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A Comparative Study of Apoptosis and Necrosis in HepG2 Cells: Oxidant-Induced Caspase Inactivation Leads to Necrosis

Afshin Samali, Helena Nordgren, Boris Zhivotovsky, Elizabeth Peterson, and Sten Orrenius[1]
Institute of Environmental Medicine, Division of Toxicology, Karolinska Institute, S-171 77 Stockholm, Sweden

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Apoptosis and necrosis are two distinct forms of cell death that can occur in response to various agents. In the present study the HepG2 cell line was used for a comparative study of CD95-mediated apoptosis and menadione-induced necrosis. Apoptosis coincided with the release of cytochrome *c* from mitochondria, activation of caspases, cleavage of cellular proteins, and also involved nuclear condensation and DNA fragmentation. Necrosis was not accompanied by DNA fragmentation, caspase activation or cleavage of caspase target proteins, despite cytochrome *c* release from mitochondria. In fact, the addition of menadione to cells undergoing CD95-mediated apoptosis blocked their caspase activity. Inhibition of caspases coincided with an accumulation of reactive oxygen species (ROS) and ATP depletion. In order to determine the predominance of either of these events in the inhibition of caspase, cells were either co-incubated with antioxidant enzymes or their ATP level was manipulated to maintain it at a relatively high level during the experiments. Co-incubation with catalase, but not Cu/Zn superoxide dismutase, substantially reduced the levels of ROS and reversed the inhibitory effect of menadione on caspase activity. In contrast, increasing cellular ATP level had little effect on restoring caspase activity. These data suggest that menadione inhibits caspase activity by the generation of hydrogen peroxide through redox cycling and that caspase inactivation by this mechanism may prevent cell death by apoptosis in this oxidative-stress model.

Cell death can follow two distinct pathways, apoptosis or necrosis. However, the early biochemical events that dictate the mode of cell death are still unclear. Necrosis appears to be the result of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents. It is a relatively passive process and is associated with a rapid cellular ATP depletion. Necrosis is characterized morphologically by a dramatic increase in cell volume and ultimately rupture of the plasma membrane spilling the cellular contents into the intercellular milieu (1). This release of the dying cells' contents into the extracellular spaces can cause further tissue damage by affecting neighboring cells or by attracting pro-inflammatory cells to the lesion (2, 3). There are some proteases associated with necrosis, mainly calcium-activated or lysosomal proteases (4, 5). Apoptosis is a physiological form of cell death (6), that occurs during development of multicellular organisms or during the immune response (for review see 7). In addition, the apoptotic program can be activated in response to stress conditions, toxins, chemicals and other physical or xenobiotic agents including heat, radiation and heavy metals. Apoptosis is a complex process characterized by cell shrinkage, chromatin condensation and internucleosomal DNA fragmentation (8, 9, 10). Several protease families are implicated in apoptosis (for review see 11), the most prominent being caspases (12). Caspases are cysteine-containing aspartic acid-specific proteases and all have similar site specific proteolytic activity. Caspases are divided into three distinct groups based on their substrate specificities. Group I (YVADase) includes caspase-1, -4 and -5 which are involved in cytokine production. The group II (DEVDase) caspases, e.g. caspase-3 and -7 are the main effector caspases during apoptosis. These are cleaved by group III (IETDase) caspases (i.e. caspase-6, -8, -9 or -10) early during the onset of apoptosis (13). Upon activation, group II caspases act on various cellular proteins including poly(ADP-ribose) polymerase (PARP) and fodrin (13). Interestingly, most events in apoptosis appear to require a caspase-mediated proteolytic step.

One of the main mechanisms by which caspase-8 is activated during apoptosis is through engagement of

[1] To whom correspondence shoul be addressed. Fax: +46-8-32 90 41. E-mail: sten.orrenius@imm.ki.se.

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the CD95 receptor with CD95-ligand or agonistic antibodies. It was recently shown that caspase cascades can be activated or amplified after release of cytochrome *c* from the mitochondria into the cytosol during the early stages of apoptosis (14, 15). Once cytochrome *c* is released from mitochondria it partakes in a series of protein-protein interactions leading to activation of caspases (16).

