ANALYSIS OF ULTRASTRUCTURAL CHANGES IN CHO-K1 CELLS AFTER THE ADMINISTRATION OF ALOE-EMODIN

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Aloe-emodin is an anthraquinone with numerous biological properties, including antibacterial, antiviral, antifungal, immunomodulative and potential antineoplastic properties. The aim of this work is to assess the effects of ultrastructural changes caused by aloe-emodin on the organelles of CHO-K1 cells. The ultrastructural study was performed using transmission electron microscopy. The analysis of submicroscopic changes was performed with a transmission electron microscope TECNAI G2 Spirit BioTwin (FEI Company). Selected cellular organelles were also measured using the TIA microscope software (TEM Imaging & Analysis 3,2 SP6). Additionally, the marking of lysosomes was performed using acridine orange (a non-enzymatic lysosome marker). The preparation was analyzed with the use of a Nikon Eclipse 80i fluorescence microscope.

The results obtained indicate that the anthraquinone used in the study has a modulating influence on the activity and differentiation of the cell nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus. Aloe-emodin should be considered as an agent with a multidirectional mechanism of action on the cell, the extent of the examined changes being dependent on the dose of the studied anthraquinone.

Key words: anthraquinones, aloe-emodin, apoptosis, autophagy, lysosomes, ultrastructure, CHO-K1 cells

INTRODUCTION

Aloe-emodin (1,8-dihydroxy-3-hydroxymethyl-9,10-anthraquinone) is an active anthraquinone isolated from the roots and rhizome of Rheum palmatum (Polygonaceae), Rhamnus frangula (Rhamnaceae) and from the leaves of Aloe barbadensis and Aloe arborescens (Liliaceae). This compound possesses antibacterial (COOPOOSAMY and MAGWA, 2006), antiviral (ZANDI et al., 2007; LIN et al., 2008), antifungal (AGARWAL et al., 2000), and immunomodulative (YU et al., 2006; CHERNG et al., 2008) properties, as well as potential antineoplastic properties. Its proapoptotic and antiproliferative action has also been observed in neoplastic cell lines, including the liver (KUO et al., 2002; LU et al., 2007), lungs (LAI et al., 2007), central nervous system (MILATOVIC et al., 2005), and stomach (CHEN et al., 2007; LIU et al., 2007).

At present, the mechanism of aloe-emodin action is poorly known, and it is probably associa-
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ted with its biological properties. Due to the lack of detailed information in the available literature on the influence of aloe-emodin on the ultrastructure of cells, an attempt to assess the extent of ultrastructural changes in CHO-K1 cells has been undertaken, special consideration being given to qualitative and quantitative changes.

MATERIALS AND METHODS

The morphological studies were done on the CHO-K1 cell line (Chinese hamster ovary) isolated from the ovary of a Chinese hamster. The cells were grown in cell culture dishes (Nunc) at a temperature of 37°C and in a 5% carbon dioxide atmosphere, in a CO₂ incubator (Thermo Scientific). The culture was grown on a DMEM base enriched with 10% fetal bovine serum (FBS), with the addition of a mixture of antibiotics, including penicillin (10,000 U/ml), streptomycin (10 mg/ml) and amphotericin B (25 μg/ml). The reagents were obtained from PAA Laboratories (Austria).

Twenty-four hours after incubation the growth liquid was exchanged for a complete medium with the addition of aloe-emodin (Sigma-Aldrich St.Louis, USA) at concentrations of 1 μM, 15 μM, 60 μM and 100 μM. The research was performed on CHO-K1 cells at a density of 3 x 10^5 cells/ml, which, after 24-hour exposure to aloe-emodin, were prepared for electron microscopy study according to the modified method of MARZELLA and GLAUMANN (1980)

Ultrathin slices obtained with the use of an EM UC7 ultramicrotome (Leica) were additionally contrasted in uranyl acetate and lead citrate. The analysis of submicroscopic changes was performed with the use of a transmission electron microscope TECNAI G2 Spirit BioTwin (FEI Company). Measurements of selected cell organelles were performed with the use of the TIA microscope software (TEM Imaging & Analysis 3.2 SP6). For each of the doses of the experimental agent, the diameter of mitochondria and autophagic vacuoles was measured in 50 cells from each group. The average values of the morphometric measurements were statistically analyzed using the Kruskal-Wallis nonparametric ANOVA test (Statistica 10.0, StatSoft, Poland). The differences were considered statistically significant at P<0.05.

