Tumour necrosis factor- α induces superoxide anion generation in mitochondria of L929 cells

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Within a few minutes after addition to L929 cells, tumour necrosis factor- α (TNF α) induced an increase in lucigenin-enhanced chemiluminescence that could be inhibited by superoxide dismutase. The generation of superoxide anion ($O_2^{\cdot-}$) was sensitive to treatment with rotenone, antimycin A and cyanide, indicating that the signal originated from mitochondria. The mechanism of production of $O_2^{\cdot-}$ was shown to be independent of ATP synthesis, as uncoupling of this event from mitochondrial electron transport did not alter the generation of $O_2^{\cdot-}$ induced by TNF α . Chemiluminescence was further dependent on the presence of extracellular calcium, suggesting a role for this cation as a second messenger. This hypothesis was supported by the finding that inhibition of

mitochondrial calcium uptake by Ruthenium Red exerted a protective effect on TNF α -treated L929 cells. Increased $O_2^{\cdot-}$ generation was followed by a marked decrease in mitochondrial dehydrogenase activity and cellular ATP levels, while cell membrane permeability was moderately increased. A role for mitochondrial $O_2^{\cdot-}$ generation in TNF α cytotoxicity was further supported by the finding that resistant L929 cells had decreased ability to produce $O_2^{\cdot-}$ in response to TNF α . In addition, we detected a decreased activity of the mitochondrial enzyme succinate dehydrogenase in these cells, suggesting that this component of the respiratory chain might be an important contributor to the TNF α -induced generation of $O_2^{\cdot-}$.

INTRODUCTION

Originally known for its capacity to cause the regression of certain tumours (Carswell et al., 1975), evidence has accumulated that tumour necrosis factor- α (TNF α) participates in other aspects of the immune response. *In vitro*, TNF α is cytotoxic for certain cell lines. Several modes of cell killing have been suggested, such as oxidative stress (Matthews et al., 1987; Meier et al., 1989), induction of endogenous nucleases (Schmid et al., 1987) and depletion of ATP/NADH stores (Agarwal et al., 1988). Oxidative stress in mitochondria may be the common denominator of these events. The participation of mitochondria in TNF α -induced cytotoxicity was inferred from the observation that these organelles were altered in target cells soon after treatment with TNF α (Matthews and Neale, 1987). In support of this, the same authors and subsequently others (Lancaster et al., 1989; Schulze-Osthoff et al., 1992) detected impaired respiration in target cells treated with TNF α . A possible role of mitochondria in the TNF-induced cytotoxicity is further suggested by the finding that Mn-superoxide dismutase (Mn-SOD), an enzyme localized in the mitochondria, is synthesized in response to TNF in target cells (Wong and Goeddel, 1988; Wong et al., 1989).

As mitochondria are the main source of reactive oxygen species (ROS) in non-phagocytic cells (Chance et al., 1979), we investigated whether TNF induces ROS generation in these organelles. We report here that the TNF α -sensitive cell line L929 generates elevated amounts of superoxide anion ($O_2^{\cdot-}$) upon treatment with TNF α . As shown by the response to various inhibitors, mitochondria are the main source of $O_2^{\cdot-}$ in these cells. Calcium might be a potential mediator of this reaction, as it is essential for the TNF α -induced generation of $O_2^{\cdot-}$. It has

also been demonstrated that blocking the uptake of Ca^{2+} by mitochondria decreased the cytotoxic action of TNF α on L929 cells. The increase in $O_2^{\cdot-}$ formation was followed by a decrease in mitochondrial dehydrogenase activity and subsequently by a decrease in cellular ATP levels. The importance of the mitochondrial respiratory chain in TNF α cytotoxicity is supported by our observation that TNF α -resistant cells produced less $O_2^{\cdot-}$ in response to the cytokine and displayed a lower basal activity of the mitochondrial enzyme succinate dehydrogenase (SDH) compared with TNF α -susceptible cells.

