Acta Biochimica Polonica

Vol. 51 No. 4/2004

1023–1038

QUARTERLY

Partial characterization of human choriocarcinoma cell line JAR cells in regard to oxidative stress✪

Anna Hallmann[1], Jerzy Klimek[1], Makoto Masaoka[2], Marcin Kamiński[3], Jakub Kędzior[3], Anna Majczak[3], Edyta Niemczyk[3], Michał Woźniak[4], Piotr Trzonkowski[5] and Takashi Wakabayashi[3]✉

[1]*Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, Gdańsk, Poland;* [2]*Department of Anatomy and Cell Biology, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [3]*Department of Cell Biology and Molecular Pathology,* [4]*Department of Medical Chemistry, and* [5]*Department of Histology, Medical University of Gdańsk, Gdańsk, Poland*

Received: 22 June, 2004; revised: 12 October, 2004; accepted: 26 October, 2004

Key words: placenta, JAR cells, oxidative stress, menadione, H_2O_2, flow cytometry

Characterization of free radical-induced cell injury processes of placenta cells is of vital importance for clinical medicine for the maintenance of intrauterine fetal life. The present study has analyzed cell injury processes in cells of the choriocarcinoma cell line JAR treated with menadione, an anticancer drug, and H_2O_2 in comparison to osteosarcoma 143B cells using electron microscopic and flow cytometric techniques. Flow cytometry on JAR cells exposed to 100 μM menadione and double-stained with Annexin V and propidium iodide (PI) detected apoptotic cells reaching the maximum after 4 h of incubation with a rapid decrease thereafter. Viable cells became decreased to 46% of the control after 2 h of incubation, reaching 5% after 4 h. Cells stainable with both Annexin V and PI began to increase distinctly after 2 h of incubation, reaching 55% after 4 h. Electron microscopy showed that cells stainable with both dyes specified above had condensed nuclei and swollen cytoplasm, suggesting that they were undergoing a switch of the cell death mode from apoptosis to necrosis. On the other hand, 90% of 143B cells remained intact after 4 h

✪This work was supported in part by grants from State Committee for Scientific Research KBN, within the project ST-40 to A.H. and J.K. and within the project W-48 to A.H.

✉Correspondence to: Takashi Wakabayashi, Department of Cell Biology and Molecular Pathology, Medical University of Gdańsk, Dębinki 1, 80-210 Gdańsk, Poland; tel. & fax: (48 58) 349 1456; e-mail: twakaba@amg.gda.pl; twakaba@med.nagoya-u.ac.jp

Abbreviations: DHE, dihydroethidium; EGF, epidermal growth factor; FASL, Fas ligand; H_2DCF-DA, dichlorodihydrofluorescein diacetate; LDH, lactate dehydrogenase; MEN, menadione; PI, propidium iodide; ROS, reactive oxygen species.

of menadione treatment although the intracellular levels of superoxide were always higher than those of JAR cells treated with the drug. In contrast, JAR cells were more resistant than 143B cells to H_2O_2-induced cytotoxicity. These results may suggest that cytotoxicity of menadione cannot be explained simply by oxygen free radicals generated from the drug. The resistance of JAR cells to oxygen free radical-induced cytotoxicity may be advantageous for intrauterine fetal life.

Data have accumulated demonstrating that apoptosis, a form of cell death, plays a crucial role in normal development and differentiation of human placenta (Smith *et al.*, 1997a; Chan *et al.*, 1999; Mayhew *et al.*, 1999). At an early stage of gestation, villous trophoblasts proliferate markedly and form the outer villous syncytiotrophoblasts and the inner cytotrophoblasts that finally form the placenta. Recent advances in the study of syncytiogenesis have revealed that a series of early steps of the apoptosis cascade precede syncytial fusion in human trophoblasts (Huppertz *et al.*, 1998; 1999). Phosphatidylserine translocation from the inner to the outer plasmalemmal leaflet, which is an early sign of apoptosis, is a prerequisite for syncytial fusion (Leyden *et al.*, 1993; Adler *et al.*, 1995). In contrast to other cellular systems, the apoptotic cascade in syncytial systems does not proceed immediately to apoptotic death. Instead, upon syncytial incorporation of a trophoblast cell into the neighboring syncytrophoblast, high levels of apoptosis inhibitor (Mcl-1, Bcl-2) block progression of the apoptotic cascade (Pőtgens *et al.*, 2002).

Characterization of apoptosis in placenta has been achieved by several investigators besides those cited above, including Fas expression (Runić *et al.*, 1998) and expression of apoptosis-inducing Fas ligand (FASL) (Bamberger, 1997). Recently, Crocker *et al.* (2001) have shown that, upon exposure to TNFα/TNFγ, dexamethasone, staurosporine, or hypoxia, cytotrophoblasts isolated from whole term placenta, syncytiotrophoblasts obtained by treating cytotorphoblasts by recombinant human epidermal growth factor (EGF), and human extravillous trophoblast cell line, SGHPL-4, showed increased apoptosis. The typical morphological features of apoptosis were observed in cytotrophoblasts and SGHPL-4 cells, but only chromatin condensation was observed in syncytiotrophoblasts. Enhanced levels of reactive oxygen species (ROS) and decreased levels of antioxidants have been postulated to contribute to the development of pre-eclampsia, which is one of the most significant diseased conditions associated with human pregnancy along with intrauterine growth restriction (Wilson & Wang, 1993; Walsh & Wang, 1995; Wang & Walsh, 1996; Ishihara *et al.*, 2002). Actually, increased apoptosis has been found in pre-eclampsia and intrauterine growth restriction (Smith *et al.*, 1997b; Ishihara *et al.*, 2002). Thus, it is urgent to characterize further responses of placenta to oxidative stress and analyze the apoptotic processes in placenta.

In the present study we attempted to characterize apoptosis in cells of the human choriocarcinoma cell line JAR in comparison to that in human osteosarcoma 143B cells using menadione (2-methyl-1,4-naphthoquinone, MEN) and hydrogen peroxide as experimental models for the induction of oxidative stress and apoptosis. MEN, a redox cycling reagent, is one of the most popular chemicals which are known to induce cell death, and has been extensively studied by many investigators (DiMonte *et al.*, 1984a; 1984b; McConkey *et al.*, 1988; Dypbukt *et al.*, 1994; Sata *et al.*, 1997; Yamaguchi *et al.*, 1997; Sun *et al.*, 1999; Derfuss *et al.*, 1998; Turner *et al.*, 1998; Carcchio *et al.*, 1999; Samali *et al.*, 1999; Hollensworth *et al.*, 2000; Jones *et al.*, 2000; Laux & Nel, 2001; Ma *et al.*, 2002; Gerasimenko *et al.*, 2002). MEN is known to generate superoxide ($O_2^{\cdot -}$) during its metabolism (Thor *et al.*, 1982) which is then con-

verted into hydrogen peroxide (H_2O_2) by the action of superoxide dismutase. The drug induces either apoptosis or necrosis depending on experimental conditions, such as its concentration, duration of treatment and source of tissues (McConkey *et al.*, 1988; Dypbukt *et al.*, 1994; Sata *et al.*, 1997; Samali *et al.*, 1999). Recently, we have carried out a detailed analysis on time-dependent ultrastructural and flow cytometric changes with annexin V/PI staining of human osteosarcoma cell line 143B cells using 100 μM MEN, and found that transition of the cell death mode from apoptosis to necrosis occurs before the apoptotic processes are completed. In the electron microscope, 143B cells treated with MEN for 6 h consisted of a mixed population of cells with typical apoptotic features and those with a mixture of apoptotic and necrotic features and the population of the latter type of cells became predominant when the incubation time with MEN was prolonged further. Cells with a mixture of apoptotic and necrotic features observable by electron microscopy and stainable both with Annexin V and PI were designated as "intermediate cells", indicating that they were a transitional state from apoptosis to necrosis (Kamiński *et al.*, 2003).

