Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro

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Captopril; desethylamiodarone; type II pneumocyte; angiotensin-converting enzyme inhibitor

Amiodarone (AM) is a benzofuran derivative with class III antiarrhythmic activity that is effective in controlling intractable cardiac arrhythmias (13, 35). Evidence suggests that the drug may have a role in reducing the relative risk for arrhythmic or sudden death and overall mortality in survivors of myocardial infarction (5, 28) and in heart failure patients (10, 15, 34). Its clinical use, however, is limited by AM-induced pulmonary toxicity (AIPT), which occurs with an incidence of 6% and an estimated mortality of 5–10% in affected patients (26, 28). The pathology of AIPT, which includes alveolitis, phospholipidosis, and, in its advanced stages, irreversible fibrosis is well described, but the underlying mechanisms remain unclear (32, 44). A number of theoretical pathogenic mechanisms are suggested by animal and cell culture studies, including alterations in membrane properties, increases in intracellular free calcium (29), generation of radicals (41), lung phospholipidosis (24, 33), immunologic mechanisms (1), and direct cytotoxicity (32). Although the latter potential mechanism has been studied in isolated lung fibroblasts, endothelial cells, and alveolar macrophages (32), no studies to date have explored the possibility that AM or its primary metabolite desethylamiodarone (Des) might be capable of inducing apoptosis.

The type II alveolar epithelial cell (AEC) accomplishes many lung functions known to be affected in AIPT, including antioxidant defense, synthesis of the important lung-specific phospholipids comprising pulmonary surfactant, and local immunomodulation (36). In addition, the AEC is the stem cell for alveolar epithelial repair after lung injury and during normal AEC turnover (17, 36). Animal studies demonstrating that incomplete or delayed alveolar repair leads to acceleration of collagen deposition and lung fibroblast proliferation after injury (45) have led to the theory that AECs play a critical role in the healing of lung injury without fibrosis (19, 36, 40, 45). More recent investigations suggest an important role for apoptosis of AECs in animal models of lung fibrosis (12) and in the advanced stages of fibrotic lung disease in humans (2, 19, 39, 40). For these reasons, we hypothesized that AM and its primary metabolite Des might be potent cytotoxic for AECs by mechanisms involving both necrosis and apoptosis. We report here that AM or Des induces apoptosis and necrosis of both primary rat AECs and a...
human AEC-derived cell line and does so in primary AECs at concentrations significantly lower than those known be cytotoxic for other pulmonary cell types. Furthermore, we show that a significant component of the cytotoxicity is inhibitable by antagonists of the renin-angiotensin (ANG) system.

METHODS

Reagents and materials. AM and Des were kindly provided by Wyeth/Ayerst Research (Princeton, NJ). Captopril and saralasin were obtained from Sigma (St. Louis, MO). All other materials were obtained from sources described earlier (38, 43) or were of reagent grade.

Cell culture. The human lung adenocarcinoma cell line A549 was obtained from American Type Culture Collection and was cultured in Ham’s F-12 medium supplemented with 10% fetal bovine serum. Primary AECs were isolated from adult male Wistar rats as described earlier (37, 43). The primary cells were studied on day 2 of culture, at a time at which they are type II cell-like by accepted morphological and biochemical criteria (36). All primary cell preparations were of >90% purity as assessed by acridine orange staining as previously discussed (37). All cells were seeded in 24- or 6-well chambers, and all experiments were conducted at confluent 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 and necrosis. Detection of apoptotic cells with propidium iodide (PI) was conducted as described earlier (38, 43) after digestion of ethanol-fixed cells with DNase-free RNase in PBS containing 5 µg/ml of PI. Cells with discrete nuclear fragments containing condensed chromatin were scored as apoptotic; in the primary rat AEC preparations, apoptotic cells were scored only if they also displayed immunoreactivity with anti-cytokeratin antibodies but not the spindle shape of fibroblasts (37, 43). In replicate culture vessels, necrosis was detected as the loss of exclusion of PI after application of the vital dye directly to the cell culture medium at 5 µg/ml final concentration. Red fluorescent (PI-positive) cells (>570 nm) were scored over the subsequent hour as a percentage of the total cells in any given field. A minimum of four separate fields were scored in each of three separate culture vessels per experiment condition; all experiments were performed at least twice, and the data were compiled. Although all necrotic cells are PI positive, some apoptotic cells may also take up the dye, but these comprise a minor fraction of the total dead cell population (see RESULTS).

