Human lung myofibroblast-derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides

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Human lung myofibroblast-derived inducers of alveolar epithelial apoptosis identified as angiotensin peptides. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1158–L1164, 1999.—Earlier work from this laboratory found that fibroblasts isolated from fibrotic human lung [human interstitial pulmonary fibrosis (HIPF)] secrete a soluble inducer(s) of apoptosis in alveolar epithelial cells (AECs) in vitro (B. D. Uhal, I. Joshi, A. True, S. Mundle, A. Raza, A. Pardo, and M. Selman. Am. J. Physiol. 269 (Lung Cell. Mol. Physiol. 13): L819–L828, 1995). The cultured human fibroblast strains most active in producing the apoptotic activity contained high numbers of stellate cells expressing α-smooth muscle actin, a myofibroblast marker. The apoptotic activity eluted from gel-filtration columns only in fractions corresponding to proteins. Western blotting of the protein fraction identified immunoreactive angiotensinogen (ANGEN), and two-step RT-PCR revealed expression of ANGEN by HIPF fibroblasts but not by normal human lung fibroblasts. Specific ELISA detected angiotensin II (ANG II) at concentrations sixfold higher in HIPF-conditioned medium than in normal fibroblast-conditioned medium. Pretreatment of the concentrated medium with purified renin plus purified angiotensin-converting enzyme (ACE) further increased the ELISA-detectable ANG II eightfold. Apoptosis of AECs in response to HIPF-conditioned medium was completely abrogated by the ANG II receptor antagonist saralasin (50 µg/ml) or anti-ANG II antibodies. These results identify the protein inducers of AEC apoptosis produced by HIPF fibroblasts as ANGEN and its derivative ANG II. We show here that purified angiotensin (ANG II) or purified angioten-}

INTACT ALVEOLAR EPITHELIUM is critical in maintaining many lung functions including gas exchange, water balance, surfactant synthesis, and local immunomodulation (15, 21). Evidence from both animal models (1, 8) and human lung biopsies (13) supports the concept that efficient repair of the alveolar epithelium after damage is critical to lung healing without fibrosis, whereas inefficient epithelial repair promotes fibrogenesis (32). Repair of damaged alveolar epithelium is accomplished by the proliferation and differentiation of alveolar epithelial type II pneumocytes, the stem cells for replacement of both type II and type I alveolar epithelial cells (AECs) (21). The many antifibrotic roles of type II and type I AECs have been summarized by Simon (19).

Previously, Uhal et al. (26) reported the identification of a soluble inducer(s) of apoptosis and necrosis in cultured AECs that was produced by fibroblasts isolated from fibrotic human lungs but not by fibroblasts isolated from normal human lungs (26). The apoptotic activity was present in serum-free conditioned medium prepared from primary isolates of human lung fibroblasts that were cultured for as many as 12 passages after isolation from biopsy tissue. Subsequent examinations of the same biopsy samples used for fibroblast isolation revealed that AEC apoptosis and necrosis in situ were localized primarily adjacent to foci of α-smooth muscle actin (α-SMA)-positive myofibroblasts (25). These findings suggested that the soluble inducer of epithelial apoptosis identified in vitro (26) was also active in vivo in the advanced fibrotic human lung.

More recent work in this laboratory found potent inhibition of AEC apoptosis by the angiotensin-converting enzyme (ACE) inhibitor captopril (24) and dose-dependent induction of apoptosis in the same cells by purified angiotensin II (ANG II) or purified angiotensinogen (ANGEN) (29). In light of these findings, we hypothesized that the soluble inducer of AEC apoptosis produced by "fibrotic" human lung fibroblasts might be related to the expression of ANGEN by myofibroblasts, a phenomenon recently observed in rat cardiac muscle (11). We show here that α-SMA-positive fibroblasts isolated from fibrotic human lungs express ANGEN and demonstrate that this protein and its product ANG II are the soluble inducers of AEC apoptosis identified in the earlier study by Uhal et al. (26).

