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Antiarrhythmic Amiodarone Mediates Apoptotic Cell Death of HepG2 Hepatoblastoma Cells through the Mitochondrial Pathway

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The antiarrhythmic amiodarone is known to cause hepatic toxicity. Recently, much attention has been devoted to the role of apoptosis in the pathogenesis of drug-induced cytotoxicity. The aim of this study is to investigate whether apoptosis contributes to hepatic toxicity caused by amiodarone and, if so, by which mechanism. HepG2 human hepatoblastoma cells were incubated for 48 hours with various concentration of amiodarone. To determine apoptotic cells, concentration of cytochrome c in the cytosol fraction, caspase-9 and -3 activities, and the percentage of the cells with hypodiploid DNA were measured quantitatively by flow cytometric assay or ELISA. The expression of Bcl-2-related proteins was examined by Western blot analysis. Amiodarone induced cytochrome c release into the cytosol fraction, activation of caspase-9 and caspase-3, and the occurrence of hypodiploid cells beginning at 10 μg/ml in the HepG2 cells. However, 2.5 μg/ml of amiodarone, a clinically attainable serum level, did not significantly. The expression of Bcl-xL but neither Bcl-2 nor Bax was decreased in the amiodarone-treated cells. Thus, amiodarone-induced cell death related with cytochrome c release and caspases in the HepG2 cells, suggesting that the drug causes hepatic toxicity in part through the induction of apoptosis. It is our conclusion that the amiodarone-induced apoptosis of HepG2 cells proceeds via the mitochondrial pathway, and is mediated by the downregulation of Bcl-xL.

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Introduction

Amiodarone is now used as an extremely potent antiarrhythmic drug. When chronically given orally, this drug prolongs action potential duration and refractoriness of all cardiac fibers. Amiodarone has been used widely in attempts to prevent sudden death and thereby reduce mortality in patients with malignant ventricular tachyarrhythmias and in other high-risk groups, particularly patients with recent myocardial infarction and congestive heart failure. During long-term therapy with amiodarone, however, its side effects can be a major problem, which limits the use of this drug. In CASCADE,[1] 25% of patients had to stop amiodarone within 2 years of therapy because of its side effects. The most serious adverse reaction is pulmonary toxicity, which occurs with an incidence of 6% and an estimated mortality of 5-10% in affected patients.[1,2] Amiodarone can also cause a number of relatively common adverse effects, e.g., hyper- or hypothyroidism, accumulation of corneal microdeposits, complain of photosensitivity, gastroenterological disturbances, and liver dysfunction.[3-6]

Recently, much attention has been devoted to the role of apoptosis in the pathogenesis of drug-induced cytotoxicity. The pathological activation of apoptosis is now thought to contribute to a variety of disease processes. Recent investigators demonstrated that amiodarone induces apoptosis of several types of cells in vitro, including human alveolar epithelial cell-derived A549 cell line, primary rat alveolar epithelial cells,[7] and human thyroid TAD-2 cell line.[8] Thus, we hypothesized that amiodarone might also cause hepatic toxicity through the induction of apoptosis. The aim of this study

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is to investigate whether amiodarone induces apoptosis of hepatocytes, and if so, by which mechanism.

Materials and Methods

Cell culture

Human hepatoblastoma cell-line HepG2 was obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco). The culture media were exchanged every 2-3 days, and HepG2 cells were maintained as monolayer culture at 37°C in 5% CO_2 at 95% humidity.

Induction of HepG2 cell apoptosis by amiodarone

HepG2 cells were cultured with varying concentrations of amiodarone (Sigma, St. Louis, MO) for 48 hours in DMEM containing 10% FBS. After cultivation, apoptotic cell death of HepG2 cells was evaluated by release of cytochrome c into the cytoplasm, activation of caspases and DNA fragmentation as previously described.[9-11]

Accumulation of cytochrome c into the cytoplasm in HepG2 cells was evaluated by the use of sandwich enzyme-linked immunosorbent assay (ELISA) kit for cytochrome c (MBL, Nagoya, Japan). Briefly, cytoplasmic extracts from HepG2 cells were prepared according to the manufacture's protocol, and the absorbance at 450 nm was calculated (Multiskan JX, LABSYSTEMS, Tokyo, Japan).

