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TIME-DEPENDENT APOPTOSIS OF ALVEOLAR MACROPHAGES FROM RATS EXPOSED TO BLEOMYCIN: INVOLVEMENT OF TNF RECEPTOR 2

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Tumor necrosis factor-alpha (TNF-α) is produced by alveolar macrophages (AM) in response to bleomycin (BLM) exposure. This cytokine has been linked to BLM-induced pulmonary inflammation, an early drug effect, and to lung fibrosis, the ultimate toxic effect of BLM. The present study was carried out to study the time dependence of apoptotic signaling pathways and the potential roles of TNF receptors in BLM-induced AM apoptosis. Male Sprague-Dawley rats were exposed to saline or BLM (1 mg/kg) by intratracheal instillation. At 1, 3, or 7 d postexposure, AM were isolated by bronchoalveolar (BAL) lavage and evaluated for apoptosis by ELISA. The release of cytochrome c from mitochrondria, the activation of caspase-3, -8, and -9, the cleavage of nuclear poly(ADP-ribose) polymerase (PARP), and the expression of TNF receptors (TNF-R1/p55 and TNF-R2/p75), TNF-R-associated factor 2 (TRAF2), and cellular inhibitor of apoptosis 1 (c-IAP1) were determined by immunoblotting. The results showed that BLM exposure induced AM apoptosis, with the highest apoptotic effect occurring at 1 d after exposure and gradually decreasing at 3 and 7 d postexposure, but still remaining significantly above the control level. The maximal translocation of cytochrome c from mitochondria into the cytosol was observed at 1 d postexposure, whereas the activation of caspase-9 and caspase-3 and caspase-3-dependent cleavage of PARP was found to reach a peak level at 3 d postexposure. BLM exposure had no marked effect on AM expression of TNF-R1 or caspase-8 activation, but significantly increased the expression of TNF-R2 that was accompanied by a rise in c-IAP1 and a decrease in TRAF2. This induction of TNF-R2 by BLM was significant on d 1 and increased with greater exposure time. In vitro studies showed that pretreatment of naive AM with a TNF-R2 antibody significantly inhibited BLM-induced caspase-3 activity and apoptosis. These results suggest that BLM-induced apoptosis involves multiple pathways in a time-dependent manner. Since maximal BLM-induced AM apoptosis (1 d postexposure) preceded maximal changes in caspase-9 and -3 (3 d postexposure), it is possible that a caspase-independent mechanism is involved in this initial response. These results indicate that the sustained expression of TNF-R2 in AM by BLM exposure may sensitize these cells to TNF-α-mediated toxicity.

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Bleomycin (BLM) is an antineoplastic antibiotic used in the treatment of a variety of tumors. However, the clinical usage of this drug has been limited due to its severe, life-threatening pulmonary toxicity. BLM-induced pulmonary toxicity is attributed to a lack of BLM hydrolase, which is required for inactivation of BLM in the lung (Chandler, 1990; Jules-Elysee & White, 1990; Sleijfer, 2001). It has been well established that BLM induces DNA damage and apoptosis (Harrison et al., 1989; Tounekti et al., 1993) in lung cells and leads to the progressive development of pulmonary fibrosis. Apoptosis, or genetically programmed cell death, results in cellular plasma and nuclear membrane blebbing, cell shrinkage, dissolution of nuclear lamina, and the activation of proteases and endonucleases (Zimmermann et al., 2001). Apoptosis is considered to be a protective mechanism that limits lung injury. However, overexpression of apoptotic cells may contribute to the inflammatory burden in the injured lung. Recent studies have shown that intratracheal instillation of apoptotic alveolar macrophages (AM) resulted in the development of pulmonary fibrosis in rats (Wang et al., 2003). Thus, BLM-induced fibrosis may also involve the induction of apoptosis and the clearance of apoptotic cells in the lung.

The mechanisms through which BLM induces apoptosis have been the subject of many studies, and both the intrinsic (through mitochondrial-mediated events) and the extrinsic (through death receptor signaling) apoptotic pathways have been implicated. Kuwano and colleagues (2000) have shown that BLM activates the Fas-Fas ligand receptor signaling pathway, leading to excessive apoptosis of lung epithelial cells. This BLM-induced apoptosis, lung inflammation, and fibrosis can be significantly attenuated by treatment with a caspase inhibitor (Kuwano et al., 2001). In a previous study, it was demonstrated that BLM induced intracellular oxidation in AM, and this action was accompanied by a rise in intracellular calcium levels (Bhat et al., 1994). Both reactive oxygen species and intracellular calcium are known to alter mitochondrial functions (Green & Reed, 1998), suggesting that BLM may produce apoptosis through a mitochondrial pathway.

Studies have shown that AM from BLM-exposed rats exhibit increased production of tumor necrosis factor α (TNF-α), suggesting that this cytokine may play an important role in BLM-induced pulmonary injury (Piguet et al., 1989; Ma et al., 1999). TNF-α plays an important role in a wide range of cellular effects, including apoptosis, chemotaxis, cell proliferation and differentiation, and inflammation (Vassalli, 1992). The biological activities of TNF-α are mediated through the TNF receptors TNF-R1 (p55) and TNF-R2 (p75). These two receptors have a similar extracellular domain but a completely unrelated intracellular domain, indicating that TNF-R1 and TNF-R2 may initiate distinct signal transduction pathways by interacting with different signaling proteins (Ledgerwood et al., 1999; Gupta, 2001). TNF-R1 is the main receptor mediating TNF-induced inflammatory responses and apoptosis through its death domain in the intracellular region (Ashkenazi & Dixit, 1998). Unlike TNF-R1, TNF-R2 does not possess a cytoplasmic death signaling domain, and the role of TNF-R2 in cell death remains unclear. Studies have suggested that TNF-R2 may regulate