METHODS

Reagents and materials. Purified ANG II, lisinopril, saralas-
lated at the National Institute of Respiratory Diseases (Tlalpan, Mexico) (26) with methods described below. Of these, fibrotic lung fibroblast strains [human interstitial pulmonary fibrosis (HIPF)-X, where X is the patient number] were derived from patients with two different types of interstitial lung disease (ILD): idiopathic pulmonary fibrosis (IPF) and chronic hypersensitivity pneumonitis (CHP); strains HIPF-8 and HIPF-28, respectively. In both diseases, patients had clinical, functional, and radiological features that fulfill the diagnostic criteria for an ILD (18). Briefly, all of them had progressive dyspnea, bilateral reticulonodular images on chest roentgenogram, restrictive lung functional impairment with decreased lung volume and compliance, and hypoxemia at rest that worsened with exercise. In addition, patients with CHP had domestic exposure to pigeons that predated the development of respiratory symptoms, specific serum antibodies against avian antigens as determined by ELISA, and a morphological study consistent with the diagnosis. Briefly, the tissue samples showed a diffuse interstitial inflammation of mononuclear predominance, mainly lymphocytes, and frequent multinucleated giant cells in terminal and respiratory bronchioles as well as in the alveolar spaces. Small and loosely arranged granulomas were observed in the interstitium, and there were no histological changes suggestive of infection or another ILD. Patients with IPF had neither antecedents of any occupational or environmental exposure nor other known cause of ILD. Morphological study showed patchy alveolar septal fibrosis and interstitial inflammation consisting mostly of mononuclear cells but also of neutrophils and eosinophils. A variable macrophage accumulation was consisting mostly of mononuclear cells but also of neutrophils and eosinophils. A variable macrophage accumulation was observed in the air spaces as was cuboidalization of the alveolar epithelium. Biopsies lacked granulomas, vasculitis, microorganisms, and inorganic material as determined with polarized light microscopy (2). Control fibroblasts were obtained from individuals having a lobectomy for removal of a primary lung tumor. No morphological evidence of disease was found in the tissue samples used for isolation of control cells (N-X).

Lung fibroblasts from normal or fibrotic tissue were isolated by trypsin digestion of tissues minced to 1-mm fragments (26). Fibroblast strains were established in Dulbecco's modified Eagle's medium (or in Ham's F-12 medium) supplemented with 10% fetal calf serum, 200 U/ml of penicillin, and 200 mg/ml of streptomycin. All cells were cultured at 37°C in 95% air-5% CO2 until just before confluence, which generally occurred in 1–2 wk. After passage 1, essentially 100% of all fibroblast isolates were immunoreactive with monoclonal antibodies to vimentin (23, 26). All strains also exhibited the morphological characteristics of fibroblasts, although fibrotic strains generally had a more "stellate" morphology (see Fig. 1). Analyses of immunoreactivity to α-SMA antibodies were performed on cells harvested from the same culture vessels in which conditioned medium was prepared (see Flow cytometry and microscopy). Equivalent cell "densities" for the preparation of conditioned medium were defined as the point at which normal or fibroblastic cells reached 100% confluence. However, because of the larger cell size of fibroblastic fibroblasts (up to five times the volume of normal fibroblasts; data not shown), normal fibroblast isolates contained more cells per culture vessel at 100% confluence. Regardless, all comparisons of α-SMA reactivity and functional assays of conditioned medium were made on cells at the equivalent conditions of 100% confluence.

For preparation of conditioned medium, fibroblasts (taken on the same day that confluence was reached) were incubated for 24 h in serum-free Ham's F-12 medium, and the medium was collected and centrifuged to remove detached cells. The cell-free conditioned medium was lyophilized or used directly. Only passage 3–12 primary cell cultures were used for the preparation of conditioned medium.

