Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor

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The same laboratory (5) later showed that intratracheal administration of Fas-activating antibodies caused alveolar epithelial apoptosis and pulmonary fibrosis in mice.

The angiotensin-converting enzyme (ACE) inhibitor captopril is known to inhibit experimental lung fibrosis induced by the plant alkaloid monocrotaline (12) or by gamma irradiation (26). Recently, Uhal et al. (21) found that captopril is also a potent inhibitor of Fas-induced apoptosis in human or rat AECs in vitro (21).

On this basis, we hypothesized that captopril might inhibit experimental lung fibrogenesis in part through its ability to abrogate apoptosis of lung epithelial cells. We further hypothesized that a synthetic inhibitor of lung epithelial cell apoptosis with no capacity to inhibit ACE also would attenuate lung fibrogenesis as potently as captopril.

Experimental lung fibrosis induced by Bleo is a well-studied model of fibrogenesis supported by ample literature (6, 20, 31). Although the ability of Bleo to induce apoptosis in alveolar macrophages has been documented (7), little is known about the direct effect of Bleo on apoptosis or its regulation in lung epithelial cells. We report here that exposure of isolated rat AECs to Bleo induces apoptosis that can be inhibited with either the ACE inhibitor captopril or the caspase inhibitor N-benzylcarboxy-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk). We also demonstrate that the administration of either agent to rats exposed to Bleo both attenuates apoptosis of alveolar and airway wall cells and prevents the subsequent accumulation of lung collagens.

METHODS

Reagents and materials. Bleo (bleomycin sulfate) and captopril were obtained from Sigma (St. Louis, MO). The peptide inhibitor of the interleukin-1β-converting enzyme family of caspases, ZVAD-fmk, was obtained from Kamiya Biomedical (Seattle, WA). The peptide caspase inhibitors Asp-Glu-Val-Asp-fluoromethylketone (DEVD-fmk) and Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk) were obtained from PharMingen (San Diego, CA). Alkaline phosphatase...
(AP)-conjugated streptavidin, digoxigenin (Dig)-labeled dUTP, and biotin dUTP were obtained from Boehringer Mannheim (Indianapolis, IN). Reagents for detection of alkaline phosphatase and other secondary reagents for in situ end labeling (ISEL) of DNA were obtained from sources described earlier (22).

Animals and induction of pulmonary fibrosis. Adult male Wistar rats, 175–200 g, were housed in the Department of Laboratory Animal Medicine, Michael Reese Hospital (Chicago, IL). Forty animals were divided into four groups; three groups received a single intratracheal instillation of 8 U/kg of Bleo in sterile saline. Two days before the administration of Bleo, one group received 500 mg/l of captopril in the drinking water. Another group received daily intraperitoneal injections of 1 mg/kg of ZVAD-fmk in 10% DMSO-PBS. Control animals received administration of the vehicles only. Captopril and ZVAD-fmk administration were continued for 14 days after instillation of Bleo, at which point all animals were killed for histology, detection of collagen, and DNA fragmentation in epithelial cells.

Fig. 1. Inhibition of Fas-induced apoptosis of primary rat alveolar epithelial cells (AECs) by captopril (CAPTO) or caspase inhibitors in vitro. Primary AECs isolated from adult Wistar rats were exposed to recombinant human Fas ligand (50 ng/ml) for 20 h in the presence and absence of captopril (500 ng/ml) or the indicated caspase inhibitors, all at 60 μM. Apoptosis was detected by quantitation of fragmented nuclei (see METHODS). ZVAD-fmk, N-benzylocarboxy-Val-Ala-Asp-fluoromethylketone, a caspase inhibitor with selectivity toward caspases 1 and 4; DEVD-fmk, Asp-Glu-Val-Asp-fluoromethylketone, selective for caspase 3; YVAD-cmk, Tyr-Val-Ala-Asp-chloromethylketone, an inhibitor selective for caspase 1. **P < 0.01 vs. control by ANOVA and Student-Newman-Keuls test.

