Glutathione and the rate of cellular proliferation determine tumour cell sensitivity to tumour necrosis factor *in vivo*

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Low rates of cellular proliferation are associated with low GSH content and enhanced sensitivity of Ehrlich ascites-tumour (EAT) cells to the cytotoxic effects of recombinant human tumour necrosis factor (rhTNF- α). Buthionine sulfoximine, a selective inhibitor of GSH synthesis, inhibited tumour growth and increased rhTNF- α cytotoxicity *in vitro*. Administration of sublethal doses (10^6 units/kg per day) of rhTNF- α to EAT-bearing mice promoted oxidative stress (as measured by increases in intracellular peroxide levels, $O_2^{\cdot-}$ generation and mitochondrial GSSG) and resulted in a slight reduction (19%) in tumour cell number when controls showed the highest rate of cellular

proliferation. ATP (1 mmol/kg per day)-induced selective GSH depletion, when combined with rhTNF- α administration, afforded a 61% inhibition of tumour growth and resulted in a significant extension of host survival. Administration of *N*-acetylcysteine (1 mmol/kg per day) or GSH ester (5 mmol/kg per day) abolished the rhTNF- α - and ATP-induced effects on tumour growth by maintaining high GSH levels in the cancer cells. Our results demonstrate that the sensitivity of tumour cells to rhTNF- α *in vivo* depends on their GSH content and their rate of proliferation.

INTRODUCTION

Tumour necrosis factor α (TNF- α) is a macrophage/monocyte-derived cytokine that plays a key role in infection and immunity [1]. TNF- α shows a variety of biological effects including cytotoxic and cytostatic anti-tumour activity [2]. However, all tumour cells are not equally susceptible to TNF- α [3]. The reason for the different sensitivity to TNF- α and the mechanisms by which cancer cells resist TNF- α are still unclear.

Tumour cell resistance and sensitivity to TNF- α is related to its intracellular antioxidant-buffering capacity [4]. Reactive oxygen intermediates (ROIs) are involved in the intracellular mechanism of TNF- α -induced cell killing [5], and correlations have been found between high intracellular GSH levels and *in vivo* tumour resistance to TNF- α [4,6]. GSH, the most prevalent non-protein thiol in mammalian cells, plays a key role in determining the sensitivity of cells to radiation and drug-induced cytotoxicity [7–9]. Previously, it has been reported that elevation of intracellular GSH levels is associated with mitogenic stimulation [10], that GSH regulates DNA synthesis [11], and that GSH controls the onset of tumour-cell proliferation by regulating protein kinase C activity and intracellular pH (pH_i) [12]. The onset of severe tumour-related weight loss (cachexia) in the host is accompanied by a decrease in the rate of cancer cell proliferation and a decrease in GSH in the tumour [12,13]. This is due, at least in part, to the fall in protein synthesis affecting γ -glutamylcysteine synthetase activity [14], the control step in GSH synthesis [15]. Thus changes in the rate of cancer-cell proliferation are accompanied by changes in their intracellular GSH levels, and consequently these could be reflected by changes in tumour-cell sensitivity to TNF- α . In the present studies, we have used Ehrlich ascites tumour (EAT)-bearing mice to investigate the relationship between TNF- α -induced oxidative stress and glutathione metabolism *in vivo*, and its correlation with tumour growth and cell proliferation.

MATERIALS AND METHODS

Animals and tumour inoculation

We used adult male mice OF1 from Iffa Credo (Madrid, Spain). The animals were fed *ad libitum* on a stock laboratory diet (Leticia, Barcelona, Spain) and kept on a 12 h light/12 h dark cycle with the room temperature maintained at 22 °C. All experiments were carried out or started between 10:00 and 12:00 h to minimize the effects of diurnal variation. The EAT was kindly provided by the Department of Pathology (Universidad de Valencia) and inoculated intraperitoneally as previously described [13]. All injected mice developed an ascites tumour. The animals died 15.0 ± 0.5 days ($n = 12$) after inoculation of the tumour.

Isolation, culture and compartmentation of tumour cells

Cells were collected from tumour-bearing mice, and then isolated as previously described [13]. Synchronized cells were plated (at a density of 2×10^4 cells/cm²) and grown in 75 cm² polystyrene flasks (Falcon Labware) in Dulbecco's modified Eagle's medium (Gibco Laboratories), pH 7.4, supplemented with 10% fetal bovine serum, 10 mM Hepes, 40 mM NaCHO₃, 100 units/ml penicillin and 100 μ g/ml streptomycin as previously described [14]. To detach the cells, the cultures were incubated for 5 min with 0.05% (w/v) trypsin (Sigma) in PBS (10 mM sodium phosphate, 4 mM KCl, 137 mM NaCl), pH 7.4, containing 0.3 mM EDTA, followed by the addition of 10% calf serum to inactivate the trypsin. Cellular integrity was assessed by measuring Trypan Blue exclusion (cell viability was always greater than 97%) and leakage of lactate dehydrogenase activity (always less than 2% of the total activity). To separate cellular compartments, samples from the detached cells were washed out twice by low-speed centrifugation. The supernatant, containing

Abbreviations used: EAT, Ehrlich ascites tumour; TNF, tumour necrosis factor; rmTNF, recombinant mouse tumour necrosis factor; rhTNF, recombinant human tumour necrosis factor; ROIs, reactive oxygen intermediates; GSH ester, glutathione monoisopropyl(glycyl) ester; BSO, L-buthionine (S,R)-sulfoximine; pH_i , intracellular pH.