Epithelial cell culture and detection of apoptosis. Primary type II AECs were isolated from adult male Wistar rats by a trypsin digestion and density gradient protocol described earlier (22). All primary epithelial cell isolates were >90% pure as determined by acridine orange uptake into lamellar bodies and were used on culture day 2, a time at which they were type II-like by accepted morphological and biochemical criteria (23). Epithelial cell apoptosis was detected by the assessment, under fluorescence microscopy, of chromatin condensation and nuclear fragmentation within cytoplasmic positive cells after ethanol fixation and staining with propidium iodide (24, 26). In these assays, detached cells were retained by centrifugation of the culture vessels during fixation with 70% ethanol. For testing of conditioned medium, cultured epithelial cells were incubated for 20 h with each conditioned medium (serum-free Ham's F-12 medium; see Fibroblast isolation, culture, and preparation of conditioned medium) diluted 1:5 in serum-free Ham's F-12 medium; the initial study by Uhal et al. (26) of the conditioned medium showed that this concentration provided a maximal response at the least expense of conditioned medium.

The A549 human lung carcinoma cell line was cultured with methods previously described (24, 26, 29) and was used for testing no less than 48 h after passage. All epithelial cells were were used at culture densities of no more than 70–90% confluence because pilot experiments indicated that density-arrested cells were less sensitive than subconfluent cultures to the induction of apoptosis by HIPF medium or other inducers (data not shown). All test agents (saralasin and ANGs) were dissolved directly in culture medium except for DEVD-fmk, which was first suspended in dimethyl sulfoxide. Control experiments found no significant effect of the small molecule volume on apoptosis or its inhibition (data not shown).

Flow cytometry and microscopy. Flow cytometric analyses were performed on a Partec CA-III flow cytometer equipped with a 25-mW argon-ion laser for excitation at 488 nm. Propidium iodide fluorescence data were acquired through a 610-nm long-pass filter, and FITC fluorescence was acquired...
through an EM520 band-pass filter. After standardization with fluorescent microspheres (Coulter, Hialeah, FL), amplifier gains were not changed throughout an experiment. Flow cytometric analyses of fibroblast ploidy (DNA content) versus α-SMA immunoreactivity (α-SMA-FITC) were conducted as described earlier (27). Briefly, cells were lightly trypsinized from the culture dishes, dispersed by agitation, and fixed in 70% ethanol. The fixed cells were incubated with FITC-conjugated anti-α-SMA antibody diluted 1:400 in 1% bovine serum albumin in phosphate-buffered saline, pH 7.3; the cells were washed and resuspended in phosphate-buffered saline containing DNase-free RNase and 5 µg/ml of propidium iodide. Anti-α-SMA fluorescence and DNA content data were acquired in log and linear scales, respectively.

Protein methods. Gel filtration was performed on lyophilized serum-free conditioned medium resuspended in deionized water at 20% of its original volume. The concentrated medium was loaded onto Sephadex PD-10 columns (Pharmacia Biotech, Uppsala, Sweden) that were preequilibrated with serum-free Ham’s F-12 medium, and fractions were eluted with the same Ham’s F-12 serum-free medium. Collected fractions were diluted 1:3 with serum-free Ham’s F-12 medium and were tested for apoptotic activity as described in Methods. After centrifugation for 10 min at 100,000 g, supernatants were collected and subjected to gel filtration. The dialysate was lyophilized, resuspended in gel-specific loading buffer, and electrophoresed on 16% Tricine ready gels (pH 8.3), 75 mM KCl, 3 mM MgCl2, 0.01 mM dithiothreitol, 10 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 0.01 mM dithiothreitol, 0.2 mM each deoxyribonucleotide triphosphate, 1 U/µl of RNase inhibitor (RNasin), and 2 U of avian myoblastosis virus reverse transcriptase (Promega, Madison, WI) in a total volume of 30 µl. The reaction was performed for 1 h at 45°C, followed by 20 cycles of PCR amplification as described below. PCR amplification was performed with 10-µl aliquots of cDNA obtained as described above, equivalent to 1 µg of the starting RNA. The identity of the expressed genes was determined by the expected size of the PCR product on 1.6% agarose gels.