MATERIALS AND METHODS

Materials

Recombinant mouse TNF α was purchased from Genzyme (Boston, MA, U.S.A.). The activity was 4×10^7 units/mg of protein. Bovine milk xanthine oxidase (XO) was from Calbiochem (La Jolla, CA, U.S.A.), and ascorbic acid was from Aldrich (Steinheim, Germany). All chemicals were from Sigma (St. Louis, MO, U.S.A.) unless specified.

Cell culture

L929 cells were obtained from the ATCC (Rockville, MD, U.S.A.). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland, U.K.), supplemented with 2 mM glutamine, 10 mM Hepes, pH 7.4, penicillin (100 units/ml), streptomycin (100 μ g/ml) and 7% heat-inactivated fetal calf serum. For chemiluminescence assays, cells were cultured on glass coverslips in 24-well plates (Costar, Cambridge, MA, U.S.A.) at a density of 1×10^5 cells/ml (1 ml/well). Cells were

Abbreviations used: TNF α , tumour necrosis factor- α ; SOD, superoxide dismutase; ROS, reactive oxygen species; HBSS, Hanks' balanced salt solution; X/XO, xanthine/xanthine oxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine; SDH, succinate dehydrogenase; COX, cytochrome c oxidase; PMA, phorbol 12-myristate 13-acetate.

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cultured in 96-well plates (Costar) without coverslips for the cytotoxicity assays. TNF-resistant cells were obtained by culturing in medium containing increased TNF concentrations (up to 1000 pg/ml). After 10 passages, clones were isolated with cloning rings. Only the clones with a stable TNF resistance were compared with TNF-sensitive cells.

Chemiluminescence

Cells on coverslips were transferred to glass vials containing 50 μ M bis-*N*-methylacridinium nitrate (lucigenin) in Hanks' balanced salt solution (HBSS), pH 7.4. Cells were further incubated for 5 min at 37 °C and subsequently transferred to a chemiluminescence analyser thermostatically controlled at 37 °C. TNF α and other chemicals were added to a total volume of 2 ml. Chemiluminescence was measured in a modified Kontron Betamatic I liquid spectrometer running in the out-of-coincidence mode (Bertoni et al., 1985). $O_2^{\cdot-}$ was generated by adding xanthine (X)/XO (50 μ M/0.8–4.2 mg/ml) to lucigenin (50 μ M) in HBSS, pH 7.4.

Cytotoxicity assays

Cells were incubated with TNF α and actinomycin D (1 μ g/ml) at 37 °C in Iscove's modified Dulbecco's medium (Gibco) supplemented with 0.2% bacto-peptone (Difco, Detroit, MI, U.S.A.), glutamine (2 mM), pyruvate (1 mM), penicillin (100 units/ml), streptomycin (100 μ g/ml), non-essential amino acids (Gibco), MEM-vitamin solution (Gibco) and 10 mM Hepes, pH 7.4. Mitochondrial dehydrogenase activity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cleavage assay as described by Mosmann (1983). Lactate dehydrogenase (LDH) activity in the supernatant was determined by monitoring the disappearance of supplemented NADH at 340 nm as previously described (Madden and Storrie, 1987). ATP was analysed based on the luciferin/luciferase reaction with a commercially available assay kit (Calbiochem). Luminescence was measured in a bioluminometer equipped with injector (LB-950; Berthold, Wildbad, Germany).

Oxygen consumption assays

Cells were trypsin-treated and resuspended to a density of 6×10^6 /ml in 25 mM Tris/HCl, pH 7.4, 0.25 M sucrose, 2 mM EDTA, 5 mM MgCl₂ and 10 mM K₂HPO₄. Cells were then incubated under constant stirring at 37 °C. Oxygen consumption was monitored with a Clark-type electrode (YSI model 5331) after consecutive addition of the chemicals (final concentrations): digitonin (0.2 mg/10⁷ cells), rotenone (0.2 μ M), succinate (5 mM), antimycin A (0.2 μ M) and *NNN'*-tetramethyl-*p*-phenylenediamine (TMPD)/ascorbate (0.8 mM/4 mM). SDH activity was defined as the rate of oxygen uptake after addition of the substrate, succinate. Similarly, the respiration rate in the presence of the electron donors TMPD/ascorbate was used as a measure of cytochrome *c* oxidase (COX) activity (Granger and Lehninger, 1982). Enzyme activity was expressed in ng-atoms of O/min per 10⁶ cells.