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non-viable cells, was discarded. Then viable cells were re-suspended in Krebs–Henseleit bicarbonate medium, pH 7.4, and centrifuged immediately through a layer of silicone oil (AR200/SF96:100, 6:1, v/v) (AR200 was from Serva, Heidelberg, Germany, and SF96:100 was from Klöckner, Duisburg, Germany) by the method of Zuurendonk and Tager [16]. When the cells were fractionated with digitonin (1.6 mM) to separate cytosolic and mitochondrial compartments [16], up to 96% of the total lactate dehydrogenase activity was found in the supernatant (cytosolic fraction). The cytosolic compartment contained maximally 9 and 0% of the total amount of glutamate dehydrogenase and cytochrome *c* oxidase respectively. The high percentage of the total lactate dehydrogenase found in the supernatant after centrifugation shows that complete rupture of the plasma membrane had occurred. On the other hand, digitonin fractionation did not damage the mitochondria, as shown by the very low percentages of glutamate dehydrogenase and cytochrome *c* oxidase found in the supernatant. Corrections were made for contamination of the cytosol by the mitochondrial pellet, based on the assumption that the metabolites measured shared the same cytosolic space as the lactate dehydrogenase.

Recombinant human TNF (rhTNF)

rhTNF- α was obtained from Sigma and had a specific activity of 2×10^7 units/mg of protein (lyophilized from a 0.2 μ m-filtered solution of 10 mM sodium phosphate buffer containing 50 μ g of BSA/ μ g of cytokine), as determined *in vitro* by the murine L929 cytolytic assay [17]. Stock solutions of rhTNF- α (10^6 units/ml) were diluted in sterile physiological saline solution (0.9% NaCl), adjusted to pH 7.0, and stored at 4 °C.

For *in vivo* administration, normal or tumour-bearing mice were given a single intravenous injection (via the saphenous vein) of rhTNF- α (or excipient), under light ether anaesthesia, in 0.25 ml of physiological saline, adjusted to pH 7.0, containing 0.1% BSA. Serum TNF activity (units/ml) was measured by the L929 cytolytic assay [17].

Preparation of GSH ester

GSH monoisopropyl(glycyl) ester was obtained as previously described [18].

Sample preparation, metabolite and enzyme assays

GSH was measured by using the glutathione S-transferase reaction as previously described by Akerboom and Sies [19]. GSSG was determined by HPLC as described by Asensi et al. [20]. Lactate dehydrogenase activity and ATP were measured as described by Bergmeyer [21].

Flow cytometry

Cell suspensions were diluted to approx. 400 000 cells/ml. Flow-cytometric analysis was performed with an EPICS PROFILE II (Coulter Electronics, Hialeah, FL, U.S.A.). Fluorochromes were from Molecular Probes Europe BV (Poortgebouw, Leiden, The Netherlands), except for dihydroethidium which was from Sigma. Fluorochromes were excited with an argon laser tuned at 488 nm. Forward-angle light scatter and right-angle light scatter were measured, and fluorescence was detected through a 488 nm-blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass or a 575 nm long-pass. Samples were acquired for 10 000 individual cells. Cell viability was determined by using the fluorescent dye propidium iodide (final concentration 5 μ g/ml) at 630 nm fluorescence emission and by light-scattering proper-

ties. The rest of the studies were limited to viable cells. Mitochondrial membrane potential was determined using the fluorescence dye Rhodamine 123 [22] (1 μ g/ml final concentration) at 525 ± 5 nm fluorescence emission; the mitochondrial membrane mass was measured with nonyl-Acridine Orange [23] (10 μ g/ml final concentration) at 525 ± 5 nm fluorescence emission; intracellular peroxide levels were measured with 2',7'-dichlorodihydrofluorescein diacetate [24] (10 μ g/ml final concentration) at 525 nm fluorescence emission; $O_2^{\cdot -}$ generation was determined with dihydroethidium [24] (2 μ g/ml final concentration) at 630 nm fluorescence emission.

Measurement of pH_i

pH_i in tumour cells was determined by using the [14 C]dimethylloxazolidine-2,4-dione (New England Nuclear) distribution-ratio method [25], as previously described [12].

Measurement of protein synthesis

Fractional rates of protein synthesis were based on the flooding-dose technique of Garlick et al. [26] and obtained as previously described [13].

Expression of results and statistical significance

The results are expressed as means $\pm$ S.D. for the indicated number of different experiments. The statistical significance of differences was assessed by Student's *t* test.

RESULTS

Tumour growth and GSH content *in vitro*

As shown in Figure 1, proliferation was very rapid between days 3 and 5, and then decreased until confluency (when non-synchronized cells were cultured, no exponential rates of proliferation could be achieved; results not shown). GSH levels increased in the tumour cells during the period of active proliferation to reach a maximum 4 days after plating, but then gradually decreased over the next four days as the cell number increased and the growth rate declined (Figure 1). Indeed, when cultures reached confluency on day 6, GSH levels had decreased to practically control values (day 0) from the peak value on day

Figure 1 Effect of tumour growth *in vitro* on the intracellular GSH content of cancer cells

Synchronized cancer cells obtained from mice bearing the tumour for 7 days were cultured as described in the Materials and methods section. All points are means $\pm$ S.D. for six independent experiments.