Quinone compounds, such as menadione, are known to have cytotoxic effects which are mediated through oxidative stress and alterations in cellular Ca^{2+} homeostasis. Metabolism of menadione leads to the generation of reactive oxygen species (ROS) (17, 18) and to the oxidation of GSH to GSSG (19). Production of ROS and the resulting oxidative stress are implicated in cell death; however, the extent of oxidative stress may determine the mode of cell death. Therefore, in the present study we have used the HepG2 cell line to compare the events involved in CD95- and menadione-induced cell death with particular emphasis on the mechanisms which decide the mode of cell death.

MATERIALS AND METHODS

Cell culture and induction of cell death. HepG2 cells (ECACC) were cultured in Modified Eagle's Medium (MEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 1% non-essential amino acids, 1 mM pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents were obtained from Gibco). Cells were maintained in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Cell death was induced after treatment with 250 ng/ml anti-CD95 antibody (αCD95) (MBL Co. Ltd. Japan) in the presence of 1 μg/ml cycloheximide (CHX) to sensitize the cells, or with 250 μM of either menadione or pyrogallol. In antioxidant studies cells were treated as above in the presence or absence of 500 U/ml of catalase or 400 U/ml Cu/Zn-SOD (all from Sigma). The pan-caspase inhibitor, zVAD-fmk (Enzyme Systems Products) was used at 20 μM.

Vital dye exclusion assay and Hoechst-staining. Plasma membrane integrity of cells was tested by staining with 0.2% trypan blue (Sigma). The trypan blue positive cells were detected using a light microscope. For identification of cells with nuclear changes typical of apoptosis, cells were stained with 10 μg/ml of Hoechst 33342 (Molecular Probes), for 10 min at 4°C in the dark and analyzed using a UV microscope.

Field inversed pulse gel electrophoresis (FIGE). For the detection of HMW DNA fragments, samples were prepared and analyzed as previously described (20).

Caspase activity assays. The activities of group I, group II and group III caspases were determined fluorometrically using YVAD-AMC, DEVD-AMC or IETD-AMC, respectively, in a buffer containing 100 mM Hepes, 10% sucrose, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, CHAPS, 5 mM DTT, 10^{-6}% Nonidet P-40 (21).

Preparation of cytosol for detection of cytochrome c release. After appropriate treatments, cytosolic fractions of cells were prepared as previously described and the presence of cytochrome *c* was analyzed by Western blotting (22).

Western blotting. For Western blot analysis cells were lysed in a buffer containing 0.02 M Hepes, 0.33 M NaCl, 0.1 μM EGTA, 1.0 μM EDTA, 1.0 μM $MgCl_2$, 0.1% Triton X-100, pH 7.4 for 30 min on ice. Equal amounts of protein were boiled in Laemmli's buffer for 5 min and were resolved on a 10% SDS-PAGE. After transblotting, the nitrocellulose membranes were blocked in 5% non-fat milk and then probed using antibodies against PARP (Biome), fodrin (Affinity), cytochrome *c*, caspase-3 or caspase-8, for 1 h. After incubation with primary and appropriate HRP-conjugated secondary antibodies (Pierce), membranes were developed using the ECL-reagent (Amersham).

Measurement of intracellular ROS. Superoxide anion levels were measured using the dye dihydroethidium (Molecular Probes) as previously described (23, 24) while peroxide levels were determined using the dye DCFH/DA (24).

ATP measurements. For ATP studies, cells were grown in glucose-free medium supplemented with pyruvate (1 mM) or glucose/pyruvate (20 mM/1 mM). Intracellular ATP was determined luminometrically using the ATP Bioluminescence Assay kit CLS II (Boehringer Mannheim), according to the manufacturer's instructions. Values were corrected for the amount of protein. Addition of glucose (20 mM) resulted in ~20% rise in ATP level in comparison to the cells cultured in the presence of pyruvate alone.