RT-PCR. RT-PCR assay of ANGEN gene products was performed as described earlier (29). Briefly, total RNA was isolated by the RNeasy Mini protocol (Qiagen, Santa Clarita, CA). To synthesize cDNA by RT-PCR, 3 µg of purified RNA were reverse-transcribed with 2 U of Avian dCTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 0.01 mM dithiothreitol, 0.2 mM each deoxyribonucleotide triphosphate, 1 U/µl of RNase inhibitor (RNasin), and 2 U of avian myoblastosis virus reverse transcriptase (Promega, Madison, WI) in a total volume of 30 µl. The reaction was performed for 1 h at 45°C, followed by 20 cycles of PCR amplification as described below. PCR amplification was performed with 10-µl aliquots of cDNA obtained as described above, equivalent to 1 µg of the starting RNA. The identity of the expressed genes was determined by the expected size of the PCR product on 1.6% agarose gels.

For RT-PCR from the human fibroblast strains, the following primers for ANGEN were used: “outer” primer of a two-step nested assay, coding, 5′-GCTTTCAACACTACGTCCA-3′, and uncoding, 5′-AGCTGTGGGATACCTGT-3′; and “inner” primer of the nested assay, coding, 5′-TTCCTCCTGCTGGCCGAG-3′, and uncoding, 5′-GGGGCTCTCTCTCATCCGC-3′. These primers yielded a final PCR product of 447 bp (13). For β-actin, a single-step RT-PCR was used with the following primers: coding, 5′-AGGCCAACCGC-GAGAAGATGACC-3′, and uncoding, 5′-GAAGTCCAGGGC-GACGTAGC-3′. These primers produced a PCR product of 332 bp (17). The ANGEN-to-β-actin ratio was calculated from densitometric scans of PCR products detected by ethidium bromide staining of the 1.6% agarose gels.

Microscopy and image analysis. Photomicroscopy was performed on an Olympus EMT-2 epifluorescence phase-contrast microscope equipped with band-pass filters for detection of FITC and propidium iodide and fitted with both color and gray-scale charge-coupled device cameras.

RESULTS

Fibroblast isolates from fibrotic human lungs are designated as “HIPF” and were derived from IPF or CHP patients (see Methods). In culture, these cells displayed morphological characteristics typical of myofibroblasts, including flattened and stellate morphology with occasional lipid inclusions (Fig. 1, D–F). Because of size differences, the number of HIPF fibroblasts per culture vessel at early confluence was roughly one-half that of the normal fibroblast strains (data not shown).

Flow cytometric (fluorescence-activated cell sorting) analysis of the expression of α-SMA indicated that most HIPF strains contained significantly higher numbers of α-SMA-positive cells than the normal strains. Figure 2 displays the fluorescence-activated cell sorting method as applied to one normal (N-11) strain (A) and one fibrotic (HIPF-112) strain (B); the bivariate analysis determined DNA content versus immunoreactivity to fluorescent antibodies specific for α-SMA as described earlier (27). In a previous study (26), it was shown that all fibroblast isolates were heavily immunoreactive with monoclonal antibodies to vimentin (data not shown).

Table 1 reports the flow cytometric data together with the potency of conditioned medium from each strain to induce apoptosis in AECs as determined as described earlier (26). All strains with apoptotic activity also contained α-SMA-positive cells in higher proportions than in the normal isolates. Regardless of passage number, the HIPF strains displayed a higher proportion of α-SMA-positive cells than did normal strains at any passage (data not shown), suggesting preferential recovery of the abundant α-SMA-positive myofibroblasts from the fibrotic tissue biopsies (13). Over three years of study, only one normal strain (N-12) in one
passage (passage 6) exhibited modest apoptotic activity and concurrently increased expression of α-SMA (data not shown); conversely, one fibrotic strain (HIPF-61) exhibited both no apoptotic activity and a low percentage of cells expressing α-SMA (Table 1). The remainder of this study will focus on the strains listed in Table 1.

