (as the range of detection can vary from blot to blot) and was used to generate a standard curve. **A:** The staining of purified EGF protein (**1**, 120 ng; **2**, 24 ng; **3**, 4.8 ng; **4**, 960 pg; **5**, 192 pg) detected with rabbit anti-EGF antibody decreased with decreasing protein concentration. **B:** Dots of purified EGF (120 ng) and TGF-α (1.5 μg) immunoblotted with rabbit anti-EGF or sheep anti-TGF-α to demonstrate antibody specificity. The TGF-α antibody did not cross-react with the purified EGF and the EGF antibody did not cross-react with purified TGF-α. **C** and **D:** EGF (**C**) and TGF-α (**D**) protein in right lung homogenates ($n = 3$) did not reveal any statistically significant protein changes in whole lung homogenates as compared with control values. Levels measured in each treatment group are normalized to their respective controls run on the same blot, and data are expressed as a percentage of the control value ± SD.

Although the immunohistochemistry demonstrates striking focal changes in growth factor expression after naphthalene injury, these alterations were not detectable by immunoblots of whole-lung homogenates (Figure 9). This may be due to limitations in the methods as well as to the focal nature of the injury and growth factor changes. In contrast to previous studies showing pronounced changes in growth factor expression in whole-lung homogenates in response to bleomycin injury,[15] we were not able to detect significant changes in either EGF or TGF-α protein levels. This may be due to the fact that, relative to bleomycin, naphthalene treatment results in a characteristic focal injury that includes cells of and near the terminal bronchioles (which occupy only approximately 5% of the total lung volume[24]). It may be that the lung homogenate dilutes the focal changes thereby rendering them undetectable. This underscores the necessity for site-specific assays to evaluate focal injury.

Evidence for the involvement of EGFR, EGF, and TGF-α in lung cell proliferation is derived from studies of lung cancer, of lung cells *in vitro* and, recently, of lung injurants that cause fibrotic lesions. Increased levels of EGFR and TGF-α have been found in some forms of lung cancer.[25,26] Growth of normal tracheobronchial and nasal epithelial cells in culture has been shown to require the presence of TGF-α or EGF in the medium.[27–30] Fibroblasts isolated from hamster lungs exposed to oxidant stress are induced to produce greater than normal levels of TGF-α.[31] EGFR (and TGF-α) have been detected in macrophages, alveolar septal cells, and airway epithelium of both bleomycin-treated and control rats and are up-regulated in response to injury.[15] TGF-α stimulates the proliferation of epithelial cells, fibroblasts, and endothelial cells *in vitro*.[32–35] TGF-α is also chemotactic for epithelial cells in culture[36] and accelerates burn wound re-epithelialization.[33]

The changes in EGFR, EGF, and TGF-α protein distribution and abundance after Clara cell injury by naphthalene are similar to the changes seen in bleomycin-treated rats.[15] EGFR, EGF, and TGF-α co-localize to sites of increased cell proliferation in both studies, indicating common regulation of the cellular responses after epithelial or mesenchymal cell injury. Furthermore, expression of these peptides is increased in the same sorts of cells after both types of injury: peribronchiolar interstitial cells (fibroblasts and macrophages) and epithelial cells. However, our study differs in that the epithelial cell with the most profound change in growth factor expression was the squamous bronchiolar cell, whereas in the study by Madtes et al,[15] it was alveolar cells. This is expected because these are the epithelial cell types located in the primary target zones of their respective injurants (naphthalene *versus* bleomycin).