RESULTS

Induction of apoptosis and necrosis. Treatment of the HepG2 cells with αCD95 resulted in a gradual loss of the trypan blue exclusion ability of cells' over time (up to 24 h), but this loss did not exceed 15% (Fig. 1A). However, the effects of menadione were much more dramatic in that more than 90% of the cells were stained with trypan blue by 6 h and in contrast to CD95-mediated cell death it was not inhibitable by the pan-caspase inhibitor, z-VAD-fmk (Fig. 1B).

A study of the nuclear changes by staining with Hoechst 33342 dye demonstrated that αCD95 induced nuclear changes typical of apoptosis in 76% of the cells by 24 h (Fig. 2A). However, the percentage of apoptotic nuclei in menadione treated cells did not deviate from that in the control cells (Fig. 2B). The lack of apoptotic nuclei and rapid loss of plasma membrane integrity after menadione treatment were all indicative of a necrotic mode of cell death.

The pattern of DNA fragmentation was used as another criterion to differentiate between apoptosis and necrosis. In αCD95-treated cells HMW DNA degradation into 50 kbp fragments indicative of apoptosis were visible using FIGE (Fig. 3). In menadione-treated cells on the other hand there was no cleavage of DNA that could be detected by FIGE (Fig. 3). Oligonucleosome-sized DNA fragments were undetectable after treatment with either αCD95 or menadione, using conventional gel electrophoresis (data not shown).

Cytochrome c translocation and caspase activation during cell death. In recent years, activation and/or amplification of the caspase cascade has been shown to involve release of cytochrome *c* from mitochondria (14, 15, 25). Necrosis has also been shown to involve mitochondrial damage (26, 27) and release of cytochrome *c* (22, 25). Western blot analysis of the cytosolic fraction demonstrated rapid release of cytochrome *c* from mitochondria after treatment of cells with either αCD95 or menadione (Fig. 4A).

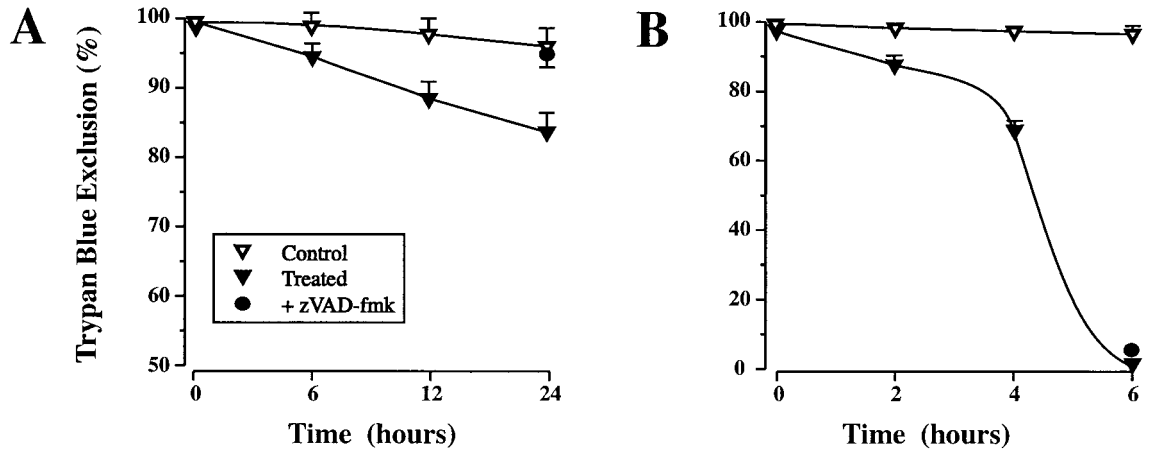

FIG. 1. A comparison of the loss of plasma membrane integrity, as determined by trypan blue exclusion, of HepG2 cells after treatment with (A) 250 ng/ml αCD95 for up to 24 h, or (B) 250 μM menadione for 6 h. The effect of the caspase inhibitor, zVAD-fmk (20 μM) on loss of plasma membrane integrity was also tested in each case.

