Tumour necrosis factor-induced cytotoxicity is accompanied by intracellular mitogenic signals in ME-180 human cervical carcinoma cells

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Tumour necrosis factor-α (TNF) induced a cytotoxic response in ME-180 human cervical carcinoma cells *in vitro*. This cytotoxic response was accompanied by a temporal series of intracellular signals that are commonly triggered by a mitogenic stimulus: increased c-*fos* (20–30 min) and c-*myc* (40–60 min) expression, increased activity of ornithine decarboxylase (3 h), increased intracellular polyamine content (7 h) and increased thymidine incorporation into DNA (14 h). A cytotoxic response independent of these mitogenic signals could not be explained by an induction of interleukin-6, which is an autocrine cytotoxic agent in some cell types; nor by a biphasic, dose-dependent response in which low concentrations of TNF are mitogenic and higher concentrations are cytotoxic. Conversely, a dependent role of these mitogenic signals was suggested by the absence of a TNF-promoted increase in thymidine incorporation into DNA in an ME-180 clone that is resistant to TNF-induced cytotoxicity. A decrease in the proliferation rate of TNF-sensitive cells induced by either α-difluoromethylornithine treatment (resulting in polyamine depletion) or serum starvation rendered the cells insensitive to TNF-induced cytotoxicity, further suggesting a role for mitogenic signals and cell division in TNF-mediated cytotoxicity. However, inhibiting proliferation with cycloheximide resulted in increased sensitivity to TNF, implying that mitogenesis itself was not essential for a cytotoxic response. TNF induced DNA fragmentation in sensitive cells, suggesting that cytotoxicity occurred via apoptosis.

INTRODUCTION

Tumour necrosis factor-α (TNF) is a product of activated macrophages that plays a central role in integrating and amplifying the host response to infection and malignancy (Old, 1985; Kunkel et al., 1989). Through interactions with macrophages, fibroblasts and endothelial cells, TNF promotes the immune response, local inflammatory processes and wound repair. TNF is also proposed to mediate the wasting (cachexia) that often accompanies disease states (Old, 1985; Kunkel et al., 1989).

TNF was first identified as an oncolytic agent that promotes the haemorrhagic necrosis and regression of some malignancies by inducing an inflammatory response in tumour capillary beds (Old, 1985; Kunkel et al., 1989). TNF also acts directly on transformed cells, eliciting a cytotoxic response from some and inhibiting the proliferation of others; however, many transformed cells are resistant to the cytotoxic and cytostatic activities of TNF (Old, 1985; Kunkel et al., 1989). In order to fully realize the immunotherapeutic potential of TNF, it is important to identify the mechanisms which render transformed cells responsive or resistant to its cytotoxic activity.

Our goal has been to identify the intracellular events associated with the sensitivity or resistance of transformed cells to TNF-mediated cytotoxicity. Whereas the suppression of early growth-associated signals such as c-*fos* and c-*myc* expression accompanies the TNF-induced inhibition of cell proliferation in some cells (Yarden and Kimchi, 1986; Kronke et al., 1987), the present study identifies the opposite phenomenon in ME-180 human cervical carcinoma cells. TNF-treated ME-180 cells displayed a number of intracellular events associated with cell division prior to undergoing apoptosis. Experiments have been conducted to determine whether the mitogenic signals are a component of the cytotoxic response to TNF or are associated with a coincident but unrelated signal transduction mechanism.

MATERIALS AND METHODS

Materials

Recombinant human TNF-α (5.02×10^7 units/mg) was a gift from Genentech (San Francisco, CA, U.S.A.). [*methyl*-^{3}H]Thymidine (85.6 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.); L-[1-^{14}C]-ornithine (59 mCi/mmol) was from Amersham (Arlington Heights, IL, U.S.A.). Poly(I)·poly(C) and cycloheximide (CHX) were from Sigma (St. Louis, MO, U.S.A.); Nytran membranes were from Schleicher and Schuell (Keene, NH, U.S.A.). α-Difluoromethylornithine (DFMO) was generously provided by Merrell Dow (Cincinnati, OH, U.S.A.).