Additional lysosomal marking was performed with the use of acridine orange (a non-enzymatic lysosomal marker) according to the modified method of HARJI et al., (2007). For this purpose the cells were grown, under the conditions given above, on microscope slides in cell culture dishes. Twenty-four hours after the exposure of CHO-K1 cells to aloe-emodin at a dose of 15 μM the growth liquid was removed, the cells were rinsed in PBS and incubated with acridine orange solution (5 μg/ml PBS) under growth conditions for 15 minutes. The control preparation was similarly made, however the control CHO-K1 cells were not exposed to aloe-emodin. The preparations were analyzed with the use of a fluorescence microscope Nikon Eclipse 80i.

RESULTS

The obtained results are shown on electronograms (Fig.1a-1f). The ultrastructure of cells from the control group was used as the correct image of the cell (Fig.1a). Cell nuclei with a normal membrane, rough endoplasmic reticulum and oval-shaped mitochondria with regular crest arrangement were observed.

The 24-hour exposure of CHO-K1 cells to aloe-emodin at a dose of 1 μM caused slight changes in the cell ultrastructure. The changes concerned slight growth of the rough endoplasmic reticulum, statistically insignificant swelling of mitochondria and an increased number of Golgi complexes (Fig.1b).

The 24-hour exposure of the CHO-K1 cells to aloe-emodin at a dose of 15 μM (Fig.1c) effectuated a significant modification of the Golgi apparatus profile.

These cells also showed statistically significant swelling of mitochondria, the diameter of which was 0.87 μm after incubation with aloe-emodin, in comparison with the size of mitochondria from the control group [0.50 μm (Fig.3)]. The mitochondria were characterized by a spherical shape and shorter crests, and the brightening of the mitochondrial matrix was observed. The lysosomal compartment was represented by an increased number of primary lysosomes, secondary lysosomes and autophagic vacuoles, with a significantly increased size of autophagic vacuoles,
to 0.93 μm, in comparison with the size of the vacuoles from the control group [0.50 μm (Fig.4)].

Encumbering the cells with the studied anthraquinone at a dose of 15 μM also caused changes in the shape of the cell nucleus (Fig.1c). The analysis of CHO-K1 cells after 24-hour exposure to aloe-emodin at doses of 45 μM (Fig.1d) and 60 μM (Fig.1e) showed cytotoxic changes related mainly to the deformation of the normal structure of the nucleus, nuclear contour irregularity and

![Fig. 1. (a) – The CHO-K1 control cell not exposed to aloe-emodin; (b) – The CHO-K1 cell after 24-hour exposure to aloe-emodin at a dose of 1 μM; (c) – The CHO-K1 cell after 24-hour exposure to aloe-emodin at a dose of 15 μM; (d) – The CHO-K1 cell after 24-hour exposure to aloe-emodin at a dose of 45 μM; (e) – The CHO-K1 cell after 24-hour exposure to aloe-emodin at a dose of 60 μM; (f) – The CHO-K1 cell after 24-hour exposure to aloe-emodin at a dose of 100 μM. N- nucleus; M-mitochondria, ER- rough endoplasmic reticulum, AG-Golgi apparatus, VA- autophagic vacuole, L-lysosomes.](image-url)
its fragmentation. Intensification of autophagy was also observed, which manifested itself in the increased number of autophagic vacuoles containing fragments of cytosol and cell organelles in various phases of digestion. The greatest number of autophagic vacuoles, often with a vastly increased size as compared with the size of the control group vacuoles, was observed after the 24-hour exposure of CHO-K1 cells to aloe-emodin at a dose of 100 μM (Fig.1f). The size of the autophagic vacuoles was as much as 464 % (2.32 μm) larger then the size of the vacuoles in the cells taken from the control groups (0.50 μm). Other changes included degradation of endoplasmic reticulum and changes in the Golgi apparatus, characterized by a reduction in the number of dictyosomes (Fig.1f). Mitochondria of the cells studied after 24-hour exposure to aloe-emodin at doses ranging from 45 to 100 μM showed a significant (about 20%) increase in volume as compared with the mitochondria from the control group cells.