We estimated the enzymatic activity of caspase-3 and caspase-9 of HepG2 cells by the use of peptide substrates as previously described.[9-11] The activity of intracellular Leu-Glu-His-Asp ase (LEHDase; caspase-9 like activity) in HepG2 cells was examined by colorimetric protease assay kit (MBL), and that of Asp-Glu-Val-Asp ase (DEVDase) activity (caspase-3 like activity) by flow cytometric protease assay kit (MBL). Briefly, cell lysates from HepG2 cells were mixed with LEHD substrate containing rhodamine at 37°C for 60 min. After incubation, the activity of LEHDase was evaluated by a spectrophotometer at O.D. of 405 nm (Multiskan JX). For determination of DEVDase activity of HepG2 cells, HepG2 cells were incubated with DEVD substrate containing rhodamine for 60 min at 37°C in 5% CO_2 incubator, washed, and the percentage of intracellular $DEVDase^{1}$ cells was examined by flow cytometer.

DNA fragmentation in HepG2 cells was expressed as the percentage of hypodiploid DNA cells as previously described.[9-11] In brief, HepG2 cells were fixed with 70% ethanol and treated with RNAase (100 μg/ml, Sigma Chemical Co., St. Louis, MO), and then stained with propidium iodide (100 μg/ml, Sigma) for 30 min on ice. The stained cells were analyzed by a flowcytometer to detect the percentage of cells with hypodiloid DNA.

Western blot analysis for the expression of Bcl-2-related proteins in HepG2 cells

Expression of Bcl-2, Bcl-xL and Bax in HepG2 cells was examined by western blotting. HepG2 cells were collected and lysed by the addition of lysis buffer (50 mM Tris buffer, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% NP-40, and 0.5% sodium deoxycholate) for 20 min at 4°C, and insoluble material was removed by centrifugation at 13,000 rpm for 30 min at 4°C. The supernatant was collected and protein concentration was determined by the Bio-Rad (Melville, NY) protein assay kit. 10 μg each of the lysate from HepG2 cells was subjected to 15% SDS-PAGE, and transferred to a nitrocellulose filter. The filter was incubated at room temperature for 1 hr in the presence of each antibody (*anti-Bcl-xL:* mouse monoclonal, Trevigen, Gaithersburg, CA; *anti-Bcl-2:* mouse monoclonal, Dako Japan, Kyoto, Japan; *anti-Bax:* rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), and further incubated with 1: 1000 dilution of anti-mouse IgG or anti-rabbit IgG coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL) was used for detection.

Results

Determination of apoptosis in HepG2 cells during amiodarone treatment

Mitochondria is a central organelle in response to apoptogenic stimuli.[12-14] As shown in Figure 1 A, the accumulation of cytochrome c into the cytosol fraction, a major apoptogenic molecule translocating from the mitochondria during apoptosis, was determined in HepG2 cells treated with higher concentration of amiodarone. The level of the cells treated with 10 μg/ml or more of amiodarone increased twofold greater than that of untreated cells. The enzyme activity of caspase-9, which is related with mitochondrial pathway in apoptosis, was increased in amiodarone-treated HepG2 cells (Figure 1 B). The

