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Respiration and Mitochondrial Membrane Potential Are not Required for Apoptosis and Anti-apoptotic Action of Bcl-2 in HeLa Cells

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Abstract—The release of cytochrome *c* from intermembrane space of mitochondria into cytosol is one of the critical events in apoptotic cell death. The important anti-apoptotic oncoprotein Bcl-2 inhibits this process. In the present study it was shown that apoptosis and release of cytochrome *c* induced by staurosporine or by tumor necrosis factor-α in HeLa cells were not affected by inhibitors of respiration (rotenone, myxothiazol, antimycin A) or by uncouplers (CCCP, DNP) that decrease the membrane potential at the inner mitochondrial membrane. The inhibitors of respiration and the uncouplers did not affect also the anti-apoptotic activity of Bcl-2.

Key words: apoptosis, necrosis, cytochrome *c*, mitochondria, respiration, membrane potential

Programmed cell death, or apoptosis, is a complex cascade of biochemical processes that leads to characteristic morphological changes of the cell. Apoptosis plays a critical role in development, maintenance of homeostasis, and different pathologies in multicellular organisms. A variety of natural ligands (cytokines, hormones) binding to their receptors as well as various stresses trigger the genetically programmed pathways of apoptosis (see review [1]).

Mitochondria play a key role in many variants of apoptosis. They are the target for the action of specialized pro- and anti-apoptotic proteins of the Bcl-2 family, some other proteins, which also have other functions (p53, TR3), and low molecular weight mediators of apoptosis (ceramide, reactive oxygen species (ROS), etc.). The main response of mitochondria to apoptotic stimuli is the release of a number of soluble proteins, such as cytochrome *c*, apoptosis-inducing factor (AIF), procaspases 2, 3, and 9 (inactive precursors of apoptotic proteases), and protein SMAC/Diablo (an activator of caspases that blocks the action of natural inhibitors of caspases) from the intermembrane space of mitochondria into the cytosol (see reviews [2, 3]). Overproduction of ROS in mitochondria is probably one of the other consequences of their bioenergetic malfunctioning, which can play an important role in apoptosis. Apoptosis in some cell types can be triggered by mitochondrial inhibitors that cause the reduction of the cellular ATP level. Reduction of ATP concentration at the same time can block apoptosis and/or switch the apoptotic death mechanism to necrosis [4]. The complexity in the effects of mitochondrial inhibitors on apoptosis prevent making definite conclusions about the role of respiration and mitochondrial membrane potential in cytochrome *c* (and other proteins) release into cytosol and also in realization of the other elements of the apoptotic program.

Abbreviations: ANT) adenine nucleotide translocator; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; DMEM) Dulbecco's modified Eagle's medium; DNP) 2,4-dinitrophenol; DOG) 2-deoxy-D-glucose; MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; STS) staurosporine; TNF-α) tumor necrosis factor-α.

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MATERIALS AND METHODS

Cell culture and chemicals. The human carcinoma cell lines HeLa and HeLa-Bcl-2 were maintained in DMEM medium supplemented with high glucose

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(5 mM), 0.08 mg/ml gentamycin sulfate and 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C and 5% CO_2. The human bcl-2 gene was introduced into HeLa cells using pLPC-bcl-2 vector for transfection. Control clone was prepared similarly using an empty vector. Expression of Bcl-2 protein in transfected cells was confirmed by Western blot analysis using anti-human Bcl-2 monoclonal antibody. The HeLa and HeLa-Bcl-2 cell lines were provided by Dr. G. A. Belov, M. P. Chumakov Institute of Poliomyelitis and Viral Encephalites, Moscow, Russia.

Cell viability was determined by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reductase activity [5]. Necrotic cells were detected by failure of trypan blue exclusion. Apoptotic cells were stained with fluorescent dye Hoechst 33342 (Sigma, USA) after fixation with 4% paraformaldehyde. Cells in Vectashield® (Vector, USA) medium were analyzed under a non-confocal fluorescence microscope (Axiophot, Zeiss, Germany).

