GM-CSF enhances lung growth and causes alveolar type II epithelial cell hyperplasia in transgenic mice

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GM-CSF was used to direct expression of mouse granulocyte macrophage colony-stimulating factor (GM-CSF; SP-C-GM mice) in lung epithelial cells in GM-CSF-replete (GM-CSF) in pulmonary surfactant metabolism. Mice deficient in GM-CSF (GM–/–) or the GM-CSF receptor β-subunit disrupted surfactant metabolism characterized by accumulation of surfactant phospholipids and proteins similar to that seen in the human disease pulmonary alveolar proteinosis (10, 20, 27, 28). Surfactant lipid and protein concentrations were corrected when GM-CSF was expressed under control of the human SP-C gene promoter in lung epithelial cells of GM–/– mice, demonstrating that local expression of GM-CSF was sufficient to correct the proteinosis phenotype (2). In the present study, morphometric and immunohistochemical analyses were used to determine the effects of GM-CSF on lung growth and cellularity in transgenic mice expressing GM-CSF in lung epithelial cells. High levels of GM-CSF expression in the lung increased lung size and caused type II epithelial cell hyperplasia.

**MATERIALS AND METHODS**

The alveolar surface of the mammalian lung is lined by type II and type I epithelial cells, providing an extensive surface area required for gas exchange. Type II cells synthesize and catabolize pulmonary surfactant and serve as progenitor cells for repair after alveolar injury (15). Type I epithelial cells are terminally differentiated, squamous cells that cover ~90% of alveolar surfaces. The basal surfaces of type I cells are closely apposed to an encapsulating capillary network surrounding each alveolus, enhancing efficient O2–CO2 exchange between alveolar gases and blood. After alveolar injury, type II cells proliferate and differentiate into type I cells to repopulate the denuded basal lamina. The mechanisms involved in alveolar repair and remodeling in vivo are not known but are likely mediated by various growth factors and their receptors that direct proliferation or differentiation of type II epithelial cells (22, 23, 30, 34).

Type II cells synthesize, secrete, and recycle the components of pulmonary surfactant (32). Pulmonary surfactant is a complex mixture of phospholipids and proteins enriched in dipalmitoylphosphatidylcholine and associated surfactant proteins (SP)-A, -B, -C, and -D and is critical for reducing surface tension at the air-liquid interface in the alveolus. Recent gene-targeting experiments demonstrated the vital role of granulocyte macrophage colony-stimulating factor (GM-CSF) in pulmonary surfactant metabolism. Mice deficient in GM-CSF (GM–/–) or the GM-CSF receptor β-subunit disrupted surfactant metabolism characterized by accumulation of surfactant phospholipids and proteins similar to that seen in the human disease pulmonary alveolar proteinosis (10, 20, 27, 28). Surfactant lipid and protein concentrations were corrected when GM-CSF was expressed under control of the human SP-C gene promoter in lung epithelial cells of GM–/– mice, demonstrating that local expression of GM-CSF was sufficient to correct the proteinosis phenotype (2). In the present study, morphometric and immunohistochemical analyses were used to determine the effects of GM-CSF on lung growth and cellularity in transgenic mice expressing GM-CSF in lung epithelial cells. High levels of GM-CSF expression in the lung increased lung size and caused type II epithelial cell hyperplasia.
obtain homozygote mice of either the GM+/+;SP-C-GM+/+ or GM−/−;SP-C-GM−/− genotype. Lines of GM+/+ or GM−/− control mice were derived by backcrossing SP-C-GM−/− littermates. Mice used in this study were of generation F3 or greater.

Bronchoalveolar lavage and measurement of proteins by enzyme-linked immunosorbent assay. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium and were killed by aortic exsanguination. Bronchoalveolar lavage (BAL) was performed using three 1-ml aliquots of phosphate-buffered saline (PBS), which were pooled, measured for recovery volume, and stored at −20°C until assay. GM-CSF was measured using the Endogen GM-CSF Minikit enzyme-linked immunosorbent assay (ELSA; Endogen, Cambridge, MA). Horseradish peroxidase (HRP)-streptavidin (Zymed Laboratories, South San Francisco, CA) was diluted to 1:8,000. Substrate was the Dako TMB One-Step Substrate System (Dako, Carpinteria, CA). Plates were read at 450 nm using a Dynatech plate reader (Dynatech Laboratories, Chantilly, VA). The lower limit of detection with this system is <5 pg/ml; thus any samples falling below this were reported as not detectable. All samples >500 pg/ml were diluted and repeated.

