mtDNA-depleted U937 cells are sensitive to TNF and Fas-mediated cytototxicity

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Abstract **It has been proposed that TNF cytotoxicity is mediated by reactive oxygen intermediates generated by uncoupling of mitochondrial respiration. We have compared sensitive U937 cells and derived cell lines depleted of mtDNA for their ability to undergo TNF- and Fas-induced apoptosis. Cells lacking around 98% of mtDNA were still sensitive to TNF-induced apoptosis. U937 cells devoid of mtDNA (U937-ρ°) were resistant to TNF, but this was due to the loss of its 55 kDa receptor. U937-ρ° cells were also resistant to docosahexaenoic acid, which causes U937 cell death by lipid peroxidation. These cells were sensitive to anti-Fas toxicity. The results indicate that TNF and Fas-induced toxicity occurs by a mechanism mostly independent of mitochondrial free radical generation.**

Key words: Polyunsaturated fatty acid; Apoptosis; Tumor necrosis factor; Fas; mtDNA

1. Introduction

The 55 kDa TNF-receptor and Fas/APO-1 (CD95) belong to a family of surface receptors that also include the NGF receptor, CD40, CD27, 4-1BB and OX40 [1]. While most receptors of this family are implicated in proliferative processes, TNF receptor and Fas, when cross-linked by antibodies or their physiological ligands (TNF or the Fas ligand, respectively), frequently induce apoptosis [2–5]. TNF has a main role in inflammatory responses [4] while Fas-mediated apoptosis participates in the effector function of cytotoxic T-lymphocytes [6,7], as well as in the auto-elimination of activated mature T-cells, and hence, in peripheral tolerance [5,8,9].

Oxidative damage has been implicated in some forms of TNF-mediated cytotoxicity [4]. Between 1% and 5% of the oxygen consumed by mitochondria is converted to damaging free radicals [10] and, at least in TNF-induced death of L929 cells, the lack of mitochondrial function abolish TNF toxicity [11]. However, it should be noted that TNF induces necrotic death in L929 cells, while in U937 cells it induces apoptosis [12]. Previous work of our group showed that docosahexaenoic acid (DHA) enhanced TNF toxicity on U937 cells by increasing the amount of lipid peroxidation products. However, although this potentiating effect was prevented by antioxidant treatments, TNF cytotoxicity itself was not abolished [13].

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Abbreviations: TNF, tumor necrosis factor; anti-Fas, cytotoxic monoclonal anti-Fas antiboby; DHA, docosahexaenoic acid; MTT, 3-[4,5-dimethylthiazol]-2,5-diphenyl-tetrazolium bromide.

The mechanism by which Fas ligation triggers apoptosis, as well as a general biochemical mechanism for the apoptosis process itself, are unknown [5]. Some reports have suggested, however, the lack of implication of oxidative damage in cytotoxicity induced with anti-Fas, using antioxidants [14,15] or anaerobic conditions [16].

In the present work, we have studied the effect of TNF and anti-Fas on U937 cells and in derived cell lines depleted of mtDNA by prolonged exposure to low doses of ethidium bromide. Results indicate that impairment of mitochondrial function and lipid peroxidation do not play a key role in the apoptosis induced by both molecules in human myeloid cells.

2. Materials and methods

2.1. Materials

4,7,10,13,16,19-Docosahexaenoic acid (DHA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethylsulfoxide, ethidium bromide (EtBr), amytal, rotenone and thenoyltrifluoroacetone (TTFA) were products from Sigma (Spain). Tumor necrosis factor-α (TNF) was kindly provided by The National Biological Standards Board (UK). Mouse monoclonal IgM antibody (clone CH-11), specific for human Fas [17], was from UBI (Lake Placid, USA).

