Effects of Amiodarone on Elastin Biosynthesis in Primary Hamster Lung Cell Cultures (43209)

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Abstract. **Amiodarone is a Class III antiarrhythmic agent that has been implicated as a cause of human pulmonary fibrosis. Pulmonary fibrosis is associated with increased levels of connective tissue proteins such as collagen and elastin. The purpose of this investigation was to determine whether elastin synthesis would be altered by *in vitro* amiodarone administration.**

Primary hamster lung cell cultures were utilized. Cultures were treated with 2, 10, and 20 μg/ml amiodarone. Following treatment, elastin synthesis was monitored by a biochemical tracer assay based on the presence of the cross-linking amino acids: desmosine/isodesmosine. These cross-links are found only in elastin. Addition of [^{14}C] lysine to cultures results in uptake of the radiolabel into the cross-links. Cross-links were isolated and identified using chromatography and electrophoresis. At all doses of amiodarone, elastin synthesis was seen to increase above control levels.

Light and electron microscopy confirmed the presence of an extracellular matrix. The morphologic studies also revealed the presence of cytoplasmic inclusion bodies and vacuoles that are often associated with cationic, amphiphilic drugs such as amiodarone. [P.S.E.B.M. 1991, Vol 196]

Elastin is a connective tissue protein found largely in areas of the body subjected to periodic stress. It comprises 20–30% of the connective tissue of the lung (1). In the lung, elastin is found in the parenchyma, pleura, blood vessels, trachea, and bronchi and around the openings of alveoli (2).

Elastin plays a vital role in maintaining lung structure. Any alteration in elastin or its synthesis may create uneven stresses on lung parenchyma. These stresses may lead to uneven inflation, perfusion, and diffusion (3).

Several disease states are known to affect the connective tissue components of the lung (4). Interstitial pulmonary fibrosis, defined as the generation of excessive amounts of fibrous connective tissue with a resulting decline in pulmonary elasticity, is one such disease (5).

Amiodarone, a benzofuran derivative categorized as a Class III antiarrhythmic drug, is an agent capable of inducing pulmonary fibrosis (6). Once fibrosis is initiated by amiodarone, the condition may progress despite discontinuation of the drug (7).

Today, amiodarone is often employed experimentally to induce fibrosis in animals (8). This experimentally induced fibrosis provides a means to explore possible mechanisms of fibrosis and to evaluate possible treatments.

However, there are disadvantages (9) associated with *in vivo* study of fibrosis and elastin synthesis. Disadvantages include incomplete extraction of elastin and difficulty in measuring specific activity and reutilization rate of isotopic precursors.

To circumvent these problems, *in vitro* studies involving newly synthesized, metabolically labeled elastin have been conducted. Although there exist over 42 cell types in the lung and airways, to date synthesis of insoluble elastin has been confirmed in only four of these cell types: chondrocytes (10), mesothelial cells (11), endothelial cells (12), and smooth muscle cells (13). No evidence for the formation of insoluble elastin

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has been noted from cultures of fibroblasts except for those grown on an elastin matrix (14). None of the cell types mentioned have been monitored for elastin synthesis following amiodarone treatment.

This investigation is the first to employ an *in vitro* system (primary hamster lung cell cultures) to study elastin synthesis following amiodarone treatment. Since primary cultures contain a variety of lung cells, these cultures more accurately reflect *in vivo* conditions than do cultures of isolated lung cells, especially those having undergone serial passages.

Materials and Methods

Establishment of Primary Cultures. Female Syrian hamsters were killed by a lethal dose of pentobarbital, and the lungs were removed aseptically and placed in cold Ham's F-12 medium (Gibco, Grand Island, NY). Lungs were cleared of extraneous blood vessels, minced into pieces approximately 1 mm^3 in size, and placed in 25-cm^2 Corning tissue culture flasks containing 2 ml of supplemented medium (Ham's F-12 with 10% heat-inactivated fetal bovine serum, 1 mg of streptomycin/ml, and 100 units of penicillin/ml). Cultures were maintained at 37°C in an atmosphere containing 95% air-5% CO_2. Once cellular outgrowth from the tissue was apparent, tissue pieces were removed from the flasks, and cells were allowed to reach confluency. Cells were then subcultivated at a density of 160,000 cells/ml into T-25 tissue culture flasks containing a total of 5 ml. Preliminary studies confirmed the production of insoluble elastin by these cultures. Confluent subcultivated cultures were treated and analyzed for elastin synthesis.