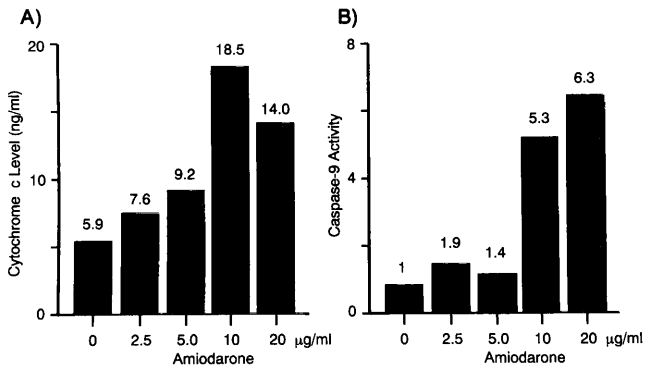

Figure 1. Effects of amiodarone on mitochondrial apoptotic pathway of HepG2 cells. HepG2 cells were cultured with varying concentrations of amiodarone for 48 hours. After cultivation, cytochrome c relase (A) and caspase-9 activity (B) in HepG2 cells were examined as described in Materials and Methods. Note that cytochrome c relase and caspase-9 activity in HepG2 cells were dose-dependently induced by amiodarone.

activity of the cells treated with 10 μg/ml or more of amiodarone increased 5-fold greater than that of untreated cells.

Activation of caspase-3 leading to DNA fragmentation is believed to be irreversible, executive apoptotic process.[12-14] As shown in Figure 2 A, the use of 10 to 20 μg/ml of amiodarone significantly activated DEVDase in HepG2 calls, indicating the activation of caspase-3 by the drug. The nuclear alterations are often associated with internucleosomal cleavage of DNA,[15] and considered as a biochemical hallmark of apoptosis. To determine the percentage of apoptotic nuclei or recognize the apoptotic cells in a heterogeneous cell population, we used a flow cytometric method for measuring the percentage of cells with hypodiploid DNA after propidium iodide staining. This procedure has been shown to have an excellent correlation with colorimetric and electrophoretic methods for the detection DNA fragments.[16] As shown in Figure 2 B, amiodarone increased the percentage of hypodiploid cells. The apoptotic cells treated with 10 μg/ml or more of amiodarone increased 3-fold greater than that of untreated cells.

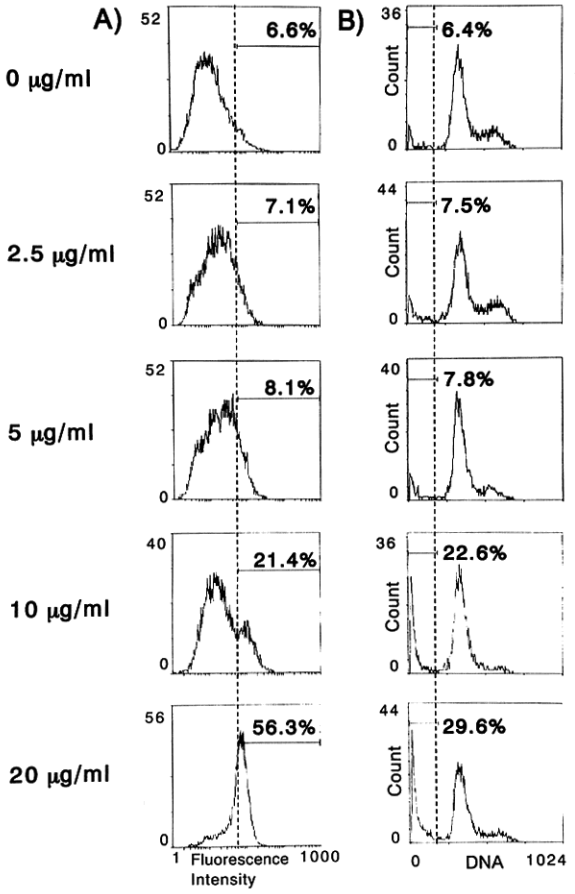

Figure 2. Both caspase-3 activation and DNA fragmentation are managed in HepG2 cells by amiodarone. HepG2 cells were cultured with varying concentrations of amiodarone for 48 hours. After cultivation, caspase-3 activity (A) and the detection of hypodiploid cells (B) were examined as described in Materials and Methods. Note that caspase-3 activity and the percentage of cells with hypodiploid DNA in HepG2 cells were dose-dependently induced by amiodarone.