For immunostaining, cells were grown on glass cover slips and were fixed by a 15-min incubation with ice-cold methanol at –20°C. Probes were incubated with monoclonal anti-cytochrome *c* antibody (0.001 mg/ml, 7H8.2C12, PharMingen) and were stained with FITC-tagged secondary anti-mouse IgG antibody (Molecular Probes, USA). Images were analyzed under the Axiophot non-confocal fluorescence microscope equipped with an MTI CCD camera and the Scion Image computer program.

Mitochondrial inhibitors, emetine, TNF-α, MTT, and STS were from Sigma.

RESULTS AND DISCUSSION

To test the importance of respiration, oxidative phosphorylation, and mitochondrial membrane potential in apoptosis, we have chosen the HeLa carcinoma cell line. This type of cells (as a number of other rapidly growing, non-differentiated tumor cell lines) has a high level of both respiration coupled to ATP synthesis and of glycolysis. The measurements of O_2 consumption by these cells in cultivation medium (at concentration of glucose 5 mM) have shown that the inhibitor of oxidative phosphorylation oligomycin (5 μg/ml) strongly inhibited respiration and uncouplers (5 μM CCCP, 0.2 mM DNP) stimulated it. Inhibitors of respiration (2 μM rotenone, 1 μM myxothiazol, 2 μM antimycin A) almost completely prevented consumption of oxygen by HeLa cells (data not shown). Crabtree's effect (inhibition of respiration and oxidative phosphorylation in the presence of substrate of glycolysis) was pronounced in HeLa cells under higher concentrations of glucose and was mainly caused by competition for ADP. The inhibitors of mitochondrial respiration, ATP synthase (oligomycin) and protonophoric uncouplers did not inhibit proliferation of HeLa cells for more than 24 h in the presence of glucose. 2-Deoxyglucose (DOG, 5 mM), a competitive inhibitor of glycolytic ATP production, was also nontoxic but caused rapid necrotic cell death (more than 80% in 24 h) in combination with any one of the mitochondrial inhibitors mentioned above. These data indicate that both mitochondrial and glycolytic ATP production are dispensable for survival of HeLa cells.

Staurosporine (STS), a general inhibitor of protein kinases, is a well-known pro-apoptotic agent. STS (1 μg/ml) caused rapid (80% cells in 8 h) morphological changes of HeLa cells, appearance of blebs on their surface, and condensation and fragmentation of chromatin, which are characteristic features for apoptosis (Fig. 1). The last feature was used for quantitative estimation of apoptosis. Apoptotic changes were correlated with the fall of MTT-reduction activity in the cells. This test did not distinguish apoptosis from necrosis, but the level of necrosis was not more than 3-5% in our experiments and it was not taken into account. Both the inhibitors of respiration and the uncouplers mentioned above did not affect apoptotic cell death. Inhibition of staurosporine-induced apoptosis by Bcl-2 was also insensitive to the mitochondrial inhibitors (Fig. 2).

Staurosporine-induced release of cytochrome *c* from mitochondria into cytosol was observed using an immunostaining technique (Fig. 3). There were different structures (thread-like and round) in intact cells in which cytochrome *c* was localized. These structures were stained by rhodamine 123 and mitotracker red, specific mitochondrial dyes (data not shown). Staining of cytochrome *c* became diffuse in apoptotic cells, which indicated the release of the protein into cytosol. Brightness of the immunostaining visibly grew in apoptotic sells, apparently due to their rounding. The inhibitors of respiration and the uncouplers did not prevent the release of cytochrome *c* into the cytosol. The release of cytochrome *c* was prevented by Bcl-2, and the mitochondrial inhibitors did not interfere with this effect. In control experiments it was shown that expression of Bcl-2 did not decrease the effectiveness of the uncouplers and the inhibitors of respiration. When this work was in preparation it was reported that apoptosis and cytochrome *c* release induced by UV or actinomycin D also were not inhibited by uncouplers [6].

