Ethanol Potentiates Doxorubicin-induced Inhibition of Cell Survival in Cultured Chinese Hamster Ovary Cells

Patricia E. Ganey and Ronald G. Thurman

Laboratory of Hepatobiology and Toxicology, Department of Pharmacology and Curriculum in Toxicology, University of North Carolina, Chapel Hill, North Carolina 27599-7365

ABSTRACT

Doxorubicin is an antineoplastic drug which undergoes oxidation-reduction cycling and produces toxicity to some cancer cell lines. Since oxidation-reduction cycling requires reducing equivalents and because ethanol metabolism via alcohol dehydrogenase (ADH) increases NADH, the effect of ethanol on doxorubicin toxicity was examined in cultured cells. Since some cells exhibit resistance to anthracyclines such as doxorubicin, two different Chinese hamster ovary cell lines were used, one sensitive (AUX B1) and one resistant (CH*C5) to doxorubicin. Studies were designed to determine if ethanol could decrease resistance to doxorubicin. Cells were treated for 24 h with doxorubicin in the presence or absence of ethanol, and the number of viable cells was estimated spectrophotometrically. Ethanol (60–150 mM) potentiated the doxorubicin-induced decrease in cell number in both cell lines. In AUX B1 cells the concentration of doxorubicin required for half-maximal inhibition of cell survival was reduced 20-fold by ethanol, and a completely nontoxic concentration of doxorubicin decreased the number of surviving cells to 30% in the presence of ethanol. Addition of ethanol to the medium also increased doxorubicin-induced inhibition of cell survival in CH*C5 cells, but the effect was less dramatic than in AUX B1 cells. The effect of ethanol on cell number was concentration related; the half-maximal response was observed with about 1 mM ethanol. The hypothesis that ethanol potentiates doxorubicin toxicity by generation of NADH during metabolism by ADH was strengthened by the observations that both cell lines possess ADH activity (30–400 units/10^12 cells) and that ethanol (0.1–0.5 mM) increased NADH fluorescence 15–80% over basal values in cultured cells. Further, the effect of doxorubicin on cell number was also potentiated by another substrate for ADH, 2-ethylhexanol. Desferrioxamine, an iron chelator, increased survival in cells treated with doxorubicin plus ethanol by up to 60% (half-maximal effect, 1 mM), and (+)-catechin, a radical scavenger, abolished the decrease in cell number due to doxorubicin plus ethanol at concentrations greater than 0.1 mM. Allropurinol, an inhibitor of xanthine oxidase with radical scavenging properties, diminished the effect of doxorubicin plus ethanol on cell number by 45% (P < 0.05). Taken together, these data are consistent with the hypothesis that ethanol potentiates toxicity due to doxorubicin by providing reducing equivalents for oxidation-reduction cycling which produce toxic reduced oxygen species.

INTRODUCTION

Clinical use of the anthracycline antitumor agent doxorubicin is limited by the cardiotoxicity it produces at cumulative doses exceeding 500–550 mg/m^2 body surface area (1). The mechanism by which doxorubicin produces toxicity is unknown, but production of deleterious free radicals during oxidation-reduction cycling of the quinone nucleus has been implicated (2–6). Oxidation-reduction cycling of quinones requires oxygen and reducing equivalents, and in a previous study we demonstrated that toxic effects of doxorubicin were oxygen dependent in the perfused rat liver (7). Oxidation-reduction cycling, assessed from a stimulation of oxygen uptake, was inhibited by diminishing the supply of reducing equivalents and was stimulated by fructose, which increases NAD(P)H. These observations led to the hypothesis that oxygen tension influences doxorubicin toxicity indirectly by regulating the supply of reducing equivalents for oxidation-reduction cycling. This hypothesis was tested by elevating the NADH oxidation-reduction state with ethanol. In perfused liver, ethanol enhanced the toxicity both of the model quinone, menadione (8), and doxorubicin (9).

The purpose of these studies was to evaluate whether ethanol increases toxicity of doxorubicin to cultured cells. In addition, it was of interest to determine if ethanol could increase cell killing of tumor cells resistant to doxorubicin. Therefore, experiments were performed using CHO cell lines sensitive (AUX B1) and relatively resistant (CH*C5) to the toxic effects of doxorubicin (10, 11).

