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Mitochondrial respiratory chain inhibitors induce apoptosis

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Abstract

In this paper the specific mitochondrial respiratory chain inhibitors rotenone and antimycin A and the highly specific mitochondrial ATP-synthase inhibitor oligomycin are shown to induce an apoptotic suicide response in cultured human lymphoblastoid and other mammalian cells within 12–18 h. The mitochondrial inhibitors do not induce apoptosis in cells depleted of mitochondrial DNA and thus lacking an intact mitochondrial respiratory chain. Apoptosis induced by respiratory chain inhibitors is not inhibited by the presence of Bcl-2. We discuss the possible role of mitochondrial induced apoptosis in the ageing process and age-associated diseases.

Key words: Human Lymphoblastoid cell; Melanoma cell; Proto-oncogene product Bcl-2

1. Introduction

Apoptosis is a widespread and morphologically distinct process of cell death. Cells undergoing apoptosis shrink, the plasma membrane forms blebs, the nucleus condenses and nuclear DNA is degraded to oligonucleosomal fragments by a calcium activated endonuclease [1–4]. Apoptosis is a normal physiological process eliciting no inflammatory response, in contrast to necrotic cell death and is involved in the selective and desired destruction of cells and tissues, also referred to as programmed cell death [5]. It has been suggested that the failure of cells to undergo apoptosis is associated with diseases like cancer whereas the aberrant induction of apoptosis of cells that are supposed to survive could be involved in, for example, the destruction of the immune system by HIV or underlie neurological diseases like Parkinson's syndrome and other age-related diseases [6].

The ageing process is characteristically associated with a general decline in bioenergy and involution of muscle and organ tissues. We have previously proposed [7] that the accumulation of mitochondrial DNA mutations with time makes a significant contribution to the age-associated bioenergy decline through a progressive reduction of the oxidative phosphorylation capacity of the mitochondria. In this paper we show that highly specific inhibitors of mitochondrial bioenergetic function induce apoptosis in different cultured mammalian cells independently of whether the cells express the proto-oncogene product Bcl-2, a protein which is generally found to inhibit apoptosis [8]. We suggest that apoptosis induced through interference with mitochondrial bioenergy may also occur in vivo and may underlie age-associated diseases.

2. Materials and methods

2.1. Cell lines and culture conditions

The human lymphoblastoid cell lines Namalwa ρ^+ and Namalwa ρ^0, BM 13674 and BMX 13674, Daudi, BL 29, K562, the human melanoma cell lines MEL 3, MEL 28 and MM 96 and the mouse fibroblast cell line L 929 were maintained in logarithmical growth phase using RPMI 1640 medium (Gibco, Melbourne, Australia) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% (v/v) foetal calf serum, 5 mM MOPS-KOH (pH 6.8), 50 μg/ml uridine, 1 mM sodium-pyruvate and 2 mM L-glutamine. Cultures in 75 or 25 ml culture flasks were incubated at 37°C in a humidified gas mixture containing 5% CO_2 balanced with air.

2.2 Morphological quantification of apoptosis

1 ml lymphoblastoid cells (5×10^6 cells/ml) were mixed with 1 ml fresh medium containing twice the concentration of inhibitor (purchased from Sigma, St. Louis, MO, USA) desired and cultured in a 25 ml culture flask. After 12 or 18 h a 200 μl aliquot was drawn from the suspension and transferred to slides coated with 10 μl poly-L-lysine (10 μg/ml) using a cytospin (5 min at 1,500 rpm). The cells were fixed for 10 min in a mixture of ethanol/acetic acid (3:1), washed for 1 min in distilled water and subsequently air dried and stained with a drop of 0.5 μg/ml 4,6-diamidino-2-phenylindole for 10 min. The adhered cells were washed twice with distilled water, air dried and mounted with anti-fading medium (10 mg/ml *p*-phenyldiamine in 90% glycerol, pH 9.0). The slides were observed under a fluorescence microscope (Nikon, microphot-FX) at an excitation wavelength of 280 nm. The percentage of apoptotic nuclei was determined by counting more than 500 cells from at least 3 separate determinations.