Figure 2 Time course of cytotoxicity in EAT cells treated with different concentrations of rhTNF- α *in vitro*

Synchronized cancer cells obtained from mice bearing the tumour for 7 days were cultured as described in the Materials and methods section. Cells were either untreated (●) or treated with different concentrations of rhTNF- α (○, 10 units/ml; ■, 100 units/ml; □, 1000 units/ml). After the cells had been plated out, rhTNF- α was present in the culture medium from day 3. The experiments were finished 24 h later. Data are means $\pm$ S.D. for five independent experiments.

Table 1 Time course of cytotoxic activity of rhTNF- α on EAT cells growing *in vitro* at different rates of proliferation

Control cells (98% viability) were left untreated for 5 days of culture, whereas parallel cell cultures were treated with 1000 units/ml rhTNF- α for either 12 or 24 h. The results are means $\pm$ S.D. for five different observations. * P < 0.01 compared with controls (no treatment).

Time of rhTNF- α administration (days of culture)	Cell viability (%)	
	12 h	24 h
2	28.3 $\pm$ 7.1*	4.7 $\pm$ 2.3*
4	91.2 $\pm$ 5.5	62.4 $\pm$ 9.6*

4. Therefore, as occurs *in vivo* [12], we found a close correlation between high rates of cellular proliferation and increased levels of intracellular GSH in the tumour cells.

Time course of cytotoxic activity of rhTNF- α on tumour cells *in vitro*

Cytotoxicity of rhTNF- α was studied by exposing cultured tumour cells to rhTNF- α over a concentration range of 10–1000 units/ml (Figure 2). rhTNF- α was present in the culture medium from day 3 over a 24 h incubation period, precisely when tumour cells show the highest rate of cellular proliferation and reach, under control conditions, the highest levels of intracellular GSH (see Figure 1). The number of viable cells was measured every 6 h. As shown in Figure 2, rhTNF- α showed no cytotoxic effects on EAT cells when added at a dose of 10 units/ml. However, cell survival decreased significantly in a time-dependent manner to 75 and 32% respectively when the cells were treated with rhTNF- α at doses of 100 of 1000 units/ml over the 24 h incubation period.

In order to test if cytotoxicity of rhTNF- α in tumour cells is related to their rate of proliferation, we measured cell viability

Table 2 Effect of GSH depletion on rhTNF- α cytotoxicity against EAT cells *in vitro*

After the cells had been plated out, BSO (100 μ M) and GSH ester (200 μ M) were added to the culture medium on day 2. GSH ester (200 μ M) was added every 24 h. rhTNF- α (1000 units/ml) was added on day 4. The experiments were finished 24 h after addition of rhTNF- α . Data are means $\pm$ S.D. for five independent experiments. * P < 0.05, ** P < 0.01 compared with controls (no additions); † P < 0.05, †† P < 0.01 refers to the comparison between results obtained in the presence and in the absence of GSH ester.

Additions	$10^{-5} \times$ Cell number (cells/ml)	Cell viability (%)	GSH (μ mol/g)
None	19.6 $\pm$ 2.0	99.0 $\pm$ 1.0	2.3 $\pm$ 0.4
BSO	7.7 $\pm$ 1.3**	90.2 $\pm$ 3.5*	0.7 $\pm$ 0.2**
rhTNF- α	15.9 $\pm$ 1.5*	94.3 $\pm$ 7.1	1.2 $\pm$ 0.3**
BSO + rhTNF- α	6.5 $\pm$ 0.7**	12.6 $\pm$ 5.5**	0.4 $\pm$ 0.1**
BSO + GSH ester	18.4 $\pm$ 1.6††	97.3 $\pm$ 3.4†	2.5 $\pm$ 0.5††
rhTNF- α + GSH ester	18.8 $\pm$ 2.2	95.5 $\pm$ 3.0	2.2 $\pm$ 0.4††
BSO + rhTNF- α + GSH ester	16.7 $\pm$ 1.9††	92.8 $\pm$ 4.4*††	2.0 $\pm$ 0.3††

when the cytokine was added (1000 units/ml) to the culture medium on days 2 or 4 (see Figure 1). As shown in Table 1, over a 24 h incubation period, rhTNF- α -induced cell death is much higher when the rate of cell proliferation is lower.

GSH depletion increases the sensitivity of tumour cells to rhTNF- α *in vitro*

As shown in Table 2, L-buthionine (S,R)-sulphoximine (BSO), a non-toxic and selective inhibitor of γ -glutamylcysteine synthetase [7], depletes GSH and inhibits cell growth to 30 and 39% of the controls respectively (see [12]). rhTNF- α also depletes GSH and inhibits growth to 52 and 81% of the controls respectively (Table 2). On the other hand, when BSO and rhTNF- α were added to the culture medium, an additive effect was evident since both GSH levels and cell number were lower than in the presence of BSO alone (Table 2). Moreover, cell survival in BSO and rhTNF- α -treated cells decreased sharply (to only 12%) as compared with either controls, BSO-treated or rhTNF- α -treated tumour cells (Table 2).