To determine if cytochrome *c* release from mitochondria lead to the activation of caspases in our experimental model, two different approaches were taken. These were caspase activity assay using the fluorogenic substrates and Western blotting to determine the cleavage of pro-caspases and their cellular substrates. Using fluorometric assays there was no detectable induction of either group I (YVADase) or group III (IETDase) activities in cells treated with αCD95 (data not shown). However, caspase-3-like DEVDase activity increased from 4 pmol/min in untreated cells to 64 pmol/min in cells treated with αCD95 for 12 h (Fig. 4B). Menadione, however, did not only fail to induce activity of any of the group-specific caspases monitored in these cells (Fig. 4B, data not shown), but also reduced the background DEVDase activity to below that of untreated cells (Fig. 4B).

Western blotting of protein samples clearly demonstrated cleavage of pro-caspase-8 and -3 in cells treated with αCD95, but not with menadione (Fig. 4C). The processing of caspases to their active forms led to the cleavage of caspase target proteins, PARP and Fodrin (Fig. 4C).

Mechanism of menadione induced necrosis: ROS generation versus ATP-depletion. It has been reported that excessive generation of ROS (28, 29), as well as depletion of cells of ATP (30, 31) may interfere with the apoptotic death program. Furthermore, inhibition of caspase activity has been shown to drive cells into necrosis (32). Therefore, we hypothesized that menadione may cause necrosis through inactivation of caspases, either via oxidation-mediated events or as a result of ATP depletion. To this end, cells were first treated with αCD95 and were then incubated with menadione for 2 h. The DEVDase activity after 12 h

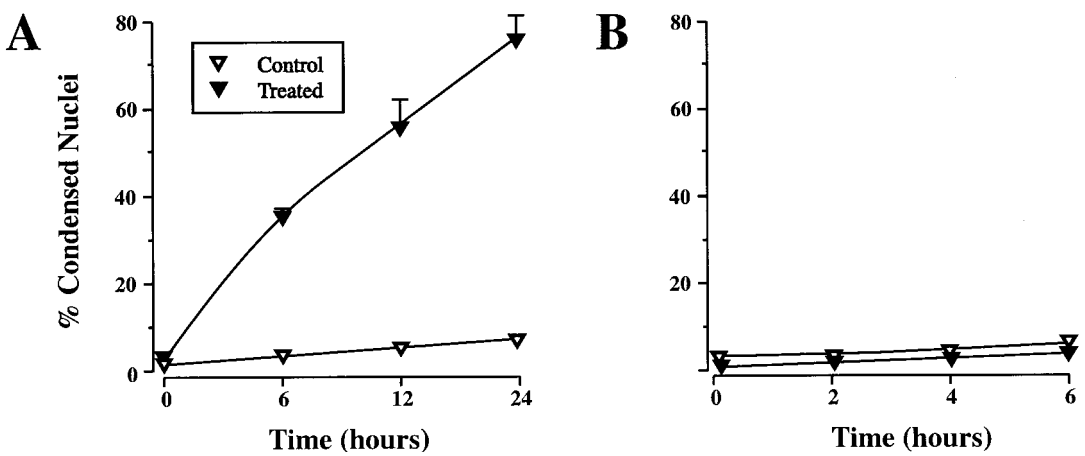

FIG. 2. A comparison of nuclear changes during apoptosis and necrosis using Hoechst 33342 in cells treated with (A) 250 ng/ml αCD95 for up to 24 h, or (B) 250 μM menadione for 6 h.