In the present study we tried to characterize apoptosis in human choriocarcinoma JAR cells in comparison to that in human osteosarcoma 143B cells using MEN and hydrogen peroxide (H_2O_2) as experimental models for the induction of apoptosis caused by oxidative stress. We demonstrate here that intracellular levels of superoxide in MEN-treated JAR cells are distinctly lower than those in MEN-treated 143B cells and yet the apoptotic and necrotic changes of the former cells are more distinct than of the latter. JAR cells are more resistant to H_2O_2-induced cell injury. These results may suggest that other factors besides superoxide may be involved in the MEN-induced cell injury processes and that JAR cells are more resistant than 143B cells to oxidative stress.

MATERIALS AND METHODS

Cell culture. The choriocarcinoma cell line JAR (ATCC HTB-144) was cultured in a humidified atmosphere with 5% CO_2 in RMPI-1640 (Sigma Chemical Co., St. Louis, MO, U.S.A.) containing 1 mM sodium pyruvate, 10 mM Hepes (Sigma Chemicals, U.S.A.), supplemented with 10% heat-inactivated fetal bovine serum (Gibco), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma Chemicals, U.S.A.). Human osteosarcoma cell line 143B cells (ATCC CRL-8303) were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Eagle medium (Nissui Co. Ltd, Tokyo, Japan) containing 1 mM pyruvate (Sigma Chemicals, U.S.A.) supplemented with 10% fetal bovine serum (Gibco) and kanamycin (50 μg/ml) (Sigma Chemicals, U.S.A.) as described before (Karbowski *et al.*, 2001). 143B cells were kindly provided by Dr. M. Tanaka, Department of Gene Therapy, Gifu International Institute of Biotechnology (Yagi Park, Gifu, Japan).

Treatment of cells with inducers of free radicals. Cells were cultured in the presence of inducers of oxygen free radicals: menadione (2-methyl-1,4-naphthoquinone, MEN) (Sigma Chemicals, U.S.A.) or hydrogen peroxide (Sigma Chemicals, U.S.A.). Cells cultured on 6-well culture dishes (5×10^5–1×10^6 cells/well) were treated for 2, 4, 6, 9, 15 or 24 h with 100 μM MEN or 1 mM H_2O_2 in the culture medium. Recently we observed that MEN at a concentration of 100 μM causes transition of the cell death mode from apoptosis to necrosis in 143B cells (Kamiński *et al.*, 2003) and this concentration of MEN was adopted also in the present study.

Assessment of cell viability. Viability of cells cultured in the presence of MEN or H_2O_2 for various times, specified above, was assessed by the leakage of lactate dehydrogenase (LDH) from the cell essentially according to the method of Gallo *et al.* (1999). Aliquots of cell suspension were centrifuged

for 3 min at 15 000 × ***g***, and the supernatant thus obtained was used for the assay. To a cuvette containing 0.05 M Tris/HCl, pH 7.4, 1 mM sodium pyruvate and 0.15 mM NADH and 10 μl sample of the assay solution was added and the decrease in absorbance at 340 nm resulting from the conversion of NADH to NAD^+ was measured spectrophotometrically. The extent of LDH release was expressed as the percentage of the total release, i.e., the release of LDH after cell lysis with 1% Triton X-100.

Flow cytometric detection of the intracellular levels of oxygen free radicals. The overall intracellular levels of superoxide ($O_2^{\cdot -}$) were measured following the conversion of dihydroethidium (DHE) (Molecular Probes Inc., Eugen, OR, U.S.A.) into ethidium by the method of Mancini *et al.* (1997) as described before (Spodnik *et al.*, 2002). Cells growing on 6-cm culture dishes were stained with DHE (final concentration, 10 μM) for 30 min at 37°C in a humidified atmosphere with 5% CO_2. Intracellular levels of H_2O_2 were measured using dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes) essentially by the method of Garcia-Ruitz *et al.* (1997) as described previously (Karbowski *et al.*, 1999a; 1999b). Cells growing on 6-cm culture dishes were stained with H_2DCF-DA (final concentration, 5 μM) for 1 h at 25°C. Cells stained with DHE or H_2DCF-DA collected from the culture dish by trypsinization were washed in phosphate-buffered saline (PBS) (Sigma Chemicals, U.S.A.), pH 7.4, re-suspended in PBS and submitted immediately to FACSCAN (Coulter Corporation, Coulter, France).

Annexin V and PI binding assay. Loss of phospholipid asymmetry and exposure of phosphatidylserine in the outer leaflet of the cell membrane of experimental cells was assayed using FITC conjugated Annexin V (BioSource Int., CA, U.S.A.) and propidium iodide (PI) (Molecular Probes) double staining. PI can be used to assess plasma membrane integrity and cell viability. PI is excluded from cells with intact plasma membranes. Cells growing on 6-cm culture dishes were collected by trypsinization, washed with PBS, spooled with floating cells and suspended in 50 μl of Annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). In some experiments, cells attached to the culture dish excluding those floating in the culture medium were used. Then, FITC-Annexin (2.5 μl) and PI (final concentration, 2 μg/ml) were added and the mixture was kept for 30 min at 20°C in the dark. The sample volume was adjusted to 0.5 ml with the Annexin V binding buffer and submitted to flow cytometric analysis using Coulter FACSCAN.

Intracellular levels of ATP. The intracellular level of ATP was measured by high performance liquid chromatography (HPLC). Extraction of nucleotides from the cells was carried out essentially according to the method of Kalsi *et al.* (1999). Cells (5×10^6 cells/ml) were collected from the culture dishes by trypsinization, centrifuged together with those floating in the culture medium and were washed with PBS. The cells were then suspended in 0.3 ml of cold 1.4 M $HClO_4$ and kept for 15 min on ice for the extraction of nucleotides. The acid extracts were neutralized to pH 7.5 by the addition of 0.1 ml of cold 3 M K_2HPO_4 and centrifuged (14000 × ***g***). The supernatants obtained were subjected to analysis by HPLC according to the method of Smoleński *et al.* (1990). Protein was determined using Bradford method (1976) after dissolving the perchloric acid precipitates in 0.5 M NaOH.

Electron microscopy. An equal volume of a fixative containing 4% glutaraldehyde, 4% formaldehyde and 0.2 M Na-cacodylate, pH 7.4, was added to the culture medium to fix attached and floating cells. When cells attached to culture dishes and those floating in the culture medium were fixed separately, the culture medium was removed from the culture dish, transferred to a test tube and fixed by the addition of an equal volume of the alde-

hyde solution, specified above. The surface of the culture dish was washed gently with PBS and a fixative containing 2% glutaraldehyde, 2% formaldehyde and 0.1 M Na-cacodylate, pH 7.4, was added to the culture dish to fix the attached cells. After fixation the samples were post-fixed with osmium tetroxide. Dehydration was initiated with 25% ethanol containing 1% uranyl acetate followed by graded series of ethanol. The samples were then treated with propylene oxide and processed for electron microscopy as described before (Karbowski *et al.*, 1997).

Statistical analysis. Data presented are means ± standard deviation (mean ± S.D.) of at least three different experiments. Statistical comparisons of data were made using paired *t*-test. Values of experimental groups are statistically different from those of the control group at: a ($P < 0.001$), b ($0.001 < P < 0.01$), c ($0.01 < P < 0.02$), d ($0.02 < P < 0.05$).

RESULTS

Viability of JAR cells treated with MEN or H_2O_2

In Figs. 1A and 1B both JAR cells and 143B cells were treated with 100 μM MEN and 1 mM H_2O_2, respectively, for various lengths of time and the viability of cells was measured using LDH release. In the case of MEN, the rates of LDH release from both cell lines were similar to each other for up to 4 h of the treatment, and they became distinctly higher in JAR cells than in 143B cells when the treatment lasted for 6 h or longer reaching 75% and 60%, respectively, at 24 h of the treatment. On the other hand, LDH release from JAR cells remained less than 5% for up to 9 h of H_2O_2-treatment reaching 12% at 24 h while that from 143B cells was 11.5% after 4 h of the treatment reaching 77.5% at 24 h. These data suggest that JAR cells are more resistant than 143B cells to H_2O_2-induced oxidative stress while they are more susceptible than the latter cells to MEN-induced cell injury. Thus, time-dependent changes in the intracellular levels of oxygen free radicals in MEN- or H_2O_2-treated JAR cells were analyzed in comparison to those of 143B cells.