The expression of Bcl-2-related proteins

The maintenance of mitochondrial homeostasis during apoptogenic stimuli is regulated by Bcl-2-related protein family.[12-14] We examined the expression of antiapoptotic proteins, Bcl-2 and Bcl-xL, and a proapoptotic protein, Bax, in HepG2 cells by Western blotting. As shown in Figure 3, HepG2 cells expressed not Bcl-2 and Bax but Bcl-xL, which was consistent with previous observations. As compared with untreated HepG2 cells, Bcl-xL expression was clearly inhibited by amiodarone treatment. Amiodarone did not induce the expression of Bcl-2 and Bax in HepG2 cells.

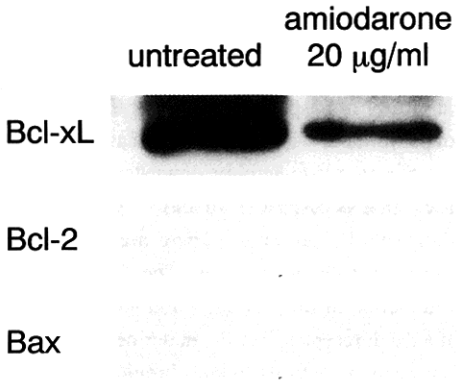

Figure 3. Western blot analysis of Bcl-2 in HepG2 cells. HepG2 cells were cultured with or without 20 μg/ml of amiodarone for 48 hours. After cultivation, Bcl-2 expression in HepG2 cells was examined by western blotting as described in Materials and Methods. Results shown are the representative experiment from three performed. Note that Bcl-2 expression in HepG2 cells was clearly inhibited by amiodarone treatment.induced by amiodarone.

Discussion

Hepatotoxicity is a well-recognized adverse reaction to amiodarone, requiring regular liver enzyme assays and often liver biopsies. Elevations of serum aminotransferase levels has been detected in 14 to 82% of patients with orally administered amiodarone.[3,4] The degree of abnormality is generally mild, and the levels may normalize after the dose of amiodarone is lowered or when the drug is stopped. Although symptomatic hepatic disease caused by amiodarone occurs less frequently, patients who have hepatomegaly, jaundice, cirrhosis, or chronic active hepatitis have been reported.[5,6] Acute hepatotoxicity was characterized by sharp and fairly high elevations of serum aminotransferase levels, typically in the range of thousands of units, that occurred within several weeks of drug administration and that were followed by a rapid decline in the next few days, as expected after an acute liver injury. On the other hand, chronic toxicity is characterized by a 1.5-4-fold increase in the aminotransferases, which occurrs after months or even years of therapy. The classic histologic appearance of amiodarone-induced toxicity resembles alcoholic liver disease, so-called "pseudoalcoholic hepatitis."[17,18] Steatosis, both macrovesicular and microvesicular, was

the most frequent histopathologic feature. Ballooning of hepatocytes, Mallory bodies, and fibrosis were also common. Other changes in alcoholic hepatitis including nuclear unrest, acidophilic bodies, foam cells, glycogenated nuclei, and portal inflammation were also found. Phospholipidosis, which is characterized by whorled lamellated inclusions within expanded lysosomes, has been observed in a number of patients taking amiodarone ho showed no evidence of pseudoalcoholic hepatitis.[3,19] Thus, amiodarone induces functional and histopathologic changes in varying degrees, suggesting that amiodarone-induced hepatic toxicity occurs by various mechanisms.