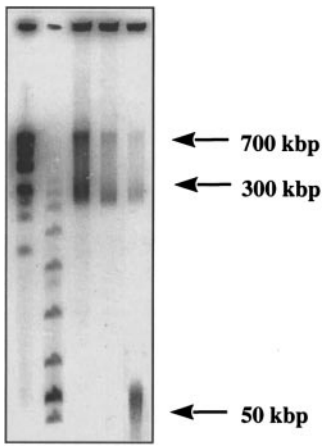

FIG. 3. The pattern of DNA fragmentation in HepG2 was determined by FIGE. Lanes 1 and 2, markers; lane 3, untreated control; lane 4, cells treated with 250 μM menadione for 6 hours; and lane 5, cells treated with 250 ng/ml αCD95 for 12 h.

incubation with αCD95, reached 64 pmole/min which was reduced to 3.5 pmole/min in cells co-incubated with menadione (Fig. 5A). A similar, pattern of caspase inhibition was observed using pyrogallol, another ROS

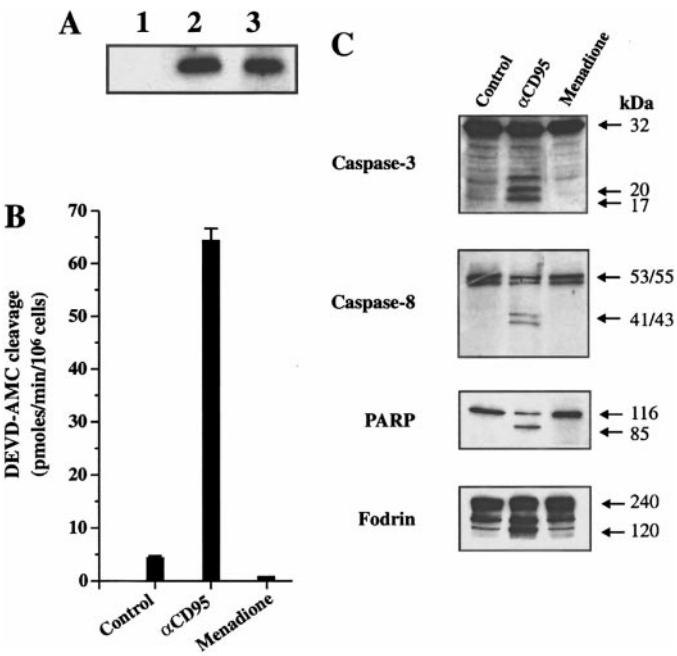

FIG. 4. The release of cytochrome *c*, determination of caspase activity and cleavage of caspase substrates during cell death. Cells were treated with 250 ng/ml αCD95 or 250 μM menadione. (A) Western blot analysis of cytochrome *c* release into the cytosolic fraction of cells undergoing apoptosis or necrosis. Control cells (lane 1); cells treated for 2 h with αCD95 (lane 2); or menadione (lane 3). (B) The rate of DEVD-AMC cleavage (AMC release) was measured fluorometrically in cells treated with αCD95 for 12 h or menadione for 4 h; (C) the protein extracts from these cells were then analyzed by Western blotting using antibodies against caspase-3, caspase-8, PARP or fodrin.

