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Inhibition of target cell mitochondrial electron transfer by tumor necrosis factor

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Using digitonin permeabilization to assay mitochondrial electron transfer, we have found that respiratory activity (succinoxidase and cytochrome oxidase) in three mouse fibroblast lines is completely eliminated by incubation with human recombinant tumor necrosis factor-α (hrTNF). As with cytotoxicity, hrTNF-induced mitochondrial dysfunction occurs in resistant cells upon inhibition of protein synthesis, whereas sensitive cells exhibit spontaneous respiratory inhibition. In C3HA cells, inhibition is detectable 1.5–2 h after hrTNF addition, preceding cell lysis by at least 5 h (as measured by dye exclusion), and is approximately coincidental with morphological changes we have previously reported for this cell line. LM cells also exhibit inhibition of electron transfer, coincidental with morphological changes. These results suggest that bioenergetic dysfunction may be involved in the cytotoxic mechanism of TNF.

Tumor necrosis factor-α; Cytotoxicity, immunological; Bioenergetics; Mitochondria; Electron transport; (Murine fibroblast)

1. INTRODUCTION

Tumor necrosis factor-α (TNF) is a soluble protein produced by cells of the immune system which was originally identified and assayed by its ability to cause necrosis of tumors when injected into animals [1,2]. It is now recognized that TNF belongs to a class of molecules called monokines, which are secreted by macrophages upon activation by a variety of stimuli, including exposure to certain bacterial products [3]. In addition to induction of tumor necrosis in vivo, TNF possesses multiple immunoregulatory and inflammatory functions [4]; in vitro, this 17 kDa protein (active as a trimer [5]) is preferentially cytotoxic to many transformed (as well as virally infected [6]) cell lines, although this specificity is not absolute [7,8]. The mechanism that determines sensitivity vs resistance to TNF may reside intracellularly, since binding to high-affinity receptors, internalization and degradation occur in both cell types [9,10]. In addition, it has been shown that TNF treatment of resistant cells induces production of a 'protective' response, involving synthesis of a relatively small set of proteins [11–13]; pretreatment with protein synthetic inhibitors prevents this response and thus confers TNF sensitivity [9,8].

It is thus reasonable to suspect that insight into the selective action of TNF toward cells can be gained by investigating the mechanism of cytotoxicity. We report that an early and dramatic intracellular event during TNF-induced cytolysis is inhibition of the bioenergetic machinery of the target cell, specifically a virtually complete loss of mitochondrial electron transfer.

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Abbreviations: hrTNF, human recombinant tumor necrosis factor-α; TMPD, tetramethylphenylenediamine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; CHX, cycloheximide

2. MATERIALS AND METHODS

2.1. *Cells and media*

Three cell lines were used, PSC3H (an SV40-transformed fibroblast line [14]), C3HA (a 3T3-like line [14,15]) and LM (a

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transformed mouse fibroblast line). Cells were maintained in Dulbecco's modified Eagle's medium (DME) (Gibco) supplemented with 10% fetal calf serum (FCS) (Hyclone Labs, Logan, UT) and 1% glutamine, at 37°C in 5% CO_2.

2.2. *Assays*

All cells were counted and plated overnight prior to addition of hrTNF. The following morning additions of hrTNF (100 U/ml) and/or cycloheximide (CHX; 25 μg/ml) were made as designated in the figure legends, and the plates were incubated at 37°C. At the indicated times, cells were harvested by trypsinization, combined with the decanted media from the plate to include cells which were no longer adherent, washed, and finally resuspended to $2–10 \times 10^6$ cells per ml in 2.7 ml respiration medium [0.25 M sucrose, 20 mM K^+-Hepes (pH 7.1), 10 mM $MgCl_2$] with or without (as indicated in the figure legends) 1 mM ADP and 2 mM potassium phosphate, and kept on ice. Oxygen consumption was measured with a Clark oxygen electrode (YSI model 5300) at 37°C with constant stirring, using the digitonin procedure for determining mitochondrial electron transfer in permeabilized cells [16,17]. Oxygen concentration was calibrated with air-saturated buffer, assuming 390 ngatom/ml O_2 [17]. Additions were made with a Hamilton syringe (volumes <1% total) as indicated in the figures. Cell viability was determined by trypan blue exclusion.

2.3. *Reagents*

Digitonin was crystallized from ethanol as in [18] and added in dimethyl sulfoxide. hrTNF was generously supplied by Cetus Corp. (spec. act. 1.3×10^7 U/mg). One unit is the amount required to cause 50% lysis of L929 cells in the presence of 1 μg/ml actinomycin D. All other compounds were from standard commercial sources.

