

Detection of Apoptotic Changes in HeLa Cells after Treatment with Paracetamol and Sodium Fluoride

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Abstract. Apoptosis is genetically programmed cell death, an irreversible process of cell senescence with characteristic features (cell shrinkage, chromatin condensation, DNA fragmentation, apoptotic bodies) different from necrosis. Several effective *in vitro* methods for qualitative and quantitative detection of apoptotic events have been developed. Chromatin degradation, reductions in cell volume and other apoptosis-associated changes in cell morphology and physiology can be quickly analysed by multiparametric flow cytometry (FC) using small numbers of intact cells. One further method used for morphological determination of apoptotic changes is the fluorescent microscopic technique (FM) based on labeling of cells with fluorochromes acridine orange and ethidium bromide.

In our experiments FC was used for determination of DNA changes in HeLa cells based on staining of DNA by fluorochrome propidium iodide (PI). Among the tested chemicals (paracetamol and sodium fluoride) apoptotic process could only be detected in paracetamol-treated cells. Apoptosis was induced mainly in cells treated with paracetamol (concentration range 4–5 mg/ml) for 8 h and following incubation for 18 h in fresh medium without paracetamol. The results obtained by the FM method correlated with the results obtained by FC.

Key words: Apoptosis — Flow cytometry — Fluorescence microscopy — Paracetamol — Sodium fluoride

Introduction

The mechanisms of cell proliferation and differentiation as well as maintenance of homeostasis in living organisms have been the subject of intense research through-

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out the history of biological studies. Disregulation of cell death mechanisms is involved in the pathogenesis of an increasing number of diseases. Defective apoptosis can participate in malignant transformation, viral latency, autoimmune diseases etc. (Solary et al. 1996). Though complex, apoptosis appears amenable to therapeutic intervention. The range of modern pharmaceutical strategies available to treat such disregulated gene-directed processes seems promising with respect to advances in the control of cancer, immune system and neurodegenerative disorders, heart disease, and perhaps even the process of aging itself (Barr and Tomei, 1994; Rauko et al. 1998).

The multiparametric flow cytometric method (FC) is an assay widely used in *in vitro* conditions because of the rapid analysis of large numbers of cells and the identification and quantification of subpopulations of heterogenous cell systems. In our experiments we used FC for determination of chromatin degradation based on DNA staining with fluochrome propidium iodide (PI). The fluorescence microscopic (FM) assay was the other method used to confirm the results obtained by FC. The latter technique is based on morphological determination of the cell death type after exposure to tested chemicals by labeling cells with fluorochromes acridine orange and ethidium bromide.

The tested chemicals (paracetamol and sodium fluoride) were selected on the basis of our previous results of their cytotoxic and/or genotoxic effect on mammalian cells *in vitro* (Slameňová et al. 1992; Ruppová et al. 1996). While the cytotoxic effects of both chemicals were obvious in our experiments, their genotoxicity was not confirmed unambiguously. Paracetamol (acetaminophen) is characterised by anti-pyretic and analgesic features. The dangerous effects of paracetamol overdosage are well recognised (Hawton et al. 1995). The human population is exposed to inorganic fluorides through fluoridation of water, through dental products, and through fluoride naturally occurring in water, soil and food (Li et al. 1988; Slameňová et al. 1997). Sodium fluoride (NaF) has been used for decades, either systematically or topically, for the prevention of dental caries (Slameňová et al. 1996). Any environment with a low pH, such as the stomach, will favour NaF uptake and enhance its cytotoxic effect (Slameňová et al. 1997). Paracetamol and sodium fluoride are used because of their therapeutic benefit, and not only their acute and chronic genotoxic potential but also their potential to induce the process of apoptosis should therefore be considered.

The objective of our investigations was to find out whether the two chemicals chosen were able to induce the process of apoptosis in our *in vitro* system, and to compare results obtained by two different methods, flow cytometry (FC) and fluorescent microscopy (FM).