the rate of TNF association with TNF-R1 in the ligand-passing model to enhance the TNF-R1 death signal (Tartaglia et al., 1993; Ashkenazi & Dixit, 1998; Chan & Lenardo, 2000) or could mediate cell death independently when overexpressed (Haridas et al., 1998; Declercq et al., 1998). Although the intracellular domains for TNF-R1 and TNF-R2 are unrelated, these two receptors share some activities, such as the induction of apoptosis and activation of NF-κB (Gupta, 2001; Ledgerwood et al., 1999). The intracellular domain of TNF-R2, when activated through receptor stimulation, is capable of binding with molecules known as TNF-R-associated factors (TRAF 1 and 2) followed by the recruitment of cellular inhibitors of apoptosis (c-IAP) 1 and 2 (Rothe et al., 1995). Both TRAF2 and c-IAP1 are known to play important roles in TNF-R1- and TNF-R2-mediated apoptosis and cell survival programs (Li et al., 2002; Shu et al., 1996). Activation of TNF-R1 on one hand induces apoptosis through a death domain activated caspase cascade and, on the other hand, inhibits apoptosis by activating TRAF2-mediated induction of NF-κB (Ashkenazi & Dixit, 1998; Beg & Baltimore, 1996). Occupancy of TNF-R2 may induce c-IAP1 and c-IAP1-mediated degradation of TRAF2, thus enhancing apoptosis (Li et al., 2002).

Increased production of TNF-α by AM upon BLM stimulation (Ma et al., 1999) may play a major role in the development of apoptotic injury and pulmonary fibrosis (Ortiz et al., 1999). Using double TNF receptor knockout mice, Ortiz and colleagues (1998a) showed that the BLM-induced increase in TNF production preceded AM apoptosis and correlated with the pathogenesis of BLM-induced lung injury. Studies have also demonstrated that BLM exposure upregulates TNF-R2 but not TNF-R1 mRNA in the mouse lung (Ortiz et al., 1999). The objective of the present study was to (1) characterize the major apoptotic pathways in AM associated with BLM exposure, (2) determine the time-dependent induction of TNF-R1 and TNF-R2 by BLM, and (3) elucidate the role of TNF-R2 in modulating BLM-mediated apoptosis. These studies should provide more insight into the role of apoptosis in the development of inflammatory lung injury and pulmonary fibrosis.

MATERIALS AND METHODS

Chemicals and Reagents

BLM sulfate was obtained from Sigma Chemical Company (St. Louis, MO). Caspase-3 fluorogenic substrate, Ac-DEVD-AMC, was obtained from BD Pharmingen (San Diego, CA). Cell death detection enzyme-linked immunosorbent assay (ELISA) kits were from Roche Diagnostic Corporation (Indianapolis, IN).

The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): caspase-8 p20 (H-134), TNF-R1 (H-271), TNF-R2 (D-2), c-IAP1 (H-83), and TRAF2 (C-20). Anti-caspase-3 and anti-cytochrome *c* antibodies were purchased from Biosource International (Camarillo, CA). Antibodies for caspase-9 and poly(ADP-ribose) polymerase (PARP) were from Cell Signaling Technology, Inc. (Beverly, MA).

Animal Exposure

Specific-pathogen-free male Sprague-Dawley rats [Hla:(SD)CVF] from Hilltop Lab Animals (Scottdale, PA), weighing approximately 200 g, were used for all experiments. Rats were kept in facilities that are AAALAC-accredited, specific pathogen free, and environmentally controlled. Rats were provided HEPA-filtered air and tap water ad libitum, under a controlled light cycle (12-h light/dark cycle) and temperature (22–24°C) and were acclimated for 1 wk before use. Rats were anesthetized with methohexital sodium (35 mg/kg body weight, ip, Jones Pharma, St. Louis, MO) and then placed on an inclined restraint board. BLM (1 mg/kg body weight in 0.25 ml sterile saline) was intratracheally instilled with a curved ball-tipped cannula (18 gauge). Control animals received the same amount of vehicle (sterile saline).

Isolation of Alveolar Macrophages

At 1, 3, and 7 d post BLM exposure, animals were anesthetized with sodium pentobarbital (Fort Dodge Animal Health, Fort Dodge, IA) at 0.2 g/kg body weight and exsanguinated by cutting the renal artery. AMs were obtained by bronchoalveolar lavage (BAL) with Ca^{2+}/Mg^{2+}-free phosphate-buffered solution (PBS: 145 m*M* NaCl, 5 m*M* KCl, 1.9 m*M* NaH_2PO_4, 9.35 m*M* Na_2HPO_4, and 5.5 m*M* glucose; pH 7.4). A total of 80 ml BAL fluid was collected from each rat and centrifuged at 500×g for 10 min at 4°C. Cell pellets were combined and resuspended in 1 ml PBS. The number of AM was determined according to their unique cell diameters using an electronic cell counter equipped with a cell-sizing unit (Coulter Electronics, Hialeah, FL). Aliquots of cell suspensions containing 5×10^6 AM were added to each well of 6-well tissue culture plates containing Eagle's minimum essential medium (EMEM) supplemented with 10% heat inactivated fetal calf serum, 1 m*M* L-glutamine, 100 U/ml penicillin–streptomycin, and 10 m*M* HEPES buffer. The cells were incubated in a humidified incubator (37°C and 5% CO_2) for 2 h to allow AM attachment to the plastic plates. The nonadherent BAL cells were removed by rinsing the plates with PBS. AM-enriched cells were detached by scraping and centrifuged at 500×g for 10 min at 4°C, and the cell pellets were collected and counted for further analysis.