In all assays, detached cells were retained by centrifugation of the culture vessel at 300 g for 10 min with a centrifuge rotor designed for culture plates. For detection of necrosis (dye exclusion of PI), PI was added directly to the cell culture medium immediately before centrifugation of the culture vessel. For detection of apoptosis, ethanol was added directly to the cell culture medium at a final concentration of 70% immediately before the centrifugation. Afterward, the ethanol was carefully removed, and the total cell population (attached plus sedimented detached cells) was stained with PI as described earlier (37).

RESULTS

By dye exclusion assay, AM and Des caused concentration-dependent necrosis of human A549 cells beginning at 10 and 5 µg/ml, respectively (Fig. 1); at the higher doses of DES, the cytotoxicity was essentially 100%.

AM and Des also induced apoptosis in A549 cells beginning at 10 and 5 µg/ml, respectively (Fig. 3); at doses of Des > 5 µg/ml, the A549 cell number remaining on the culture vessels was so low as to preclude the accurate scoring of apoptotic cells. In the primary AECs (Fig. 4), AM and Des induced significant apoptosis beginning at 2.5 µg/ml for each agent (P < 0.05), a concentration essentially the same as the therapeutic serum level of 1.8 µg/ml (4). In both cell types, the concentration dependence of the induction of apoptosis was bimodal; fewer apoptotic cells were observed at the highest drug concentrations at which necrosis was overwhelmingly high (compare with Figs. 1 and 2).

Scoring of the total cell number at the end of the 20-h incubation revealed that both drugs caused significant cell loss per culture vessel in both A549 and primary AEC cultures (Figs. 5 and 6, respectively). Importantly, unattached cells were recovered and included in the analyses (see METHODS), and thus the data represent respectively (both P < 0.05; Fig. 2), concentrations at or below the known therapeutic serum concentration of AM (see Fig. 1) in patients receiving the drug for 9 mo (4). At the higher doses of DES, the cytotoxicity was essentially 100%.
net decreases in cell number rather than simple detachment from the substratum. In cultures that were not incubated with the drug (Figs. 5 and 6), the cell number did not change significantly during the 20-h incubation (data not shown).

In both A549 cells and primary rat AECs (Fig. 7, left), apoptosis in response to AM was inhibited by 81 and 97%, respectively, by the broad-spectrum caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk), confirming the specificity of the nuclear fragmentation assay for apoptosis under the conditions employed. Apoptosis of both cell types also was inhibited by the angiotensin-converting enzyme (ACE) inhibitor captopril (500 ng/ml) or by the nonselective ANG II-receptor antagonist saralasin (50 µg/ml); these data are consistent with earlier demonstrations that captopril could block Fas-induced apoptosis of the same cells (8, 38). Moreover, the same three agents significantly inhibited the AM-induced decrease in total cell number of either cell type (Fig. 7, right). The caspase inhibitor ZVAD-fmk abrogated 46 and 75%, respectively, of the net cell loss by A549 and primary rat AECs, indicating that a significant component of the cell loss was due to apoptosis per se.