2.2. Generation of U937 cells without mtDNA (U937-ρ°)

Human promonocytic leukemia U937 cells were kindly provided by Dr. Jeremy Brock (Department of Immunology, University of Glasgow, UK). Cells were routinely cultured at 37°C, in a humidified atmosphere containing 5% CO_2, using RPMI 1640 medium plus 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (hereafter complete medium). Selective elimination of mtDNA was achieved by long-term exposure to low concentrations (50 ng/ml) of EtBr essentially as described by King and Attardi [18]. Selection and culture medium of U937-ρ° cells was also supplemented with glucose (4.5 mg/ml), sodium piruvate (0.1 mg/ml) and uridine (50 μg/ml). After 15 weeks of incubation with EtBr, the sensitivity of cells to TNF was analyzed by the MTT-reduction method and their mtDNA content by PCR as described below. Treatment with EtBr was prolonged for another 20 weeks. The extent of cell differentiation was measured by the nitro-blue tetrazolium (NBT) reduction method [19].

2.3. mtDNA analysis

Cells were harvested and the DNA was extracted by the phenol/chloroform method, as described elsewhere [20]. mtDNA amplification was performed by PCR, using the following set of primers: *np 3148–3167 (forward)* 5′-CCTACTTCACAAAGCGCCTT-3′ and *np 3531–3550 (reverse)* 5′-CGATGGTGAGAGCTAAGGTC-3′ (nucleotide numbering according to [20]). The 403 bp amplified fragment was analyzed by electrophoresis on 2% agarose. The bands were visualized under UV light in the presence of EtBr and photographed.

2.4. Cell proliferation assays

U937 and U937-ρ° cells were seeded in flat-bottomed, 96-well plates at an initial density of 2×10^5 cells/ml (100 μl/well), and cultured for 48–72 h in control medium or medium containing different concentrations of one or several of the following molecules: DHA (60–120 μM),

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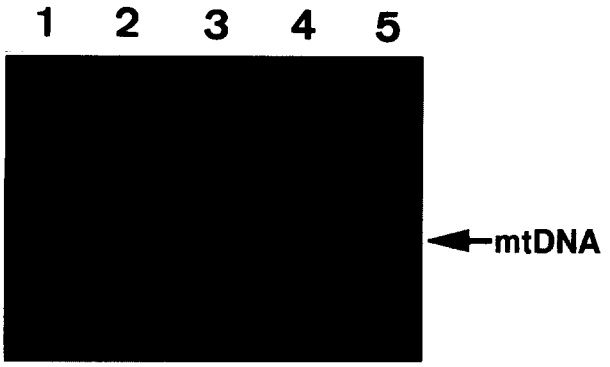

Fig. 1. Agarose gel electrophoresis of PCR-amplified products from mtDNA. Total DNA was purified and amplified using specific primers as described in section 2. 1 = control (no template); 2 = DNA molecular weight markers; 3 = amplified products from U937-ρ° cells; 4 = amplified products from U937 cells; 5 = amplified products from U937 cells treated with 50 ng/ml EtBr for 15 weeks.

TNF (5–100 U/ml), anti-Fas (500 ng/ml). In the latter case, wells were coated with anti-Fas overnight prior to the addition of the cell suspension. DHA was added to the medium bound to bovine albumin as described in detail in [21]. Mitochondrial electron-chain uncouplers, dissolved in ethanol (amytal, TTFA) or dimethyl sulfoxide (rotenone), were added to cultures 1h before the addition of TNF. Final concentration of organic solvents was 0.1% and did not affect cell growth. Cultures containing the same amounts of drugs, without TNF, were used as controls. Cell proliferation was determined by a modification of the MTT-reduction method, as previously described [21,22]. U937-ρ° cells, in spite of the lack of functional mitochondria, can also reduce MTT [23].