Intracellular levels of oxygen free radicals in MEN- or H_2O_2-treated JAR cells

In Figs. 2A and 2B, intracellular levels of $O_2^{\cdot -}$ and H_2O_2, respectively, were measured in JAR cells treated with MEN for various lengths of time, in comparison to those in

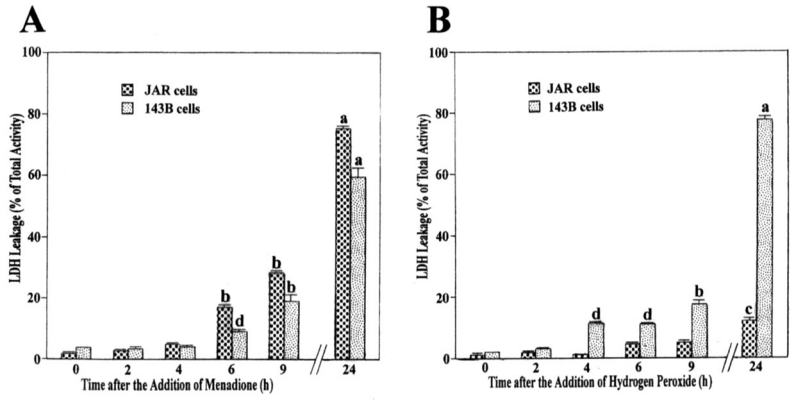

Figure 1. Time-dependent changes in the viability of JAR cells and 143B cells treated with MEN or H_2O_2 judged by LDH release.

Cells were treated with 100 μM MEN (A) or 1 mM H_2O_2. Values are expressed as the percentage of the total enzyme activity obtained by treating cells with Triton X-100. Values are expressed as the means ± S.D. of three experiments. Data on experimental groups are statistically different from the corresponding control at: a ($P < 0.001$), b ($0.001 < P < 0.01$), c ($0.01 < P < 0.02$), d ($0.02 < P < 0.05$).

143B cells. The intracellular level of $O_2^{\cdot -}$ in JAR cells became remarkably increased immediately after MEN treatment reaching the maximum after 4 h of the treatment with a rapid decrease thereafter (Fig. 2A). Also in the case of 143B cells, MEN caused a rapid and distinct increase in the intracellular level of $O_2^{\cdot -}$ reaching the maximum after 6 h of the treatment. The maximum intracellular level of $O_2^{\cdot -}$ in JAR cells, however, was much higher than that in 143B cells. The time-dependent changes in the intracellular level of H_2O_2 in JAR cells treated with MEN were similar to those in 143B cells. Namely, it decreased rapidly after 2 h of the treatment, and remained low thereafter (Fig. 2B). The changes in the intracellular level of H_2O_2 in H_2O_2-treated JAR cells were distinct from those of 143B cells: the intracellular levels of H_2O_2 in H_2O_2-treated JAR cells remained essentially the same as that of the control throughout the experiment while those of JAR cells increased distinctly immediately after the addition of H_2O_2, reaching the maximum after 6 h of the treatment followed by a rapid decrease (Fig. 2C). Typical examples of flow cytometric charts obtained for MEN- and H_2O_2-treated JAR cells and those for 143B cells are shown in Figs. 3A–3C and 3D–3F, respectively.

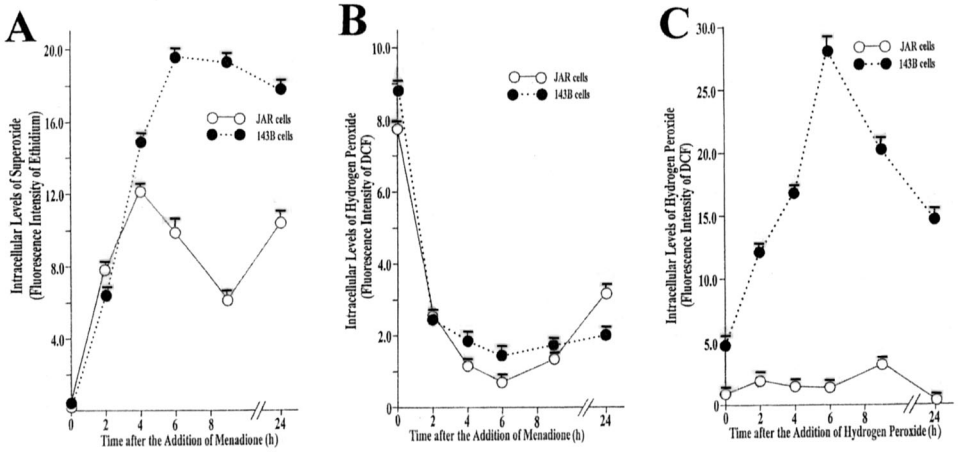

Figure 2. Changes in the intracellular levels of reactive oxygen species in JAR cells and 143B cells treated with MEN or H_2O_2.

Cells were treated with 100 μM MEN and intracellular levels of $O_2^{\cdot -}$ (A) and of H_2O_2 (B) were measured using dihydroethidium for the former and H_2DCF-DA for the latter. Cells were treated with 1 mM H_2O_2 and intracellular levels of H_2O_2 were measured using H_2DCF-DA (C). Data are the means ± S.D. of three experiments.

Changes in the ultrastructure of JAR cells treated with MEN or H_2O_2

For electron microscopic examination of cells, they were treated with MEN or H_2O_2 for various lengths of time and cells attached to the culture dish were collected avoiding the contamination with floating cells in the culture medium, as described before (Kamiński *et al.*, 2003). JAR cells were characterized by the presence of irregular shaped nuclei with prominent nucleoli (Fig. 4A) and scarce mitochondria were scattered in the cytoplasm (Fig. 4B). After 4 h of the MEN treatment, typical apoptotic cells with highly condensed nuclei and cytoplasm were occasionally detected (Fig. 4C). Although the majority of cells did not show such typical apoptotic features, aggregation of chromatin in the nucleus was evident and depositions of electron dense materials were frequently seen in mitochondria (Fig. 4D). After 6 h of the MEN treatment, aggregation of chromatin in the nucleus became further evident and the cyto-

