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Original Research Communication

Regulation of Tumor Necrosis Factor-Induced, Mitochondria- and Reactive Oxygen Species-Dependent Cell Death by the Electron Flux through the Electron Transport Chain Complex I

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ABSTRACT

Tumor necrosis factor (TNF) induces a caspase-independent but mitochondria-dependent cell death process in the mouse fibrosarcoma cell line L929. Mitochondria actively participate in this TNF-induced necrotic cell death by the generation of mitochondrial reactive oxygen species (ROS). The aim of this study was to identify the mitochondrial components involved in TNF-induced production of ROS and their regulation by bioenergetic pathways. Therefore, we analyzed the bioenergetic characteristics in two metabolic L929 variants that exhibit different sensitivities to TNF. L929^{gln} cells use glutamine as respiratory substrate and are far more susceptible to TNF-induced ROS generation and cell death as L929^{glc} cells that use glucose as respiratory substrate. We show that the higher levels of reducing NAD(P)H equivalents, detected in the desensitized L929^{glc} cells, do not cause diminished ROS generation. To the contrary, TNF increases the levels of NAD(P)H, probably altering complex I activity. A multiparameter analysis of electron flux through the mitochondrial electron transport chain, TNF-induced ROS levels, and cell death convincingly demonstrates a dependence of TNF signaling on complex I activity. Also, the sensitizing effect of glutamine metabolism correlates with an enhanced contribution of complex I to the overall electron flux. This participation of complex I activity in TNF-induced cell death is regulated by substrate availability rather than by a direct modification of complex I proteins. From the results presented in this paper we conclude that TNF-induced ROS generation and cell death are strongly regulated by bioenergetic pathways that define electron flux through complex I of the electron transport chain. Antiox. Redox Signal. 1, 285–295.

INTRODUCTION

DISRUPTION OF THE INTRACELLULAR REDOX-BALANCE often participates in the process of cell death triggered by death receptors of the tumor necrosis factor (TNF) receptor superfamily such as the 55-kD tumor necrosis factor receptor (TNF-R1) and CD95. These receptors are characterized by the presence of a death domain (DD) in the cytoplasmic region (Schulze-Osthoff *et al.*, 1998). Clustering of the DD results in recruitment of DD-containing signaling molecules that, together with the clustered receptor form the death-inducing signaling com-

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plex (DISC) (Hsu *et al.*, 1996; Varfolomeev *et al.*, 1996). Downstream of the DISC, the signaling pathways may vary between cell types and stimuli. In most cells, engagement of a death receptor activates cell death pathways that exhibit an apoptotic phenotype, including activation of execution caspases, membrane blebbing, nuclear condensation, and DNA fragmentation (Schulze-Osthoff *et al.*, 1998). However, there are conditions where death receptors activate pathways with a necrotic phenotype, characterized by cell swelling, membrane permeabilization, and absence of caspase activation and DNA fragmentation (Grooten *et al.*, 1993; Kawahara *et al.*, 1998; Vercammen *et al.*, 1998b). Morphologically most cell death processes are categorized in one of these two types of cell death.

Although it is widely accepted that reactive oxygen species (ROS) and redox changes can regulate various components of the cell death machinery, the causal relationship between redox changes and cell death has to be studied on a cell- and stimulus-specific basis. In the case of TNF-induced cell death in L929 cells, we showed that a caspase-independent but ROS- and mitochondria-dependent pathway to necrosis-like cell death is activated (Goossens *et al.*, 1995; Vercammen *et al.*, 1998a). The causal relationship between TNF-induced ROS production and cell death was established by the protective effect of the synthetic free radical scavengers butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). TNF-induced ROS generation was no immediate, oxidative burst-like effect but instead was a late event, preceding cell death by 1–2 hr. The mitochondrial origin of TNF-induced ROS was demonstrated in cells that were permeabilized with digitonin, a mild detergent that disrupts the plasma membrane and single membrane organelles but leaves mitochondria intact (Goossens *et al.*, in press). In this cytosol-free condition, mitochondria exhibit similar or even more pronounced fluorescence derived from the ROS-sensitive fluorogenic marker DHR123, illustrating that mitochondria are the main intracellular source of TNF-induced ROS.

In most situations, mitochondrial ROS production is due to a leak of electrons from the electron transport chain. In TNF-treated cells, an increased ROS production is accompanied by an increase in mitochondrial membrane potential (Goossens *et al.*, in press), suggesting that TNF induces changes in the mitochondrial electron transport chain activity. Also, previous results from our laboratory showed that inhibition of complex I and II of the electron transport chain inhibits TNF-induced cell death (Higuchi *et al.*, 1992; Schulze-Osthoff *et al.*, 1992). Other convincing data implicating the energy metabolism in ROS production are the sensitizing effect of tumor cell-specific bioenergetic pathways, which is characterized by the consumption of glutamine instead of glucose for oxidative phosphorylation, on TNF-induced ROS production and cell death (Goossens *et al.*, 1996). Forcing these cells to switch to glucose as a respiratory substrate desensitizes them to TNF-induced cell death.