RESULTS

The inhibition of RNA transcription or protein expression enhances the sensitivity of L929 cells towards TNF α (Wallach,

1984). In the presence of actinomycin D (1 μ g/ml), a TNF α concentration of 20 pg/ml caused a 50% loss of viability when given to L929 cells, and all cells were killed by amounts of TNF α above 300 pg/ml. TNF α concentrations in the picogram range caused an immediate increase in $O_2^{\cdot-}$ generation in L929 cells as measured by lucigenin-enhanced chemiluminescence (Figure 1). The chemiluminescence signal, reflecting the rate of the reaction, increased linearly over the first 60 min after addition of TNF α to the cells, indicating that the production of $O_2^{\cdot-}$ followed exponential kinetics over this time period. The chemiluminescence signal was inhibited more than 90% in the presence of SOD (30 μ g/ml), indicating that it largely reflected $O_2^{\cdot-}$ generation (Figure 1). We obtained the same signal whether or not actinomycin D was added to the cells. In contrast to TNF α , phorbol 12-myristate 13-acetate (PMA) (30 nM) or the calcium ionophore A23187 (1 μ M), two activators of NADPH oxidase in phagocytic cells, did not induce a significant chemiluminescence signal (Figure 1, inset). The generation of chemiluminescence was dependent on bioactive TNF α , as heat treatment (56 °C for 60 min) abrogated its stimulatory effect (results not shown). Cells were found to be negative for mycoplasma contamination, thus excluding this potential source of chemiluminescence in our system (Meier and Habermehl, 1990). Further, treatment of cells with bacterial lipopolysaccharide (10–200 ng/ml) before or simultaneously with TNF α did not modify the chemiluminescence signal (results not shown).

The role of the mitochondrial electron transport chain was investigated using a panel of specific inhibitors. Chemiluminescence was abrogated in the presence of rotenone (10 μ M), an inhibitor of mitochondrial NADH dehydrogenase (complex I), and by cyanide (100 μ M), which inhibits COX (complex IV), whereas antimycin A (20 μ M), an inhibitor of the *bc*₁-site of complex III, failed to completely inhibit chemiluminescence (Figure 2). Chemiluminescence generated by X/XO was not affected by rotenone, antimycin A or cyanide, indicating that the effect of these inhibitors was not due to their interference with the

Figure 1 TNF α -induced chemiluminescence in L929 cells

Cells adherent on glass coverslips were incubated in HBSS, pH 7.4, containing lucigenin (50 μ M). Chemiluminescence was measured after addition of TNF α (final concentration 100 pg/ml) (●). The signal was inhibited by more than 90% in the presence of SOD at 30 μ g/ml (○). The inset shows chemiluminescence of L929 cells alone (◇), after addition of PMA (final concentration 30 nM) (□) or after addition of calcium ionophore A23187 (final concentration 1 μ M) (△). Each curve is the average of two measurements. Shown are results of a typical experiment out of 21.

Figure 2 Inhibition of TNF α -induced chemiluminescence by inhibitors of mitochondrial electron transport

Cells were stimulated with TNF α (100 pg/ml) (●). (a) Rotenone (final concentration 10 μ M) was added to cells simultaneously with TNF α (○) or alone (△). (b) Antimycin A (final concentration 20 μ M) in combination with TNF α (○) or alone (△). (c) NaCN (final concentration 100 μ M) with TNF α (○) or alone (△). Each panel represents a typical result out of four experiments.

Figure 3 Effect of dinitrophenol on TNF α -induced chemiluminescence

Chemiluminescence was monitored after addition of TNF α (100 pg/ml) to L929 cells (●). The effect of dinitrophenol (30 μ M) on chemiluminescence was monitored when given to L929 cells alone (△) or in combination with TNF α (100 pg/ml) (○). Each curve is the average of duplicates. Shown are results from a typical experiment out of three.

