GM-CSF provides autocrine protection for murine alveolar epithelial cells from oxidant-induced mitochondrial injury

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Sturrock A, Seedahmed E, Kasimov MM, Boltax J, McManus ML, Paine R 3rd. GM-CSF provides autocrine protection for murine alveolar epithelial cells from oxidant-induced mitochondrial injury. Am J Physiol Lung Cell Mol Physiol 302: L343–L351, 2012. First published December 2, 2011; doi:10.1152/ajplung.00276.2011.—Exposure of mice to hyperoxia induces alveolar epithelial cell (AEC) injury, acute lung injury and death. Overexpression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the lung protects against these effects, although the mechanisms are not yet clear. Hyperoxia induces cellular injury via effects on mitochondrial integrity, associated with induction of proapoptotic members of the Bcl-2 family. We hypothesized that GM-CSF protects AEC through effects on mitochondrial integrity. MLE-12 cells (a murine type II cell line) and primary murine type II AEC were subjected to oxidative stress by exposure to 80% oxygen and by exposure to H2O2. Exposure to H2O2 induced cytochrome c release and decreased mitochondrial reductase activity in MLE-12 cells. Incubation with GM-CSF significantly attenuated these effects. Protection induced by GM-CSF was associated with Akt activation. GM-CSF treatment also resulted in increased expression of the antiapoptotic Bcl-2 family member, Mcl-1. Primary murine AEC were significantly more tolerant of oxidative stress than MLE-12 cells. Incubation with GM-CSF further increased Akt activation and Mcl-1 expression in primary AEC. Conversely, suppression of AEC GM-CSF expression by use of GM-CSF-specific small interfering RNA resulted in decreased tolerance of oxidative stress. Furthermore, silencing of Mcl-1 prevented GM-CSF-induced protection. We conclude that GM-CSF protects alveolar epithelial cells against oxidative stress-induced mitochondrial injury via the Akt pathway and its downstream components, including Mcl-1. Epithelial cell-derived GM-CSF may contribute to intrinsic defense mechanisms limiting lung injury.

acute lung injury; apoptosis; growth factors; lung; granulocyte-macrophage colony-stimulating factor

ACUTE LUNG INJURY RESULTING in the acute respiratory distress syndrome (ARDS) remains a devastating condition impacting large numbers of individuals each year. Despite recent advances in ventilator management and critical care, the morbidity and mortality associated with ARDS remain unacceptably high (43). Although ARDS is the result of a variety of different insults, oxidative stress and alveolar epithelial cell injury are common features of many of these processes (12). Injury of the alveolar epithelium is a key aspect of many instances of lung injury, contributing to early leak of fluid and protein into the alveolar space and associated with later inflammatory and fibrotic changes (34, 35). In animal models of acute lung injury, strategies to limit alveolar epithelial cell injury or enhance epithelial cell recovery following injury have been associated with improved survival (32, 33). Oxidative stress is a common mechanism in many forms of lung injury, whether generated locally in the lung from cellular sources or due to toxic inhalation (12). In response to oxidative stress, reactive oxygen species are generated in mitochondria (20). Mitochondrial integrity is impaired, leading to release into the cytoplasm of cytochrome c and initiation of pathways to cell death (2, 30).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is an endogenous growth factor found in the normal lung that is a product of alveolar epithelial cells (AEC) (5, 9, 13). GM-CSF is critical for functional maturation of alveolar macrophages and for normal pulmonary innate immune responses (31, 36, 41). In addition to this role in host defense, GM-CSF is a mitogen for AEC in vivo and in vitro (22, 32). Using exposure to increased concentrations of oxygen to induce lung injury, we have found that GM-CSF is protective against lethal lung injury both in normal mice (32) and in mice rendered more susceptible to lung injury by infection with Pneumocystis murina (4). In these experiments, treatment with GM-CSF or overexpression of GM-CSF in the lung was associated with decreased apoptosis of AEC following exposure to hyperoxia. The present study was undertaken to further define the mechanisms by which GM-CSF protects against AEC injury and death. Because hyperoxia induces significant mitochondrial stress, we have focused on the effects of GM-CSF on AEC mitochondrial integrity in the setting of oxidative stress. We have found that GM-CSF induces activation the phosphatidylinositol 3-kinase (PI3)-kinase/Akt pathway, resulting in induction of a mitochondria-associated antiapoptotic member of the Bcl-2 family, Mcl-1. Using RNA interference, we have confirmed that the protective effects of GM-CSF are mediated at least in part via Mcl-1.