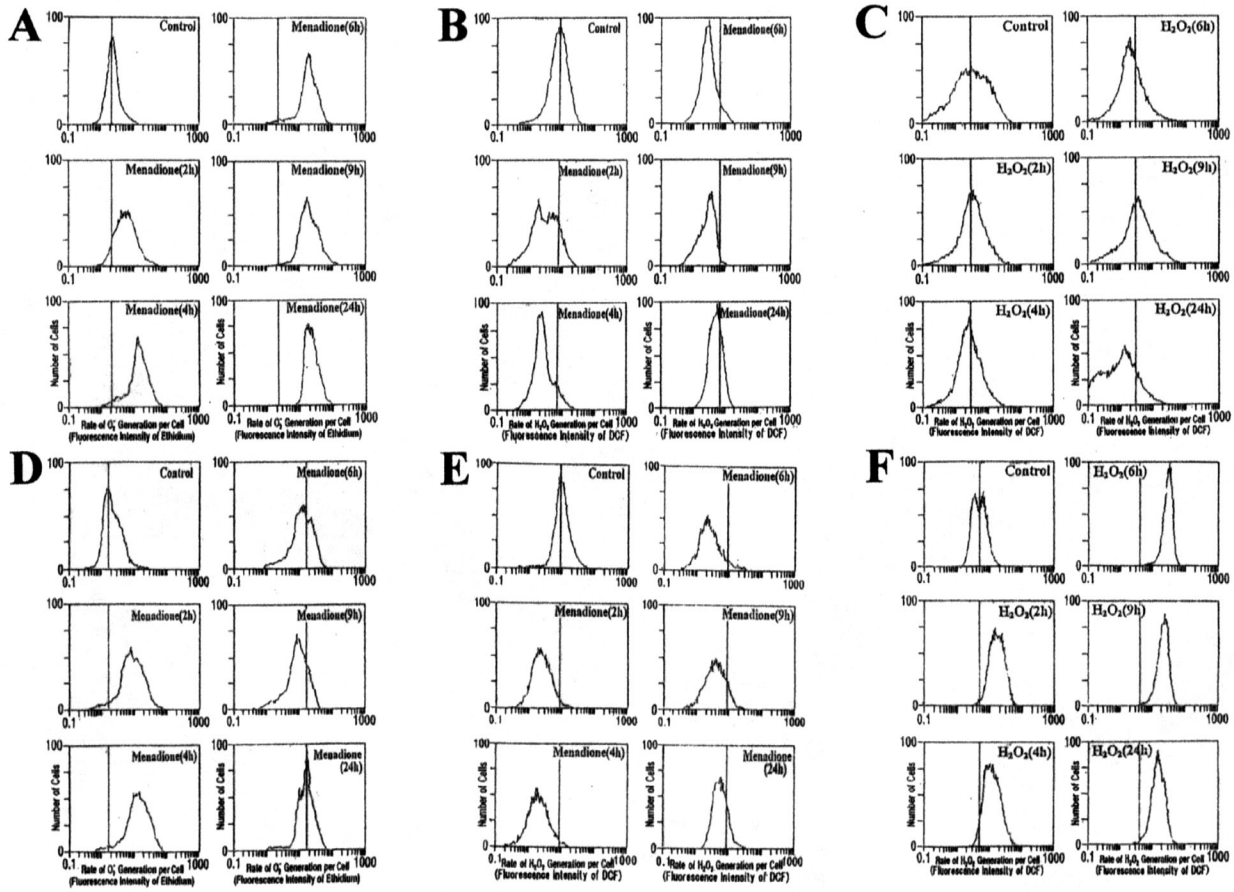

Figure 3. Flow cytometric charts demonstrating time-dependent changes in the intracellular levels of $O_2^{\cdot -}$ and H_2O_2 in MEN-treated JAR cells (A, B) and 143B cells (D, E) and levels of $O_2^{\cdot -}$ and H_2O_2 in H_2O_2-treated JAR cells (C) and 143B cells (F).

Data were obtained from the experiments shown in Fig. 2.

plasm became highly vacuolated (Fig. 4E). High magnification electron micrographs showed swelling of cytoplasm with distinct degenerative changes of organelles, and mitochondria were no longer detected (Fig. 4F). After 24 h of the MEN treatment the degenerative changes in the cytoplasm proceeded further, and plasma membranes often became ruptured (electron micrographs are not shown).

Results obtained with 143B cells were in agreement with the data reported previously (Kamiński *et al.*, 2003). Namely, typical apoptotic cells and those with moderately condensed nuclei and swollen cytoplasm co-existed at 6 h of the MEN treatment (electron micrographs are not shown). After 9 h of the treatment, apoptotic cells were no longer seen and cells with moderately condensed nuclei and swollen cytoplasm dominated (electron micrographs are not shown). Next, effects of H_2O_2 on the ultrastructure of JAR cells were examined. The majority of JAR cells treated with H_2O_2 for up to 9 h remained intact (Fig. 5A), and mitochondria appeared normal (Fig. 5B). When the duration of the treatment of cells with H_2O_2 was prolonged for 24 h, moderate aggregation of chromatin in the nucleus was observed occasionally (electron micrographs are not shown). On the other hand, H_2O_2 exerted more distinct effects on 143B cells: cells with numerous autophagic vacuoles in the cytoplasm and aggregation of chromatin in the nucleus were of-

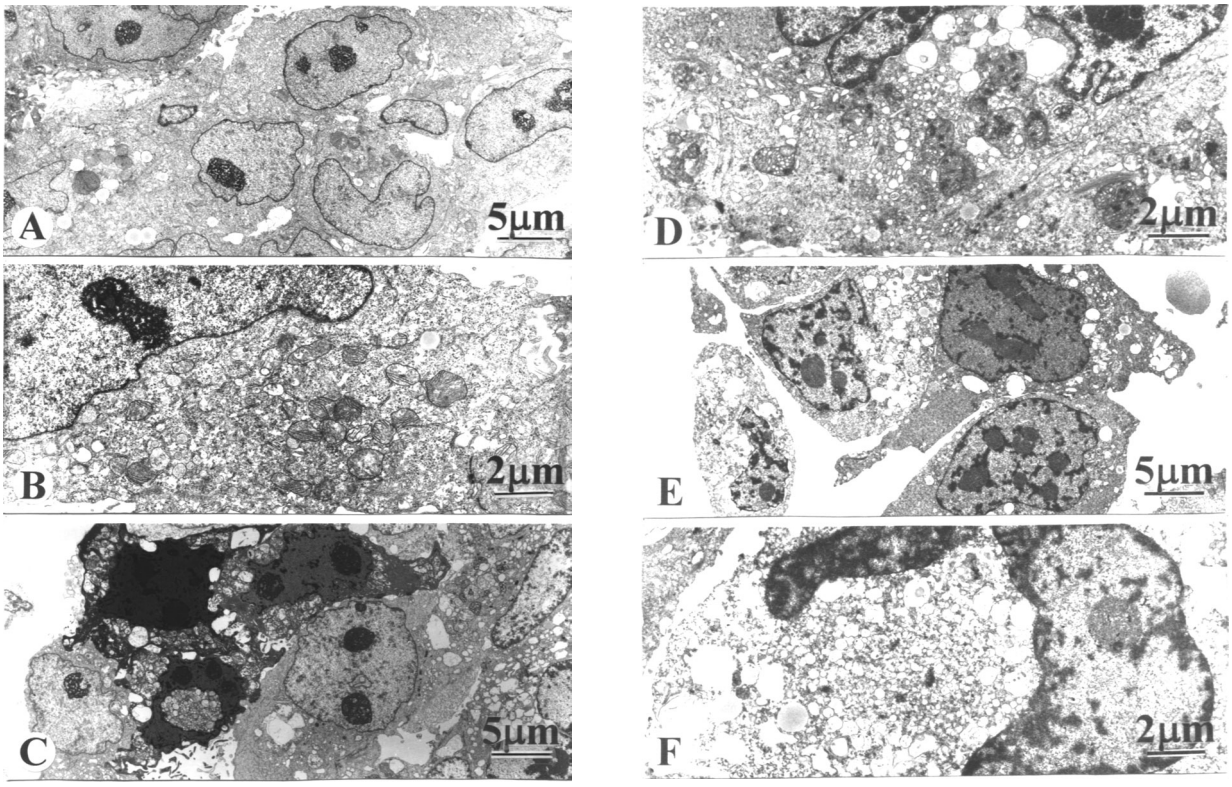

Figure 4. Ultrastructural changes of MEN-treated JAR cells.

JAR cells attached to the culture dish after the treatment with 100 μM MEN for 4 h (C, D) or for 6 h (E, F) were fixed for electron microscopy. A: control. Magnification: A, C, E: ×3500; B, D, F: ×8750.

ten detected after 2 h of the treatment. Typical apoptotic cells were encountered occasionally (Fig. 5C). When 143B cells were treated with H_2O_2 for 6 h or longer, cells with numerous autophagic vacuoles (Fig. 5D) were more common (Fig. 5E) and with aggregated chromatin scattered in the nucleus. Highly vacuolated mitochondria with depositions of electron dense materials were often encountered after 24 h of the treatment (Fig. 5F).

Detection of apoptotic and necrotic changes in MEN- and H_2O_2-treated cells by Annexin V and PI double staining

FITC-conjugated Annexin V and PI staining was used in the present study to detect apoptotic and necrotic changes in MEN- and H_2O_2-treated JAR cells. When cells double-stained with the dyes were analyzed by flow cytometry, four different groups of cells were obtained based on their stainability: those unstainable with Annexin V and PI (Annexin V(–)/PI(–)): viable cells; those stainable with Annexin V but unstainable with PI (Annexin V(+)/PI(–)): apoptotic cells; those stainable with both Annexin V and PI (Annexin V(+)/PI(+)): late apoptotic or necrotic cells; and those unstainable with Annexin V but stainable with PI (Annexin V(–)/PI(+)): necrotic cell debris or apoptotic bodies. Fig. 6A shows JAR cells treated with MEN for various lengths of time stained with the dyes and subjected to flow cytometry. The population of apoptotic cells reached a maximum after 4 h of the treatment and rapidly decreased thereafter while that of necrotic or late apoptotic cells began to increase after 2 h of the treatment and continued to increase thereafter reaching 95% after 24 h. On the other hand, most of 143B cells treated with MEN for up to 4 h remained intact (Fig. 6B).

