УДК 616-006.441:616.056+577.1:547.943:611.018.15

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HOW MITOCHONDRIA IS TRANSFORMED FROM GENERATOR OF ENERGY FOR CELL INTO CELL KILLER

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Structure-functional changes in cells which die by apoptosis mechanism have systemic character and occur at different levels of cell organization, namely, in plasma membrane, cytoplasm, and nucleus. It should be noted that mitochondria play a dual role there. In normal state, these organelles support most processes of cellular life with energetic resources, while at the action of extreme agents (toxic substances, for example, anticancer drugs, or x-ray irradiation) they turn to killers of self cells. Such dualism in mitochondria effects is expressed in various forms. The mitochondrial membrane contains Bcl-2 and Bcl-XL proteins whose increased level blocks apoptosis, while an elevation of the amount of other proteins, like Bax and Bid related to Bcl-2, oppositely, facilitates the apoptosis. Mitochondrial matrix contains cytochrome C which is vitally important for energy generation by these organelles, while simple release of this protein from mitochondria to cytoplasm is absolutely deadly for cells. It activates a cytosolic protein Apaf-1 whose complex with cytochrome C further activates the proteolytic enzyme pro-caspase-9 which gets a capability to split so-called effector caspases 2, 3, 6, 7 and 10. The last degrade the intracellular proteins which are critically important for supporting cellular life processes. The reactive oxygen species are important mediators for realization of apoptosis program, and blocking their generators or neutralization of the action of those highly reactive agents also suppresses apoptosis development. The article presents literature data, as well as the results of studies of collaborators of the Department headed by the author, regarding potentials of enhancing or inhibiting cell death by the apoptosis mechanism.

Keywords. Cell death, apoptosis, mitochondria, mechanisms, reactive oxygen species.

Up to recent time, investigation of the mechanisms of cell death did not attract big attention of scientists, since cell death was considered only as a consequence of evident cell damage or aging. Massive death of cells during development of multi-cellular organisms was observed, however, switching mechanisms of that phenomenon were not known, since not only old and impaired cells, but also «young» cells possessing high potential to reproduction are dieing [1]. In this article, we have addressed the role of specific cellular macromolecules and role of changes in functioning of energy generating intracellular organelle, the mitochondria, in cell death mechanisms. It is accepted that mitochondria is an ancient endosymbiotic organism which lost most of its genetic apparatus during co-evolution with its host organism and has been specialized for energy generation for host cell [2]. Since membrane potential of mitochondria is critical for its functioning as cellular «electric power station», the ultra-structural and molecular events in this organelle can affect processes of cell death.

There are two principal ways of cell death – programmed and un-programmed [3]. The programmed cell death, also called apoptosis, is genetically determined, and the protein products of specific genes are responsible for switching on processes of destruction of specific macromolecules (proteins and nuclear DNA) and cell structures (plasma membrane, cytosol, mitochondria, and nucleus). Autophagy is another form of the programmed cell death that is accompanied by cellular self-digestion [3]. The un-programmed cell death, also called cell necrosis, is mainly induced by the pathological agents, and it is accompanied by the inflammation development, while the apoptosis is not [3]. It should be noted that the apoptotic or necrotic effects strongly depends on duration of action of stressing agent, as well as its dose. The pro-apoptotic agent acting for a longer time and in higher dose can induce the necrotic effects in the same target cells. Although apoptosis, opposite to necrosis, demands energy for its realization, if the mitochondria functions are impaired, cell death processes are also enhanced. The reactive oxygen species are among the most damaging agents affecting cellular structures and functions [4], and mitochondria are both a generator of these agents and their target.

In this article, we have analyzed a dual role of mitochondria in apoptosis – as a generator of energy needed for life processes, and as a source of bio-molecules that participate in realization of cell death program.