METHODS AND MATERIALS

Animals. C57Bl/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions and were monitored daily by veterinary staff. The animal care committee at the Salt Lake City Veterans Affairs Medical Center approved these experiments.

Isolation and purification of primary alveolar epithelial cells. Murine type II alveolar epithelial cells were isolated and purified using a modification of published methods (14, 25). Following anesthesia with Avertin (Sigma, St. Louis, MO) and heparinization, mice were exsanguinated and the pulmonary vasculature was perfused with saline. The trachea was cannulated and the lungs were filled with 1–2
were utilized for experiments. Changes of room temperature sterile PBS to remove nonadherent cells.

streptomycin and 10% fetal calf serum in wells coated with fibronectin was 3–6% on technology, Santa Cruz, CA). Secondary staining was performed with anti-vimentin, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary staining was performed with FITC-conjugated anti-mouse IgM. Routinely, fibroblast contamination was 3–6% on day 3 after isolation. Cells were maintained in DMEM 10% FCS unless otherwise stated.

MLE-12 cells. MLE-12 cells, an immortal murine alveolar epithelial type-II cell line (44) were obtained from ATCC (Manassas, VA) and maintained in DMEM 10%. The original culture was expanded and stocks frozen within 7 days of receipt to maintain early passage.

In vitro exposure to oxidative stress. Oxidative injury was induced by exposure to H2O2 or to hyperoxia in vitro. Experiments using H2O2 as the source of oxidative stress were carried out in serum-free DMEM, using freshly diluted H2O2. For hyperoxia exposure, MLE-12 cells or primary murine AEC were placed in a sealed self-contained chamber (Billups-Rothenberg, Del Mar, CA) and exposed to an atmosphere of 80% oxygen-5% CO2 for 48 h. The chamber was humidified and maintained at 37°C and was flushed daily with a commercially available gas mixture of CO2 and oxygen, adjusted to maintain a fractional concentration of oxygen of 0.80 as measured in real time with an oxygen analyzer within the chamber (maxO2, Maxtec, Salt Lake City, UT).

Cell fractionation for cytochrome c release. Cytochrome c release was measured as the ratio of cytochrome c in cytoplasmic and mitochondrial fractions. Cytoplasmic and mitochondrial fractions were obtained by cell fractionation by using the protocol and reagents in the Mitochondrial Isolation Kit for Mammalian Cells (Thermo Scientific). For each sample, 2×10 cm dishes of MLE-12 cells were used at 70–80% confluence. Protein levels of the fractions were determined by use of the Bio-Rad BCA assay. Levels of cytochrome c in each fraction were expressed as mean densities as a ratio to actin levels.

Cell injury: LDH release. The release of lactate dehydrogenase (LDH) into the culture medium was determined as a measure of relative AEC injury. Briefly, the media were collected and spun free of debris prior to assay. LDH was determined by ELISA (Roche), and the values were corrected to reflect total media volume. The cell layer was washed with PBS then incubated in RIPA buffer (Thermo Fisher) for 20 min on ice to lyse cells. RIPA-insoluble material was removed by centrifugation and LDH was determined in the RIPA-soluble cytoplasmic fraction. Total cytoplasmic LDH was calculated by correction for volume. Percent LDH release was used as a measure of injury = LDH in medium/LDH medium + LDH cytoplasm × 100.