Endotoxin treatment of mice. Lung injury was induced by in vivo administration of intratracheal lipopolysaccharide (LPS) to GM+/+ FVB/N mice. LPS from Salmonella typhimurium (Sigma Chemical, St. Louis, MO) was diluted to a working concentration of 100 µg/ml in sterile saline. LPS (100 µl) was instilled intratracheally, as described previously (35). Mice were anesthetized by intraperitoneal injection of pentobarbital sodium and were killed by aortic exsanguination at 2, 4, 6, 8, or 12 h posttreatment. BAL was performed, and GM-CSF was measured as described above.

Processing and staining of tissues for histopathology. Lungs were inflation fixed, as described previously (3). Briefly, animals were anesthetized with pentobarbital sodium injected intraperitoneally and were killed by aortic exsanguination. The trachea was exposed and cannulated; 4% paraformaldehyde-PBS, pH 7.4, was instilled from a height of 23 cm for 1 min, and the trachea was ligated. Lungs and heart were dissected out of the chest cavity, placed in cold 4% paraformaldehyde-PBS, pH 7.4, and stored at 4°C for 24 h. Tissue was then washed in cold PBS and was cryoprotected in 30% sucrose for frozen sections or dehydrated in a series of alcohols and embedded in paraffin. Hematoxylin and eosin and Masson’s trichrome staining were used for histological analysis of 5-µm paraffin sections. Cytospin preparations of BAL cells were stained using a Leukocyte Acid Phosphatase kit (Sigma Diagnostics) to identify tartrate-resistant cells.

Immunofluorescence and immunohistochemistry. Paraffin sections (5 µm) were used for immunohistochemical staining of pro-SP-C. Anti-pro-SP-C (68514) is a rabbit polyclonal antibody raised against the NH2-terminal domain of the propeptide, as described previously (31). Tissues were deparaffinized, and endogenous peroxidases were quenched using 3% H2O2-methanol for 1 h. Tissues were rinsed with 0.1 M PBS, pH 7.4, and 2% Triton X-100 (PBS-Triton), blocked with 2% normal goat serum (NGS) in PBS-Triton for 2 h at room temperature, and incubated at 4°C overnight with anti-pro-SP-C diluted 1:1,000. Slides were washed in PBS-Triton. Anti-pro-SP-C was detected using the Vectastain ABC goat anti-rabbit immunohistochemical horseradish peroxidase (HRP) kit (Vector Laboratories, Burlingame, CA) and nickel diaminobenzaldehyde (Ni-DAB) substrate. Tissues were counterstained with tris(hydroxymethyl)aminomethane (Tris)-cobalt and nuclear fast red.
incubated in anti-goat secondary antibody (described above) for 30 min. Anti-GM-CSF-R

were expanded for this study. Line 59 mice were bred to (12). Two separate founder lines, designated 48 and 59, cDNA in lung epithelial cells, as reported previously were used to construct a chimeric SP-C-GM-CSF gene promoter sequences (3.7SP-C) from the human SP-C gene. Specific primers were then used to amplify a 309-nucleotide (nt) polymerase chain reaction (PCR) product corresponding to nucleotides 642–951 of GM-CSF-R (Genbank accession no. M85078). The forward primer sequence was 5'-TCGGGGCCAGTGGTTCT-3'; the reverse primer sequence was 5'-CAGTGGTCTACCTCCTGTCC-3'. PCR was performed according to the PCR SuperMix protocol (GIBCO-BRL, Gaithersburg, MD) with 35 cycles of 45 s at 94°C, 30 s at 61°C, and 2 min at 72°C.

