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Transfection of cells with *transforming growth factor-α* leads to cellular resistance to the antiproliferative effects of tumor necrosis factor

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Abstract Tumor necrosis factor (TNF) is a growth-modulatory cytokine that inhibits the growth of certain cell lines, stimulates the growth of some, and has no effect on the growth of still others. The molecular basis for this differential regulation of growth by TNF is not understood. We postulate that the growth of normal or tumor cells is determined by the balance between growth-stimulatory and -inhibitory signals. In the present study, we demonstrate that the transfection of cells with the *transforming growth factor* (*TGF*)-α gene induces resistance to TNF. Colon carcinoma cell lines that express elevated levels of *TGF*-α were also found to be resistant to this cytokine. Exogenous addition of the growth factor was also effective in decreasing the antiproliferative effects of TNF. Transfection of cells with the *TGF*-α gene led to downmodulation of TNF receptors but an increase in intracellular glutathione levels. Thus, these results support our hypothesis that expression of growth factors by certain tumor cells can lead to resistance to antiproliferative agents such as TNF.

Key words: Transforming growth factor (TGF)-α; TNF; Kinase; Receptor; Cytokine resistance; Glutathione; Cell proliferation

1. Introduction

Tumor necrosis factor (TNF) is a highly pleiotropic cytokine with both a transmembrane and secreted form of 213 and 157 amino acid residues, respectively [1,2]. Depending on the cell line, it has differential growth-modulatory effects, inhibiting the growth of some while stimulating that of others in culture [3]. While several cell lines are highly sensitive to the effects of TNF, others are quite resistant. The molecular basis for this resistance is not understood but roles for manganese superoxide dismutase, growth factors, pp60$^{v\text{-}src}$, HER2/neu/erb B2, and glutathione have been demonstrated [4–8]. Interestingly, the expression of TNF itself by certain tumor cells has been shown to cause resistance to TNF [9].

Transforming growth factor-α (TGF-α) is a potent mitogen that is synthesized as a 160 amino acid glycosylated and palmitoylated transmembrane precursor. It is released from the cell surface by proteolysis (between alanine and valine) of the extracellular domain as a 50-amino acid mature peptide (for references see [10]). Like TNF, the transmembrane (unprocessed) form of TGF-α can transduce signals. TGF-α is a member of the epidermal growth factor (EGF) family of proteins, and it shares structural and functional characteristics with EGF. TGF-α also binds to the EGF receptor that is a protein tyrosine kinase. Elevated levels of TGF-α have been associated with cellular transformation [11,12], and functioning as an autocrine growth factor in human renal cell carcinoma, colon carcinoma, breast adenocarcinoma, pancreatic carcinoma, and prostatic carcinoma [13–19]. Overexpression of this cytokine in transgenic mice has been shown to cause epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast [20].

The differential growth-modulatory activities of TNF against different cell types may be the result of differences in the balance between growth-stimulatory and -inhibitory signals. Previously, we have shown that cells transfected with the human protein tyrosine kinase genes *EGF receptor-2* (*HER-2/erb B/neu*), or with pp60$^{v\text{-}src}$ are resistant to TNF [6,7]. In the present study, we investigated the effects of TGF-α transfection on the anticellular activity of TNF. We found that it also induced resistance to TNF. In contrast to pp60$^{v\text{-}src}$ but like *Her2* kinase, resistance induced by TGF-α was accompanied by the modulation of TNF receptors. Cellular resistance to TNF correlated with an increase in intracellular glutathione levels.

2. Materials and methods

2.1. Materials

Gentamicin and fetal calf serum (FCS) were obtained from GIBCO, Grand Island, NY. DMEM was obtained from Whittaker MA Bioproducts, Walkersville, MD. Bacteria-derived recombinant human TNF and murine interferon-γ (IFN-γ) purified to homogeneity were kindly provided by Genentech Inc., South San Francisco, CA. Other chemical agents were purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Cells

Human colon carcinoma cell lines, HCT116 and JVC which secrete elevated levels of TGF-α protein [14], were kindly provided by Dr. S. Chakrabarty from M.D. Anderson Cancer Center, Houston. Human *TGF*-α-transfected NIH3T3 cells and the *neomycin* (*neo*)-transfected control lines were the gift of Dr. Stuart A. Aaronson of the National Cancer Institute, Bethesda, MD. Cells were transfected with plasmid pSVTGFa containing a 595-bp fragment comprising the entire coding region for *TGF*-α but lacking a potential polyadenylation signal [12]. Cells were routinely grown in DMEM supplemented with glutamine (2 mM), gentamicin (50 μg/ml), and FBS (10%) in a humidified incubator in 5% CO_2 in air. Occasionally, the transfected cells were checked for neomycin resistance by including G418 (500 μg/ml) in the medium.