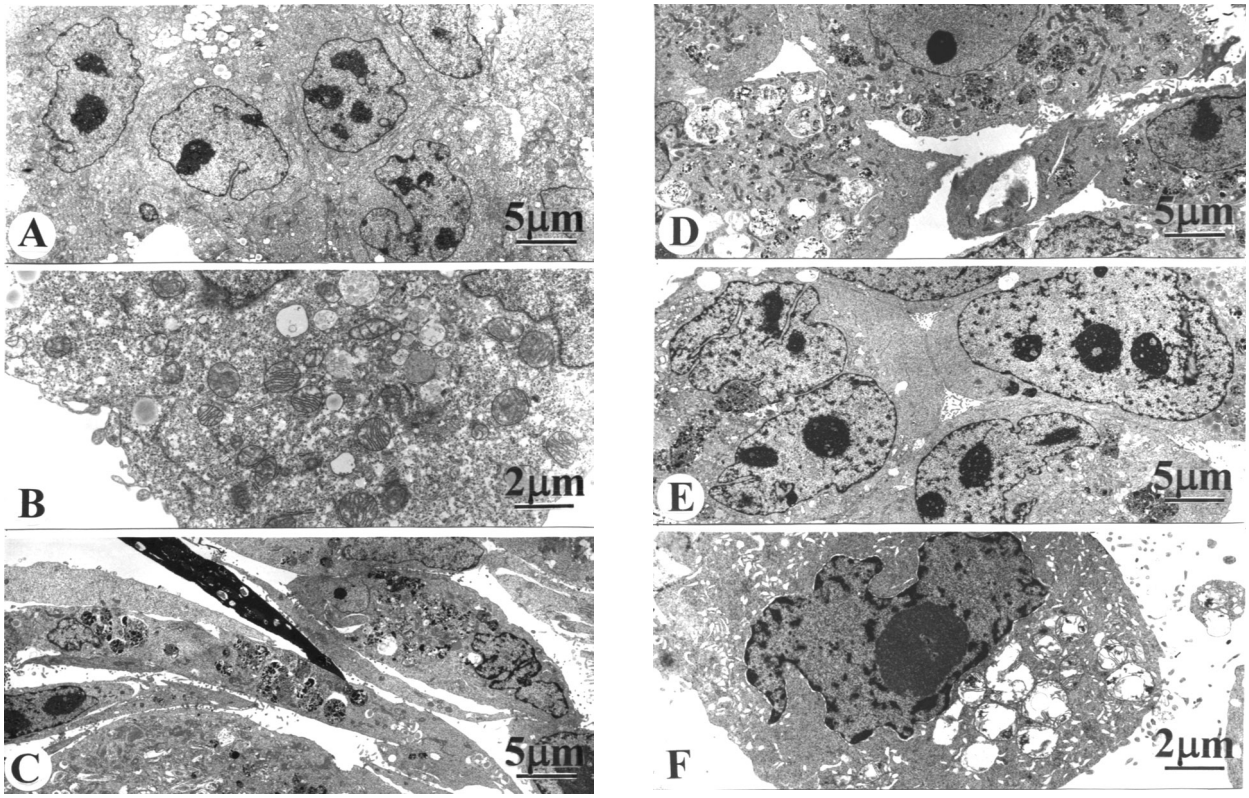

Figure 5. Ultrastructural changes of H_2O_2-treated JAR cells and 143B cells.

JAR cells attached to the culture dish after the treatment with 1 mM H_2O_2 for 9 h (A, B) and 143B cells treated for 2 h (C), 6 h (D, E) or 24 h (F) were fixed for electron microscopy. Magnifications: A, C–E: ×3500; B, F: ×8750.

The population of apoptotic cells reached the maximum after 6 h of the treatment and decreased thereafter. The frequency of late apoptotic or necrotic cells was larger than that of apoptotic cells after 9 h of the treatment, reaching 80% after 24 h, in agreement with the data from our laboratory published before (Kamiński *et al.* 2003). Next, flow cytometric analysis was performed on H_2O_2-treated JAR cells (Fig. 6C) and 143B cells (Fig. 6D). As was expected from the electron microscopic results described in the previous section, the toxic effects of H_2O_2 on both JAR and 143B cells, especially the former, were less distinct compared to those of MEN. Namely, about 70% of JAR cells treated with H_2O_2 for 24 h remained unstainable with Annexin V and PI. The frequencies of both apoptotic and necrotic cells were less than 5% throughout the course of the experiment, excepting the population of late apoptotic or necrotic cells, which reached about 10% after 24 h of the experiment (Fig. 6C).

In the case of 143B cells, 52% of the H_2O_2-treated cells remained unstainable with the dyes after 24 h of the treatment, although the frequency of both apoptotic and necrotic cells was much higher than for JAR cells (Fig. 6D). The results described above were obtained for cells attached to the culture dish spooled with those floating in the culture medium. When only the cells attached to the culture dish were used, similar results were obtained, although Annexin V(+)/PI (+) cells were somewhat less frequent.

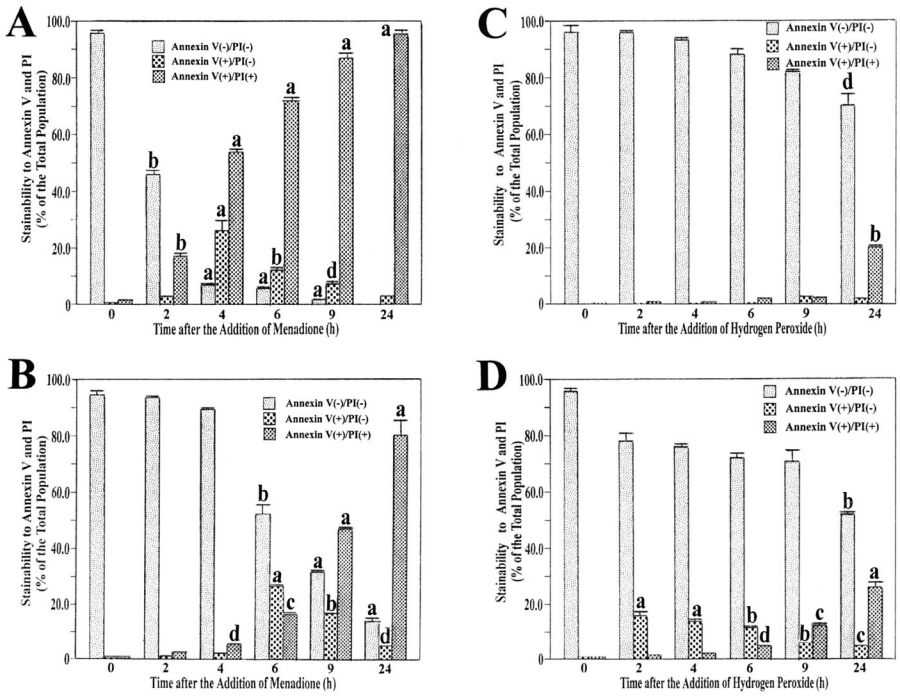

Figure 6. Viability of MEN- and H_2O_2-treated JAR cells and 143B cells.

Cells were treated with 100 μM MEN (A: JAR cells; B: 143B cells) or 1 mM H_2O_2 (C: JAR cells; D: 143B cells) for up to 24 h, and stained with Annexin V and PI for flow cytometry. Annexin V(–)/PI(–): intact cells; Annexin V(+)/PI(–): apoptotic cells; Annexin V(+)/PI(+): late apoptotic or necrotic cells; data are the means ± S.D. of three experiments. Data on experimental groups are statistically different from the corresponding control at: a ($P < 0.001$), b ($0.001 < P < 0.01$), c ($0.01 < P < 0.02$), d ($0.02 < P < 0.05$).