Gel-filtration analyses of concentrated HIPF medium indicated that the epithelial apoptotic activity eluted exclusively in the protein fraction (Fig. 3); no activity was found in the low-molecular-mass fractions (compare with 390 kDa; Fig. 3A) or in organic extracts of the medium (data not shown).

A specific ELISA for ANG II detected the peptide at concentrations that were significantly higher in HIPF-conditioned medium than in normal fibroblast medium (Fig. 4A). Furthermore, preincubation of the medium with purified renin and purified ACE increased the ELISA-detectable ANG II concentration roughly eightfold (Fig. 4B), confirming that ANGEN is present in the conditioned medium in much higher abundance than ANG II.

Western blotting of concentrated medium conditioned by the HIPF-8 fibroblast strain revealed one major band that was immunoreactive with rabbit antiserum raised against human ANG I as the immunogen (Fig. 5). The size of this band was 58 kDa, the known molecular mass of human ANGEN. Although no immunoreactive bands corresponding to ANG I and ANG II (1.4 and 1.0 kDa, respectively) were observed, peptides of this small size were found to pass completely through the polyvinylidene difluoride membrane, precluding their analysis by this assay (data not shown).

RT-PCR of total RNA isolated from HIPF fibroblast strains with primers specific for human ANGEN (Fig. 6A) generated a single PCR product of 447 bp, the size expected in conditioned medium that was significantly greater than that of serum-free media, P < 0.05.

Values are means ± SE. α-SMA, α-smooth muscle actin; HIPF, human interstitial pulmonary fibrosis; AEC, alveolar epithelial cell; N, normal. Percentage of α-actin-positive cells was determined from same T-flask in which serum-free conditioned medium was generated (see Fig. 2 and METHODS). Apoptosis of rat AECs was determined by propidium iodide assay (26) of primary AEC exposed to serum-free conditioned medium; percent increase is relative to serum-free medium alone. NS, no significant increase above activity of serum-free medium alone. All activities of HIPF media were significantly greater than those of serum-free media, P < 0.05.

![Fig. 3. Gel-filtration analysis of HIPF-derived apoptotic activity. A: elution profile of FITC (390 Da) and protein (FITC-insulin) from Sephadex PD-10 column. B: elution of apoptotic activity (see METHODS) from PD-10 column loaded in identical fashion as in A. Note activity exclusively in fractions corresponding to small proteins.](http://ajplung.physiology.org/)

![Fig. 4. ELISA for ANG II in human lung fibroblast-conditioned medium. Conditioned medium from 3 normal (NORM; strains N-A, N-11, and N-13) and 3 HIPF (strains HIPF-19, HIPF-28, and HIPF-112) primary fibroblast isolates were concentrated and assayed by specific ELISA for ANG II content. A: untreated medium. B: same medium after treatment with purified renin plus purified angiotensin-converting enzyme (see METHODS). Values are means ± SE. Significantly different from corresponding NORM value: *P < 0.05 vs. NORM (by ANOVA and Student-Newman-Keul's test).](http://ajplung.physiology.org/)