2.3. Antiproliferation assays

For growth inhibition assays, cells (5×10^3/well) were plated over-

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Abbreviations: TNF, tumor necrosis factor; TGF-α, transforming growth factor; IFN, interferon; EGF, epidermal growth factor; FBS, fetal bovine serum; GSH, glutathione.

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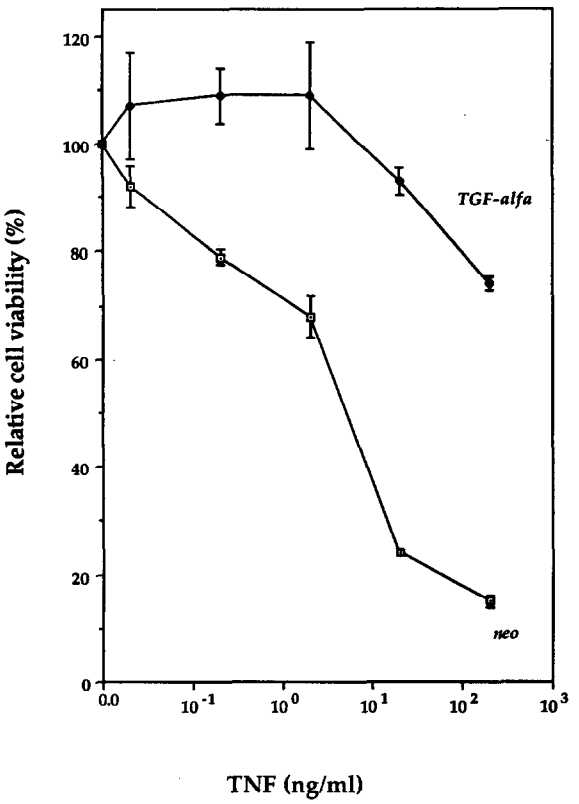


Fig. 1. Antiproliferative effects of different concentrations of TNF against *neo* and *TGF-α*-transfected NIH3T3 cells. 5×10^3 cells/well in 0.1 ml were incubated with various concentrations of human TNF in 96-well plates at 37°C for 72 h. The relative cell viability was determined by staining with Crystal violet as described in section 2. All determinations were in triplicate.

night in 0.1 ml of DMEM with 10% FBS in 96-well Falcon plates. Thereafter, the medium was removed and a serial dilution of human TNF was layered in 0.1 ml of the medium. After a 72-h incubation at 37°C, the viable cells were monitored by crystal violet staining according to the procedure as described [3]. The relative cell viability was calculated as optical density in the presence of the test sample divided by optical density in the absence of the test sample (medium) and expressed as a percentage.

2.4. Receptor-binding assay

Binding assays were performed in 96-well microplates (Falcon 3911, Becton Dickinson Labware, Oxnard, CA) as described [21,22].

2.5. Determination of intracellular glutathione levels

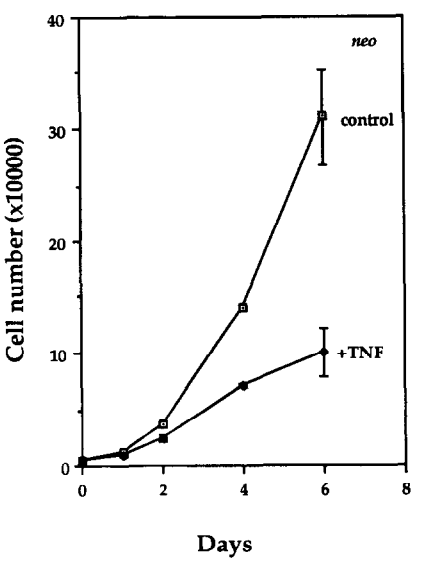
The intracellular glutathione levels were determined by ion-exchange high-performance liquid chromatography as described previously [23]. Briefly, 3×10^6 cells were homogenized in 10% $HClO_4$ and centrifuged. The pellet was analyzed for protein by the method of Lowry, and the supernatant was analyzed for glutathione content. All determinations were made in triplicate, and the results were expressed as nanomoles of glutathione per mg of protein.