Cells and cell culture

ME-180 human cervical carcinoma cells sensitive to the cytotoxic activity of TNF (ME-180/SEN) were obtained from Dr. Lloyd Old (Memorial Sloan-Kettering Cancer Center). The TNF-resistant subclone (ME-180/RES) was isolated after continuous culture of ME-180/SEN cells with 1 nM TNF for 2 months. ME-180/RES cells retained the resistant phenotype during subsequent culture in the absence of TNF. FS-4 human foreskin fibroblasts were obtained from Dr. Lester May (Rockefeller University). Cells were grown in Dulbecco's modified Eagle's medium containing 1 × non-essential amino acids, 2 mM L-glutamine, penicillin (50 i.u./ml), streptomycin (50 μg/ml) (Cellgro, Mediatec,

Abbreviations used: TNF, tumour necrosis factor-α; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IL-6 interleukin-6; ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; CHX, cycloheximide.

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Washington, DC, U.S.A.) and 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, UT, U.S.A.), unless otherwise noted.

Cellular cytotoxicity

ME-180/SEN and ME-180/RES cells were incubated in the absence or presence of 1 nM TNF for various times as described in the Results and discussion section. In studies using CHX, cells were incubated without or with 10 μg/ml CHX and the indicated concentrations of TNF for a brief 24 h period, because of the rapid potentiation of cytotoxicity by CHX. After incubations, monolayers were washed twice with PBS. Adherent cells were trypsinized (0.05% trypsin/0.5 mM EDTA; Gibco, Grand Island, NY, U.S.A.) and counted with a Coulter counter. The viability of adherent cells was verified by Trypan Blue exclusion. Non-adherent cells were not viable.

Isolation of RNA and Northern blot analysis

Total cellular RNA was extracted from 1.5×10^7 cells into 4 M guanidinium isothiocyanate, 0.1 M sodium acetate, pH 5.0, 5 mM EDTA and 0.1 M β-mercaptoethanol, and isolated by centrifugation on a caesium chloride density gradient (Chirgwin et al., 1979). RNA (10 μg) was fractionated by electrophoresis on 1.1% agarose/3.3% formaldehyde phosphate-buffered gels, transferred on to Nytran membranes by capillary action and then hybridized to 50–200 ng of probes labelled with [^{32}P]dCTP (5×10^5 Čerenkov c.p.m./μg) by random priming (Prime Time C; International Biotechnologies, New Haven, CT, U.S.A.) (Wahl et al., 1981). The genomic probe for human c-*myc* in pBR322 (Alitalo et al., 1983) was purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). The v-*fos* DNA probe (1 kb fragment) (Curran et al., 1982) was a gift from Dr. R. A. Rifkind (Memorial Sloan-Kettering Cancer Center). The cDNA probe for human β_2-microglobulin in pUC-9 (Suggs et al., 1981) was a gift from Dr. Yvon Cayre (College of Physicians and Surgeons, Columbia University). The full-length cDNA probe for human interleukin-6 (IL-6) in pBR322 (May et al., 1983) was a gift from Dr. Lester May (Rockefeller University).

Ornithine decarboxylase (ODC) and polyamines

Exponentially growing cells were treated with 1 nM TNF for various times, washed with PBS and collected by gentle scraping after a 10 min incubation with 2 mM EDTA in PBS at 37 °C. After an aliquot was removed for determination of cell number, the remaining cells were collected by centrifugation (3 min, 500 *g*) and resuspended in 1 ml of 50 mM potassium phosphate, pH 7.4, 1 mM dithiothreitol and 0.1 mM EDTA. ODC activity was measured by CO_2 trapping (Beaven et al., 1978) in cell supernatants obtained by freeze–thaw fracture and centrifugation (30000 *g*, 1 h). Briefly, cell supernatants were incubated with L-[1-^{14}C]ornithine, the released $^{14}CO_2$ was trapped by 0.3 M potassium hydroxide and the trapped radioactivity was measured by scintillation counting. Protein was determined by the method of Bradford (1976) using rabbit γ-globulin as the standard. Polyamines were measured in cell supernatants using the method of Seiler and Knodgen (1985) by first acidifying with an equal volume of 0.4 M perchloric acid. Polyamines were fractionated by reverse-phase ion-pair h.p.l.c. on a 5 μm C-18 column eluted with a gradient of 0.1 M sodium phosphate (pH 2.5)/2% acetonitrile to 0.1 M sodium phosphate (pH 3.1)/30% acetonitrile. Polyamines were quantified by fluorescence following post-column derivatization with *o*-phthalaldehyde.

[^{3}H]Thymidine uptake and incorporation into DNA

Exponentially growing cells (2×10^6 cells/10 cm culture dish) were incubated for 10–20 h in the absence or presence of 1 nM TNF. [^{3}H]Thymidine (1 μCi/ml) was added to cell cultures for the final hour of incubation. Cells were washed five times with cold Hanks' balanced salt solution and then harvested. Cells were lysed by addition of 0.25 vol. of 1 M NaOH and then duplicate aliquots were spotted on to Whatman GF/C filters (Whatman International Ltd., Maidstone, Kent, U.K.) for quantification of [^{3}H]thymidine uptake.