Fig. 2. Bleomycin (Bleo)-induced apoptosis of AECs in vitro and pharmacological inhibition. A: primary AECs isolated from adult Wistar rats were exposed to Bleo at the indicated concentrations for 20 h. B: primary AECs were exposed to Bleo (50 mU) for a total of 20 h beginning after a 1-h pretreatment with CAPTO (+CAP; 500 ng/ml), ZVAD-fmk (+ZVA; 60 μM), or aurintricarboxylic acid (+ATA; 10 μM). CTL, control. Apoptosis was detected as in Fig. 1. **P < 0.01 vs. control. *P < 0.01 vs. Bleo 50 (both by ANOVA and Student-Newman-Keuls test).
Immediately before death, the animals were given intraperitoneal injections of pentobarbital sodium, and the trachea was cannulated. The middle right lobe was ligated at the hilus, excised distal to the ligation, and immediately frozen in liquid N₂ for hydroxyproline assay of total collagen (see Identification of collagen). The remaining lung tissues were carefully removed and instilled with 4% paraformaldehyde in PBS at 20 cmH₂O constant pressure, then immersed in the same fixative for 2 h. The fixed tissues were washed with PBS three times for 15 min each and then embedded in paraffin. Five-micrometer sections of lung were deparaffinized by passing them through xylene, 1:1 xylene-alcohol, 100% alcohol, and 70% alcohol for 10 min each. Ethanol was removing by rinsing with distilled water.

ISEL of fragmented DNA. ISEL of fragmented DNA was conducted with a modification of the method of Mundle et al. (13). Briefly, ethanol was removed from deparaffinized lung sections by rinsing in distilled water for at least 10 min. The slides were then placed in 3% hydrogen peroxide (Sigma) for 30 min at 20°C, rinsed with PBS, and incubated with proteinase K (Sigma) in saline-sodium citrate (0.3 M NaCl and 30 mM sodium citrate in water, pH 7.0) for 15 min at 37°C. The samples were rinsed once in water and three times in 0.15 M PBS for 4 min each and then incubated in salinesodium citrate at 80°C for 20 min. After four rinses in PBS and four rinses in buffer A (50 mM Tris·HCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, and 0.005% BSA in water, pH 7.5), the sections were incubated at 18°C for 2 h with ISEL solution (0.001 mM Dig-dUTP, 20 U/ml of DNA polymerase I, and 0.01 mM each dATP, dCTP, and dGTP in buffer A). Afterward, the sections were rinsed thoroughly five times in buffer A and three additional times in PBS. Detection of incorporated dUTP was achieved by incubation for 2 h at 37°C with AP-conjugated anti-Dig (Boehringer Mannheim) at a 1:400 dilution. Bound AP-antibody was then detected with the fast blue chromogen system, and the sections were mounted with Fluoromount solution (Southern Biotechnology, Birmingham, AL).

Identification of collagen. Collagens were localized through the staining of lung sections by the picrosirius red (PR) technique (15), which has absolute specificity for collagen. Briefly, sections deparaffinized with xylene were rehydrated through a descending series of ethanol to distilled water. The hydrated sections were immersed in 0.2% aqueous phosphomolybdic acid for 2 min, rinsed in distilled water, and then stained for 110 min with 0.1% sirius red F3BA (Pfaltz and Bauer, Stamford, CT) in saturated aqueous picric acid, pH 2.0. The sections were washed for 2 min in 0.01 N HCl, rinsed for 45 s in 70% ethanol, dehydrated in three changes of absolute alcohol, cleared in xylene, and mounted in a non-aqueous mounting medium. Fibrotic foci were detected by polarization microscopy under which collagen fibers appear yellow-white (22) or white in gray-scale images. Quantitation of collagens in alveolar septa and peribronchial parenchyma by digital imaging is described in detail below.

Fig. 3. Detection of apoptotic cells by in situ end labeling (ISEL) of fragmented DNA after intratracheal administration of Bleo. Male Wistar rats received Bleo (bleomycin sulfate; 8 U/kg) intratracheally; lung tissues were harvested 1, 7, and 14 days after Bleo and subjected to ISEL coupled to a fast blue detection system (see METHODS). Positive reaction is blue. A: at 1 day after Bleo, ISEL-positive nuclei were observed in cells within the alveolar walls (arrowheads) immediately adjacent to small airways or vessels. Some airway epithelial cells also were ISEL positive by 1 day after Bleo (arrows) but less frequently than at 8 h after Bleo (data not shown). Magnification, ×200. B: at higher magnification (×400), many ISEL-positive nuclei were observed in septal wall cells at the alveolar corners (arrows); in other areas, ISEL-positive nuclei were observed in squamous cells lining the alveolar septa (C, arrowheads). D: by 7 days after Bleo, ISEL of alveolar wall cells (arrows) and airway epithelial cells (arrowheads) were more prominent throughout the parenchyma. Magnification, ×200. E: daily intraperitoneal administration of ZVAD-fmk blocked airway and alveolar ISEL in response to Bleo (see METHODS and Fig. 5 for ISEL on day 14).
For quantitation of total lung collagen, tissues frozen in liquid N₂ (see above) were dried at 80°C to a constant weight in preweighed tubes. The weighed dry tissue was hydrolyzed in 6 N HCl and subjected to determination of hydroxyproline as described earlier by Woessner (29). The efficiency of the hydrolysis was verified with rat tail collagen by comparison to standard hydroxyproline (Sigma). The hydroxyproline analysis was applied to samples from a minimum of six animals per treatment group.