The adhering melanoma and mouse fibroblast cell lines were grown to the desired cell density in 8-well chamber slides 2 days before each experiment. At the start of the experiment, the medium was exchanged for fresh medium containing inhibitor. The cells were subsequently treated as described above.

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2.3. Purification of cellular DNA

Cells were harvested by centrifugation and the pellet was immediately lysed in a medium containing 1% (w/v) SDS, 0.5 mg/ml proteinase K (Boehringer/Mannheim, Germany) and 10 mM Tris-HCl (pH 8.0) and incubated for 12 h at 37°C. Protein was subsequently precipitated by addition of 5 volumes saturated NaCl solution. The DNA in the supernatant was precipitated with 3 volumes of ethanol at −20°C, taken up in 10 mM Tris-HCl (pH 8.5) and stored at −70°C.

2.4. Labelling and visualisation of fragmented DNA

The amount of DNA was determined in each sample with the fluorescent dye Hoechst 33258 [10]. Subsequently 0.5–1.0 μg DNA was examined for DNA fragmentation as described by Rösl [9]. An *Eco*RI–*Hin*dIII digest of λ phage DNA was used as a standard.

2.5. Immunoblotting

1×10^7 cells were suspended in lysis buffer (1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2% aprotinin, 2 mM EDTA and 10 mM Tris-HCl, pH 8.0), left on ice for 15 min and subsequently centrifuged for 10 min in an Eppendorf centrifuge. An aliquot of the supernatant containing 50–100 μg protein was boiled in sample buffer [11] for 5 min. The proteins were separated on a 15% SDS-polyacrylamide gel as described by Laemmli [11] and electrotransferred (Sartoblot, 30 V for 1–2 h) to a polyvinylidenefluoride membrane (Biorad, Hercules, CA). The filters were blocked over night at 4 °C with blocking buffer (5% non-fat milk (Blotto) plus 2% foetal calf serum in phosphate-buffered saline). The filters were incubated for 1 h with a 1:1 dilution of the supernatant of hybridoma cell line Bcl-2/100 (kindly provided by David Mason (Oxford) [12]) in blocking buffer, subsequently incubated with a 1:2,000 dilution of sheep anti-mouse alkaline phosphatase conjugate (Silenus, Hawthorn, Australia) in blocking buffer for 1 h and stained for alkaline phosphatase activity as described in [13]. Between each step the filters were washed three times with phosphate buffered saline containing 0.05% Tween-20 for 10 min each.

3. Results

Several different cell lines were cultured in complete medium in the presence of the mitochondrial respiratory chain inhibitors rotenone (final concentration 5 μg/ml, complex I inhibitor) or antimycin A (final concentration 5 μg/ml, complex III inhibitor) or the mitochondrial ATP synthase inhibitor oligomycin (final concentration 10 μg/ml) for a period of 18 h and analysed for morphologically detectable apoptosis. Since the inhibitors were dissolved in ethanol control incubations with an appropriate amount of ethanol (final concentration 0.5% (v/v)) were carried out. The culture medium was always supplemented with pyruvate [14] and uridine [15] in order to prevent direct necrotic cell death through a decline in intracellular ATP-levels and to prevent a reduction of