In the present paper, we extended this analysis of the regulatory role of bioenergetic pathways on TNF-induced ROS production to the level of its impact on electron flow through complex I and II of the electron transport chain. Thus, various metabolic parameters and their modulation by TNF were monitored in L929 cells growing on glucose as mayor respiratory substrate and L929 cells using glutamine as respiratory substrate, and we correlated these measurements with ROS-production and sensitivity to cell death upon TNF treatment.

MATERIALS AND METHODS

Cell culture

L929, a murine fibrosarcoma cell line, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 m*M* glutamine (Gln), 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin. Adaptation of the cells to Gln-free culture conditions was performed by a stepwise decrease in Gln concentration in the medium to zero over a 2-month period (Goossens *et al.*, 1996). The resulting $L929^{glc}$ population was routinely maintained in DMEM free of Gln (and glutamate) (Gibco Bio-Cult, Paisley, UK), supplemented with 10% dialyzed heat-inactivated FCS (Gibco Bio-Cult) and antibiotics. To discriminate on the basis of

respiratory substrate, the parental L929 cells were termed $L929^{gln}$ cells. Suspension cultures of the normally adherent-growing $L929^{glc}$ and $L929^{gln}$ cells were set up when required for flow cytometry or for oxygen consumption measurements. To that end, adherent cells were harvested from tissue culture flasks by trypsinization at 37°C, washed, and resuspended in culture medium. The cells were seeded in 90- or 30-mm diameter bacterial-grade Petri dishes at 3–5 × 10^5 cells/ml and incubated overnight at 37°C in a humidified, 5% CO_2 incubator. Under these conditions, the cells no longer adhered to the plastic surface and remained in suspension. TNF sensitivity of the cells was not altered in these suspension cultures (Grooten *et al.*, 1993).

TNF and reagents

Recombinant murine TNF was produced in *Escherichia coli* and purified to at least 99% homogeneity. The preparation had a specific activity of 2.2 × 10^8 IU/mg protein and contained <4 ng endotoxin/mg of protein. TNF activity was determined as described previously (Ostrove and Gifford, 1979), using an international standard TNF preparation (code no. 88/532, obtained from the Institute for Biological Standards and Control, Potters Bar, UK) as a reference. Unless otherwise mentioned, TNF was added at a final concentration of 1,000 IU/ml. DHR123 from Molecular Probes (Eugene, OR) was prepared as a 5 m*M* stock solution in dimethylformamide, stored at −20°C and used at a final concentration of 1 μM. Rotenone from Sigma Chemical Co (Deisenhofen, Germany) and thenoyltrifluoroacetetone (TTFA) from Serva (Heidelberg, Germany) were prepared as a 25 m*M* and 250 m*M* stock solution in DMSO, stored at −20°C, and used at a final concentration of 25 μM or 250 μM, respectively. Propidium iodide (PI) from Molecular Probes (Eugene, OR) was prepared as a 3 m*M* stock solution in phosphate-buffered saline (PBS), stored at 4°C, and used at a final concentration of 20 μM. Digitonin was purchased from Sigma Chemical Co. (Deisenhofen, Germany) and used at 0.03% to open cells. Respiration buffer contained 220 m*M* mannitol, 68 m*M* sucrose, 2 m*M* NaCl, 2 m*M* $MgCl_2$, 2.5 m*M* PO_4H_2K, 0.5 m*M* EGTA, 10 m*M* HEPES-NaOH, pH 7.4, and the respective substrates glutamate/malate at 5 m*M* and succinate at 10 m*M*.

PI exclusion assay and quantitative flow cytometry

Cell samples were taken from suspension cultures and PI was added 3–10 min before analysis on a FACStar plus (Beckton Dickinson, San Jose, CA). Cell debris and cell aggregates were gated out electronically. The PI dye was excited with a water-cooled argon-ion laser (Enterprise II, Coherent, Santa Clara, CA) at 488 nm. PI fluorescence was measured above 590-nm wavelength using a long-pass filter. Routinely, 5,000 cells were analyzed. S.D. on duplicate samples was consistently <5%.

Measurement of ROS formation by flow cytometry

The ROS-sensitive probe DHR123 was added to suspension cultures at the onset of the experiment. Cell samples were taken from the suspension cultures at regular time intervals and analyzed on a FACStar plus (Beckton Dickinson) as described before. The DHR123-derived R123 fluorescence was excited with a water cooled argon-ion laser at 488 nm and detected with a 530-nm wavelength band-pass filter. Cell debris and aggregates were electronically gated out. R123 fluorescence was measured on the viable PI-negative cell population and was measured on 5,000 viable cells/sample.

Measurement of intracellular NAD(P)H content by flow cytometry

Cell samples were taken from the suspension cultures at regular time intervals and analyzed on a FACStar plus (Beckton Dickinson). NAD(P)H levels are measured as the cell's blue autofluorescence. Cells were excited with a water cooled multi-line UV laser (INNOVA 90, Coherent, Palo Alto, CA) at 330–360 nm and detected between 400 and 470-nm wavelength. Cell debris and aggregates were electronically gated out. Blue autofluorescence was measured on the viable PI-negative cell population and was measured on 5,000 viable cells/sample.