The analysis of morphological changes in CHO-K1 cells with the use of fluorescence microscopy, both in relation to the control group of CHO-K1 cells and in relation to the cells exposed for 24 hours to aloe-emodin at a dose of 15 μM, showed that both the cell nucleus and the cell cytoplasm exhibited a green color (Fig.2a). The characteristic components of cells exposed to aloe-emodin at the above-mentioned dose, marked with acridine orange, were numerous granules with an intensive red-orange fluorescent color against a background of the green-colored cytoplasm, which indicated activation of the lysosomal compartment (Fig.2b).

DISCUSSION

Cytotoxic and cytostatic substances have multidirectional effects on the cells, and they may disturb numerous metabolic pathways, as well as the synthesis of nucleic acids. The cellular response to a cytotoxic agent may manifest itself in stopping the cell life cycle or inducing death through, among other possibilities, apoptosis or autophagy (McConkey et al., 1996; Król, 2002; Ricci and Zong, 2006; Skierski, 2008).

Therefore, an important task in searching for new antineoplastic compounds is to study their influence not only on cancer cells but also on healthy cells. Anthraquinones, including aloe-emodin, are an example of compounds displaying a multidirectional mechanism of action on the cell. Both the results presented in this paper and the results of previous research show (Trybus et al., 2011) that aloe-emodin is an agent with a modulating influence on the activation and differentiation of the lysosomal compartment. On the transmission electron microscopy level, an increased number of primary and secondary lysosomes was observed, which could indicate an intensification of lysosomal degradation (Fig.1e and Fig.1f). Activation of the lysosomal compartment was also confirmed by fluorescent micros-

Fig. 2. (a) – The CHO-K1 control cells not exposed to aloe-emodin (stained with acridine orange); (b) - The CHO-K1 cells after 24-hour incubation with aloe-emodin at a dose of 15 μM (stained with acridine orange). ↑-numerous lysosomes in the cytoplasm.
copy, since the cells encumbered with anthraquinone displayed intensification of the red-orange fluorescent hue in the cytoplasm, indicating an increase in the number of lysosomes containing protonated acridine orange which had been introduced into the lysosomes at a high concentration together with hydrogen ions (Fig. 2b).

This happens because the lysosomal system is one of the first systems in the cell to respond to stress factors through an increase in the number of lysosomes together with the number of hydrolases contained within them (Klönsky and Emr, 2000; Król, 2002). A relationship between the dose of aloe-emodin and the increase in the number and size of autophagic vacuoles has been observed. The greatest increase in the size of autophagic vacuoles (containing degraded cytosol elements together with organelles), in comparison with the control group cells, was observed after the application of aloe-emodin at a dose of 100 μM (Fig. 1f, Fig. 4). The results obtained suggest that the intensification of autophagic processes observed in the studied cells is the response of the cells to aloe-emodin. The literature indicates that excessive activation of autophagy, during disturbed cell homeostasis, may, in consequence, even lead to cell death (Kundu and Thompson, 2005; Ricci and Zong, 2006; Thorburn, 2008; Hsieh et al., 2009). The obtained results showed that irregularly shaped nucleus visible in Fig. 1c, and the nuclear fragmentation visible in Fig. 1d are typical morphological changes characteristic of apoptosis, one of the indicators of which are the changes starting in the cell nucleus. These changes are associated with chromatin at an early stage, and at a later stage fragmentation of the cell nucleus occurs (Häcker, 2000). The changes observed in the nuclei of cells encumbered with aloe-emodin can be connected with its influence on nuclear DNA. This is confirmed by the studies of Pecere et al. (2000, 2003) who demonstrated the ability of aloe-emodin to enter the cytoplasm and later to penetrate into the nucleus and interact with DNA.