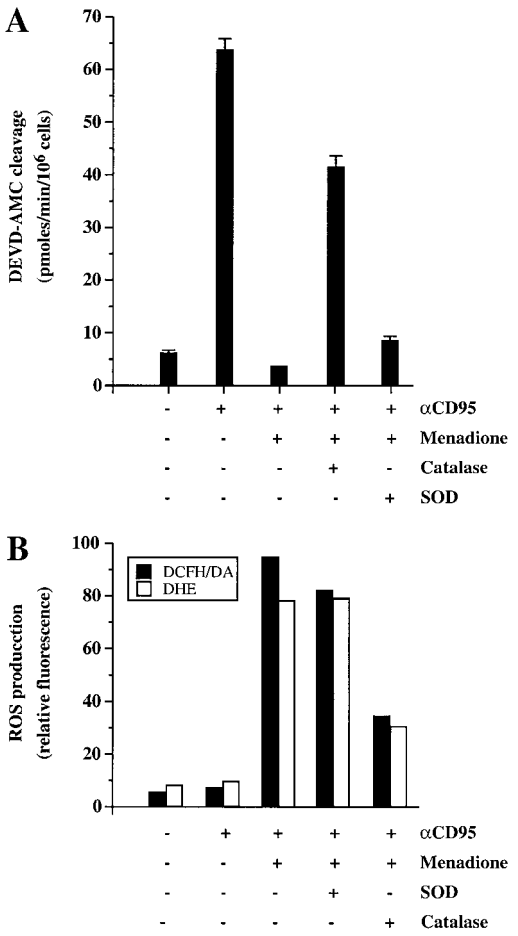

FIG. 5. The effect of modulation of cellular redox status on caspase activity. Cells were cultured in presence of catalase (500 U/ml) or Cu/Zn-SOD (400 U/ml) and caspase activity was induced after treatment of cells with 250 ng/ml of αCD95 for 12 h. Menadione was added to these cells during the last 2 h of incubation in the presence or the absence of antioxidants. (A) The alterations in caspase was measured fluorometrically prior or after addition of menadione in presence of antioxidants. (B) Changes in intracellular peroxides or superoxide was determined by measuring oxidation of DCFH/DA and dihydroethidium (DHE), respectively, with a flow cytometer.

generator (data not shown). Preincubation of αCD95-stimulated cells with catalase, but not with Cu/Zn-SOD, for 30 min was able to reverse the inhibitory effect of menadione on caspase activity (Fig. 5A). The restoration of caspase activity coincided with a reduction in menadione-induced ROS levels (Fig. 5B).

Since a substantial loss of ATP may also contribute to inhibition of caspases by menadione, intracellular ATP levels were measured in cells treated with αCD95, menadione or both. As demonstrated in Fig. 6A, both menadione and αCD95 reduced cellular ATP levels, however, the effects of menadione were more pronounced. Addition of menadione to αCD95-treated cells reproduced the same effect on ATP levels and caspase activity as menadione alone (Figs. 6A and 6B). Main-

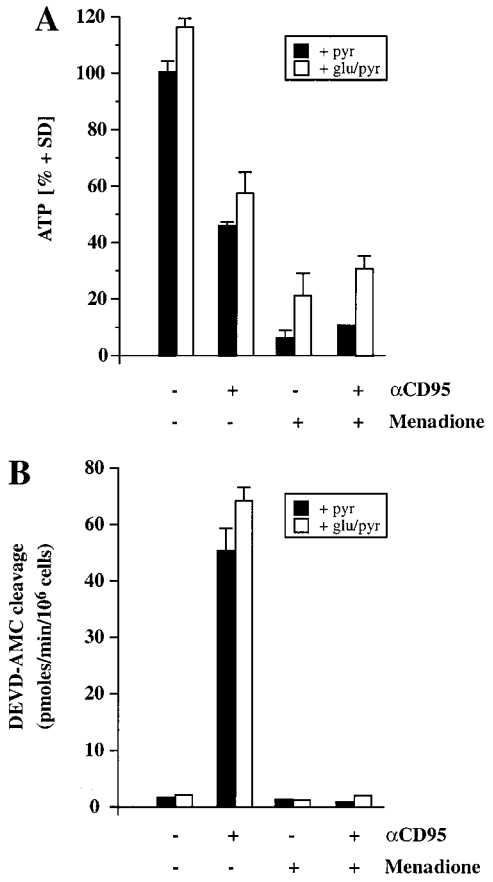

FIG. 6. The effect of modulation of cellular ATP level on caspase activity. Cells were cultured in glucose-free medium containing pyruvate (1 mM) or glucose (20 mM) and pyruvate. Cells were subjected to αCD95, menadione or both as mentioned in the text. Cellular ATP levels (A) and caspase activity (B) were then measured.

tenance of ATP at a relatively high level by culturing cells in a glucose-enriched medium had no apparent effect on restoring caspase activity (Figs. 6A and 6B). These results suggest that the generation of ROS, rather than ATP depletion, plays a key role in caspase inactivation and lack of apoptotic morphology in cells treated with menadione.