Immunoblot for proteins. Whole cell lysates were prepared for Western blotting by disrupting cells in RIPA buffer containing a broad-spectrum proteinase inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). The protein content of the lysate was determined by use of Bio-Rad (Hercules, CA) BCA Protein Assay. Proteins were separated by SDS-PAGE, loading equal amounts of protein onto preformed SDS-PAGE gels (Criteron Precast 8–16% gels, Bio-Rad). After transfer to polyvinylidene difluoride membrane (Bio-Rad), the protein of interest was detected using a specific primary antibody and a horseradish peroxidase-conjugated secondary antibody raised against the species in which the primary antibody was developed. The signal was developed by using ECL Western Blotting Detection reagents (GE Lifescience/Thermo Fisher, Pittsburgh, PA). Each immunoblot was stripped by use of Restore Plus Western Blot Stripping Buffer (Thermo Fisher), blocked, and probed for actin (as above). Densitometry readings were performed on a BioSpectrum MultiSpectral Imaging System (BioSpectrum, Upland, CA) and the mean density minus mean background taken for each band. Data are expressed as mean densities normalized to actin levels (or total Akt, in the instance of phospho-Akt). Antibodies were from the following suppliers: cytochrome c and actin (Santa Cruz Biotechnology); phospho-Akt/Akt, phospho-GSK-3α/β/GSK-3α/β (Cell Signaling, Danvers, MA); Mcl-1 (R&D Systems, Minneapolis, MN).

Real-time PCR. Total cellular RNA was isolated from cultured cells using RNaseasy (Qiagen, Valencia, CA). First strand cDNA was reverse transcribed from 1 μg of total RNA using a high capacity cDNA kit (Applied Biosystems, Foster City, CA). Gene specific primers were designed using the Roche Applied Science Universal Probe Library Assay Design Center and synthesized/PC purified by the University of Utah DNA/Peptide Core. Specific PCR products were generated from cDNA (100 ng) using Brilliant SYBR Green QRT-PCR 2-step (Stratagene, La Jolla, CA) and an Mx3000P real-time computerized cycler from Stratagene. The two-step cycle program (melting temperature = 60°C) with a dissociation analysis was used as recommended by Stratagene. Appropriate controls (no template control and Rox reference dye) were included in each experiment. The threshold cycle from GAPDH was used as a calibrator to normalize the specific RNA quantitation. Results are expressed as fold change over control values after correction for GAPDH, with the first biological control set at 100%.

GM-CSF protein measurements. After the appropriate perturbation (e.g., 48 h in hyperoxia or small interfering RNA treatment) fresh medium was added to the cultures for 18–24 h. Culture supernatants then were collected, centrifuged to remove cell debris, and either immediately assayed for GM-CSF levels by ELISA (R&D Systems), or frozen at −80°C and assayed later, care being taken to ensure only one freeze-thaw cycle for each sample.

GM-CSF RNA interference and antibody neutralization. GM-CSF and Mcl-1 were inhibited by use of RNA interference technology. Accell SMART pools each consisting of four short, or small, interfering RNAs (siRNA) for GM-CSF or Mcl-1 were obtained from Dharmacon/Thermo Fisher (Lafayette, CO). Accell siRNA is specifically modified for use without a transfection reagent and works at a higher concentration than conventional siRNA, using passive delivery in Accell serum-free media. Experiments were carried out according to the Dharmacon protocol, using 1 μM GM-CSF or Mcl-1 Accell SMARTpool siRNAs. The siRNAs were added to cultures on day 3 after isolation (when they had been adherent for 48 h). The cells were then incubated for 48 or 72 h with the siRNA followed by a 24-h recovery (see Accell siRNA delivery protocol, Dharmacon). In experiments to neutralize endogenous GM-CSF, cultures were treated with neutralizing rat anti-murine GM-CSF polyclonal antibody (100
same stress (Fig. 4B). When exposed to H2O2 at 250 ng/ml, primary AEC compared with MLE-12 cells exposed to the same concentration of rat IgG. Control cells were treated with the same concentration of rat IgG.

**Statistical analysis.** Data are presented as means ± SE. Statistical analysis was carried out with GraphPad Prism v4C software (GraphPad). Differences between groups were compared by one-way ANOVA. Comparisons were deemed statistically significant for P values <0.05.