Figure 4 Inhibition of TNF α -induced chemiluminescence by Ca²⁺ deprivation

Cells were stimulated with TNF (100 pg/ml) (●). The same treatment was effected in the presence of EGTA (1 mM) in the assay medium (○) or by incubating cells in a Ca²⁺-free assay medium (△). Each curve is the average of two measurements. Shown are results from a typical experiment out of five.

reaction between O₂^{•-} and lucigenin, but rather reflected their metabolic action (results not shown). Treatment of L929 cells with dinitrophenol (30 μ M), an uncoupler of electron transport from energy-requiring reactions, had no effect on the chemiluminescence signal induced by TNF α . However, dinitrophenol alone was able to increase the production of O₂^{•-} in L929 cells (Figure 3).

We then investigated the effects of Ca²⁺ on the generation of O₂^{•-} induced by TNF α . This cation is a common denominator of many signalling cascades and it exerts various effects on mitochondrial functions. Ca²⁺ regulates several mitochondrial dehydrogenases (Denton and McCormack, 1985), and an increased mitochondrial Ca²⁺ concentration is detrimental for these organelles as this leads to inhibition of ATP synthesis (Tuena De Gomez-Puyou et al., 1980). We found that chelation of extracellular Ca²⁺ by EGTA (1 mM) or incubating cells in a Ca²⁺-deficient buffer completely blocked the TNF-induced generation of O₂^{•-} in L929 cells (Figure 4). To assess the role of mitochondrial Ca²⁺ uptake, we studied the effect of the inhibitor Ruthenium Red. Since Ruthenium Red does not penetrate efficiently into L929 cells, we needed to permeabilize them with digitonin. Digitonin alone caused a certain degree of cytotoxicity (Figure 5, inset), while Ruthenium Red alone failed to alter the effect of TNF α . However, it can be seen from Figure 5 that Ruthenium Red clearly protected the cells at elevated concentrations of TNF α when it could interact with mitochondria.

We were also interested to know whether alterations of mitochondrial functions occurred rapidly after the onset of O₂^{•-} generation. To this end, we measured mitochondrial dehydrogenase activity, cellular ATP levels and cell membrane permeability, a parameter of cell death that should increase over time. Figure 6(a) shows that mitochondrial dehydrogenase activity, mainly represented by NADH dehydrogenase and SDH, as measured by the cleavage of MTT into insoluble formazan (Mosmann, 1983), was already significantly decreased at 2.5 h after addition of TNF α to the L929 cells. Minimal dehydrogenase activity was reached at 10 h after TNF α treatment, and activity thereafter remained at low levels until 20 h. Cellular ATP levels decreased from 6 h onwards and reached minimal concentrations at 20 h. In contrast, as assessed by LDH release, the permeability of the plasma membrane increased only slightly and reached 20% by 20 h after addition of TNF α to the cells (Figure 6a).

Figure 5 Modulation of TNF α cytotoxicity by Ruthenium Red

L929 cells were incubated for 20 h with various concentrations of TNF α in the presence of actinomycin D (1 μ g/ml) (●). Ruthenium Red (25 μ M) was added 1 h before TNF α (○). Alternatively, the same concentration of Ruthenium Red was added in combination with digitonin (0.1 μ M) (□) or digitonin (1 μ M) (△). The inset shows the effect of digitonin (1 μ M) on TNF α cytotoxicity (○) in comparison to TNF α alone (●).

Figure 6 Mitochondrial dehydrogenase activity, ATP levels and cell permeability in L929 cells treated with TNF α

(a) TNF-sensitive cells; (b) TNF-resistant cells. Cells were incubated at 37 °C with TNF α (100 pg/ml) and actinomycin D (1 μ g/ml). Mitochondrial dehydrogenase activity (□) is expressed as the intensity of absorption at 590 nm after a 1 h incubation with MTT. ATP concentrations in 1×10^5 cells (○) are expressed in nM. LDH release was used as a marker of cell permeability (△) and is expressed as % of maximal release (when cells were permeabilized with 0.1% Triton X-100). Results are expressed as the means of three measurements.