MATERIALS AND METHODS

Cell Culture. The adenosine-, thymidine-, and glycine-requiring auxotroph AUX B1 and the multidrug-resistant mutant CH*C5 derived from line CHO (kindly provided by Dr. R. Juliano of the Department of Pharmacology, University of North Carolina at Chapel Hill) were used in this study. Monolayer cultures were routinely maintained at 37°C in α-minimum essential medium (Gibco) supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotic mixture (Gibco) in humidified air containing 5% CO_2. Cells from exponentially growing cultures were added to 24-well tissue plates (2.5 x 10^6 cells/well) and were incubated overnight before experiments were performed. Drugs prepared in α-minimum essential medium were added to wells in the concentrations indicated in the figure legends. In preliminary experiments where doxorubicin was incubated with cells for various lengths of time, 24 h of incubation provided the most consistent results; therefore, this time period was used in further experiments.

Estimation of Cell Number. The fraction of cells surviving after 24 h of incubation was determined by using the MTT dye assay (12). Cells were incubated for 2 h at 37°C with 2.4 mM MTT, the dye was removed, and dimethyl sulfoxide was added. The culture plates were shaken gently for 15 min, and absorbance at 540 nm was measured. The number of live cells was determined by comparison to a standard curve generated by measuring the absorbance of a known number of cells treated with MTT in a similar manner. A decrease in cell number after 24 h of treatment is referred to as a decrease in cell survival.

Determination of Ethanol Concentration. The concentration of ethanol in culture medium was determined as described in detail elsewhere (13). Samples (1 ml) of culture medium were removed at various times and were incubated at 37°C in stopped flasks for 1 h. Subsequently, 1 ml of the equilibrated headspace was injected into a Hewlett-Packard 5720A gas chromatograph fitted with a Poropak Q column. Concentration was determined relative to ethanol standards treated similarly. Ethanol was routinely added to cultured cells at a concentration of 150 mM, and was about 75 mM after 24 h of incubation.

Fluorescence of NADH in Cultured Cells. Fluorescence of reduced pyridine nucleotides was monitored in cells grown in culture. The tip...
(diameter, 2 mm) of a bifurcated, fiber-optic light guide was immersed in the medium directly over the cells. One end of the light guide was attached to a light source, and cells were illuminated with 366 nm light. The other end of the light guide was attached to a photomultiplier tube. A filter that excluded light of wavelengths shorter than 450 nm was attached to a light source, and cells were illuminated with 366 nm light. Fluorescence was measured before and after the addition of ethanol (0.1-0.5 mM) as nA of photomultiplier current (14).

Measurement of Alcohol Dehydrogenase Activity. Cells were homogenized in Krebs-Henseleit buffer and spun at 100,000 \( \times g \) (4°C) for 45 min, then the supernatant was incubated with ethanol (5 mM) and NAD\(^+\) (0.28 mM) at 38°C. ADH activity was determined from the increase in absorbance at 366 nm as a function of time (15).

Assessment of Drug Accumulation. Accumulation of doxorubicin in cells was determined as described by Daoud and Huang (16). Cells were seeded at 1.5 \( \times 10^7 \) cells in 6-well plates 24 h before experiments. Cells were incubated at 37°C in α-minimum essential medium (without serum) containing doxorubicin (15 nM for AUX B1 cells; 26 μM for CHC5 cells) in the absence or presence of ethanol (150 mM) for up to 180 min. At various times, cells were removed from the incubator and washed twice with ice-cold phosphate-buffered saline. Cells were detached by gently scraping with a rubber spatula, and intracellular doxorubicin was extracted with 0.3 N HCl in 50% ethanol. Doxorubicin was measured fluorometrically (excitation at 485 nm, emission at 580 nm), and the concentration in cells was determined by comparison with standards.

Statistical Analysis. Values are presented as mean ± SEM of 4–6 determinations per group. The data were analyzed by using a Student's t test or a 2-way analysis of variance. When the data were analyzed by analysis of variance, individual means were compared by Tukey's omega procedure. In all cases, the criterion for significance was \( P < 0.05 \).

RESULTS

Potentiation of Doxorubicin Toxicity by Ethanol. The decrease in cell number due to doxorubicin was concentration related in both AUX B1 and CHC5 cell lines (Fig. 1). Cell survival was inhibited in AUX B1 cells by concentrations of doxorubicin above 0.015 μM, and the half-maximal effect required about 0.15 μM (Fig. 1A). In contrast, doxorubicin-induced inhibition of cell survival occurred at concentrations above 5 μM in CHC5 cells, with the half-maximal effect observed with about 15 μM (Fig. 1B). In the presence of ethanol, 50% inhibition was observed in AUX B1 cells with about 0.07 μM doxorubicin, a 20-fold reduction compared to drug alone. Further, concentrations of doxorubicin which alone did not affect cell survival (0.007–0.015 μM) decreased cell number in the presence of ethanol. In CHC5 cells, ethanol decreased cell survival only at concentrations of doxorubicin that alone decreased cell number. For this reason, subsequent attention was directed at studies with AUX B1 cells.