![Fig. 5. Western blotting of HIPF fibroblast-derived proteins. Medium conditioned by HIPF-8 fibroblast strain was dialyzed against water, lyophilized, and electrophoresed on 16% Tricine gels. Proteins transferred to polyvinylidene difluoride membrane (see METHODS) were immunoblotted with rabbit antiserum against angiotensin peptides. 2X and 1X, protein equivalents loaded. Single major band corresponds to known molecular mass (nos. on left) of angiotensinogen (58 kDa; arrowhead).](http://ajplung.physiology.org/)
cells expressing the propeptide (14). By densitometry (Fig. 6B), this PCR product was roughly eightfold more abundant in total RNA isolates from HIPF fibroblast strains than in those from the normal strains. The epithelial apoptotic activity of HIPF-conditioned medium was completely abrogated by the sequence-specific peptide inhibitor of caspase 3, DEVD-fmk (Fig. 7, top). These results are consistent with the earlier demonstration by Uhal et al. (26) that the endonuclease inhibitor aurantricarboxylic acid could abrogate HIPF-induced alveolar epithelial apoptosis and confirm that the bioassay employed is a reliable measure of apoptosis. The HIPF-induced activity was also eliminated by the nonselective ANG II receptor antagonist saralasin at a concentration of 50 µg/ml. Furthermore, HIPF-induced apoptosis could be abrogated by antibodies specific for ANG II. The same antibody preparation also eliminated apoptosis induced by either purified ANG II or purified ANGEN (Fig. 7, bottom), but a nonspecific isotype-matched control antibody had no such effect. Bioassay of the fibroblast-conditioned medium thus confirmed the identity of the HIPF-derived apoptotic activity as ANGEN and its product ANG II.

DISCUSSION

ANG II has been well documented to induce apoptosis of cultured endothelial cells (5) and cardiac myocytes (9). Recent work from this laboratory found potent induction of apoptosis by ANG II in both human and rat AECs at concentrations of the peptide within the physiologically relevant range (29). The same study found that well-differentiated primary isolates of rat AECs could undergo apoptosis induced by purified ANGEN; moreover, the ANGEN-induced apoptosis could be blocked by an ANG II receptor antagonist or by inhibitors of ACE. These findings indicate that well-differentiated AECs express sufficient reninlike and ACE-like activities to convert ANGEN and ANG I to ANG II, at least in the rat. Whether AECs within the intact human lung possess a similar capacity will be an important subject for further investigation.

Myofibroblasts isolated from injured rat cardiac muscle were recently found to be capable of producing ANG peptides in vitro, in contrast to normal rat cardiac fibroblasts that do not express ANGEN (11). The same cells also expressed ACE and cathepsin D but not renin, suggesting a limited capacity to convert some of the synthesized ANGEN to the processed peptides ANG I and ANG II. In the present study, the ELISA data of

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Fig. 4 indicate the presence of a small amount of ANG II in HIPF fibroblast-conditioned medium, but pretreatment of the medium with purified converting enzymes generated significantly more ANG II. These data suggest that human lung myofibroblasts, like rat heart myofibroblasts, also express limited enzymatic activities for ANGEN conversion. The determination of which converting enzymes are expressed by these cells is currently underway.

Regardless, the ability of primary AECs to undergo ANG II receptor-dependent apoptosis in response to purified ANGEN (29) suggests that enzymatic cleavage of ANGEN by myofibroblasts may not be required for the induction of apoptosis in the adjacent epithelia because the epithelial cells can accomplish the conversion by themselves. In biopsy tissues from patients with advanced pulmonary fibrosis, alveolar epithelial apoptosis and necrosis were found immediately adjacent to foci of α-actin-positive myofibroblasts (25); furthermore, the biopsy specimens examined in that study were the same as those used for the isolation of the HIPF fibroblast strains used in the present study. For these reasons, we suggest that the production of ANGEN by myofibroblasts is a plausible mechanism to explain the colocalization of AEC apoptosis adjacent to these foci in situ (25). On the other hand, the number of fibroblast strains examined in the present study constitutes a relatively small sample size that is limited to patients with IPF and CHP (see METHODS). Although the myofibroblast is found in a wide variety of injured and repairing tissues (11, 27), a larger evaluation of patients with diverse forms of lung disease will be necessary before these results can be extrapolated to all fibrotic lung disorders.

