Angiotensin II induces apoptosis in human and rat alveolar epithelial cells

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programmed cell death; type II pneumocyte; angiotensin-converting enzyme; pulmonary fibrosis; granular pneumocytes

THE TYPE II ALVEOLAR EPITHELIAL CELL (AEC) is the stem cell for alveolar epithelial repair after injury and during normal tissue turnover (13, 14). Proliferation and differentiation of these cells replace lost type II and type I epithelial cells, the terminally differentiated progeny of type II cell division (17). Recent investigations suggested an important role for apoptosis in the alveolar epithelium as a determinant in the pathogenesis of pulmonary fibrosis in animal models (8, 9) and in the advanced stages of lung disease in humans (3, 20). Elucidation of the regulation of apoptosis in these cells is thus of critical importance in understanding lung homeostasis and in the design of potential strategies to block apoptosis in pathologies to which it contributes.

A recent study from this laboratory (19) demonstrated potent inhibition of apoptosis in the human AEC-derived A549 cell line by the angiotensin-converting enzyme (ACE) inhibitor captopril. The inhibition of apoptosis exhibited a concentration dependence similar to that for captopril inhibition of ACE in other cell types (15). For this reason, we hypothesized that apoptosis of AECs might be induced by ANG II or angiotensinogen through pathways involving ACE and the ANG II receptor. We report here that both primary rat AECs and the A549 human AEC-derived cell line express reninlike and ACE-like activities as well as functional ANG II receptors capable of inducing apoptosis.

METHODS

Reagents and materials. Purified ANG II, angiotensinogen, lisinopril, saralasin, and antibodies to ANG II and angiotensinogen were obtained from Sigma (St. Louis, MO). Fluorescein-conjugated annexin V was obtained from PharMingen (San Diego, CA), and PCR primers were synthesized by Genemed Synthesis (San Francisco, CA). All other materials were from sources described earlier (19, 22) or were of reagent grade.

Cell culture. The human lung adenocarcinoma cell line A549 was obtained from American Type Culture Collection and cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS). Primary AECs were isolated from adult male Wistar rats as described earlier (18, 21). 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 (17), and all preparations were of >90% purity as assessed by acridine orange staining as previously described (18). All cells were seeded in 24- 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 were applied for 20 h at 37°C in a 5% CO2 incubator.

Detection of apoptosis. Detection of apoptotic cells with propidium iodide (PI) was conducted as described earlier (19) after digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 µg/ml of PI. Assay of annexin V binding by flow cytometry was performed essentially as previously described (22) for human lung fibroblasts after trypsinization of cells from culture vessels. Assay of caspase-3 activity was performed on viable suspension cultures as reported earlier (19). In all assays, detached cells were retained by centrifugation of the culture vessels during fixation with 70% ethanol or by retention of culture medium and recovery by centrifugation before assay.

For microscopy, the cells were cultured on glass coverslips, incubated with test reagents, and fixed with 70% ethanol. The fixed cells were immunolabeled overnight at 4°C with monoclonal antibodies to cytokeratins 7 and 19 (Chemicon International, Temecula, CA), and then for 1 h at 37°C with antiserum IgG-rhodamine and DNase-free RNase (Boehringer Mannheim, Indianapolis, IN). The coverslips were then rinsed in distilled water three times and mounted under Fluoromount solution (Southern Biotechnology Associates, Birmingham, AL) containing 5 µg/ml of PI. Photomicroscopy was performed as described earlier (19). Cells exhibiting nuclear fragmentation and chromatin condensation (see Fig. 1) were scored in four randomly selected fields per culture well in at least three culture vessels per experimental condition and are expressed as a percentage of the total cells within the same field (minimum of 100 cells/field). See Statistics for details of statistical analyses.

RT-PCR. Total RNA was isolated by the RNeasy mini protocol (Qiagen, Santa Clarita, CA). To synthesize cDNA by RT-PCR, 3 µg of purified RNA was reverse transcribed with 2 µM oligo d(T), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 0.01 mM DTT, 0.2 mM each deoxynucleotide 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, equivalent to 1 µg of the starting RNA, obtained as described above. The reaction was performed in 50 µl of PCR buffer containing 5 mM MgCl2, 5 µg/ml of each 5’ and 3’ primer (see below), 2 µl of 10 mM deoxynucleotide triphosphates, and 1 U of Taq polymerase (Promega) with a Perkin-Elmer PCR amplifier. The PCRs included denaturation at 95°C for 30 s, annealing of the primers at 45°C for 30 s, and elongation of the chain at 72°C for 1 min with Taq DNA polymerase. The samples were stored at 4°C; negative controls lacked DNA. The identity of the expressed genes was determined by the expected size of the PCR product in 1.6% agarose gels.

