Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway

Shulamit B. Wallach-Dayan,1* Gabriel Izbicki,1* Pazit Y. Cohen,1 Regina Gerstl-Golan,1 Alan Fine,2 and Raphael Breuer1,2,3
1Lung Cellular and Molecular Biology Laboratory, Institute of Pulmonology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; and 2Pulmonary Center and 3Department of Pathology, Boston University School of Medicine, Boston, Massachusetts

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Bleomycin is a neoplastic drug known to produce interstitial pulmonary fibrosis in humans (46) as well as in experimental models (38). This redox active agent is capable of producing electron-deficient substances such as hydroxyl radicals, superoxide, and hydrogen peroxide (29). These reactive oxygen species (ROS) are capable of damaging important biological molecules and changing the redox state of cells (29), including lung cells (19), and have been implicated in apoptotic cell death (21, 30).

Apoptosis is implemented by molecular pathways that ultimately result in activation of a family of cysteine proteases termed caspases (14). In mammals, two main caspase cascades have been delineated. The first pathway links death receptors (e.g., Fas) and caspase-8 (34), whereas the second pathway, the mitochondrial pathway, occurs through diverse extracellular stresses, such as hypoxia, and involves procaspase-9 activation (15), which in turn cleaves and activates downstream effector caspases (27). However, caspase-8 has also been shown to be activated independently of death receptor induction (51). The role of Fas/Fas ligand (Fas/FasL) pathway in lung fibrosis is controversial (4, 7, 17, 18, 23–25). Fas and FasL are upregulated in fibrosing lung diseases in humans (7, 25) and in murine bleomycin-induced fibrosis (7, 18, 24). Lung epithelial cells are the main target of in vivo bleomycin- or Fas/FasL-induced apoptosis, which terminates in fibrosis (17, 18, 23, 37). To further investigate the pathways involved in bleomycin-induced apoptosis, we studied apoptotic signaling in a murine lung epithelial (MLE) cell line in vitro.

We report that bleomycin causes an increase in ROS levels with sequential activation of caspase-8, mitochondrial leakage, activation of caspase-9, and subsequent cell apoptosis. Moreover, bleomycin upregulates Fas and sensitizes cells to Fas-mediated apoptosis. However, the Fas/FasL pathway is probably not directly involved, since the initiation of apoptosis was inhibited in the presence of reduced glutathione (GSH) and caspase-8 inhibitors, but not by antibodies interfering with the Fas/FasL pathway.

MATERIALS AND METHODS

Monoclonal antibodies and other reagents. We used the following monoclonal antibodies (MAbs) and reagents: 1 mg/ml propidium iodide (PI) stock solution (Calbiochem, La Jolla, CA) in phosphate-buffered saline (PBS); annexin V-FITC (PharMingen, San Diego, CA); 1 mCi/ml sterile [3H]methylthymidine (Amersham Biosciences, Rehovot, Israel); GSH (Sigma, St. Louis, MO); 2',7'-dichlorofluorescin diacetate (DCFH/DA) and MitoTracker RedCMXRos mitochondrial membrane potential (MTP) tracker (Molecular Probes, Eugene, OR); bleomycin (ASTA Medica) and caspase-8 [z-IETD-fluoromethyl ketone (fmk)] and caspase-9 (z-LEHD-fmk) inhibitors (R&D Systems, Minneapolis, MN); Faslantagonist, anti-mouse FasL Ab, clone-MFL3, and Jo2-Fas agonist (PharMingen): IgG control, clone-RGE53 (Chemicon International, Temecula, CA); caspase-8 and -9 activity assays kit (BioVision, Mountain View, CA).