As discussed earlier (29), very little is known about the local concentrations of ANGEN or ANG II in extravascular compartments of the lung. In patients with acute respiratory distress syndrome (31), the mean concentration of ANG II in arterial plasma (85 pg/ml or 0.1 nM) is just below the lowest concentration of ANG II found in an earlier work by Uhal et al. (29) to elicit a significant induction of apoptosis in primary AECs. In light of this information, it seems reasonable to hypothesize that the local concentration of ANG II in the microenvironment surrounding cells that synthesize ANGEN might readily exceed the threshold necessary to induce apoptosis of adjacent epithelial cells. On the other hand, ANG II has a biological half-life of <1 min (28), and thus the microenvironment in which apoptosis of epithelial cells might be induced would be expected to be of limited size. Such a scenario is consistent with the observations by Uhal et al. (25) of focal AEC apoptosis and necrosis in the vicinity of α-actin-positive fibroblastic foci. In situ, these regions are often found to consist primarily of cuboidal rather than squamous epithelial cells, suggesting that type I AECs might be more susceptible to myofibroblast-induced apoptosis. This hypothesis might be testable through future comparisons of early (type II-like) versus late (type I-like)-stage cultures of primary rat AECs exposed to fibroblast-conditioned medium.

The induction of ANGEN expression has been linked to several profibrotic processes. ANG II is known to increase type I collagen synthesis in myofibroblast-like cultured valvular interstitial cells of the heart (12), and ANG II receptor antagonists are known to attenuate the formation of fibrous tissue at the site of experimental myocardial infarction (20). As discussed by Katwa et al. (11), the myofibroblast is known to be one of the key sources of ANGEN, and thus ANG II, at these sites. In the lung, the elimination of AECs by ANG II-induced apoptosis would be expected to reduce the tonic suppression of fibroblast proliferation exerted by epithelium-derived prostaglandin E2 (6) and to compromise the immunomodulatory functions of the intact epithelium (4, 10). Furthermore, the resulting loss of portions of the epithelial barrier would increase the exposure of underlying mesenchymal cells to proinflammatory cytokines released by free alveolar cells (19).

The important role of intact alveolar epithelium in the prevention of lung fibrogenesis was first suggested by the work of Haschek and Witschi (8) and is supported by subsequent investigations of both animal models (1, 32) and human lung biopsies (13). A critical role for AEC apoptosis in the pathogenesis of lung fibrosis is supported by the demonstration that activation of epithelial Fas by intratracheal administration of receptor-activating antibodies induced pulmonary fibrosis in mice (7). For these reasons, we speculate that myofibroblast-induced apoptosis in the alveolar epithelium is an important component in the progression of fibrogenesis in advanced fibrotic lung (25) and may also play a role in the initiation of nascent fibrotic foci. As previously discussed (21, 26), exposure of primary cultures of AECs to the same HIPF fibroblast-conditioned medium tested herein resulted in the loss of nearly 75% of the total cell number in only 20 h despite a seemingly modest apoptotic indexes of <15% (see Figs. 5 and 9 in Ref. 26). In the present study, replicate aliquots of the same conditioned medium yielded similar apoptotic indexes of 1.9 ± 0.4 and 8.6 ± 0.2% for control and HIPF-stimulated samples, respectively (Fig. 7). The finding of such apparently "low" apoptotic indexes in cultures undergoing significant and cumulative cell loss is consistent with the findings of others (3) and supports the contention that the induction of apoptosis is a minor fraction of AECs is sufficient to significantly reduce the cell population size in a surprisingly short period of time.

In summary, primary fibroblasts isolated from fibrotic human lung synthesize ANGEN and limited amounts of ANG II. Apoptosis of cultured AECs in response to serum-free medium conditioned by these cells was completely abrogated by antagonists of the renin-angiotensin system and by specific antibodies to ANG II. These studies have identified the fibroblast-derived soluble inducer of AEC apoptosis (26) as ANGEN and its product ANG II. They also suggest a mechanism for the previously documented ability of ACE inhibitors to abrogate experimental lung fibrogenesis in animal models (16, 30).
REFERENCES


