Immunology 1995 **84** 543–548

DNA damage induced by tumour necrosis factor-α in L929 cells is mediated by mitochondrial oxygen radical formation

Y. SHOJI, Y. UEDONO, H. ISHIKURA, N. TAKEYAMA & T. TANAKA *Department of Emergency and Critical Care Medicine, Kansai Medical University, Moriguchi, Osaka, Japan*

SUMMARY

Treatment of L929 cells with tumour necrosis factor-α (TNF-α) plus actinomycin D induced DNA damage (indicated by the appearance of a sub-G_1 peak due to extracellular leakage of low molecular weight DNA following DNA fragmentation) before significant cell lysis occurred. The DNA damage occurred in parallel with a decrease of the intracellular total glutathione content and an increase of intracellular reactive oxygen intermediates (ROI), as indicated by increased dihydrorhodamine 123 oxidation. Because the inhibition of mitochondrial respiration suppressed the increase of dihydrorhodamine 123 oxidation and DNA damage as well as the decrease in the total glutathione content, it was suggested that increased mitochondrial formation of ROI was responsible for DNA damage after TNF treatment. Deferoxamine (a ferric iron chelator) and dithiothreitol (a sulfhydryl reagent) both prevented DNA damage and cell killing, indicate that hydroxyl radicals generated from O_2^- and H_2O_2 produced by the mitochondria in a process catalysed by iron contributed to DNA damage and that this pathway may be involved in TNF-α-induced cytotoxicity. An inhibitor of poly(ADP)-ribose polymerase (3-aminobenzamide), worsened DNA damage, but was protective against cell lysis, suggesting that DNA repair subsequent to injury was more important than DNA damage *per se* in development of TNF-α cytotoxicity.

INTRODUCTION

Tumour necrosis factor (TNF) is a cytokine primarily produced by members of the mononuclear phagocyte system in response to particulate and soluble inflammatory stimuli, which elicits a large number of biological actions including hemorrhagic necrosis of transplanted tumours, immunoregulatory effects, and an antiviral response.[1,2] Recently, TNF has been shown to act as a critical mediator of systemic inflammatory responses, both by a direct effect on cells at sites of inflammation and by influencing other cells of the immune system, such as neutrophils.

Although the direct cytotoxic effect of TNF on many types of tumour cell lines has been studied extensively, the mechanisms involved in the induction of TNF cytotoxicity after binding to its target cell-surface receptors are not fully understood.[2] Many intracellular processes, such as DNA fragmentation, adenosine diphosphate (ADP) ribosylation, phospholipase activation, oxidative stress, an increase in cytosolic Ca^{2+}, and induction of endogenous nucleases, are possibly involved in TNF-induced cell lysis.[3] A recent study by Hennet *et al.*[4] using L929 cells showed that TNF induces superoxide anion (O_2^-) production from the mitochondria and suggested an important contribution of the mitochondrial respiratory chain to TNF cytotoxicity.[4]

TNF induces DNA strand breaks in L929 cells not only at a dose that causes the subsequent loss of cell viability but also at a lower dose that allows continuing cell viability.[5,6] Since DNA fragmentation is believed to be the hallmark of apoptosis, sometimes referred to as programmed cell death, TNF cytotoxicity has been proved to be due to apoptosis in some cell lines.[5,7] Although oxidative stress can result in DNA damage and is assumed to be a mediator of apoptosis, the interrelationship between TNF cytotoxicity, DNA damage, and the generation of mitochondrial reactive oxygen intermediates (ROI) remains uncertain.[8].

Accordingly, the aim of this study was to assess the relative contribution of mitochondrial ROI to TNF-induced cytotoxicity and DNA damage using a TNF-sensitive cell line (L929 cells). We found that TNF increases mitochondrial ROI generation and DNA damage in L929 cells before apparent cell

Received 21 September 1994; accepted 19 December 1994.

Abbreviations: 3AB, 3-aminobenzamide; DHR, dihydrorhodamine 123; DPPD, N,N',-diphenylphenylene-diamine; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; MMP, mitochrondrial membrane potential; O_2^-, superoxide; PBS, phosphate-buffered saline; PI, propidium iodide; ROI, reactive oxygen intermediates; TNF, tumour necrosis factor.

Correspondence: Yoshikazu Shoji, Department of Emergency and Critical Care Medicine, Kansai Medical University, Fumizono-cho 1, Moriguchi, Osaka, 570 Japan.

damage develops. As shown by the response to mitochondrial respiratory blockers, mitochondrial respiration represents an important source of ROI following TNF treatment, and increased ROI production may be responsible for both DNA damage and subsequent cell damage.