2.5. Flow cytometry analysis

The expression of Fas and the 55 kDa TNF-receptor on the membrane of U937 and U937-ρ° cells was analysed by flow cytometry. For TNF-receptor expression, cells were incubated on ice for 1 h with the mouse anti-55 kDa TNF-receptor antibody H398 (Bender Medsystems, Germany) and revealed with a FITC-labeled goat anti-mouse IgG (Caltag, San Francisco, CA). For Fas expression, cells were stained for 1 h with the CH-11 anti-Fas, and revealed with a FITC-labeled goat anti-mouse IgM (Sigma). After fixing in 1% paraformaldehyde, 5,000 cells were analysed by flow cytometry using a FACScan (Becton and Dickinson) and Lysis-II software.

3. Results

3.1. Characterization of U937-ρ° cells

To test the involvement of mitochondrial function in apoptotic processes, U937 cells were depleted of mtDNA by continuous exposure to 50 ng/ml EtBr. After an initial period of reduced cell growth, cells regained progressively the proliferation capacity and by the 15th week of treatment growth rate was only slighty lower than that of wild-type U937 cells. They exhibited a normal morphology by light microscopy (data not shown). The mtDNA content in these cells was reduced by around 98% when compared with normal U937 cells (Fig. 1). After further culture in the presence of EtBr for another 20 weeks, cells were entirely devoid of mtDNA (Fig. 1) and were designated as U937-ρ°. These cells showed some signs of cell differentiation, such as adhesion to plastic (Fig. 2D) and superoxide generation (31% of U937-ρ°cells were positive for NBT-staining). They were also critically dependent on anaerobic glucolysis for survival, since addition of the anti-metabolite 2-deoxyglucose (10 mM) to the culture medium caused a progressive cell death, whereas it had no effect on U937 cells (data not shown).

3.2. Effect of docosahexaenoic acid on growth of U937 and U937-ρ° cells

Polyunsaturated fatty acids, and especially DHA, are cytotoxic to human leukemic cell lines through the induction of lipid