3. RESULTS

Fig.1 presents a diagram depicting in the form of a scheme the principal components of the mitochondrial electron-transfer chain, which is responsible for cellular ATP synthesis coupled to the oxidation of respiratory substrate. For most of the data reported here, rotenone and ADP plus P_i (or FCCP) were added prior to initiation of measurement, the former to inhibit oxidation of endogenous, NADH-linked substrate and the latter to induce maximal ('state 3') electron-transfer activity. Upon initiation of respiration by the addition of succinate and digitonin (added to permeabilize the plasma membrane [16] and outer mitochondrial membrane [19] and thus allow accessibility of the added substrate to the respiratory chain), oxygen uptake is a measure of electron transfer through complexes II, III and IV ('succinoxidase' activity), and is independent of energy-coupled effects (e.g. respiratory control). In the absence of uncoupler (FCCP), a decrease in the rate of oxygen uptake upon subsequent addition of the ATPase inhibitor oligomycin is indicative of coupling between electron transfer and ATP synthesis ('respiratory control'). Addition of antimycin A (which inhibits electron transfer through complex III) and subsequent addition of the artificial electron donor system ascorbate/TMPD results in electron transfer exclusively through complex IV (cytochrome-*c* oxidase), since electrons from these carriers enter the chain at the level of cytochrome *c*. In this way, differential inhibition of two segments of the chain (complexes II plus III and complex IV) can be examined, as well as an indication of the general integrity of the mitochondrial membrane (as exhibited by respiratory control).

PSC3H cells are an SV40-transformed mouse line which is normally resistant to hrTNF but becomes sensitive in the presence of protein synthesis inhibitors (not shown). Fig.2 shows tracings from a typical mitochondrial oxygen uptake assay, using a Clark electrode to measure dissolved oxygen in the cell suspension. Fig.2A (top tracing,

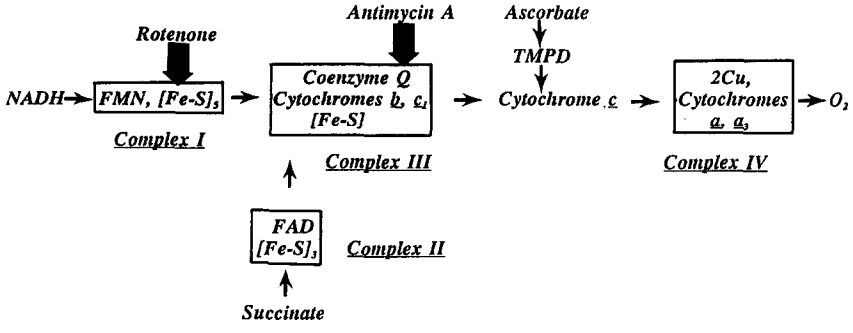

Fig.1. Scheme depicting the electron flow through the mitochondrial respiratory chain. [Fe-S], iron-sulfur center. Bold arrows indicate site of action of inhibitors; thin arrows represent electron transfer.

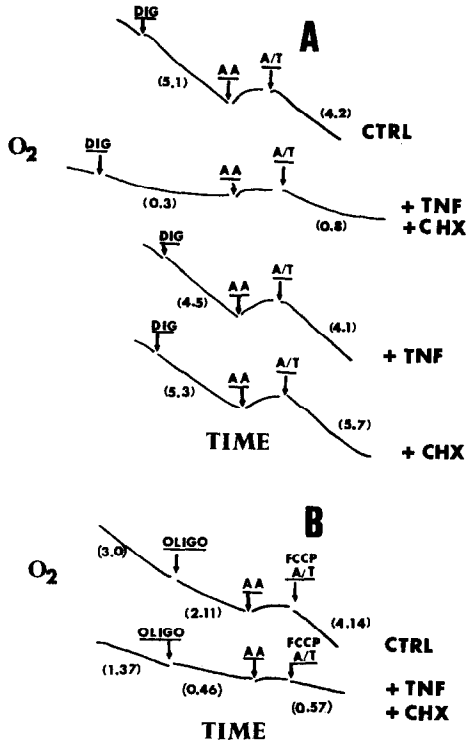

Fig.2. Inhibition of mitochondrial electron transfer by hrTNF in mouse (A) PSC3H and (B) C3HA cells. Cells were prepared and oxygen uptake was assayed as described in section 2 in respiration medium containing ADP, P_i and rotenone (0.1 μM). Initial electron donor was succinate (5 mM). Additions (indicated by arrows): DIG, digitonin (0.015% final); AA, antimycin A (20 nM final); A/T, ascorbate/TMPD (1 mM and 0.2 mM final, respectively); OLIGO, oligomycin (0.1 μM final); FCCP, 80 nM final. Where indicated, hrTNF (200 U/ml) or cycloheximide (25 μg/ml) were added to the culture and incubated for (A) 7 or (B) 3 h before harvest and assay. Rates of oxygen uptake (in ngatom/min per 10^6 cells) are in parentheses.