To determine [^{3}H]thymidine incorporation into DNA, macromolecules were precipitated from duplicate aliquots of the lysed cell suspension with trichloroacetic acid (final concentration 10%) and vacuum-filtered (Whatman GF/C filters). Filters were washed three times with 5% trichloroacetic acid and twice with 90% ethanol, air-dried and assayed for [^{3}H]thymidine by liquid scintillation counting.

IL-6 expression

Exponentially growing ME-180/SEN cells and confluent FS-4 fibroblasts were incubated in the absence or presence of 1 nM TNF for 6 h or were treated with 100 μg/ml poly(I)·poly(C) + 50 μg/ml CHX for 1 h and then with 50 μg/ml CHX for 5 h. With FS-4 cells, these reagents were applied in medium conditioned by the fibroblasts for 6 days. Total cellular RNA was isolated and probed for IL-6 mRNA by Northern blotting.

DNA fragmentation

ME-180/SEN cells were incubated in the absence or presence of 1 nM TNF for the indicated times. Adherent cells were collected after trypsin treatment and combined with cells collected from the medium. Cells were washed in PBS, pelleted (3 min, 500 *g*) and incubated overnight at 37 °C in 400 μl of lysis buffer (200 mM Tris/HCl, pH 8, 100 mM EDTA, 50 μg/ml proteinase K, 1% SDS). DNA was extracted with equal volumes of phenol and chloroform/3-methylbutan-1-ol (24:1, v/v) and precipitated with 0.1 vol. of 3 M sodium acetate, pH 5, and 2.5 vol. of cold 100% ethanol. Pelleted DNA was resuspended in 400 μl of TE (10 mM Tris/HCl, pH 8, and 1 mM EDTA) containing 0.1% SDS and 50 μg/ml RNAase A (previously heat-treated at 100 °C for 15 min), and incubated for 3 h at 37 °C. DNA was again extracted with equal volumes of phenol and chloroform/3-methylbutan-1-ol and precipitated with ethanol. Pelleted DNA was resuspended in TE and aliquots were electrophoresed on a 1.5% agarose gel in TAE buffer (40 mM Tris/acetate, 1 mM EDTA). The gel was stained in 1 μg/ml ethidium bromide in TAE for 30 min, destained in TAE and photographed under u.v. illumination.

RESULTS AND DISCUSSION

Much of the interest in TNF derives from its ability to elicit a cytotoxic response from some types of transformed cells. Unfortunately, not all transformed cells are responsive to TNF-mediated cytolysis. *In vitro* studies with transformed cell lines have shown that TNF can induce cytotoxicity, inhibit proliferation, have no apparent effect on cell growth or act as a mitogen and increase proliferation, depending upon the cell chosen (Sugarman et al., 1985). These disparate responses require

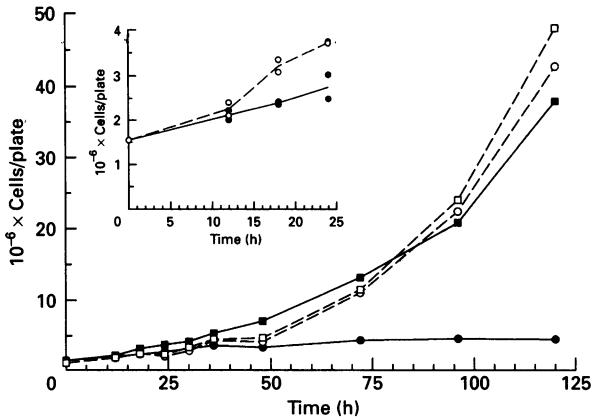

Figure 1 Effect of TNF on the growth of ME-180/SEN and ME-180/RES cells

ME-180/SEN (○, ●) and ME-180/RES (□, ■) cells were incubated in the absence (○, □) or presence (●, ■) of 1 nM TNF for the indicated times. Adherent cells were washed with PBS, harvested by trypsinization and counted. The data are the means of results from duplicate samples. The inset illustrates cell growth of ME-180/SEN cell cultures during the first 24 h of incubation.