Two types of immunopositive interstitial cells were detected in increased abundance after Clara cell injury. The first type were large round cells located between the injured airway epithelium and the adjacent blood vessels. These are probably alveolar macrophages similar to those described by Madtes et al[15] after alveolar injury by bleomycin in rats.[23] Cultured alveolar macrophages and peripheral blood monocytes are known to express TGF-α,[37,38] and although Clara cell injury by naphthalene is not characterized by an inflammatory influx, transient increases in macrophages/monocytes and their expression of TGF-α may occur in injured regions. The second type of growth-factor-positive interstitial cell was characterized by a flat profile and their location. These cells were located directly adjacent to the basal lamina and juxtaposed to clusters of positive squamous epithelial cells. These cells may be the correlate of the interstitial cells with the long cytoplasmic extensions that are de-

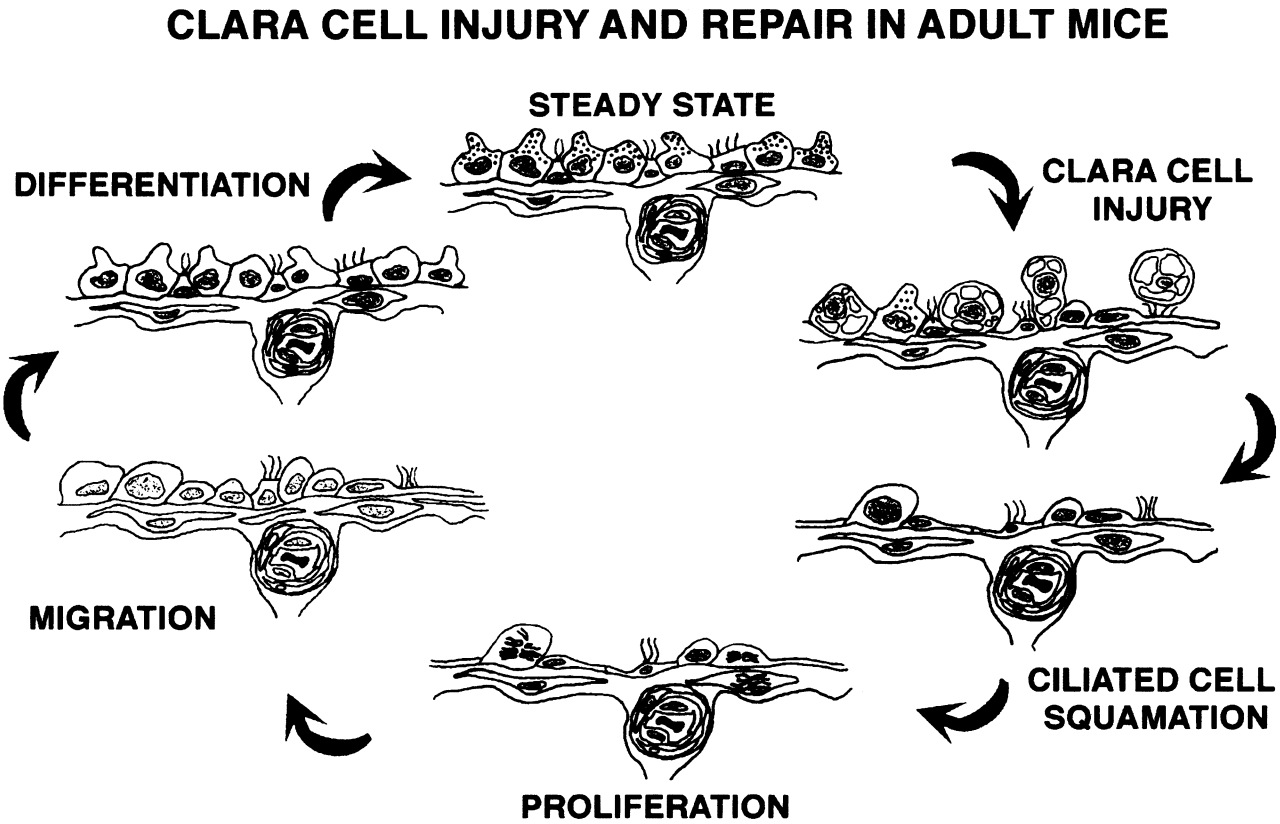

Figure 10. After naphthalene-induced Clara cell injury in adult mice, epithelial repair occurs in two distinct phases that include the overlapping stages of injury, squamation, proliferation, migration, differentiation, and steady state. The first phase occurs 1 to 2 days after injury and includes concurrent vacuolization and exfoliation of injured Clara cells and squamation of remaining ciliated cells to cover the basal lamina as well as cell proliferation in the interstitium and the epithelium. The second phase of repair takes place 4 to 21 days after naphthalene injury and includes migration and re-differentiation of the epithelial cells to return the epithelium to steady state.