**DISCUSSION**

The widespread use of AM for the treatment of ventricular and supraventricular cardiac arrhythmias has led to increased interest in its side effects, particularly pulmonary toxicity. It is now known that pulmonary toxicity is the most significant, most limiting, and potentially life-threatening side effect associated with AM use (21, 23, 29). It has been shown previously that AM and its active metabolite Des are directly cytotoxic to cell types other than AECs such as bovine arterial endothelial cells (22), alveolar macrophages (22, 27), human pulmonary arterial endothelial cells (30), interstitial lung fibroblasts (22), bronchial epithelial cells (6), and hepatocytes (11). To our knowledge, this is the first study to examine the possibility that AM or Des might be capable of inducing apoptotic cell death and only the second assessment of AM cytotoxicity toward cultured alveolar epithelial cells (14).

Several mechanisms underlying the adverse pulmonary effects of AM have been proposed. In addition to the direct cellular damage referred to above, these include derangements in lipid metabolism associated with the induction of phospholipidosis (3), immune-mediated mechanisms such as the activation of natural killer cell activity (16), and the development of edema associated with an increased production of superoxide anion (18). The tissue level of reduced glutathione was also found to be increased significantly. In contrast, pretreatment with antioxidant agents such as butylated hydroxyanisole, vitamin E, and N-acetylcysteine or ventilation with 40% oxygen protected against AM-induced edema (18). However, the relationship of these proposed mechanisms to the induction of lung fibrogenesis is unclear.

**Fig. 3. AM and Des induce apoptosis in human AECs. Human A549 cells were incubated for 20 h in Ham's F-12 medium with 1% FBS supplemented with purified AM or Des at indicated concentrations. At 20 h, cells were fixed with 70% ethanol, and fragmented nuclei were detected with PI (see METHODS). Arrows, [AM]serum in patients receiving AM for 9 mo (4). *P < 0.05 vs. V.**

**Fig. 4. AM and Des induce dose-dependent apoptosis in primary rat AECs. Rat AECs were treated in a manner identical to that described in Fig. 3. Note the greater sensitivity of well-differentiated rat AECs to AM-induced apoptosis compared with that of A549 cells (Fig. 3) and the bimodal response with increasing drug concentration. *P < 0.05 vs. V.**

**Fig. 5. AM and Des cause dose-dependent cell loss of human AECs. Human A549 cells were incubated as described in Fig. 1 with purified AM or Des at indicated concentrations. At 20 h, culture vessels were centrifuged to retain detached cells, cells were fixed with 70% ethanol, and total cell number (attached + detached) was counted (see METHODS). C, control. *P < 0.05 vs. C.**

**Fig. 6. AM and Des cause dose-dependent cell loss of rat AECs. Primary rat AECs were incubated with purified AM or Des at indicated concentrations. Total cell number (attached + detached) was scored as described in Fig. 5. Arrows, [AM]serum in patients receiving AM for 9 mo (4). *P < 0.05 vs. C.**
In this study, we have shown that AM and Des induce apoptosis in A549 cells and primary rat type II cells, two models of the type II AECs critical to a variety of important lung functions. The concept that AEC integrity and the ability to repair damage are critical determinants in the pathophysiology of pulmonary fibrosis is well supported (7, 45). In our study, significant cell death began at 10 µg/ml for AM and 5 µg/ml for Des in A549 cells (Fig. 1), consistent with the earlier observation that Des is more cytotoxic than AM (22). The well-differentiated primary AECs, however, were much more sensitive to either agent, with toxicity beginning at 1 and 0.1 µg/ml for AM and Des, respectively, in these cells. These doses are significantly lower than those shown earlier to induce cytotoxicity in other lung cell types (22, 27). Interestingly, AM and Des had equal potency for the induction of apoptosis in primary AECs (Fig. 4), but Des was more toxic for the induction of necrosis (Fig. 2). This discrepancy might be explained by the expression of the cytochrome P-450 monooxygenase system by AECs (20, 31), which is believed to metabolize AM to its more toxic metabolite Des (32). Two types of pneumocytes in vivo, type II AECs and bronchiolar nonciliated Clara cells, exhibit substantial cytochrome P-450-dependent monooxygenase activity, (9, 20, 31). To our knowledge, A549 cells do not express these enzymes, and this may explain the observation that A549 cells are less sensitive to AM-induced toxicity than primary AECs.