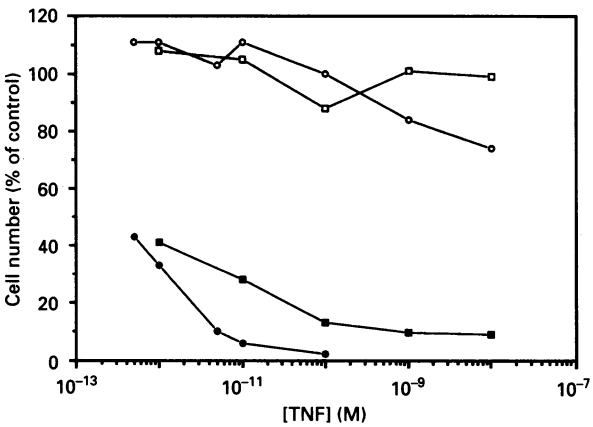

Figure 2 Effect of CHX on the TNF cytotoxic response in ME-180/SEN and ME-180/RES cells

ME-180/SEN (○, ●) and ME-180/RES (□, ■) cells were incubated in the absence (○, □) or presence (●, ■) of 10 μg/ml CHX and the indicated concentrations of TNF for 24 h. Adherent cells were washed with PBS, harvested by trypsinization and counted. The data are means of duplicate samples expressed as percentages of untreated controls.

elucidation for full utilization of the therapeutic potential of TNF.

The present study was conducted with ME-180 cells from which TNF elicits a dose-dependent cytotoxic response (ME-180/SEN; Marino et al., 1989) and a TNF-resistant clone (ME-180/RES). Figure 1 shows that treatment with 1 nM TNF effectively inhibited proliferation of ME-180/SEN cultures in exponential-phase growth. After 18 h of exposure, the cell number was 75% of control; after 5 days, this was decreased to 10% of control. Conversely, TNF did not elicit a cytotoxic or cytostatic response in cultures of ME-180/RES cells. When simultaneously treated with 10 μg/ml CHX and various concentrations of TNF for 24 h, both ME-180/SEN and ME-180/RES cells were sensitive to the cytotoxic effects of TNF (Figure 2).

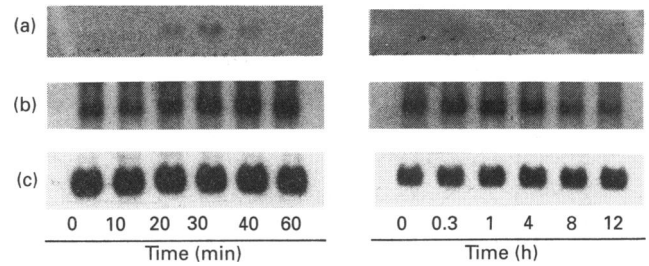

Figure 3 Northern blot of proto-oncogene expression in ME-180/SEN cells

Cells in exponential growth were treated with 1 nM TNF and collected at the times indicated. Total cellular RNA was isolated and 10 μg of each sample was fractionated by agarose/formaldehyde gel electrophoresis. A Nytran blot of the fractionated RNA was sequentially probed for c-*fos* (**a**) and c-*myc* (**b**) expression. Probe-labelled β_2-microglobulin (β_2-m) mRNA (**c**) confirmed that each lane of the gel was equally loaded with non-degraded mRNA.

Treatment with CHX alone essentially stopped cell growth; after 24 h the cell number was 48% and 41% of control for ME-180/SEN and ME-180/RES cells respectively. The resistant and sensitive ME-180 cells expressed similar numbers of high-affinity receptors (ME-180/SEN, 2900 ± 600, $K_d = 0.1$ nM, $n = 4$; ME-180/RES, 4100 ± 700, $K_d = 0.2$ nM, $n = 4$; Scatchard analysis not shown). Therefore we have sought insight into the basis for the sensitivity or resistance of these cell lines through characterization of post-receptor processes.

The TNF-induced antiproliferative effects in both HeLa cells (Yarden and Kimchi, 1986) and HL-60 cells (Kronke et al., 1987) were accompanied by diminished c-*myc* expression. We wondered whether diminished proliferation in TNF-treated ME-180/SEN cells would be accompanied by a similar decrease in the expression of the c-*myc* and c-*fos* proto-oncogenes. To test this, we isolated RNA from exponentionally growing ME-180/SEN cells treated with 1 nM TNF and analysed it for expression of c-*fos* and c-*myc* by Northern blotting (Figure 3). Surprisingly, TNF promoted an initial rapid increase in c-*fos* mRNA; this effect was greatest at 20–30 min and returned to the basal, undetectable, level within 1 h. TNF also increased c-*myc* mRNA approx. 2-fold ($n = 5$); the greatest stimulation was detected 40–60 min after TNF addition, with a subsequent slow return to the basal level. The response of these proto-oncogenes to TNF was remarkably similar to that occurring in an epidermal growth factor (EGF)-, platelet-derived growth factor (PDGF)- or serum-triggered mitogenic response in other cell types (Müller et al., 1984; Greenberg and Ziff, 1984).