Cell culture systems. We used the murine type II lung epithelial cell line (ATCC, MLE-15), an extensively used model (5, 6, 10, 16, 20, 35, 48, 49, 52). Cells were maintained in hydrocortisone insulin transferrin estradiol selenium (HITES) medium (Industrial Laboratories, Beit HaEmek, Israel) that was supplemented with 2 mM t-glutamine, 10% heat-inactivated FBS (Sigma, St. Louis, MO), and 5 mM Pen-Strep (Industrial Laboratories, Beit HaEmek, Israel). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO2. MLE cells (0.5 × 104) were cultured in HITES medium, with or without 0.1 mM of benzoyl peroxide and with or without 0.1 mM reduced glutathione, or 30–60 mM caspase inhibitors, and were assayed at 0.1, 1, 48 h. ROS levels, caspase activities, and MTP disruption were assayed at 24 h. To trigger Fas-induced apoptosis, Fas was treated with 10 nM of 2,7'-dichlorofluorescin diacetate (DCFH/DA) and MitoTracker RedCMXRos mitochondrial membrane potential (MTP) tracker (Molecular Probes, Eugene, OR); bleomycin (ASTA Medica) and caspase-8 [z-IETD-fluoromethyl ketone (fmk)] and caspase-9 (z-LEHD-fmk) inhibitors (R&D Systems, Minneapolis, MN); Faslantagonist, anti-mouse FasL Ab, clone-MFL3, and Jo2-Fas agonist (PharMingen); IgG control, clone-RGE53 (Chemicon International, Temecula, CA); caspase-8 and -9 activity assays kit (BioVision, Mountain View, CA).

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upregulated in MLE cells by pretreatment with 5 mU/ml of bleomycin. At 24 h, this medium was replaced by medium containing 1 μg/ml agonist Fas and/or 20 μM antagonist anti-MFL3 FasL. MAb. For fluorescence-activated cell sorting (FACS) analysis, cells were maintained in 3% FCS in PBS (FACS buffer).

**Trypan blue exclusion.** Cells were exposed to trypan blue dye (0.04% in 1× PBS), placed on a hemocytometer, and examined under light microscopy. We counted 200 random cells.

\[^{3}H\]thymidine-labeled DNA release by dead cells. Cells (3–5 × 10^5/ml) were labeled with 5 μCi/ml (final concentration) at their exponential phase of growth for 4–6 h after treatment with different bleomycin concentrations; cells were harvested and counted in a \( \beta \)-counter.

**DNA fragmentation analysis.** DNA fragmentation assay was described previously (40). In brief, 5 × 10^5 cells were lysed in 30 μl of lysis buffer [80 mM EDTA, 200 mM Tris (pH 8.0), 1.6% sodium lauril sarcosinate, and 1 mg/ml protease K], incubated at 50°C for 4 h followed by incubation with RNase A (0.2 mg/ml), at 37°C for 4.5 h, fractionated by aagarose gel electrophoresis, visualized under UV, and confirmed by digitized densitometry using a tabletop scanner (Bio-Rad Multi-Analyist/PC version 1.1). Data was analyzed with Fluon-S-Multimager (Bio-Rad).

**DNA content and cell cycle flow cytometry analysis.** Genomic DNA integrity was also analyzed by determining the DNA content in the nuclei with flow cytometry as described previously (36). Cells were fixed with 70% ethanol for 30 min at 4°C, washed, and incubated in PBS containing PI (50 μg/ml) and 0.1 mg/ml RNase (Boehringer Mannheim, Indianapolis, IN) for 30 min at room temperature. DNA content was determined by flow cytometry on FACStar (Becton Dickinson, Mountain View, CA).

**In vitro detection of apoptotic cells by annexin V affinity labeling.** FITC-conjugated annexin V (1 μg) was added to 0.5 × 10^5 MLE adherent cells in the culture flask and incubated for 30 min. Cells were pelleted (400 g, 5 min) and resuspended in FACS buffer. PI (5 μg/ml) was added for 15 min incubation on ice. Flow cytometry analysis by plotting green fluorescence (FL1)/FITC-annexin V vs. red fluorescence (FL2)/PI-positive cells was performed with a FACStar (Becton Dickinson, Mountain View, CA). PI- and PI/annexin V-negative cells were excluded from the presented data.

**ROS measurement.** MLE cells were treated with bleomycin (100 mU) at different time points, and the peroxide anion levels were estimated by staining with DCFH/DA, final concentration 5 μM (Eastman Kodak, Rochester, NY), for 50 min at 37°C. Cells were harvested and immediately underwent flow cytometric analysis with excitation and emission settings of 488 and 530 nm, respectively.

**Assay for caspase activation.** Caspase-8 and caspase-9 activities were measured using the BioVision FLICE/Caspase-8/9 Colorimetric Assay Kit (BioVision). Cells (0.3–1 × 10^6) were lysed on ice for 10 min and centrifuged (10,000 g, 1 min). Cytosolic extracts (supernatant) were incubated for 1–2 h at 37°C with IETD-p-nitroanilide (pNA) or LEHD-pNA color substrates (200 μM), respectively. Samples were analyzed by spectrophotometer (400 nm).