Rat type II cell isolation and culture Type II epithelial cells were isolated from rat lungs as described previously for bromoethylxuridine (Brdu) labeling studies (26). Briefly, animals were anesthetized with pentobarbital sodium, and the lungs were perfused via the pulmonary artery. Elastase (Worthington Biochemicals, Freehold, NJ) was used to digest the type II cells from the basement membrane. Type II cells were obtained by the panning method described by Dobbs and colleagues (9). Cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 1% antibiotic-antimycotic (GIBCO-BRL) and then were plated at a density of 2 × 10^4 cells per well in 96-well tissue culture plates. Cultures prepared using this method generally contained 90–95% type II cells. Type II cells were then treated with 1 × 10^{-11} to 1 × 10^{-7} M mouse GM-CSF (Peprotech, Rocky Hill, NJ) for 48 h; Brdu was added, and the cells were allowed to incubate for an additional 24 h. Control plates received no GM-CSF treatment. A colorimetric immunoassay (Boehringer Mannheim, Indianapolis, IN) was used to assess Brdu incorporation. Fixed cells were incubated with anti-BrdU antibody complex in PBS (1:100) for 90 min. Cells were washed, and colorimetric substrate solution was added. Sulfuric acid (1 M solution) was added to stop the substrate enzyme reaction after 10 min, and absorbance was measured at 450 nm using a Dynatech plate reader.

RESULTS

Production of SP-C-GM-CSF transgenic mice. Promoter sequences (3.7SP-C) from the human SP-C gene were used to construct a chimeric SP-C-GM-CSF gene (SP-C-GM) directing expression of mouse GM-CSF cDNA in lung epithelial cells, as reported previously (12). Two separate founder lines, designated 48 and 59, were expanded for this study. Line 59 mice were bred to homozygosity in both GM−/− and GM+ + backgrounds; line 48 SP-C-GM+ + homozygote mice were bred only in the GM−/− background. Line 48 and 59 mice appeared healthy, bred normally, and produced litters of 8–12 pups. Pups grew at a normal rate and required no special care. The majority of SP-C-GM mice lived a normal life span, with some surviving at least two years. Three other founder mice, killed at 12 mo of age for histological analysis, failed to transmit the SP-C-GM transgene to the F1 generation. One additional founder mouse that failed to breed was found moribund and was killed at 4 mo of age. No changes in SP-C-GM mice compared with control mice were noted in the number or relative cell counts of peripheral blood white cells, peritoneal macrophages, or liver and spleen histology, as assessed by hematoxylin and eosin staining, which was described previously (12).

GM-CSF protein production. GM-CSF concentrations were measured in the BAL fluid by ELISA (Fig. 1A). In line 48 and 59 GM−/−, SP-C-GM+ + mice and line 59 GM+ +, SP-C-GM+ + mice, GM-CSF concentrations in BAL fluid ranged from 215 to 1,825 pg/ml. GM-CSF levels in BAL fluid from line 48 mice, although higher on average, were more variable than those in line 59 animals. In mice from line 59, GM-CSF concentrations were similar in GM−/−, SP-C-GM+ + and GM+ +, SP-C-GM+ + BAL fluids. In comparison, GM-CSF concentrations in the BAL fluid from GM−/− control mice, although detectable, were near the lower limit of detection (5 pg/ml) by ELISA and so are reported as not detectable. GM−/− mice were completely deficient in GM-CSF.