TNF can act as a mitogen in several cells (e.g. FS-4 human foreskin fibroblasts and Hos-1 human osteosarcoma cells) and has been shown to induce c-*fos* and c-*myc* expression prior to increasing the proliferation rate (Lin and Vilcek, 1987; Kirsten and Baglioni, 1988). Kohase et al. (1986) have suggested that mitogenesis is the true physiological response to TNF and that cytotoxicity is an aberration of transformed cells. This led us to assay for other intracellular mitogenic responses in TNF-treated ME-180 cells. In ME-180/SEN cells, the activity of ODC increased 2-fold within 3 h after addition of 1 nM TNF (Figure 4a). Within 7 h of TNF addition, intracellular putrescine (the product of the ODC-catalysed reaction) was also increased 2-fold (Figure 4b). There was no net increase in the cellular content of spermidine or spermine, which are metabolites of putrescine (results not shown). However, the amount of N^1-acetylspermidine was increased 1.6-fold (Figure 4c), which suggests that conversion of spermidine to putrescine was also increased. Donato et al. (1989) have reported similar changes in ODC activity in their TNF-treated ME-180 cells; however, in A375

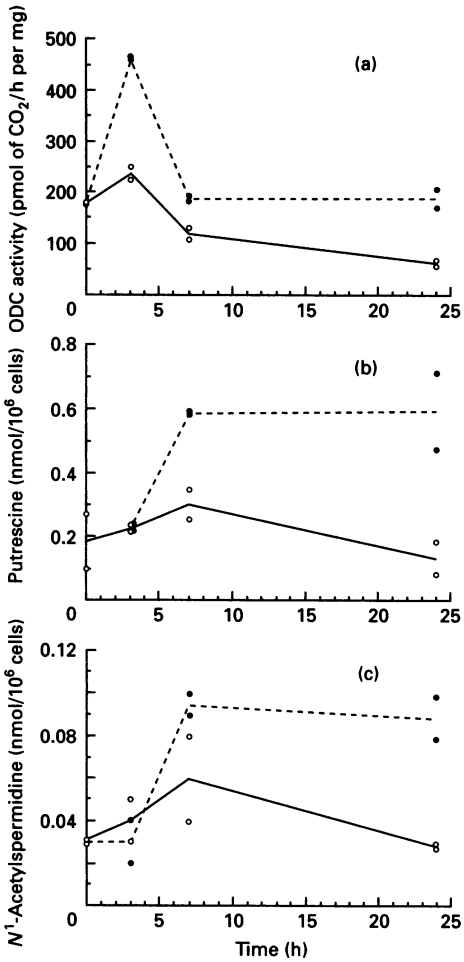

Figure 4 Effect of TNF on cellular polyamines

ME-180/SEN cells were incubated in the absence (○) or presence (●) of 1 nM TNF for the indicated times and then collected. Cells were assayed for ODC activity (**a**) or putrescine (**b**) and N^1-acetylspermidine (**c**) content as described. Data are from duplicate samples and are representative of results from three experiments.

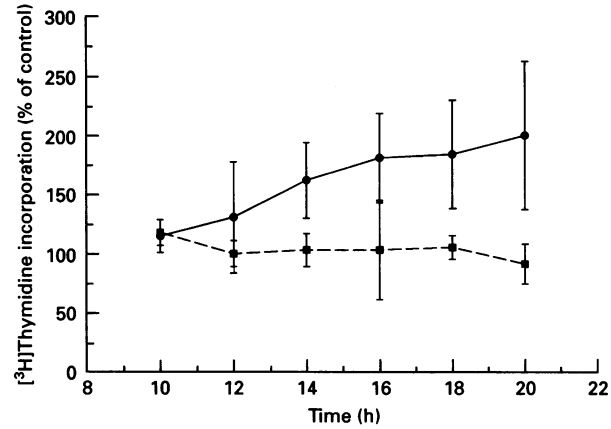

Figure 5 Effect of TNF on [^{3}H]thymidine incorporation into DNA

ME-180/SEN (●) and ME-180/RES (■) cells were incubated in the absence or presence of 1 nM TNF for the indicated times. [^{3}H]Thymidine incorporation into DNA was then assayed. The effect of TNF is presented as a percentage of the incorporation in control cultures at each time point. Results are expressed as the means ± S.D. of values from three separate experiments.