Materials and Methods

Chemicals. Paracetamol (acetaminophen; APAP, *N*-acetyl-*p*-aminophenol, $C_8H_9NO_2$, MW 151.16), propidium iodide (PI) and ethidium bromide (EB) were obtained from Sigma (Deisenhofen, Germany), sodium fluoride (NaF, MW 41.99) was produced by Slo-

vakofarma Hlohovec (Slovakia) and acridine orange (AO) and RNA-ase (20 U/mg) were from Merck (Germany)

Cell lines. Heteroploid human transformed cell line HeLa (obtained from Institute of Virology, Slovak Academy of Sciences) was cultured on glass in minimum essential medium (MEM) with addition of glutamine (33 mg/ml), 10% donor bovine serum and gentamycin K (40 mg/ml) in a humidified 5% CO₂ atmosphere at 37°C

Treatment of cells Paracetamol was first dissolved in dimethyl sulphoxide and then diluted in complete phosphate buffered saline (PBS, 2 h treatment) or in MEM without serum (8 h and 24 h treatment). NaF was diluted in PBS (pH 4, 60 min treatment) or in MEM without serum (pH 7, 8 h and 20 h treatment). Samples were collected and processed 18 h after exposure

Flow cytometry (FC) We used the modified basic procedure of single DNA staining by PI without fixation of the cells (Telford et al 1994). Analytic multiparametric flow cytometry measurements were performed using a COULTER Epics XL flow cytometer with argon laser. Red fluorescence (DNA) was detected through 620 nm bandpass filter. Ten thousand cells in each sample were analyzed. The DNA degradation of chromatin was estimated from red fluorescence histograms

Fluorescence microscopy (FM) Morphology of exposed cells was determined under fluorescent microscope (Opton Axiophot, Germany) after labeling with acridine orange and ethidium bromide. Live cells were determined by the exclusion of EB stain. Live and dead apoptotic cells were identified by nuclear condensation of chromatin stained by AO (green colour) or EB, respectively. Necrotic cells were identified by uniform labeling of the cell with EB (red colour) (Piazza et al 1995). Fluorescence was detected through 525 nm bandpass filter. Photographs were taken on Kodak Gold 400 ASA type film. For each sample 100 cells were visually evaluated and percentages of vital, apoptotic and necrotic cells were calculated

Statistics Statistical analysis of the data was carried out by the unparametric Kruskal Wallis analysis and Wilcoxon test ($\alpha = 0.05$)

Results

Paracetamol FC and FM methods were used to test the effects of paracetamol on cells in different conditions: 2 h for concentrations 7.5, 10; 12.5 and 15 mg/ml, 8 h for 3; 4; 5; 6 and 7 mg/ml and 24 h for 1, 2.5; 5 and 7.5 mg/ml. Unlike NaF, paracetamol induced significant changes in all the concentrations used. After 2 h exposure no significant increase of apoptotic cells in comparison to untreated control cells was observed, and a significant increase of vital and necrotic cells was detected in concentrations 12.5 and 15 mg/ml of paracetamol by both the FC and the FM methods. The evaluation of 8 h paracetamol exposure revealed the most interesting results: a significant increase of apoptotic cells at concentrations of up to 5 mg/ml obtained by FM (Fig. 1, lower part) and by the FC method up to 6 mg/ml (Fig. 1, upper part). The amounts of vital cells significantly decreased almost in all concentrations in comparison with the control using both methods. The percentages of necrotic cells increased significantly compared to controls for the 7 mg/ml concentration obtained by the FC and the FM methods (Fig. 1). After 24 h exposure to paracetamol, a significant increase of the percentages of necrotic cells was noticed in comparison to untreated cells for all the concentrations used obtained