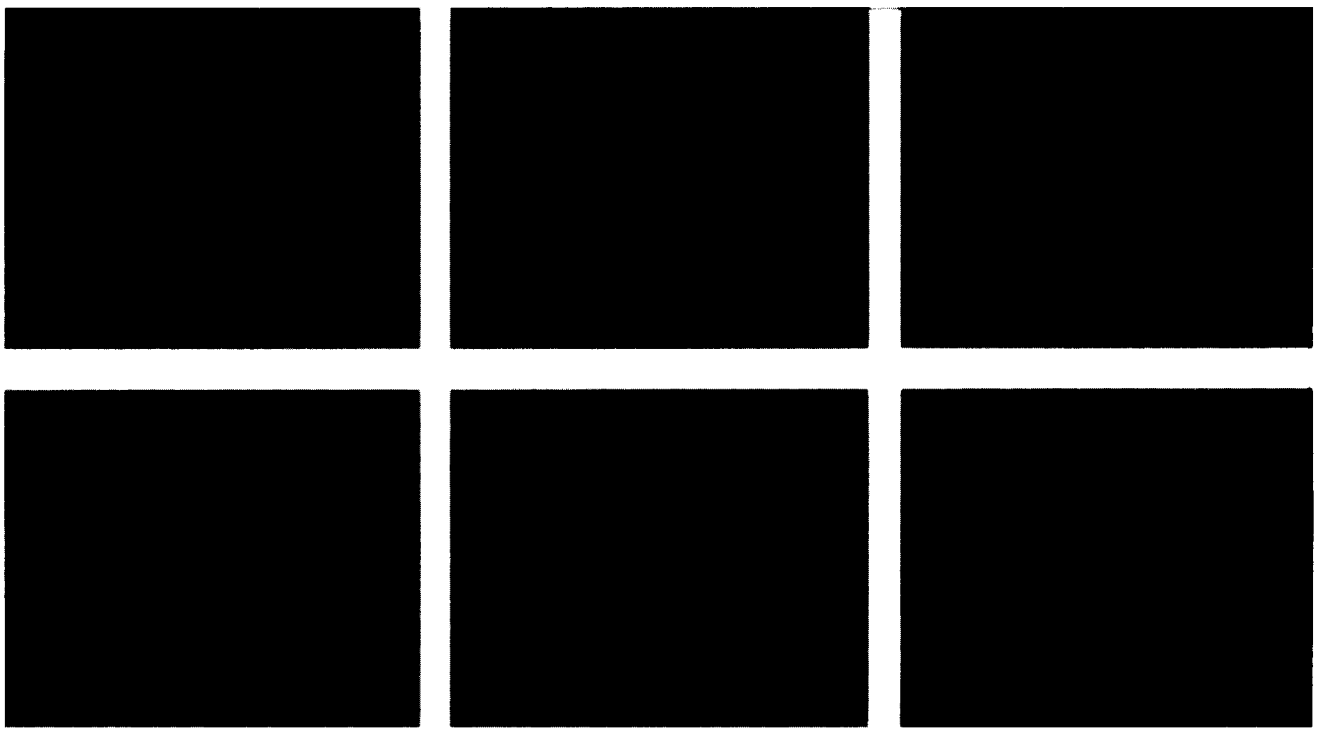

Fig. 2. Effect of incubation with TNF and DHA on growth of U937 (A–C) and U937-ρ° (D–F) cells. (A and D) Control cells. (B and E) Cells treated with 60 μM and 120 μM DHA, respectively, for 72 h. (C) Cells incubated with TNF (20 U/ml) for 72 h; and (F) with 100 U/ml TNF for 96 h.

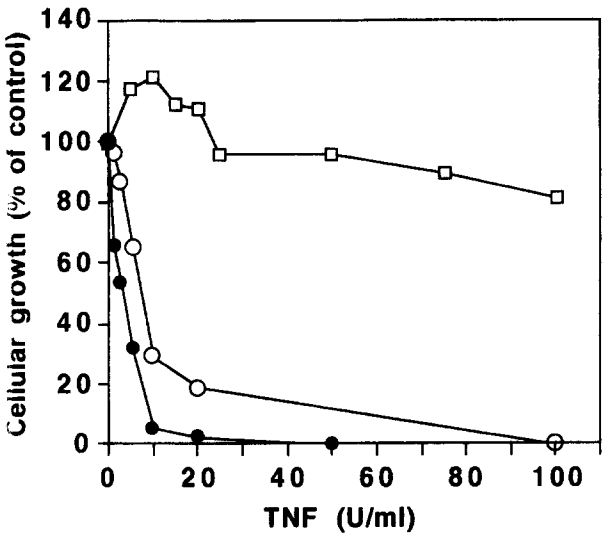

Fig. 3. Effect of TNF on proliferation of U937 cells (closed circles), U937 cells treated with EtBr for 15 weeks (open circles) and U937-ρ° cells (open squares). Cells were incubated in the presence of TNF for 72 h and cellular growth estimated by the MTT-reduction assay.

peroxidation processes [13,21]. Supplementation of U937 cells with 60 μM DHA for 72 h caused massive cell death (Fig. 2B). This effect was prevented by the simultaneous supplementation with 25 μM vitamin E ([13], and data not shown). However, U937-ρ° cells were completely insensitive to the toxic effect of DHA (up to 120 μM), which only induced a typical fusiform morphology in these cells (Fig. 2E).

3.3. Effect of TNF and anti-Fas on growth of U937 and U937-ρ° cells

U937 cells were highly sensitive to TNF-induced apoptosis (Figs. 2C and 3). Inhibitors of mitochondrial-chain complex I (amytal, rotenone) did not offer a significant protection while the complex II inhibitor TTFA partially, but significantly ($P < 0.005$, two-tailed, Student t-test), lowered TNF-induced toxicity on U937 cells (Fig. 4). Cells treated with EtBr for 15 weeks, containing around 2% of the amount of mtDNA present in wild type cells, were still sensitive to TNF cytotoxicity (Fig. 3) However, TNF did not inhibit but rather stimulated at low doses the growth of U937-ρ° cells (Fig. 3). After 96 h of incubation with 100 U/ml TNF, U937-ρ° cells appeared lengthened but no dead cells were observed in cultures (Fig. 2F). The expression of the cytotoxic 55 kDa TNF-receptor was analyzed in these cells by flow cytometry. Most U937 and U937 cells treated with EtBr for 15 weeks expressed similar amounts of 55 kDa TNF-receptor (mean fluorescence intensity 12.1 and 11.9, respectively), while less than 1% of U937-ρ° cells were specifically stained, indicating the loss of surface expression of this receptor.