Amiodarone Treatment. Amiodarone (LaBaz Laboratories, Ambarès, France) was prepared fresh prior to each dosing period. The drug was dissolved in Ham's F-12 at a concentration of 100 μg/ml. Cells were exposed to 2, 10, and 20 μg/ml, respectively, for 18 hr. Since injectable amiodarone is dissolved in a vehicle of 1% benzyl alcohol, 2% Tween 80, control cells were treated with these percentages of vehicle dissolved in Ham's F-12. Experiments were performed in triplicate.

Labeling of Cross-Links. Following treatment, cells were rinsed free of drug solutions, provided with fresh media containing 4 μCi of [^{14}C]lysine (Amersham, Arlington Heights, IL)/5 ml media, and pulsed for 7 days.

Elastin Assay. Elastin production was analyzed according to the method of Keller *et al.* (15). Briefly, following the pulsing period, cells were scraped, pooled, and dialyzed against several changes of distilled water (d-H_2O). Nondiffusable material was lyophilized, and equal amounts of each sample were hydrolyzed in 6 *N* HCl. Hydrolysates were evaporated three times, reconstituted in d-H_2O, filtered through Whatman no. 4 filter paper, and lyophilized once again. Lyophilized hydrolysates were reconstituted in d-H_2O, spotted on Whatman 3 MM paper strips, and subjected to descending chromatography three times in a solution of butanol:acetic acid:water (4:1:1). Origins of the chromatograms were cut out and eluted in d-H_2O. Eluates were centrifuged, and supernatants were removed and lyophilized. Lyophilized material was reconstituted in d-H_2O and spotted on 20- × 20-cm 0.1-mm cellulose thin-layer plates (Brinkmann Instruments, Westbury, NY). Standards of desmosine and isodesmosine (Elastin Products, St. Louis, MO) were spotted alongside the samples. Plates were subjected to one-dimensional electrophoresis in a solution of pyridine:acetic acid:water (1:10:189) at 600 V for 90 min.

Identification of Cross-Links. Plates were air-dried, and standards were sprayed with ninhydrin and developed at 45°C. Samples were scraped from the plates using the location of the standards as a guide, placed into liquid scintillation vials, and read in a Packard TriCarb 460CD liquid scintillation spectrophotometer.

Protein Determination. Protein concentrations were measured by the Pierce bicinchoninic acid protein assay (16). Bovine serum albumin served as the standard.

Examination of Primary Cultures. Primary cultures were examined by phase contrast microscopy and photographed at various magnifications.

Electron Microscopy. Cells were scraped, pelleted, and fixed for 1 hr in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4). Pellets were minced into pieces 1 mm^3 or smaller, rinsed with cacodylate buffer, and postfixed in osmium tetroxide for 1 hr at 4°C. Following fixation, pellets were rinsed, stained for 1 hr with 0.5% uranyl acetate, dehydrated in increasing concentrations of acetone, and embedded in an LX-112/Araldite mixture. Semi-thin sections were cut and stained with toluidine blue. Thin sections were cut, double-stained with uranyl acetate and lead citrate, and examined at 75 kV with a Hitachi 11E electron microscope.

