Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1721 (2005) 55–64

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

Induction of apoptosis by electrotransfer of positively charged proteins as Cytochrome *C* and Histone H1 into cells

I. Tsoneva[a,*], B. Nikolova[a], M. Georgieva[b], M. Guenova[c], T. Tomov[a], M.-P. Rols[d], M.R. Berger[e]

[a]*Institute of Biophysics, Bulg. Acad. Sci., Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria*
[b]*Department of Oncogenesis, National Oncological Center, Unit of Toxicology and Chemotherapy, Plovdivsko Pole 6, 1756 Sofia, Bulgaria*
[c]*National Centre of Clinical and Transfusion Hematology, Lab. of Cytopathology and Flow Cytometry, Plovdivsko Pole 6, 1756 Sofia, Bulgaria*
[d]*Institute of Pharmacologie, CNRS (UNRS5089), 205, Route de Narbonne, 31077 Toulouse Cedex 4, France*
[e]*German Cancer Research Center, Unit of Toxicology and Chemotherapy, Im Neunheimer Feld 280, 69 120 Heidelberg, Germany*

Received 20 April 2004; received in revised form 21 July 2004; accepted 7 October 2004
Available online 22 October 2004

Abstract

Cytochrome *C* (Cyt. *C*) is a mitochondrial protein inducing apoptosis when it is accumulated in the cytosol by a currently unknown mechanism, but regulated by the bcl-2 family of proteins. The linker Histone H1 is another basic protein with highly conservative structure, composition, and equal molecular weight, not changed during the evolution.

An attempt was made to understand better the apoptotic processes by electroloading of leukemic cells, such as K562, HL-60, and SKW3, and human lymphocytes with positively charged proteins, such as Cyt. *C*, Histone H1, and methylated BSA albumin (mBSA). The triggering apoptotic processes followed by MTT test, FACS analysis, and DNA fragmentation after the electrotransfer of these proteins into the cells were observed.

Histone H1 and mBSA induce the release of Cyt. *C* from rat liver mitochondria. Cytochrome *C* release was higher when mitochondria were in "high-energy" state. It is supposed that release of Cyt. *C* from mitochondria is due to the mechanical rupture of the outer mitochondrial membrane, rich in negatively charged groups, predominately due to cardiolipin. The reason for the morphological rupture of the outer mitochondial membrane could be the rigidification and segregation of the membrane and the destroyed membrane asymmetries of both monolayers in the presence of positively charged proteins at higher linear charges such as Histone H1. We suggested that Histone H1, at a given moment of activated signaling for apoptosis, could be not transported to the nucleus and could lead to the release of Cyt. *C* from the mitochondria in the cytoplasm. It is temping to speculate that Histone H1 has other physiological extranuclear functions involved in apoptosis.

Keywords: Apoptosis; Cytochrome *C*; Histone H1; methylated BSA (mBSA); Electroporation; Mitochondria

1. Introduction

Apoptosis (programmed cell death) is an important process for maintaining homeostasis in multicellular organisms. All cells in such organisms have the capacity to undergo this form of death. The dysregulation of apoptosis is implicated in a number of human diseases, including cancer, autoimmune diseases, viral infections, neurodegenerative diseases, AIDS, and cardiovascular diseases [1].

Cyt. *C* is a basic protein composed of 104 amino acids, including 19 lysine residues. Cyt. *C* is a mitochondrial protein inducing apoptosis when it is accumulated in the cytosol by a currently unknown mechanism, but regulated by the bcl-2 family of proteins. Recent reports demonstrated that the electroloaded or microinjected Cyt. *C* into the cells could activate apoptosis [2–5]. There are data that apoptosis induction could be observed without accumulation of Cyt. *C* in the cytosol and the above process is a cell type- and inducer-dependent phenomenon [6].

On the other hand, the linker Histone H1 is another basic protein with highly conservative structure, composition, and

* Corresponding author. Tel.: +35 92 9792622; fax: +35 92 9712493.
E-mail address: itsoneva@obzor.bio21.bas.bg (I. Tsoneva).

0304-4165/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2004.10.002

equal molecular weight not changed during the evolution. There is growing evidence that Histone H1 belongs to a larger family of proteins having numerous biological functions involving other non-chromatid functions [7,8]. Evidence on the hormonal properties of histones comes from Comsa et al. [9]. Furthermore, the existence of a cytoplasmic pool of Histone H1 was shown, which contrasts with the lack of detectable amounts of core histones in the cytoplasm. This indicates that the observed H1 pool is not just a reflection of its cytoplasmic synthesis, but probably has some functional significance [10]. Rose et al. [11] give data that the cytoplasmically expressed Histone H1 has antimicrobial activity in the epithelial cells.