LPS was administered intratracheally to nontransgenic GM+ + FVB/N mice, and GM-CSF was measured in the BAL fluid (Fig. 1B). GM-CSF was detected readily in BAL fluid at 2, 4, 6, 8, and 12 h after exposure, demonstrating that GM-CSF is secreted into the alveolar lumen after LPS-mediated lung injury. The concentrations of GM-CSF at 2, 4, 6, and 8 h after LPS exposure were comparable to concentrations measured in BAL obtained from SP-C-GM mice. Atypical lung histology in SP-C-GM mice. In both GM+ + and GM−/− backgrounds, lungs from line 48 and line 59 SP-C-GM mice had sparse regions of thickened hyperplastic alveolar walls and slightly increased numbers of alveolar macrophages compared with GM+ + and GM−/− control mice as early as 25 days of age. Alveolar wall hyperplasia was noted first in the peripheral, subpleural regions of the lung parenchyma. By 2–5 mo of age, regions of marked alveolar wall hyperplasia were noted throughout the lung, although some regions appeared relatively normal (Fig. 2). The identity of alveolar macrophages was confirmed by tartrate-resistant acid phosphatase staining (not shown). Some alveolar macrophages were multinucleated, consistent with other studies of alveolar macrophage morphology after GM-CSF treatment (5, 16). In some mice, enlarged alveolar macrophages filled the alveolar airspaces, with the greatest aggregations noted in areas most affected by alveolar hyperplasia. By 9–12 mo of age, hyperplastic alveolar walls were found throughout the lung parenchyma. Alveolar spaces contained hypertrophic alveolar macrophages that were frequently multinucleated. At 12 mo of age, alveolar macrophages were also noted in larger bronchioles in some SP-C-GM mice. Collagen deposition, as assessed by Masson's trichrome stain, was not increased in animals examined at any age (not shown). No increase in eosinophils or granulocytes was observed in airways.
of any of the animals. Additionally, four separate GM-/-, SP-C-GM +/- founder mice that failed to breed or to pass on the SP-C-GM transgene were killed at 4 or 12 mo of age for histological assessment. Hyperplastic alveolar walls and increased numbers of alveolar macrophages were noted in each of these animals, similar to the findings in line 48 and 59 mice. Because levels of GM-CSF in the BAL fluid and alveolar wall hyperplasia were most consistent in mice from line 59, subsequent experiments used mice derived from this line.

Type II cell hyperplasia in SP-C-GM mice. Type II cells were identified in the hyperplastic alveolar walls of SP-C-GM lungs by staining with anti-pro-SP-C, an antibody specific for the amino terminal domain of pro-SP-C. Pro-SP-C is synthesized only by type II cells. Immunoperoxidase labeling of pro-SP-C demonstrated marked type II cell hyperplasia in the SP-C-GM mice compared with control mice (Fig. 3). Type II cells in SP-C-GM mice were also larger and stained more intensely than type II cells of GM-/- mice. Immunofluorescent anti-pro-SP-C staining also demonstrated increased numbers of type II cells in the SP-C-GM mice (Fig. 4). Furthermore, colocalization of anti-pro-SP-C and anti-PCNA immunofluorescent staining was consistently observed in SP-C-GM mice compared with GM-/- and GM +/- controls. The colocalization of anti-pro-SP-C and anti-PCNA indicates increased proliferation of type II cells, which may contribute to the increased numbers of type II cells seen in SP-C-GM mice. Increased numbers of PCNA-staining alveolar macrophages were also found in the alveolar spaces and interstitium of SP-C-GM compared with GM-/- and GM +/- lungs.

Increased lung weight and volume in SP-C-GM mice. At 4–5 mo of age, lungs from mice of each genotype were removed and weighed (Table 1). Lung weight was significantly increased in SP-C-GM mice compared with those from GM-/- and GM +/- mice. Fluid-filled lungs of SP-C-GM mice were 30–40% heavier than those of GM-/- and GM +/- control mice. Correspondingly, the total volume (mm$^3$) of inflated SP-C-GM lungs was 25–40% larger than GM-/- and GM +/- lungs. The increased weight and lung size detected in the morphometric analysis were consistent with increased lung DNA and total protein noted in SP-C-GM mice at 2 mo of age [Ikegami et al. (13a)]. To quantitate the total number of type II cells per lung, type II cells were counted in representative fields (1 $\times$ 10$^3$ mm$^3$ of lung parenchyma) from each lung lobe. The number of type II cells was more than doubled per parenchymal unit and was increased fourfold per lung in SP-C-GM mice compared with GM-/- and GM +/- mice (Table 1).

GM-CSF receptors on type II cells and alveolar macrophages. Immunohistochemical staining was used to identify GM-CSF-R subunits. The GM-CSF receptor complex consists of GM-CSF-R and a shared subunit (β) that also is part of the interleukin (IL)-3 and IL-5 receptor complexes. Type II cells and bronchial epithelial cells stained with antibodies to GM-CSF-R, identifying these cell types as potential targets of GM-CSF in mouse lung (Fig. 5). Alveolar macrophages and endothelial cells were also stained (not shown), consistent with previous studies demonstrating GM-CSF receptors or GM-CSF responses in these cell types (12, 14).