The "receptor-dependent" apoptosis in HeLa cells was induced by tumor necrosis factor-α (TNF-α, 5-20 ng/ml) in combination with the protein synthesis inhibitor emetine (1 μg/ml). This treatment caused apoptosis in 30-65% of cells during 5-8 h. TNF-α interacts with the receptors on the cell surface and induces both apoptosis and the expression (mediated by transcription factor NF-κB) of anti-apoptotic proteins (see review [7]). The last effect prevails in HeLa, so TNF-α did not cause apoptosis *per se*. No necrotic death was detected during 8-15 h in the presence of TNF-α and

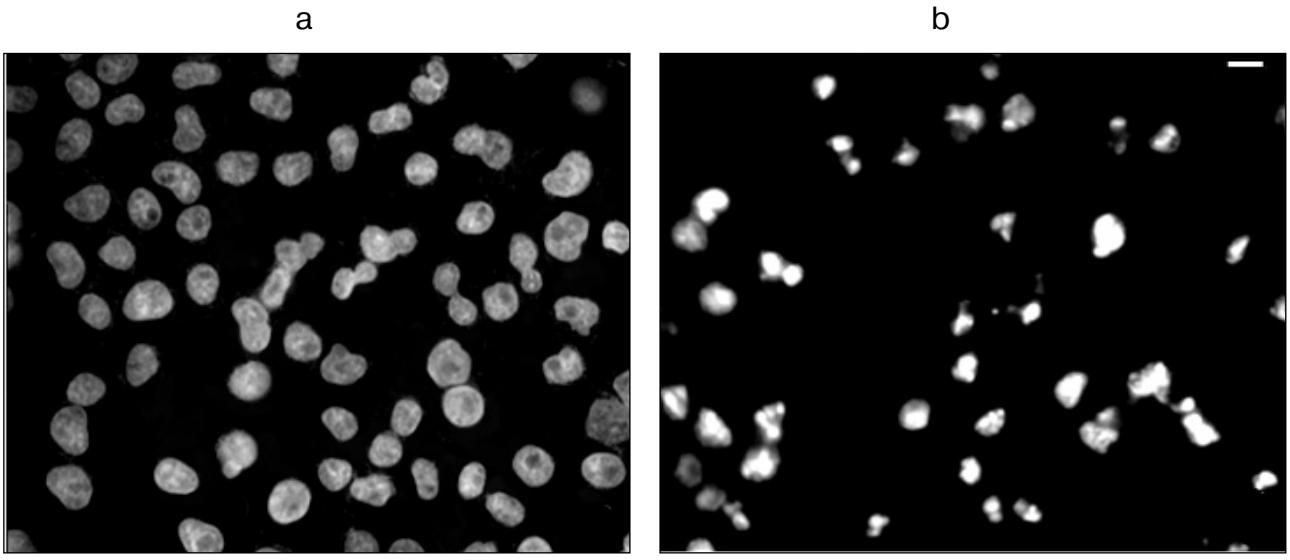

Fig. 1. Changes in the nucleus of HeLa cells during apoptosis induced by STS. Cells were fixed and stained with Hoechst 33342 (1 μg/ml) as described in "Materials and Methods": a) control cells; b) cells incubated for 8 h with 1 μg/ml STS. Bar is 100 μm.