The previous experiments suggested that mitochondria were the source of $O_2^{\cdot -}$ in TNF α -treated L929 cells and that the decrease in mitochondrial electron transport was an early event

Figure 7 Oxygen consumption by TNF-sensitive and TNF-resistant L929 cells

SDH and COX activity were measured in TNF-sensitive cells (lower trace) and TNF-resistant cells (upper trace) as described in the Materials and methods section. SDH activity is given by the rate of O_2 uptake after addition of succinate, and COX activity is given by the rate of O_2 uptake after ascorbate/TMPD addition. The inset represents the chemiluminescence signal induced by TNF α (100 pg/ml) in TNF-sensitive L929 cells (●) and in TNF-resistant L929 cells (○). Each curve is the average of two measurements. Shown are results from a typical experiment out of five.

in the cytotoxic action of the cytokine. To assess the possible significance of these alterations, we compared TNF-susceptible and -resistant cells; the latter were obtained by prolonged culture in the presence of TNF α . When subjected to the cytokine at concentrations that were toxic for susceptible cells, mitochondrial dehydrogenase activity also decreased in TNF-resistant cells but remained at about 40% of control values by 20 h (Figure 6b). ATP levels paralleled the changes in dehydrogenase activity. LDH activity in the cell medium increased to a lower extent in TNF-resistant than -susceptible cells (Figure 6b). Using chemiluminescence measurements, we further observed that TNF-resistant cells had a much lowered $O_2^{\cdot -}$ production compared with TNF-sensitive cells when treated with TNF α (Figure 7, inset). We then compared the activities of respiratory chain enzymes in resistant and susceptible cells by measuring oxygen consumption with a Clark-type electrode. The plasma membrane and mitochondrial outer membrane were permeabilized by digitonin to allow added substrates and inhibitors to interact with mitochondrial enzymes. The addition of rotenone blocked electron flow at the level of NADH dehydrogenase (complex I) and allowed the measurement of SDH activity after addition of the substrate succinate. To measure COX activity, the electron flow was blocked at the bc_1 -site of complex III by antimycin A, and ascorbate/TMPD were used as electron donors for COX. We detected an SDH activity of between 0.8 and 1.3 ng-atoms of O /min per 10^6 cells in TNF-resistant cells ($n = 4$), while enzyme activity ranged from 2.1 to 3.2 ng-atoms of O /min per 10^6 cells in TNF-susceptible cells ($n = 4$). In contrast, COX activity did not change significantly in TNF-resistant cells (Figure 7).

DISCUSSION

The cytotoxic effect of TNF on malignant cells *in vitro* has been shown previously to be independent of the density of TNF receptors on the surface of target cells (Matthews and Neale, 1987). In most cell types the cytotoxic action of TNF is enhanced when protein synthesis is blocked (Wallach, 1984), indicating that target cells synthesize protective proteins such as Mn-SOD (Wong and Goeddel, 1988). The involvement of mitochondria

(Matthews et al., 1987) and of enhanced oxidative mechanisms (Matthews et al., 1987; Meier et al., 1989) were previously proposed, but mitochondrial ROS generation in TNF-treated cells has not so far been documented. By measuring lucigenin-enhanced chemiluminescence, we show in this work that TNF α stimulated the generation of superoxide anion in L929 fibrosarcoma cells. In the presence of rotenone, antimycin A and cyanide the chemiluminescence signal was abolished or strongly inhibited. This indicates that the mitochondrial electron transport chain is the likely source of $O_2^{\cdot-}$. The fact that dinitrophenol treatment did not alter TNF α -induced $O_2^{\cdot-}$ generation indicates that this reaction is not coupled to ATP synthesis in mitochondria. Dinitrophenol was able to stimulate $O_2^{\cdot-}$ production alone, but did not further enhance the TNF α -induced reaction. This suggests that TNF α might itself act as an uncoupler, maybe via ion-channel formation. A similar property of TNF α has been recently described by Kagan et al. (1992).