3. Results

3.1. NIH-3T3 cells transfected with TGF-α *are resistant to TNF*

First we examined the antiproliferative effects of TNF by crystal violet dye uptake on NIH3T3 cells transfected with either the *neo* (control) or *TGF-α* gene. The control cells were growth inhibited by TNF in a dose-dependent manner (Fig. 1), whereas *TGF-α*-transfected cells were relatively resistant. At 20 ng/ml TNF, the growth inhibition of control and *TGF-α*-transfected cells was 78% and 5%, respectively.

Since *TGF-α*-transfected cells grow faster than control cells, we also examined the effect of TNF on the growth rate of cells as determined by the change in viable cell number over time. The results of this experiment, shown in Fig. 2, indicated that in the presence of TNF the growth of control cells was inhibited by almost 3-fold for up to 6 days (upper panel), whereas *TGF-α*-transfected cells continued to proliferate in the presence of TNF at a rate identical to untreated cells (lower panel). These results further confirm the role of *TGF-α* transfection in inducing resistance to TNF. NIH-3T3 cells transfected with the



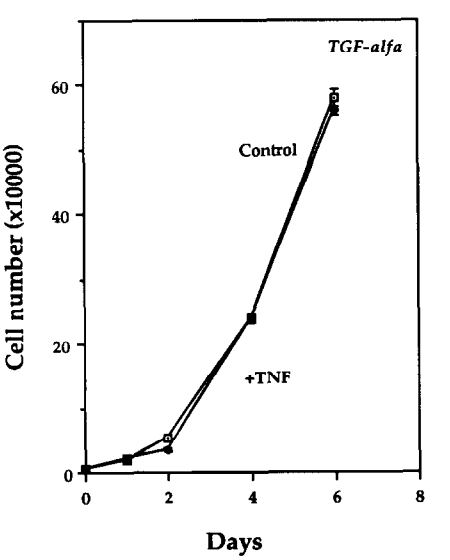


Fig. 2. Effect of TNF on the growth rate of *neo*- (upper panel) and *TGF-α*-transfected (lower panel) NIH3T3 cells. 5×10^3 cells/well in 1 ml were incubated in the presence or absence of TNF (20 ng/ml) in 24-well plates at 37°C. At indicated times cell number was determined by the Trypan blue exclusion method as described in section 2. All determinations were in duplicate.

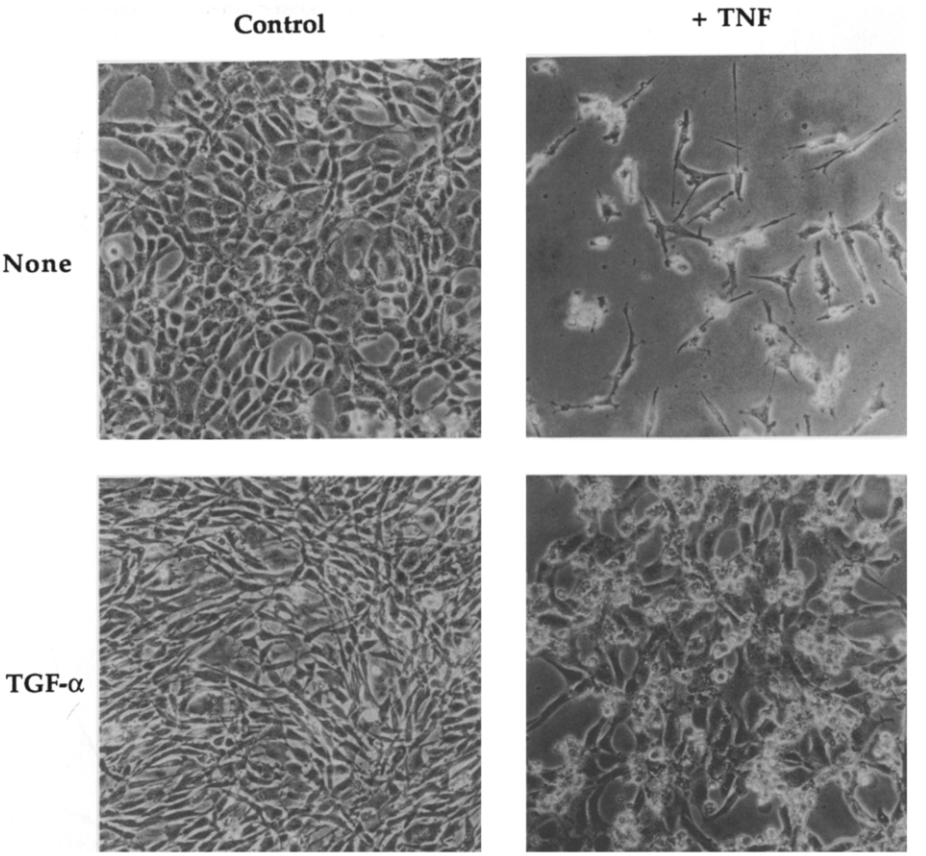
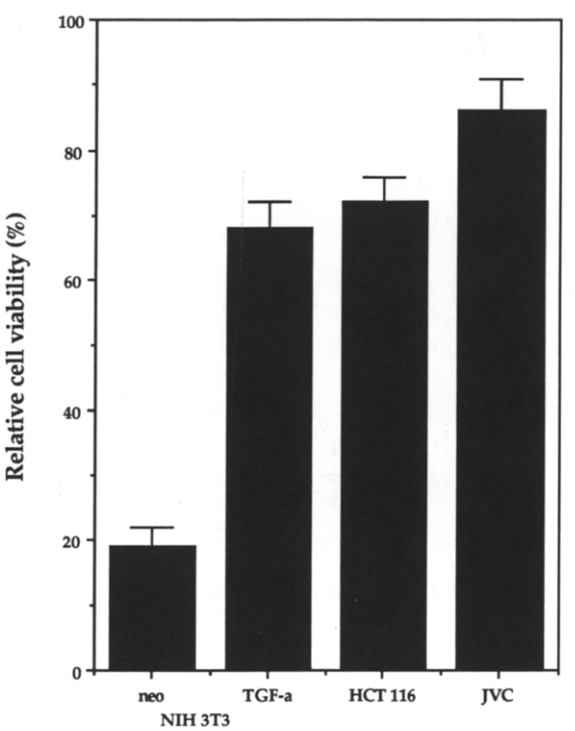