Cytotoxicity by Microtitration. Cells were plated on 96-well plates (Costar) and exposed to various treatment groups according to the method of Ende *et al.* (17). Cells were plated at a density of 2500/well and incubated at 37°C in a 95% air-5% CO_2 humidified atmosphere for at least 48 hr in the presence of 50 μl of supplemented medium and amiodarone. Amiodarone concentration in the first well was 12,500 μg/ml. A serial 2-fold dilution of the drug was performed across the plate resulting in dilution factors of 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, and 4096. Following incubation, the contents of the first row were gently aspirated, and each well was rinsed with phosphate-buffered saline to remove traces of serum. The phosphate-buffered saline was then removed and 0.4% try-

pan blue was added to each well. Cells were stained for 10 min. Following removal of the stain and rinsing with phosphate-buffered saline, cells were assayed for increased membrane permeability as demonstrated by uptake of the supravital dye, trypan blue. The number of stained cells per total number of cells per field, as viewed at ×100, was recorded. Since dye uptake increases with time, only one row at a time was stained and assayed. Results are based on the mean counts (±SD) from three fields and three separate experiments.

Statistical Methods. Statistical analysis of data included a post hoc one-way analysis of variance.

Results

Figure 1 represents the results of a cytotoxicity by microtitration assay. Normal serum therapeutic levels of amiodarone range between 1 and 3 μg/ml. However, amiodarone concentration in tissues such as the lung, heart, and liver may be 10–1000 times higher than blood concentrations (18, 19). The amiodarone concentrations used in this investigation account for normal serum therapeutic levels as well as for elevated levels of the drug found in the lung.

According to the cytotoxicity study (Fig. 1), at doses of amiodarone ranging between 2 and 24 μg/ml, the viability of cells was 97.9% or greater. Therefore, the drug doses used in this investigation (20, 10, and 2 μg/ml) had no significant effect on the cells' ability to survive.

Figure 2 depicts the results of multiple elastin assays in cpm/mg protein percent of controls. Figure 2 indicates an increase in elastin synthesis at all doses of amiodarone used. Cultures treated with 2 μg/ml of amiodarone exhibited an average 81% increase in elastin synthesis over controls. At a dose of 10 μg/ml, amiodarone-treated cultures exhibited an average 52% increase in elastin production over controls. This dose was significant at the $P < 0.05$ level. Finally, cultures treated with 20 μg of amiodarone/ml exhibited an

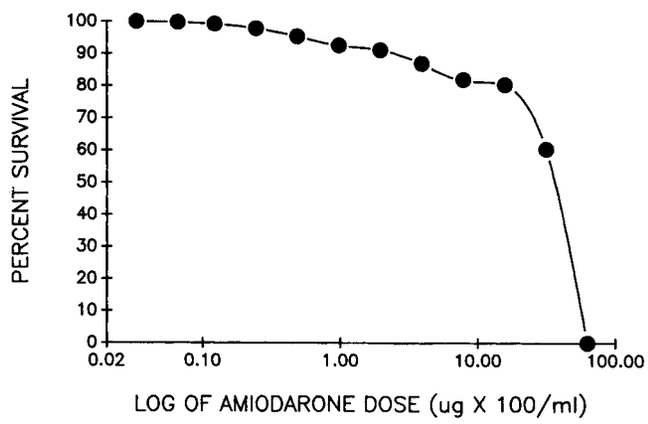

Figure 1. Cytotoxicity by microtitration assay. Note that the experimental doses result in cell viability of 97% or greater.

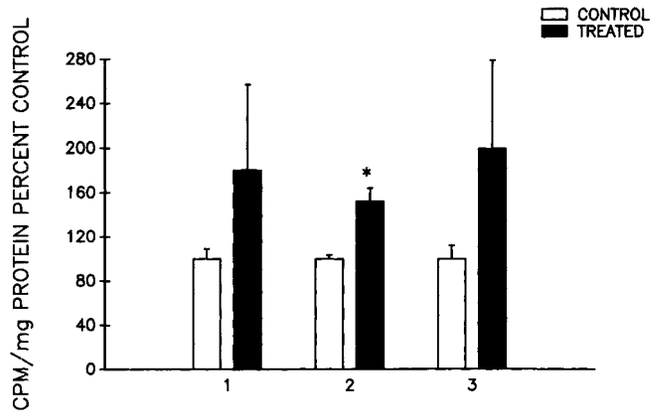

Figure 2. Amiodarone-induced increases of elastin synthesis in cpm/mg protein percentage of controls. Data represent mean ± SE of experiments performed in triplicate. Histograms 1, 2, and 3 represent, respectively, 2, 10, and 20 μg/ml doses of amiodarone. These doses correspond, respectively, to 81%, 52%, and 99% increases in elastin synthesis above controls. * Significance at the $P < 0.05$ level.