Oxygen consumption

Oxygen consumption was measured with a Clark-type microelectrode in a 1-ml cell (Strathkelvin Instruments, Glasgow, UK) equilibrated at 37°C. For intact cells: cells were grown in suspension culture overnight, harvested, counted, and resuspended in fresh medium saturated with O_2. O_2 consumption was followed for 5–15 min, depending on the rate of consumption. For open cells: cells were grown in suspension culture overnight, harvested, counted, and resuspended in respiration buffer containing 0.03% digitonin. Depending on the conditions, open cells were supplied with 5 m*M* glutamate/malate in the presence of TTFA, with 10 m*M* succinate in the presence of rotenone or with both substrates in the absence of inhibitor. Then 5 m*M* ADP was added to start state 4 respiration. Background O_2 consumption in cell-free medium was 0.25% per min. S.D. on independent preparations was <5%.

RESULTS

TNF-induced cell death is not regulated by levels of intracellular NAD(P)H

Substantial evidence indicates that the balance of reduced over oxidized pyridine nucleotides, especially NADPH/NADP, is an important regulator of cellular redox status (Pandolfi *et al.*, 1995; Tian *et al.*, 1998). Reducing equivalents in the form of NADPH are cofactors of enzymes that recycle the thiol redox buffers glutathione (GSH) and thioredoxin (TRX). In addition, NADH is the substrate for complex I of the electron transport chain and, as a consequence, controls the electron flow rate through this complex. NADPH is one of the products of glucose metabolism through the pentose phosphate pathway, while NADH is mainly produced through dehydrogenases in the mitochondria. Accordingly, different levels of NAD(P)H in L929 cells growing on glucose ($L929^{glc}$) instead of on glutamine ($L929^{gln}$) as major respiratory substrate may cause the decreased TNF-induced ROS generation and cell death through the redox-regulating feature of NADPH or through an altered complex I activity. To verify this possibility, we measured intracellular NAD(P)H levels in $L929^{glc}$ and $L929^{gln}$ cells by flow cytometry. Cellular blue autofluorescence is almost completely due to reduced pyridine nucleotides NADH and NADPH (Thorell, 1981, 1983). In a metabolic steady state, the NAD(P)H/NAD(P) ratio is constant and the resulting blue fluorescence intensity is often characteristic for a particular cell type under given external conditions of substrate concentration and oxygen tension (Koziol, 1971). In agreement with this, we observed a significant difference in NAD(P)H levels between $L929^{glc}$ and $L929^{gln}$ cells. $L929^{glc}$ cells contain about 20% more NAD(P)H than $L929^{gln}$ (Fig. 1A).

To verify if this increased NAD(P)H level directly correlates with an enhanced redox buffering capacity and the decreased sensitivity of $L929^{glc}$ cells to ROS and cell death, we measured the effect of TNF on intracellular NAD(P)H levels in both L929 cell types. Death cells were excluded from this analysis on the basis of uptake of the polar exclusion marker PI, indicative of membrane permeabilization and irreversible damage to the cells (Grooten *et al.*, 1993). Surprisingly, we found that TNF induces a significant increase in cellular NAD(P)H levels in $L929^{gln}$ but especially in $L929^{glc}$ cells, instead of the expected ROS-driven consumption of reducing equivalents (Fig. 1A). Furthermore, the two parameter histograms of Fig. 1B that display the R123 fluorescence derived from the ROS-sensitive fluorogenic marker DHR123 versus NAD(P)H fluorescence, reveal that the ROS-producing cell population coincides with cells containing high NAD(P)H levels, indicating that TNF-treated cells contain high NAD(P)H levels shortly before cell death. These results are contradictory to a role of NADPH as redox buffer in either of the metabolic L929 variants. Rather, they suggest that TNF signaling interferes with the metabolic state of the cell, leading to increased NAD(P)H production or decreased consumption.

Characterization of substrate-specific electron flow through the respiratory chain and its modulation by TNF

The conclusion that the reduced sensitivity to TNF of $L929^{glc}$ cells that switched from glu-