RT-PCR was used to amplify nucleotides 642–951 of the GM-CSF-R subunit mRNA from isolated mouse type II cells (Fig. 6). The amplified band comigrated with a band generated in a mouse alveolar macrophage cell line but, as expected, was smaller than the product generated from genomic DNA. An additional larger
band was consistently amplified from type II cell mRNA. The precise identity of that band is unknown. The presence of GM-CSF-Rα mRNA in mouse type II cells corroborates the immunohistochemical staining in mouse lung tissue sections and provides further evidence for GM-CSF receptor expression in type II cells.

GM-CSF stimulates BrdU uptake by type II cells in vitro. Freshly isolated rat type II cells were incubated with mouse GM-CSF for 72 h. BrdU was added to the cultures for the final 24 h. BrdU uptake increased in a dose-dependent manner, indicating that DNA synthesis was increased in the presence of GM-CSF (Fig. 7). The number

Fig. 2. Lung histology. Lung sections were stained with hematoxylin and eosin. At 4–5 mo of age, lungs from line 59 GM-CSF gene-targeted mice also homozygous for the SP-C-GM transgene (GM−/−,SP-C-GM+; A) and mice homozygous for GM-CSF and the SP-C-GM transgene (GM+/+,SP-C-GM+; B) had hyperplastic alveolar walls and increased numbers of alveolar macrophages compared with GM−/− (C) and GM+/+ (D) mice. A is representative of marked alveolar hyperplasia found in regions of all SP-C-GM lungs, and B is representative of the appearance of both hyperplastic and relatively normal regions in all SP-C-GM mice at 2–5 mo. Similar histological changes were found in mice from line 48 GM−/−,SP-C-GM+ and GM+/−,SP-C-GM+ founder mice as described in MATERIALS AND METHODS. Short arrows, alveolar walls; long arrows, alveolar macrophages. Bar = 50 µm.
of cells per well was not increased by the addition of GM-CSF, as assessed by cell counts (data not shown).

DISCUSSION

Transgenic mice expressing GM-CSF in lung epithelial cells were generated in GM-CSF null mutant and GM-CSF-replete backgrounds. Expression of GM-CSF had a profound effect on lung growth in the SP-C-GM mice. Lungs from SP-C-GM mice 4–5 mo of age were 30–40% larger than lungs of GM−/− or GM+/+ control mice, as assessed by weight or morphometric analysis, and numbers of type II cells per lung were increased three- to fourfold. Lung epithelial cell-specific GM-CSF expression corrected alveolar proteinosis in the GM−/− mice, enhanced lung growth, and caused type II cell hyperplasia in mice from either GM−/− or GM+/+ backgrounds.

Lung injury initiates a complex cascade of inflammatory responses that limit tissue damage and enhance...
the cell proliferation required to repair the lung parenchyma (23). Alveolar repair is dependent on both proliferation and differentiation of type II cells and is thought to be influenced by various growth factors. For example, hepatocyte growth factor and keratinocyte growth factor are increased after lung injury and are known to stimulate type II cell DNA synthesis and proliferation (17, 22, 23, 30). The present findings suggest that GM-CSF may also play a role in type II cell proliferation. Because lung size and alveolar morphology were not perturbed in GM−/− mice, GM-CSF is not required for lung morphogenesis. However, GM-CSF mRNA is rapidly induced in the lung after endotoxin exposure, suggesting that GM-CSF may be an early
mediator of the repair process after alveolar injury (24). The enlarged lungs and increased type II cell proliferation seen in SP-C-GM mice support the hypothesis that GM-CSF influences postnatal lung growth and/or differentiation and is consistent with a potential role in repair and remodeling after injury.

