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Mitochondria-targeted antioxidants do not prevent tumour necrosis factor-induced necrosis of L929 cells

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Abstract
Mitochondrial production of reactive oxygen species (ROS) is widely reported as a central effector during TNF-induced necrosis. The effect of a family of mitochondria-targeted antioxidants on TNF-induced necrosis of L929 cells was studied. While the commonly used lipid–soluble antioxidant BHA effectively protected cells from TNF-induced necrosis, the mitochondria-targeted antioxidants $MitoQ_3$, $MitoQ_5$, $MitoQ_{10}$ and MitoPBN had no effect on TNF-induced necrosis. Since BHA also acts as an uncoupler of mitochondrial membrane potential, two additional uncouplers were tested. FCCP and CCCP both provided dose-dependent inhibition of TNF-induced necrosis. In conclusion, the generation of mitochondrial ROS may not be necessary for TNF-induced necrosis. Instead, these results suggest alternative mitochondrial functions, such as a respiration-dependent process, are critical for necrotic death.

Keywords: *Tumour necrosis factor, reactive oxygen species, mitochondria, antioxidants, L929 cells, necrosis*

Abbreviations: *BHA, butylated hydroxyanisole; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; FCCP, carbonyl cyanide* p*-trifluoromethoxyphenylhydrazone; H_2O_2, hydrogen peroxide; MitoPBN, [4-[4-[[(1,1-dimethylethyl)oxidoimino]methyl]-phenoxy]butyl]triphenylphosphonium bromide; MitoQ, mixture of mito-quinol [10-(3,6-dihydroxy-4,5-dimethoxy-2-methyl-phenyl)decyl]triphenylphosphonium bromide and mito-quinone [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)decyl]triphenylphosphonium bromide; MitoVit E, [2-(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)ethyl]triphenylphosphonium bromide; NAC, N-acetylcysteine; PCD, programmed cell death; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; TNF, tumour necrosis factor α; TPMP, methyltriphenylphosphonium cation*

Introduction

TNF is a pleiotropic cytokine involved in a broad range of physiological and pathological processes. TNF regulates pro-survival and pro-apoptotic pathways through the activation of signaling pathways mediated by MAP kinases, nuclear factor-κB, phospholipases and caspases [1,2]. TNF induces cell death that is either apoptotic (caspase-dependent) or necrotic (caspase-independent) [1]. Signaling to cell death involves the recruitment of TRADD (TNF Receptor Associated Death Domain protein) to the cytosolic death domain of TNFR1. TRADD in turn recruits either Fas Associated Death Domain protein (FADD) or Receptor Interacting Protein (RIP) to this complex. Recruitment of FADD leads to activation of

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the caspase cascade and apoptotic cell death in a reasonably well understood process. Recruitment of RIP to TRADD is a necessary step in the necrotic pathway [3–5], but very little is known about how RIP then signals to necrotic cell death.

Much attention has been paid to the role of reactive oxygen species (ROS) in the signal transduction pathways activated by TNF and TNF is now widely regarded as a cytokine that uses redox regulation as part of its signal transduction machinery [1,4,6]. Investigation of TNF-induced death of the murine fibrosarcoma L929 cell line provided the first evidence that a receptor-mediated cell death might involve and require the intracellular production of ROS [7]. It is now well established that L929 cells undergo a caspase-independent necrotic programmed cell death (PCD) when treated with TNF and this has become a major paradigm for investigating the mechanisms underlying TNF-induced necrosis [8]. Cellular ROS generation can occur at a number of sites including the mitochondrial respiratory chain, plasma membrane NADPH oxidase and lipoxygenase, and TNF treatment has been reported to increase ROS production from all three sites [1,9–11]. A large number of studies have implicated mitochondrial ROS in TNF-induced necrosis of L929 cells. Early studies demonstrated that inhibition of the mitochondrial respiratory chain complexes I and II prevented TNF-induced necrosis, whereas inhibition of complex III augmented death. This suggested that TNF stimulates ROS generation between complex I/II and complex III and that ROS production is a critical effector of TNF-induced necrosis [7]. Subsequently, the use of radical scavengers, antioxidants, anaerobic growth conditions and genetic deficiencies in mitochondrial replication have further implicated ROS, and specifically mitochondrial ROS, in the necrotic signalling pathway [1,6,7,12–14]. However, many of these results can be interpreted differently. For example, differences in the efficacy of various free radical scavengers, while not easily being explained by differences in reactivity, may be due to them acting by non-antioxidant mechanisms. Furthermore, a number of workers have failed to find evidence of mitochondrial ROS production following TNF treatment of L929 cells [11,15]. Nevertheless, the pervading view in the literature is that mitochondrial ROS production is causally related to TNF-induced necrosis of L929 cells.