Ethanol alone did not affect cell survival. On the other hand, the potentiation of doxorubicin-induced decrease in cell number by ethanol was concentration related (Fig. 2); maximal potentiation was observed at concentrations above 50 μM. Since the concentration of ethanol could not be controlled during cell incubations, values are plotted versus amount of ethanol added.

To determine whether ethanol enhanced doxorubicin-mediated inhibition of cell survival by increasing accumulation of drug, intracellular doxorubicin was measured in cells incubated in the absence and presence of ethanol. In AUX B1 cells, intracellular doxorubicin increased within 60 min to about 150 ng/10^6 cells, where it remained for 180 min of incubation (Fig. 3A). In CHC5 cells, intracellular doxorubicin reached a plateau concentration of about 60 μg/10^6 cells by 30 min (Fig. 3B). Ethanol had no effect on accumulation of doxorubicin in AUX B1 or CHC5 cells.

Role of Alcohol Dehydrogenase. ADH activity was detected in both sensitive and resistant cell lines. Values in AUX B1 cells were 300 units/10^12 cells and 4000 units/10^12 in CHC5 cells. In AUX B1 cells ethanol increased NADH fluorescence in steps proportional to the concentration of ethanol added between 0.1 and 0.5 mM ethanol (Fig. 4). Fluorescence also increased in response to ethanol in CHC5 cells; however, in the absence of cells, addition of ethanol to the medium did not change the fluorescence signal (data not shown). These results...
Role of Free Radicals. Oxidation-reduction cycling of doxorubicin produces reactive oxygen species which have been implicated in toxicity (2, 3, 18, 19). Therefore, agents which scavenge radicals or prevent their production were tested for their ability to inhibit the effect of doxorubicin plus ethanol on cell number. Desferrioxamine, an iron chelator, inhibited the decrease in cell survival by up to 60%. Half-maximal inhibition was observed with about 1 mM desferrioxamine (Fig. 6A). Catechin, a radical scavenger, also increased cell survival in the presence of doxorubicin plus ethanol at all concentrations tested and inhibited the decrease in cell number completely at concentrations above 0.05 mM (Fig. 6B). In addition, allopurinol (1 mM), an inhibitor of xanthine oxidase with radical scavenging properties (20), increased cell survival in the presence of doxorubicin plus ethanol by 60% ($P < 0.05$), whereas catalase (11,000 units) plus superoxide dismutase (17,000 units) caused a reduction in toxicity of about 35% that was not statistically significant (data not shown).

**DISCUSSION**

**Ethanol Potentiates Effect of Doxorubicin on Cell Survival.** The mechanisms proposed to explain how doxorubicin kills
cells are numerous and include intercalation into DNA (21), interaction with topoisomerase II (22), alterations in cell membranes (23), and generation of free radicals during oxidation-reduction cycling (2, 3, 18, 19). We demonstrated that toxicity due to oxidation-reduction cycling of the model quinone menadione was potentiated by ethanol in perfused rat liver (8). This effect was also observed in livers from deer mice with ADH, but not in deer mice genetically deficient in ADH, supporting the hypothesis that ethanol enhanced toxicity through metabolic generation of reducing equivalents via ADH. Further support for this hypothesis came from experiments in which other agents that generate NADH (e.g., xylitol) also increased oxidation-reduction cycling due to menadione (8). Thus, metabolism of ethanol by ADH increased the supply of reducing equivalents (NADH) necessary for oxidation-reduction cycling and thereby enhanced toxicity.

The present study was undertaken to determine whether ethanol increases toxicity due to doxorubicin in cell lines characterized as sensitive and insensitive to the drug. In AUX B1 cells, ethanol potentiated doxorubicin-induced inhibition of cell survival in a concentration-related manner (Fig. 2), and concentrations of doxorubicin that alone did not diminish cell number reduced the number of surviving cells by up to 70% when incubated in the presence of ethanol (Fig. 1A). Ethanol also enhanced doxorubicin toxicity to the resistant cell line (Fig. 1B), but the concentration of doxorubicin required for half-maximal reduction of cell survival was still about 50-fold greater than the concentration required in the sensitive cell line, indicating that ethanol did not overcome resistance. This suggests that while ethanol may increase the effectiveness of doxorubicin to damage tumor cells sensitive to the anthracycline, it is not more beneficial in cells resistant to doxorubicin.