A cell undergoing apoptosis exhibits characteristic morphological and biochemical changes that are distinct necrosis.[16,20] The morphological features of apoptosis include chromatin margination along the nuclear membrane, nuclear condensation, budding and fragmentation. These changes reflect complex biochemical events carried out by a family of caspases. Caspases can be broadly divided into two groups: initiator caspases, such as caspase-8 and caspase-9, whose main function is to activate downstream caspases, and executor caspases such as caspase-3, -6 and -7, which are responsible for dismantling cellular proteins.[21] There are two main pathways leading to the activation of caspases. The first of these depends upon the participation of mitochondria and the second involves the interaction of a death receptor, like the tumor necrosis factor receptor-1 and the Fas receptor, with its ligand. Previous studies have indicated that chemotherapeutic drugs, industrial chemicals and environmental contaminants cause the mitochondrial inner membrane permeability transition and pore formation, resulting in membrane depolarization and translocation of cytochrome c from the mitochondria into the cytosol.[22,23] Cytochrome c release from the mitochondria initiates a cascade that leads to the activation of caspase-9. Once active, caspase-9 then activates executor caspase-3. The activation of caspase-3 usually leads to cleavage of cytoplasmic substrates for the manifestation of apoptotic morphological changes.[24,25] In this study, we demonstrated in HepG2 cells that amiodarone induced cytochrome c release into the cytosol fraction, and activation of caspase-9 and its downstream caspase-3 prior to the occurrence of hypodiploid DNA in the cells. From these findings, amiodarone appears to induce apoptosis through the mitochondrial pathway.

Recent experimental evidence has suggested that Bcl-2-related proteins, including Bcl-2, Bcl-xL and Bax, are involved in the apoptotic process. Both Bcl-2 and Bcl-xL inhibit apoptosis, while Bax accelerates the apoptotic process.[12] A number of studies proved that drug-induced apoptosis in various types of cells is mediated by Bcl-2-related proteins.[26,27] However, the role of the proteins in amiodarone-induced apoptosis is still not clear. Di Matola et al.[8] indicated that human thyroid TAD-2 cells treated with amiodarone underwent apoptosis, whose process was not mediated by modulation of Bcl-2, Bcl-xL, or Bax protein expression. In this present study, we demonstrated that Bcl-xL was highly expressed, but Bcl-2 and Bax were not in the untreated HepG2 cells. During the apoptotic process in the HepG2 cells treated with amiodarone, the level of Bcl-xL protein was down-regulated, and Bcl-2 and Bax remained unexpressed. Therefore, Bcl-xL might play an important role in the apoptotic process by amiodarone at least in liver.

Recent technological advances in the use of flow cytometry and ELISA have made it possible to quantify apoptosis.[28,29] For quantitative determination of apoptosis, we applied these techniques, and measured concentration of cytochrome c in the cytosol fraction, caspase-9 and -3-like activities, and the percentage of the cells with hypodiploid DNA. We demonstrated thus amiodarone-induced apoptosis of HepG2 cells in a dose-dependent manner. Amiodarone-induced cell death related with mitochondrial dysfunctions and activation of caspases in the HepG2 cells beginning at 10 μg/ml of the drug, although 2.5 μg/ml, a clinically attainable serum level, did not significantly. The concentrations at which amiodarone-induced hepatic toxicity were only four times as high as the usual serum drug levels in patients given antiarrhythmic therapy with the drug. Therefore, high-dose amiodarone may induce irreversible liver damage via apoptosis.

This is the first observation that amiodarone mediates apoptotic cell death of hepatocytes. Amiodarone-induced cell death related with mitochondrial dysfunctions and activation of caspases in the HepG2 cells, suggesting that the drug causes hepatic toxicity in part through the induction of apoptosis, which was mediated by the downregulation of Bcl-xL. Amiodarone-induced apoptosis of HepG2 cells showed a dose-dependent manner, suggesting that high-dose amiodarone may induce irreversible liver damage via apoptosis. Our data again demonstrated the importance of monitoring in serum amiodarone concentration to avoid the adverse effects including hepatotoxicity.

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