average 99% increase in elastin synthesis over controls. Both the 2- and the 20-μg/ml amiodarone-treated cultures exhibited a trend toward an increase in elastin synthesis. In general, increase in elastin production does not occur in a dose-dependent fashion.

In this investigation, in addition to employing a biochemical assay for elastin, both light and electron microscopy were performed. Other investigators (20) have reported on the appearance of cytoplasmic inclusion bodies and vacuoles in cells treated with amiodarone. The light and electron microscopy performed in this study confirm this finding.

Figure 3 is a composite of photomicrographs of control and amiodarone-treated cultures. Figure 3A is a photomicrograph of a vehicle-treated control culture of primary hamster lung cells. Cells appear to be normal and there is no evidence of inclusion bodies of vacuoles. This photomicrograph is representative of all control cultures. Figure 3B–D are representational photomicrographs of 2-, 10-, and 20-μg/ml amiodarone-treated cells, respectively. As the dose of amiodarone is increased, the appearance of inclusion bodies and vacuoles increases. Cultures treated with 20 μg of amiodarone/ml displayed a dramatic number of inclusion bodies and vacuoles as compared with lower doses of the drug.

Figure 4 is a composite of electron micrographs of control and treated cultures. Regardless of cell types observed, electron microscopy revealed cells to be resting on a thick layer of extracellular matrix (ECM). Figure 4A is an electron micrograph of a cell from a vehicle-treated control. Control cells possessed a homogenous cytoplasm with normal-appearing organelles. In addition, control cells exhibited few vacuoles or inclusion bodies.

Figure 4B is an electron micrograph of a cell from a culture treated with 2 μg of amiodarone/ml. The