MATERIALS AND METHODS

Reagents

Recombinant murine TNF-α was purchased from Gibco Laboratories (Grand Island, NY). Dihydrorhodamine 123 (DHR) was purchased from Molecular Probes (Eugene, OR). Actinomycin D, rhodamine 123, propidium iodide (PI), deferoxamine mesylate, rotenone, antimycin A, N,N′-diphenylphenylene diamine (DPPD), 3-aminobenzamide (3AB), and other reagents were purchased from Sigma (St Louis, MO).

Cell culture

L929 cells (NCTC clone 929) from the ATCC (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (Gibco) with 10% heat-inactivated newborn calf serum, 100 U/ml of penicillin, 0·1 mg/ml of streptomycin and 0·25 μg/ml of amphotericin B. The cells were maintained at 37° in a humidified atmosphere of 5% CO_2/95% air. For all experiments, cells were seeded in a 9·2 cm^2 dish at 1·7 × 10^5 cells/ml (2 ml/dish) and cultured for 36–48 hr just before reaching confluence. The cells were washed with phosphate-buffered saline (PBS), replenished with fresh medium, treated with actinomycin D (0·5 μg/ml) and TNF-α (1 ng/ml), and then further incubated at 37° for up to 12 hr. In some experiments, cells were pretreated with various compounds at 30 min before TNF treatment.

TNF cytotoxicity assay

Cell viability was quantified by measurement of lactate dehydrogenase (LDH) release into the medium. The released LDH was expressed as a percentage of total cellular LDH, which was determined after complete lysis of the cells with 0·2% Triton-X 100 (Wako Pure Chemicals, Tokyo, Japan). The LDH activity of each sample was measured by a standard spectrophotometric assay.[9]

Glutathione assay

We measured the intracellular content of glutathione (γ-glutamylcysteinylglycine, GSH) in L929 cells after treatment with TNF-α to determine the level of intracellular oxidative stress. After incubation for 4 hr with TNF-α and actinomycin D, the cells were washed twice with PBS, trypsinized, centrifuged, and the pellet was resuspended in 800 μl of PBS. Various test compounds were added 30 min before TNF-α treatment. An aliquot of each sample was diluted and the number of cells was counted using a hemocytometer. The remainder of each sample was extracted with 4 ml of 5% sulfosalicylic acid, centrifuged at 10 000 ***g*** for 10 min at 4°, and stored at −60° until measurement. Intracellular total glutathione (GSH + 2GSSG) was measured by high-performance liquid chromatography (HPLC) using the assay of Neuschwander–Tetri.[10] Briefly, 500 μl of acid extract was mixed with 250 μl of 0·1 M Tris (pH 8·5), 200 μl of 0·8 N NaOH, and 250 μl of 50 mM dithiothreitol to reduce GSSG and the acid-soluble mixed disulfides to GSH, and incubated for 30 min. After 30 min at room temperature, GSH was derivatized with orthophthalaldehyde and the resulting highly fluorescent GSH-orthophthalaldehyde adduct was separated and quantified by HPLC (LC-6A, Shimadzu, Kyoto, Japan). Chromatography was performed on a Wakosil–ODS column (4 × 250 mm I.D., 5 μm particle size, Wako Pure Chemicals, Osaka, Japan) using an isocratic mobile phase of 7·5% methanol/92·5% 0·15 M sodium acetate (pH 7·0, 40°), at a flow rate of 0·7 ml/min. Peaks were detected by the measurement of fluorescence at 420 nm after excitation at 340 nm (RF-540, Shimadzu). The GSH content was quantitated by comparison with a standard curve generated with known amounts of GSH, and was expressed as nanomoles per 10^6 cells.

Intracellular ROI generation

Intracellular ROI generation was quantified by flowcytometric measurement of cellular metabolism of DHR to rhodamine 123.[11,12] Non-fluorescent DHR is taken up by the cells and converted by intracellular ROI into green fluorescent rhodamine 123. Rhodamine 123 preferentially accumulates within the mitochondria, and thus is sequestered intracellularly. Conversion from the non-fluorescent to the fluorescent form of this probe is relatively specific for ROI, since there is a low rate of oxidation due to spontaneous and non-ROI dependent reactions.[11] Cells were treated with TNF-α and actinomycin D, together with 5 μM DHR for 3 hr. In some experiments, rotenone and antimycin A were added 30 min before TNF-α treatment. After incubation, cells were washed twice with PBS, trypsinized, resuspended in PBS, and analysed by flowcytometry on an Epics Profile-II cell analyser (Coulter, Hialeah, FL) using an argon laser (150 mW, 488 nm). Green fluorescence was collected through a 530 nm bandpass filter set and the data were presented as a linear scale. Forward-scatter and side-scatter data were also collected and used to eliminate fluorescent signals associated with cell debris. For determination of the mitochondrial membrane potential (MMP), cell suspensions were loaded with 500 nM of rhodamine 123 for 10 min, and directly assayed by flowcytometry as described above.