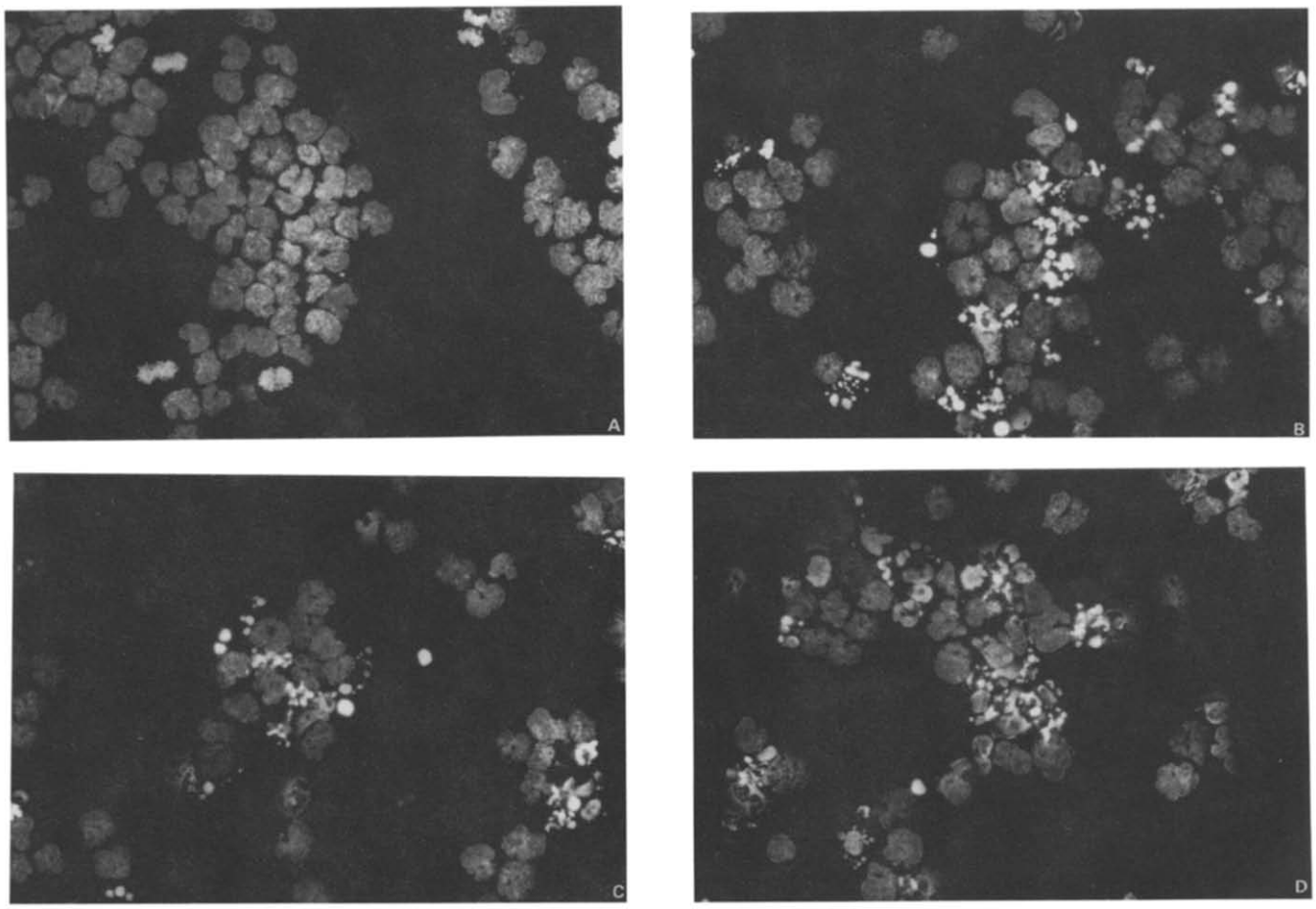

Fig. 1. The effect of mitochondrial respiratory chain inhibitors on the nuclear morphology of lymphoblastoid cells. Human lymphoblastoid BM 13674 cells were incubated with ethanolic solutions of 5 μg/ml rotenone (B), 5 μg/ml antimycin A (C) or with 10 μg/ml of the ATP-synthase inhibitor oligomycin (D) for a period of 12 h. The control (A) contained only ethanol. Cells were cytospun onto a glass slide, fixed and nuclear DNA was stained with 4,6-diamidino-2-phenylindole and subsequently analysed under a fluorescence microscope.

pyrimidine biosynthesis by inhibition of the mitochondrial dihydroorotate dehydrogenase complex, respectively.