GM-CSF is produced in normal lung and was first isolated from lung cell-conditioned media (4). A variety

Table 1. Lung weights and morphometric analysis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body Weight, g</th>
<th>Lung Wet Weight, g</th>
<th>Inflated Lung Weight, g</th>
<th>Total Lung Volume, mm³</th>
<th>Type II Cells Per 1 x 10⁵ µm³</th>
<th>Type II Cells Per Lung</th>
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<tr>
<td>GM−/−, SP-C-GM +/+</td>
<td>28.2 ± 1.1</td>
<td>0.32 ± 0.03*</td>
<td>0.94 ± 0.04</td>
<td>764 ± 28†</td>
<td>12.5 ± 1.6†</td>
<td>9.5 ± 1.2 x 10⁷†</td>
</tr>
<tr>
<td>GM+/+, SP-C-GM +/+</td>
<td>23.5 ± 0.7</td>
<td>0.29 ± 0.02*</td>
<td>0.83 ± 0.08</td>
<td>694 ± 52†</td>
<td>12.7 ± 2.0†</td>
<td>8.8 ± 1.9 x 10⁷†</td>
</tr>
<tr>
<td>GM−/−</td>
<td>25.3 ± 0.8</td>
<td>0.26 ± 0.01</td>
<td>0.61 ± 0.02</td>
<td>521 ± 3</td>
<td>4.4 ± 0.4</td>
<td>2.3 ± 0.2 x 10⁷†</td>
</tr>
<tr>
<td>GM+/+</td>
<td>28.5 ± 2.1</td>
<td>0.23 ± 0.01</td>
<td>0.54 ± 0.01</td>
<td>446 ± 24</td>
<td>4.7 ± 0.2</td>
<td>2.1 ± 0.5 x 10⁷†</td>
</tr>
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Values are means ± SE. Lungs from 4- to 5-mo-old mice (n = 11) were removed and weighed. *Differences in lung wet weights between surfactant protein (SP)-C granulocyte macrophage (GM) transgenic mice (SP-C-GM mice) and GM colony-stimulating factor (CSF) gene-targeted mice (GM−/− mice) or wild-type mice homozygous for GM-CSF (GM+/+ mice) were statistically significant as determined by analysis of variance (ANOVA; P < 0.03) and Student-Newman-Keuls test (P < 0.05). Three mice of each genotype were killed at 4–5 months of age for morphometric analysis. Lungs were inflation-fixed and weighed, and total lung volume (mm³) was determined using the Cavalieri method as described in MATERIALS AND METHODS. Type II cells were counted per 1 x 10⁵ µm³ of lung parenchyma, and total number of type II cells per lung was calculated. Lungs of SP-C-GM mice were 30–40% larger, and total number of type II cells was increased 3- to 4-fold compared with controls. †Differences between SP-C-GM mice and GM−/− or GM+/+ control mice were statistically significant as determined by ANOVA (P < 0.005) and Student-Newman-Keuls test (P < 0.05). Differences between GM−/−, SP-C-GM +/+ and GM+/+, SP-C-GM +/+ mice or between GM−/− and GM+/+ mice were not statistically significant.

Fig. 5. Immunohistochemical staining with anti-GM-CSF receptor α-subunit (GM-CSF-Rα) antibodies. Type II cells and bronchiolar epithelial cells in GM+/+ mice were stained with GM-CSF-Rα antibodies (A). Alveolar macrophages and endothelial cells were also stained (not shown). Staining in GM+/+ tissue sections was blocked when primary (anti-GM-CSF-Rα) antibody was preincubated with an immunizing peptide (B). Staining of tissue sections incubated without primary antibody was indistinguishable from staining seen in B. Staining in GM−/−, SP-C-GM +/+ lungs (not shown) was similar to the pattern observed in GM+/+ lungs. Short arrows, type II cell; long arrows, bronchiolar epithelium. Bar = 40 µm.
of pulmonary cells express GM-CSF, including mouse and human alveolar macrophages, endothelial cells, and fibroblasts, rat type II cells, and human bronchial and tracheal cells (1, 6, 8, 19). However, the normal lung GM-CSF concentrations in BAL fluid from GM+/+ mice were below the limit of detection by ELISA. Although GM-CSF was not readily detected in the normal lung, intratracheal administration of endotoxin in GM+/+ FVB/N mice increased BAL fluid GM-CSF concentrations to levels similar to the those measured in SP-C-GM mice. In recent studies, type II cell hyperplasia was observed in rat lungs 3–7 days after exposure to endotoxin (29). However, it is unknown at present whether the hyperplastic response to LPS was mediated directly by GM-CSF or by other factors influenced by endotoxin. The tightly regulated expression pattern of endogenous GM-CSF mRNA and protein contrasts with the high levels of GM-CSF measured in BAL fluid from the SP-C-GM mice. The continuous high-level production of GM-CSF in the transgenic mice was associated with marked type II cell hyperplasia and infiltration of alveolar macrophages, generating lung histology that is quite distinct from acute changes seen after endotoxin.