In order to prove that GSH depletion is responsible, by itself, for growth inhibition and cell death, we cultured tumour cells in the presence of BSO and/or rhTNF- α and GSH ester (Table 2). Previous studies showed that GSH esters, in contrast with GSH itself, are effectively transported into many tissues, including EAT cells [14], and converted into GSH intracellularly [27]. In all cases, GSH ester increased GSH levels, cell number and cell viability to practically control values (Table 2).

Dose–response relationship for rhTNF- α -induced lethality *in vivo*

It has been shown that the rhTNF- α doses required to cure mice resulted in significant toxicity [28]. Thus it is essential to find ways to avoid rhTNF- α toxicity while preserving its therapeutic efficacy. The fact that GSH depletion can increase the sensitivity of tumour cells to rhTNF- α *in vitro* (see Table 2) prompted us to search for a similar effect *in vivo*, without challenging host survival.

First, we studied the dose–response relationship for rhTNF- α -induced lethality by giving the rhTNF- α challenge 4 days after inoculation of the tumour, when the tumour begins to grow exponentially [12] (Table 3). Control tumour-bearing mice treated with physiological saline had a 100% 48 h survival rate, whereas lethality appears when rhTNF- α is administered at doses of

Table 3 Dose–response relationship for rhTNF- α -induced lethality in EAT-bearing mice

Mice bearing the tumour for 4 days were given a bolus injection of 0.25 ml of rhTNF- α (see the Materials and methods section). Controls received 0.25 ml of physiological saline alone. The number of mice in each experimental group was ten.

$10^{-6} \times$ Treatment dose of rhTNF- α (units/kg)	No. surviving after treatment dose
0	10
0.5	10
1	10
2	3
4	1

Table 4 TNF activity in serum from EAT-bearing mice injected intravenously with rhTNF- α

rhTNF- α (10^6 units/kg) was administered on day 4 after inoculation of the tumour. Data are means $\pm$ S.D. for five different animals. * $P < 0.001$ compared with control group (top line). Limits of TNF detection for serum were about 50 units/ml.

Time after rhTNF- α administration (h)	Serum TNF activity (units/ml)
0	< 50
0.5	21 859 $\pm$ 1922*
1	11 117 $\pm$ 1388*
2	4867 $\pm$ 1006*
3	1781 $\pm$ 414*
6	< 50
12	< 50

Table 5 Effect of administration *in vivo* of TNF on growth of EAT in mice

rhTNF- α (10^6 units/kg) was administered daily, starting 24 h after inoculation of the tumour. The tumour was allowed to grow for 7 days. Results are expressed as means $\pm$ S.D. for nine different animals. * $P < 0.05$ compared with control group (mice treated with saline alone).

Parameter	0	Time after inoculation of the tumour (days)	
		7	
		Saline	rhTNF- α
Animal weight (g)	27.3 $\pm$ 1.1	33.6 $\pm$ 1.5	30.4 $\pm$ 1.3*
Carcass weight (g)		26.9 $\pm$ 0.8	22.6 $\pm$ 0.7*
Tumour volume (ml)		6.7 $\pm$ 0.4	7.8 $\pm$ 0.5*
$10^{-6} \times$ Tumour cell density (cells/ml)		514 $\pm$ 74	355 $\pm$ 66*
Fractional rate of protein synthesis in tumour cells (% per day)		69 $\pm$ 8.1	56.7 $\pm$ 6.5*
pH _i in tumour cells		7.33 $\pm$ 0.05	7.20 $\pm$ 0.04*
Intracellular GSH (μ mol/g)		3.2 $\pm$ 0.5	2.3 $\pm$ 0.3*

2×10^6 units/kg or higher (Table 3). All mice that did not survive rhTNF- α treatment were dead 24–48 h after rhTNF- α administration (Table 3). On the other hand, we found no significant difference in life expectancy between controls and mice treated with sublethal doses of rhTNF- α (10^6 units/kg; Table 3), since both groups died about 15–17 days after tumour inoculation (results not shown).

Table 6 Flow-cytometric analysis of peroxides and $O_2^{\cdot-}$ generation by cancer cells in EAT-bearing mice treated with rhTNF- α

Intracellular content of peroxides and generation of $O_2^{\cdot-}$ were estimated in individual cells as indicated in the Materials and methods section and are expressed as fluorescence intensity as a percentage of control, with the control value set at 100%. rhTNF- α (10^6 units/kg) was administered daily (see the legend to Table 5). The tumour was allowed to grow for 7 days. Results are expressed as a mean $\pm$ S.D. for five different animals. *Significantly different from controls (mice treated with saline alone) ($P < 0.01$).

	$O_2^{\cdot-}$	Peroxides
Controls	100 $\pm$ 49	100 $\pm$ 40
rhTNF- α	241 $\pm$ 73*	358 $\pm$ 136*

Serum TNF activity in EAT-bearing mice injected with rhTNF- α

Mice bearing the tumour for 4 days that received a single intravenous injection of rhTNF- α (10^6 units/kg) showed very high initial levels of serum TNF, which then decreased to control values by 3–6 h after the injection (Table 4). Serum TNF activity in both tumour- and non-tumour-bearing mice was undetectable with this assay (< 50 units/ml; $n = 7$).