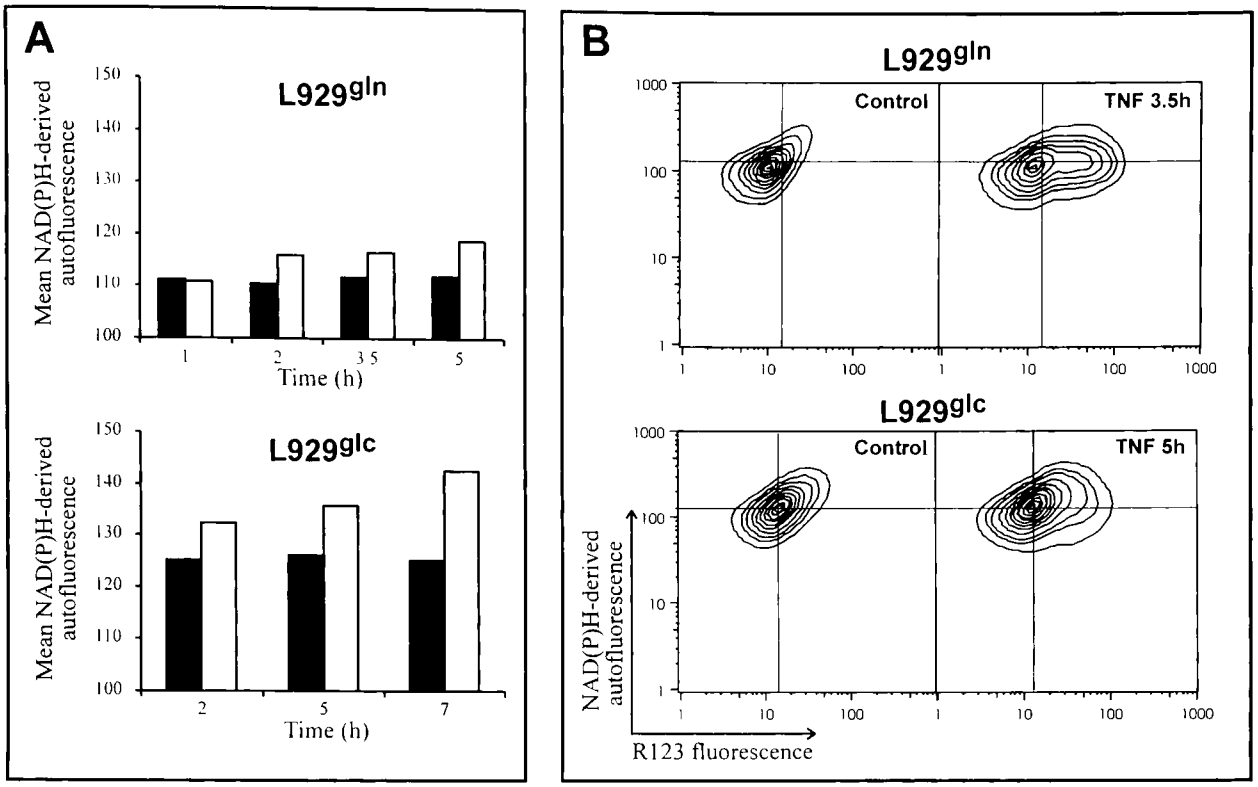

FIG. 1. TNF induces an increase in intracellular NAD(P)H concentrations. A. Mean NAD(P)H-dependent fluorescence intensity in viable, PI-negative cells of untreated (open bars) and TNF-treated (filled bars) L929^{gln} (top) and L929^{glc} cells (bottom) analyzed at different time points after TNF treatment. **B.** Parameter contour maps of ROS-dependent DHR123-derived R123 fluorescence and NAD(P)H-dependent autofluorescence in viable PI-negative cells of untreated (left panels) and TNF-treated (right panels) L929^{gln} (top) and L929^{glc} cells (bottom). L929^{gln} cells were treated with 1,000 IU/ml TNF for 3.5 hr; L929^{glc} cells were treated for 5 hr. The reference lines are drawn through the respective mean fluorescence channel of the untreated population, showing that in TNF-treated cells the ROS-positive cells show high NAD(P)H content compared to untreated cells.

tamine to glucose as respiratory substrate does not reflect an increased NAD(P)H redox capacity led us to determine how the respiratory substrate affects the electron flux through the different complexes of the electron transport chain and if the observed differences could explain the differential TNF responsiveness of L929^{glc} and L929^{gln} cells. As shown in Fig. 2, mitochondrial oxygen consumption was similar or slightly increased in L929^{glc} cells as compared to L929^{gln} cells. However, the relative contribution of complexes I and II to this overall oxygen consumption is different in L929^{glc} and L929^{gln} cells. In L929^{glc} cells, a partial reduction of oxygen consumption was observed by the complex I inhibitor rotenone and by the complex II inhibitor TTFA, whereas in L929^{gln} cells rotenone inhibits nearly all oxygen consumption but TTFA only marginally reduced oxygen consumption. Apparently, in L929^{glc} cells both electron transport chain complexes contribute to a similar degree to the overall electron flow, whereas in L929^{gln} cells the majority of the electrons come from NADH and flow through complex I. These data show that glucose metabolism supplies the cells with complex I as well as complex II substrates while glutamine metabolism sustains a high electron flux through complex I and prevents electron flow through complex II.

As shown in Fig. 2, treatment with TNF of L929^{gln} cells induced a significant increase in oxygen consumption after 2 hr when 10% of the cells exhibited cell death. This TNF effect was completely blocked by the complex I inhibitor rotenone and only marginally by the complex II inhibitor TTFA. Contrary to the TNF effect in glutamine-dependent cells, TNF did