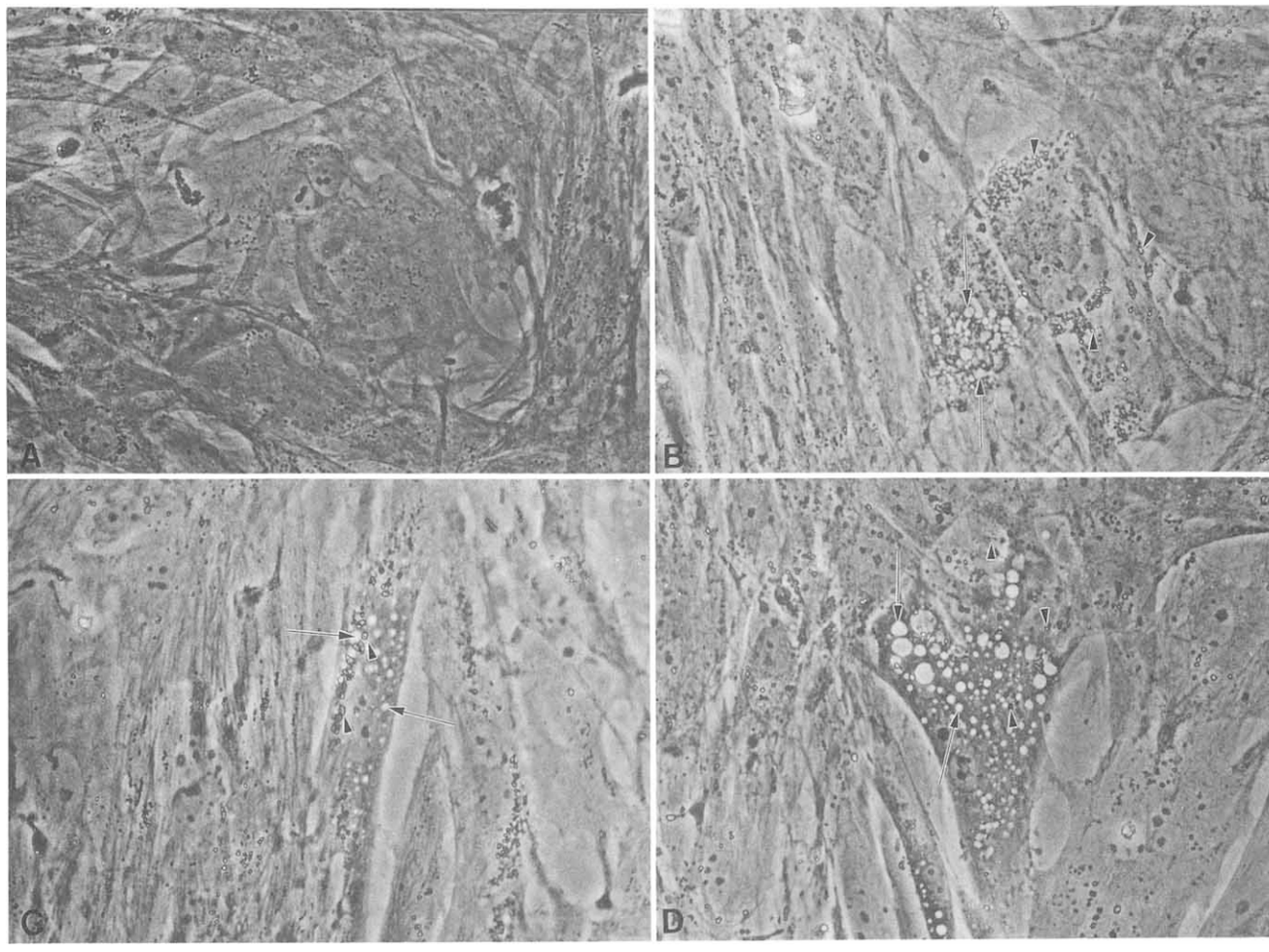

Figure 3. Phase contrast photomicrographs from control and treated cultures. Labels include vacuoles (arrows) and inclusion bodies (arrowheads) (original magnification ×250). (A) Vehicle-treated (control) primary culture of hamster lung cells. (B) primary culture of hamster lung cells treated (18 hr) with 2 μg of amiodarone/ml. Cells at this dose exhibit moderate numbers of cytoplasmic inclusion bodies and vacuoles. (C) Primary culture of hamster lung cells treated (18 hr) with 10 μg of amiodarone/ml. Cells at this dose exhibit increasing numbers of cytoplasmic inclusion bodies and vacuoles. (D) Primary culture of hamster lung cells treated (18 hr) with 20 μg of amiodarone/ml. Cells at this dose exhibit a large number of cytoplasmic inclusion bodies and vacuoles. The cells appear "perforated" since the majority of the cytoplasm is occupied by vacuoles.

juxtanuclear region of this cell presents large vacuoles and inclusion bodies. This cell is typical of cells treated with 2 μg of amiodarone/ml. The myofilaments in the cytoplasm indicate that this cell resembles a smooth muscle cell.

Figure 4C is an electron micrograph from a culture treated with amiodarone at a dose of 10 μg/ml. It is of interest to note that the cytoplasm is filled with numerous vacuoles and large inclusion bodies. Upon closer examination, the inclusion bodies are observed to contain material arranged in the typical whorl-like pattern associated with lamellar inclusion bodies. The cell type represented in Figure 4C is fibroblast-like.

Finally, Figure 4D depicts an electron micrograph of a cell from a culture treated with 20 μg of amiodarone/ml. Again, the cytoplasm is filled with numerous vacuoles and inclusion bodies.