Effect of rhTNF- α on tumour growth *in vivo*

In a previous report [13], we suggested the possibility that humoral factors, such as TNF, could have direct effects on glutathione status in tumour cells. Indeed, as shown in Table 2, rhTNF- α depletes GSH and may inhibit tumour growth *in vitro*. In addition, we showed that BSO-induced GSH depletion is accompanied by tumour-growth inhibition [12]. Therefore we studied whether rhTNF- α administration to EAT-bearing mice affects GSH contents in cancer cells and/or the rate of tumour cell proliferation *in vivo*. We found that a single dose of rhTNF- α (10^6 units/kg; see Tables 3 and 4) depletes GSH in cancer cells to approx. 80 % of its control value (results not shown). Control values were recovered 6 h after rhTNF- α administration. However, a single dose of rhTNF- α did not promote any significant change in the rate of tumour cell proliferation (results not shown). To investigate whether rhTNF- α exposure for a longer period affects cancer growth, we used multiple rhTNF- α dosing [see the legend to Table 5; the use of two doses/day instead of one dose/day was of no use as the former increased lethality up to 50 % ($n = 10$), whereas the use of one dose/day was safe (0 % lethality for $n = 10$)]. By this procedure, GSH content and tumour cell number fell to approx. 72 and 80 % of control values respectively (Table 5) in mice treated with rhTNF- α . Moreover, all parameters measured to evaluate tumour growth *in vivo* (tumour volume, cell density, protein synthesis, pH_i) were significantly lower in rhTNF- α -treated than in saline-treated tumour-bearing mice (Table 5).

rhTNF- α -induced oxidative stress *in vivo*

In order to test whether these TNF-induced effects are related to an intracellular increase in ROIs, we used flow-cytometric analysis to measure intracellular peroxide levels and $O_2^{\cdot-}$ generation. We found that 60 min after rhTNF- α administration, peroxide levels in tumour cells from rhTNF- α -treated mice were approx. 3.6 times higher than those found in cells from controls (EAT-bearing mice treated with physiological saline) (Table 6). $O_2^{\cdot-}$ generation was also increased in rhTNF- α -treated mice

Table 7 Effect of ATP and rhTNF- α administration *in vivo* on tumour growth

rh TNF- α (10^6 units/kg per day) was administered for 6 days (see the Materials and methods section), starting 24 h after inoculation of the tumour. ATP was administered intraperitoneally daily (1 mmol/kg per day for 7 days; the volume injected into either control or ATP-treated mice was never higher than 0.015 ml/g of mouse), starting 2 h after inoculation of the tumour, in sterile physiological saline solution adjusted to pH 7.0. GSH ester (5 mmol/kg; 0.5 ml) was injected intraperitoneally in isomolar solution (pH 6.6–6.8) daily, starting 6 h after inoculation of the tumour. *N*-Acetylcysteine (NAC; Zambon) was administered intraperitoneally daily (1 mmol/kg per day for 7 days; the volume injected either into controls or into NAC-treated mice was never higher than 0.01 ml/g of mouse), starting 6 h after inoculation of the tumour, in sterile physiological saline. To allow calculation of the tumour cell number and GSH content of tumour cells for each experimental condition, EAT-bearing mice ($n = 20$) were randomly divided into two groups ($n = 10$ for each). One group was used to test host survival and the other to determine the indicated parameters; the latter were killed on day 7. Data are means $\pm$ S.D. ($n = 10$) for each experimental condition. *Significantly different from controls (mice treated with saline alone, indicated as None in left column) ($P < 0.05$).

Treatment	$10^{-8} \times$ Cell number	GSH (μ mol/g)	Host survival (days)
None	36.7 ± 4.7	3.4 ± 0.6	14.7 ± 0.6
ATP	$18.5 \pm 5.2^*$	$1.5 \pm 0.3^*$	$19.5 \pm 2.0^*$
rhTNF- α	$29.8 \pm 4.5^*$	$2.3 \pm 0.5^*$	$17.6 \pm 1.5^*$
ATP + rhTNF- α	$14.3 \pm 3.6^*$	$0.8 \pm 0.4^*$	$21.3 \pm 2.4^*$
NAC + ATP + rhTNF- α	32.1 ± 6.3	2.7 ± 0.7	15.0 ± 1.0
GSH ester + ATP + rhTNF- α	30.6 ± 3.9	2.6 ± 0.5	15.5 ± 0.7

(Table 6). On the other hand, flow-cytometric analysis showed that the mitochondrial membrane potential and the mitochondrial size of cancer cells were not affected by administration of rhTNF- α to EAT-bearing mice (results not shown).

Selective GSH depletion and rhTNF- α -induced cytotoxicity against tumour cells *in vivo*

We have demonstrated inhibition of cancer growth and selective GSH depletion in EAT cells *in vivo* by extracellular ATP [14], and recently we reported that administration of ATP in combination with diethylmaleate, a thiol-depleting agent, and X-rays leads to complete regression of EAT in mice [9]. Thus we used the ATP-induced selective GSH depletion in combination with rhTNF- α in order to improve the therapeutic effect of this cytokine. As shown in Table 7, the combination of ATP and rhTNF- α produces the best survival rate and the highest inhibition of tumour growth. Moreover, ATP and rhTNF- α , when combined, decreased GSH contents in the cancer cells to a lower level than that produced by ATP or rhTNF- α alone (Table 7).