GM-CSF caused progressive pulmonary type II cell hyperplasia in the SP-C-GM mice. The hypercellularity of the alveolar walls was largely due to an increase in both size and number of type II cells. Although it is possible that changes in other cell types also contributed to the increased lung size observed in the SP-C-GM mice, type II cell hyperplasia and macrophage infiltration predominated the histological findings. The increase in type II cells per lung was attributed to both increased numbers of type II cells per parenchymal unit and increased lung volume. Immunofluorescent staining for both PCNA and pro-SP-C in SP-C-GM mice was increased, supporting the concept that type II cell proliferation, at least in part, accounts for the histological findings in the lungs. Although PCNA staining suggests an increased rate of mitosis, GM-CSF may also influence type II cell numbers by altering differentiation or apoptotic pathways in type II cells. The progressive changes in lung histology suggest that hyperplasia was also related to the prolonged duration of exposure to GM-CSF.

The accumulation of alveolar macrophages observed in SP-C-GM mice is consistent with the known mitogenic effects of GM-CSF. Metcalf (18) observed increased numbers of macrophages, eosinophils, and granulocytes in peritoneal and lung compartments after intraperitoneal injection of GM-CSF and in transgenic mice expressing GM-CSF and particularly noted increased mitosis in macrophages. Increased numbers of alveolar macrophages were associated with increased PCNA staining in lungs of SP-C-GM mice, suggesting a proliferative effect of GM-CSF on local macrophage precursors. Local expansion of the macrophage population is consistent with in vitro studies that demonstrated that alveolar macrophages proliferate when stimulated with GM-CSF (5). Chemotactic properties of GM-CSF may also contribute to the increased number of alveolar macrophages in lungs of the SP-C-GM mice. The morphology of the SP-C-GM alveolar macrophages, including enlarged cytoplasm and multinucleated cells, is consistent with in vitro studies of morphological changes in alveolar macrophages treated with GM-CSF (16). No changes were seen in peripheral blood leukocytes, and neither granulocytes nor eosinophils were increased in lungs of SP-C-GM mice. These findings support the hypothesis that increased numbers of alveolar macrophages in the SP-C-GM mice result from local production of GM-CSF within the lung. Furthermore, increased numbers and activity of macrophages in the SP-C-GM mice likely contribute to the resolution of alveolar proteinosis in the GM−/−, SP-C-GM+/+ mice.

Type II cell hyperplasia was consistently observed in SP-C-GM mice and occurred in the absence of pulmonary fibrosis. The present findings therefore are distinct from those in which a recombinant adenovirus was used to direct expression of GM-CSF in the mouse lung (33). GM-CSF concentrations after adenoviral vector delivery may differ from those generated in the

Fig. 6. Reverse transcriptase (RT)-polymerase chain reaction (PCR) of GM-CSF-Rα/β from isolated mouse type II cells. RNA was harvested from a mouse alveolar macrophage cell line (MH-S cells) or primary type II cells isolated from GM−/− mice and was used for RT-PCR to detect GM-CSF-Rα/β transcripts. A band of the expected size (309 nucleotides) was generated from MH-S and type II cell (TII) RNA. In addition, a slightly larger band was consistently generated from type II cell RNA. No band was observed in the reaction mix without template (Ø). A band of ∼0.7 kb was produced in the reaction containing genomic DNA as template.