Materials and Methods of Investigation

Reagents. *Chelidonium majus L.* alkaloids sanguinarine, chelidonine, chelerythrine were prepared in our lab by Dr. Maxym Lootsik as described in [5]. Anti-caspase-8, anti-caspase-9 antibodies were supplied by Beckman Coulter (Miami, FL), anti-Bcl-2, anti-caspase-3 and cleaved PARP antibodies – Santa Cruz Biotechnology (USA), anti-actin antibody – Sigma Chem. Co. (USA), anti-cytochrome *c* antibodies – BD Pharmingen (USA), anti-Bax – Immunotech (France), proteinase K – Fluka (Germany), RNase A – Sigma Chem. Co. (USA), lauroylsarcosinate – Fluka (Germany), EDTA – Serva (Germany), Trizma base – Sigma Chem. Co. (USA), $NADP^+$ (Reanal, Hungary), glucose-6-phosphate dehydrogenase (Ferak Berlin, Germany), hexokinase (Ferak Berlin, Germany), epoxide resin epon-812 (Fluka, Germany), cacodilate buffer Sigma Chem. Co. (USA), NP-40 – Sigma Chem. Co. (USA), glutaric aldehyde – Sigma Chem. Co. (USA), protease inhibitor mixture – Roche (Germany), ECL reagents – Sigma Chem. Co. (USA), N-acetyl-cysteine (Sigma Chem. Co. (USA), cyclosporine A (Sigma Chem. Co. (USA).

Cells and their culturing. CEM T-leukemia human cells (obtained from cell collection at the Institute of Experimental Pathology, Oncology and Radiobiology NASU, Kyiv, Ukraine) were cultured in suspension in RPMI-1640 medium (Sigma Chem. Co, USA) supplemented with 10 % fetal bovine serum (Sigma Chem. Co, USA) in 5 % CO_2 – 95 % air atmosphere at 37 °C and 100 % humidity. Cells were grown in 35 mm plastic culture dishes and their growth and viability was measured after staining dead cells with 0.1 % trypan blue.

Determination of cell death and apoptosis. Cells were stained with propidium iodide for study the membrane integrity and with Hoechst 33342 – for study of cell nucleus intactness. Cell suspension was transferred to Eppendorf tube, Hoechst 33342 (final concentration 1.5 μM) and propidium iodide (final concentration 1 μg/ml) were added for 15 min at 37 °C, and fluorescent microscopy using blue and red excitation filter at magnification x400 was applied. Digital images were captured using the digital camera.

DNA isolation and electrophoresis. Isolation of DNA from the apoptotic cells and DNA electrophoresis were performed according to Gong et al [6]. Briefly, cells were centrifuged at 2,000 rpm for 5 min, washed with PBS and fixed in 70 % ice-cold ethanol. Then the cells were centrifuged at 2,000 rpm for 5 min, and cell pellet was resuspended in 40 μl of phosphate-citrate buffer (pH 7.8) for 30 min at room temperature. After centrifugation at 3,000 rpm (5 min), supernatant was transferred to the Eppendorf tube and 3 μl of 0.25 % NP-40 solution and 2 μl of RNase A (10 mg/ml) were added. After 1 h incubation at 37 °C, 5 μl of proteinase K (1 mg/ml) was added and the mixture was incubated for 1 h at 37 °C. Separation of apoptotic DNA fragments was performed for 2 h at 4 V/cm in 1.5 % agarose gel supplemented with 0.5 μg/ml ethydium bromide. DNA was visualized in UV-transilluminator and photographed by digital camera.

DNA comet assay. DNA comet assay was performed at moderate alkaline conditions as described [5]. Briefly, cells were pelleted by centrifugation, and re-suspended in PBS. Cell suspension was gently mixed with low melting point agarose and pipetted on microscope slide. Then slides were immersed in cold (4 °C) lysis buffer (0.5 M EDTA, 2 % lauroylsarcosinate, 0.3 mg/ml proteinase K, pH 7.5) for 60 min at 4ºC. The cells were lysed for 20 h at 37 °C and then were transferred into the electrophoretic chamber filled up with freshly prepared electrophoresis buffer (90 mM tris, 90 mM boric acid, 2 mM EDTA, pH 8.5). Electrophoresis was performed at 0.6 V/cm for 25 min [77]. Samples were stained with 2 μg/ml ethydium bromide. The DNA impairment was evaluated by a visual score using an arbitrary scale ranging from 0 (no DNA damage) to 3–4 (intensive DNA damage), which was based on the length of comet tail and relative proportion of DNA in the comet head and tail [88]. Minimally 300 cells were evaluated in each sample at magnification x200.