For RT-PCR from the human A549 cell line, the following primers were used: 1) ANG II receptor type 1, coding = 5’-AAGAAGGAAAGGAAAGAAACCCAGTA-3’ and uncoding = 5’-AGGAAGAGAAAGAAGAAACCCAGTA-3’, which yielded a PCR product of 414 bp (4); 2) ANG II receptor type 2, coding = 5’-CCTTTTGTCTCTTCTCTTATGG-3’ and uncoding = 5’-TTGGTCAGCGGTATGCTCTTCTC-3’, which also yielded a product of 414 bp (4); and 3) ACE, coding = 5’-AGTGGCCATTATTTGGAAGC-3’ and uncoding = 5’-AGTTGCCATTATTTGGAAGC-3’, which led to a PCR product of 499 bp (2).

For RT-PCR of rat-specific gene products, the primers used were 1) ANG II receptor type 1, coding = 5’-GAGAGGTGTTGAG-3’ and uncoding = 5’-GAGACAGATTGTGTTGAG-3’, which provided a PCR product of 235 bp (6); 2) ANG II receptor type 2, coding = 5’-ATGAAGGACAACTCAGGTTCTC-3’ and uncoding = 5’-CAAGGGGACTACATAAAGATGTCG-3’, which yielded a product of 499 bp (16); and 3) ACE, coding = 5’-GTCAAGTTTCACTCAACAG-3’ and uncoding = 5’-AGAAAGGAGCAGAGCGCTCAG-3’, which led to a PCR product of 304 bp (12).

Statistics. All values reported are means ± SE. Significant differences between treatment means were determined by ANOVA followed by the indicated post hoc analysis. All experiments were performed at least two times, and the data were combined except as indicated in Figs. 2–5.

RESULTS

Apoptosis in AECs was quantitated by fluorescence detection of chromatin condensation and nuclear fragmentation in alcohol-fixed cells stained with PI (19). Double labeling with PI and monoclonal antibodies to cytokeratins 7 and 19 identified cells with both apoptotic nuclear morphology and positive anti-cytokeratin immunoreactivity (Fig. 1). Although apoptotic cells with negative immunoreactivity and the spindle-shaped morphology of fibroblasts were occasionally observed within the primary rat lung cell preparations, these were not scored.

Purified ANG II induced apoptosis in a concentration-dependent manner in both the human A549 cell line and primary rat AECs (Fig. 2). The observed EC50 for apoptosis by ANG II was 50 nM for A549 cells and 10 nM for primary rat AECs; the presence or absence of medium growth factors (Fig. 2) had no influence on maximal induction of apoptosis by ANG II. To confirm the induction of apoptosis, annexin V binding and activity of caspase-3/cysteine protease protein-32/Yama were assessed in both human and rat AECs exposed to the purified peptide. Incubation with 5.0 µM ANG II increased the percentage of annexin V-positive primary
rat AECs from 10.0 to 40.9% (Fig. 3, A and B) and significantly increased the activity of caspase-3 in intact AECs of either human or rat origin (Fig. 3 C).

Apoptosis was also induced in primary AECs by purified angiotensinogen (Fig. 4); at the maximal concentration of 5 µM, angiotensinogen induced apoptosis with the same potency as ANG II. The nonselective ANG II-receptor antagonist saralasin (50 µg/ml) completely inhibited AEC apoptosis induced by either ANG II or purified angiotensinogen, suggesting a mechanism mediated through ANG II interaction with one or both of its receptors. The nonthiol ACE inhibitor lisinopril (500 ng/ml) also blocked apoptosis in response to angiotensinogen but not in response to purified ANG II (Fig. 4). Purified angiotensinogen was not capable of inducing apoptosis in the human A549 cell line (data not shown), suggesting that these cells may not express ACE-like or reninlike activities sufficient to convert angiotensinogen to ANG II. However, apoptosis induced by purified ANG II in the A549 cell line was completely inhibited by saralasin (data not shown) at the same concentration (50 µg/ml) that abrogated both ANG II- and angiotensinogen-induced apoptosis of the primary AECs (see Fig. 4).