It is currently difficult to accurately measure mitochondrial-specific ROS production. The fluorescent probes most commonly used to assay cellular ROS production are of questionable value, being generally non-specific and often reflecting other cellular changes, such as cytochrome *c* release, rather than an absolute increase in cellular ROS levels [16–18]. Therefore, we have re-addressed the specific role of mitochondrial ROS in TNF-induced necrosis of L929 cells making use a series of mitochondria-targeted derivatives of the antioxidant ubiquinol (MitoQ) and the spin trap phenyl-*N*-*tert*-butylnitrone (MitoPBN). MitoQ is the best characterized of the mitochondria-targeted reagents. Within mitochondria, MitoQ is reduced by complex II of the respiratory chain to its active ubiquinol form. In blocking oxidative damage the ubiquinol is oxidized to a ubiquinone, which is then reduced back to the active ubiquinol [19]. This selective accumulation and continual recycling by mitochondria makes MitoQ several hundred-fold more potent at preventing mitochondrial oxidative damage than antioxidants that are not accumulated in the matrix. The original MitoQ (now $MitoQ_{10}$) was synthesized with a 10-carbon linker chain between the antioxidant moiety and the targeting cation. Subsequently, a series of MitoQ's with different chain lengths have been developed, including $MitoQ_3$ and $MitoQ_5$, with three and five-carbon linker chains, respectively [20]. In contrast, the targeted spin trap MitoPBN reacts rapidly with carbon-centred radicals but is unreactive with superoxide and most lipid peroxidation intermediates [21]. Surprisingly, these reagents did not attenuate TNF-induced necrosis, implying that mitochondrial ROS are not a critical modulator of this process. Instead, pharmacological doses of mitochondrial uncouplers were effective inhibitors of cell death.

Materials and methods

Cells and reagents

The murine fibrosarcoma cell line L929 (obtained from ATCC) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 1% glutamine, 4.5 g/l glucose, 100 units/ml penicillin and 100 μg/ml streptomycin. Recombinant murine TNF was from R&D Systems Inc, Minneapalis, MN, USA. The mitochondria-targeted antioxidants $MitoQ_3$, $MitoQ_5$, $MitoQ_{10}$ and MitoPBN were synthesized as described previously [20,21]. Other reagents were obtained from Sigma-Aldrich, New Zealand.

Analysis of cell death

Cell death induced by TNF was assayed by determining plasma membrane integrity using the membrane impermeant dye propidium iodide (PI). L929 cells were seeded at 1×10^5 cells/well in a 24 well plate and allowed to adhere for 24 h. Cells were treated with 5 ng/ml mTNF in the presence or absence of antioxidants or mitochondrial uncouplers. After varying incubation times the cells were harvested with trypsin, washed and stained with PI (2 μg/ml in PBS). Analysis was performed on a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) with CellQuest Pro software. PI stained cells were defined as necrotic.

Statistics

ANOVA with Tukey–Kramer post-test was performed using GraphPad InStat version 3.0b for Macintosh (GraphPad Software, San Diego, CA). A p-value $<$ 0.05 was considered to be statistically significant.

Results

TNF induces necrosis in L929 cells which is inhibited by BHA

Conflicting data on the involvement of mitochondrial ROS in TNF-induced necrosis of L929 cells may be due to clonal variations in the L929 cells used. The majority of studies supporting such a role for mitochondrial ROS use L929 cells that are susceptible to TNF on its own. Reports that failed to find a role for mitochondrial ROS treated cells with TNF in the presence of a protein synthesis inhibitor did not clarify whether the cells were dying by necrosis or apoptosis [22,23]. We confirmed that TNF alone caused a dose-dependent cell death of our L929 cells with morphological characteristics of necrosis (cell swelling, no chromatin condensation), which was inhibited by the lipid–soluble antioxidant BHA (Figure 1). This is consistent with previous reports that claim to demonstrate a role of mitochondrial ROS in TNF-induced necrosis [1] and so the L929 cells used in this study are a valid model for further investigation of this question.

Mitochondria-targeted antioxidants do not prevent TNF-induced necrosis

To directly determine whether the protective effect of BHA was due to its antioxidant activity in mitochondria, we used the mitochondria-targeted derivatives of ubiquinol ($MitoQ_3$, $MitoQ_5$, $MitoQ_{10}$) and PBN (MitoPBN), with the targeting cation TPMP as the control. None of these compounds showed any cytotoxicity at concentrations up to 50 μM and all subsequent experiments were performed using concentrations up to 10 μM. There was no significant protection against TNF-induced death in L929 cells treated with increasing concentrations of $MitoQ_3$, $MitoQ_5$ or $MitoQ_{10}$, MitoPBN or TPMP (Figure 2(A)). To determine whether these compounds could delay death a time course experiment was carried out (Figure 2(B)). TNF caused a slight increase in PI-labeled cells at 6 h, with a significant increase in PI labeling by 12 h ($p < 0.001$). TNF-induced death was not significantly altered by any of the mitochondria-targeted compounds at any time. In contrast, BHA inhibited cell death at all time points. Overall these data demonstrate that mitochondrial production of ROS is not required for execution of the necrotic death programme in L929 cells.