Determination of intracellular level of ATP. The ATP concentration was determined enzymaticaly using hexokinase and glucose-6-phosphate dehydrogenase. Produced NADPH was measured spectrophotometricaly at 340 nm. Briefly, cells were cultured with alkaloids, washed twice with ice-cold PBS and 20 μl of 0.6 M ice-cold perchloric acid was added to 10^6 cells. Perchloric acid was neutralized with 2 M KOH and pellets were removed by centrifuged at 10,000 rpm for 5 min. For enzymatic reaction, sample were mixed with buffer (0.2 M Tris-HCl (pH 7.5), 1.4 мМ $NADP^+$, 8 мМ $MgCl_2$, 65 мМ glucose, glucose-6-phosphate dehydrogenase). In 5 min the absorption E_1 at 340 nm was measured using the NanoDrop spectrophotometer. Then hexokinase suspension was added and left for 30 min at 37 °C. Finally, absorption E_2 at 340 nm was measured and calculated value $E_2 - E_1$ was used for determination of ATP concentration from the calibration curve.

Western blot analysis. Cells were treated with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 % Triton X-100, 150 mM NaCl with freshly added protease inhibitor mixture for 30 min at 4 °C and supernatants with protein samples were collected after centrifuged at 12,000 g for 15 min. Protein concentration was determined by Peterson method [9]. Equal amounts of protein (30–40 μg) were subjected to electrophoresis in 12 % polyacrylamide gel with 0.1% SDS. The proteins were transferred by electro-phoresis onto the nitrocellulose membrane. The membranes were blocked by 5% nonfat dry milk in PBS containing 0.05% Tween 20 at 20 °C for 1 h, and then probed with an appropriate dilution of primary antibody overnight at 4 °C. The blots were washed twice for 5 min with PBS-Tween-20 and incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated secondary antibody (Amersham Life Science, Inc., Arlington Heights, IL) in 5 % milk/PBS-Tween 20 at 4 °C for 1 h. After washing twice in PBS-

Tween 20 for 5 min, the proteins were visualized by the ECL reagents. The following primary antibodies were used: anti-caspase-8, anti-caspase-9; anti-Bax (1:1,000; mouse monoclonal); anti-caspase-3, anti-cleaved PARP, anti-Bcl-2 (1:1,000; rabbit polyclonal). For each immunoblot, equal loading of protein was confirmed by stripping the blot, and its reprobing with anti-actin antibody (1:300; rabbit polyclonal).

Measurement of cytosolic and mitochondrial cytochrome *c, Bax, and Bcl-2*. After treatment with the alkaloids, cells were washed twice with cold PBS, collected by centrifugation at 1,500 g for 5 min, and incubated for 5 min in ice-cold buffer (250 mM sucrose, 70 mM KCl, 100 μg/ml digitonin in PBS) according to [1010]. Then the cells were pelleted for 5 min at 1,000 g, and the supernatant was collected, as a cytosolic fraction. Mitochondria fraction was prepared by lysis of the pellet in the immune-precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2 % Triton X-100, 0.3 % NP-40 with freshly added complete protease inhibitor cocktail from Roche). 20 μg of cytosolic or mitochondrial proteins, as determined by Lowry protein assay, were loaded onto a 12 % SDS-polyacrylamide gel. Proteins were transferred onto the nitrocellulose sheets as described above. Cytochrome *c*, Bax, and Bcl-2 were detected with appropriating antibodies, and then re-probed with anti-β-actin antibody. After incubation with 1:5,000 dilution of horseradish peroxidase-conjugated anti-mouse Ig, the blots were developed by ECL.

Rhodamine-123 accumulation in mitochondria. To check intactness of cellular mitochondria, the cells were loaded with rhodamine-123 fluorescent dye used for measuring mitochondrial membrane potential (MMP) [11]. Cells were cultured for 30 min with rhodamine-123 (1 μg/ml). After incubation, the cells were centrifuged for 3 min at 2,000 rpm, resuspended in PBS to final concentration $3 \cdot 10^6$ cells per ml, and photographed under fluorescent microscope using green excitation filter at magnification ×400.

