The Role of Mitochondria in Apoptosis Induced *in vitro*

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Abstract. Cell death remains the focus of *in vitro* toxicology. Xenobiotics are capable of bringing about two types of cell death: apoptosis and necrosis. From our previous study we know that cells treated with xenobiotics showed very dynamic changes in their morphology, particularly vigorous movement of the plasma membrane. Such changes probably depend on adequate energy supply. This observation stands in contradiction with published data showing that generation of ATP in mitochondria is altered very early in apoptosis. In this study we analysed the relationship between mitochondrial activity and cell death induced by Etoposide, a selective inhibitor of topoisomerase II, treatment (10 μg/ml). As a model system we used stabilised cell line Hep2. Several markers of apoptosis, including typical cell morphology and DNA ladder formation were measured. The dynamics of morphological changes was recorded by the time-lapse videomicroscopy. We measured mitochondrial membrane potential with a specific fluorochrome DASPMI, quantification was done by microfluorometric assessment. Our data show that mitochondrial activity was maintained during the first 6 hours after the treatment with Etoposide, at the same time substantial changes in cell morphology as well as typical DNA fragmentation were observed.

Key words: Apoptosis — Mitochondria – *In vitro* toxicology

Introduction

Cells cultured *in vitro* represent indispensable experimental model objects in modern toxicology. In the cytotoxicity assessment the cell death is a very frequently used end point. Sufficient understanding of the cellular mechanism of toxic cell death is therefore of great importance. Due to this fact cell death has also become much-studied phenomenon in the frame of toxicology.

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It is generally accepted that there are two distinct types of cell death: apoptosis and necrosis. Apoptosis is a regulated process responsible for the removal of superfluous, aged, or damaged cells. It appears that all cells possess the intrinsic capacity of undergoing apoptosis. Apoptosis involves the action of specific proteases (caspases) and activation of nucleases, resulting in a typical, regular DNA fragmentation pattern (formation of mono- and oligomers 180-200 base pairs long, so called DNA ladder). Necrosis is an unregulated process; it is usually an outcome of a severe injury involving the damage of the plasma membrane and subsequent swelling of the cytoplasm and organelles (oncosis) (Wilson 1998).

There are chemicals capable of inducing both apoptosis and necrosis in different concentrations (Kroemer et al. 1998). In our previous study both types of cell death were observed in treated cultured cells (Červinka et al. 1997). Based on these facts, it would be therefore more appropriate to assume that apoptosis and necrosis represent two extremes of a broad spectrum of cell death modes.

Recent evidence appears to point to the fact that the cellular system of energy production localised in mitochondria is a frequent target for many toxic substances (Bereiter-Hahn 1990) This finding is strengthened further by the observations of a decreased mitochondrial potential during the process of apoptosis. The mechanisms responsible for this change were proposed to be the opening of the inner membrane pore. The opening of this pore is thought to destroy the chemiosmotic proton gradient which is required for the maintenance of mitochondrial energy production (Kroemer et al. 1997). From our previous study we know that cells treated with xenobiotics showed very dynamic changes in their morphology, in particular vigorous movements of the plasma membrane (Červinka and Půža 1995). These movements depend on adequate energy supply. This observation stands in contradiction with published data showing that generation of ATP in mitochondria is altered very early in apoptosis (Mignotte and Vayssiere 1998).

So far the relationship between morphological changes and mitochondrial functions remains a bit unclear. We have therefore designed and carried out experiments to analyse one aspect of this problem, i.e. the relationship between mitochondrial activity and cell death induced by a selective inhibitor of topoisomerase II (Etoposide). As a model cell system we used stabilised cell line Hep2. Several markers of cell death, including typical cell morphology and DNA ladder formation were measured. The dynamics of morphological changes was recorded by the time-lapse videomicroscopy. Mitochondrial membrane potential was monitored with a fluorochrome DASPMI, quantification was done by microfluorometric assessment.

Materials and Methods

Cell lines

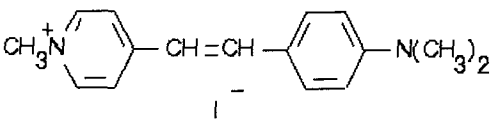
The continuous cell line Hep2 (European Collection of Animal Cell Cultures Ref No 86030501) was used in this study Stock cultures are continuously maintained as stationary monolayers The cells were grown in a humidified 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagles medium (Sevapharm, Prague, Czech Republic) supplemented

with 10 % bovine serum (Bioveta Ivanovice, Czech Republic), penicillin G (100 U/ml) and streptomycin (100 μg/ml) Cells were routinely tested for the presence of mycoplasma employing the fluorescence method, and only mycoplasma-free cultures were used Cells in stock cultures were detached by 0 25 % trypsin (Bioclot, Brno, Czech Republic) in Dulbecco's phosphate buffered saline without calcium chloride and magnesium chloride, pH 7 3 (Sigma D 5527) and resuspended in culture medium After dilution with culture medium to 10^5 cells per ml, one millilitre of the suspension was seeded into 60-mm plastic Petri dishes (Gamedium, Prague, Czech Republic)