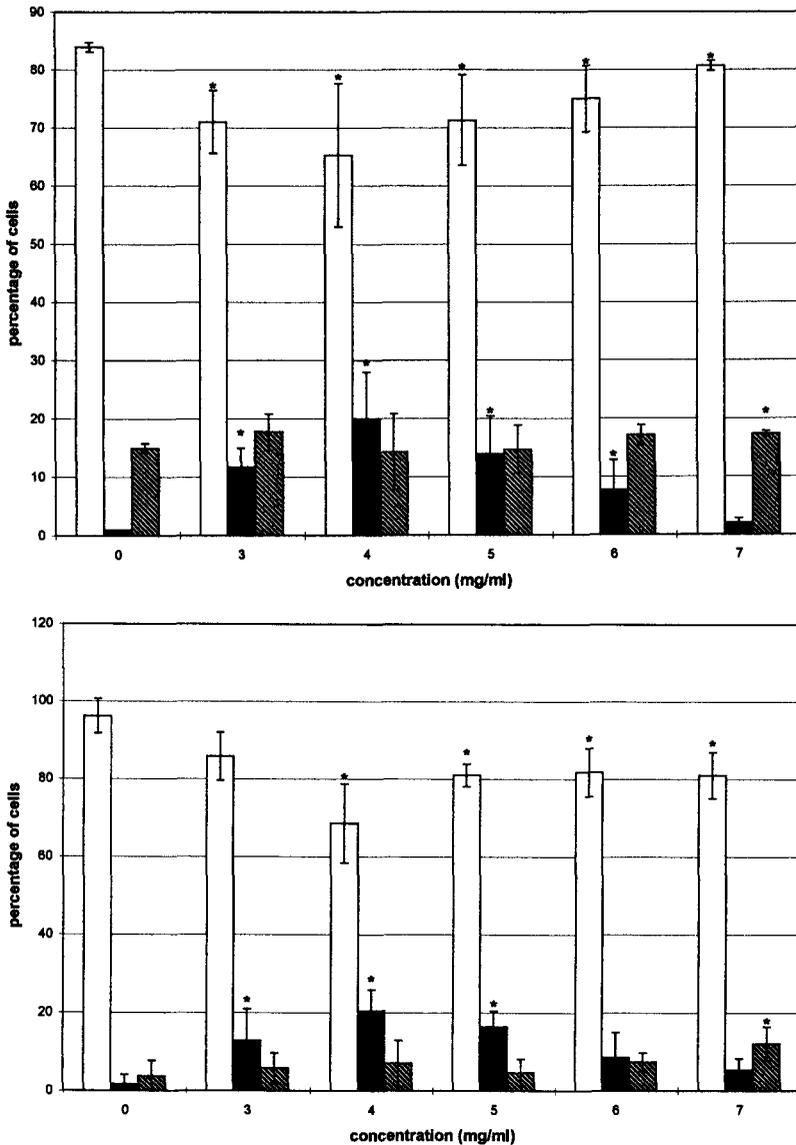


Figure 1. Dose-dependent effects after 8 h exposure of paracetamol on HeLa cells. Time of samples collecting was 18 h after paracetamol removal. Upper panel: Flow cytometric analysis; Lower panel: Fluorescence microscopic analysis of vital (□), apoptotic (■) and necrotic (▨) cells. * = $p < 0.05$ compared to control.

by FM, and for 5 mg/ml by FC; using the latter method, the exposure to 7.5 mg/ml was too injurious for the cells. The increase of necrotic cells was accompanied by a decrease of vital cells in cell populations exposed to concentrations of paracetamol

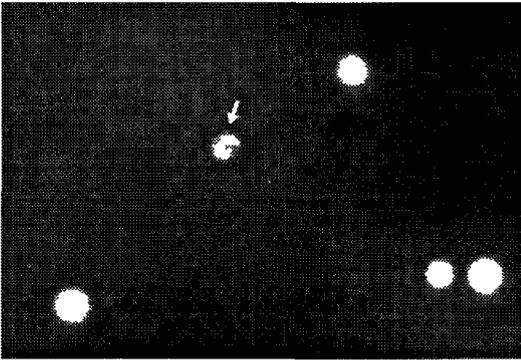


Figure 2. Representative photomicrograph of normal and apoptotic (arrow) HeLa cells obtained by fluorescence microscopy

mentioned above. The amounts of apoptotic cells did not increase significantly in cells exposed to 5 mg/ml in comparison to control cells.