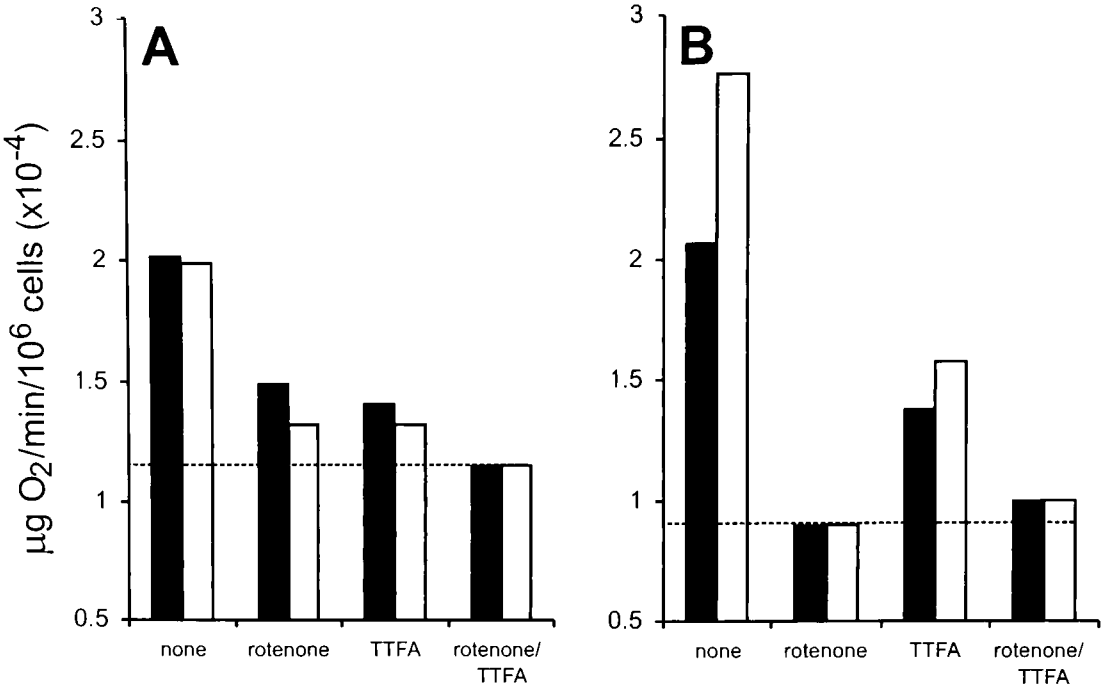

FIG. 2. Substrate-dependent electron flux through complex I and II of the electron transport chain and its modulation by TNF. Oxygen consumption in untreated (filled bars) and TNF-treated (open bars) $L929^{glc}$ **(A)** and $L929^{gln}$ cells **(B)** pretreated for 1 hr with inhibitors of complex I (rotenone) or complex II (TTFA). Nonmitochondrial oxygen consumption was determined by addition of the complex IV inhibitor N_3 (broken line). Combined inhibition of complex I and II results in complete inhibition of mitochondrial oxygen consumption in both cell types.

not alter oxygen consumption in $L929^{glc}$ cells at a time point when 5% of the cells died. These results suggest that TNF induces an increased electron flux through the electron transport chain and that this increased flux occurs through complex I. The glutamine-driven metabolism, already using complex I predominantly, sustains this increased electron flux through complex I while the glucose-dependent metabolism delays or suppresses it.

The electron flux through complex I regulates TNF-induced ROS generation and cell death

To substantiate the involvement of substrate-regulated electron flux through complex I in TNF-induced ROS generation and cell death, the effect of complex-specific inhibitors on both parameters were analyzed in $L929^{glc}$ and $L929^{gln}$ cells (Fig. 3). In agreement with our previous report, TNF-induced ROS generation and cell death were greatly delayed in $L929^{glc}$ (Fig. 3B) as compared to $L929^{gln}$ cells (Fig. 3A). Pretreatment of the cells with rotenone completely inhibited TNF-induced ROS generation and drastically delayed cell death in both cell types, indicating that complex I activity is an important contributor to both responses. In fact, in the presence of the complex I inhibitor, the difference in sensitivity to TNF-induced cell death between $L929^{glc}$ and $L929^{gln}$ cells is abolished. Thus, the different electron flux through complex I observed in these metabolic variants—high complex I activity supported by glutamine metabolism but low complex I electron flux supported by glucose metabolism—underlies the more efficient inhibition of TNF-induced ROS generation and cell death by rotenone in $L929^{gln}$ cells. Pretreatment of the cells with TTFA inhibited TNF-induced ROS generation and cell death equally well as rotenone in $L929^{glc}$ cells but only marginally in $L929^{gln}$ cells.

Rotenone alone induced an increment of basal ROS levels in both L929 metabolic variants (Fig. 3), raising the question if this feature may be responsible for the inhibitory activity on TNF-induced ROS formation and cell death. However, experiments in open cells, depleted of cytosol, showed that these rotenone-induced

radicals are mainly produced by redox-cycling of rotenone in the cytosol and are not of mitochondrial origin (data not shown). The absence of a TNF-induced ROS signal in the presence of rotenone is not associated with the high amounts of ROS generated by redox-cycling of rotenone because menadione, another redox-cycling drug, does not affect TNF-induced ROS nor cell death (data not shown). Thus, we conclude that the inhibitory activity of rotenone on TNF-induced ROS generation and cell death reflects inhibition of complex I activity in L929^{glc} as well as in L929^{gln}.