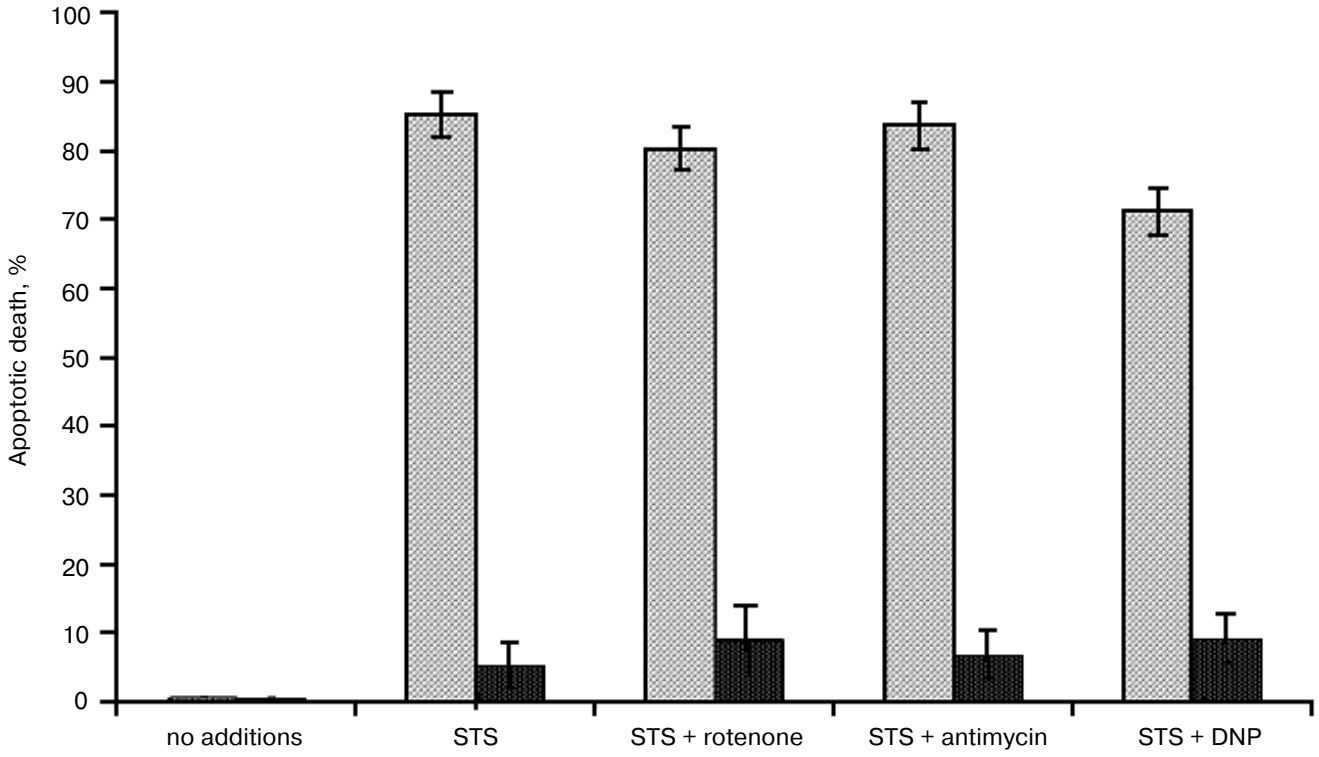

Fig. 2. Effects of mitochondrial inhibitors on STS-induced apoptosis in HeLa and HeLa-Bcl-2 cells (dark bars). Cells were incubated with STS (1 μg/ml) and with STS in combination with rotenone (2 μM), antimycin A (2 μM), and DNP (0.2 mM). The apoptotic cells were counted after staining with Hoechst 33342 (mean values obtained in five-eight independent experiments are presented).