Drugs used

For induction of apoptosis we used Etoposide (Vepesid inj , Bristol-Myers Squibb) a topoisomerase II inhibitor (molecular weight 589) in the concentration of 10 μg/ml

For fluorometric determination of mitochondrial activity we used the isomeric styryl dye DASPMI (4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide (4-Di-1-ASP), (D-288), molecular weight 366 24 (Molecular Probes)



Scheme 1. Structure of the styryl dye DASPMI (4-(4-(dimethylamino)styryl)-*N*-methylpyridinium iodide (4-Di-1-ASP), (Molecular Probes, D-288)

Dynamic morphology analysis

After 24 hours of cultivation the standard medium was replaced with a medium supplemented with Etoposide and the cultures were recorded by time-lapse videomicroscopy for several days The time-lapse video-microscopy apparatus consists of an inverted microscope Olympus IMT-2, equipped with a long-working-distance condenser, phase contrast objective (20×) and 4 1 photo-eyepiece A CCD colour video camera (Mitsubishi CCD-100E) and time-lapse video recorder (Mitsubishi HS-S5600E), with slow-down recording (120 times) are connected to this apparatus The microscope was situated in a chamber with temperature maintained at 37 °C

Microfluorometry

Intact mitochondria in living cells were stained with DASPMI Stock solution of 0 5 mmol/l in distilled water was diluted to the working solution of 5 μmol/l in the cultivation medium Equilibrium was obtained after one hour of cultivation Observation and measurements were carried out by a Zeiss Axiovert microscope (immersion objective 100×), with excitation and emission wavelength similar to FITC 450–490 nm and 510–520 nm, respectively In addition to the microscope, photometer, photoenhancer and a computer for automatic data collection as well as computer-controlled specimen movement were employed Fluorescent intensity at 100 different places along the specimen was measured in each measurement

DNA ladder assay

After 24 hours of cultivation the standard medium was replaced with a medium supplemented with Etoposide at 10 μg/ml concentration. Cells were treated by this drug for 1, 3, 12, 18 and 24 hours, respectively. After the treatment the cells were harvested by means of a cell scraper and resuspended in the medium to the final concentration of approximately 10^6 cell/ml. Next, the cells were centrifuged for 5 min (JOUAN MR 22, rotor SWM 180.5) at 157 × g at 4°C. The resulting pellet was resuspended in 0.5 ml sodium azide (0.1%), pipetted into 1.5-ml tubes and again centrifuged for 5 min (JOUAN MR 22, rotor AM 2.19) at 80 × g at 4°C. The pellets were stored at −80°C. DNA isolation and electrophoresis were adapted and performed according to Wolfe et al. (1994) and Eastman (1995). 300 μl lysis buffer containing 500 mmol/l Tris-HCl (pH 8), 16 mmol/l ethylenediamine tetracetic acid (EDTA), 10 mmol/l NaCl plus 10 μl proteinase K (20 mg/ml) and 20 μl 10% sodium dodecyl sulphate (SDS) were added to the cells and the whole solution was incubated for 16 hours at 37°C. The solution was centrifuged for 10 min (JOUAN MR 22, rotor AM 2.19) at 13,790 × g at 4°C, supernatant collected, and DNA isolated by phenol/chloroform extraction and ethanol precipitation. Isolated DNA was resuspended in TE buffer containing 10 mmol/l Tris-HCl and 1 mmol/l EDTA (pH 8.0) and separated by horizontal gel electrophoresis on 2% agarose gel stained with ethidium bromide. Running time 2 hours at 50V.

Results

The dynamics of morphological changes after the treatment with Etoposide is very well documented on our video sequences. During this study we managed to record more than 20 video-sequences. For illustration we selected typical pictures showing morphology of Hep2 cells before treatment and 12 hours after the treatment with Etoposide (Fig. 1). In the period of 4–8 hours after beginning of the treatment the cell becomes rounded and the plasma membrane is rapidly moving, numerous pseudopodia are formed. After another 6–8 hours other typical changes can be observed – disruption of plasma membrane and release of the cell contents. These changes are quite typical for apoptosis, we have seen similar ones after treatment with other apoptosis inducers (Červinka et al. 1997). Nevertheless, we wanted to prove that the cell death we observed is really apoptosis.