Changes in intracellular levels of ATP in MEN- and H_2O_2-treated JAR cells

The electron microscopic and flow cytometric results suggested that the switch of the cell death mode from apoptosis to necrosis took place in MEN- and H_2O_2-treated JAR cells. Thus, temporal changes in the intracellular level of ATP in cells treated with MEN or H_2O_2 were examined, since a decrease in the intracellular level of ATP plays a key role in cell injury processes (Trump *et al.*, 1971; Trump & Berezesky, 1994) and also in the switch mechanism of the cell death mode

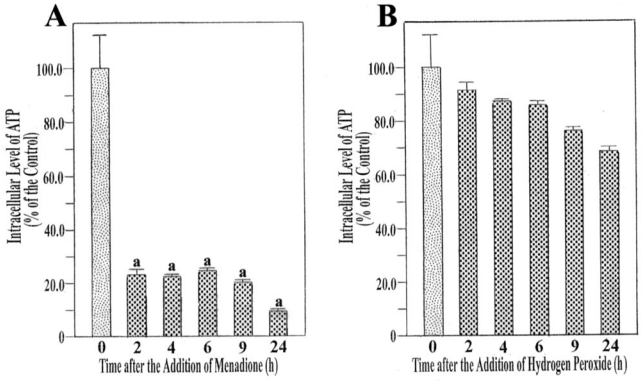

Figure 7. Time-dependent changes in intracellular levels of ATP in MEN- and H_2O_2-treated JAR cells.

Cells were treated with 100 μM MEN (A) or 1 mM H_2O_2 (B) for up to 24 h and ATP was measured by HPLC. Data on experimental cells are expressed as the percentage of the values of the corresponding controls. Values are the means ± S.D. of three experiments. Data on experimental groups are statistically different from the corresponding control at a ($P < 0.001$), b ($0.001 < P < 0.01$), c ($0.01 < P < 0.02$), d ($0.02 < P < 0.05$).

from apoptosis to necrosis (Leist *et al.*, 1997; Ferrari *et al.*, 1998; Ha & Synder 1999). JAR cells were treated with MEN or H_2O_2 for various lengths of time for up to 24 h, and the intracellular levels of ATP were measured. As shown in Fig. 7A, the intracellular levels of ATP in JAR cells decreased to 26.6% of the original level (control 10 ± 5 nmoles ATP/mg protein) after 2 h of the MEN treatment reaching 9.8% after 24 h, while those in 143B cells remained at about 60% of the original level (control 8 ± 5 nmoles ATP/mg protein) for up to 6 h of the MEN treatment and decreased to almost zero after 9 h. The intracellular level of ATP in JAR cells was 83% after 2 h of H_2O_2 treatment and 69.2% after 24 h (Fig. 7B), while in 143B cells it decreased to 10% of the original level after 2 h of H_2O_2 treatment and remained at a similar level for up to 24 h.

DISCUSSION

The present study demonstrated that JAR cells from a human choriocarcinoma cell line are more susceptible than the 143B cells derived from a human osteosarcoma cell line to MEN-induced oxidative stress while they are more resistant than the latter to H_2O_2-induced oxidative stress. This was supported by electron microscopic and flow cytometric data together with those on changes in the intracellular level of ATP.

Transition of cell death mode from apoptosis to necrosis in MEN-treated JAR cells

The flow cytometric analysis on MEN-treated JAR cells showed that the population of apoptotic cell reached its maximum after 4 h of the incubation followed by a gradual decrease. On the other hand, the population of late apoptotic or necrotic cells began to increase after 2 h of the incubation and continued to increase distinctly thereafter. In the electron microscope, the major population of cells after 6 h of the MEN treatment consisted of those with condensed nuclei and swollen cytoplasm. Recently, we showed that the switch of the cell death mode from apoptosis to necrosis took place in 143B cells when they were exposed to MEN, and the major population of cells after 9 h of the incubation consisted of those with condensed nuclei and swollen cytoplasm, which were designated as "intermediate cells", indicating that they were in a transitional state from apoptosis to necrosis (Kamiński *et al.*, 2003). The electron microscopic features of JAR cells after 6 h of MEN treatment detected in the present study were essentially the same as those of the 143B cells, cited above, indicating that the switch from apoptosis to necrosis took place also in JAR cells. As to the mechanism involved in the switch of the cell death mode from apoptosis to necrosis, increases in the intracellular level of free radicals have been stressed by several investigators (Bonfoco *et al.*, 1995; Nobel *et al.*, 1997; Hampton & Orrenius, 1997). A massive increase in the intracellular level of oxygen free radicals may cause caspase inactivation and affect mitochondria resulting in a decreased ability to produce ATP. In the present study, we showed that the intracellular level of $O_2^{\cdot -}$ in JAR cells began to increase immediately after MEN treatment, reaching maximum at 4 h. The intracellular level of ATP decreased to less than 30% of the control 2 h after the treatment. It is tempting to assume that the increase in the intracellular level of $O_2^{\cdot -}$ is the triggering factor for the induction of apoptosis and above certain levels of $O_2^{\cdot -}$, ATP generation from mitochondria drastically decreases resulting in the switch from apoptosis to necrosis detected in JAR cells. However, other functions of MEN than the generation of oxygen free radicals must be taken into account. MEN is known to cause GSH depletion and arrylation of proteins thereby altering the activity of key thiol enzymes in ATP metabolism (this will be dis-

cussed later), and exact mechanisms of the switch of the cell death mode from apoptosis to necrosis detected in the present study await further investigation.

Difference between JAR cells and 143B cells in susceptibility to MEN- and H_2O_2-induced cytotoxicity

The present study showed that JAR cells were more susceptible than 143B cells to MEN-induced cytotoxicity while they were more resistant than the latter to H_2O_2-induced cytotoxicity, based on the data on LDH release, electron microscopy and flow cytometry. However, the MEN-induced elevation of the intracellular level of $O_2^{\cdot -}$ was much more distinct in 143B cells than in JAR cells. The cytotoxic effects of quinines including MEN are thought to be mediated by NADPH-cytochrome P450 reductase through their one electron reduction to semiquinone radicals, which subsequently enter the redox cycle with molecular oxygen to produce $O_2^{\cdot -}$ which dismutates either spontaneously or in a reaction catalyzed by superoxide dismutase (SOD) to form H_2O_2. H_2O_2 is then converted into H_2O and O_2 catalyzed by catalase, or participates in the Fenton reactions generating hydroxyl radicals (Hassan & Fridovich, 1979). MEN also generates $O_2^{\cdot -}$ through nonenzymatic interaction with thiols such as reduced glutathione (GSH) or protein-SH (Werfers & Sies, 1983; Ross *et al.*, 1985; Takahashi *et al.*, 1987; Bellomo *et al.*, 1987; Tzeng *et al.*, 1994). In addition, MEN inhibits glutathione reductase and induces severe depletion of GSH (Bellomo *et al.*, 1987; Tzeng *et al.*, 1994). These properties of MEN suggest that, although MEN shares to some extent a signaling pathway with H_2O_2 for cell death induction, it may affect cells differently to H_2O_2 through modification of protein-SH and GSH by oxidation (Thor *et al.*, 1982; Werfers & Sies, 1983; Takahashi *et al.*, 1987) or arylation (DiMonte *et al.*, 1984a) or by influencing cellular Ca^{2+} homeostasis (DiMonte *et al.*, 1984b; Gerasimenko *et al.*, 2002). Recently, McAmis *et al.* (2003) have shown that MEN causes endothelial barrier failure by a direct effect on intracellular thiols, independently of H_2O_2 production. Namely, using pulmonary artery endothelial cells and diffusional permeability to fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) as a measure of barrier function, they found a profound barrier dysfunction with a parallel fall in glutathione almost to depletion, while H_2O_2 gave only a slight decrease in GSH concentration. Interaction of MEN with GSH has been found to differ depending on the cell type: in hepatocytes 15% of GSH is conjugated to MEN to form MEN-SG and 75% is oxidized to GSSG (Chung *et al.*, 1997). It has been also shown that glutathione depletion occurs rapidly in MEN-exposed platelets, before the plasma membrane and intracellular membrane damage (Cho *et al.*, 1997). Thus, although MEN may exert toxic effects on 143B and JAR cells by various mechanisms described above including glutathione depletion, disturbance in Ca^{2+} homeostasis, inactivation of thiol enzymes, the degree of cell injury could be different between the two cell lines used in the present study. It showed that the cytotoxic effects of MEN on both JAR and 143B cells were much more pronounced than those of H_2O_2. Furthermore, the elevation of the intracellular level of $O_2^{\cdot -}$ by MEN was much higher in 143B than in JAR cells, although the former were less severely injured. These results could be explained by the other effects of MEN, described above, than the direct action of $O_2^{\cdot -}$ generated from the drug.