Fas-mediated apoptosis was evaluated in wild-type or mtDNA-depleted U937 cells. Contrary to others [14,15], we did not use the inhibitor of transcription, actinomycin D, to potentiate anti-Fas toxicity on U937 cells, as treatment with actinomycin D alone was highly toxic for those cells, inducing a typical apoptotic process (data not shown). After 24 h incubation with anti-Fas, most of U937 cells died, while growth of U937-ρ° cells was only slightly depressed (Fig. 5). However, growth of U937-ρ° cells became progressively inhibited at longer times, reaching a 60% inhibition of cellular growth after 72–96 h of culture with anti-Fas. Flow cytometry analysis revealed no significant differences in Fas surface expression between U937 and U937-ρ° cells (data not shown).

4. Discussion

The currently proposed mechanism of signal transduction for the intracellular propagation of the cytotoxic signal of TNF is mainly based in the works of Fiers et al., carried out with the murine fibrosarcoma cell line L929 [2,4,11]. Essentially, this mechanism proposes that TNF-induced events interfere with normal electron flow in the mitochondria, leading to the generation of reactive oxygen intermediates. These oxygen radicals would cause cell damage by inducing membrane lipid peroxidation and triggering the program of cell death. According to this hypothesis, TNF cytotoxicity on L929 cells can be partially prevented by BHA, a synthetic lipid antioxidant, some types of iron chelators and inhibitors of mitochondrial electron transport complexes I and II [2]. Furthermore, L929 cells depleted of mtDNA are resistant to TNF-induced necrosis [11]. However, it is unclear if this mechanism could be extrapolated to cell types which undergo apoptosis in response to TNF, such as U937 promonocytic cells [12].

Our present results show that U937-ρ° cells, devoid of functional mitochondria, are resistant to the cytotoxic effects of DHA, a highly polyunsaturated fatty acid that induces lipid peroxidation and kills cells by necrosis [13,21]. These, as well as other data [10], support the implication of mitochondria in necrotic cell death induced by oxidative stress.

Contrary to that observed in L929 cells [2,4,11], treatment with mitochondrial inhibitors and reduction in the mtDNA content by around 98% did not prevent TNF toxicity in U937 cells. However, it should be noted that there was no correlation between the amount of mtDNA and the sensitivity to TNF among the different mtDNA defficient L929 subclones and no evaluation of the amount of TNF-receptors in wild-type and L929-ρ° cells was performed [11]. U937-ρ° cells were insensitive to TNF, but this cannot be attributed to the loss of mitochondrial function but to the complete down-modulation of the 55 kDa TNF receptor. The loss of the 55 kDa TNF-receptor

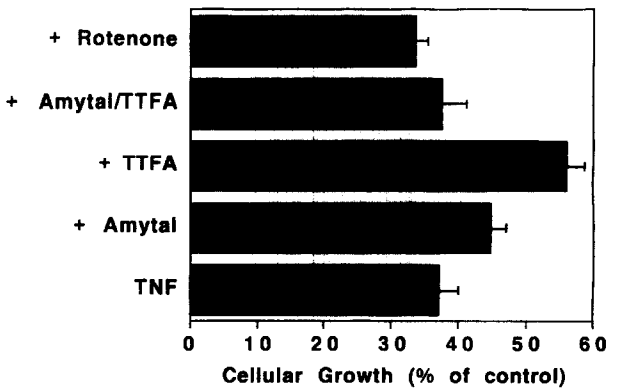

Fig. 4. Effect of mitochondrial chain inhibitors on TNF cytotoxicity. U937 cells were preincubated for 1 h with either amytal (200 μM), TTFA (25 μM) or rotenone (100 nM), as indicated. Then, TNF (10 U/ml) was added to the cultures and cell proliferation was determined by the MTT-reduction assay after 47 h of further incubation.