Meier et al. (1989) reported previously that TNF α increased the production of $O_2^{\cdot-}$ in human fibroblasts. As rotenone, azide and cyanide did not modify the rate of $O_2^{\cdot-}$ production, these authors excluded mitochondria as a source of elevated $O_2^{\cdot-}$ generation. However, the lowest TNF α concentration at which they could observe $O_2^{\cdot-}$ production was about 30-fold higher than that used in our experiments. These differences may also be explained by the method of $O_2^{\cdot-}$ measurement employed in this study. In our hands, lucigenin-enhanced chemiluminescence was some 100-fold more sensitive than cytochrome *c* reduction, the method used by Meier et al. (1989). Previous work showed that the inhibition of the respiratory chain by rotenone (Takeshige and Minakami, 1979) or antimycin A (Konstantinov et al., 1987) leads to increased $O_2^{\cdot-}$ generation at the sites proximal to the blockade. We were able to reproduce these results in isolated mitochondria, but not when intact cells were stimulated with TNF α .

ROS damage mitochondria themselves. These organelles have no DNA repair system, rendering mitochondrial DNA very susceptible to oxidative damage (Richter et al., 1988). In addition, most components of the respiratory chain have been shown to be inactivated by small amounts of $O_2^{\cdot-}$ (Zhang et al., 1990), a ROS otherwise considered as a mild oxidant. TNF α -induced $O_2^{\cdot-}$ generation was Ca^{2+} -dependent, suggesting that the cation may be a second messenger in this process. A role for Ca^{2+} in the induction of mitochondrial $O_2^{\cdot-}$ production and in cytotoxicity was demonstrated by the protective effect exerted by Ruthenium Red at high concentrations of TNF α . In support of this interpretation, Ca^{2+} was recently shown to play a similar role in mitochondrial $O_2^{\cdot-}$ generation induced by doxorubicin, a cytotoxic drug used in cancer therapy (Chacon and Acosta, 1991). It is nevertheless unclear whether Ca^{2+} acts alone or in conjunction with other intracellular mediators. For example, lipoxygenase products such as hydroperoxyeicosatetraenoic acids have been reported to induce a cycling of Ca^{2+} by mitochondria (Richter et al., 1987) that leads to irreversible change to these organelles (Richter and Kass, 1991).

Our observation that TNF α -resistant L929 cells had lowered SDH activity as well as a decreased ability to generate $O_2^{\cdot-}$ after TNF α challenge is interesting in light of observations made during the cytotoxic action of TNF α . We showed that our TNF-resistant cells were still responsive to TNF α , indicating that a loss of TNF receptors might be excluded as a cause of resistance. The comparison of changes in dehydrogenase activity, cellular ATP levels and cell membrane permeability between TNF-sensitive and TNF-resistant cells revealed that a rapid fall in mitochondrial dehydrogenase activity was prevented in the latter cells. Consequently, cellular ATP levels remained at about 40 %

of control values, paralleling the changes in dehydrogenase activity. Lancaster et al. (1989), and more recently Schulze-Osthoff et al. (1992), have reported that the activity of SDH decreases within a few hours of addition of TNF α to the cells. These observations are in accordance with our finding about the changes in mitochondrial dehydrogenase activity. In addition, we were able to confirm these results by measuring of oxygen consumption of TNF α -treated L929 cells (not shown). Taken together, these observations raise the question of whether SDH has the role of an effector or a target (or both) in TNF α -induced cytotoxicity. As we observed a decreased TNF α -induced $O_2^{\cdot-}$ production in TNF-resistant cells, it is tempting to speculate that this difference is related to the lower SDH activity in these cells. It seems possible that, in TNF-treated cells, SDH could show activity in addition to the reported dehydrogenase activity, perhaps similar to the situation with XO, which is transformed to an oxidase producing $O_2^{\cdot-}$ upon exposure to oxidants. Whether a decreased flow of electrons through this site is protective because of decreased ROS generation further down the electron transport chain cannot be excluded. However, in contrast to others (Schulze-Osthoff et al., 1992), we failed to observe an enhancement of cytotoxicity when antimycin A was applied to TNF α -treated cells (results not shown). This finding does not support a role of the ubiquinone site of complex III in the generation of $O_2^{\cdot-}$, as hypothesized by Schulze-Osthoff et al. (1992).

In conclusion, our results suggest that TNF α induces elevated $O_2^{\cdot-}$ production in mitochondria. This leads to inactivation of dehydrogenase activity and hence to a subsequent decrease in cellular ATP levels, which is fatal for target cells.

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