**RESULTS**

Effect of GM-CSF on oxidant-induced mitochondrial injury. To begin to explore the effects of GM-CSF on mitochondrial injury in the setting of oxidative stress, we first used a model of oxidative injury in the type II cell-like MLE-12 cell line. Exposure of MLE-12 cells to H2O2 (250 μM) resulted in significant mitochondrial injury, as indicated by increased release of cytochrome c (Fig. 1A) and decreased mitochondrial NADPH-dependent dehydrogenase (MTT) activity (Fig. 1B). Treatment with GM-CSF resulted in significant decrease in oxidant-induced MTT suppression. This mitochondrial injury was associated with impaired cellular integrity, as indicated by increased release of LDH into the culture medium. Treatment with GM-CSF also protected MLE-12 cells against LDH-release in response to oxidative stress (Fig. 1C).

Activation of PI3-K/Akt signaling pathway by GM-CSF. We postulated that GM-CSF might protect MLE-12 cells against oxidative injury through its ability to activate the PI3-K/Akt survival pathway. Under baseline conditions phospho-Akt was not detected in MLE-12 cells. Upon exposure to GM-CSF (20 ng/ml), Akt phosphorylation was briskly induced within 15 min (Fig. 2). Levels remained high through 30 min and were still measurable 60 min after addition of GM-CSF. In pro-B cells and myeloid progenitor cells, GM-CSF regulates viability via the PI3-K/Akt pathway, in part through increased expression of the mitochondrial protective prosurvival protein, Mcl-1 (11, 42). Treatment of MLE-12 cells with GM-CSF more than doubled expression of Mcl-1 protein. This increased expression was prevented by preincubation with PI3-kinase inhibitors, wortmannin and LY294002 (Fig. 3, A and B). Mcl-1 protein expression is regulated in part posttranslationally via glycogen synthase kinase-3 (GSK3), a known substrate of Akt that is involved in metabolic signaling, cell cycle regulation, and cell survival. Phosphorylation of Mcl-1 by GSK3 initiates ubiquitination and rapid degradation of Mcl-1. Phosphorylation of GSK3 by Akt inactivates GSK3, thereby increasing Mcl-1 protein expression. Treatment of MLE-12 cells with GM-CSF induced GSK3 phosphorylation (Fig. 3, C and D). These data suggest that GM-CSF protects type II AEC-like cells from oxidative mitochondrial damage via induction of a survival pathway involving PI3-K/Akt, GSK3, and Mcl-1.

Oxidative injury in primary murine AEC. We next compared the susceptibility to oxidative injury of primary murine AEC to MLE-12 cells. Primary AEC were significantly more resistant to oxidative cellular injury compared with MLE-12 cells, as indicated by decreased LDH release in response to exposure to 80% oxygen for 48 h (Fig. 4A). In response to this oxidative stress, mitochondrial activity was diminished significantly less in primary AEC compared with MLE-12 cells exposed to the same stress (Fig. 4B). When exposed to H2O2 at 250 μM for 15 h, primary AEC demonstrated significantly less reduction in MTT activity compared with MLE-12 cells. Full MTT activity was preserved in primary AEC at a concentration of H2O2, at which this activity was decreased by 50% in MLE-12 cells (Fig. 4C).

Having shown that GM-CSF activates the Akt survival pathway in MLE-12 cells, we hypothesized that the relative