Apoptosis Assay

Apoptosis in AMs was determined by measuring the levels of cytosolic histone-bound DNA fragments using a cell death ELISA kit (Roche Diagnostics Corp). The assays were carried out according to the protocol provided by the manufacturer. Briefly, 1×10^5 AM were lysed with 200 µl of lysis buffer at room temperature for 30 min, centrifuged at 500×g for 10 min, and 20 µl of the lysed supernatant (cytoplasmic fraction) was added to the streptavidin-coated microplate for analysis. Appropriate amounts of anti-histone-biotin and anti-DNA-peroxidase were added to the sample wells, incubated at room temperature for 2 h, and then washed 3 times with washing buffer. The substrate ABTS, supplied in the kit, was added to each well, incubated at room temperature

for 10–20 min, and the reaction was stopped. Apoptosis was determined by measuring the optical density at 405 nm using a microplate spectrophotometer reader (Spectra Max 250; Molecular Devices Co., Sunnyvale, CA).

To determine the role of TNF-R2 in BLM-induced apoptosis, naive AM (1×10^5) were incubated with TNF-R2 antibody at various concentrations (1:10, 1:50, and 1:100) in EMEM supplemented with 10% heat inactivated fetal calf serum for 1.5 h (37°C, 5% CO_2). BLM was added to the cells at a final concentration of 35 μg/ml and incubated for an additional 24 h. Apoptosis in AM was determined using a cell death ELISA kit.

Cell Extracts and Western Blotting

Whole-cell extracts, cytosol, mitochondria, and the nuclear fraction of AMs were prepared as described previously, with minor modification (Rocha et al., 2000; Yang et al., 1997). Briefly, the AM pellets collected after attachment were resuspended in 5 volumes of lysis buffer (20 m*M* HEPES-KOH, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* sodium EDTA, 1 m*M* sodium EGTA, 1 m*M* dithiothreitol, and 0.1 m*M* phenylmethylsulfonyl fluoride, 250 m*M* sucrose, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 5 μg/ml pepstain A). After placing on ice for 15 min, the cell suspensions were homogenized with 15 strokes of a Teflon homogenizer. Aliquots of cell homogenates were saved as whole-cell extracts and used for TNF-R1 and TNF-R2 assays. To obtain nuclei, the cell homogenates were centrifuged at 1000×g for 10 min at 4°C. The resulting nuclear pellets were resuspended in lysis buffer and used for PARP cleavage assays. The supernatants were centrifuged at 10,000×g for 15 min at 4°C, and the resulting mitochondrial pellets were resuspended in lysis buffer and used for cytochrome *c* assay. The supernatants of the 10,000×g spin were further centrifuged at 100,000×g for 1 h at 4°C, and the resulting supernatants, that is, the cytosolic fraction, were used for cytochrome *c*, caspase-3, caspase-8, caspase-9, TRAF2, and c-IAP1 assays. The protein concentration in each fraction was determined using a BCA protein assay kit with bovine serum albumin as the standard (Pierce, Rockford, IL).

Equal amounts of protein were subjected to electrophoresis in 4–20% Tris-glycine precast gels (Invitrogen Corp., Carlsbad, CA) in a mini-gel apparatus. Resolved proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience, Inc., Keene, NH). The membrane was blocked with 5% nonfat dry milk in 20 m*M* Tris-HCl buffer (pH 7.5) containing 0.15 *M* NaCl-buffered saline with 0.1% Tween 20 (TBS-T) at room temperature for 1 h, then washed with TBS-T 3 times (5 min each). The membrane was incubated with appropriate first antibody (at 1:500–1:1000 dilution) in the blocking buffer overnight at 4°C, then washed 3 times with TBS-T. The membrane was subsequently incubated with a peroxidase-linked second antibody at a 1:1000 dilution in blocking buffer for 1 h at room temperature followed by 3 TBS-T washes. The antigen-antibody complex was detected on Fuji medical x-ray film using enhanced chemiluminescence (ECL) reagents (Amersham Biosciences, Piscataway, NJ).

Caspase-3 Activity Assay

Caspase-3 activity was assayed with a cell-permeable synthetic substrate, Ac-DEVD-AMC. AM were lysed in cell lysis buffer (10 m*M* Tris-HCl, pH 7.5, 10 m*M* NaH_2PO_4, 130 m*M* NaCl, 1% Triton X-100, 10 m*M* sodium pyrophosphate). Ac-DEVD-AMC (20 μ*M*) was added to the cell lysate and the reaction mixture was incubated at 37 °C for 1 h in the dark. Caspase-3 activity was determined by measuring the AMC liberated from the substrate spectrofluorometrically using a CytoFluor multiwell plate reader series 4000 (PerSeptive Biosystem, Framingham, MA) with excitation at 380 nm and emission at 440 nm.

Statistical Analysis

Data are presented as mean ± standard error (SE) from at least three separate experiments. Statistical analyses were carried out with the GB-Stat (Dynamic Microsystems, Inc.). The time-dependent responses of AM after BLM exposure were analyzed using two-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc test. The in vitro effects of TNF-R2 antibody on BLM-exposed AM were analyzed using one-way ANOVA followed by Dunnett's test. The significance was set at $p < .05$.

RESULTS

Time-Dependent Induction of Apoptosis and Caspase-3 Activity by BLM

An earlier study using a BLM-sensitive murine model showed that apoptosis occurred in AM during lung injury induced by intravenously injected BLM (Ortiz et al., 1998b). One objective of the current study was to examine the initiation and duration of AM apoptosis in rats following the direct delivery of BLM to the lung by intratracheal instillation. The time-course studies reveal that BLM exposure induced an increase in the percentage of apoptotic AM (Figure 1), with highest occurrence of apoptotic cells at 1 d after BLM treatment. At 3 and 7 days post BLM exposure, the number of apoptotic AM decreased when compared to that of day 1, but was still higher than that of the controls.