Six lymphoblastoid cell lines (Daudi, BL-29, Namalwa ρ^0, Namalwa ρ^+, BM 13674 and BMX 13674), one myeloid leukemia cell line (K 562), three melanoma cell lines (MEL 3, MEL 28 and MM 96) and one mouse fibroblast cell line (L 929) were analysed for the induction of apoptosis by the inhibitors of mitochondrial energy metabolism. The fluorescence microscope pictures in Fig. 1 show the nuclear morphology of a representative lymphoblastoid cell line (BM 13674) incubated for 12 h either with ethanol alone (Fig. 1A) or with rotenone (Fig. 1B), antimycin A (Fig. 1C) or oligomycin (Fig. 1D). In each incubation containing an inhibitor a large percentage of the cell nuclei showed the characteristic condensed and fragmented appearance of apoptotic cells. In control incubations only a very small, albeit clearly recognisable, percentage of the nuclei showed the morphological characteristic of apoptosis (c.f. Table 1). DNA extracted from BM 13674 lymphoblastoid cells which had been incubated with rotenone, antimycin A or oligomycin for 12 h displayed the typical DNA-ladder of multiples of 200 basepairs characteristic for apoptotic cells (Fig. 2, lanes 1 to 3). Similar results (not shown) were obtained with the other cell lines investigated.

The quantisation of the effect of the inhibitors on nine other cell lines (lymphoblastoid, melanoma, myeloid leukemia and mouse fibroblast) is summarised in Table 1; all of the nine cell lines cell lines investigated were induced to undergo apoptosis in the presence of the inhibitors of mitochondrial energy production, as judged from both the morphological appearance of the nuclei and the characteristic DNA-laddering pattern.

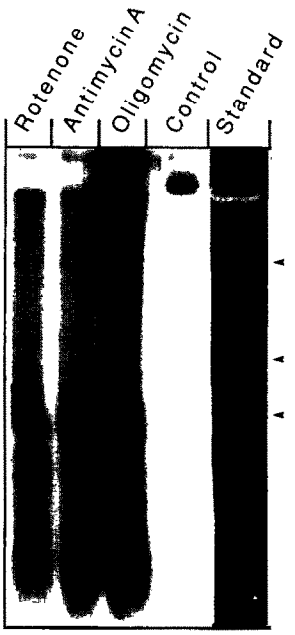

Fig. 2. Agarose gel electrophoresis of DNA extracted from lymphoblastoid cells. Human lymphoblastoid BM 13674 cells were incubated with 5 μg/ml rotenone (lane 1), 5 μg/ml antimycin A (lane 2) or with 10 μg/ml of the ATP-synthase inhibitor oligomycin (lane 3) over a time period of 18 h. To the control only ethanol was added (lane 4). Subsequently DNA was extracted, labelled and analysed in an agarose gel as described in Section 2. In lane 5 an *Eco*RI–*Hin*dIII digest of λ phage DNA was loaded as a molecular weight standard, arrows indicate DNA fragments of 5.148, 2.023 and 1.375 kb.

In our laboratory we have established a Namalwa ρ^0

Table 1
Apoptosis induced by mitochondrial respiratory chain inhibitors in various cultured cell lines.

Cell line	Bcl-2 expression	Apoptotic cells (%)			
		Inhibitors			
		none	Antimycin A 5 μg/ml	Rotenone 5 μg/ml	Oligomycin 10 μg/ml
BM13674	–	4 (9)	28 (2)	37 (6)	51 (2)
BMX13674	+	2 (8)	28 (2)	33 (5)	36 (2)
Namalwa ρ^+	+	2 (7)	22 (3)	28 (4)	n.d.*
Namalwa ρ^0	+	5 (2)	7 (2)	7 (2)	n.d.
BL-29	–	4 (5)	33 (2)	40 (3)	34 (2)
Daudi	–	4 (7)	26 (3)	29 (5)	n.d.
K 562	+	3 (3)	27 (1)	32 (2)	n.d.
MEL 3	+	1 (5)	n.d.	24 (3)	n.d.
MEL 28	+	3 (3)	n.d.	31 (3)	n.d.
MM 96	+	3 (4)	n.d.	36 (3)	n.d.
L 929	+	2 (3)	56 (1)	37 (1)	n.d.