Discussion

The results of this investigation indicate that elastin synthesis is increased when primary hamster lung cell cultures are exposed to various concentrations of the fibrotic agent amiodarone. The assay performed to determine formation of insoluble elastin is based on a technique developed by Keller *et al.* (15). Production of insoluble elastin is considered to accompany the appearance of ^{14}C-radiolabeled elastin-specific cross-links desmosine and isodesmosine. This technique is designed to radiolabel, isolate, and identify these cross-links. The radiolabeling period was chosen to be 7 days since pilot studies indicated this time period to be sufficient to ensure development of cross-links. Sufficient time for development of cross-links is important since it is known that once allysine is formed, equilibrium to the stable end-products desmosine/isodesmosine is a relatively slow process (21). Increases in cpm/

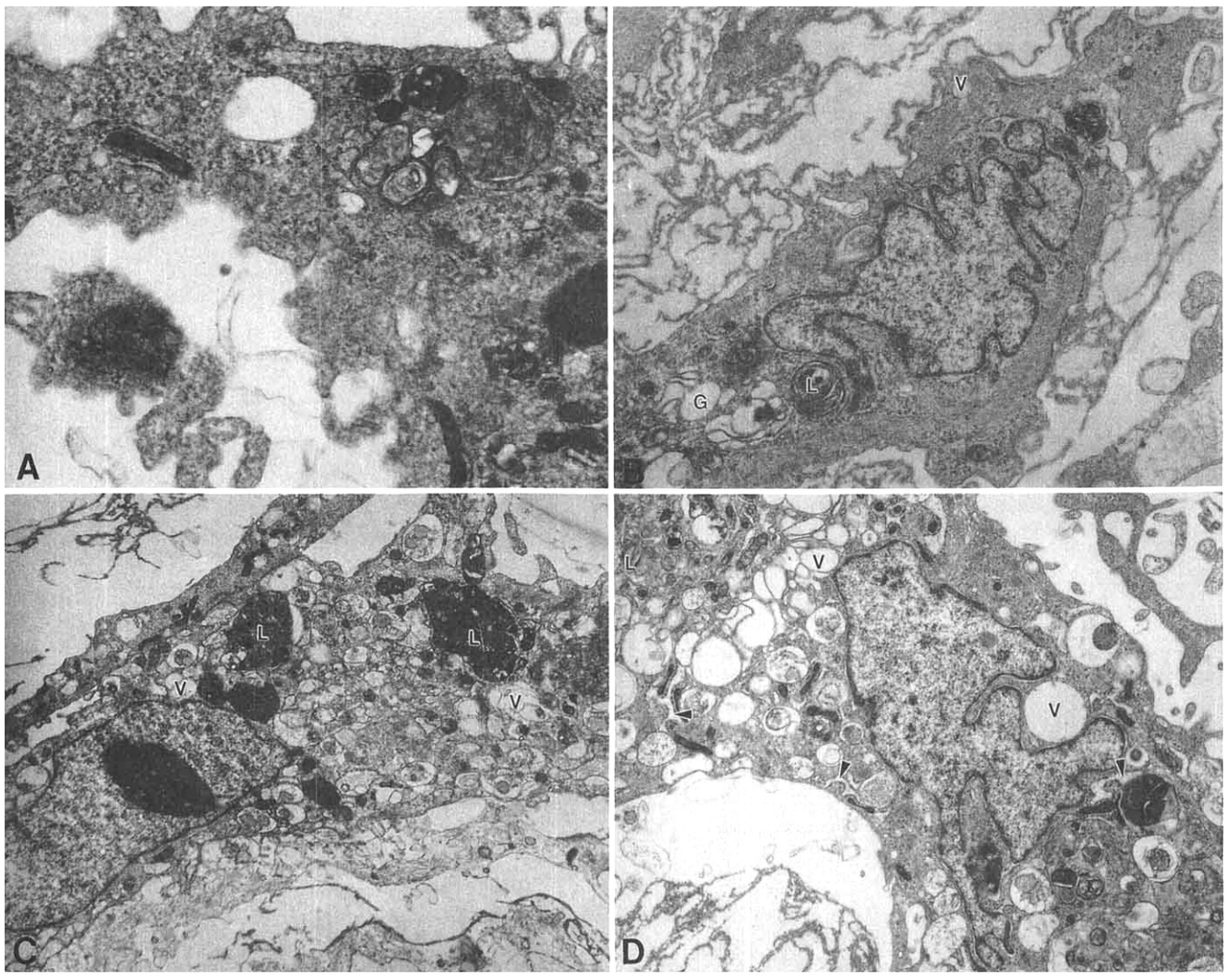

Figure 4. Composite of electron micrographs of control and treated cultures. Labels include vacuoles (V), lamellar inclusion bodies (L), and Golgi (G) (original magnification ×18,500). (A) Vehicle-treated (control) culture of primary hamster lung cells. (B) A primary culture of hamster lung cells treated with amiodarone at a concentration of 2 μg/ml. The micrograph is of a smooth muscle cell with large numbers of myofibrils within the cytoplasm. vacuoles, lamellar inclusion bodies (L), and dilated Golgi appear in the juxtanuclear region. (C) A cell from a primary culture of hamster lung cells treated with amiodarone at a dose of 10 μg/ml. Cells contain increasing numbers of vacuoles and lamellar inclusion bodies. (D) A primary culture of hamster lung cells treated with amiodarone at a concentration of 20 μg/ml. The cell contains numerous vacuoles, lamellar inclusion bodies, and dilated regions of endoplasmic reticulum (arrowheads).

mg protein percentage of controls indicated an increase in elastin synthesis.

The cultures employed in this investigation consisted of a heterogenous population of lung cells. However, to ensure that the seeded populations of both control and treated cultures were consistent, each experiment had its own control cultures derived from the same animals. The morphologic studies following treatment with amiodarone did not reveal any change in observable cell type, indicating that amiodarone did not select for a specific subpopulation of cells.