A typical hallmark of apoptosis is formation of DNA ladder due to an internucleosomal hydrolysis of the DNA. Results of our DNA-ladder assay are illustrated in Fig. 2. Typical ladder formation is already detectable 1 hour after the beginning of the Etoposide treatment. The DNA ladder persisted even 24 hours after the treatment. From this data we can conclude that Etoposide at a dose of 10 μg/ml is an efficient inducer of apoptosis in our model system with Hep2 cells.

The next part of our work was devoted to the measurement of mitochondrial activity after the treatment with Etoposide. First, we had to demonstrate that DASPMI is mitochondria specific. Therefore, we stained the cells with DASPMI and studied the morphology of them with a high magnification. Fig. 3 shows a control cell stained with DASPMI, the left part in phase contrast, the right part in fluorescence. The mitochondria are very well stained, the localisation, size and shape of fluorescence are quite typical for mitochondria. Thus we conclude that

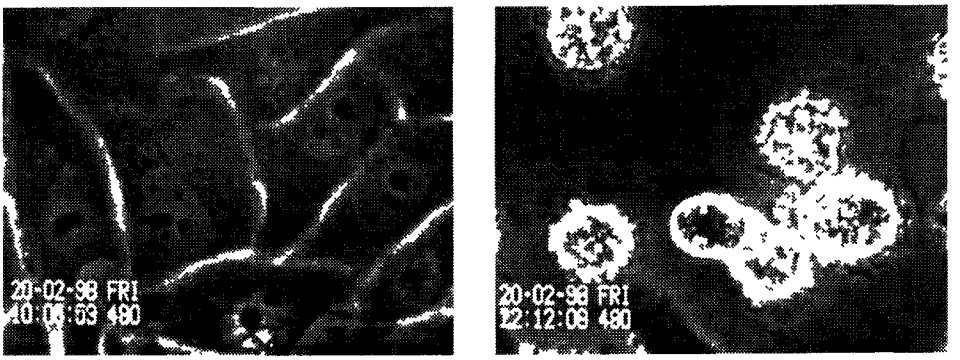


Figure 1. Morphology of Hep2 cells before (left panel) and 12 hours after the treatment with Etoposide (10 μg/ml) (right panel) Phase contrast, 500×, prints were made from time-lapse video-recordings

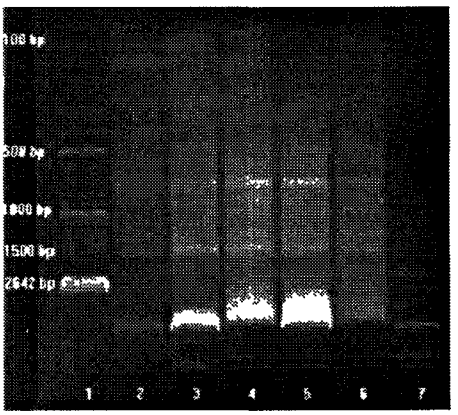


Figure 2. DNA fragmentation after the treatment with Etoposide in different concentrations 1) The Molecular Weight Marker Boehringer Mannheim XIV – 100 bp ladder, 2) Hep2 cells treated with Etoposide for 1 h, 3) Hep2 cells treated with Etoposide for 3 h, 4) Hep2 cells treated with Etoposide for 12 h, 5) Hep2 cells treated with Etoposide for 18 h, 6) Hep2 cells treated with Etoposide for 24 h, 7) Untreated Hep2 cells

DASPMI is suitable for selective staining of mitochondria. After the treatment with Etoposide we saw a very bright fluorescence even in the rounded cells with typical apoptotic morphology. Only in the completely disintegrated cell the fluorescence was very weak (Fig 4)

Finally, we tried to quantify the fluorescence after DASPMI staining. It was not possible to measure the fluorescence during prolonged time intervals (more than 2 hours) because the intensity of fluorescence decreased too rapidly and this did not permit to follow the fate of mitochondria in individual cells. Therefore we measured fluorescence in parallel cultures Results of microfluorometric quantification (intensity of fluorescence in arbitrary units) in control cultures were calculated as 2218 ± 148 (mean $\pm$ S.D). After 8 hours of cultivation in the presence of Etoposide (10 μg/ml) we found similar values (2179 ± 163).