In order to prove that GSH depletion by itself is responsible for changes in survival rate and in the rate of tumour cell proliferation, we administered *N*-acetylcysteine or GSH ester to EAT-bearing mice treated with ATP and rhTNF- α . In previous studies, we have shown that *N*-acetylcysteine [13] and GSH ester [14] increase GSH levels in EAT cells *in vitro*. As shown in Table 7, both compounds increased GSH contents significantly in the cancer cells and, in addition, reversed the inhibition of tumour growth and decreased host survival to control values.

DISCUSSION

TNF- α was originally described as a protein that causes haemorrhagic necrosis and regression of experimental tumours [17,29].

However, the TNF- α doses required to cure tumour-bearing mice lead to not only tumour necrosis but also injury of normal tissue and may eventually cause a lethal shock syndrome similar to endotoxic shock [29]. This toxicity implies severe limitations for its therapeutic use.

TNF- α has shown cytostatic or cytolytic effects on sensitive tumour cells, but not normal cells [2,28]. These selective effects on tumour cells are not due to receptor absence, since TNF- α -resistant cells (e.g. human diploid fibroblasts) have sufficient numbers of high-affinity receptors, internalize TNF- α and degrade it in a similar fashion to TNF- α -sensitive cells [30]. Moreover, the fact that serum TNF activity is undetectable in EAT-bearing mice is not surprising. Indeed, patients with different cancers show very low or undetectable levels of TNF in the blood [31]. Possibly, the absence of detectable levels of TNF in different tumour-bearing animals and cancer patients may be due to very rapid formation of a complex between TNF and its receptor [31]. However, the nature of the signal transduced by the receptor remains unknown, and the molecular mechanisms by which tumour cells offer resistance to TNF- α are still poorly understood. ROIs have been implicated in the cytotoxic activity of TNF- α , and various different studies are consistent with the hypothesis that tumour cell sensitivity to TNF- α is related to its capacity to buffer oxidative attack [5,6,32]. Recently, direct evidence for TNF- α -induced mitochondrial ROIs and their involvement in DNA damage [33] and cytotoxicity [34] in the murine fibrosarcoma cell line L929, has been reported. Essentially, this mechanism proposes that TNF- α -induced events interfere with normal electron flow in the mitochondria, leading to generation of ROIs which cause cell damage. In accord with this idea, recombinant mouse (rm)TNF- α cytotoxicity on L929 cells can be partially prevented by lipid antioxidants, iron chelators and inhibitors of the mitochondrial electron-transport complexes I and II [35]. The involvement of mitochondria in necrotic cell death is further supported by the fact that L929 cells depleted of mitochondrial DNA are resistant to lipid peroxidation and rmTNF- α -induced necrosis [36], although U937 cells lacking 98% of mitochondrial DNA were still sensitive to rhTNF- α -induced apoptosis [37].

Previously, we have shown that rapid GSH synthesis in tumour cells is associated with high rates of cellular proliferation (Figure 1; [13]) and that GSH depletion is, by itself, sufficient to sensitize cancer cells to the cytotoxic effects of radiation-induced oxidative stress [9]. However, cells may survive unless GSH falls to very low levels [9]. Indeed, cytotoxic activity of rhTNF- α depends on the rate of tumour cell proliferation (Table 1), which is associated with the activity of γ -glutamylcysteine synthetase [14], the control step in GSH synthesis [7]. In accord with this, Kirstein et al. [38] reported that L929 cells, growing *in vitro*, were more sensitive to rhTNF- α toxicity when they approached high density. Thus a possible explanation for the spectacular decrease in cell survival found in BSO- and rhTNF- α -treated cells (Table 2) is that, by combining BSO and rhTNF- α , very low levels of GSH can be achieved in the cancer cells. In fact, Ehrlich tumours growing in mice can be eliminated by X-rays if a combined therapy capable of decreasing GSH sufficiently in the tumour, without placing normal tissues at a disadvantage, is previously applied [9]. This supports the idea that a critical threshold of GSH must be reached within cells for lethal injury to be caused [9]. However, a major problem when using BSO *in vivo* is that it decreases GSH levels in tumour and in non-tumour cells [12]. To solve this problem, we reported that ATP-induced tumour growth inhibition is accompanied by a selective decrease in the content of GSH within the cancer cells *in vivo* [14]. Indeed, by combining ATP and rhTNF- α , it is possible to deplete GSH further and, in

addition, to increase host survival significantly (Table 7). Nevertheless, the lowest level of intracellular GSH that we could achieve by using ATP and rhTNF- α was not as low as that obtained by combining ATP and diethylmaleate (less than 5% of the controls), an α,β -unsaturated carbonyl compound that reacts with free thiol groups [9]. Thus it appears that for rhTNF- α to be therapeutically effective, it may need to be combined with conventional chemotherapy or radiation or possibly other cytokine(s). Interestingly, it has been reported that, when administered together with interferon γ , rhTNF- α showed enhanced anti-proliferative effects [2]. The enhanced cytotoxicity observed in the presence of these two cytokines suggests that they may act through convergent cellular response pathways. In addition, the combination of recombinant interleukin 1 and rhTNF- α may be clinically useful by inducing tolerance to the lethal effects of TNF- α [39]. Furthermore Van der Zee et al. [40] have observed thermal enhancement of both rhTNF- α -induced systemic toxicity and tumour cure in rats.