![Fig. 1. Effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on the susceptibility of MLE-12 cells to oxidative injury in response to H2O2](http://ajplung.physiology.org/)
resistance of primary AEC to oxidative injury might be due to Akt pathway activation as a result of ongoing autocrine GM-CSF expression. GM-CSF mRNA and protein were highly expressed in unstimulated primary AEC, whereas transcripts were expressed at only very low level and protein was at the limit of detection in unstimulated MLE-12 cells (Fig. 5). Similarly, in contrast to MLE-12 cells, primary AEC express phosphorylated Akt at baseline. As in MLE-12 cells, Akt phosphorylation was further increased upon addition of GM-CSF to primary AEC (Fig. 6). We examined Mcl-1 expression in primary AEC, to determine whether its enhanced expression might contribute to the resistance of primary AEC to mitochondrial injury due to oxidative stress. Primary AEC under basal conditions had approximately twofold greater expression of Mcl-1, normalized to actin, compared with MLE-12 cells under the same conditions (Fig. 6A; compare Fig. 3A). Treatment with GM-CSF further increased primary AEC Mcl-1 protein expression. Similarly, GM-CSF induced increased GSK3 phosphorylation in these cells (Fig. 6C). Taken together, these data suggest that the decreased susceptibility of primary AEC to oxidative injury compared with MLE-12 may be a consequence of ongoing constitutive GM-CSF expression, resulting in Akt activation and increased expression of Mcl-1.

Suppression of GM-CSF expression in primary murine AEC. To confirm the role of autocrine GM-CSF for protection of primary AEC in the setting of oxidative stress, we used siRNA to suppress GM-CSF expression in these cells. Treatment of primary AEC with siRNA for GM-CSF resulted in 75% suppression of GM-CSF mRNA expression, and 60% reduction in GM-CSF protein expression (Fig. 7). Neither siGM-CSF nor nontargeting siRNA affected transcription of GAPDH, suggesting that the treatment was not significantly toxic (data not shown). Gene-specific silencing of GM-CSF in primary AEC resulted in a 40% reduction in Mcl-1 transcripts compared with nontargeting siRNA controls, whereas BAX transcript levels were unaffected by siGM-CSF (Fig. 8A), indicating a direct role for GM-CSF in preventing oxidative injury in primary AEC via involvement of Mcl-1 in mitochondrial protection. To confirm the role of constitutive GM-CSF in protection of AEC against oxidative injury primary murine AEC were treated with GM-CSF-specific siRNA or with nontargeting siRNA prior to exposure to H2O2. Silencing GM-CSF rendered primary AEC significantly more sensitive to oxidative

Fig. 2. Effect of GM-CSF on Akt activation in MLE-12 cells. MLE-12 cells were exposed to GM-CSF (20 ng/ml) or control medium. Cells were harvested at the times shown. A: representative Western blots showing total Akt and phospho-Akt, loading samples from duplicate plates. B: densitometry data, showing the ratio of phospho-Akt to total Akt. Data are expressed as means ± SE; *P < 0.05 vs. control, n = 3.

Fig. 3. Induction of Mcl-1 and phospho-glycogen synthase kinase-3(GSK3) by GM-CSF in MLE-12 cells. MLE-12 cells were exposed to GM-CSF (20 ng/ml) or control medium for 2 h. The PI3 kinase inhibitors wortmannin (10 μM) and LY294002 (100 μM) were added to selected dishes 30 min prior to addition of GM-CSF. Mcl-1 protein and actin were detected by Western blot analysis. A representative blot (A) and densitometry data (B), showing the ratio of Mcl-1 to actin, are shown. *P < 0.01 vs. control, n = 3. C: Western blot analysis showing the time course of expression of phospho GSK3 following treatment of MLE-12 cells with GM-CSF (20 ng/ml). D: densitometry data showing the ratio of phospho-GSK3 to actin are shown. *P < 0.01 vs. control. **P < 0.001 vs. control. n = 3.
MLE-12 cells were exposed to either room air or 80% oxygen in 5% CO2 for 48 h in DMEM 10%. A: LDH release was expressed as % of total LDH. Data are expressed as means ± SE, n = 4. *P < 0.001 vs. MLE-12 in hyperoxia; for both MLE-12 and primary AEC, values in hyperoxia are significantly different from those in normoxia (P < 0.001). B: mitochondrial function was measured as MTT activity (% control). Data are expressed as means ± SE, n = 5. *P < 0.001 vs. AEC hyperoxia; for both MLE-12 and primary AEC, values in hyperoxia are significantly different from those in normoxia (P < 0.001). C: primary AEC and MLE-12 cells were exposed to control medium or medium with H2O2 (250 μM) for 15 h before mitochondrial function was measured as MTT activity (OD at 495 nm). Data are expressed as means ± SE, *P < 0.001 vs. MLE-12 in control medium and **P < 0.001 vs. AEC in H2O2; n = 4.