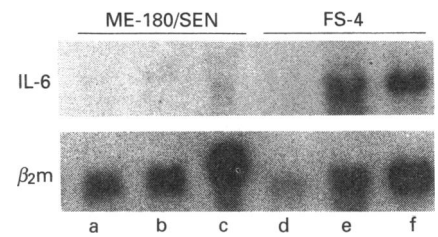

Figure 6 Northern blot of IL-6 mRNA

ME-180/SEN and FS-4 fibroblast cells were incubated in the absence or presence of 1 nM TNF for 6 h, or in the absence or presence of 100 μg/ml poly(I)·poly(C) + 50 μg/ml CHX for 1 h followed by 50 μg/ml CHX for 5 h. Total cellular RNA was then isolated and IL-6 mRNA assayed by Northern blotting. Lane a, ME-180/SEN control; lane b, ME-180/SEN + TNF; lane c, ME-180/SEN + poly(I)·poly(C) and CHX; lane d, FS-4 control; lane e, FS-4 + TNF; lane f, FS-4 + poly(I)·poly(C) and CHX. Labelled β_2-microglobulin (β_2m) mRNA was used as a control.

melanoma cells, Endo et al. (1988) found a decrease in ODC activity preceding the TNF-induced decrease in proliferation.

Increased ODC activity and polyamine production generally precede DNA replication and cell division. For this reason, [^{3}H]thymidine uptake and incorporation into DNA were quantified at a time when stimulated cells would be in S phase (i.e. 10–20 h after addition of TNF). Cellular uptake of thymidine in TNF-treated ME-180/SEN cells was 100% of that in control cells at 10 h. However, incorporation of thymidine into DNA in TNF-treated cells was 160–200% of that in control cultures of exponentionally growing ME-180/SEN cells 14–20 h after addition of 1 nM TNF (Figure 5).

In ME-180/SEN cells, we have found that 1 nM TNF elicited a series of events generally associated with mitogenesis: increased expression of c-*fos* (20–30 min) and c-*myc* (40–60 min), increased ODC activity (3 h) and intracellular polyamine content (7 h), and increased thymidine incorporation into DNA (14 h). By 18 h, however, there was already a 25% decrease in cell number. We have also characterized the properties of ME-180 cells that are resistant to TNF-promoted cytotoxicity. As with the sensitive line, TNF promoted similar increases in the expression of c-*fos* and c-*myc*, ODC activity and cellular polyamine content in the ME-180/RES line (results not shown). In contrast to the sensitive line, however, TNF did not augment [^{3}H]thymidine incorporation into DNA in ME-180/RES cells (Figure 5). Thus an incomplete complement of these mitogenic signals was associated with resistance to TNF-mediated cytotoxicity.

One potential growth-inhibitory signal induced by TNF is IL-6. Autocrine stimulation by IL-6 has been shown to attenuate TNF-induced mitogenesis in FS-4 cells (Kohase et al., 1986). However, Northern blot analysis of total RNA from control or TNF-treated ME-180/SEN cells did not detect IL-6 mRNA (Figure 6, lanes a and b), though it easily detected it in TNF- or poly(I)·poly(C) + CHX-treated FS-4 cells (lanes e and f). Thus IL-6 production cannot account for the growth-inhibitory response to TNF in ME-180/SEN cells.

Lewis et al. (1987) reported that very low concentrations of TNF promoted a growth response in their ME-180^{neo} cells, whereas greater than 60 pM TNF resulted in a cytotoxic response. We tested a wide range of concentrations of TNF on our ME-180/SEN cells using ODC activity at 3 h post-treatment as a sensitive indicator of the 'mitogenic' response. We found no disproportionate increase in response at very low TNF concentrations (Figure 7). ODC activity increased proportionately with

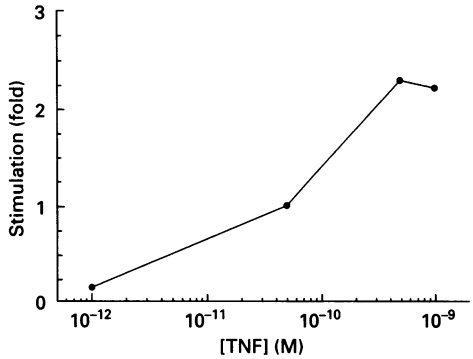

Figure 7 Activity of ODC as a function of TNF concentration

ME-180/SEN cells were treated with the indicated concentrations of TNF for 3 h. Cells were collected and assayed for ODC activity as described. Results are expressed as a fold stimulation over control cultures.