Why are very low levels of intracellular GSH (see above) necessary to cause lethal injury? Goossens et al. [34] have suggested that the mitochondrial glutathione system may play a role in cell protection against TNF- α by scavenging most of the ROIs produced, and that depletion of the cytosolic and mitochondrial GSH pools, but not depletion of the cytosolic pool alone, causes a major increase in rmTNF-induced free radicals. In fact, GSH is required for normal mitochondrial function [41]. Furthermore mitochondria do not possess γ -glutamylcysteine synthetase or GSH synthetase activity [42], indicating that mitochondrial GSH arises from the cytosol. Indeed, mitochondria take up and retain GSH through a multicomponent transport system [41]. At external levels of less than 1 mM, GSH is transported into the mitochondrial matrix by a high-affinity component (K_m 60 μ M), which is saturated at levels of 1–2 mM and stimulated by ATP [41]. Moreover, decreasing cytosolic GSH levels (e.g. by administering BSO) decreases net export of GSH from mitochondria to the cytosol [41]. In addition, mitochondria can take up GSH effectively even when the cytosolic GSH levels are low [41]. In EAT cells, we found that mitochondria contain 89 ± 10 nmol of GSH/g ($n = 6$) (approx. 2.7% of total intracellular GSH) and 7 ± 2 nmol of GSSG/g ($n = 6$) (approx. 15.5% of total intracellular GSSG). In tumour-bearing mice treated with rhTNF- α (see legend to Table 7), mitochondrial GSH content in cancer cells was not significantly different from controls; however, mitochondrial GSSG content increased to 15 ± 4 nmol/g ($n = 6$; $P < 0.01$). This increase in mitochondrial GSSG is consistent with the hypothesis that GSH plays a key role in scavenging TNF-induced ROIs. Mitochondrial GSH and GSSG contents in cancer cells from EAT-bearing mice treated with rhTNF- α and ATP were not significantly different from those from tumour-bearing mice treated with rhTNF- α alone (results not shown). However, in EAT-bearing mice treated with ATP and diethylmaleate (as reported in [9]), where intracellular GSH is about 3.0% of controls, mitochondrial GSH and GSSG contents were of 26 ± 7 nmol/g ($n = 6$; $P < 0.01$) and 39 ± 10 nmol/g ($n = 6$; $P < 0.01$) respectively. The rate of death of cancer cells from ATP- and diethylmaleate-treated mice increases dramatically ($50.2 \pm 4.7\%$ of all cells are dead 1 h after treatment; $n = 7$). However, the percentage of dead cells in EAT-bearing mice treated with physiological saline, ATP, rhTNF- α or ATP plus rhTNF- α (see legend to Table 7) was never higher than 5% ($n = 7$) in all cases (results not shown). These facts demonstrate that depletion of mitochondrial GSH, which appears to be critical in the cascade of events that lead to cell death, requires that low levels of GSH in the cytosol are reached. Furthermore our results suggest that the decrease in tumour cell number found

in EAT-bearing mice treated with ATP, rhTNF- α or ATP plus rhTNF- α (Table 7) is mainly due to inhibition of cell growth and not to an increase in the rate of cell death.

In conclusion, the relevant facts reported in this paper are: (1) GSH levels in tumour cells increase during the period of active cell proliferation and then decrease as growth rate declines; (2) low GSH content in tumour cells increases rhTNF- α cytotoxicity; (3) administration of sublethal doses of rhTNF- α decreases GSH content and growth of cancer cells, and extends survival of EAT-bearing mice; (4) rhTNF- α - and ATP-induced tumour-growth inhibition and GSH depletion extend host survival further; (5) administration of *N*-acetylcysteine or GSH ester abolishes the rhTNF- α - and ATP-induced tumour-growth inhibition by maintaining high GSH levels in cancer cells; (6) glutathione is implicated in the mediation of rhTNF- α -induced cytotoxicity. The results suggest possible clinical applications in cancer therapy.