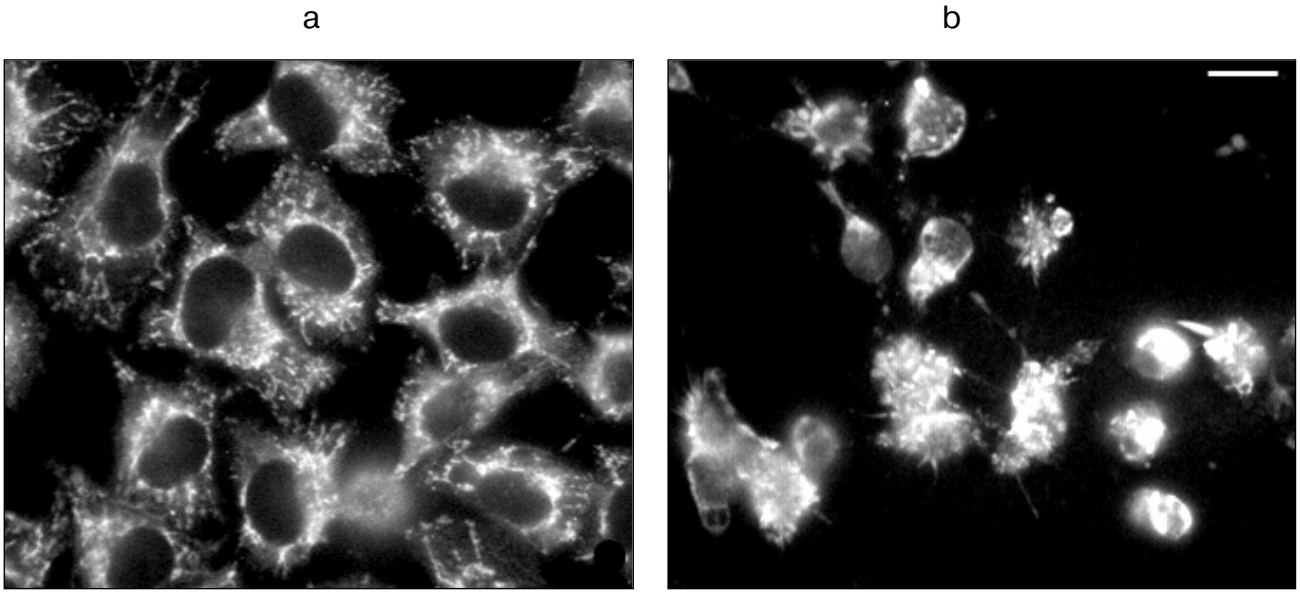

Fig. 3. Immunofluorescent staining of cytochrome *c* in HeLa cells: a) control cells; b) cells incubated with STS (1 μg/ml, 8 h); release of cytochrome *c* into cytoplasm. Bar is 100 μm.

emetine. Apoptotic changes in chromatin were correlated to cytochrome *c* release into cytosol and were prevented by Bcl-2. The inhibitors of respiration and the uncouplers did not interfere either with cytochrome *c* release, with apoptosis, or with the protective action of Bcl-2 (data not shown).

The mechanism of cytochrome *c* (and some other proteins) release from mitochondria into cytosol during apoptosis is not clear. Three hypotheses have been suggested (see reviews [2, 3]): 1) in the inner membrane of mitochondria the permeability transition pore opens. The permeability transition is a sudden increase of inner mitochondrial membrane permeability to ions and solutes, which causes dissipation of $\Delta\psi_m$ and diffusion of solutes down gradients of their concentrations. This is followed by water flux across the inner membrane, by passive osmotic swelling, rupture of the outer mitochondrial membrane and cytochrome *c* release; 2) an electrochemical gradient of protons at the inner membrane of mitochondria increases (due to cessation of ATP synthesis), which causes K^+ accumulation, swelling of the mitochondrial matrix and further see (1); 3) proapoptotic members of the Bcl-2 family (such as Bax and Bid) themselves or these proteins in complex with the resident mitochondrial proteins form large pores for cytochrome *c* (and other proapoptotic proteins) in the outer membrane. The last hypothesis is the most popular but it includes the elements of active transmembrane transport of proteins, which hardly agrees with the finding that the release of cytochrome *c* (if it is already begun) is practically temperature-independent [8]. The mechanism that included hyperpolarization of mitochondria is based on the measurements of membrane potential with fluorescent indicators [9], which were not quantitative estimations and were repeatedly exposed to grounded criticism. The data obtained in the present work exclude this mechanism from consideration in the described models of apoptosis. Our results do not contradict the hypothesis of the permeability transition pore (PTP) opening [10]. The effect of some permeability transition pore inhibitors confirmed its participation in TNF-α-dependent apoptosis of hepatocytes [11] and HeLa (Shchepina et al., unpublished data) and also necrotic death of L929 fibrosarcoma cells [12]. Our observations exclude some mechanisms of the pore induction. In particular, one of them is based on excessive Ca^{2+} accumulation in the matrix, which is impossible when the membrane potential is decreased. The other mechanism of the PTP opening is based on its sensitivity to membrane potential [13]. The decrease of potential (for instance under the effect of Bax and Bid) could cause pore opening, but this scenario also cannot be realized in the presence of the uncouplers. Finally, we have suggested that the opening of the permeability transition pore during TNF-α-dependent apoptosis occurs by a mechanism that does not depend on membrane potential, accumulation of Ca^{2+} in the matrix, and the state of the respiratory chain. One of the possible mechanisms includes the conformational change of adenine nucleotide translocator (ANT) in the inner mitochondrial membrane [14]. Changes in the properties of ANT were recently demonstrated in several models of apoptosis [15]. Further investigations are needed to test this suggestion and reveal the possible mecha-

nisms that cause the changes in ANT and the role of this effect in different types of apoptosis.

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