The present study shows that JAR cells are more resistant than 143B cells to H_2O_2 cytotoxicity. Shibukawa *et al.* (2003) have reported that overexpression of *N*-acetylglucosaminyltransferase (GnT-III) suppresses H_2O_2-induced apoptosis. It has been shown that human choriocarcinoma cell lines, including JAR used in the present study, have a high expression of *N*-acetylglucosaminyl-

transferase IV (GnT-IV) (16-66-fold) and GnT-III (15-25-fold) as compared with those in human placenta (Takamatsu *et al.*, 1999). Thus, the resistance of JAR cells to H_2O_2 cytotoxicity observed in the present study could be explained by the above described lines of evidence although we did not analyze the activity of GnT-III in the present study. The fact that JAR cells are more resistant than 143B cells to H_2O_2 cytotoxicity might be important for the intrauterine life of the fetus. We are now repeating the experiments using human placenta to verify the data obtained in the present study and the results will be reported soon.

REFERENCES

Adler RR, Ng AK, Rotes NS. (1995) Monoclonal antiphosphatidylserine antibody inhibits intracellular fusion of the choriocarcinoma line, JAR. *Biol Reprod.*; **53**: 905–10.

Bamberger A-M, Schulte HM, Thuneke I, Erdmann I, Bamberger CM, Asa SL. (1997) Expression of the apoptosis-inducing Fas ligand (FasL) in human first and third trimester placenta and choriocarcinoma cells. *J Clin Endocrinol Metab.*; **82**: 3173–5.

Bellomo G, Mirabelli F, DiMonte D, Richelmi P, Thor H, Orrenius C, Orrenius S. (1987) Formation and reduction of glutathione-protein mixed disulfides during oxidative stress: A study with isolated hepatocytes and menadione (2-methyl-1,4-naphthoquinone). *Biochem Pharmacol.*; **36**: 1313–20.

Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. (1995) Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell culture. *Proc Natl Acad Sci USA.*; **92**: 7162–6.

Bradford MM. (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.*; **72**: 248–4.

Carcchio R, Kovalenko D, Kaufmann WK, Cohen PL. (1999) Apoptosis provoked by the oxidative stress inducer menadione (vitamin K_3) is mediated by the Fas/Fas ligand system. *Clin Immunol.*; **93**: 65–4.

Chan CC, Lao TT, Cheung AN. (1999) Apoptotic and proliferative activities in first trimester placenta. *Placenta.*; **223**: 175–80.

Cho Y-S, Kim M-J, Lee J-Y, Chung J-H. (1997) The role of thiols in protecting against simultaneous toxicity of menadione to platelet plasma and intracellular membranes. *J Pharmacol Exp Therap.*; **280**: 135–40.

Chung JH, Seo DC, Chung SH, Lee JY, Seung SA. (1997) Metabolism and cytotoxicity of menadione and its metabolite in rat platelets. *Toxicol Appl Pharmacol.*; **142**: 378–85.

Crocker IP, Barratt S, Kaur M, Baker PN. (2001) The *in-vitro* characterization of induced apoptosis in placental cytotrophoblasts and syncytiotrophoblasts. *Placenta.*; **22**: 822–30.

Derfuss T, Fickenscher H, Kraft MS, Henning G, Lengenfelder B, Fleckenstein B, Meinl E. (1998) Antiapoptotic activity of the herpesvirus saimiri-encoded Bcl-2 homolog: stabilization of mitochondria and inhibition of caspase-3-like activity. *J Virol.*; **72**: 5897–904.

DiMonte D, Ross D, Bellomo G, Eklőw L, Orrenius S. (1984a) Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys.*; **235**: 334–42.

DiMonte D, Ross D, Bellomo G, Eklőw L, Orrenius S. **(**1984b**)** Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. *Arch Biochem Biophys.*; **235**: 343–50.

Dypbukt JM, Ankerona M, Barkitt M, Sj olm A, Ström K, Orrenius S, Nicotera P. (1994) Different prooxidant levels stimulate a growth, trigger apoptosis or produce necrosis of insulin-secreting RINm5F cells. *J Biol Chem.*; **269**: 30553–60.

Ferrari D, Stępczyńska A, Los M, Sachulze-Ostoff K. (1998) Differential regulation and ATP requirement for caspase-8 and caspase-3 activation during CD95- and anticancer drug-induced apoptosis. *J Exp Med.*; **188**: 979–84.

Gallo M, Aragno M, Gatto V, Tamagno E, Brignardello E, Manti R, Danni O, Boccuzzi G. (1999) Protective effect of dehydroepiandrosterone against lipid peroxidation in a human liver cell line. *Eur J Endocrinol.*; **141**: 35–9.

Garcia-Ruiz C, Colell A, Mari M, Morales A, Fernandez-Checa JC. (1997) Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. *J Biol Chem.*; **272**: 11369–77.

Gerasimenko JV, Gerasimenko OV, Palejwala A, Tepkin AV, Petersen OH, Watson AJM. (2002) Menadione-induced apoptosis: role of cytosolic Ca^{2+} elevation and the mitochondrial permeability transition pore. *J Cell Sci.*; **115**: 485–97.

Ha HC, Synder SH. (1999) Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc Natl Acad Sci USA.*; **96**: 13978–82.

Hampton MB, Orrenius S. (1997) Dual regulation of caspase activity by hydrogen peroxide: implication of apoptosis. *FEBS Lett.*; **414**: 552–6.

Hassan HM, Fridovich I. (1979) Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. *Arch Biochem Biopys.*; **196**: 385–95.

Hollensworth SB, Shen C-C, Sim JE, Spitz DR, Wilson GL, LeDoux SP. (2000) Glial cell type-specific responses to menadione-induced oxidative stress. *Free Radic Biol Med.*; **28**: 1161–74.

Huppertz B, Frank HG, Kingdom JC, Reister F, Kaufmann P. (1998) Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the placenta. *Histochem Cell Biol.*; **110**: 495–508.

Huppertz B, Frank H-G, Reister F, Kingdom J, Korr H, Kaufmann P. (1999) Apoptosis cascade progresses during turnover of human trophoblast: analysis of villous cytotrophoblast and syncytial fragments *in vitro*. *Lab Invest.*; **79**: 1687–702.

Ishihara N, Matsuo H, Murakoshi H, Laoag-Fernandez JB, Samoto T, Maruo T. (2002) Increased apoptosis in the syncytiotrophoblasts in human term placentas complicated by either preeclampsia or intrauterine growth retardation. *Am J Obstet Gynecol.*; **186**: 158–66.

Jones BF, Lo CR, Liu H, Pradhan Z, Garcia L, Srinvasan A, Valentino KL, Czaja ML. (2000) Role of caspases and NF-κB signaling in hydrogen peroxide- and superoxide-induced hepatocyte apoptosis. *Am J Physiol Gastrointest Liver Physiol.*; **278**: 693–9.

Kalsi KK, Zych M, Slomińska EM, Kochan Z, Yacoub MH, Smoleński RT. (1999) Adenine incorporation in human and rat endothelium. *Biochim Biophys Acta.*; **1452**: 145–50.

Kamiński M, Masaoka M, Karbowski M, Kędzior J, Nishizawa Y, Usukura J, Wakabayashi T. (2003) Ultrastructural basis for the transition of cell death mode from apoptosis to necrosis in menadione-treated osteosarcoma 143B cells. *J Electron Microsc.*; **52**: 313–25.

Karbowski M, Kurono C, Nishizawa Y, Horie Y, Soji T, Wakabayashi T. (1997) Induction of megamitochondria by some chemicals inducing oxidative stress in primary cultured rat hepatocytes. *Biochim Biophys Acta.*; **1349**: 242–50.

Karbowski M, Kurono C, Woźniak M, Ostrowski M, Teranishi M, Nishizawa Y, Usukura J, Soji T, Wakabayashi T. (1999a) Free radical-induced megamitochondria formation and apoptosis. *Free Radic Biol Med.*; **26**: 396–409.

Karbowski M, Kurono C, Woźniak M, Ostrowski M, Teranishi M, Soji T, Wakabayashi T. (1999b) Cycloheximine and 4-OH-TEMPO suppress chloramphenicol-induced apoptosis in RL-34 cells *via* the suppression of the formation of megamito-

chondria. *Biochim Biophys Acta.*; **1449**: 25–40.