**Monitoring inner MTP.** MitoTracker RedCMXRos was added to the cells for the last 30 min of bleomycin incubation. Cells were washed in PBS, and their fluorescence was measured using the FL-3 channel and analyzed by flow cytometry.

**Fas and FasL expression assayed by flow cytometry.** MLE cell surface expression of Fas and FasL were assessed by indirect immunofluorescence and analyzed by flow cytometry. In brief, 0.5 × 10^6 cells were washed with FACS buffer, incubated on ice for 45 min with 1 μg PE-conjugated anti-Fas or FasL MAb, washed, and analyzed by flow cytometry using a FACStar (Becton Dickinson).

**Data analysis and statistics.** Flow cytometry and colorimetric assayed experiments were repeated at least two or three times. Data were analyzed according to mean fluorescence intensity or optical density, respectively, presented as means ± SD, and plotted on graphs or on dot plots. Statistical analysis was performed by simple \( t \)-test for experiments with single comparisons and ANOVA with Bonferroni test for experiments with multiple comparisons.

**RESULTS**

**Bleomycin induces MLE cell death and apoptosis in a dose-response manner.** MLE cells were cultured with increasing doses of bleomycin (5–1,000 mU) and assayed at 24 h. Cell viability decreased with increasing doses of bleomycin as assayed by trypan blue exclusion and \[^{3}H\]thymidine release (Fig. 1A). To further test whether the bleomycin-induced MLE cell death was due to apoptosis induction, three assays were used to detect apoptosis: DNA ladder (Fig. 1B), annexin V staining (Fig. 1C), and DNA content analysis (Fig. 1D). As demonstrated, bleomycin caused apoptosis of MLE cells in a dose-response manner.

**Bleomycin increases ROS levels, altering mitochondrial membrane potential with sequential apoptosis.** The kinetics of bleomycin (100 mU)-induced MLE cell apoptosis was studied by annexin V staining, followed by flow cytometric analysis, as presented in Fig. 2. Apoptosis increased at 24 h, and at 48 h no viable cells were found.

To clarify the association of bleomycin-induced apoptosis and the oxidative stress, peroxide (ROS) levels (using DCFH staining followed by flow cytometry analysis) were determined over time, as demonstrated in Fig. 2. ROS was seen to increase at 0.5 h and at all other time points.

Bleomycin-induced apoptosis was attenuated by preincubation with GSH (see Fig. 7). This also supports the idea that bleomycin-induced apoptosis occurs via increased ROS.

The kinetics of change in the collapse of the inner MTP was studied to assess whether the bleomycin-induced rise in ROS levels occurs due to MTP disruption. MitoTracker Red CMXRos, whose accumulation in the mitochondria depends on the membrane potential, was used. A collapse in MTP was detected by the decrease of a cell population with high MitoTracker Red staining. Changes in MTP are presented in Fig. 2. The percentage of disrupted MTP in cells exposed to bleomycin and assayed at 0.5 or 1 h was not affected, mildly affected at 24 h, and considerably affected (11%) at 48 h.

As demonstrated in Fig. 2, changes in ROS levels occurred earlier (0.5 h) than in MTP or apoptosis (24 h).

**Bleomycin induces caspase-8 and caspase-9 activation, and their inhibition decreases bleomycin-induced apoptosis.** Caspase-8 and -9 activities were increased in MLE cells after exposure to bleomycin (100 mU, 24 h) as demonstrated in Fig. 3A. To determine whether a mitochondrial (caspase-9) or death receptor pathway (caspase-8) is required for bleomycin-induced apoptosis, MLE cells were cultured in RPMI (B0) and then were exposed to bleomycin for 24 h, following preincubation with 30 μM caspase-8 inhibitor, z-LEHD-fmk, and/or 60 μM caspase-9 inhibitor, z-IETD-fmk. These caspase-8 and -9 inhibitors, when used separately, significantly suppressed apoptosis, as detected by annexin V flow cytometry analysis (Fig. 3B), from average values of 60 ± 8.1% in bleomycin-treated cells (B1) to 36 ± 11.2% (B2) and 45 ± 6.8% (B3), respectively. However, when these inhibitors were used together, there was no additive effect on bleomycin-induced apoptosis (data not shown).
Sequential activation of caspase-8 and -9 in bleomycin-treated MLE cells. To examine the regulatory relationship between caspase-8 and -9 activities, bleomycin-treated MLE cells were pretreated with 30 μM caspase-8 inhibitor (z-IETD-fmk; Fig. 4A) or 60 μM of caspase-9 inhibitor (z-LEHD-fmk; Fig. 4B). Pretreatment with caspase-8 inhibitor markedly suppressed caspase-9 activity and attenuated MTP-disruption. In contrast, caspase-9 inhibitor had a marked effect on MTP but only a mild effect on caspase-8 activity. Therefore, caspase-8 is required for bleomycin-induced MTP-disruption and caspase-9 activation.