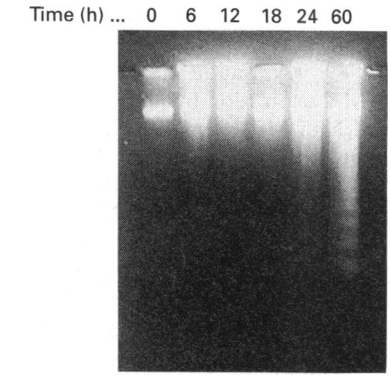

Figure 8 DNA fragmentation in TNF-treated ME-180/SEN cells

ME-180/SEN cells were treated with 1 nM TNF and collected at the indicated times. DNA was isolated and size-fractionated by electrophoresis on a 1.5% agarose/TAE gel. The gel was subsequently stained with ethidium bromide and photographed under u.v. illumination.

Table 1 Effect of polyamine depletion on responsiveness of ME-180/SEN cells to TNF

Twelve-well culture plates were seeded with 3×10^4 ME-180/SEN cells per well. After 24 h, the medium was changed without addition (untreated), with inclusion of 1 mM DFMO (DFMO-pretreated) or with inclusion of 1 mM DFMO plus 10 mM putrescine (DFMO + Putr.). After 48 h the medium was siphoned off and the cultures were washed with calcium- and magnesium-free Hanks' balanced salt solution and then incubated with fresh medium containing no additions (control), 1 nM TNF, 100 μM putrescine or 100 μM putrescine + 1 nM TNF. After 3 days the cell number was assayed, and is reported as the mean ± S.D. of results from three wells.

	$10^{-4} \times$ Cell number		
Treatment	Untreated	DFMO-pretreated	DFMO + Putr.
Control	104 ± 4	16 ± 5	130 ± 3
1 nM TNF	50 ± 4	15 ± 1	57 ± 3
100 μM Putrescine	111 ± 3	70 ± 6	124 ± 9
Putrescine + TNF	45 ± 4	28 ± 4	52 ± 7

Table 2 Effect of serum depletion on responsiveness of ME-180/SEN cells to TNF

Six-well culture plates were seeded with 5×10^4 cells in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). After 24 h the cultures were washed with PBS and then incubated with medium containing 10% or 0.1% FBS. After 2 days, 1 nM TNF was added to half of the plates. After 24 h the cell number was assayed and is reported as the mean ± S.D. of results from three wells. Values in parentheses indicate cell number as a percentage of the relevant control (no TNF) value.

Treatment	$10^{-4} \times$ Cell number
10%FBS	51 ± 1
10% FBS + TNF	37 ± 1 (72%)
0.1% FBS	9.3 ± 0.1
0.1% FBS + TNF	8.7 ± 0.4 (94%)

TNF concentration, and the maximum ODC activity was reached at 0.5 nM TNF, near the approximate K_d for the high-affinity TNF receptor (Kull, 1988). In addition, Marino et al. (1989) found no increase in cell number in response to treatment with picomolar concentrations of TNF in the line of ME-180 cells used in the present study. The difference in growth response to TNF between the ME-180/SEN cells used in the present study and the ME-180^{neo} cells used by Lewis et al. (1987) might be explained by the pSVE^{neo}Ba16 plasmid carried by the ME-180^{neo} cells or by the transfection procedure itself. Alternatively, the difference in response may be due to experimental conditions such as cell density. We and others (Fletcher et al., 1987; Patek et al., 1989) have shown that cell growth and responses to TNF can be dependent on cell density in several cell types (results not shown). In summary, neither a concentration-dependent biphasic response nor the production of IL-6 can account for the simultaneous mitogenic and antiproliferative events elicited by TNF in our ME-180/SEN cells.