scribed near regions of disturbed basal lamina by electron microscopy in bronchioles of mice 2 days after naphthalene treatment.[1] These interstitial cells may be involved in signaling epithelial cells to proliferate in a paracrine manner as they also express greatly elevated levels of EGFR, EGF, and TGF-α. Additional experiments are necessary to determine whether these cells have a role in regulation of bronchiolar epithelial repair. However, it seems likely that fibroblast-epithelial interactions will be important in repair after bronchiolar injury based on observations in previous studies of bleomycin-induced injury that show that these interactions are important regulators of alveolar type II cell repair.[39]

Given that TGF-α and EGF are potent mitogens for epithelial cells, increased expression of these two EGFR ligands in the epithelium 1 to 2 days after Clara cell injury (the time frame in which cell proliferation is maximal; see Ref. 1) is expected. EGFR expression in injured terminal bronchioles is elevated throughout the terminal bronchiole and at sites of increased cell proliferation (Figure 8). Most (but not all) BrdU-positive cells within the epithelium contain increased immunostaining for EGFR. Some EGFR-positive cells did not incorporate BrdU and this may be reflective of the 1-hour pulse label used in this study not labeling some cells that have already divided or, conversely, perhaps the cells would divide at a later time. Additional studies that include long-term labeling and three-dimensional imaging of living cells within the injury target zone are needed to answer these questions. Squamous epithelial cells clustered together in distal bronchioles 2 days after injury have elevated expression of EGFR, EGF, and TGF-α protein when compared with vehicle-treated control mice. This is consistent with the cell signaling for proliferation 2 days after injury seen previously with TGF-α in bleomycin-treated rat lungs[23] and with the timing of maximal cell proliferation previously determined after naphthalene injury.[1] We do know that the squamous, proliferating, EGFR-positive cells present in the terminal bronchiole at this time point contain very little, if any, CC10. This suggests that these cells are not fully differentiated Clara cells. Furthermore, the proliferating cells do not appear to have cilia on their cell surface. This suggests that the proliferating cells do not belong to either of the two fully differentiated cell populations found in this lung region and that they may be de-differentiated cells. This is consistant with two other previous studies of naphthalene injury in mice.[1,40]

EGF and TGF-α continue to be expressed at elevated levels after the cell proliferative stage has passed. This implies that proliferating cells in the early phase of repair from injury regulate their response to EGF and TGF-α by regulation of receptor expression. This is similar to observations made in bleomycin-treated rats where regulation of EGFR expression may modulate the effects of

TGF-α.[23] In our model, EGF and TGF-α distribution and abundance approach but do not reach steady-state levels 14 days after injury. Although the epithelium has regenerated and appears morphologically normal and fully differentiated at 14 days after injury,[1] the widespread pulmonary response to injury (altered growth factor expression) is not over. Whether continued increased expression of EGF/TGF-α is related to sequestration in extracellular matrix, to a continuing imbalance of other growth regulators, or to increased protein stability/decreased degradation is not known, but the pattern of expression of these two ligands suggests that extracellular matrix does not normally contain large amounts of these two proteins.

Repair from acute bronchiolar injury clearly involves increases in EGFR, EGF, and TGF-α protein expression. These increases are probably involved in regulation of cell proliferation after injury. Cessation of cell proliferation coincides with decreased EGFR expression but not EGF or TGF-α, suggesting that cell proliferation is regulated by decreased receptor expression. This study is the first to describe growth factor distribution and abundance in a lung injury model where Clara cells within distal bronchiolar epithelium are selectively targeted. This differs from other pulmonary injury and repair models in that the injury is cell, site, and dose specific. The present study defines patterns of growth factor expression that lay the groundwork for additional studies to define the role of these growth factors in the lung's cytokine network, as well as the intra- and extracellular regulators involved in growth factor responses in acute bronchiolar injury.

Acknowledgments

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