ROS upregulates caspase-8 activity. GSH was used to determine a regulatory effect of ROS-oxidative stress on caspase-8 activity. MLE cells were pretreated with GSH (10 mM) and then 1 h later with bleomycin (100 μU, 24 h). GSH decreased the bleomycin-induced caspase-8 activation as shown in Fig. 5.

Bleomycin upregulates Fas/FasL expression. Fas receptor, which functions to initiate a signal, causing apoptosis, was shown to be expressed in lung epithelial cells (13). Flow cytometry analysis was performed to determine whether bleomycin caused an upregulation of Fas or FasL expression and to determine their dependence on oxidative stress. MLE cells were treated with bleomycin (100 μU, 24 h), followed by FACS staining using phycoerythrin-conjugated anti-Fas or FasL MAb. Fas and FasL expression was increased (Fig. 6, A

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**Table 1.** Dose-dependent decrease in cell viability as shown in A, when assayed by trypan blue exclusion and % viable cells out of total cells; 2 - [3H] thymidine release assay (DPM)

<table>
<thead>
<tr>
<th>BLEO mU/ml</th>
<th>0</th>
<th>5</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>500</th>
<th>1000</th>
</tr>
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<tbody>
<tr>
<td>% viability</td>
<td>86</td>
<td>76</td>
<td>73</td>
<td>63</td>
<td>51</td>
<td>43</td>
<td>27</td>
</tr>
<tr>
<td>[3H] thymidine</td>
<td>3868 ±479</td>
<td>2675 ±215</td>
<td>6998 ±682</td>
<td>12711 ±820</td>
<td>42624 ±2010</td>
<td>63226 ±11300</td>
<td>74276 ±10900</td>
</tr>
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1 - % viable cells out of total cells  
2 - [3H] thymidine release assay (DPM)
Bleomycin-induced apoptosis is decreased by glutathione but not by Fas/FasL pathway antagonist. We have shown that Fas/FasL upregulation (Fig. 6) and caspase-8 activity (Fig. 5) are associated with ROS upregulation. To determine whether bleomycin-induced MLE cell apoptosis is due to oxidative stress or Fas/FasL pathway, GSH or antagonistic anti-FasL MAbs were used, respectively. MLE cells were preincubated for 1 h with MFL3 (20 μM) or GSH (10 mM) and then for 24 h with bleomycin (100 μU).

The percentage of apoptotic cells was determined by FACS annexin V staining. GSH decreased bleomycin-induced apoptosis (Fig. 7). In contrast, FasL antagonist MFL3 did not decrease bleomycin-induced MLE cell apoptosis (Fig. 7).

Fig. 3. Bleo activates caspase-8 and caspase-9, and their specific inhibitors decrease Bleo-induced apoptosis in MLE cells. A: 0.5 × 10^6 MLE cells were treated with Bleo (100 μU, 24 h). Caspase-8 and caspase-9 activities were determined by a colorimetric kit. Both caspase-8 and -9 were increased [Bleo vs. untreated (Untr)]. OD, optical density. *P < 0.05, **P < 0.01. B: 0.5 × 10^6 MLE cells were preincubated for 1 h, with or without caspase inhibitors, and cultured with Bleo (100 μU, 24 h), stained with annexin V, and analyzed by flow cytometry. MLE cells cultured in RPMI (B0), cultured with Bleo (B1), with Bleo and 30 μM caspase-8 inhibitor (z-IETD-fmk) (B2), or with Bleo and 60 μM caspase-9 inhibitor (z-LEHD-fmk) (B3). Both caspase inhibitors decreased the MLE cell apoptosis from 60 ± 8.1% (B1) to 36 ± 11.2% (B2) and 45 ± 6.8% (B3), respectively. Casp., caspase. All panels depict 1 of 2 independent experiments showing similar results, presented by dot plots. The number in each box represents means ± SD of 2 independent experiments.