Electron microscopy. Cells were fixed with 1.5% glutaraldehyde in 0.2 M cacodilate buffer (pH 7.2) for 1 h at 4 °C. Samples were washed with cacodilate buffer and additionally fixed for 1 h at 4 °C with 2 % OsO_4 solution in cacodilate buffer. Then, they were washed out of the fixating solutions, dehydration was carried out using increasing ethanol concentrations (50, 70, 90 and 100 %). Additionally, samples were dehydrated in propylene oxide and transferred in epoxide resin epon-812. Sections were prepared, contrasted for 15 min with 2 % uranil acetate, and then treated with lead citrate, according to Reynolds [12]. The sections were analyzed with electron transmission microscope PEM-100, and photographed on digital camera.

Statistical Analysis. All experiments were repeated three times. The results are presented, as mean ± standard deviation. Differences were considered statistically significant, when *P* was less than 0.05.

Results and Discussion

It is known that mitochondria play a decisive role in apoptotic cell death due to a release of cytochrome *C* and switching on caspase cascade [13]. We have applied different alkaloids of *Chelidonium majus L.* medicinal plant as inducers of apoptosis in human T-leukemia CEM cells. While sanguinarine and chelerythrine rapidly and directly damaged mitochondria structure by impairing their functioning, the effect of structurally related chelidonine towards mitochondria was considerably delayed and, probably, a secondary and indirect one [14]. We have analyzed which could be the role of mitochondria in defining rate and intensity of apoptosis. It was demonstrated that apoptosis induction is

accompanied by following principal changes in mitochondria: 1) a decrease in the anti-apoptotic proteins of Bcl-2 family; 2) an increase in the pro-apoptotic proteins of this family (ex. Bax); 3) an elevation of intra-mitochondrial Ca^{2+}; 4) a drastic decrease in trans-membrane potential ($\psi\Delta m$) and, as a result, a decrease in generated ATP; 5) a release of mitochondrial protein – cytochrome *C* [3]. In most effects listed above, the action of sanguinarine and chelerythrine was very fast (for example, these alkaloids induced a release of cytochrome *C* from mitochondria to cytosol as soon as in 2 min after the start of cell targeting), the action of chelidonine which is known from literature as the cytoskeleton inhibitor [15] was significantly delayed and observed in 12–24 hrs [14].

While sanguinarine and chelerythrine (1 μg/ml, 3 h) caused drastic changes in the ultra-structure of mitochondria (marked disordering of cristae and intra-mitochondrial vacuolization) of CEM T-cells, chelidonine was un-effective there even in 4 μg/ml dose [14]. These morphological changes are in agreement with the results of measuring the mitochondrial trans-membrane potential estimated as amount of Rhodamine-123 accumulation in mitochondria. It should be noted that chelidonine did not affect that indicator in 6 hrs, however, in longer terms (12 and 24 hrs) this alkaloid inhibited dye accumulation in mitochondria. These data also correlate well with the effect of alkaloids on the parameters of oxidative phosphorylation in the isolated mitochondria: there was a significant inhibition of oxidative phosphorylation under the action of sanguinarine and chelerythrine, while chelidonine was ineffective there. Finally, the differential effect of studied alkaloids towards mitochondrial structure and functions can be well seen when measuring of ATP content was conducted in human T-leukemia cells of CEM line. There were no statistically significant changes at 1 hr time point of alkaloids' action, however, in 3 hrs ATP level decreased by approximately 60 % at the action of sanguinarine and chelerythrine, while chelidonine did not affect ATP level in the treated T-leukemia cells.