Figure 2A shows the kinetics of caspase-3 induction in AM from rats exposed to BLM or saline for 1 to 7 days. The results show that caspase-3 activity was elevated in AM from all BLM-treated rats. The peak elevation occurred on d 3, which was later than the observed maximal apoptotic effect of BLM at 1 d postexposure as shown in Figure 1. Figure 2B shows that the active form of caspase-3 (17 kDa) appeared at 1 d after BLM-treatment and reached a maximum at 3 d after exposure. This increase in caspase-3 was accompanied by a decrease in its precursor (32 kDa), which was also maximal at 3 d. Concurrently, there was a significant increase in nuclear poly(ADP-ribose) polymerase (PARP) degradation, demonstrated as a 89 kDa fragment, at

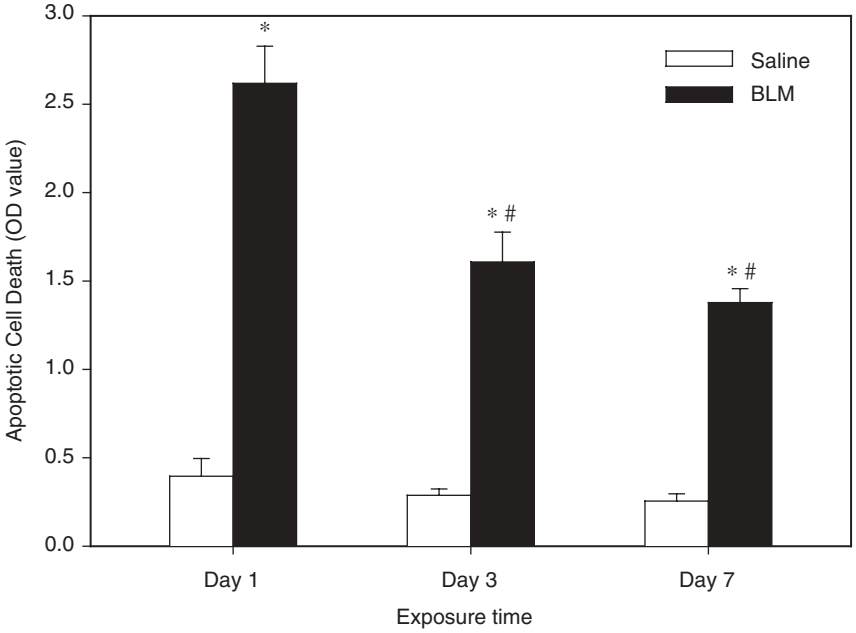

FIGURE 1. Time-dependent BLM exposure-induced apoptosis in AM. Rats were intratracheally instilled with 1 mg/kg body weight of BLM or saline as control. AM were isolated by bronchoalveolar lavage at 1, 3, and 7 d postexposure. Apoptosis in AMs was determined by measuring the levels of cytosolic histone-bound DNA fragments using an ELISA kit. Data are presented as means ± SE ($n = 5–6$). Asterisk indicates significantly different from saline control at $p < .05$; #, significantly different from BLM at 1 d postexposure at $p < .05$.

all time points post BLM exposure, which also appeared to be maximal at 3 d. These results show that the time course of the appearance of the PARP fragmentation corresponds to the activation of caspase 3 in AM after BLM exposure, suggesting a strong correlation between BLM-induced caspase 3 activation and PARP degradation.

BLM-Induced Cytochrome *c* Release and Caspase-9 Activation

To characterize the mechanisms involved in BLM-induced caspase-3 activation, cellular expression of caspase-8, caspase-9, and the release of cytochrome *c* from mitochondria in AM were monitored at 1, 3, and 7 d post BLM exposure. Figure 3 shows that the expression of caspase-8 in BLM-exposed AM was not different from that in control AM at all exposure times. In contrast, the procaspase-9 (51 kDa) was significantly reduced after BLM exposure, whereas the active form of caspase-9 (38 kDa) was markedly elevated at 1 d, peaked at 3 d after BLM-exposure, and had returned to baseline by d 7. Figure 3 further shows that at 1 d after BLM treatment there was a maximal increase in the cytosolic cytochrome *c* level when compared to the saline control, and this was accompanied by a corresponding decrease of the level in mitochondria.