Fig. 4. Dependence of caspase-9 and MTP on caspase-8 activation. We treated 0.5 × 10^6 MLE cells with Bleo (100 μU, 24 h), with Bleo and preincubation with 30 μM caspase-8 inhibitor (A) or with 60 μM caspase-9 inhibitor (B). Caspase activities were determined by using the colorimetric caspase activity assay kit. MTP changes were determined by MitoTracker assay. Caspase activities and MTP changes are presented as fold ratios, determined by dividing each experimental value by the average of control untreated cells. Caspase-9 (A) and caspase-8 (B) activities were increased in MLE cells treated by Bleo. Caspase-9 was inactivated by caspase-8 inhibitor (A, Bleo + inh8), whereas caspase-8 was slightly inactivated by caspase-9 inhibitor (B, inh9). MTP was reduced by both caspase inhibitors (A and B). Inhibition of increased Bleo-induced caspase-8 and -9 activities by their specific inhibitors was confirmed (insets: Bleo vs. Bleo + inh8 or inh9). *P < 0.05, **P < 0.01.
to bleomycin increased Fas-induced apoptosis. This effect was decreased with GSH (10 mM), thereby demonstrating that bleomycin causes sensitization to Fas-induced apoptosis, which is also ROS dependent.

**DISCUSSION**

Apoptosis of alveolar epithelial cells has been implicated as a mechanism leading to pulmonary fibrosis (18, 23, 37). We used an MLE cell line to investigate bleomycin-induced apoptosis of lung epithelial cells. Bleomycin caused ROS accumulation, MTP disruption, apoptosis, increased caspase-8 and -9 activity, and increased expression of cell surface Fas and FasL. The onset of ROS occurred earlier than that of MTP or apoptosis, indicating that the increased ROS evolves directly from the bleomycin effect and not secondary to cell apoptosis and/or a subsequent mitochondrial leak.

Our data show inhibition of apoptosis by glutathione and not by Fas/FasL pathway antagonists. This confirms that bleomycin functions via ROS-induced mitochondrial cell death (1, 28, 42) and that the Fas/FasL pathway is not predominant. Fas/FasL antagonists in hepatocytes were shown to interfere with bleomycin-induced apoptosis (32, 33). However, results from

![Fig. 5. Bleo-induced upregulation of caspase-8 activation is inhibited by GSH. Caspase-8 activity, determined by a caspase activity assay kit, was increased after being cultured with 100 nM Bleo for 24 h compared with untreated MLE cells (Untr). Addition of 10 mM glutathione 1 h before (Bleo + GSH) inhibited Bleo-induced caspase-8 activation. *P < 0.05.](image)

![Fig. 6. Bleo causes an upregulation of Fas/FasL expression. CD95 (Fas, A) and CD95L (FasL, B) receptor expression, using 1 μM of CD95-PE- or CD95L-PE-conjugated MAb, respectively, were analyzed by flow cytometry and are presented by segregated histograms. Fas and FasL expression was increased in Bleo-treated (100 nM, 24 h) 0.5 x 10⁶ MLE cells compared with untreated cells. Glutathione (10 mM) inhibited the increased Bleo-induced Fas expression (Bleo + GSH), as shown in A. There was no inhibition of FasL expression by GSH (data not shown). Data presented are representative of 3 different experiments.](image)

![Fig. 7. GSH but not antagonist anti-FasL MAb decrease bleomycin-induced MLE cell apoptosis. MLE cells (0.5 x 10⁵) were preincubated for 1 h with MFL3 anti-FasL (20 μM) or GSH (10 mM) and then for 24 h with Bleo (100 nM). The percentage of apoptotic cells was determined by FACS analysis of annexin V staining. GSH decreased Bleo-induced apoptosis (Bleo + GSH vs. Bleo). In contrast, addition of anti-FasL (Bleo + α-FasL) resulted in no change from cells treated with Bleo alone. To confirm the inhibitory effect of antagonist MFL3 anti-FasL, MAb, 0.5 x 10⁵ Jurkat cells (Ctrl-Jurkat) were treated with agonist Jo2 anti-Fas (1 μg/ml) to induce apoptosis, which was inhibited by MFL3 anti FasL (inset: segregated histograms, Bleo vs. Untr). *P < 0.05.](image)