DNA staining

After incubation for 4 and 6 hr with TNF-α and actinomycin D, cells were washed twice with PBS, and fixed in 70% ice-cold ethanol for 30 min at 4°. In some cases, several test compounds were added 30 min before TNF-α treatment. After fixation, cells were washed twice, incubated for 30 min with 1 mg/ml of RNase, and stained with 50 μg/ml of PI for 10 min.[13] Red fluorescence from PI was collected through a 630 nm bandpass filter set and the data were presented as a linear scale. The cell-cycle distribution was analysed by the Multicycle program (Phoenix Flow Systems, San Diego, CA) after all data had been stored.[14]

Statistical analysis

All data were expressed as the mean ± standard error (SE) or standard deviation (SD). Statistical significance was determined by ANOVA. If the analysis gave a significant result ($P < 0·05$), further comparison of individual data was done by Scheffé's test.

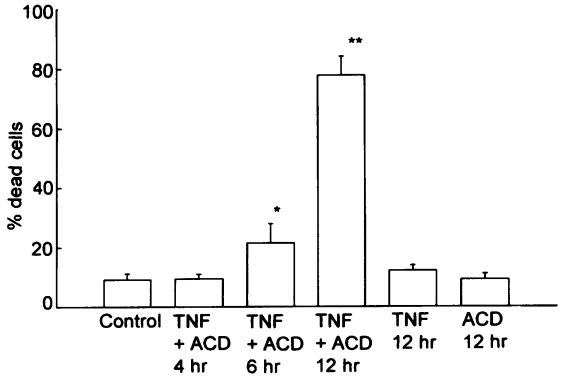

Figure 1. Time course of the death of cultured L929 cells after treatment with 1 ng/ml TNF-α and 0·5 mg/ml actinomycin D (ACD). Cell death was assessed by the release of lactate dehydrogenase into the culture medium. Values are the mean ± SD of determinations from 5–6 separate cultures. $*P < 0.01$, $**P < 0.0001$ compared with control.

RESULTS

Cytotoxic effect of TNF

Treatment of L929 cells with 1 ng/ml TNF-α and 0·5 μg/ml actinomycin D for 4 hr did not increase LDH leakage when compared with control cultures (Fig. 1). However, LDH leakage was slightly increased after 6 hr of TNF treatment and became more pronounced after 12 hr. At 12 hr, almost all of the cells were detached from the culture dish. When applied alone, neither TNF-α nor actinomycin D caused any increase of LDH leakage. The effects of pretreatment with various agents on TNF cytotoxicity were evaluated at 12 hr after treatment with 1 ng/ml TNF-α and 0·5 μg/ml actinomycin D (Table 1). An iron chelator (deferoxamine), a sulfhydryl reagent (dithiothreitol), and an inhibitor of poly(ADP–ribose) polymerase (3AB), all effectively prevented TNF cytotoxicity. The contribution of mitochondrial respiration to TNF cytotoxicity was evaluated using specific respiratory chain inhibitors. An inhibitor of complex I, rotenone, which blocks the electron flow from nicotinamide adenine dinucleotide phosphate (NADH) dehydrogenase to the ubiquinone pool, also prevented cell killing. On the other hand, antimycin A, an inhibitor of the bc_1-site of complex III, failed to inhibit LDH leakage. Rotenone and antimycin A did not influence LDH leakage from L929 cells for up to 12 hr in the absence of TNF and actinomycin D (data not shown). No protection against TNF cytotoxicity was observed in the presence of a lipid-soluble antioxidant (DPPD).

Table 1. Effect of various agents on the cytotoxicity of TNF-α

Agents		*n*	% Dead cells
TNF		8	77·9 ± 6·3
TNF			
+ Deferoxamine	20 mM	6	35·6 ± 7·4*
+ Dithiothreitol	2 mM	5	45·2 ± 13·2*
+ N,N′,-diphenylphenylene diamine	5 μM	5	83·2 ± 5·9
+ Rotenone	22·5 μM	5	40·6 ± 9·5*
+ Antimycin A	18 μM	5	68·2 ± 2·4
+ 3-Aminobenzamide	30 mM	5	32·8 ± 10·1*

The death of cultured L929 cells after treatment for 12 hr with 1 ng/ml TNF-α and 0·5 mg/ml actinomycin D was assessed by the release of lactate dehydrogenase into the culture medium. Some cultures were pretreated with various agents for 30 min prior to TNF treatment. Values are the mean ± SD of the determinations for the indicated number (*n*) of separate cultures. $*P < 0.001$ compared with TNF.