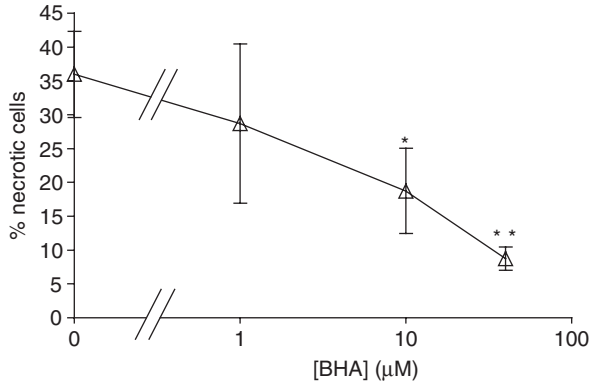

Figure 1. BHA inhibits TNF-induced necrosis. L929 cells were incubated with 5 ng/ml mTNF in the presence of the antioxidant BHA. After 20 h cells were stained with PI and analysed by flow cytometry. * $p < 0.05$, ** $p < 0.01$ compared with 0 μM BHA. Data represent means ± SD ($n = 4$).

TNF-induced necrosis is inhibited by uncouplers of mitochondrial respiration

We next sought an alternative explanation for the efficacy of BHA. In addition to its antioxidant activity, BHA is an effective mitochondrial uncoupler at the concentrations used to inhibit necrosis [24]. Therefore, we compared the effectiveness of BHA with two additional mitochondrial uncouplers, FCCP and CCCP (Figure 3). FCCP and CCCP significantly inhibited TNF-induced necrosis in a dose-dependent manner similar to BHA.

Discussion

Caspase-independent PCD occurs as part of physiological processes activated by TNF, including hyperacute shock and antiviral responses [3,25,26]. Furthermore, although much has been made of therapeutic opportunities for apoptosis inhibition, it is becoming apparent that inhibition of apoptosis often results in cell death by a caspase-independent pathway. Therefore, understanding the signalling pathways leading to caspase-independent cell death is of increasing interest. L929 cell culture is the best characterized model of TNF-induced caspase-independent PCD and in recent years significant progress has been made towards understanding the immediate post-receptor events that enable TNF to induce a PCD with a necrotic phenotype [3–5].

ROS are widely regarded as an important second messenger for TNF signalling and in particular ROS derived from the mitochondrial respiratory chain have been strongly implicated as a critical factor in TNF-induced necrosis. Consistent with these previous reports, BHA was an effective dose-dependent inhibitor of TNF-induced necrosis. However, the mitochondria-targeted antioxidants and spin trap used in this study did not prevent cell death. As we and

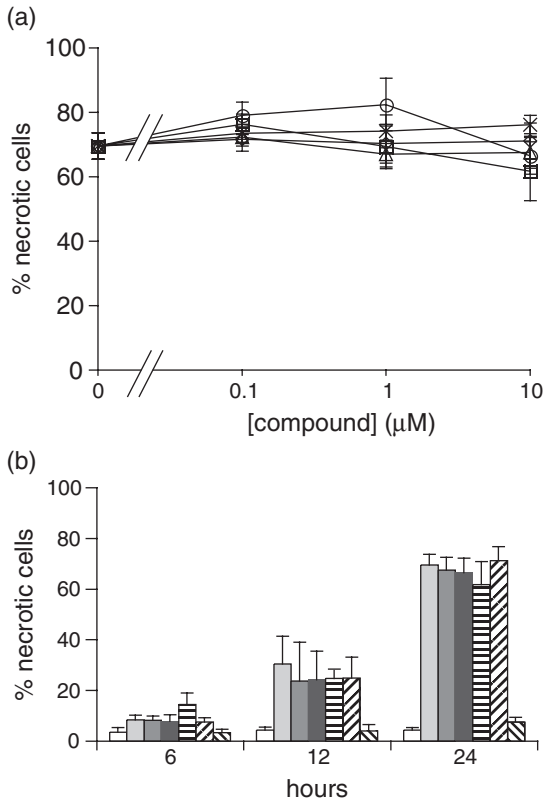

Figure 2. Mitochondrial targeted antioxidants do not inhibit TNF-induced necrosis. (A) L929 cells were incubated with 5 ng/ml mTNF in the presence of $MitoQ_3$ (△), $MitoQ_5$ (○), $MitoQ_{10}$ (□), MitoPBN (◇) or TPMP (X). After 24 h cells were stained with PI and analysed by flow cytometry. Data represent means ± SD ($n = 9$). (B) L929 cells were left untreated (□) or incubated with TNF alone (▨) or in the presence of 10 μM $MitoQ_3$ (▩), $MitoQ_5$ (■), $MitoQ_{10}$ (▤), MitoPBN (▨) or 25 μM BHA (▧) for 6, 12 or 24 h. Cells were stained with PI and analysed by flow cytometry. Data represent means ± SD ($n = 9$).