In this manuscript we have further defined the mechanisms underlying the protective effects of GM-CSF for the alveolar epithelium in the setting of oxidative stress. We found that treatment of AEC in vitro with GM-CSF resulted in increased tolerance of oxidative stress following exposure to hyperoxia or H2O2, manifest as preserved mitochondrial activity and decreased cellular injury and death. Intracellular events associated with this effect included activation of Akt, increased expression of Mcl-1, and phosphorylation of GSK3. Compared with the MLE-12 cell line, primary murine AEC in vitro were more resistant to oxidative injury, and demonstrated constitutive expression of GM-CSF and activation of Akt. Silencing GM-CSF expression in primary AEC increased the vulnerability of these cells to oxidative injury.

A wide variety of insults that lead to acute lung injury involve oxidative stress in the lung, often as a result of endogenously generated reactive oxygen species from inflammatory cells or due to toxic inhalation (12). Supplemental oxygen therapy can be a life-sustaining modality for individuals with respiratory illness. However, high-dose oxygen therapy can itself contribute to lung injury (18). Animal models of acute lung injury due to sustained exposure to >90% oxygen have provided clinically relevant information concerning the pathobiology of oxidant-induced lung injury. Both in vivo and in vitro, hyperoxia induces alveolar epithelial cell and pulmonary capillary endothelial cell injury and death. These processes may involve apoptosis, necrosis, or intermediate pathways (2, 18). In selected instances, short-term exposure to H2O2 in vitro may mimic the oxidative stress caused by more prolonged exposure to high concentrations of oxygen.

GM-CSF is a growth factor produced in the normal lung. Although best known for its important role in the functional maturation of alveolar macrophages, GM-CSF also has been shown to have effects on alveolar epithelial cells. These cells both produce GM-CSF and express GM-CSF receptors (22).

Silencing of Mcl-1 in primary murine AEC. Having determined that GM-CSF induced Mcl-1 expression, experiments were performed to investigate the role of this increased expression in the protective effect of GM-CSF against oxidative injury. Treatment of primary AEC with siRNA for Mcl-1 resulted in significant suppression of Mcl-1 mRNA expression (Fig. 9A). When AEC treated with control (nontargeting) siRNA were exposed to oxidative stress there was significant reduction in MTT activity, that was almost completely reversed by treatment with GM-CSF. However, this protective effect of GM-CSF was lost in cell treated with Mcl-1 siRNA (Fig. 9B). These data suggest that the protective effect of GM-CSF against oxidant-induced injury requires Mcl-1.

DISCUSSION

In this manuscript we have further defined the mechanisms underlying the protective effects of GM-CSF for the alveolar epithelium in the setting of oxidative stress. We found that treatment of AEC in vitro with GM-CSF resulted in increased tolerance of oxidative stress following exposure to hyperoxia or H2O2, manifest as preserved mitochondrial activity and decreased cellular injury and death. Intracellular events associated with this effect included activation of Akt, increased expression of Mcl-1, and phosphorylation of GSK3. Compared with the MLE-12 cell line, primary murine AEC in vitro were more resistant to oxidative injury, and demonstrated constitutive expression of GM-CSF and activation of Akt. Silencing GM-CSF expression in primary AEC increased the vulnerability of these cells to oxidative injury.

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Treatment with GM-CSF in vivo or in vitro results in proliferation of alveolar epithelial cells (22). In previous work, we have shown that overexpression of GM-CSF in the lung protects mice from lung injury and death due to hyperoxia (32). This protective effect was associated with decreased apoptosis of alveolar lining cells. Alveolar cell apoptosis was also decreased in wild-type mice treated with systemic GM-CSF during exposure to hyperoxia and in a complex model of lung injury induced by transient exposure to hyperoxia in immuno-compromised mice infected with *Pneumocystis murina* (4). However, in the complex environment of the lung, GM-CSF might be acting either directly on epithelial cells or indirectly, through effects on other cell types such as alveolar macrophages. In the present study we have now shown that one component of this protection involves direct effects of GM-CSF on alveolar epithelial cells to decrease susceptibility to injury induced by oxidative stress. Whether GM-CSF exerts additional indirect effects mediated by other cells and mediators is the subject of ongoing investigation.