DISCUSSION

Menadione and αCD95 are known to cause cell death in hepatocytes. The CD95-mediated cell death occurs via apoptosis, while menadione has been regarded as a classical inducer of necrosis in hepatic-derived cells. In the present comparative study, αCD95 induced apoptosis in HepG2 cells that involved caspases and was inhibitable with the pan-caspase peptide inhibitor zVAD-fmk. Menadione, on the other hand, induced necrosis which was associated with rapid generation of ROS, ATP depletion and loss of plasma membrane integrity, and which was not inhibitable with zVAD-fmk. The only common feature of αCD95-induced apoptosis and menadione-induced necrosis observed in the present study was the release of cytochrome *c* from mitochondria.

The release of cytochrome *c* from mitochondria into the cytosol leads to the activation of a caspase cascade. In this study, pro-caspase-3 cleavage and activity was markedly increased after ligation of CD95 receptor which coincided with pro-caspase-8 cleavage and cytochrome *c* release. Although pro-caspase-8 itself was found to be cleaved, there was no detectable IETDase activity in apoptotic HepG2 cells (see Fig. 4B), possibly due to a low level of activity below the detection threshold of the assay. In contrast to αCD95-treated cells, menadione-induced cytochrome *c* release was not accompanied by caspase activation. Furthermore, addition of menadione to apoptotic cells quenched their caspase activity and promoted necrosis. These observations lend further support to the hypothesis that caspase inactivation in lethally injured cells switches the mode of cell death from apoptosis to necrosis (32). Two possible mechanism by which menadione could inhibit caspases were ATP depletion or generation of ROS. It has been reported that apoptotic cell death induced in Jurkat T cells by staurosporine and αCD95, could be switched to necrosis, when cells were depleted of ATP (30). In the same report repletion of the extramitochondrial ATP-pool with glucose restored the ability of the cells to undergo apoptosis. However, in the present study an increase in ATP levels as a result of culturing the HepG2 cells in glucose-enriched medium did not restore caspase activity (see Fig. 6). This observation suggested that in menadione-treated cells caspases were rendered inactive irrespective of ATP levels, possibly as a result of the oxidative stress which accompanies the redox cycling of this compound.

Caspases contain an active site cysteine nucleophile (12) which is prone to oxidation or thiol alkylation (28, 33). It is therefore not surprising that activity of caspases is optimal under reducing environments. Any deviation from such reducing conditions within the dying cell could be detrimental to caspases and render them inactive. It was recently demonstrated, by our group that hydrogen peroxide suppresses both the activation and activity of caspases, possibly through modulation of the redox status of the cell and the oxidation of cysteine residues in caspases (29). Furthermore, the oxidation of dithiocarbamates to dithiocarbamate disulphides has been shown to inhibit apoptosis (28), through covalent binding to active site cysteine of caspases and inhibition of their enzymatic activity (34). Also S-nitrosylation of thiol group within the active site cysteine of caspases and tissue transglutaminase, has been shown to inactivate these enzymes (35). Inhibition of caspase activity in cells that would otherwise undergo apoptosis, has recently been shown to drive cells into necrosis (32, 35). In this study the

inhibitory effect of menadione on caspases was shown to be mimicked by pyrogallol, another ROS generator. Furthermore, coincubation with catalase, but not Cu/Zn-SOD, significantly reduced the level of ROS produced by menadione and restored caspase activity. Taken together, these results strongly suggest that the inhibitory effects of menadione are due to production of hydrogen peroxide and not to a direct effect of menadione on caspase thiol groups.

The toxic effects of menadione and production of ROS have been shown in various cell types, however, in some cells it appears to induce necrosis while in others apoptosis is the predominant form of cell death (17, 36). Although differences in concentration of menadione used in each case may explain the mode of cell death, a more plausible explanation for these discrepancies may be the differential rate of redox cycling of menadione which appears to be cell type dependent. In conclusion, this study has shown that menadione is an inhibitor of caspase activity and apoptotic cell death in HepG2 cells by a mechanism that is reversible by catalase. This observation suggests that caspase activity is required for the apoptotic phenotype and that strong oxidants inhibit caspases.

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