Cells were cultured in the presence or absence of the mitochondrial respiratory chain inhibitors rotenone or antimycin A or in the presence of the mitochondrial ATP-synthase inhibitor oligomycin over a time period of 18 h and analysed for morphologically detectable apoptosis, as described in Section 2. The expression of Bcl-2 in the cell lines was investigated by Western blot analysis with a monoclonal antibody raised against Bcl-2, as shown in Fig. 3. The percentage of cells undergoing apoptosis are the mean values of the number of experiments shown in between parentheses.
* n.d., not done

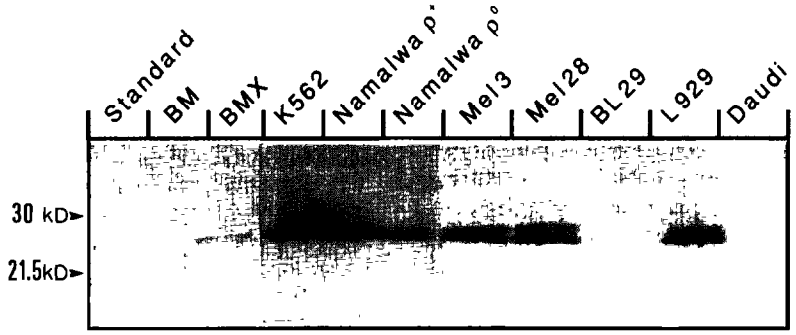

Fig. 3. Western blot analysis of various cultured cell lines with a monoclonal antibody against Bcl-2. Lymphoblastoid cells were harvested, lysed and boiled in sample buffer as described in Section 2. 100 μg of protein was loaded in each well of a 15% SDS-polyacrylamide gel, the proteins were separated and electrotransferred to a polyvinylidenefluoride membrane which was subsequently incubated with a monoclonal antibody against Bcl-2. Cross reacting antibodies were visualised with a sheep anti-mouse alkaline phosphatase conjugate. Lanes 1 to 11 contain protein from molecular weight standard, BM, BMX, K562, Namalwa Namalwa ρ^+, Namalwa ρ^0, Mel 3, Mel 28, BL-29, L929 and Daudi cells, respectively.

cell line, which was derived from Namalwa ρ^+ lymphoblastoid cells by treatment with ethidium bromide to ablate mitochondrial DNA [16]. These ρ^0 cells therefore lack a functional mitochondrial respiratory chain [16]. Our observation that these cells did not undergo apoptosis upon incubation with the mitochondrial respiratory chain inhibitors, in contrast to the parent Namalwa ρ^+ cell line (Table 1), clearly demonstrates the involvement of mitochondrial respiratory chain function in the induction of apoptosis as described in this communication.

The proto-oncogene product Bcl-2 has been found to prevent apoptotic cell death in many types of cells both in vitro and in vivo [8]. Western blot analysis of our cell lines with a monoclonal antibody raised against Bcl-2 indicated that except for three lymphoblastoid cell lines, which expressed no detectable (Daudi and BL 29 (Fig. 3 lanes 9 and 11, respectively)) or only very low (BM 13674 (Fig. 3, lane 2)) amounts of Bcl-2, all other cell lines strongly expressed Bcl-2 (Fig. 3 and Table 1). In accordance with the results from other groups, cell lines that expressed Bcl-2 were found to be resistant to the induction of apoptosis by UV-irradiation, serum removal or treatment with glucocorticoids (results not shown). Since, with the exception of the Namalwa ρ^0 cell line, all cell lines described in this paper were found to undergo apoptosis upon incubation with rotenone, antimycin A or oligomycin, we conclude that apoptosis induced by inhibition of mitochondrial energy metabolism proceeds independently of the level of expression of Bcl-2.