others have found these reagents to be effective in a wide variety of signalling and oxidative stress paradigms involving mitochondrial ROS production (reviewed in [27]), the results described here challenge the conclusion that mitochondrial ROS are essential mediators of TNF-induced necrosis. Consistent with our findings, over-expression of either Mn SOD or mitochondria-targeted catalase in L929 cells is also unable to prevent TNF-induced death [28]. It has previously been suggested that the lack of efficacy of these two antioxidant enzymes is due to their intra-mitochondrial location [1]. BHA is a lipid–soluble antioxidant and therefore will be present in the mitochondrial inner membrane close to the site of ROS production, whereas the over-expressed proteins were present in the mitochondrial matrix. Therefore, if the role of mitochondrial ROS in TNF-induced necrosis was membrane-specific (e.g. lipid peroxidation), only BHA would be effective. However, this seems an unlikely explanation for the lack of efficacy of the mitochondria-targeted antioxidants as these reagents are very effective inhibitors of membrane oxidation events.

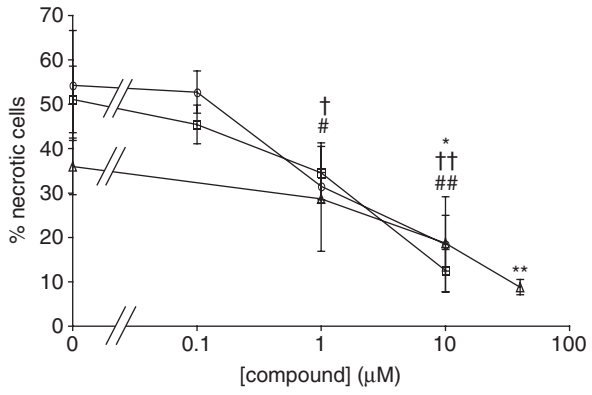

Figure 3. Mitochondrial uncouplers inhibit TNF-induced necrosis. L929 cells were incubated with 5 ng/ml mTNF in the presence of BHA (△), FCCP (○) or CCCP (□). After 20 h cells were stained with PI and analysed by flow cytometry. ⋆ $p < 0.05$ BHA vs. 0 μM, ⋆⋆ $p < 0.01$ BHA vs. 0 μM, † $p < 0.05$ CCCP vs. 0 μM, †† $p < 0.001$ CCCP vs. 0 μM, # $p < 0.05$ FCCP vs. 0 μM, ## $p < 0.01$ FCCP vs. 0 μM. Data represent means ± SD ($n = 4$ for BHA, $n = 3$ for FCCP, CCCP).

In addition to its antioxidant activity BHA is an effective uncoupler of mitochondrial respiration [24]. As the two commonly used mitochondrial uncouplers, FCCP and CCCP, significantly inhibited TNF-induced necrosis, the ability of BHA to inhibit TNF-induced necrosis may in part be due to its uncoupling activity and not its antioxidant activity. Mitochondrial uncouplers increase the proton permeability of the inner mitochondrial membrane and dissipate the proton electrochemical potential gradient built

up by respiration. This uncouples ATP synthesis from respiration leading to decreased ATP production and potentially altered ROS production. It is not yet clear which, if any, of these effects is responsible for the protective effect of these compounds. In addition, a recent report [29] proposes that the mode of action of BHA involves both inhibition of the mitochondrial respiratory chain complex I and inhibition of lipoxygenases. Thus, multiple activities of BHA, including scavenging of non-mitochondrial ROS, may contribute to its ability to block TNF-induced necrosis. Our results, while restricting the involvement of mitochondrially-produced ROS, do suggest that mitochondrial respiratory function is necessary for TNF-induced necrosis. This is consistent with the numerous studies showing correlations between interference with respiratory chain function and TNF-induced necrosis and the reduced sensitivity of L929 cells to TNF when grown on a glycolytic substrate [30]. Further investigations, including the development of additional mitochondria-specific reagents, will be required to understand how mitochondrial respiration contributes to TNF-induced necrosis specifically and caspase-independent PCD in general.

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