Thus, the onset of the process of apoptosis must be under strict physiological control.

In this report, we attempt to better understand the apoptotic processes by cellular uptake of exogenously added positively charged proteins as Cyt. *C*, Histone H1 and mBSA. The loading of cells was done by the method of electroporation. By the method of electropermeabilisation, the macromolecules, such as drugs and DNA, could be successfully transferred into the cells [12–16].

We concentrated our efforts on the cytotoxic effects, morphological changes, DNA fragmentations, and FACS analysis of electroloaded cells with positively charged proteins. It was shown that primary lymphocytes are less sensitive to Cytochrome *C* exogenously introduced into the cells. The incubation with Histone H1 or mBSA resulted in cell death by destruction of the integrity of the plasma membranes of cancer lines K562, SKW3 and HL-60. Electroporation in the presence of these proteins showed some additional increase of the dead cells. It was observed that Histone H1 and mBSA increased the permeability of mitochondria isolated from rat liver and release of Cyt. *C* in the incubation medium was found. It was suggested that Histone H1 has extra nuclear functions involved in the processes of apoptosis. The effect of Cyt. *C* release in the presence of Histone H1, in respect to mBSA, was higher when mitochondria are in "high energy" state.

2. Materials and methods

2.1. Cells and culture conditions

K562: DSMZ no. ACC 10; cell type: human chronic myeloid leukemia (CML) in blast crisis; origin: established from the pleural effusion of a 53-year-old woman with chronic myeloid leukemia in blast crisis in 1970; doubling time ca. 30–40 h; cytogenetics: human hypotriploid karyotype. K562 has Bcr-Abl rearrangement and expresses the fusion protein p210.

SKW 3 cell line: DSMZ no. ACC 53; cell type: human T-cell leukemia; origin: established from the peripheral blood of a 61-year-old man with T-cell chronic lymphocyte leukemia in 1977; doubling time ca. 30–40 h; cytogenetics: human near diploid karyotype with polyploidy.

HL-60 myeloid cell line: DSMZ no. ACC 3; cell type: human acute myeloid leukemia; origin: established from the peripheral blood of a 35-year-old woman with acute myeloid leukemia in 1976; doubling time ca. 25 h; cytogenetics: human flat-model hypotetraploid karyotype with hypodiploid sideline and 1.5% polyploidy.

All cell lines were grown as suspension culture (RPMI-1640 medium, supplemented with 10% fetal calf serum) at 37 °C in an incubator with humid atmosphere and 5% CO_2. Cells were passaged two or three times per week to keep them in log phase.

2.2. Isolation of human lymphocytes

Human peripheral blood from healthy donors was obtained from the National Centre of Blood Transfusion in Sofia. Peripheral blood lymphocytes were prepared by Ficoll Paque gradient centrifugation. Briefly, 15-ml anticoagulated blood was diluted with 25-ml PBS and underplayed with 10-ml Ficoll-Paque Solution (Pharmazia), centrifuged for 20 min at 800×*g*, 20 °C. The interface band with lymphocytes was aspirated and washed three times with PBS. Finally, the lymphocytes were resuspended in culture medium RPMI-1640 (without phenol red) supplemented with 10% FCS, Pen Strep (1%) at a concentration of 10^7 cells/ml. The lymphocytes were activated by adding 3 μg/ml Phytochemagglutinin dissolved in RPMI 1640.

2.3. Compounds and chemicals

2.3.1. Cytochrome C

Cytochrome *C* was from bovine heart, C-3131, Sigma.

2.3.2. Histone H1

Histone H1 with molecular weight (MW)=21 kDa was kindly supplied by Prof. L. Srebreva, Institute of Molecular Biology, BAS, Sofia. Histone H1 was a lysine-rich histone, p*K*=10.5, and was isolated from mouse liver. The method of isolation is given in detail by Srebreva and Zlatanova [17] and Srebreva et al. [18].

2.4. Methylated albumin (mBSA)

The method for methylation of BSA (fraction V, Sigma) is given in Ref. [19]. Briefly, 5-g BSA was dissolved in 500-ml methanol, and 4.2-ml 12 N HCl was added. The suspension obtained was left for 3 days in the dark at room temperature (about 20 °C) and shaken from time to time. The protein pellet was collected by centrifugation and subsequently washed with absolute methanol until the yellow was lost (generally two to three times). For this purpose, the protein was mixed in methanol and pelleted by centrifugation. The protein obtained was washed twice with ether.