Table 2. Effect of various agents on the cellular glutathione content of TNF-treated cells

Agents		*n*	Total glutathione (nmol/10^6 cells)
Saline control		7	20·08 ± 2·23
TNF		6	12·51 ± 1·53*
TNF			
+ Rotenone	22·5 μM	5	18·58 ± 2·91**
+ Antimycin A	18 μM	6	18·50 ± 2·78**
+ Deferoxamine	20 mM	5	18·08 ± 1·13***
+ Dithiothreitol	2 mM	6	17·79 ± 2·11***

The total glutathione (GSH + GSSG) content of cultured L929 cells treatmented with 1 ng/ml TNF-α and 0·5 mg/ml actinomycin D for 4 hr was measured as described in the Materials and Methods. Some cultures were pretreated with various agents for 30 min prior to TNF treatment. Values are the mean ± SD of determinations for the indicated number (*n*) of separate cultures. $*P < 0.001$ compared with control. $**P < 0.05$, $***P < 0.01$ compared with TNF.

Changes in intracellular GSH

When the intracellular total GSH level in cells was measured at 4 hr after the addition of TNF and actinomycin D, there was a 62·3% fall of the total GSH level compared with the control (Table 2). Pretreatment with mitochondrial inhibitors, rotenone (22·5 μM) and antimycin A (18 μM), prevented this decrease of intracellular total glutathione. The iron chelator (Deferoxamine)

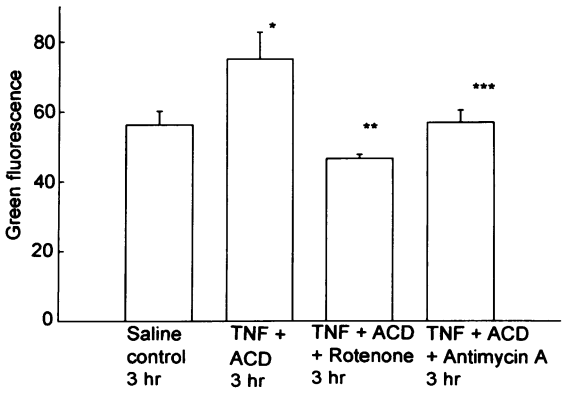

Figure 2. Dihydrorhodamine 123 oxidation by L929 cells at 3 hr after incubation with 1 ng/ml TNF-α and 0·5 mg/ml actinomycin D (ACD). Cells were pretreated with 22·5 μM rotenone or 18 μM antimycin A for 30 min before TNF-α treatment. The green fluorescene intensity of rhodamine 123 was measured as described in the Materials and Methods. Values are the mean ± SD from the determinations on 4–5 separate cultures. $*P < 0.0005$ compared with control. $**P < 0.0001$, $***P < 0.001$ compared with TNF-α treatment.

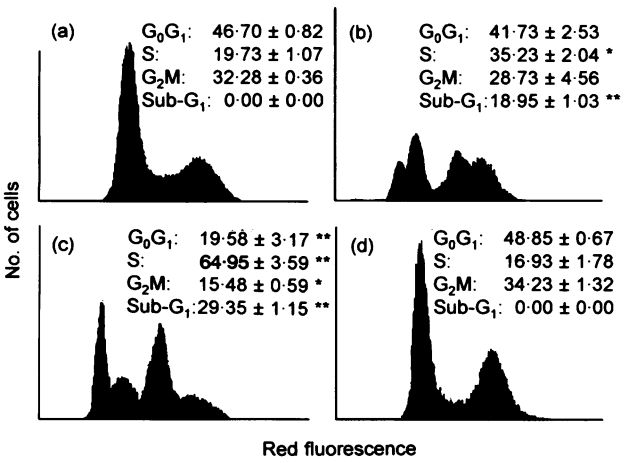

Figure 3. Changes in the cell-cycle distribution of L929 cells. DNA frequency histograms (after staining with propidium iodide) of cells from the control (time 0) cultures (a), from cultures treated with 1 ng/ml TNF-α and 0·5 mg/ml actinomycin D for 4 (b), and 6 (c) hr, and from cultures treated with 0·5 mg/ml actinomycin D alone for 6 hr (d). The percentage of healthy cells in each phase is shown as the mean ± SE of the determinations for 4–5 separate cultures. $*P<0·01$, $**P<0·0001$ compared with control.

and the sulfhydryl reagent (dithiothreitol) were also effective in maintaining the intracellular glutathione level.