As noted above, the reactive oxygen species (ROS) can be an important player in apoptosis scenario, particularly when the mitochondria are involved. For measuring the effect of specific alkaloids on ROS production dihydroethidium reagent was used, and the product was measured by FACS on FL3-H channel. It is know that N-acetyl-cysteine (NAC) is an effective scavenger of ROS [16], and, thus, it was used for defining if the alkaloids under study can realize their pro-apoptotic effects via induction of ROS generation. We found that NAC was capable of blocking most effects of sanguinarine and chelerythrine mentioned above, namely, a release of cytochrome *C*, activation of caspases, DNA damage, level of mitochondrial trans-membrane potential, while this ROS scavenger was not effective towards chelidonine induced proapoptotic effects. The effect of sanguinarine and chelerythrine on ROS production was time and dose dependent, and 30 min pretreatment of cells with NAC (5 mM) suppressed significantly the effect of following 60 min treatment of cells with sanguinarine or chelerythrine. Chelidonine did not induce rapid generation of ROS, and its very late effects here could be secondary ones.

Thus, a multi-target action of specific alkaloids as apoptosis-inducing agents was demonstrated. Blocking mitochondria as principal energy generating machine of cell is leading to a start of apoptosis scenario expressed via different actions, such as morphological changes in mitochondrial ultra-structure, various effects leading to a drop of trans-membrane potential and a decrease in oxidative phospholyration and ATP level, a release of cytochrome *C* and Ca^{2+} flow into mitochondria. Altogether, these changes lead to cell death. A principal question appears – why the eukaryotic cell entrusted its

ancient endosymbiont – the mitochondria which is not capable of independent life – diagnosing cell death and, moreover, cell killer role?

Acknowledgement. The author thanks Vitaly Kaminskyy, PhD whose data were cited in the manuscript, as well as other collaborators of the Department of Regulation of Cell Proliferation and Apoptosis at the Institute of Cell Biology, NAS of Ukraine, who are working in that Department headed by the author.

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РЕЗЮМЕ

Ростислав СТОЙКА

ЯК МІТОХОНДРІЯ ПЕРЕТВОРЮЄТЬСЯ ІЗ ЕНЕРГОГЕНЕРАТОРА КЛІТИНИ У ВБИВЦЮ КЛІТИНИ

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Структурно-функціональні зміни в клітинах, що гинуть шляхом апоптозу, мають системний характер і відбуваються на різних рівнях клітинної організації, а саме в плазматичній мембрані, цитозолі, мітохондріях і ядрі. При цьому необхідно відзначити, що мітохондрії відіграють тут двояку роль. За нормальних умов ці органели забезпечують енергетичними ресурсами більшість процесів життєдіяльності клітини, тоді як за дії екстремальних чинників (токсичні речовини, у т.ч. протипухлинні препарати, чи рентгенівське випромінювання) вони стають «вбивцями» власних клітин. Цей дуалізм у дії мітохондрій проявляється у різних формах. До складу мітохондріальної мембрани входять білки Bcl-2 і Bcl-XL, підвищений рівень яких протидіє апоптозу, тоді як переважання там інших білків, Bax і Bid, споріднених до Bcl-2, навпаки, сприяє апоптозу. У матриксі мітохондрій міститься цитохром С, який є життєво важливим для здійснення цими органелами функції енергозабезпечення клітини, і в той же час просте «витікання» цього білка з мітохондрій у цитоплазму стає абсолютно смертельним для клітин. Активуючи в цитоплазмі білок Apaf-1, його комплекс із цитохромом С у подальшому активує ще й протеолітичний ензим прокаспазу-9, яка при цьому набуває здатності розщеплювати так-звані ефекторні каспази 2, 3, 6, 7 і 10. Останні руйнують внутрішньоклітинні білки, які є критично важливими для забезпечення життєдіяльності клітин. Серед посередників у реалізації програми апоптозу є активні форми кисню. Тому блокування дії їхніх генераторів чи знешкодження самих цих сполук із високою реакційною здатністю також гальмує прояв ознак апоптозу. У роботі наведені дані літератури і результати досліджень співробітників відділу, керованого автором, щодо можливостей впливу (стимулювання чи гальмування) на відмирання клітин шляхом апоптозу.

Ключові слова. Смерть клітини, апоптоз, мітохондрія, механізми, активні сполуки кисню.

Стаття надійшла: 20.06.2014.
Після доопрацювання: 14.12.2014.
Прийнята до друку: 15.01.2015.