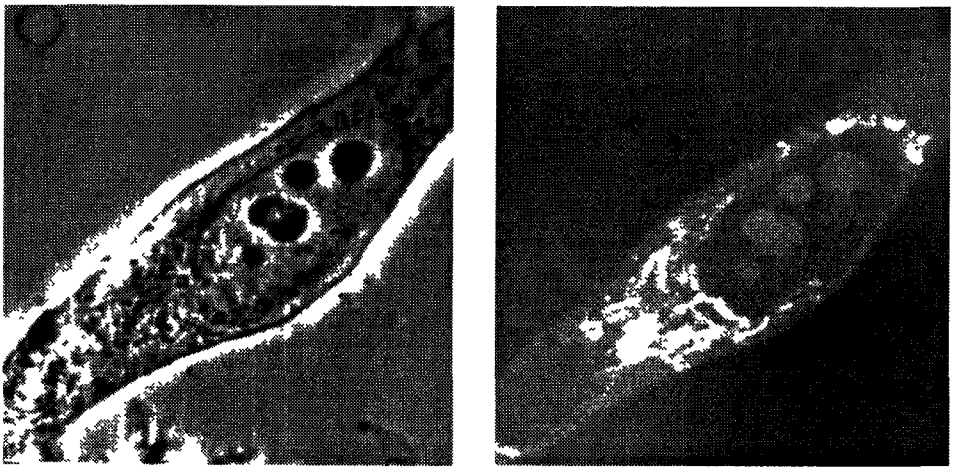


Figure 3. Control Hep2 cells after staining with DASPMI Left panel – phase contrast, right panel – fluorescence microscopy, 1000×

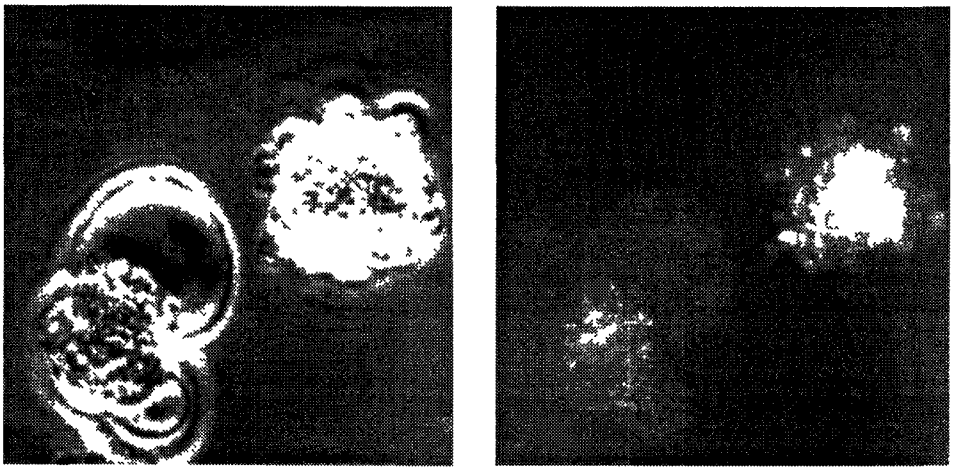


Figure 4. Hep2 cells treated with Etoposide after staining with DASPMI Left panel – phase contrast, right panel – fluorescence microscopy, 1000×

From our results it is obvious that the mitochondrial transmembrane potential in cells is maintained during 6–8 hours after the treatment with Etoposide. This very probably means that at this time the mitochondria are intact.

Discussion

The role of the mitochondria in cell death was currently summarised by Green and Reed 1998. In principle, there are two ways how mitochondria can influence the cell death. Firstly, it is the disruption of membrane potential with a subsequent drop in ATP production. Secondly, it is the release of caspase activation proteins (for example, cytochome c) from mitochondria with subsequent activation of apoptotic pathway.

In our recent study we focused our attention on the relationship between mitochondrial membrane potential and cell death. This phenomenon has been evaluated in several studies (Castedo et al. 1996; Masaki et al. 1989; Salvioli et al. 1998) but no unequivocal conclusions have been made. One approach to resolve this problem is to study the time course of the cell death In our recent study we made a parallel observation of cell morphology, mitochondrial potential and DNA-ladder formation. We could demonstrate that changes in mitochondrial potential do not come first in the cascade of events leading to apoptosis The typical morphological changes and the DNA ladder formation can be detected several hours earlier than changes in mitochondrial membrane potential. This evidence is in agreement with the latest investigations suggesting that apoptosis runs independently of mitochondrial ATP synthesis (Jia et al 1997).

The discrepancies of the findings reported in the literature could be partly explained by the heterogeneity of the cell population. In our video sequences it is clearly demonstrated that not all the cells behave exactly the same way. Cytofluorimetric analysis, which was performed simultaneously, allows the discrimination of mitochondrial potential in each single cell, thus giving further information about the cellular heterogeneity. Therefore, we believe that our approach of monitoring of mitochondrial potential (visualisation of mitochondria in living cells and measurement of fluorescence by CCD camera) is favourable to integrated data obtained by flow cytometry.

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