Oxidation of DHR

Intracellular ROI generation following TNF treatment of L929 cells was quantified by the oxidation of non-fluorescent DHR to fluorescent rhodamine 123 (Fig. 2). At 3 hr after the addition of TNF and actinomycin D, the fluorescence intensity was increased about 1·34-fold when compared with the control. The increase of fluorescent intensity was restrained to control levels by pretreatment with rotenone and antimycin A. This finding suggested that accelerated ROI formation following TNF treatment may arise from the mitochondrial electron transport system. To assess the influence of TNF treatment on the MMP, which is a critical determinant of the intracellular sequestration of DHR, we estimated this potential by rhodamine 123 staining at 3 hr after TNF treatment. There was no signfiicant difference between control and TNF-treated cells (data not shown).

Cell-cycle distribution

Figure 3 shows the time course of changes in the cell-cycle distribution following TNF and actinomycin D treated of L929 cells. There were no cells with DNA damage after 3 hr (data not shown), but cells with poor DNA staining [weaker than that of G_1 cells (sub-G_1 peak)[13,14]] began to appear at 4 hr, although no LDH leakage was observed (Table 1). At 6 hr after TNF treatment when LDH leakage began to occur, cells with DNA damage increased considerably. Treatment with actinomycin D alone for 6 hr had no effect on the cell-cycle distribution.

Table 3 shows the effect of various agents on the cell-cycle distribution following TNF and actinomycin D treatment of L929 cells. Both rotenone and antimycin A, which suppress the decrease of intracellular glutathione and the increase of DHR oxidation, reduced the development of DNA-damaged cells. Pretreatment with both DFX and dithiothreitol also suppressed the emergence of DNA-damaged cells.

DISCUSSION

This study showed that treatment of L929 cells with TNF-α produced a rapid increase of intracellular ROI and a marked diminution of the total GSH content. The inhibitory effect of specific mitochondrial respiratory chain inhibitors such as rotenone and antimycin A on the enhancement of DHR oxidation by TNF-α strongly indicates that this cytokine caused accelerated leakage of ROI from the mitochondrial electron transport system. It has long been known that O_2^- and H_2O_2 can be generated during mitochondrial respiration, and it has been hypothesized that this phenomenon may be responsible for reperfusion injury[15] and hyperbaric oxygen

Table 3. Effect of various agents on the cell-cycle distribution of L929 cells treated with TNF

Agents		n	Cell cycle (%) G0G1	S	G2M	DNA-damaged cells (%)
Saline control		4	46·7 ± 0·8	19·7 ± 1·1	32·3 ± 0·3	0·0 ± 0·0
Actinomycin D		4	48·9 ± 0·7	16·9 ± 1·8	32·4 ± 1·3	0·0 ± 0·0
TNF + Actinomycin D		4	41·7 ± 2·5	35·2 ± 2·0*	28·7 ± 4·6	19·0 ± 1·0**
TNF + Actinomycin D						
+ Deferoxamine	20 mM	4	51·0 ± 0·5	27·0 ± 2·1	22·0 ± 1·7	0·0 ± 0·0***
+ Dithiothreitol	2 mM	5	53·4 ± 0·8	29·8 ± 1·4	16·9 ± 1·5	0·0 ± 0·0***
+ Rotenone	22·5 μM	4	43·3 ± 2·5	36·6 ± 2·2	20·1 ± 2·2	1·5 ± 1·5***
+ Antimycin A	18 μM	5	42·8 ± 2·4	36·6 ± 1·4	20·6 ± 2·8	5·6 ± 2·3****
+ 3-Aminobenzamide	30 mM	4	42·4 ± 2·9	33·9 ± 3·2	23·8 ± 2·7	25·9 ± 2·5

The cell-cycle distribution of cultured L929 cells treated with 1 ng/ml TNF-α and 0·5 mg/ml actinomycin D for 4 hr was estimated by flow cytometry as described in the Materials and Methods. Some cultures were pretreated with various agents for 30 min prior to TNF treatment. Values show the percentage of cells (± SE) in different phases of the cell cycle (for the healthy cell population) as well as the percentage of DNA-damaged cells in 4–5 separate cultures. $*P<0·005$, $**P<0·0001$ compared with control. $***P<0·0001$, $****P<0·0005$ compared with TNF.

toxicity.[16] The present findings are in good agreement with a recent study of Hennet *et al.*,[4] who demonstrated that TNF-α induced an increase in the signal originating from L929 mitochondria when using lucigenin-enhanced chemiluminescence for the detection of O_2^-. A similar hypothesis has also been proposed by Schulze–Othoff *et al.*[17] However, an opposite view is held at by Meier *et al.*,[18] who reported that TNF-α enhanced the extra-mitochondrial O_2^- production (measured by cytochrome *c* reduction) in human fibroblasts. This discrepancy is probably due to differences in the cells used and the method of O_2^- measurement.