Possible Mechanisms of Potentiation of Doxorubicin Toxicity by Ethanol. By what mechanism does ethanol increase toxicity of doxorubicin to cultured CHO cells? One possibility is that ethanol increases uptake or decreases efflux of doxorubicin, resulting in increased intracellular accumulation of the drug. However, doxorubicin accumulation was not different in the presence or absence of ethanol over a 3-h period (Fig. 3), allowing this possibility to be ruled out. Another possibility is that ethanol increases the generation of free radicals from doxorubicin. In perfused liver, ethanol potentiates toxicity of menadione and doxorubicin by increasing the supply of reducing equivalents necessary for oxidation-reduction cycling (8, 9). Doxorubicin causes oxidation of NADH, and in perfused liver this occurs only in sublobular regions where cell death is observed (7). Furthermore, in the presence of ethanol, the time course of oxidation is consistent with the generation of reactive oxygen species measured as an increase in oxygen uptake (8).

Both AUX B1 and CHC5 cells have ADH; therefore, NADH should be produced during metabolism of ethanol. Indeed, ethanol increased fluorescence of NADH in both cell lines (Fig. 4). In addition, another agent which produces NADH during metabolism by ADH, ethylhexanol, also potentiated doxorubicin toxicity (Fig. 5). Therefore, it is likely that ethanol enhances toxicity of doxorubicin through generation of NADH, which causes oxidation-reduction cycling and production of damaging free radicals to occur at higher rates. Given the higher level of activity of ADH in CHC5 compared to AUX B1 cells, it is difficult to understand why the effect of ethanol on doxorubicin-induced inhibition of cell survival was less dramatic in the resistant than in the sensitive cell lines. One possible explanation is that the production of oxygen radicals is not important in the mechanism by which doxorubicin causes toxicity in CHC5 cells, but that other mechanisms involving topoisomerase II, alterations in cell membranes, or intercalation into DNA are more important. Consistent with this hypothesis, doxorubicin stimulated hydroxyl radical formation in sensitive but not in resistant MCF-7 human breast tumor cells (24). The surface P-glycoprotein of CHC5 cells is thought to modulate drug permeability (10); however, intracellular accumulation of doxorubicin was not affected by the presence of ethanol (Fig. 3). Therefore, the reason why ethanol had a more pronounced effect of doxorubicin toxicity in the sensitive than in the resistant cell line remains unclear.

Involvement of Free Radicals in Decrease in Cell Number Due to Doxorubicin plus Ethanol. Oxidation-reduction cycling of doxorubicin generates superoxide anion and hydroxyl radicals. Radical scavengers have been shown to protect against doxorubicin toxicity in a number of systems, including MCF-7 human breast tumor cells (19, 24), heart and liver microsomes (5), and kidney microsomes (25). In this study, both the iron chelator, desferrioxamine, and the radical scavenger, (+)-catechin, increased cell survival in the presence of doxorubicin plus ethanol significantly (Fig. 6). It has been suggested that oxygen radical formation due to doxorubicin occurs both in the extracellular as well as intracellular spaces (24, 26) and that hydroxyl radical formation due to doxorubicin is in part iron mediated (24). This may explain why desferrioxamine, which does not readily enter cells, afforded protection against doxorubicin plus ethanol. Desferrioxamine may have inhibited iron-catalyzed production of deleterious reactive oxygen species in the extracellular space. Allopurinol also provided protection against toxicity of doxorubicin plus ethanol, an effect which may be due to inhibition of xanthine oxidase or to scavenging of radicals. It is not known whether these cells have xanthine oxidase or whether xanthine oxidase is important in doxorubicin-induced toxicity in CHO cells. Catalase plus superoxide dismutase, neither of which readily enters cells, did not alter cell survival statistically. Thus, the exact nature of the role oxygen radicals play in the potentiation of doxorubicin-induced toxicity in CHO cells remains unclear.

In conclusion, ethanol potentiated doxorubicin-mediated inhibition of cell survival in cultured CHO cells by a mechanism involving generation of NADH. Toxicity was inhibited by free radical scavengers and an iron chelator, suggesting that reactive oxygen species are involved in the mechanism of cell killing.

REFERENCES

ETHANOL POTENTIATES DOXORUBICIN TOXICITY

Ethanol Potentiates Doxorubicin-induced Inhibition of Cell Survival in Cultured Chinese Hamster Ovary Cells

Patricia E. Ganey and Ronald G. Thurman


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/51/8/2036

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.