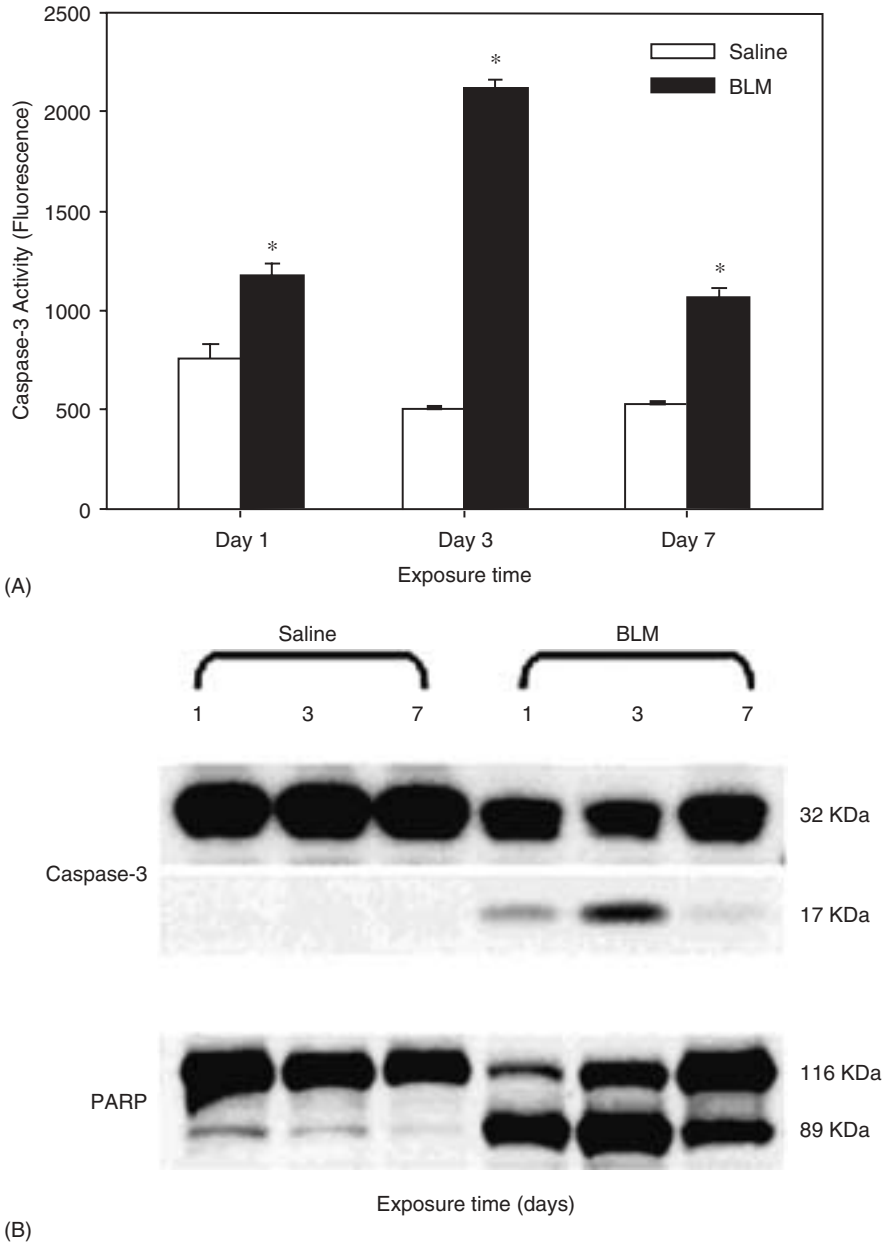

FIGURE 2. Time course of caspase-3 activation and PARP cleavage in AM. AM were isolated from BLM- or saline-exposed rats by bronchoalveolar lavage at 1, 3, and 7 d postexposure. (A) Caspase-3 activity was assayed by monitoring the fluorescence intensity of AMC liberated from the substrate, Ac-DEVD-AMC, spectrofluorometrically at an excitation of 380 nm and an emission of 440 nm. Data are presented as means ± SE from three experiments. (B) Western blot analysis of caspase-3 and PARP protein levels. The blots are representative of three separate experiments. Asterisk indicates significantly different from the control, $p < .05$.

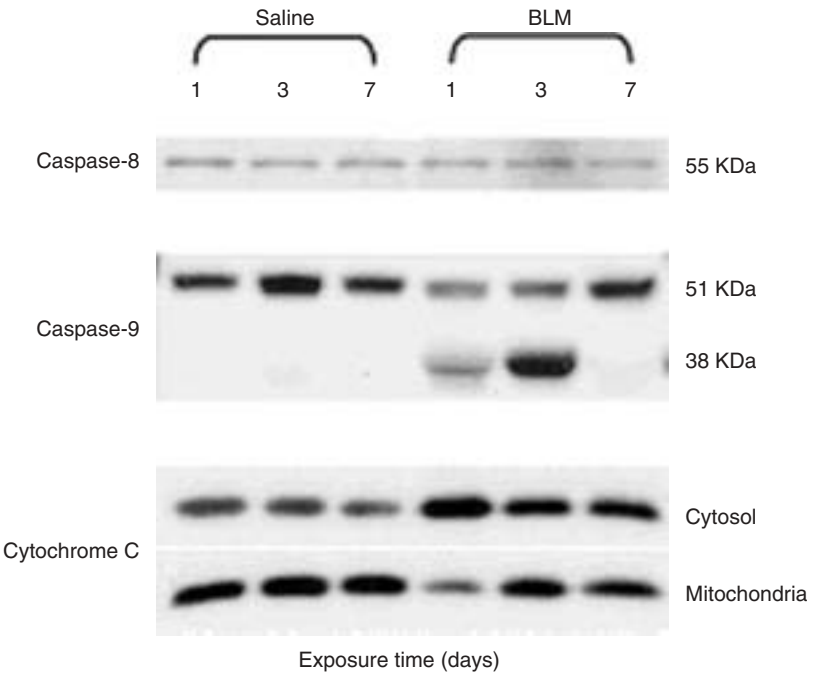

FIGURE 3. BLM-induced cytochrome *c* release and caspase-8 and caspase-9 activation in AM. Mitochondrial and cytosolic extracts were isolated from AM at different time points after BLM (1 mg/kg body weight) or saline exposure by differential centrifugation. Western blot analysis of the protein levels of cytochrome *c* and caspase-8 and -9 was conducted using appropriate antibodies. These blots are representative of three separate experiments.

The peak release of cytochrome c after BLM-exposure was decreased in AM by 3 and 7 d. These results suggest that BLM-induced AM apoptosis may involve mitochondrial release of cytochrome *c* and caspase-9 activation, but not caspase-8.

Role of TNF Receptors in BLM-Induced Apoptosis in AM

Figure 4 shows that BLM exposure did not change AM expression of TNF-R1 but markedly increased the TNF-R2 level in a time-dependent manner. BLM-induced TNF-R2 increased on d 1 and continued to increase even at 7 d after exposure, suggesting that the potential apoptotic effect mediated by TNF-R2 may have long term consequences. The absence of TNF-R1 induction coupled with the absence of caspase-8 activation (as shown in Figure 3) suggests that BLM does not induce AM apoptosis through a TNF-R1-dependent pathway.