**Lung cell isolation and detection of apoptosis in vitro.** Primary AECs were isolated from adult male Wistar rats as described earlier (24). The primary cells were studied on day 2 of culture, a time at which they are type II cell-like by accepted morphological and biochemical criteria (14), and all preparations were of >90% purity as assessed by acridine orange staining as previously discussed (25). All cells were seeded in 24-well or 6-well chambers, and all experiments were conducted at subconfluent densities of 80–90% in serum-free Ham’s F-12 medium. Test reagents were diluted with Ham’s F-12 medium and applied for 20 h at 37°C in a 5% CO₂ incubator.

Detection of apoptotic cells with propidium iodide was conducted as described earlier (25) after digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 µg/ml of propidium iodide. Cells exhibiting nuclear fragmentation and chromatin condensation (see Fig. 1) were scored in four randomly selected fields per culture well in at least four culture vessels per experimental condition and are expressed as a percentage of the total cells within the same field (minimum of 100 cells/field).

**Microscopy and image analysis.** The prepared sections were photographed under transmitted or polarized light on an Olympus BH2 epifluorescence microscope fitted with automatic photographic equipment. Color photographic slides
null nuclei (see Fig. 4) were scored as a percentage of the total epithelial

null airway (B) epithelial cells after Bleo, CAPTO, and ZVAD-fmk admin-

null B

null the adjacent parenchyma detectible in a

null was determined as a percentage of the total pixel area.

null number of white pixels (specific for collagen, see Ref. 15) per

null was defined as the average PR pixel percentage in regions that included a large-airway wall and

null 1,000; SE of the pixel area.

null that captopril at 500 ng/ml and the active site-specific
caspase inhibitors ZVAD-fmk, DEVD-fmk and YVAD-cmk, all at 60 μM, had comparable efficacy to inhibit apoptosis induced by recombinant Fas ligand. However, direct comparisons of the ability of the three caspase inhibitors to attenuate Fas- or tumor necrosis factor-induced liver damage in vivo found that ZVAD-fmk was more potent than the other caspase inhibitors, presumably because it is more permeable to some cell membranes (11). For these reasons, we chose ZVAD-fmk, an inhibitor of the interleukin-1β-converting enzyme family of caspases, for use in the in vivo experiments described below.

null Related in vitro experiments revealed that Bleo also was capable of inducing apoptosis in primary AECs isolated from adult Wistar rats (Fig. 2A). With the use of an assay of nuclear fragmentation (25), induction of apoptosis was significant at 5 μM Bleo and was concentration dependent. At the concentration of 50 μM, Bleo-induced apoptosis of AECs in vitro was inhibited 76% by 500 ng/ml of captopril (Fig. 2B), consistent with an earlier demonstration by Uhal et al. (21) that captopril inhibits Fas-induced apoptosis of AECs. Furthermore, Bleo-induced apoptosis of AECs in vitro was reduced 78% by the caspase inhibitor ZVAD-fmk (Fig. 2B) and was inhibited 59% by the endonuclease inhibitor aurintricarboxylic acid (Fig. 2B).

null In the in vivo model of Bleo-induced fibrosis, the fragmented DNA of apoptotic cells was detected by ISEL of numerous alveolar and airway epithelial nuclei (Fig. 3). In agreement with the observations of Hagimoto et al. (6), ISEL was observed almost imme-
diately after Bleo administration (at 8 h; data not shown) and was diminished but still present in airway epithelia at 24 h post-Bleo (Fig. 3A, arrows). However, the same specimens revealed focal but heavy ISEL within alveolar wall cells (Fig. 3A, arrowheads), which were found primarily adjacent to respiratory bronchiolo-
es, alveolar ducts, and small vessels. Higher magnifi-
cation revealed that many of the ISEL-positive cells at 24 h were in the alveolar corners (Fig. 3B, arrows), whereas other ISEL-positive nuclei were found in squamous cells lining the alveolar septa (Fig. 3C, arrowheads). By 7 days after Bleo administration (Fig. 3D), ISEL labeling was found again to be heavy within the airway epithelium (arrowheads) and more extensive in alveolar wall cells (arrows) throughout the parenchyma. Concurrent treatment of the animals with daily intraperitoneal ZVAD-fmk eliminated the ISEL of both airway epithelial and alveolar wall cells on day 7 post-Bleo (Fig. 3E).