TNF modulates electron flux through complex I by an indirect mechanism

To verify whether the effects of TNF and oxidative substrate on complex I result from direct modification of complex I proteins, we measured substrate-dependent oxygen consumption in opened, untreated, and TNF-treated cells. Cells were opened by digitonin treatment in respiration buffer, thus allowing leaking out of cytosolic metabolites and proteins while mitochondrial membranes and functionality stay intact (Single *et al.*, 1998). The open cells were supplied with glutamate/

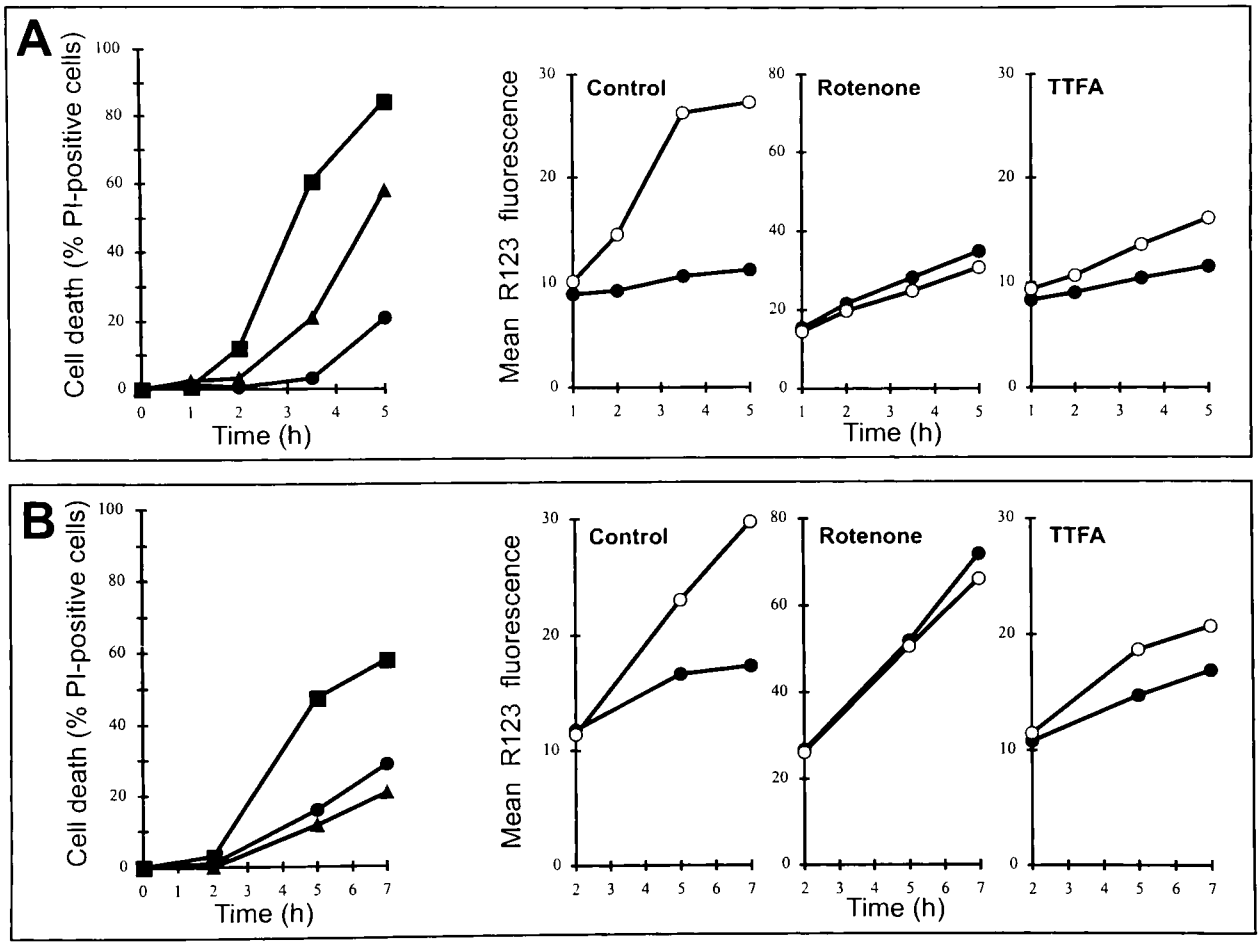

FIG. 3. Effect of complex I and II inhibitors on TNF-induced cell death and ROS generation. A. Left panel. Time-dependent TNF-induced cell death in L929^{gln} cells without pretreatment (square), pretreated for 1 hr with rotenone (circle) or with TTFA (triangle). **Right panel.** Mean ROS dependent DHR123-derived R123 fluorescence of viable, PI-negative, untreated (filled circles) and TNF-treated (open circles) L929^{gln} cells without pretreatment (left) pretreated for 1 hr with rotenone (middle) or with TTFA (right). **B. Left panel.** Time-dependent TNF-induced cell death in L929^{glc} cells without pretreatment (square), pretreated for 1 hr with rotenone (circle) or with TTFA (triangle). **Right panel.** Mean ROS-dependent DHR123-derived R123 fluorescence of viable, PI negative, untreated (filled circles) and TNF-treated (open circles) L929^{glc} cells without pretreatment (left) pretreated for 1 hr with rotenone (middle) or with TTFA (right).