It is estimated that under normal conditions 1–2% of oxygen utilized by the mitochondria is converted to O_2^- and H_2O_2.[19] This physiologic level of ROI can be normally detoxified by endogenous intracellular scavenger systems such as GSH/glutathione peroxidase, catalase and superoxide dismutase. However, TNF-α may alter the balance between mitochondrial generation of ROI and these scavengers. In this study, a decrease of the intracellular GSH content occurred in parallel with the increase of DHR oxidation. It is not clear whether the TNF-α-mediated alteration of intracellular GSH resulted from an increase in glutathione-S conjugates and GSSG efflux from the cells after the oxidation of GSH during the detoxification of excess H_2O_2 derived from the mitochondria,[9] or whether it was due to other mechanisms independent of mitochondrial ROI generation. In the latter case, as a result of the depletion of scavengers, the cells would become more sensitive to low levels of ROI. Increased susceptibility to intracellular and extracellular oxidative stress secondary to the depletion of cellular GSH has been reported in the case of pulmonary vascular endothelial cells exposed to TNF,[9] as well as ethanol-induced[20] and endotoxin-induced[21] hepatic damage. However, the former hypothesis would seem to be more likely. In the present study, the addition of respiratory chain inhibitors lessened not only the enhancement of DHR oxidation but also the reduction of total GSH levels by treatment with TNF-α. Thus, it is suggested that the depletion of cellular total GSH is a consequence of oxidative stress produced by mitochondrial ROI during TNF-α exposure.

Subjecting cells to oxidative stress can result in severe metabolic dysfunction, including the peroxidation of lipid membranes,[22] an increase of cytosolic Ca^{2+},[23,24] the mitochondrial inner membrane permeability transition,[25] oxidation of protein thiol groups,[23,26] and DNA damage with subsequent repair.[8] All of these factors may play an important role in TNF cytotoxicity. This assumption is supported by the observation that TNF cytotoxicity does not occur under anaerobic conditions,[27] as well as by the protective effect of an iron chelator (the present study and 17) and free radical scavengers[17] against TNF-mediated cytolysis. Because TNF-induced cytolysis and alterations in the intracellular GSH content are largely prevented by pretreatment with an iron chelator, it seems likely that the highly toxic hydroxyl radical (which is produced from H_2O_2 by the iron-catalysed Harber–Weiss reaction[28] has a major role in the cytotoxicity of TNF-α. The antioxidant DPPD, which inhibits lipid peroxidation without any effect on ROI formation or ROI scavengers,[29] failed to prevent TNF cytotoxicity, indicating that lipid peroxidation was not involved in this toxicity under our experimental conditions. The protective effect of the sulfhydryl reagent dithiothreitol on TNF-induced cytolysis indicates that perturbation of the redox transition of protein sulfhydryl groups induced by mitochondrial ROI, may at least partly contribute to the loss of cell viability. Changes of protein sulfhydryl groups and subsequent disturbance of the intracellular Ca^{2+} distribution during oxidative stress have been already reported.[26]

We observed that DNA damage after exposure to TNF-α occurred in parallel with the enhancement of mitochondrial ROI generation before the onset of definite cytolysis, i.e. cells showing S-phase blocking and a sub-G_1 peak appeared from 4 hr after TNF-α treatment in association with increased mitochondrial ROI generation, while the more marked increase of DNA damaged cells at 6 hr after TNF-α treatment. The appearance of a sub-G_1 peak generally means the extracellular leakage of low molecular weight DNA due to DNA fragmentation.[13,14] More importantly, we found that pretreatment with an inhibitor of mitochondrial respiration, an iron chelator, and a sulfhydryl reagent, ameliorated DNA damage and cytolysis due to TNF-α. These results suggests that hydroxyl radicals generated from O_2^- and H_2O_2 produced by the mitochondria in a process catalysed by iron are a contributing cause of DNA damage and that this biochemical pathway may be involved in TNF-α-induced cell killing.

