Amiodarone-Induced Lung Toxicity

In Vitro Evidence for the Direct Toxicity of the Drug

W. J. MARTIN II, MD, and D. M. HOWARD, BS

Amiodarone, a new antiarrhythmic agent, is associated with serious lung toxicity. This study indicates that in vitro amiodarone can directly induce bovine pulmonary artery endothelial cells to form cytoplasmic lamellar inclusions characteristic of the lung biopsy findings described in vivo. These morphologic changes occur as soon as 4 hours after incubation with the drug and with as little as 1 µg/ml (within the therapeutic range). Amiodarone-induced endothelial cell injury, monitored by 51Cr release, occurs with as little as 10-20 µg/ml. The data suggest that amiodarone toxicity to the lung may be primarily related to its direct toxic effect on lung cells, and that the characteristic morphologic changes of cytoplasmic inclusions may represent an early sign of the drug's effect. (Am J Pathol 1985, 120:344-350)

AMIODARONE is a new investigational agent which holds great promise as an effective antiarrhythmic agent.1-3 The major factor limiting its widespread use is associated serious and sometimes fatal lung toxicity.4-8 The mechanism of this adverse lung reaction, however, is poorly understood and virtually unstudied.

Three key factors of amiodarone toxicity in human subjects are recognized: 1) There is evidence of a dose-dependent effect.2,4,7,9 2) Amiodarone concentrations in tissue such as fat, liver, heart, and lung may be 10-1000-fold higher than the concentration in blood.10,11 3) A characteristic pathologic feature of toxicity in various tissues including skin, cornea, liver, and lung is the presence of intracytoplasmic lamellar inclusions.9,6,12-14

Lung biopsies of subjects with amiodarone toxicity reveal a spectrum of changes from the presence of characteristic lamellar inclusions in lung parenchymal cells, including endothelial, alveolar epithelial, and interstitial cells, to diffuse end-stage pulmonary fibrosis. Whereas the presence of lamellar inclusions represents an interesting marker for the effect of amiodarone on the lung, it is not established that amiodarone can directly induce these changes in lung cells, nor is it established what relevance these changes have to amiodarone-induced lung cell injury. Currently, there is no in vitro model of amiodarone toxicity which would permit an assessment of the direct effect of the drug on lung cells.

To address these questions, we developed an in vitro cell culture model of amiodarone toxicity to assess the direct effect of the drug on bovine pulmonary artery endothelial (BPAE) cells. These cells have been well-defined in culture and are intended to approximate the pulmonary capillary endothelial cells.15,16 In this study, we have established that amiodarone at therapeutic blood concentrations of 1-5 µg/ml11 can directly induce lamellar inclusion formation in the cytoplasm of BPAE cells. These changes occur as early as 4 hours after incubation with the drug. The direct toxicity of amiodarone to BPAE cells (monitored by 51Cr release) can be demonstrated with as little as 10-20 µg/ml and appears to be first evident at 18 hours. This in vitro model of amiodarone toxicity demonstrates for the first time the direct effect of amiodarone on lung cells with dose- and time-dependent effects of the drug on lamellar inclusion formation and cytotoxicity.

Materials and Methods

BPAE cells were isolated as previously described with only minor modifications.15-16 Bovine pulmonary arteries were obtained from a local slaughterhouse and arrived in the laboratory within 30 minutes after the

Supported in part by Grant HL 282029 from the National Heart, Lung, and Blood Institute and the Rappaport Clinical Investigator Award.

Accepted for publication April 3, 1985.

Address reprint requests to W. J. Martin II, MD, East 18A, Mayo Clinic, 200 First Street Southwest, Rochester, MN 55905.
animals were killed. The lumen of each artery was treated with 0.5% collagenase for 30 minutes at 37 C, and the cells released from the artery were washed, resuspended in Medium 199 with 10% fetal calf serum (FCS) (Sterile Systems, Logan, Utah), 0.5 µg/ml amphotericin (Squibb, Princeton, NJ), and 4 µg/ml gentamicin (Schering, Kenilworth, NJ). Cells were plated in a 24-well culture plate (Co-Star, Cambridge, Mass) sufficient to achieve 80-100% confluency in 3-5 days. BPAE cells were confirmed as endothelial in origin on the basis of typical polygonal growth patterns, the presence of factor-VIII-related antigen, and the presence of angiotensin-converting enzyme.15,16

**Morphology of Amiodarone-Treated BPAE Cells**

BPAE cells in monolayer culture were exposed to amiodarone (LaBaz Laboratories, Ambares, France) at concentrations of 1, 2, and 5 µg/ml for 4, 8, and 18 hours. These concentrations were chosen because the normal serum therapeutic level of amiodarone is 1-3 µg/ml.14 Control cells were maintained in either culture medium alone or culture medium plus appropriate concentrations of benzyl alcohol and Tween-80 (trace constituents of the supplied diluent in which amiodarone is dissolved). There were no morphologic differences between the control populations. Monolayer cultures were examined by phase microscopy, and the presence of cytoplasmic inclusions was monitored and quantified. Cell differentials were performed (minimum of 200 cells counted), the cells exhibiting cytoplasmic inclusions defined as demonstrating >3 inclusions per cell or 1 or 2 inclusions occupying as much as 50% of the endothelial cell cytoplasm. All data are expressed as means ± the standard error of the mean (SEM).