malate as complex I substrates and succinate as complex II substrate. These substrates were given combined or separately to measure overall respiration or respiration driven by complex I or II, respectively. ADP was added to obtain maximal respiration rates. The results are summarized in Fig. 4. As described above, intact L929^{gln} cells, included in this experiment as a positive control, exhibited increased oxygen consumption upon TNF treatment whereas oxygen consumption in intact L929^{glc} cells was not affected. The TNF effect on oxygen consumption in intact cells was abolished in open cells, irrespective of the substrate supplied. Apparently, TNF does not alter the intrinsic activity of complex I but modulates its activity indirectly, probably at the level of NADH substrate availability. Also, the difference in complex I and II activity between L929^{glc} and L929^{gln} cells is abolished in open cells, indicating that substrate availability rather than an altered stoichiometry or enzyme activity of the electron transport chain underlies this difference in intact cells.

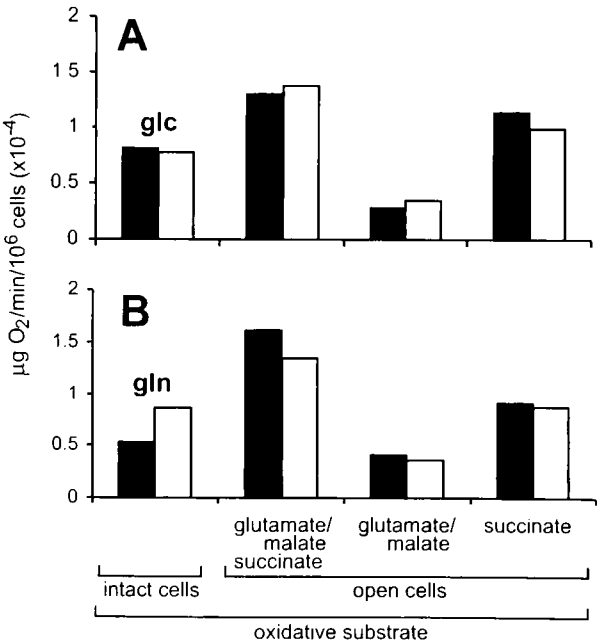

FIG. 4. Effect of TNF and respiratory substrate on respiration rates through complex I and II. Oxygen consumption in untreated (filled bars) and TNF-treated (open bars) L929^{glc} **(A)** and L929^{gln} cells **(B)**. Cells were either intact or opened with digitonin (0.03%) in respiration buffer containing the indicated respiratory substrates. Maximal respiration was achieved by addition of 5 m*M* ADP.

DISCUSSION

In this paper, we identified the mitochondrial components involved in TNF-induced production of ROS and their regulation by bioenergetic pathways. On the basis of the identification of the electron transport chain as the source of the TNF-induced ROS (Higuchi *et al.*, 1992; Schulze-Osthoff *et al.*, 1992) and on our previous observation that the respiratory substrate used by the cells—glutamine vs. glucose—affects ROS production and cell death (Goossens *et al.*, 1996), we analyzed the electron transport chain activity in L929 cells that are dependent on glucose or glutamine for their oxidative energy metabolism.

A link between cellular energy metabolism and oxidative stress was provided by Lee *et al.*, who showed that glucose deprivation increased intracellular H_2O_2 production and oxidized GSH levels (Lee and Corry, 1998). A decreased formation of NADPH, and thus a decreased ability to recycle oxidized GSH, were proposed by the authors as likely causes. In agreement with this mechanism, conditions of oxidative stress are accompanied by an elevated activity of glucose-6-phosphate dehydrogenase (G6PDH) and the pentose phosphate pathway (Slekar *et al.*, 1996; Ursini *et al.*, 1997). The pentose phosphate pathway is the major cellular source of NADPH, which is essential for protection against even mild oxidative stress (Pandolfi *et al.*, 1995). However, our current study shows that although higher NAD(P)H levels are detected in the desensitized L929^{glc} cells, TNF increases the levels of NAD(P)H in both cell types, ruling out a TNF-induced depletion of reducing equivalents as a cause for ROS production. To the contrary, these results show that TNF either stimulates the production or inhibits the consumption of NAD(P)H.

On the basis of the identification of a TNF-induced increased electron flux through complex I, a stimulated NAD(P)H production by TNF appears to be the correct alternative. Thus, a multiparameter analysis of electron flux through the mitochondrial electron transport

chain, ROS levels, and cell death convincingly demonstrated a dependence on complex I and not, or to a marginal degree, on complex II activity. Also, the sensitizing effect of glutamine metabolism as opposed to glucose metabolism correlated with a differential contribution of complex I to the overall electron flux. Indeed, glucose metabolism conveyed electrons through both complexes I and II while glutamine oxidation channeled electrons nearly exclusively through complex I, even preventing electron flux through complex II. In $L929^{glc}$ cells, oxidation of glucose through the citric acid cycle gives rise to complex I and II substrates, NADH, and succinate. $L929^{gln}$ cells feature a tumor-specific energy metabolism, characterized by high efflux of lactate from glucose and oxidation of glutamine to support oxidative phosphorylation. Overexpression of glutaminase, high transaminase activity converting glutamate to the citric acid intermediate α-ketoglutarate, and a very active malate/aspartate shuttle producing oxaloacetate support this glutamine (Moreadith and Lehninger, 1984; Matsuno, 1987). In this metabolic condition, succinate, even if present, cannot be efficiently oxidized because succinate dehydrogenase is inhibited by oxaloacetate (Garland *et al.*, 1967).