Mitochondria are key participants in cellular injury induced in the setting of hyperoxia. Hyperoxia induces increased mitochondrial reactive oxygen species production (20). These mitochondrial reactive oxygen species are critical for hyperoxia-induced cell injury and death (10, 45), in part through activation of mitochondria-dependent pathways to cell death. Bax is translocated to mitochondria, and cytochrome c is released into the cytoplasm (3, 7, 30). Alterations in the balance of pro- and antiapoptotic members of the Bcl-2 family influence the outcome of hyperoxic stress. In vitro, overexpression of antiapoptotic Bcl-2 prevents hyperoxia-induced cell death through effects in mitochondria (8, 26). Similarly, mutant mice lacking proapoptotic Bcl-2 family members are protected against death due to hyperoxia (8).

Mcl-1 is a member of the Bcl-2 family that was initially identified in myeloid leukemia cells (15, 23). It acts as a prosurvival factor by binding to proapoptotic Bcl-2 proteins, such as Bak and Bax, to prevent their polymerization and thus to prevent the formation of pores in the mitochondrial membrane.
brane for release of cytochrome c (27, 28, 40). This antiapoptotic activity of Mcl-1 requires localization to the mitochondrial membrane, regulated by the first 79 amino acids of Mcl-1 protein (21). Mcl-1 is expressed in a wide variety of cells, but its function has been most extensively studied in neurons and hematopoietic cells, where it is induced by GM-CSF (11).

Mcl-1 expression is regulated at both the transcriptional and posttranslational levels (29, 38, 40). There are multiple phosphorylation sites on the protein, many of which may be involved in regulation of degradation. Phosphorylation of serine residues at 155 and 159 by GSK3 has resulted in decreased Mcl-1 expression due to enhanced protein degradation (17, 40). GSK3 activity in turn is controlled by its phosphorylation, which results in its inactivation (16, 19). We have now shown in AEC that treatment with GM-CSF results in increased Mcl-1 expression, in association with both increased Mcl-1 mRNA expression and phosphorylation of GSK3. The result of these effects is decreased release of cytochrome c into the cytoplasm and decreased epithelial cell death.

PI3-K/Akt is a pluripotent signaling molecule that influences a number of cellular pathways. Gene transfer of a constitutively active form of Akt to the lung is protective against lung injury in the setting of hyperoxia (24). We have shown that GM-CSF rapidly induces Akt phosphorylation in alveolar epithelial cells. Furthermore, induction of Mcl-1 by GM-CSF is blocked in the presence of the kinase inhibitors wortmannin or LY294002. Suppression of Mcl-1 expression with siRNA blocks the protection provided by GM-CSF in the setting of oxidative stress. Although it is possible that GM-CSF may act to inhibit injury by additional mechanisms, these data support

![Graph](https://via.placeholder.com/150)

**Fig. 8.** Effect of diminished GM-CSF expression in primary AEC on Mcl-1 relative mRNA expression and AEC sensitivity to oxidant-induced mitochondrial injury. Primary AEC were treated with GM-CSF-specific siRNA or nontargeting siRNA (100 nM) as described in METHODS AND MATERIALS. Additional control cells were cultured in medium alone. A: Mcl-1 (dark shaded bars) and Bax (light shaded bars) mRNA expression were determined by real-time PCR. Data are presented as means ± SE; n = 3; the experiment shown is representative of 3 similar experiments. *P < 0.001 vs. control and nontargeting siRNA and vs. GM-CSF siRNA Bax expression. There was no significant difference for Bax among the 3 groups. B: mitochondrial function of primary AEC cells was determined as MTT activity after an additional 15-h incubation with H2O2 (500 μM) or medium alone. Data presented are means ± SE, n = 5. *P < 0.001 vs. nontargeting siRNA and vs. GM-CSF siRNA. C: primary AEC were treated with control IgG (100 ng/ml) or neutralizing anti-GM-CSF antibody for 24 h prior to exposure to H2O2 (250 mM). MTT activity was determined after an additional 15-h incubation with H2O2 (250 μM) or medium alone. Data presented are means ± SE, n = 4. *P < 0.001 vs. control IgG; **P < 0.001 vs. control IgG and vs. H2O2 + IgG.