null Histology of whole lung sections obtained 14 days after intratracheal Bleo administration revealed extensive lesions, especially adjacent to large airways, in the Bleo-treated group (Fig. 4). The lesions contained many lymphocytes, collapsed alveolar spaces, and thickening of nearby alveolar septa. In both the captopril- and ZVAD-fmk-treated animals, lesions were present but were much less extensive and contained fewer inflammatory cells (Fig. 4, C and D).
Detection of fragmented DNA by ISEL on day 14 after Bleo administration found positive nuclei within both the AEC and airway epithelial cell populations throughout the parenchyma (Fig. 5, B and F, respectively). In the airways, ISEL-positive nuclei were often observed within “sheets” of epithelia that were detached from the underlying stroma (Fig. 5, F). In both captopril-treated (Fig. 5, C and G) and ZVAD-fmk-treated (Fig. 5, D and H) animals, ISEL-labeled epithelial cells were rare, even within small lesions. Quantitation of ISEL-positive nuclei in both cell populations (Fig. 6) confirmed the ability of captopril and ZVAD-fmk to reduce the frequency of epithelial DNA fragmentation. Furthermore, the architecture of both the alveolar and airway epithelia appeared to be significantly preserved by both captopril and ZVAD-fmk, even within the small lesions present in these animals (compare Fig. 5, G and H to F).

With the PR method (15), the distribution of collagen in the alveolar septa of Bleo-treated animals appeared thicker, even in regions distal to airways (Fig. 7B). Quantitation of the total number of white pixels by digital imaging found that both captopril-treated and ZVAD-fmk-treated animals accumulated less collagen per unit tissue in these areas (Fig. 8A). The inhibition was also observed in areas adjacent to the bronchi; control lungs had a well-defined collagen matrix surrounding the bronchi (Fig. 7E), but in Bleo-treated lungs, this was the area of most severe collagen accumulation (Fig. 7F). Captopril- and ZVAD-fmk-treated
lungs did accumulate collagen in these regions (Fig. 7, G and H, respectively), but the deposition was less severe and did not extend as far into the adjacent parenchyma. Scoring of PR staining by digital imaging in the peribronchial parenchyma confirmed this assessment (Fig. 8B). Quantitation of total lung collagens by hydroxyproline assay confirmed the results of the PR assay and showed that total lung collagen was significantly increased 14 days after Bleo administration but not at 24 h or 7 days post-Bleo (Fig. 9). The hydroxyproline assay also verified that the administration of captopril or ZVAD-fmk inhibited lung collagen accumulation at 14 days post-Bleo.

DISCUSSION

The concept that the integrity of the alveolar epithelium and its capacity to repair injury constitute a critical checkpoint in the pathways leading to lung fibrosis is supported by a variety of studies of both animal models (1, 2, 8) and human lung biopsies (10). This laboratory (23) and others (6, 18, 19) have suggested that inappropriate apoptosis in the alveolar epithelium promotes fibrogenesis by eliminating its “antifibrotic” functions, which include the inhibition of lung fibroblast proliferation and intra-alveolar fibrinolysis. Furthermore, a previous study by this laboratory (23) found that human and rat lung fibroblasts isolated from fibrotic tissues produce a factor(s) capable of inducing apoptosis in the alveolar epithelium, suggesting that the altered fibroblast phenotypes that accumulate in fibrotic foci may continue to kill the epithelial cells that normally suppress their generation. Subsequent work (22) documented alveolar epithelial apoptosis adjacent to abnormal fibroblasts in the fibrotic human lung; the apoptosis-inducing factor(s) produced by the abnormal cells was recently identified as angiotensin peptides (24).

Those findings suggest a profibrotic mechanism in which epithelial cell death leads to expanding populations of abnormal lung fibroblasts, which, in turn, promote the further death of adjacent epithelial cells to create a hypothetically autonomous process. Expression of Fas by the epithelium and of Fas ligand by infiltrating inflammatory cells are also believed to contribute to the profibrotic nature of the microenvironment (6). The demonstration that intratracheal administration of Fas-activating antibodies can induce epithelial apoptosis and pulmonary fibrosis in mice (5) supports the contention that apoptosis in the lung epithelium is sufficient to initiate the pathways that lead to lung fibrogenesis. Moreover, the induction of epithelial apoptosis by Fas also involves angiotensin peptides, which were recently shown to be required for the signaling of apoptosis in response to Fas activation (25).