![Fig. 8. Increased sensitivity to Fas-induced apoptosis of Bleo-treated MLE cells. MLE cells (0.5 x 10⁵) were cultured in medium containing 0 (Untr) and 5 mU of Bleo, and Fas upregulation is presented (inset). In parallel, at 24 h, the media of untreated or Bleo-treated cells were substituted with media containing 1 μg/ml of Jo2 Fas agonist (α-Fas) or (Bleo + α-Fas), respectively. Flow cytometry analysis of annexin V staining was performed at 24 h, demonstrating that MLE cells exposed to Bleo are more sensitive to Fas-induced apoptosis (Bleo + α-Fas) than those exposed to Fas agonist alone (α-Fas). Glutathione (10 mM) decreased this post-Bleo sensitivity to Fas-induced apoptosis (Bleo + α-Fas + GSH vs. Bleo + α-Fas). *P < 0.05, **P < 0.01.](image)
other systems, which are consistent with ours, indicate that apoptosis is independent of this pathway (9, 11, 12, 22, 31, 47).

Although the Fas/FasL pathway was not involved, bleomycin caused an activation of caspase-8. Inhibitors of caspase-8 also inhibited bleomycin-induced caspase-9 and apoptosis, with ROS dependency, demonstrating that bleomycin triggered apoptosis through caspasas regulated by oxidative stress.

The mechanisms of caspase-8 activation by ROS, in a death receptor-independent pathway, remain to be defined. Caspase-8 activation that induces mitochondria-mediated downstream events (43) can be achieved independently from death receptor induction (26, 51, 53). Caspase-8 may help amplify mitochondria-dependent apoptosis through a feed-forward loop on which initial mitochondrial damage by ROS causes caspase-3 activation. This caspase then activates caspase-8, which further activates mitochondrial release of cytochrome c and caspase-3 (44). These studies and our results demonstrate a role for ROS in regulating caspases of the extrinsic apoptotic pathway.

The Fas/FasL pathway in our system is not directly responsible for apoptosis. Nevertheless, bleomycin-exposed MLE cells were found to be more sensitive to further apoptosis induction by agonistic anti-Fas MAb in an ROS-dependent manner (Fig. 8). The observation of a later Fas-induced apoptosis presented in this study relates to a specific epithelial cell line (MLE cells). However, Fas receptor upregulation was also tested on primary epithelial cells, isolated from lungs, at 3 but not 1 day after bleomycin treatment of C57BL/6 mice (unpublished data from our laboratory). Fas upregulation and in vivo Fas-associated apoptosis were also reported to appear in lung sections of bleomycin treated mice by day 7 (18). Fas was also overexpressed in alveolar epithelial cells in lung tissues of human idiopathic pulmonary fibrosis (25). In addition, a study by Aoshiba et al. (4) showed no change of bleomycin-induced apoptosis in alveolar epithelial cells of Fas- or FasL-deficient mice compared with wild type when cells were assayed at day 1. Moreover, administrations of a soluble form of Fas (hFas-Fc) prevented apoptosis of these cells in bleomycin-treated mice before day 7 (23). The absence of Fas/FasL involvement in the first days of bleomycin-induced murine fibrosis is also in accordance with in vivo studies showing ROS as the initial apoptotic trigger in the fibrogenic process (1–3, 27, 42). These in vivo findings are consistent with our in vitro findings and imply that two types of epithelial cell apoptosis appear in vivo after bleomycin exposure.

The extent of alveolar fibrosis is often related to the severity of epithelial cell injury (41). The importance of a first rapid apoptosis of epithelial cells in the fibrotic process may be due to their involvement in mesenchymal cell proliferation (39, 45) and their initiation and modulation of inflammatory reactions. The Fas/FasL apoptotic pathway, which appears to take place at a later time, seems essential to clear intra-alveolar granulated tissue, without induction of an acute inflammatory response (8, 50).

In summary, bleomycin induces an ROS-dependent, oxidative stress involving caspase-8 activation, terminating in apoptosis and subsequent upregulation of Fas and FasL expression that further sensitizes cells to Fas-induced apoptosis.

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