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REFERENCES

- 1 Arai, K. I., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990) *Annu. Rev. Biochem.* **59**, 783–836
- 2 Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A. and Shepard, H. M. (1985) *Science* **230**, 943–946
- 3 Watanabe, N., Niitsu, Y., Neda, H., Sone, H., Yamauchi, N., Umetsu, T. and Urushizaki, I. (1986) *Jpn. J. Cancer Res.* **76**, 1115–1119
- 4 Zimmerman, R. J., Marafino, Jr., B. J., Chan, A., Landre, P. and Winkelhake, J. L. (1989) *J. Immunol.* **142**, 1405–1409
- 5 Zimmerman, R. J., Chan, A. and Leadon, S. A. (1989) *Cancer Res.* **49**, 1644–1648
- 6 Yamauchi, N., Watanabe, N., Kuriyama, H., Neda, H., Maeda, M., Himeno, T., Tsuji, Y. and Niitsu, Y. (1990) *Int. J. Cancer* **46**, 884–888
- 7 Meister, A. (1983) *Science* **220**, 470–477
- 8 Mitchell, J. B., Cook, J. A., DeGraff, W., Glatstein, E. and Russo, A. (1989) *Int. J. Radiat. Oncol. Biol. Phys.* **16**, 1289–1295
- 9 Estrela, J. M., Obrador, E., Navarro, J., Lasso de la Vega, M. C. and Pellicer, J. A. (1995) *Nature Med.* **1**, 84–88
- 10 Shaw, J. P. and Chou, I.-N. (1986) *J. Cell. Physiol.* **129**, 193–198
- 11 Suthanthiran, M., Anderson, M. E., Sharma, V. K. and Meister, A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3329–3333
- 12 Terradez, P., Asensi, M., Lasso de la Vega, M. C., Puertes, I., Viña, J. and Estrela, J. M. (1993) *Biochem. J.* **292**, 477–483
- 13 Estrela, J. M., Hernandez, R., Terradez, P., Asensi, M., Puertes, I. R. and Viña, J. (1992) *Biochem. J.* **286**, 257–262
- 14 Lasso de la Vega, M. C., Terradez, P., Obrador, E., Navarro, J., Pellicer, J. A. and Estrela, J. M. (1994) *Biochem. J.* **298**, 99–105
- 15 Deneke, S. M. and Fanburg, B. L. (1989) *Am. J. Physiol.* **257**, L163–L173
- 16 Zuurendonk, P. F. and Tager, J. M. (1974) *Biochim. Biophys. Acta* **333**, 393–399
- 17 Carswell, E. A., Old, L. J., Kassel, R. C., Green, S., Fiore, N. and Williamson, B. D. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3666–3670
- 18 Martensson, J. and Meister, A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 471–475
- 19 Akerboom, T. P. M. and Sies, H. (1981) *Methods Enzymol.* **77**, 373–382
- 20 Asensi, M., Sastre, J., Pallardó, F. V., García de la Asunción, J., Estrela, J. M. and Viña, J. (1994) *Anal. Biochem.* **217**, 323–328
- 21 Bergmeyer, H. U. (1974) *Methods of Enzymatic Analysis*, Academic Press, New York
- 22 Chen, L. B. (1988) *Annu. Rev. Cell Biol.* **4**, 155–181
- 23 Petit, P. X., O'Connor, J. E., Grunwald, D. and Brown, S. C. (1990) *Eur. J. Biochem.* **194**, 389–397
- 24 Rothe, G. and Valet, G. (1990) *Leukoc. Biol.* **47**, 440–448
- 25 Rottenberg, H. (1979) *Methods Enzymol.* **55**, 547–569
- 26 Garlick, P. J., McNurlan, M. A. and Preedy, V. R. (1980) *Biochem. J.* **192**, 719–723
- 27 Anderson, M. E., Powrie, F., Puri, R. N. and Meister, A. (1985) *Arch. Biochem. Biophys.* **239**, 538–548

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- 28 Fiers, W. (1995) in *Biologic Therapy of Cancer*, 2nd edn., (De Vita, Jr. V. T., Hellman, S. and Rosenberg, S. A., eds.), pp. 295–327, Lippincott, Philadelphia
- 29 Beutler, B. and Cerami, A. (1888) *Endocr. Rev.* **9**, 57–66
- 30 Creasey, A. A., Doyle, L. V., Reynolds, M. T., Jung, T., Lin, L. S. and Vitt, C. R. (1987) *Cancer Res.* **47**, 145–149
- 31 Staal van den Brekel, A. J., Denterner, M. A., Schols, A. M. W. J., Buurman, W. A. and Wouters, E. F. M. (1995) *J. Clin. Oncol.* **13**, 2600–2605
- 32 Hauser, G. J., McIntosh, J. K., Travis, W. D. and Rosenberg, S. A. (1990) *Cancer Res.* **50**, 3503–3508
- 33 Shoji, Y., Uedono, Y., Ishikura, H., Takeyama, N. and Tanaka, T. (1995) *Immunology* **84**, 543–548
- 34 Goossens, V., Grooten, J., De Vos, K. and Fiers, W. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8115–8119
- 35 Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jacob, W. A. and Fiers, W. (1992) *J. Biol. Chem.* **267**, 5317–5323
- 36 Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G. and Fiers, W. (1993) *EMBO J.* **12**, 3095–3104
- 37 Gamen, S., Anel, A., Montoya, J., Marzo, I., Piñeiro, A. and Naval, J. (1995) *FEBS Lett.* **376**, 15–18
- 38 Kirstein, M., Fiers, W. and Baglioni, C. (1986) *J. Immunol.* **137**, 2277–2280
- 39 Sheppard, B. C. and Norton, J. A. (1991) *Surgery* **106**, 698–705
- 40 Van der Zee, J., Van den Ardweg, G. J. M. J., Van Rhoon, G. C., Van den Berg, A. P. and de Wit, R. (1995) *Br. J. Cancer* **71**, 1158–1162
- 41 Martensson, J., Lai, J. C. K. and Meister, A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7185–7189
- 42 Griffith, O. W. and Meister, A. (1985) *Proc. Natl. Acad. U.S.A.* **82**, 4668–4672
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