To further define the type of cytoplasmic inclusions present in amiodarone-treated cells, we used transmission electron microscopy to determine ultrastructural characteristics. BPAE cells were grown to confluency in a 100-mm tissue culture plate and were exposed to 5 µg/ml amiodarone for 4, 8, and 18 hours. To inspect many BPAE cells in cross-section, it was necessary to first remove the cells from the tissue culture plate and prepare them in suspension for electron microscopy. BPAE cells at specific time intervals were briefly exposed to trypsin-EDTA, washed, resuspended in Trump's fixative, washed in phosphate buffer, postfixed in 1% OsO₄ stained en bloc with 2% uranyl acetate, and submitted for transmission electron microscopy.

**51Cr Cytotoxicity Assay**

BPAE cells were used as target cells in a 51Cr cytotoxicity assay for assessment of the direct toxic effect of amiodarone on pulmonary endothelial cells. The assay was conducted as previously described,15,16 except that all studies were conducted with cells in monolayer culture. With BPAE cells 80-100% confluent in 24-well plates, 30 µCi/ml 51Cr (New England Nuclear, Boston, Mass) was added to the culture medium for 18 hours. BPAE cells were then washed extensively with culture medium. Amiodarone at 1, 5, 10, 20, 30, and 40 µg/ml was added to the culture medium for 18 hours, with a final volume of 1.0 ml per well. Time course studies were performed with amiodarone at 30 µg/ml at 4, 8, and 18 hours. Control cells were maintained either in culture medium alone or in culture medium plus appropriate concentrations of the trace constituents in the diluent, benzyl alcohol and Tween-80. These constituents of the diluent, comparable to 100 µg/ml amiodarone (0.002% benzyl alcohol and 0.004% Tween-80), were nontoxic.

At selected time intervals 0.5 ml of cell-free culture medium was removed from each well (A), and the remaining medium and cells were removed after the addition of 100 µl 10% Triton X-100 to the well (B), and both samples were counted in a gamma counter. The percent release (%R) of 51Cr is defined as 2A/A + B. The "spontaneous release" of 51Cr at 18 hours by control cells is 23.4 ± 0.7% (n = 18), which is well below the value of 30% maximal release considered to be the upper limit for such assays.17 BPAE cell injury was quantified in terms of a cytotoxic index (CI) defined as: (%R experimental − %R control)/(%R maximal release − %R control) ×100 where %R maximal release represents the dpm release from control cells treated with 2% Triton X-100 for the entire time interval. With this formula, a CI of 100% indicates that the toxic agent, ie, amiodarone, causes a release of all 51Cr that was "releasable" from the endothelial cells. A CI of 0% indicates no release of 51Cr beyond that due to the spontaneous release during the incubation period by control endothelial cells. All cell samples were run in duplicate with a minimum of 12 determinations.

**Results**

Amiodarone directly induces the formation of intracytoplasmic inclusions in monolayer BPAE cells (Figure 1). The inclusions first appear as small, clear vacuoles by phase microscopy and can progress to occupy as much as 50% of the size of the endothelial cell. The inclusions are most frequently multiple within a cell, although one or two inclusions per cell appear to predominate. The cells appear otherwise normal at these concentrations (1-5 µg/ml) and do not detach from the tissue culture plate.

The development of amiodarone-induced cytoplasmic inclusions in BPAE cells is a both time- and dose-dependent process (Table 1). Inclusions can be detected
in BPAE cells as early as 4 hours after exposure to amiodarone, with the number of inclusion-containing cells increasing as a function of time. For example, at an amiodarone concentration of 1 µg/ml, there are 6.3% ± 0.3% of cells containing inclusions at 4 hours, with an increase to 16.8% ± 7.0% at 18 hours (P < 0.05, compared with control cells). Similarly, at each time interval, the number of inclusion-containing cells increases as a function of amiodarone concentration (Table 1). At 18 hours, amiodarone concentrations of 0, 1, 2, and 5 µg/ml result in inclusion-containing cells of 0.6% ± 0.4%, 16.8% ± 7.9%, 38.3% ± 14.0%, and 77.3% ± 8.7%, respectively (P < 0.001). Thus, at therapeutic concentrations, pulmonary endothelial cells in vitro developed the same cytoplasmic inclusions described in lung cells with amiodarone toxicity in vivo.4,6