![Graph](https://via.placeholder.com/150)

**Fig. 9.** Effect of gene-specific siRNA silencing of Mcl-1 on primary AEC sensitivity to oxidant-induced mitochondrial injury. Primary AEC were treated with Mcl-1-specific siRNA or nontargeting siRNA (100 nM) as described in METHODS AND MATERIALS. A: Mcl-1 mRNA expression was determined by real-time PCR. Data are presented as means ± SE; n = 4; the experiment shown is representative of 2 similar experiments. *P < 0.001 vs. control and nontargeting siRNA. B: mitochondrial function of primary AEC cells was determined as MTT activity after an incubation with H2O2 (500 μM) or medium alone. Data presented are means ± SE, n = 4; the experiment shown is representative of 2 similar experiments. *P < 0.001 nontargeting siRNA vs. GM-CSF siRNA.
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a key role for GM-CSF-induced Akt activation in mitochondrial stabilization by Mcl-1.

GM-CSF may have additional, less direct, effects. In general, cells expressing the type II cell phenotype are more resistant to injury due to oxidative stress than are type I cells (37). We have not found changes in alveolar epithelial cell phenotypic markers in response to GM-CSF. Similarly, GM-CSF might act by inducing the expression of endogenous antioxidants. We found no effect of GM-CSF on the expression of mRNAs for catalase or superoxide dismutases 1–3 (data not shown). However, we cannot exclude effects of GM-CSF on mitochondrial redox balance.

These studies have employed both a type II alveolar epithelial cell line (MLE-12 cells) and primary murine AEC. Although MLE-12 cells have proven to be very useful models of the alveolar epithelium, this immortalized cell line does not recapitulate all features of the normal alveolar epithelium. Primary AEC proved more resistant to cellular injury in response to oxidative stress compare to the type II cell-like cell line. Primary AEC also demonstrated constitutive expression of GM-CSF and basal Akt activation, features that were not found in MLE-12 cells. Whether autocrine expression of GM-CSF is a characteristic of resting AEC expressing features of the type I cell or type II cell phenotype, or is a stress response in AEC is not yet known. The normal murine lung demonstrates ongoing expression of GM-CSF mRNA (1). Furthermore, studies using laser capture microdissection have demonstrated that that cells of the alveolar wall express GM-CSF mRNA in the absence of inflammatory stimulation (1), suggesting that murine AEC in primary culture indeed model appropriate aspects of the behavior of the alveolar epithelium in vivo. We found that both suppression of constitutive GM-CSF expression with specific siRNA and antibody-mediated neutralization of endogenous GM-CSF resulted in significantly diminished tolerance of oxidative stress by the primary cells. These observations strongly support an important contribution of endogenous GM-CSF expression in the defense of the alveolar epithelium against oxidative stress. Interestingly, we have shown previously that oxidative stress itself suppresses AEC GM-CSF expression through changes in GM-CSF mRNA stability (39), suggesting the potential benefit of treatment with recombinant GM-CSF in the setting of ongoing oxidative stress.

In conclusion, these studies demonstrate that GM-CSF exerts a protective effect on the alveolar epithelium against oxidative stress and that this effect is likely a manifestation of induction of Mcl-1, via activation of Akt and inactivation of GSK3. Furthermore, they demonstrate that GM-CSF produced constitutively by AEC induces an intrinsic tolerance of oxidative stress that is likely to be key in the lung. They also suggest that pharmacological treatment with recombinant GM-CSF or manipulation of endogenous GM-CSF expression may provide an opportunity to enhance defense against oxidative stress in the setting of lung injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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