The 'mitogenic' events triggered in ME-180 cells by TNF could be part of the mechanism which causes a TNF-induced increase in proliferation in other cell types and be incidental to the cytotoxic response generated in ME-180 cells. Conversely, these events could be important in generating the cytotoxic response, as the absence of increased DNA synthesis in ME-180/RES cells would suggest. If mitogenic signals and/or cell division are important components of the cytotoxic response, inhibiting mitogenesis may diminish the cells' sensitivity to TNF. Two methods of decreasing the proliferation rate were used to test this hypothesis. DFMO treatment was used to specifically inhibit cellular polyamine production by inactivating ODC (Pegg and McCann, 1982). DFMO treatment of ME-180/SEN cells greatly decreased their proliferation rate and rendered them insensitive to the cytotoxic actions of TNF (Table 1). Addition of putrescine to DFMO-treated cultures restored both the proliferation rate and responsiveness to TNF. A decrease in the proliferation rate of ME-180/SEN cells caused by serum starvation was also accompanied by a loss of sensitivity to TNF-induced cytotoxicity (Table 2). These data suggest a relationship between cellular proliferation and sensitivity to TNF in our ME-180 cells.

Intracellular signals known to be part of a mitogenic response have also been associated with apoptosis or programmed cell death (Evan et al., 1992). Increased c-*fos* and c-*myc* expression in prostate cells (Buttyan et al., 1988) and increased c-*myc* and ODC expression in 32D myeloid cells (Askew et al., 1991) preceded apoptosis triggered by withdrawal of essential factors. The cytotoxic response to TNF in several cells is through apoptosis, as shown by the characteristic DNA fragmentation into multiples of approx. 200 bp (Rubin et al., 1988; Robaye et al., 1991). ME-180/SEN cells treated with 1 nM TNF for various times exhibited the characteristic DNA fragmentation pattern of programmed cell death by 60 h of treatment (Figure 8). In addition to the early-response genes c-*fos* and c-*myc*, apoptosis is

preceded by increased expression of the testosterone-repressed prostate message-2 gene (TRPM-2) in a number of tissues (Buttyan et al., 1989; Kyprianou et al., 1991). We found no alteration in TRPM-2 expression by Northern analysis of TNF-treated ME-180/SEN or ME-180/RES cells (results not shown). Thus apoptosis is at least partially responsible for TNF-induced cytotoxicity in ME-180/SEN cells, and TRPM-2 appears not to be universally essential to cell-directed DNA fragmentation.

Previous studies (reviewed in Sporn and Roberts, 1988) have shown that hormones may be growth-stimulatory or growth-inhibitory to cells, depending upon the composition of the extracellular medium. Our observations suggest that a similar situation may exist within the cell. In so far as the cellular response to TNF is concerned, processes commonly associated with mitogenesis may elicit the opposite response when introduced into the appropriate intracellular milieu. In cultures of dividing ME-180/SEN cells, TNF stimulated c-*fos* and c-*myc* expression, ODC activity, polyamine metabolism and DNA synthesis. In spite of this supposed mitogenic stimulus, the viable cell number decreased via apoptosis. A relationship between TNF-induced cytotoxicity and the cell cycle has been reported in L929 murine fibrosarcoma cells (Darzynkiewicz et al., 1984; Coffman et al., 1989). Cell death occurred during late mitosis or just after cell division in these cells, suggesting that cell division may be essential to the cytotoxic response. When mitogenesis in ME-180 cells was disrupted by inhibition of ODC, serum starvation or the lesion in the stimulatory pathway found in ME-180/RES cells, cells were no longer sensitive to TNF-induced cytotoxicity. This demonstrates a close association between apoptosis and mitogenesis through shared intracellular signals, and implies that mitogenesis may be required for TNF-triggered apoptosis to occur. However, when cell division was prevented by treatment with the protein synthesis inhibitor cycloheximide, both ME-180/SEN and ME-180/RES cells became very sensitive to TNF-induced cytotoxicity. This indicates that, although mitogenic signals may be closely associated with the cytotoxic response, mitogenesis itself is not required. It also suggests that a proteinaceous factor may be involved in conferring resistance.

The presence of an inhibitor of apoptosis has been proposed in cells which undergo apoptosis upon withdrawal of essential factors (reviewed in Williams, 1991). Topoisomerase II has been shown to be important in repairing DNA damage resulting from TNF treatment (Baloch et al., 1990) and may thus act as a salvage protein. The presence of such an inhibitor or salvage protein in ME-180 cells could reconcile the growth and cytotoxicity data by providing a mechanism to modulate the final outcome of a shared set of signals. We inhibited both mitogenesis and TNF-induced cytotoxicity by several methods. However, by inhibiting protein synthesis, we inhibited mitogenesis and promoted cytotoxicity. A balance between TNF-induced mitogenesis/apoptosis and the cells' ability to inhibit or repair apoptosis may provide the biological flexibility necessary for TNF to elicit its varied effects on cell proliferation.

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