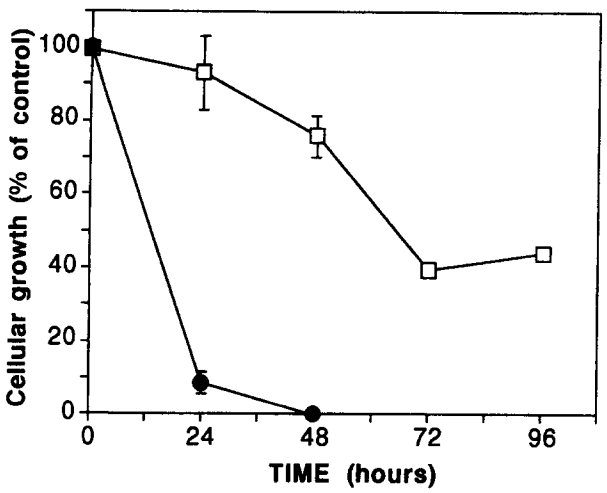

Fig. 5. Time-course effect of treatment with anti-Fas on growth of U937 (circles) and U937-ρ^0 cells (squares). Cells were incubated in 96-well plates pre-coated with anti-Fas antibody (500 ng/ml) for the times indicated. Cellular growth was estimated by the MTT-reduction assay.

occurs early during monocytic differentiation [24] and agrees with the partially differentiated phenotype of U937-ρ^0 cells.

The presence of a sequence homology in the cytoplasmic region of Fas and the 55 kDa TNF receptor necessary to induce cell death, the death domain, led to speculate that both receptors may trigger apoptosis by similar mechanisms [5,25]. However, other studies suggested that this was not the case, since some cell types expressed both molecules but were sensitive to TNF and insensitive to anti-Fas lysis, or vice versa [26]. In the present study we show that anti-Fas significantly inhibits the growth of U937-ρ^0 cells. This agrees with recent data on the lack of effect of antioxidants [14,15] or anaerobic conditions [16] on anti-Fas induced apoptosis. Nevertheless, we observe a significant difference in sensitivity to anti-Fas toxicity between wild-type and ρ^0 cells. These data suggest that, although mitochondrial free radical generation is not necessary per se for anti-Fas toxicity, there could be a contribution of this system to accelerate or potentiate it. Further studies are being conducted in our laboratory using activated T cell effectors that express the physiological Fas ligand on their surface to evaluate this question.

In summary, we demonstrate that mtDNA-depleted U937 cells are still sensitive to TNF and anti-Fas toxicity, indicating that mitochondrial generation of reactive oxygen intermediates is not the main component in the apoptotic processes initiated by those receptors. Other signalling pathways, like the activation of intracellular proteases, such as interleukin-1β converting enzyme (ICE), recently demonstrated to be involved in Fas- [27,28] and TNF- [27] induced apoptosis, could represent the necessary cytoplasmic event to trigger cell death. The activation of an sphingomyelinase that produces the lipid mediator ceramide has been described after both Fas [29] and TNF receptor engagement [30]. The connection between membrane receptor engagement and ICE activation is still unknown, although the ceramide-activated pathway could be a common candidate for both receptors. In this regard, we have observed that administration of synthetic or natural ceramide induces apoptosis in U937 and ρ^0 cells (unpublished data). Thus, ρ^0 cells, resistant to oxidative damage, posses the ceramide-activated apoptotic machinery intact and could be a suitable model to study this pathway.

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