Chemically-Induced Apoptosis in Freshly-Isolated Human Hepatocytes

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Abstract

We have investigated the use of freshly-isolated human hepatocytes to detect hepatocellular apoptosis caused by several archetypical compounds. Human hepatocytes were treated with different concentrations of galactosamine, acetaminophen, etoposide, or tamoxifen for 4, 24, and 48 hours. Caspase 3/7 activity and 3-[4,5-dimethylthiasol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) conversion were used to detect apoptosis and necrosis, respectively. At low concentrations (1 mM) and short exposure times (4-hour), galactosamine did not have any effect on caspase activity and MTT conversion. However, galactosamine at 1 mM induced apoptosis without causing necrosis following 24-hour exposure. The induction of apoptosis peaked following 48-hour exposure, which was accompanied by the induction of necrosis. Acetaminophen at the tested concentrations (0.2-20 mM) did not induce apoptosis following 4-, 24-, or 48-hour exposure. Acetaminophen at the tested concentrations did not induce necrosis following 4-hour exposure. At higher concentrations and with longer exposure (20 mM and 24-hour; 6 and 20 mM and 48-hour), induction of necrosis by acetaminophen was evident. Etoposide at the tested concentrations (33-500 µM) induced apoptosis without having effects on MTT conversion after all exposure time points, although the induction of apoptosis was not concentration-dependent and the magnitude of induction was less than 2-fold. Tamoxifen at the tested concentrations (0.5-50 µM) increased caspase activity after all exposure time points. The increase of caspase activity was not accompanied by a change in MTT conversion except at the highest tested concentration (50 µM). The induction of apoptosis was peak at 50 µM tamoxifen (3.6-, 3.9-, and 5.6-fold following treatment for 4, 24, and 48 hours, respectively). The results demonstrated that:
(1) freshly-isolated human hepatocytes can be used to detect apoptosis caused by galactosamine, etoposide, or tamoxifen; (2) the apoptotic effects of these compounds were detected at the concentrations at which no necrosis was evident; and
(3) acetaminophen was not an apoptotic agent under the experimental conditions.

Introduction

Apoptosis and necrosis are the two mechanisms by which cell death occurs. It is characterized by distinctive morphologic changes in the nucleus and cytoplasm, release of cytochrome c from the mitochondria, activation of a family of intracellular cysteine endopeptidases (caspases), chromatic cleavage at regularly spaced sites, and the endonucleolytic cleavage of genomic DNA (DNA fragmentation) at internucleosomal sites.

Although drug-induced cell death has long been considered a result of necrosis, recent evidence suggests that apoptosis rather than necrosis predominates in many cytolethal toxic injuries (1). Apoptosis could be the major contributor to xenobiotic-induced cell death with necrosis being much more rare (2).

The principal goal of the research is to investigate the utility of freshly-isolated primary human hepatocytes to detect hepatocellular apoptosis caused by four archetypical compounds. Apoptosis and necrosis were evaluated by measuring caspase 3/7 activity and MTT conversion, respectively.

Materials and Methods

Hepatocyte Cultures. Freshly-isolated human hepatocytes were obtained from BioreclamationIVT. Viability and cell counts were determined by Trypan Blue exclusion. The cell suspensions were diluted to 700,000 viable cells per mL with plating medium. A volume of 0.2 mL of the cell suspension was transferred to a collagen-coated 48-well plate. Plates were incubated overnight in a 37°C, 5% CO_2, humidified incubator to allow attachment of the hepatocytes. Each well was washed one time with incubation medium prior to the addition of drug compounds.

Preparation of stock solutions. Galactosamine, acetaminophen, and tamoxifen were prepared as 2X stock solutions in deionized water. Etoposide was prepared as 100X stock solutions in DMSO.

Preparation of dosing solutions. Solutions were prepared by diluting the stocks in incubation medium (1:2 for galactosamine, acetaminophen, and tamoxifen; and 1:100 for etoposide). DMSO was added to the dosing solutions containing galactosamine, acetaminophen, or tamoxifen to contain a final DMSO concentration of 1%. Two vehicle control (VC) solutions were prepared accordingly. Medium was removed from the 48-well plates, and dosing solutions and VC solutions were added. The plates were returned to the incubator. Cells were incubated for 4, 24, or 48 hours, and then assessed for MTT reduction and caspase 3/7 activity.