Sodium fluoride (NaF) The effect of NaF was tested using the FC and the FM methods and percentages of vital, apoptotic and necrotic cells were evaluated. We used three different exposure conditions: 60 min for concentrations of 0.2, 0.4 and 0.6 mg/ml of NaF in PBS, pH 4; 8 h for 0.01, 0.05 and 0.1 mg/ml in MEM, pH 7; and 20 h exposure for 0.01, 0.05, 0.1 and 0.2 mg/ml in MEM, pH 7 on HeLa cells. We evaluated the percentages of the resulting types of cells (i.e. vital, apoptotic and/or necrotic cells) in control and exposed cells. Using FC we found no significant statistical differences between the treated and control cells. FM gave the same results, except for the two highest concentrations (20 h exposure to NaF). In these conditions the percentages of vital cells significantly decreased and those of necrotic cells increased in comparison to control cells.

Discussion

Both paracetamol and sodium fluoride are commonly used drugs. In our previous work, we succeeded in determining the conditions for the cytotoxic effects of both tested chemicals to manifest on mammalian cell cultures (Slameňová et al 1992, 1996, 1997, Ruppová et al 1996). There are numerous serious human diseases caused by acute or chronic dysregulation of cell death. We decided to investigate which type of cell death is probably induced by paracetamol and sodium fluoride in *in vitro* conditions. The concentrations in our present experiments were selected with regards to our previous results (Slameňová et al 1992, Ruppová et al 1996). Based on the fact that the process of apoptosis occurs under normal physiological conditions or as a result of mild pathological stimuli we also used medium toxic concentrations of sodium fluoride and paracetamol in addition to low and high concentrations.

Statistical evaluation of the results obtained using FC and FM analyses showed that only paracetamol was confirmed to be an inducer of apoptosis in HeLa cells.

(Fig 1). This result correlates with the findings of Wiger et al. (1997) who investigated the effects of paracetamol on cell cycle and cell death in cultured HL-60 cells. Microscopic studies performed by these authors revealed increased numbers of cells with nuclear condensation and fragmentation, indicating that apoptosis was the dominating mode of death in this cell line after exposure to paracetamol. Within 5–10 h after the start of paracetamol exposure, they observed a marked downregulation of both *c-myc* and *bcl-2* mRNA. The authors concluded that exposure to high non-therapeutic concentrations of paracetamol (0.1–3.0 mmol/l) caused cell cycle arrest in S-phase and induction of apoptosis in HL-60 cells. These values are one order lower in comparison to our results concerning cytotoxic and apoptotic effects of paracetamol on HeLa cells. One possible explanation of this discrepancy might be that the HeLa cells used in our experiments are less sensitive to apoptosis induced by paracetamol than HL-60 cells.

In contrast to the above mentioned results concerning the ability of NaF to induce apoptosis we did not observe apoptosis induced by NaF. Our assumption was based on published results that in rat alveolar macrophages cytotoxicity of sodium fluoride was associated with apoptosis (Hirano and Ando 1996). It has been reported that calcium influx occurred in fluoride-exposed human neutrophils (English et al. 1991) and in rat kidney (Suketa et al. 1986). Intracellular calcium increase is associated with the activation of endonucleases which eventually results in cell death (Fawthrop et al. 1991; Corcoran et al. 1994). Therefore, it was conceivable that apoptotic cell death in fluoride-exposed alveolar macrophages was due to an increase of the activity of endonucleases. These conclusions were supported by the work which showed that NaF induced aberrations by an indirect mechanism involving the inhibition of DNA synthesis and/or repair (Aardema et al. 1989).

Our results support the hypothesis that apoptosis is a process manifesting itself very individually in dependence on the conditions. Therefore, a chemical which in some conditions behaves as an apoptotic inducer may, in other conditions, have no apoptotic effect, as was the case of NaF in our experiments.

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