denoted CTRL) shows oxygen disappearance for a suspension of PSC3H cells treated as described above, with digitonin (DIG) added and with succinate, rotenone, and ADP plus P_i present in the suspension buffer. The cessation in oxygen disappearance upon addition of antimycin A (AA) shows that this disappearance is due to mitochondrial succinoxidase activity, i.e. through complexes II, III and IV; the respiratory rate in this experiment is 5.1 ngatom/min per 10^6 cells, comparable in magnitude to that reported for other cultured mouse cells measured with this same technique [17]. Subsequent addition of ascorbate/TMPD (A/T) results in the resumption of oxygen uptake, through complex IV, at a rate of 4.2 ngatom/min per 10^6 cells. Elimination of this activity by addition of KCN (not shown) further confirms that this uptake is due to mitochondrial cytochrome oxidase (complex IV) activity.

The second tracing in fig.2A shows that incubation of cells for 7 h with hrTNF (100 U/ml) plus cycloheximide results in virtually complete elimination of succinoxidase activity (94% inhibition) and 81% inhibition of cytochrome oxidase (since succinoxidase activity requires complex IV function, this particular result indicates dysfunction in at least this latter segment of the electron-transfer chain, with no information as to additional sites of inhibition). The two lower traces show that (similar to cell lysis) inhibition of electron transfer requires the simultaneous presence of both hrTNF and cycloheximide. Studies of the time course of mitochondrial inhibition in this cell line (fig.3A) show that little inhibition occurs prior to approx. 5 h after addition of hrTNF plus cycloheximide, and inhibition is complete after 7 h.

In order to gain insight as to whether mitochondrial inhibition is involved with an early or late event in the lytic process, we examined respiratory inhibition in C3HA cells (a 3T3-like line [15]) which begin to show gross morphological changes within 1–2 h after addition of hrTNF plus cycloheximide, several hours before cell lysis is detected (as measured by ^{51}Cr release [20]). Fig.2B shows that preincubation of cells with hrTNF plus cycloheximide for 3 h results in significant mitochondrial dysfunction. As with PSC3H cells, this effect requires the simultaneous presence of hrTNF and cycloheximide (not shown). The decrease in the rate of oxygen uptake upon addition of oligomycin indicates that electron transfer is coupled to ATP synthesis in these mitochondria, and that the effect of hrTNF is principally upon electron transfer and not energy coupling. Fig.3B shows that hrTNF (in the presence of cycloheximide) induces virtually complete inhibition of succinoxidase activity [and also cytochrome oxidase (not shown)] detectable after 1.5–2 h (coincidental with the observed morphological changes in this cell line [20]), and that this inhibition precedes significant cell lysis (as judged by trypan blue exclusion) by at least 5 h. It is important to point out that inhibition will only be detectable by this technique if a significant fraction of the total cell population is affected; this

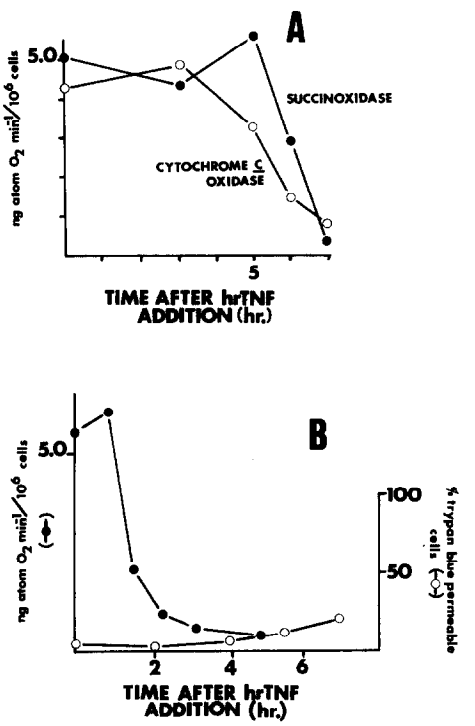

Fig.3. Time course of hrTNF-induced inhibition of mitochondrial electron transfer in (A) PSC3H and (B) C3HA cells. (A) PSC3H cells were assayed as in fig.1A. (●—●) Succinoxidase activity, (○—○) cytochrome oxidase activity. (B) C3HA cells were assayed for succinoxidase activity as described for fig.1B, except in respiration medium (no ADP or P_i) and in the presence of 80 nM FCCP. (●—●) Succinoxidase activity, (○—○) percent trypan blue-permeable cells.