To determine whether AECs express ANG II receptors and ACE in vitro, RT-PCR was performed on A549- and AEC-derived total RNA with human and rat specific primers for the type 1 and type 2 ANG II receptors and ACE. Figure 5 indicates that both A549 cells and primary rat AECs express mRNAs for both the type 1 and type 2 ANG II receptors and ACE. All major PCR products were of the correct length as predicted by earlier studies (see METHODS for details).

**DISCUSSION**

ANG II was recently shown to induce apoptosis in cardiac myocytes (10) and endothelial cells (5). To our
The effective concentration of ANG II on primary AECs by cells of epithelial origin in response to ANG peptides. To our knowledge, this is the first demonstration of apoptosis by cells of epithelial origin in response to ANG peptides.

The lowest concentration of purified ANG II capable of inducing a significant increase in apoptosis of primary AECs (5 nM; Fig. 2) is only slightly higher than the mean concentration of ANG II in arterial plasma (85 pg/ml or 0.1 nM) obtained from patients with acute respiratory distress syndrome (24). Given the relatively short half-life (0.6 min) for ANG II in plasma (23), it seems reasonable to suspect that the local concentration of ANG II in the vicinity of cells that synthesize angiotensinogen and converting enzymes might easily exceed the threshold required for the induction of apoptosis in the alveolar epithelium. Very little is known about the extravascular local concentrations of ANG II or angiotensinogen in various microenvironments of the injured lung; the definition of the parenchymal lung cell populations that are the most potent generators of these molecules in the injured and repairing lung will be important topics for future inquiry.

As previously described (21), the scoring of cells on the basis of nuclear fragmentation (Fig. 1) provides an assay much less ambiguous than the now popular terminal deoxynucleotidyl transferase dUTP nick end labeling assay, which does not discriminate between apoptotic, necrotic, and autolytic cells (7). However, because it relies on the appearance of morphological changes that occur late in apoptosis, the nuclear morphology assay underestimates the true fraction of apoptotic cells. The fact that the apoptotic indexes determined by annexin V binding (10.0% basal and 40.9% ANG II stimulated; Fig. 3) were significantly greater than those scored by the PI assay (1.87 ± 0.41% at 0.0 dose vs. 9.46 ± 0.89% at 100 µM ANG II; Fig. 2) is consistent with the known ability of annexin V to identify cells in both early and advanced stages of apoptosis (21). Even so, the fact that the observed increase in caspase-3 activity in response to ANG II (50%; Fig. 3) was somewhat lower than that observed earlier for Fas-activated apoptosis in the same cell type (fourfold; see Ref. 19) suggests that the maximum fraction of epithelial cells induced to undergo apoptosis in response to ANG II likely did not exceed 50%. Regardless, the ability of purified ANG II to induce significant increases in the binding of annexin V and in the activity of caspase-3 supports the reliability of the PI nuclear morphology assay as a specific measure of changes in the apoptotic index.

The ability of purified angiotensinogen to induce apoptosis in primary AECs (see Fig. 4) suggests that these cells also express reninlike and ACE-like activities capable of converting ANG to ANG II. The hypothesis that this conversion is required for the induction of apoptosis by angiotensinogen is supported by the ability of lisinopril to abrogate apoptosis induced by ANG but not that induced by ANG II. Although RT-PCR suggests that both A549 cells and primary AECs express ACE, it is possible that both cell types express other peptidases such as chymase or cathepsins, which also cleave ANG 1 and angiotensinogen, respectively (1). On the other hand, the inability of ANG to induce apoptosis of A549 cells, in contrast to its potent induction in the primary cell isolates (see Fig. 4), suggests that A549 cells, at least under basal culture conditions, may not express sufficient reninlike activities to convert the ANG required to elicit an ANG II-dependent response. The fact that the ACE inhibitor captopril abrogated A549 cell apoptosis induced by anti-Fas antibodies (19) suggests that renin or reninlike activities in these cells may be induced by the activation of Fas, and this hypothesis is now being tested.

Earlier work from this laboratory (21) identified soluble factors that were produced by lung fibroblasts and were capable of inducing apoptosis of A549 and primary AECs. The factors were found in medium
conditioned by cells isolated from fibrotic lung but not in those prepared from normal lung fibroblast isolates. Subsequently, Uhal and colleagues reported AEC apoptosis adjacent to myofibroblasts (20) within the same biopsies of fibrotic human lungs that were used earlier for the derivation of the fibroblast isolates (21). Myofibroblasts from cardiac tissue were recently found capable of synthesizing ANG peptides in vitro (11). The possibility that myofibroblasts within the fibrotic human lung also synthesize ANG peptides is currently under investigation.

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