Inhibitors of ACE are known to attenuate fibrogenesis in the lung (12, 27) and in other organs (16, 20, 21, 28), but the mechanisms underlying the inhibition are unclear. The antifibrotic effect of ACE inhibitors was recently linked theoretically to the concept of epithelial apoptosis by the demonstration that the ACE inhibitor captopril has a potent ability to abrogate Fas-induced apoptosis in human lung epithelial cells, at least in vitro (21). The data in this report extend those findings to demonstrate that captopril, if applied to well-differentiated primary AECs isolated from rats, also attenuates apoptosis induced by either Fas ligand (Fig. 1) or by Bleo (Fig. 2). The fact that the primary cells studied in those experiments were isolated from the same rat strain used for the in vivo studies described above supports the contention that inhibition of apoptosis by
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captopril is likely to occur in the intact animal as well as in vitro.

The data reported in Figs. 3, 5, and 6 are consistent with that hypothesis and indicate that the inhibitory effects of captopril on apoptosis affect AECs as well as cells of the alveolar walls. Furthermore, they indicate that the caspase inhibitor ZVAD-fmk has essentially the same potency as captopril at inhibition of Bleo-induced apoptosis in either lung epithelial cell population. Although both agents were more potent at inhibiting apoptosis in alveolar versus airway epithelial cells (compare Fig. 6, A and B), this result might be explained by Bleo-induced necrosis of the airways. Regardless, the successful inhibition of lung epithelial apoptosis by ZVAD-fmk is significant in consideration of the intraperitoneal route of administration (see Methods) in contrast to the more commonly used intravenous administrations (11, 17, 30).

Perhaps more importantly, the caspase inhibitor ZVAD-fmk was similar to captopril in its potency to attenuate the accumulation of lung collagen measured by either of two methods (Figs. 8 and 9). These results support the hypothesis put forth earlier (21) that the ability of captopril or other ACE inhibitors to block the death of that cell type. The caspase inhibitor ZVAD-fmk was shown to attenuate alveolar macrophage apoptosis induced by silica (9), and thus it seems likely that Bleo-induced apoptosis of macrophages also could be abrogated by this compound. In any case, apoptosis within nonepithelial cell types was not quantitated in this study, and thus the roles that inhibition of apoptosis of macrophages or other nonepithelial cells might play in Bleo-induced fibrogenesis are at this time unclear.

On the other hand, a number of profibrotic sequelae of denudation of the alveolar epithelium have been proposed. AECs express urokinase-type plasminogen activator and plasminogen activator inhibitor-I and are capable of degrading fibrin clots in vitro, and this capacity is believed to act in a tonic fashion to clear intra-alveolar fibrin in vivo (19). In addition, AECs are known to produce prostaglandins of the E series, which are documented inhibitors of lung fibroblast proliferation (3, 4), provide a physical barrier necessary for transport functions, and protect underlying interstitial cells from the cytokines released by free alveolar phagocytes. For these reasons, the loss of AECs and/or interruption of their replacement by chronic apoptosis could be envisioned as permissive for fibroblast proliferation and activation by macrophage-derived factors and for the accumulation of intra-alveolar fibrin. Many other antifibrotic functions of the alveolar epithelium were discussed in detail by Simon (18).

Several years ago, Zhang et al. (32) presented evidence from Bleo-treated rats that fibroblast subsets activated for procollagen gene expression begin to emerge first in bronchiolar adventitia, terminal bronchioles, and adjacent blood vessels before they expand to the alveolar region. Interestingly, these are the same locations in which apoptosis of airway epithelial and alveolar wall cells was observed at the earliest sampling times of the present study. Although the possibility exists that apoptosis of cell types other than epithelial might contribute to the initiation of fibrogenic reactions, the data reported herein are consistent with the hypothesis that apoptosis of nearby epithelial cells is a critical event at the most early stages of nascent fibrotic foci.

In summary, we have shown that primary cultures of well-differentiated AECs, if challenged with purified Fas ligand or Bleo, undergo apoptosis that is directly inhibitable by captopril or by the caspase inhibitor ZVAD-fmk. Intraperitoneal administration of captopril or ZVAD-fmk in adult rats abrogated Bleo-induced apoptosis of alveolar wall cells and significantly inhibited the generation of fragmented DNA within airway epithelial cells. The induction of apoptosis preceded the deposition of collagen, and neither captopril or ZVAD-fmk significantly reduced the accumulation of lung collagens by 14 days after Bleo instillation. These results support the hypothesis that the ability of captopril to attenuate experimental lung fibrogenesis is related to its ability to abrogate apoptosis in lung epithelial cells.

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