MTT Assay. Cells were incubated with 0.5 mg/ml of thiazolyl blue tetrazolium bromide (MTT) in cell medium for each time point. Acidified isopropanol was prepared by diluting hydrochloric acid (HCl) into isopropanol to a final concentration of 0.04 M HCl. Plates were incubated in a 37°C, 5% CO_2, humidified tissue culture incubator for 3 hours, then medium from all wells was removed, and 0.2 mL of acidified isopropanol was added to each well to dissolve the MTT formazan. The absorbance of MTT formazan was measured at 572 nm and 690 nm on a Wallac Victor2 multilabel counter. The corrected absorbance was determined by subtracting the 690 nm value from the 572 nm value. The average background blank was subtracted from the average of the other experimental groups to derive the adjusted absorbance. The dosing groups were compared to VC by dividing adjusted absorbance of the dosing group by the adjusted absorbance of the VC and multiplying by 100 to get the percentage of VC.

Caspase 3/7 Assay. The caspase activity was determined by using the Apo-ONE® Homogenous Caspase 3/7 Assay (Promega, Madison, WI). The assay was performed as described in the assay instructions. The plate was analyzed on the Wallac Victor2 multilabel counter with excitation at 485 nm and emission at 535 nm. The average background blank was subtracted from the average of the other experimental groups to derive the adjusted fluorescence. The dosing groups were compared to VC by dividing the adjusted fluorescence of the dosing group by the adjusted fluorescence of the VC and multiplying by 100 to get the percentage of VC.

Results

Figure 1: Galactosamine, 4 hours

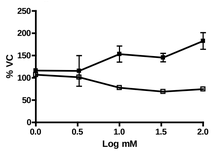


Figure 2: Galactosamine, 24 hours

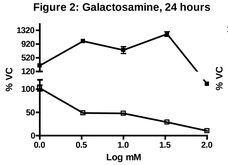
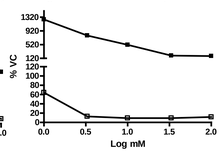
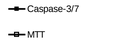


Figure 3: Galactosamine, 48 hours





Results, continued

Figure 4: Acetaminophen, 4hours

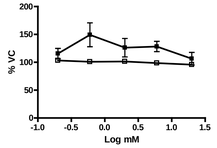


Figure 5:Acetaminophen, 24 hours

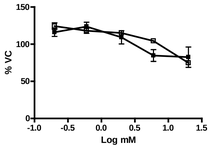
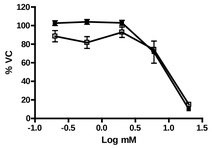


Figure 6: Acetaminophen, 48 hours



Firue 7: Etoposide, 4 hours

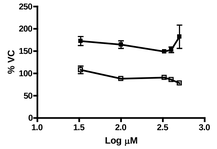


Figure 8: Etoposide, 24 hours

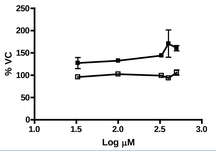


Figure 9: Etoposide, 48 hours

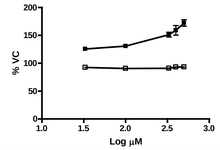


Figure 10: Tamoxifen, 4hours

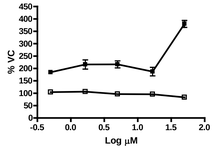


Figure 11: Tamoxifen, 24 hours

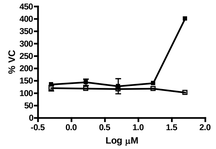
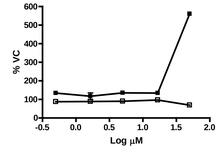


Figure 12: Tamoxifen, 48 hours



Conclusions

- Freshly-isolated human hepatocytes can be used to detect apoptosis caused by galactosamine, etoposide, or tamoxifen.
- The apoptotic effects of these compounds were detected at the concentrations at which no necrosis was evident.
- Acetaminophen was not an apoptotic agent under the experimental conditions.

References

1. Raffray M, Cohen GM. (1997) Apoptosis and necrosis in toxicology: a continuum or distinct modes of cell death? Pharmacol. Ther. 75: 153-77.
2. Gomez-Lechon ML, O'Connor E, Castell JV, and Jover RM (2002) Sensitive markers used to identify compounds that trigger apoptosis in cultured hepatocytes. Toxicol. Sci. 65: 299-308.