Fig. 3. Effect of TNF on the morphology of *neo* control and *TGF*-α-transfected NIH3T3 cells. 5×10^3 cells/well in 1 ml were incubated in the presence or absence of TNF (20 ng/ml) in 24-well plates at 37°C for 72 h, the media changed, and the cells photographed at 32× magnification.



TGF-α gene were also examined for morphology. As shown in Fig. 3, cells transfected with *TGF*-α differed significantly in morphology from that of *neo* control. Control cells had a flattish whereas *TGF*-α-transfected cells were spindle shaped.

3.2. *Tumor cell lines that overexpress* TGF-α *are resistant to TNF*

Human colon carcinoma cell lines HCT116 and JVC have been shown to secrete elevated levels of TGF-α (5.7 ± 2.2 and 9.4 ± 0.5 ng/ml) [14]. To corroborate our observation on *TGF*-α-transfected cells, we examined these cell lines for sensitivity to TNF. As shown in Fig. 4, both cell lines were as resistant to TNF as *TGF*-α transfected cells.

3.3. *Exogenous addition of EGF reduces the sensitivity of cells to TNF*

TGF-α and EGF are structurally and functionally homologous proteins that interact with a common cell surface receptor [10]. To determine if exogenous addition of EGF could reduce the sensitivity of cells to TNF, *neo* control cells were exposed to TNF in the presence and absence of EGF. As shown in Fig.

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Fig. 4. Effect of TNF on the viability of *neo*- and *TGF*-α-overexpressing cell lines. 5×10^3 cells/well in 1 ml were incubated in the presence or absence of TNF (200 ng/ml) in 96-well plates at 37°C for 72 h and then cell viability was determined by the Crystal violet method as described in section 2. All determinations were in duplicate.