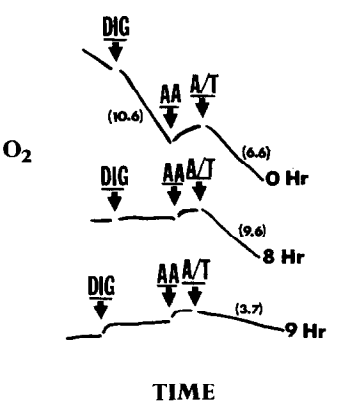

Fig.4. Inhibition of mitochondrial electron transfer by hrTNF in mouse LM cells. Mouse LM cells were assayed for succinoxidase activity after preincubation with 100 U/ml hrTNF for the indicated times before harvest and assay as described for fig.1. Rates of oxygen uptake (in ngatom/min per 10^6 cells) are in parentheses.

means that mitochondrial dysfunction may be occurring earlier in susceptible cells.

LM cells are a fully transformed mouse fibroblast line which are spontaneously sensitive to hrTNF, i.e. in the absence of inhibitors of protein synthesis [9]. Fig.4 (middle trace) shows that succinoxidase activity (but not electron transfer through complex IV) is eliminated by treatment of cells with hrTNF for 8 h (after an approx. 7 h lag, during which time cells show no mitochondrial inhibition [not shown] and which is generally coincidental with morphological degeneration [20], and exhibit no microscopically detectable effects [20]), and the bottom tracing shows that inhibition of complex IV ensues rapidly afterward (by 9 h).

4. DISCUSSION

Research to date on the mechanism of cytotoxicy induced by TNF has largely employed cell lysis assays (e.g. release of internal isotope or dye retention), which can be viewed as the end product of the entire cell death process [21] (i.e. lysis of the plasma membrane). In an effort to gain specific insight into the sequence of events which ultimately results in TNF-induced cell death, recent work from this laboratory has focussed on examination of cellular events preceding lysis, in particular morphological [20] and cytoskeletal [22] changes. Here, we report a metabolic abnormality in target cells which also precedes lysis, specifically, dysfunction in the mechanism of cellular energy production. We note that mitochondrial inhibition occurs in both of two cell types (C3HA and LM) which exhibit different morphological changes during TNF-induced cell death [20]; in addition, the onset of such inhibition is approximately coincidental with these changes.

In vitro, exposure of activated macrophages to neoplastic cells induces a nonphagocytic response, resulting in target cell cytostatis and cytotoxicity [3,23]. Studies have shown that under these conditions tumor cells develop inhibition of mitochondrial respiration [17,24], similar in pattern to that reported here for LM cells (i.e. preferential [although not completely selective] inhibition of complexes II and III as opposed to complex IV [17]). Although cell-cell contact between the macrophage and the tumor target cell has been

reported to be required [25], a soluble factor has been identified which induces mitochondrial dysfunction in murine mammary adrenocarcinoma cells [26]. It has recently been shown that TNF is a major mediator of monocyte cytotoxicity towards neoplastic cells [27–29], and that TNF is produced by macrophages as both a soluble, secreted and a membrane-bound form [30–33]; TNF-target cell interaction occurs by both cell-cell contact and by release of soluble cytotoxin. The present data thus suggest that macrophage-induced target cell respiratory dysfunction may be mediated by TNF, whether by cell-cell contact or through release of the soluble protein.

The most important question concerning the results presented here with regard to the cellular mechanism of TNF cytotoxicity is whether the mitochondrial inhibition of electron transfer is a cause or an effect. The occurrence of this inhibition in each of three cell lines investigated (and only under conditions which lead to eventual cell lysis) and the time course of inhibition in relation to cell lysis (at least in C3HA cells, where mitochondrial dysfunction precedes significant cell lysis by at least 5 h) suggest that this inhibition is an important part of the mechanism of cytotoxicity. The simultaneous occurrence of mitochondrial dysfunction and morphological changes in two lines (as described above) also supports this conclusion. Other intracellular events have been described upon treatment of sensitive cells with TNF, including activation of phospholipase A_2 [34–36], increased intracellular cAMP levels [37], and increased ADP-ribosylation activity [38]. Although further experimentation is required to delineate the relationships between these effects and the mechanism of cytotoxicity (specifically, the differentiation between signalling events and induction of cell vital damage), cellular mitochondrial dysfunction might be expected to produce abnormalities in a variety of cellular processes, including energy metabolism (and consequent effects on anabolic and regulatory functions [39]), cytoskeletal integrity [40–42], and intracellular solute and ion (including calcium [42,43]) homeostasis. In addition, the mitochondrial electron-transfer chain (primarily complex III [44]) is a major intracellular source of reactive oxygen intermediates [45,46], which have also been implicated in TNF-mediated cell cytotoxicity [47–49].

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