To determine the ultrastructural correlate of the cytoplasmic inclusions detected by phase microscopy, BPAE cells exposed to amiodarone (5 µg/ml) were assessed by transmission electron microscopy (Figure 2). The ultrastructural details of these inclusions indicate that they may appear empty or contain material which is amorphous or lamellar in appearance. Lamellar structures have been previously described as characteristic of phospholipid formation18,24 and have also been described with in vivo amiodarone toxicity.4,6,12-14 The relationship of the amorphous material to the lamellar structures is unknown, although the morphologic findings (Figure 2B) would suggest the amorphous material can be shaped into lamellar patterns by the endothelial cell. This has the morphologic appearance of “spinning wool,” and the findings suggest that both types of material within the inclusion may have a similar biochemical makeup.

Amiodarone is directly cytotoxic to BPAE cells, as determined in a 51Cr cytotoxicity assay. BPAE cell injury increases both as a function of amiodarone concentration (Figure 3A) and as a function of time (Figure 3B). At concentrations of 10, 20, 30, and 40 µg/ml the cytotoxic index increased to 5.4 ± 1.7, 17.1 ± 3.7, 33.3 ± 5.2, and 49.5 ± 5.1, respectively (P < 0.01, all comparisons). The cytotoxic effect of amiodarone (30 µg/ml), however, was not apparent after 4 and 8 hours of incubation, with a respective CI of 1.0 ± 0.9 and 3.0 ± 1.1, but was clearly evident after 18 hours, with a CI of 33.3 ± 5.2 (P < 0.001). There was no evidence of BPAE cell injury with amiodarone at 1-5 µg/ml, a concentration with which striking morphologic changes were previously noted. This suggests (within the sensitivity of the cytotoxicity assay) that the morphologic changes of cytoplasmic inclusions precede evidence of cell injury and appear to be a more sensitive index of the effect of the drug.

Discussion

Amiodarone-induced lung toxicity represents a life-threatening complication and is a major limiting factor in its widespread use as an antiarrhythmic agent.4-8 This study indicates that amiodarone toxicity in vitro can duplicate the characteristic morphologic features described with the drug in vivo, ie, the presence of cytoplasmic inclusions which give a “foamy” appearance to cells on lung biopsy and ultrastructurally are associated with the presence of lamellar structures.4,6 These morphologic changes occur at concentrations with which there is no evidence of lethal cell injury, and may represent an early sign or harbinger of serious drug toxicity. An improved understanding of amiodarone-induced lung cell injury in vitro may provide insight into the future modulation of amiodarone-induced lung injury in vivo.

Intracytoplasmic lamellar structures are typically thought to represent phospholipid accumulation within the cell. The presence of such lamellar structures may represent a normal finding in phospholipid-secreting cells such as Type II alveolar epithelial cells22 or may represent the hallmark for a disease state such as the phospholipidoses, including Niemann–Pick disease, Fabry’s disease, and Tay–Sachs disease.19-21 The evidence linking phospholipids with these cellular changes is not simply circumstantial; rather, it is known that pure phospholipids in aqueous solution can spontaneously form these characteristic lamellar patterns.23 Thus, the finding that amiodarone can directly induce

---

**Table 1 — Percentage of BPAE Cell Injury Demonstrating Amiodarone-Induced Cytoplasmic Inclusions**

<table>
<thead>
<tr>
<th>Amiodarone (µg/ml)</th>
<th>Time (hours)</th>
<th>4</th>
<th>8</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2 ± 0.4</td>
<td>1.9 ± 1.2</td>
<td>0.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.3 ± 3.0</td>
<td>8.4 ± 4.3</td>
<td>16.8 ± 7.9*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>31.6 ± 12.2*</td>
<td>32.1 ± 14.1*</td>
<td>38.3 ± 14.0*</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>60.0 ± 10.7†</td>
<td>71.5 ± 7.1†</td>
<td>77.3 ± 8.7†</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05. † P < 0.001.

---

**Figure 1 — Effect of amiodarone on BPAE cells in Medium 199 and 10% FCS for 18 hours (phase microscopy).**

A — Control BPAE cells revealing typical polygonal growth pattern with no evidence of cytoplasmic inclusions. (x 400)  
B — Amiodarone-treated (5 µg/ml) cells revealing a “foamy” appearance in endothelial cells indicative of many cytoplasmic inclusions within virtually every cell. (x 400)  
C — Amiodarone-treated (5 µg/ml) cells revealing a slightly different pattern of inclusion formation with many cells demonstrating a predominance of 1 or 2 inclusions per cell. (x 400)
Figure 2—Evidence of amiodarone's effect on monolayer BPAE cells (18 hours) fixed and prepared in suspension for transmission electron microscopy. 