The potential involvement of TNF-R2 in BLM-induced apoptosis was further evaluated by in vitro studies using normal rat AM challenged with BLM in the absence or presence of TNF-R2 antibody. Figure 5 shows that the pretreatment

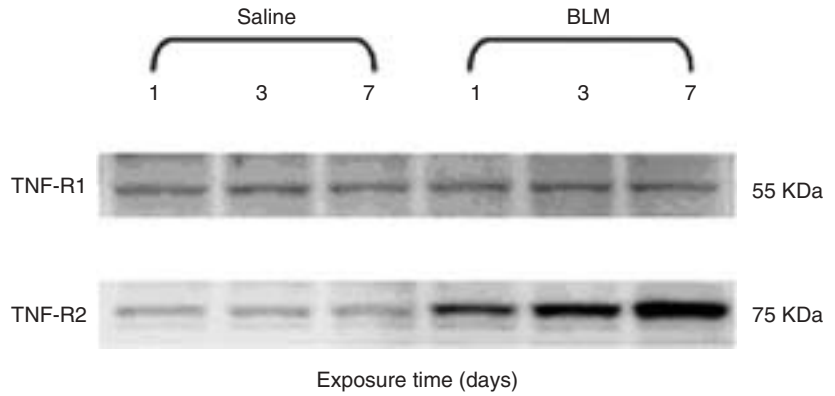

FIGURE 4. Effects of BLM on the induction of TNF-R1and TNF-R2 in AM. Expression of TNF-R1 and TNF-R2 in AM isolated from saline- or BLM-treated rats were characterized by Western blot analysis. Results are representative of three independent experiments.

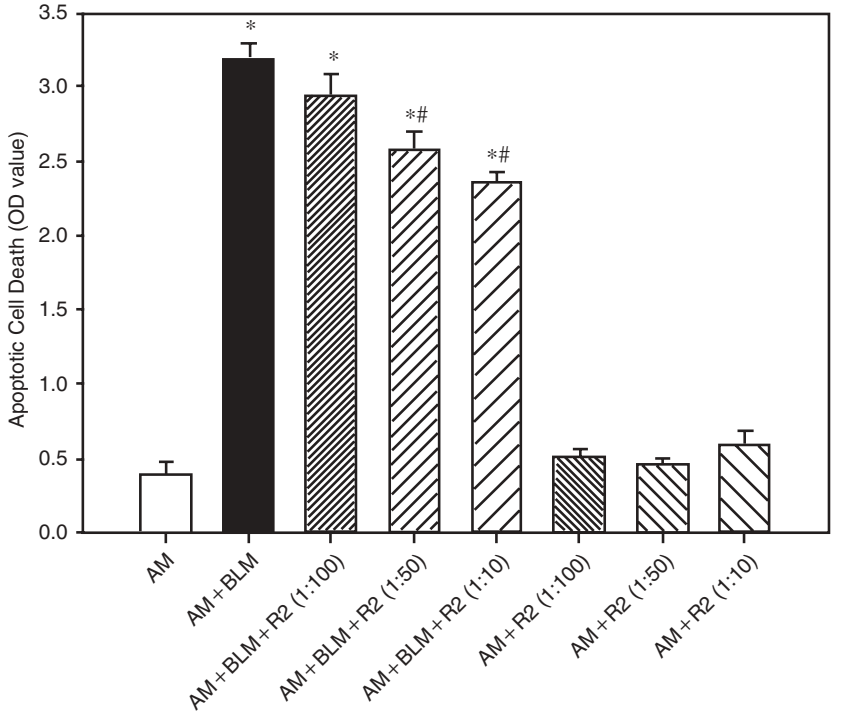

FIGURE 5. The role of TNF-R2 in BLM-induced AM apoptosis. AM (1×10^5) were isolated from normal rats and incubated in the absence or presence of various concentration of TNF-R2 antibody (at 1:100, 1:50, and 1:10 dilutions) in EMEM at 37°C, 5% CO_2, for 1.5 h, then exposed to BLM (35 μg/ml, final concentration) for a total of 24 h. AM apoptosis was determined using a cell death ELISA kit. Data represent means ± SE of three experiments. Asterisk indicates significantly different from the AM control at $p < .05$; #, significantly different from the AM + BLM group at $p < .05$.

of AM with TNF-R2 antibody significantly inhibited BLM-induced AM apoptosis in a concentration-dependent manner, while having no effect on the basal rate of apoptosis. These results suggest that TNF-R2 contributes to BLM-induced apoptotic cell death. Pretreatment of AM with the TNF-R2 antibody was also shown to inhibit BLM-induced caspase-3 activity without affecting control AM caspase-3 activity (Figure 6). These results clearly demonstrate that BLM-induced apoptosis in AM involves the induction of TNF-R2 and the activation of caspase-3.

Studies have suggested that TNF-R2 may influence TNF-α-mediated apoptosis by modulating TRAF2 through nuclear factor (NF)-κB activation (Duckett & Thompson, 1997). The expression of TRAF2 and that of c-IAP1 were examined in AM. Figure 7 shows that both TRAF2 and c-IAP1 were altered following in vivo BLM exposure. There was a significant increase of c-IAP1 level in AM from rats exposed to BLM for 1, 3, and 7 d. The increase in c-IAP1 was accompanied by a decrease in TRAF2 expression in AM.