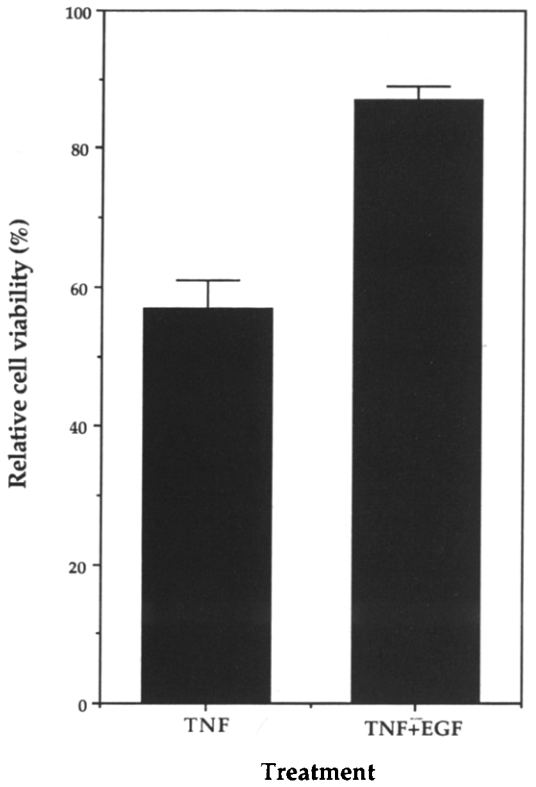


Fig. 5. Effect of exogenous epidermal growth factor on the antiproliferative effects of TNF against neo NIH3T3 cells. 5×10^3 cells/well in 0.1 ml were incubated with either human TNF (2 ng/ml) or TNF and EGF (10 ng/ml) in 96-well plates at 37°C for 72 h. The relative cell viability was determined by staining with crystal violet as described in section 2. All determinations were in triplicate.

5, EGF treatment significantly decreased the antiproliferative effect of TNF. These results are consistent with those obtained with TGF-α-transfected cells.

3.4. *Cellular resistance to TNF by* TGF-α*-transfection down modulates TNF receptors*

Previously we have shown that transfection of cells with the *HER2* oncogene leads to a resistance of cells to TNF that correlates with down modulation of TNF receptors [7]. Therefore, we examined the effect of *TGF-α* transfection of cells on TNF receptors. The results shown in Fig. 6 indicate that there was a significant decrease in the binding of TNF to *TGF-α*-transfected cells as compared to the *neo* control.

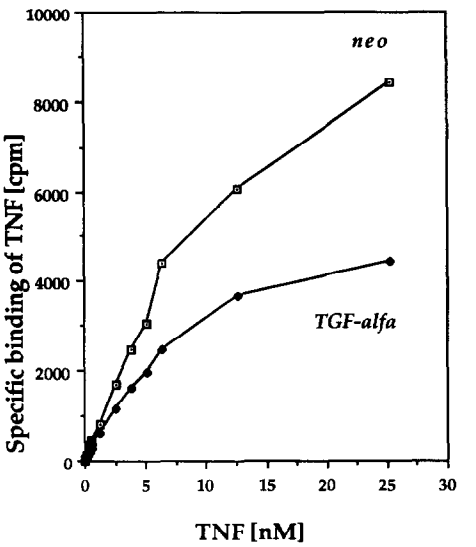
3.5. *Cellular resistance to TNF induced by TGF-α correlates with an increase in intracellular glutathione levels*

We have reported that the cell density-dependent [24] cellular resistance to TNF and that induced by $pp60^{v\text{-}src}$ [6] correlate with the decrease in intracellular glutathione levels. We, therefore, examined glutathione levels in control and *TGF-α*-transfected cells. There was about 80% more glutathione in the *TGF-α*-transfected cells than in the *neo* control (Fig. 7).

4. Discussion

We report here that transfection of NIH-3T3 cells with *TGF-α* leads to resistance to the antiproliferative effects of TNF. The induction of resistance was accompanied by modulation of TNF receptors and an increase in intracellular glutathione levels.

Why some tumor cells are sensitive and others resistant to TNF is not understood. Previously, we have shown that tumor cells overexpressing *HER2* or $pp60^{v\text{-}src}$ are resistant to TNF [6,7]. We now report that *TGF-α* overexpression also induces TNF resistance and that this may occur through the modulation of TNF receptors, as was the case for *HER2*. TGF-α mRNA is overexpressed in several different types of tumors and tumor cell lines, including glioblastomas, fibrosarcoma, colon carcinoma, pancreatic carcinoma, prostate carcinoma,