A—Control BPAE cells revealing normal morphologic features. (x 8170) B—Amiodarone-treated (5 μg/ml) BPAE cells demonstrating multiple cytoplasmic inclusions within each cell. Inclusions may appear empty, contain amorphous material, or exhibit typical lamellar structures. (x 8170) Inset—Amiodarone-treated cell with the appearance of active lamellar formation within the cytoplasmic inclusion. (x 22,230)

such changes in susceptible lung cells suggests that the drug may alter phospholipid metabolism within the cell.

Amiodarone is a cationic amphiphilic drug, and other drugs in this class have been associated with abnormalities in phospholipid metabolism. It has been proposed that amphiphilic drugs bind to lysosomal lipids and impair normal phospholipid catabolism by phospholipases. The relationship of phospholipid accumulation with lamellar structures and subsequent cell injury is not clear. Certainly, this in vitro model indicates that dramatic inclusion formation may occur at concentrations of amiodarone (1–5 μg/ml) with which there is no evidence of cell injury. However, it must be emphasized that with only a very modest increase in amiodarone concentration (10–20 μg/ml), significant injury to the endothelial cells is easily demonstrated. Furthermore, this time course of cell injury is monitored for only a relatively brief period of 18 hours, and cell injury might be evident beyond this time interval at even lower concentrations of the drug. This represents a very small margin of safety to the cell from where morphologic changes are first noted and cytotoxicity occurs.

Figure 3—Incubation of amiodarone with 51Cr-labeled BPAE cells. Cell injury is quantified as the cytotoxic index which expresses the percentage of 51Cr release from the amiodarone-treated cells where 0% represents "spontaneous release" by control cells and 100% represents maximal release by cells treated with Triton X-100. A—BPAE cells incubated with amiodarone from 1 to 40 μg/ml for 18 hours, revealing a positive dose-response effect.

B—BPAE cells incubated with amiodarone (30 μg/ml) demonstrating no significant injury at 4 and 8 hours, but with significant cytotoxicity apparent at 18 hours. All data are the mean ± SEM.

The source of the phospholipid material within the cells exposed in vivo to amphiphilic drugs is unknown. The appearance of "foamy" alveolar macrophages in drug-treated animals or human subjects has been at-
tributed to the ingestion of phospholipids secreted by Type II alveolar epithelial cells.\textsuperscript{20,24} Clearly, our study indicates that amphiphilic drugs such as amiodarone can directly induce lamellar structure formation without the need to implicate ingestion of preformed lipids made by other cell types in the lung. It is likely that phospholipid accumulation in the lung cells of amiodarone-treated subjects results from the direct effect of the drug on the target cells. Such an effect may be highly nonspecific and may result in abnormal phospholipid metabolism in amiodarone-exposed cells from different organ systems.

Unlike many adverse drug reactions, the lung toxicity associated with amiodarone may be very slow to resolve and can even progress despite discontinuation of the drug.\textsuperscript{4,5,8,9} This may be attributed in part to two factors: 1) the ability of peripheral tissue such as the lung to concentrate the drug over comparable blood levels\textsuperscript{10,11,27} and 2) the long elimination half-life of the drug, 15–45 days.\textsuperscript{10,28,29} Our study indicates amiodarone-induced cytotoxicity to pulmonary endothelial cells occurs with as little as 10–20 µg/ml. If the lung concentrates the drug only fourfold over therapeutic blood levels (1–3 µg/ml), significant cell injury might be expected. In fact, previous studies indicate lung tissue amiodarone levels may be 100 times higher than blood levels,\textsuperscript{11,27} suggesting that this \textit{in vitro} model of amiodarone toxicity is potentially relevant to the clinical effect of the drug.

It is unknown why some individuals appear to be susceptible to amiodarone-induced lung toxicity, and others are not. It is also unknown why different organ systems such as the lung, liver, skin, or eye may be spared or may be involved within a given patient. Future studies will probably focus on the extent of protein binding by the drug in the circulation as well as the affinity of the drug for certain cell types in various organs. Factors that influence these parameters may provide clues to organ and individual susceptibility. Additionally, some forms of amiodarone toxicity in the lung may be mediated in part by an inflammatory or immune response.\textsuperscript{90} Improved insight into amiodarone-induced lung injury may permit development of strategies to modulate this drug-induced insult to the lung and thereby reduce the morbidity and mortality associated with its clinical use.

References