The primary cultures in this investigation exhibited the following characteristics. Cells could only be removed from the flask by vigorous mechanical scraping. Trypsin, collagenase, hyaluronidase, or combinations of the three were not sufficient to remove cells from the flasks. One explanation for these observations is that the cells are manufacturing an ECM composed largely of elastin. In support of this explanation, elastase removed cells from the flasks, strongly implicating elastin's presence as a binding substance within the matrix.

Another characteristic of the primary cultures in question concerns the appearance of cells fixed in glutaraldehyde. The appearance of the fixed cells is unusual, considering the method of preparation. These cultures were not fixed in the flask. Rather, cells were scraped, vortexed, pelleted, and then fixed in glutaraldehyde. Despite this disruptive method of preparation, when viewed by the light microscope, thick sections stained with toluidine blue revealed sheets of cells rest-

ing on an extracellular matrix. This ECM was visible in all parts of the cultures when viewed under the electron microscope. Furthermore, the appearance of cells and ECM is similar to the morphologic arrangement of the alveolar septum. Once again, such observations strongly implicate the role of an ECM that functions by maintaining the sheet-like arrangement of the lung cells.

In addition to revealing the presence of an ECM, the light and electron microscopy revealed the presence of vacuoles and cytoplasmic inclusion bodies in cultures treated with amiodarone. These findings are in keeping with those of other investigators (20) and are postulated to be caused by altered phospholipid catabolism. Cationic, amphiphilic drugs are thought to bind to lysosomal lipids and to alter normal phospholipid catabolism by interfering with phospholipases. Amiodarone is categorized as a cationic, amphiphilic drug.

This investigation is the first to employ an *in vitro* system to study elastin synthesis following administration of the fibrotic agent, amiodarone. Results indicate an increase in elastin synthesis at all levels of amiodarone used. Light and electron microscopy revealed the presence of a definite ECM. The primary hamster lung cell cultures employed in this investigation present a unique system to study elastin biosynthesis in the lung, since these cultures more closely reflect *in vivo* conditions than do established serially passed cell lines. By employing this system to study subcellular and biochemical alterations induced by fibrotic agents, it may be possible to arrive at a better understanding of the mechanism underlying pulmonary fibrosis.

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