Karbowski M, Spodnik JH, Teranishi M, Woźniak M, Nishizawa Y, Usukura J, Wakabayashi T. (2001) Opposite effects of microtubule-stabilizing and microtubule-destabilizing drugs on biogenesis of mitochondria in mammalian cells. *J Cell Sci.*; **114**: 281–91.

Laux I, Nel A. (2001) Evidence that oxidative stress-induced apoptosis by menadione involves Fas-dependent and Fas-independent pathway. *Clin Immunol.*; **101**: 335–44.

Leist M, Single B, Castaldi AF, Kuhe S, Nicotera P. (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med.*; **185**: 1481–6.

Leyden TW, Ng AK, Rotes NS. (1993) Modulation of phosphatidylserine epitope expression by BeWo cells during forskolin treatment. *Placenta.*; **14**: 177–86.

Ma X, Du J, Nakashima I, Nagase F. (2002) Menadione biphasically controls JNK-linked cells death in leukemia Jurkat T cells. *Antioxid Redox Signal.*; **4**: 371–8.

Mancini M, Anderson BO, Caldwell E, Sedghinasab M, Patty PB, Hockenbery DM. (1997) Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in human colon carcinoma cell line. *J Cell Biol.*; **138**: 449–69.

Mayhew TM, Leach L, McGee R, Wan Ismail W, Myklebust R, Lammiman MJ. (1999) Proliferation, diffentiation and apoptosis in villous trophoblast at 13–1 weeks of gestation (including observations on annulate lamellae and nuclear pore complexes). *Placenta.*; **20**: 407–22.

McAmis WC, Schaeffer Jr RC, Baynes JW, Wolf MB. (2003) Menadione causes endothelial barrier failure by a direct effect on intracellular thiol, independent of reactive oxidant production. *Biochim Biophys Acta.*; **1641**: 43–53.

McConkey DJ, Hartzell P, Nicotera P, Wyllie AH, Orrenius S. (1988) Stimulation of endogenous endonuclease activity in hepatocytes exposed to oxidative stress. *Toxicol Lett.*; **92**: 123–30.

Nobel CS, Burgess DH, Zhivotovsky B, Burkitt MJ, Orrenius S, Slater AF. (1997) Mechanism of dithiocarbamate inhibition of apoptosis: thiol oxidation by dithiocarbamate disulfides directly inhibits processing of the caspase-3 proenzymes. *Chem Res Toxicol.*; **10**: 636–43.

Pőtgens AJG, Schmitz U, Bose P, Versmold A, Kaufmann P, Frank H-G. (2002) Mechanism of syncytial fusion: A Review. *Placenta* (Supplement A, Trophoblast Research); **16**: S107–13.

Ross D, Thor H, Orrenius S, Moldeus P. (1985) Interaction of menadione (2-methyl-1,4-naphthoquinone) with glutathione. *Chem Biol Interact.*; **55**: 177–84.

Runić R, Lockwood CJ, LaChapelle L, Dipasquale B, Demopoulos RI, Kumar A, Guller S. (1998) Apoptosis and Fas expression in human fetal membranes. *J Clin Endocrinol Metabol.*; **83**: 660–6.

Samali A, Nordgren H, Zhivotovsky B, Peterson E, Orrenius S. (1999) A comparative study of apoptosis and necrosis in $HepG_2$ cells: oxidant-induced caspase inactivation leads to necrosis. *Biochem Biophys Res Commun.*; **255**: 6–11.

Sata N, Klonowski-Stumpe H, Hau B, Hässinger D, Niederau C. (1997) Menadione induces both necrosis and apoptosis in rat pancreatic acinar AR-2J cells. *Free Radic Biol Med.*; **23**: 844–50.

Shibukawa Y, Takahashi M, Laffont I, Honke K, Taniguchi N. (2003) Down-regulation of hydrogen peroxide-induced PKCδ activation in *N*-acetylglucosaminyltransferase III-transfected HeLaS3 cells. *J Biol Chem.*; **278**: 3197–203.

Smith SC, Baker PN, Symonds EM. (1997a) Placental apoptosis in normal human pregnancy. *Am J Obstet Gynecol.*; **177**: 57–65.

Smith SC, Baker PN, Symonds EM. (1997b) Increased placental apoptosis in intrauterine growth restriction. *Am J Obstet Gynecol.*; **177**: 1395–401.

Smoleński RT, Lacho DR, Ledingham SJH, Jacoub MH. (1990) Determination of sixteen nucleotides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation. *J Chromatogr.*; **527**: 414–20.

Spodnik JH, Woźniak M, Budzko D, Teranishi M, Karbowski M, Nishizawa Y, Usukura J, Wakabayashi T. (2002) Mechanism of leflunomide-induced proliferation of mitochondria in mammalian cells. *Mitochondrion.*; **2**: 163–79.

Sun Y-L, Zhao Y, Hong X, Zhai Z-H. (1999) Cytochrome *c* release and caspase activation by menadione-induced apoptosis in plants. *FEBS Lett.*; **462**: 317–21.

Takahashi N, Schreiber J, Fisher V, Mason RP. (1987) Formation of glutathione-conjugated semiquinones by the reaction of quinone with glutathione: an ESR study. *Arch Biochem Biophys.*; **252**: 41–8.

Takamatsu S, Oguri S, Minowa MT, Nakamura K, Takeuchi M, Kobata A. (1999) Unusually high expression of *N*-acetylglucosaminyltransferase-IV in human choriocarcinoma cell lines: a possible enzymatic basis of the formation of abnormal biantennary sugar chain. *Cancer Res.*; **59**: 3949–53.

Thor H, Smith MT, Hartzell P, Bellomo G, Jewell SA, Orrenius S. (1982) The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J Biol Chem.*; **257**: 12419–25.

Trump BF, Croker BP, Mergner WJ. (1971) The role of energy metabolism, ion, and water shift in the pathogenesis of cell injury. In *Cell Membranes: Biology and Pathological Aspects.* Richter GW, Sarpelli DD. eds, pp 84–128. Williams Wilkins, Baltimore.

Trump BF, Berezesky IK. (1994) Cellular and molecular pathology of reversible and irreversible injury. In *Methods in Toxicology, vol. 1B In vitro Toxicology Indicators.* Frazier JM. ed, pp 1–122. Academic Press, San Diego, Tyson, C.A.

Turner NA, Xia F, Azhar G, Zhang X, Liu L, Wei YJ. (1998) Oxidative stress induces DNA fragmentation and caspase inactivation *via* c-Jun NH_2-terminal kinase pathway in H9c2 cardiac muscle cells. *J Mol Cell Cardiol.*; **30**: 1789–801.

Tzeng W, Chou T, Wang C, Lee J, Chen Y. (1994) Cellular thiols as a determinant of responsiveness to menadione in cardiomyocytes. *J Mol Cell Cardiol.*; **26**: 889–97.

Walsh SW, Wang Y. (1995) Trophoblast and placental villous core production of lipid peroxides, thromboxane, and prostacyclin on preeclampsia. *J Clin Endocrinol Metab.*; **80**: 1888–93.

Wang Y, Walsh SW. (1996) Antioxidant activities and mRNA expression of superoxide dismutase, catalase and glutathione peroxidase in normal and preeclamptic placentas. *J Soc Gynecol Invest.*; **3**: 179–84.

Werfers H, Sies H. (1983) Hepatic low-level chemiluminescence during redox cycling of menadione and the menadione-glutathione conjugate: relation to glutathione and NAD(P)H:quinone reductase (DT-diaphorase) activity. *Arch Biochem Biophys.*; **224**: 568–78.

Wilson SW, Wang Y. (1993) Deficient peroxidase activity in preeclampsia is associated with increased placental production of thromboxane and lipid peroxide. *Am J Obstet Gynecol.*; **168**: 456–61.

Yamaguchi M, Miyazawa K, Katagiri T, Nishimaki J, Kizaki M, Tohyama K, Toyama K. (1997) Vitamin K_2 and its derivatives induce apoptosis in leukemia cells and enhance the effect of all-*trans* retinoic acid. *Leukemia.*; **11**: 779–87.