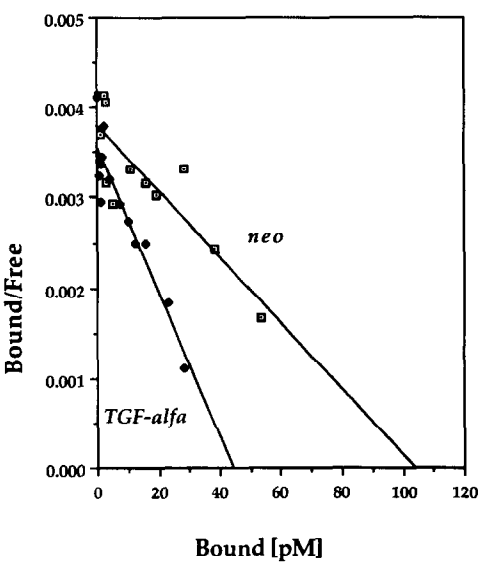


Fig. 6. Specific binding of labeled hTNF to control and *TGF-α*-transfected NIH3T3 cells. 1×10^6 cells (0.1 ml) in 96-well plates were incubated with different concentrations of labeled TNF either in the presence (nonspecific binding) or absence (total binding) of 100 nM unlabeled TNF for 1 h at 4°C. Thereafter cells were centrifuged and washed thrice, and cell-bound radioactivity counted. Specific binding was calculated as a difference between total binding and nonspecific binding. All determinations were made in triplicate.

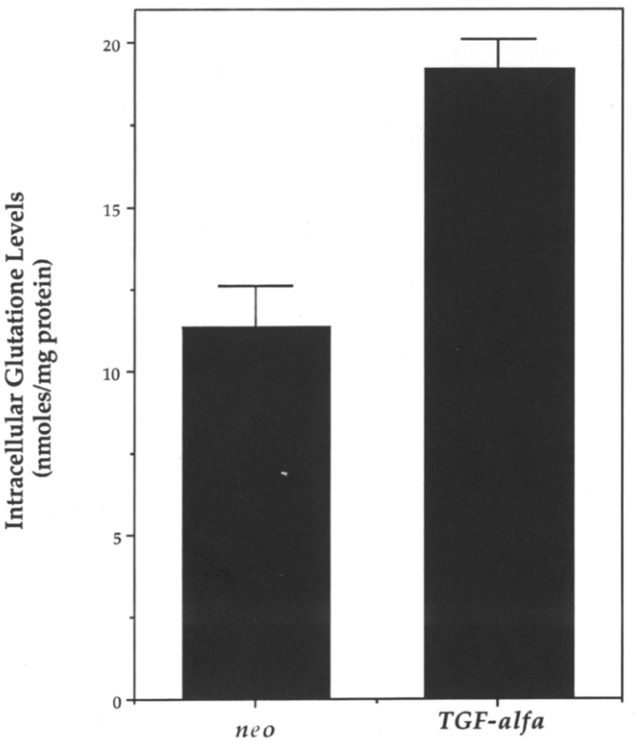


Fig. 7. Intracellular glutathione levels in control and TGF-α-transfected NIH3T3 cells. Bars represent the glutathione levels (nmoles per mg of protein) in *neo* and *TGF-α*-transfected NIH3T3 cells. All determinations were made in triplicate.

and breast carcinoma [13–19]. Human tumor cell lines HCT 116 and JVC (colon adenocarcinoma), which are known to overexpress TGF-α [14], were also found to be resistant to TNF.

How *TGF-α* induces resistance to TNF is not clear. It has been shown, however, that TNF causes the induction of TGF-α/EGF receptors and promotes the autophosphorylation of the EGF receptor in certain cell lines [25,26]. Recently we have shown that protein tyrosine phosphatases play an important role in TNF-mediated antiproliferative effects [27]. Since the proliferative effects of TGF-α are mediated through protein phosphorylation whereas the antiproliferative effects of TNF appear to be mediated through dephosphorylation, it is possible that TGF-α inactivates the protein tyrosine phosphatase pathway resulting in cellular resistance. Unlike the studies on the effect of $pp60^{v\text{-}src}$ [6], we found that resistance of *TGF-α*-transfected cells to TNF cannot be overcome by treatment of cells with IFN-γ (data not shown). We also found that *TGF-α*-mediated cellular resistance leads to an increase in intracellular glutathione levels. This result is inconsistent with our recent report showing an association between cell-density-dependent resistance to TNF and a decrease in intracellular GSH levels [24]. A decrease of GSH was also observed in cells transfected with $pp60^{v\text{-}src}$ [6]. The results are, however, consistent with elevation of intracellular GSH levels that occurs in tumor cells which develop resistance to chemotherapeutic agents [23,28]. Since tumorigenesis is a result of the altered expression of multiple oncogenes and growth factors, it is possible that different mechanisms in different tumor cells contribute to the induction of resistance to TNF.

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