It is well known that DNA damage frequently occurs when cells are exposed to oxidative stress.[8,30] This damage may be explained by the activation of Ca^{2+}-dependent endonucleases and/or the direct reaction of hydroxyl radicals with DNA.[31] An increase of 8-hydroxyguanine formation in DNA, which can specifically develop from the attack of hydroxyl radicals on guanine, had been reported in HL-60 cells subjected to oxidative stress.[32] On the other hand, it has been reported that DNA damage in hepatocytes due to oxidative stress could be inhibited by preventing the rise of Ca^{2+} with an intracellular Ca^{2+} chelator.[33] This suggests that activation of Ca^{2+}-dependent endonucleases may be involved in DNA damage during oxidative stress, but the relative importance of these two mechanisms (DNA damage by hydroxyl radicals or by endonuclease activation) is less clear.

It is also unclear whether DNA damage due to oxidative stress is the cause or result of TNF-α-induced cell death. We observed that the blocking of DNA repair by an inhibitor of poly(ADP-ribose) polymerase, 3AB, increased DNA damage and yet reduced TNF-α cytotoxicity. Moreover, DNA damage developed more rapidly than TNF-α-induced cytolysis. These findings suggest that DNA injury was not a non-specific epiphenomenon and that an influence on DNA repair subsequent to injury was more important for TNF cytotoxicity than DNA damage *per se*. Activation of poly(ADP-ribose) polymerase results in rapid depletion of the cellular NAD^+/NADH pool[34,35] and the formation of ADP-ribose from NAD^+.[35,36] The former process may upset cellular energy production, while ADP-ribose can covalently bind with various proteins and may also affect cellular metabolism.[35] It is possible that this cascade of events secondary to the activation of poly(ADP-ribose) polymerase is one of the factors involved in TNF-α cytotoxicity.

An additional problem is whether this DNA damage represents the process of apoptosis or a non-apoptotic form of DNA injury. Though no direct evidence is available, the intactness of the MMP and the occurrence of DNA damage at an early time point in our experiment suggests that it represents an apoptotic response.

In summary, our data suggest that TNF-α induces the enhancement of ROI leakage from the mitochondrial respiratory chain and that this directly or indirectly leads to DNA damage. Various events subsequent to DNA damage, such as impairment of DNA repair, may also be involved in the development of TNF-α cytotoxicity.