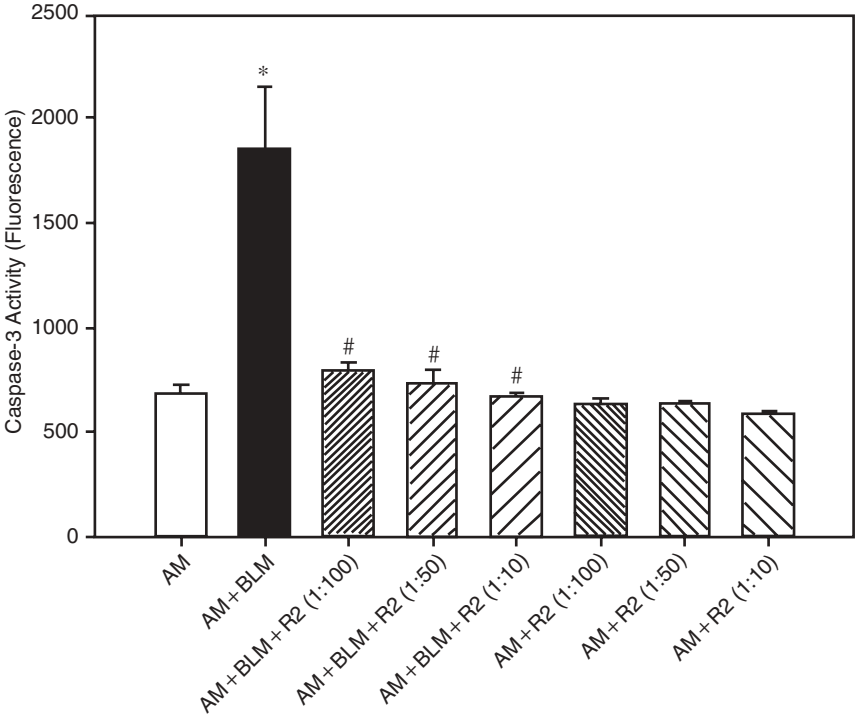

FIGURE 6. Role of TNF-R2 in BLM-induced caspase-3 activity. AM, obtained from normal rats, were pretreated with TNF-R2 antibody for 1.5 h followed by BLM (35 μg/ml) treatment for a total of 24 h. Caspase-3 activity was determined by measuring the liberation of AMC from Ac-DEVD-AMC, a caspase-3 substrate. Data represent means ± SE from four separate experiments. Asterisk indicates significant difference from AM ($p < .05$); #, significant difference from AM + BLM group ($p < .05$).

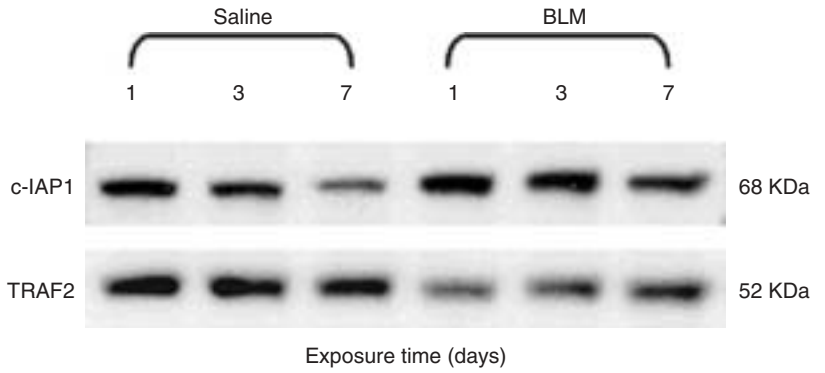

FIGURE 7. Effects of BLM on c-IAP1 and TRAF2 levels in AM. The expression of TRAF2 and c-IAP1 in AM, isolated from saline- or BLM-exposed rats at 1, 3, or 7 d postexposure, was determined using Western blot analysis. Results are representative of three independent experiments.

DISCUSSION

The inflammatory response to intratracheally instilled BLM in rats, including neutrophil infiltration and macrophage secretion of proinflammatory cytokines, has been reported in a previous study carried out in this laboratory (Ma et al., 1999). Another in vitro study showed that BLM induces a substantial rise in intracellular calcium and a significant generation of intracellular reactive oxygen species in rat AM (Bhat et al., 1994). These studies suggest that intracellular oxidative events in AM may play an important role in BLM-mediated inflammatory lung injury. Several studies have shown that apoptosis in AM is one of the earliest pulmonary alterations produced by BLM exposure (Hagimoto et al., 1997; Davis et al., 2000). The objective of the present study was to characterize the involvement of cellular factors, such as mitochondrial perturbation, caspase activation, and alteration of cellular expression of TNF receptors and effecter proteins, in BLM-induced apoptosis of AM. The results of the present study demonstrate that BLM produces AM apoptosis, which is associated in a complex fashion with time-dependent changes in several apoptotic signaling pathways.

The highest number of apoptotic AM appeared one day after BLM exposure and preceded the peak activation of caspase-3 activity on d 3. This suggests that the initial apoptosis may occur, in part, through a caspase-independent pathway. Studies by Huisman and colleagues (2002) have shown that paclitaxel, an anticancer agent, triggered cell death mainly by a caspase-independent mechanism in which the basic apoptotic pathway is merely coactivated. Although currently the details of the caspase-independent mechanism remains unclear, the initial BLM-induced apoptosis may be partially mediated through this mechanism.