The dispersion of the Golgi apparatus cisternae in the cytoplasm, which was observed in the study (Fig. 1c), could be the effect of aloe-emodin on the cell, as well as one of the signs of apoptotic changes occurring in this organelle. The Golgi complex is an organelle which is engaged in the apoptosis process together with the mitochondria. The membrane of the Golgi apparatus contains the protein caspase-2 which participates in the transduction of the proapoptotic signal which is caused by stress or by changes taking place within the endoplasmic reticulum (Sesso et al., 1999; Barr, 2000; Mancini et al., 2000). The dispersion of the Golgi apparatus cisternae indicates that, as an effect of aloe-emodin activity, the disorganization of the cell cytoskeleton may take place, which in turn may indicate inhibition of the proliferation of CHO-K1 cells. Numerous published data show that a similar extent of morphological changes was demonstrated in the studies on other cytostatic, plant-based compounds used in chemotherapy (such as vinblastine, vincristine or colchicine) which act on the mitotic spindle through the depolymerisation of

**Fig. 3.** Comparison of the average size of mitochondria in CHO-K1 cells after the exposure to different doses of aloe-emodin. ***P<0.001 compared with the control group.

**Fig. 4.** Comparison of the average size of autophagic vacuoles in CHO-K1 cells after the exposure to different doses of aloe-emodin. ***P<0.001 compared with the control group.
microtubules and whose action results in, among other effects, the dispersion of the Golgi apparatus cisternae (Pavelka and Ellinger, 1983; Turner and Tartakoff, 1989; Król et al., 1994; Król, 1996; Green and Reed, 1998; Król, 1998; Thyberg and Moskalewski, 1999; Król, 2002; Hick and Machamer, 2005).

The assessment of ultrastructural changes in CHO-K1 cells effectuated by different doses of aloe-emodin shows that anthraquinone also influences the mitochondrial structure. The relationship between the dose of the studied agent and the changes in the mitochondrial structure has been confirmed. The exposure of CHO-K1 cells to aloe-emodin at a dose of 45-100 μM (Fig.1d,1e,1f) caused an increase in the size of these organelles, which might suggest an increased energy requirement of the studied cells. Aloe-emodin added to the culture at a dose of 15 μM (Fig.1c) causes a statistically significant (in comparison with the control) increase in the size of mitochondria, which indicates high amplitude swelling. The demonstrated changes can be confirmed by numerous literature data which show that early apoptotic changes in the cell are accompanied by mitochondrial swelling (Hacker, 2000). Damage to mitochondria is one of the traits of cells which are led to the programmed cell death pathway; the opening of the mitochondrial channels may trigger the release of caspase-activating proteins from the intermembrane space into cytosol, which in turn induces the apoptosis process (Green and Reed, 1998; Wang, 2001; Manygoats et al., 2002; Green and Kroemer, 2004; Czarnecka et al., 2006; Jeong and Seol, 2008; Stanczyr and Majsterek, 2008). In turn, the removal of mitochondria – the apoptosis integrators – from the cell, as a result of autophagic process stimulation (Fig.1f) might contribute to protecting the cell from being led to the cell death pathway (Lockshin and Zakeri, 2004).

The obtained results show that aloe-emodin causes significant changes in the morphological profile of CHO-K1 cells, and the form and extent of the changes in the ultrastructure of the studied cells is dependent on the dose used.

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