Fig. 7. GM-CSF enhanced bromodeoxyuridine (BrdU) uptake by type II cells in vitro. Primary cultures of rat type II cells were prepared and treated with GM-CSF at the indicated concentrations for 72 h. BrdU was added to the cells for the final 24 h, and BrdU incorporation was quantitated by ELISA. GM-CSF significantly enhanced BrdU incorporation at concentrations >1 × 10−9 M, as assessed by analysis of variance (P < 0.05). Data represent means ± SE for 5 independent cell preparations; n = 4 for each preparation.
SP-C-GM mice. The consistent lack of pulmonary fibrosis in the SP-C-GM model also suggests that GM-CSF is not sufficient to cause fibrosis and that stimulation of additional inflammatory pathways by the adenovirus may have contributed to the fibrosis observed after adenovirus-mediated transfer of GM-CSF.

Whereas marked type II cell hyperplasia was consistently observed in the SP-C-GM mice, it remains unclear whether direct or indirect effect(s) of GM-CSF contributed to the changes in lung histology. It is possible that GM-CSF stimulates the expression of other type II cell growth regulators. GM-CSF receptors were present in type II epithelial cells but were also detected on alveolar macrophages and endothelial and bronchiolar epithelial cells. GM-CSF-Rα mediates both high- and low-affinity binding of GM-CSF to its receptor complex. GM-CSF, IL-3, and IL-5 receptor complexes share the common βc-subunit. The βc and a ligand-specific α-subunit heterodimerize to form a functional high-affinity receptor complex (14). The βc-subunit neither binds GM-CSF nor determines ligand specificity but contains a cytoplasmic domain required for signal transduction through the JAK-STAT pathway (25). The finding that GM-CSF-Rα subunits are present on type II cells suggests that GM-CSF may act directly on type II cells to regulate proliferation or differentiation or to mediate other autocrine pathways influencing type II cell proliferation.

The presence of GM-CSF receptors detected with immunohistochemical staining by GM-CSF-Rα antibodies was further confirmed by RT-PCR amplification of GM-CSF-Rα mRNA from isolated mouse type II cells. In addition to the transcript represented by the expected product of 309 nt, a slightly larger transcript was consistently detected in type II cell RNA preparations. Although the mouse type II cell preparations are highly purified (~90–95% type II cells), it remains possible that the GM-CSF-Rα 309 nt band arises from the presence of other cells contaminating the type II cell isolate.

The finding that GM-CSF stimulated BrdU uptake in rat type II cells in vitro supports the concept that GM-CSF may play a role in type II cell proliferation in vivo. Although BrdU uptake reflects increased DNA synthesis, GM-CSF alone was not sufficient to increase proliferation of type II cells in culture. The isolated rat type II cell cultures are generally >90% pure. Because contaminating alveolar macrophages are variably present in the cultures, albeit in low numbers, it is also possible that the observed stimulation of BrdU uptake by GM-CSF was mediated by paracrine effects among various cell types present in the culture.

GM-CSF plays an important role in surfactant metabolism. GM-CSF-deficient mice develop severe alveolar proteinosis related to decreased catabolism of surfactant lipids and proteins (13). SP-A and phospholipid clearance studies of GM–/–, SP-C-GM +/+ mice demonstrated that local expression of GM-CSF in respiratory epithelial cells corrected surfactant homeostasis in vivo [Ikegami et al. (13a)]. Mice homozygous for a βε receptor mutation also developed severe alveolar proteinosis that was substantially corrected by bone marrow transplantation (21). These studies support an important role for the alveolar macrophage in the pathogenesis of alveolar proteinosis. It remains unclear, however, whether donor macrophages directly corrected impaired surfactant catabolism or compensated for impaired type II cell function. Furthermore, it is not known if interactions between donor cells and type II epithelial cells also contributed to the restoration of surfactant homeostasis seen in the βε mutant mice.

GM-CSF expression in lung epithelial cells enhanced lung growth in association with proliferation of type II cells and alveolar macrophages in the SP-C-GM mice. Biochemical and histological findings in SP-C-GM mice previously demonstrated that GM-CSF was required for regulation of surfactant homeostasis. The increased lung size and type II cell hyperplasia seen in SP-C-GM mice demonstrate an unexpected effect of GM-CSF in the lung and are consistent with a potential role for GM-CSF as a regulator of type II cell proliferation and differentiation after lung injury.

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