The role of mitochondria in the apoptotic process has been the center of the debate among various studies. Caspase-3 processing and PARP cleavage

are distal events that follow the activation of selective upstream caspases such as caspase-8 and caspase-9 (Kuida, 2000). It has been suggested that activation of procaspase-9 is mitochondrial specific and cytochrome *c* dependent (Yang et al., 1997). This cytochrome *c*-dependent activation of caspase-9 is one of the pathways that can lead to caspase-3 activation and PARP degradation resulting in apoptosis (Hengartner, 2000; Wolf et al., 1999; Zimmermann et al., 2001). The present study suggests that the BLM-induced apoptosis involved the activation of caspases through the mitochondrial pathway. The release of cytochrome *c* from mitochondria peaked on d 1, which preceded the maximum induction of caspase-9 and caspase-3 protein and caspase-3 activity that occurred at 3 d postexposure. At 7 d postexposure, both the activation of caspase-9 and caspase-3 and the degradation of PARP were considerably less than levels observed at 3 d after exposure. These results suggest that the mitochondrial pathway leading to caspase-3 activation is one of the mechanisms leading to apoptotic AM in BLM-exposed rats. However, recent studies have reported a cytochrome *c*-independent activation of caspase-9 in apoptosis (Morishima et al., 2002). In addition, studies have also pointed out that although cytochrome *c* release appears to be a universal feature of apoptosis, it is, in some cases, a rather late event. For example, apoptosis induced by death receptors often bypasses the mitochondrial pathway, making cytochrome *c* release a likely result of caspase activation, rather than its cause (Hengartner, 2000). The present study, however, suggests that mitochondria play a prominent role in BLM-mediated apoptosis, even though the mitochondrial pathway does not fully account for the initial apoptotic response. BLM-induced apoptosis appears to be mediated through caspase-9 but not caspase-8, and was preceded by the mitochondrial release of cytochrome *c*. The absence of caspase-8 activation also suggests that death receptor-mediated pathways, such as by TNF-R1, do not play a major role in the initiation of apoptotic response by AM to BLM exposure.

The production of TNF-α by AM in response to BLM exposure has been linked to BLM-induced apoptosis and the development of pulmonary fibrosis (Ortiz et al., 1998b, 1999), but a direct role of TNF-α has not been demonstrated. The biological activities of TNF-α are known to be mediated through two receptors, TNF-R1 and TNF-R2 (Gupta, 2001). TNF-R1 is the primary signaling receptor, whereas TNF-R2 either enhances the TNF-R1 death signaling or mediates cell death independently when overly expressed (Ashkenazi & Dixit, 1998; Chan & Lenardo, 2000; Declercq et al., 1998; Haridas et al., 1998; Ledgerwood et al., 1999). Data showed that AM from BLM-exposed rats did not exhibit enhanced expression of TNF-R1 but instead showed a time-dependent increase in TNF-R2. The strong TNF-R2 expression observed at 7 d post BLM exposure suggests that elevated expression of TNF-R2 may occur even after longer exposure. Collectively, these results suggest that the induction of cell death by TNF-α through the TNF-R1 pathway probably does not play a major role in BLM-induced apoptosis, but the enhanced expression of TNF-R2 may increase binding of TNF-α for a prolonged modulation of AM

activity. Indeed, Ortiz and coworkers (1999) have shown that BLM exposure upregulated TNF-R2 but not TNF-R1 mRNA levels in mice, which is consistent with our observation in rats. A contentious point is whether TNF-R2 contributes to BLM-induced apoptosis. Studies by Jupp and colleagues (2001) have shown that stimuli that specifically activate TNF-R2 have the ability to stimulate cell death. Haridas and coworkers (1998) also demonstrated that TNF-R2 mediated TNF-induced cell killing, suggesting that TNF-R2 may indeed play a role in BLM-induced apoptosis. Indeed, using in vitro experiments, data demonstrated that pretreatment of AM with the TNF-R2 antibody inhibited BLM-induced caspase-3 activity with concomitant attenuation of BLM-induced AM apoptosis.

Several studies have shown that TNF-R2 may influence apoptosis by modulating the cytoplasmic levels of TRAF2 and c-IAP1. Arch et al. (1998) proposed that cytosolic TRAF2, by activating NF-κB, may prevent the formation of apoptotic signaling complexes induced by TNF-α and thereby inhibit apoptosis. Thus, a depletion of soluble TRAF2 from the cytoplasmic pool may sensitize cells to TNF-α-induced apoptosis through the TNF-R1-mediated pathway. Studies by Chan and Lenardo (2000) showed that stimulation of TNF-R2, but not TNF-R1, resulted in a reduction of the cytoplasmic TRAF2 level. Arch et al. (2000) also showed that full-length TNF-R2 induced the redistribution of TRAF2 from the soluble cytoplasmic fraction to a detergent-insoluble fraction, that is, a depletion of cytoplasmic TRAF2. c-IAP1, on the other hand, is a protein that is highly induced by TNF-α (Imanishi et al., 2003) and promotes proteasome-mediated degradation of TRAF2 (Li et al., 2002). Together, the expression of TNF-R2 and that of c-IAP1 reduce the cytoplasmic TRAF2 protein level. Our studies showed that the BLM-induced expression of TNF-R2 in vivo was indeed accompanied by a decrease in cytosolic TRAF2 and an increase in c-IAP1 protein levels, suggesting that AM from BLM-exposed rats may be sensitized for TNF-α-mediated apoptotic responses.

In summary, the current study shows that BLM induces a complex pattern of time-dependent changes in AM related to the apoptotic process. Translocation of cytochrome *c* from mitochondria to cytosol is an early event peaking 1 d after BLM exposure. However, peak activation of caspase-9 and caspase-3 and PARP degradation did not occur until 3 d postexposure. Since BLM-induced apoptosis of AM was maximal at 1 d postexposure, it is possible that a caspase-independent mechanism, perhaps involving direct drug toxicity, could be involved in this early response. TNF-R1 does not seem to play a major role in BLM-induced AM apoptosis, since BLM exposure had no marked effect on the expression of TNF-R1 and the activation of caspase-8. However, BLM did increase TNF-R2 levels, which were associated with a time-dependent decrease in TRAF2 and a rise in c-IAP1. These events were maximal at 7 d postexposure and may sensitize AM to TNF-α-mediated apoptosis. A role for TNF-R2 in BLM-induced apoptosis of AM is supported by the inhibitory effect of TNF-R2 antibodies on BLM-induced apoptosis and caspase-3 activity in vitro.

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