4. Discussion

We have shown in this communication that inhibition of the mitochondrial respiratory chain or mitochondrial ATP-synthase can elicit an apoptotic response within 12–18 h. We have further shown that this occurs in a variety of cultured eukaryotic cells, suggesting that we are dealing with a general phenomenon. Our observation that these mitochondrial inhibitors do not induce apoptosis in ρ^0 cells that are depleted of a functional mitochondrial respiratory chain shows that the induction of apoptosis described herein is due to inhibition of mitochondrial respiratory chain function and not to some unspecific side effects of the inhibitors we used. The apoptosis inducing effect of such widely used mitochondrial respiratory chain inhibitors as rotenone and antimycin A clearly has important consequences for studies using these compounds in longer-term experiments with whole cells.

In agreement with our observations, tributyltin, an alkylating agent which is described also to inhibit mitochondrial oxidative phosphorylation [17], was previously found to induce apoptosis in rat thymocytes [18]; however these authors did not infer a role for mitochondria. On the other hand Jacobsen et al. [19] recently concluded that mitochondria are not involved in apoptosis, based on the observation that ρ^0 cells, which contain no mitochondrial DNA and are therefore deficient in mitochondrial respiratory chain function, could still undergo apoptosis upon incubation with a protein kinase C inhibitor. However, we now clearly demonstrate that mitochondrial energy metabolism and apoptosis can be interlinked in at least one pathway, since acute inhibition of mitochondrial oxidative phosphorylation by various agents induces apoptosis in a wide variety of mammalian cell lines. The difference between these two studies may be explained by the way ρ^0 cells are generated. In order to obtain a complete depletion of mitochondrial DNA, cells are incubated with ethidium bromide over a time course of several weeks. We suggest that during this period the cells metabolically adapt to the slowly decreasing mitochondrial energy supply by upregulating compensatory enzyme systems such as glycolysis [14] and the plasma membrane oxidase system [20] (Martinus, R.D., unpublished results). Inhibition of mitochondrial respiratory chain activity by mitochondrial poisons, however, is immediate and directly interferes with normal mitochondrial energy metabolism. This lead

us to the suggestion that such an acute metabolic insult triggers an apoptotic response.

In this paper we also have shown that presence of Bcl-2 does not prevent the apoptotic response of cells incubated with mitochondrial respiratory chain inhibitors (Fig. 3 and Table 1), although the level of expression of Bcl-2 in these cell lines prevented apoptosis induced by growth factor removal or UV-irradiation (data not shown). Again, Jacobsen et al. [19] found that apoptosis induced in ρ^0 cells by serum removal or by incubation with the protein kinase C inhibitor staurosporin was abrogated by Bcl-2 expression. These results suggest that at least two separate pathways can elicit an apoptotic response within one cell type, one of which is Bcl-2 sensitive and the other Bcl-2 insensitive. The separation and comparison of these pathways will be the topic of forthcoming papers.

We have previously suggested that a progressive accumulation of mitochondrial DNA-mutations with time makes an important contribution to the ageing process [7,21]. Furthermore the accumulation of mitochondrial DNA-mutations in cells of aged subjects impairs mitochondrial electron transport chain activity which will eventually lead to an inability to maintain the redox balance in the cell and/or an increased production of oxygen radicals. Based on our observation that interference with mitochondrial energy metabolism induces apoptosis in a variety of cultured cells we now propose that cells with impaired mitochondrial energy metabolism due to mitochondrial DNA mutations may reach a bioenergetic threshold triggering apoptosis and thus these cells will be selectively removed from the tissue. Such a process would limit the extent of the progressive accumulation of mitochondrial DNA deletions observed in tissues of aged subjects. We suggest that the selective removal of bioenergetically compromised cells via apoptosis may be an important aspect of the pathology of age-associated diseases.

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