We established that the differential electron flux through complex I underlies the differential sensitivity of $L929^{glc}$ cells and $L929^{glc+gln}$ cells to TNF, showing that high electron flux through complex I correlates with high TNF-induced ROS production and cell death and that inhibition of electron flux through complex I abolishes the differential sensitivity of the metabolic variants to TNF-induced cell death. Our results support a mechanism whereby the TNF-induced increase in NAD(P)H levels enhances electron flux through complex I in glutamine-dependent cells but not, or to a lesser degree in glucose-dependent cells. A further argument in favor of such mechanism is the observation that neither TNF nor the respiratory substrate alter the intrinsic activity of complex I, indicating that the various levels of complex I activity are imposed by substrate (NADH) availability.

In spite of these strong arguments for an exclusive involvement of complex I in TNF-induced ROS generation and cell death, the results with the complex II inhibitor TTFA might favor a shared contribution of complex I and II to TNF signaling. However, one possible interpretation of the results considers the impact of complex II inhibition on complex I activity. From the metabolic pathways involved in glucose metabolism in $L929^{glc}$ cells, a strong impact of complex II inhibition on complex I activity could be expected as accumulation of succinate and succinyl-CoA would inhibit also NADH producing steps of the citric acid cycle, thus limiting substrate supply to complex I. In $L929^{gln}$ cells, where complex II activity is very low and substrate supply to complex I is not ruled by the citric acid cycle alone, the effect of complex II inhibition on complex I activity should be less pronounced. Such differential impact of complex II inhibition on complex I activity in both metabolic variants could explain the stronger impact of TTFA on the TNF responsiveness of $L929^{glc}$ cells and the marginal impact in $L929^{gln}$ cells. However, further experiments are required to confirm this hypothesis.

Although our results argue for a complex I-mediated ROS generation, earlier work from our laboratory provided evidence for a TNF-induced ROS generation at complex III of the electron transport chain. This conclusion was reached on the basis that complex I and II inhibitors protect L929 cells from TNF-induced cell death while complex III inhibitors (antimycin A and myxothizol) enhance TNF-induced cell death (Schulze-Osthoff *et al.*, 1992). In these experiments, cell death was estimated by measuring dehydrogenase activity through reduction of MTT. However, subsequent experiments showed that inactivation of dehydrogenase activity is a late event. Dead cells, as judged by PI uptake, still fully reduce MTT for several hours (Grooten *et al.*, 1993). Measurement of the effect on cell death of the same complex III inhibitors, but now using membrane permeabilization as parameter of cell death, showed only marginal synergism of antimycin A, while myxothiazol was neutral (data not shown). Accordingly, the previously reported synergism of complex III inhibitors with TNF-induced cell death reflects an enhancement of events in the cell death process that occur posterior to membrane permeabilization and loss

of clonogenicity, rather than an enhancement of the actual causes of cell death, namely the generation of ROS.

Recently, mitochondria have been recognized as key players in apoptotic cell death pathways. The best-characterized mitochondrial mechanisms involved in apoptosis is the opening of the permeability transition pore and release of apoptosis-activating factors such as cytochrome *c* and apoptosis-inducing factor (AIF) (Zou *et al.*, 1997; Susin *et al.*, 1999a,b). From our results, it is clear that additional mitochondrial phenomena may actively participate in cell death, including the caspase-independent mitochondria and ROS-dependent necrotic cell death pathway studied in this paper (Grooten *et al.*, 1993; Vercammen *et al.*, 1998a). Our results establish that the mitochondrial participation in this cell death pathway cannot be disconnected from interactions with the cytosol and the cellular environment that determines the activity of the complexes I and II of the electron transport chain. This observation may be more general and is also applicable on the regulation of the permeability transition pore. It has been shown that permeability transition is regulated by respiratory substrates, more specifically by the electron flux through complex I (Fontaine *et al.*, 1998). The possibility that the mitochondria and ROS-dependent cell death pathway described acts as a back-up mechanism for caspase-dependent apoptosis (Kawahara *et al.*, 1998; Vercammen *et al.*, 1998a,b) broadens the implications of this study beyond the specific cell type studied in the present paper. Because Gln is abundantly present in body fluids and its concentration may alter during the progression of neoplastic disease (Parry-Billings *et al.*, 1991), maintaining high concentrations in circulation may affect the responsiveness of certain tissues and cells to cell death signals.

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ABBREVIATIONS

AIF, apoptosis-inducing factor; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DD, death domain; DHR123, dihydrorhodamine 123; DISC, death-inducing signaling complex; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; G-6PDH, glucose-6-phosphate dehydrogenase; Glc, glucose; Gln, glutamine; GSH, glutathione; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; TNF, tumor necrosis factor; TRX, thioredoxin; TTFA, thenoyltrifluoroacetone.

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