Regardless, the observation that the total cell number of either cell culture model was significantly decreased by 20 h of exposure to AM or Des (Figs. 5–7) indicates that these agents cause significant and net cell loss over time (rather than simple detachment; see METHODS) despite that fact that the apoptotic indexes under the same conditions were seemingly low (5–15%; Figs. 3, 4, and 7). Moreover, the fact that the net cell loss was inhibitable by the caspase inhibitor ZVAD-fmk (Fig. 7) indicates that roughly 50 and 75% of the net cell loss in A549 cells and primary AECs, respectively, could be attributed specifically to apoptosis. In vivo, the normally quiescent type II AECs have an extremely high capacity to enter the cell cycle and proliferate in response to injury but in vitro do not proliferate to any measurable degree (17, 36). With this information, it is not unreasonable to hypothesize that AM and Des could induce chronic apoptosis of AECs in patients receiving AM, even at the therapeutic serum levels of the drug, that might be offset by increased type II cell proliferation. As discussed earlier (25, 36), the notion that ongoing apoptosis and proliferation can occur simultaneously in the AEC population is supported by the observation of both a high cell birth rate and high rate of cell death by apoptosis within lung carcinomas. Considered in this perspective, AM toxicity for the lung epithelium might be viewed as dependent on the capacity of the epithelium to offset ongoing apoptosis. Although no previous studies of AM pulmonary toxicity have reported apoptosis in any lung cell type, the relatively small percentage of cells undergoing apoptosis in vitro (5–15%; Figs. 3, 4, and 7) support the speculation that apoptotic cells in vivo might have gone unnoticed.

More importantly, both the induction of apoptosis and the decrease in total cell number in response to AM in vitro were blocked by the ACE inhibitor captopril or by the ANG II-receptor antagonist saralasin (Figs. 5–7). Recently, published data indicate that Fas-induced apoptosis in A549 cells or primary rat AECs is also potently inhibited by captopril, saralasin, or other antagonists of the renin-ANG system and requires the synthesis of ANG II for its execution (38, 42). In light of those findings, the data reported in this manuscript suggest that AEC apoptosis induced by AM may also require the synthesis and binding of ANG II to its receptor but may have no relationship to Fas or other cell surface receptors per se. Lung phospholipidosis involving surfactant-like lipids is a well-documented feature of AIPT (32), but whether this phenomenon is related to dysfunction of the surfactant-producing type II epithelial cells is unclear. The theory that chronic apoptosis of AECs might be a contributor to the development of AM-induced lung phospholipidosis will be an interesting topic for future inquiry.

The inhibition of AM-induced apoptosis and cell loss by an ACE inhibitor or an ANG II-receptor antagonist is particularly interesting with regard to the widespread use and proven efficacy of these agents for a variety of
thoracic and vascular diseases. Although there is little information from prospective studies to evaluate patients who have received AM and ACE inhibitors at the same time, our data suggest that AIPT may be decreased today as a result of the recent increase in administration of ACE inhibitors concurrently with AM in patients with congestive heart failure and ischemic heart disease. However, the examination of this hypothesis will require more data from patients who received these agents.

In summary, AM or Des caused dose-dependent apoptosis, necrosis, and net cell loss by human AS49 cells and primary rat AECS in vitro. In this cell type, the cytotoxicity was significant at or below therapeutic serum AM concentrations and was severe at AM concentrations known to accumulate in human lung tissue. Apoptosis and net cell loss in vitro were inhibited by the caspase inhibitor ZVAD-fmk, by the ACE inhibitor captopril, or by the ANG II-receptor antagonist saralasin. The protective effect of renin-ANG system antagonists on AM-induced apoptosis by these cells in vitro supports the hypothesis that some aspects of AIPT, particularly lung fibrosis, might be reduced by the administration of these agents concurrently. This theory is currently being tested in an animal model.

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