REFERENCES

1. Goeddel D.V., Aggarwal B.B, Gray P.W. *et al.* (1986) Tumor necrosis factors: gene structure and biological activities. *Cold Spring Harbor Symp Quant Biol* **51,** 597.
2. Tartaglia L.A., Pennica D. & Goeddel D.V. (1993) Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J Biol Chem* **268,** 18 542.
3. Larrick J.W. & Wright S.C. (1990) Cytotoxic mechanism of tumor necrosis factor-α. *FASEB J* **4,** 3215.
4. Hennet T., Richter C. & Peterhans E. (1993) Tumor necrosis factor-α induces superoxide anion generation in mitochondria of L929 cells. *Biochem J* **289,** 587.
5. Fehsel K., Kolb-Bachofen V. & Kolb H. (1991) Analysis of TNFα-induced DNA strand breaks at the single cell level. *Am J Pathol* **139,** 251.
6. Schmid D.S., Hornung R., McGrath K.M., Paul N. & Ruddle N.H. (1987) Target cell DNA fragmentation is mediated by lymphotoxin and tumor necrosis factor. *Lymphokine Res* **6,** 195.
7. Robaye B., Mosselmans R., Fiers W., Dumont J.E. & Galand P. (1991) Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro. *Am J Pathol* **138,** 447.
8. Buttke T.M. & Sandstrom P.A. (1994) Oxidative stress as a mediator of apoptosis. *Immunol Today*, **15,** 7.
9. Ishii Y., Partridge C.A., Del Vecchio P.J. & Malik A.B. (1992) Tumor necrosis factor-α-mediated decrease in glutathione increases the sensitivity of pulmonary vascular endothelial cells to H_2O_2. *J Clin Invest* **89,** 794.
10. Neuschwander-Tetri B.A. & Roll F.J. (1989) Glutathione measurement by high-performance liquid chromatography separation and fluorometric detection of the glutathione-orthophthalaldehyde adduct. *Anal Biochem* **179,** 236.
11. Royall J.A. & Ischiropoulos H. (1992) Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. *Arch Biochem Biophys* **302,** 348.
12. Banati R.B., Rothe G. Valet G. & Kreutzberg G.W. (1991) Respiratory burst activity in brain macrophages: a flow cytometric study on culture rat microglia. *Neuropath Appl Neuro* **17,** 223.
13. Zamai L., Falcieri E., Zauli G. Cataldi A. & Vitale M. (1993) Optimal detection of apoptosis by flow cytometry depends on cell morphology. *Cytometry* **14,** 891.
14. Gorczyca W., Gong J., Ardelt B., Traganos F. & Darzynkiewicz Z. (1993) The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res* **53,** 3186.
15. Ambrosio G., Zweier J.L., Duilio C. *et al.* (1993) Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem* **268,** 18 532.
16. Chance H., Sies H. & Boveris A. (1979) Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59,** 527.
17. Schulze-Osthoff K., Bakker A.C., Vanhaesebroeck B., Beyaert R., Jacob W.A. & Fiers W. (1992) Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. *J Biol Chem* **267,** 5317.
18. Meier B., Radeke H.H., Selle S., Younes M., Sies H. & Resch K. (1989) Human fibroblasts release reactive oxygen species in interleukin-1 or tumor necrosis factor-α. *Biochem J* **263,** 539.
19. Turrens J.F. & McCord (1990) Mitochondrial generation of reactive oxygen species. In: *Clinical Ischemic Syndromes: Mechanism and Consequences of Tissue Injury* (eds G.B. Zelenock, L.G. D'Alecy, Fantone J.C., M. Shlafer & J.C. Stanley) p. 203. C.V. Mosby Co., St Louis, MO.
20. Fernández-Checa J.C., García-Ruiz C., Ookhtens M. & Kaplowitz N. (1991) Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. *J Clin Invest* **87,** 397.
21. Keller G.A., Barke R., Harty J.T., Humphrey E. & Simmonds R.L. (1985) Decreased hepatic glutathione levels in septic shock. *Arch Surg* **120,** 941.
22. Carini R., Parola M., Dianzani M.U. & Albano E. (1992) Mitochondrial damage and its role in causing hepatocyte injury during stimulation of lipid peroxidation by iron nitriloacetate. *Arch Biochem Biophys* **297,** 110.
23. Boobis A.R., Fawthorp D.J. & Davies D.S. (1989) Mechanisms of cell death. *Trends Pharmacol Sci* **10,** 275.
24. Masumoto N., Tasaka K., Miyake A. & Tanizawa O. (1990) Superoxide anion increases intracellular free calcium in human myometrial cells. *J Biol Chem.* **265,** 22 533.
25. Takeyama N., Matsuo N. & Tanaka T. (1993) Oxidative damage to mitochondria is mediated by the Ca^{2+}-dependent inner-membrane permeability transition. *Biochem J* **294,** 719.
26. Di Monte D., Bellomo G., Thor H., Nicotera P. & Orrenius S. (1984) Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. *Arch Biochem Biophys* **235,** 343.
27. Matthews N., Neale M.L., Jackson S.K. & Stark J.M. (1987) Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology* **62,** 153.
28. Halliwell B. (1978) Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. Its role in degradation of hyaluronic acid by a superoxide-generating system. *FEBS Lett* **96,** 238.
29. Masaki N., Kyle M.E. & Farber J.L. (1989) tert-Butyl Hydroperoxide kills cultured hepatocytes by peroxidizing membrane lipids. *Arch Biochem Biophys* **269,** 390.
30. Brawn K. & Fridovich I. (1981) DNA strand scission by enzymically generated oxygen radicals. *Arch Biochem Biophys* **206,** 414.
31. Cohen & Duke R.C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J Immunol* **132,** 38.
32. Kolachana P., Subrahmanyam V.V., Meyer K.B., Zhang L. & Smith M.T. (1993) Benzene and its phenolic megabolites produce oxidative DNA damage in HL-60 cells in vitro and in the bone marrow in vivo. *Cancer Res* **53,** 1023.
33. Dypbukt J.M., Thor H. & Nicotera P. (1900) Intracellular Ca^{2+} chelators prevent DNA damage and protect hepatoma 1C17 cells from quinone-induced cell killing. *Free Radic Res Comms* **8,** 347.
34. Skidmore C.J., Davies M.I., Goodwin P.M. *et al.* (1979) The involvement of poly(ADP-ribose)polymerase in the degradation of NAD caused by γ-radiation and N-methyl-N-nitrosourea. *Eur J Biochem* **101,** 135.
35. Richter C. & Frei B. (1988) Ca^{2+} release from mitochondria induced by prooxidants. *Free Radic Biol Med* **4,** 365.
36. Richter C., Theus M. & Schlegel J. (1990) Cyclosporine A inhibits mitochondrial pyridine nucleotide hydrolysis and calcium release. *Biochem Pharmacol* **40,** 779.