Suppression of antiproliferative effects of tumor necrosis factor by transfection of cells with human platelet-derived growth factor B/c-sis gene

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Abstract The growth of cells is determined by the balance between growth-stimulatory and growth-inhibitory signals. In the present study, we demonstrate that the transfection of NIH 3T3 cells with a platelet-derived growth factor (*PDGF-B/c-sis*) gene induces resistance to the anticellular effects of tumor necrosis factor (TNF). Human tumor cell lines that express elevated levels of *c-sis* (e.g. epidermoid carcinoma, A-431) are also TNF resistant, whereas those that express no significant levels of this gene (e.g. breast adenocarcinoma, MCF-7) are TNF sensitive. Transfection of cells with the *c-sis* gene leads to down-modulation of TNF receptors and also a decrease in intracellular glutathione levels. Thus, our results demonstrate that over-expression of *PDGF-B/c-sis* by certain tumor cells can lead to their protection from the anticellular effects of TNF.

Key words: Platelet-derived growth factor (PDGF-B/c-sis); TNF; Kinase; Receptor; Cytokine; Resistance; Glutathione; Proliferation

1. Introduction

Tumor necrosis factor (TNF) is one of the most important growth-modulatory cytokines produced by almost all cell types of the immune system [1]. For some cells TNF is a growth factor, whereas for others it is a growth inhibitor. Although the anticellular effects of TNF are normally paracrine, the proliferative effects could be either autocrine (as in neuroblastoma, ovarian carcinoma, and B cell lymphoma) or paracrine (as in normal human fibroblasts) [2–6]. Several tumor cell lines are highly sensitive to the antiproliferative effects of TNF, and others are quite resistant [5]. The various intracellular and extracellular factors that are responsible for this resistance are unclear, but roles for manganese superoxide dismutase, transforming growth factor-α (TGF-α), pp60$^{v\text{-}src}$, and *HER-2/neu/erb B2*, and glutathione have been demonstrated [7–11]. Certain tumor cell lines that express TNF have also been found to be resistant to TNF by some undefined mechanism [12].

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

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin and consists of disulfide-linked dimers of two related (approximately 60% homologous at their amino acid sequence level) polypeptide chains designated A and B (for references see [13]). Formation of either heterodimers or homodimers of A and B chains are essential for their biological activities. A transforming gene from simian sarcoma virus (SSV), *v-sis*, has been shown to encode a protein that is structurally related to the B chain of PDGF, thus indicating a role for this growth factor in tumorigenesis. Indeed, the overexpression of *PDGF-B/c-sis* in murine fibroblasts causes cellular transformation in vitro and generation of fibrosarcoma in vivo [14]. Elevated levels of *PDGF-B/c-sis* have also been associated with cellular transformation [15,16] and shown to occur in human epithelial tumor cell lines derived from stomach, pancreas, breast, prostate, lung, glioblastomas, and fibrosarcoma (for references see [13]).

The study of factors that enhance cellular growth and those that suppress growth should lead to a better understanding of the processes involved in the regulation of normal and aberrant cell proliferation. The differential growth-modulatory activities of TNF against different cell types may be a result of the regulation of cellular growth by the balance between stimulatory and inhibitory signals. Previously, we showed that cells transfected with human protein tyrosine kinases, *HER-2* or pp60$^{v\text{-}src}$ or with growth factor TGF-α which activates tyrosine kinase, develop resistance to TNF [9,10,17]. In the present study, we investigated the effects of another gene, *PDGF-B/c-sis*, on the growth-suppressing activity of TNF. We found that it also induced resistance to TNF. In contrast to pp60$^{v\text{-}src}$, but similar to *HER-2* kinase and TGF-α, resistance induced by *c-sis* was accompanied by the modulation of TNF receptors. Cellular resistance to TNF correlated with a decrease in intracellular glutathione levels.

2. Materials and methods

2.1. Materials

Gentamicin and fetal bovine serum (FBS) 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 Co. (St. Louis, MO).

2.2. Cells

Human epidermoid carcinoma cells, A-431 (CRL 1555), and breast adenocarcinoma cells, MCF-7 (HTB 22), were obtained from American Type Culture Collection, Rockville, MD. Human *PDGF-2/c-sis*-transfected NIH3T3 cells and the neomycin-transfected control lines were a gift of Dr. Stuart A. Aaronson from National Cancer Institute,

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Bethesda, MD. These cells were transfected in the laboratory of Dr. Aaronson by the calcium phosphate method using plasmid pPDGF-B under the control of a retroviral promoter containing an entire coding region for human *PDGF-2/c-sis* [16]. NIH-3T3 cells expressing the *neo* gene (pSV-neo) were selected by their ability to grow in the presence of the neomycin analogue G418. 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. Antiproliferative assays

For growth inhibition assays, cells (5×10^3/well) were plated overnight in 0.1 ml of DMEM with 10% FBS in 96-well Falcon plates. The medium was then 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 previously described procedure [3]. The relative cell viability was calculated as the optical density in the presence of the treated sample divided by the optical density of the untreated control samples and expressed as a percentage.

2.4. Receptor-binding assay

Binding assays for TNF receptors were performed in 96-well microplates as described elsewhere [18].

2.5. Determination of intracellular glutathione levels

The intracellular glutathione levels were determined by an ion-exchange high-performance liquid chromatography method as described previously [19]. Briefly, 3×10^6 cultured 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 nmol glutathione/mg protein.

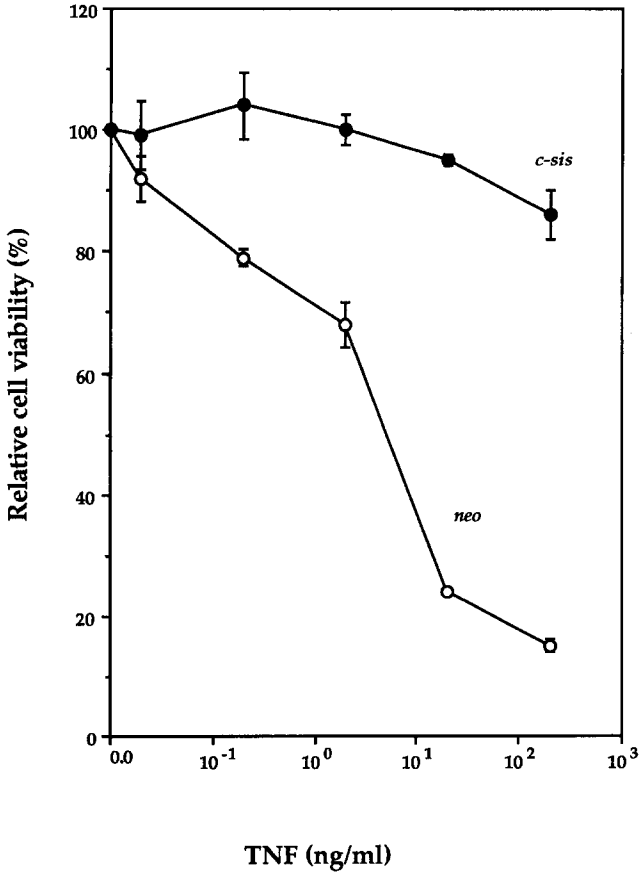


Fig. 1. Antiproliferative effects of different concentrations of TNF against *neo*- and *c-sis*-transfected NIH 3T3 cells. 5×10^3 cells/well in 0.1 ml were incubated with varying concentrations of human TNF in 96-well plates at 37°C for 72 h. The relative cell viability was determined by staining with Crystal violet as described in section 2. All determinations were in triplicate.

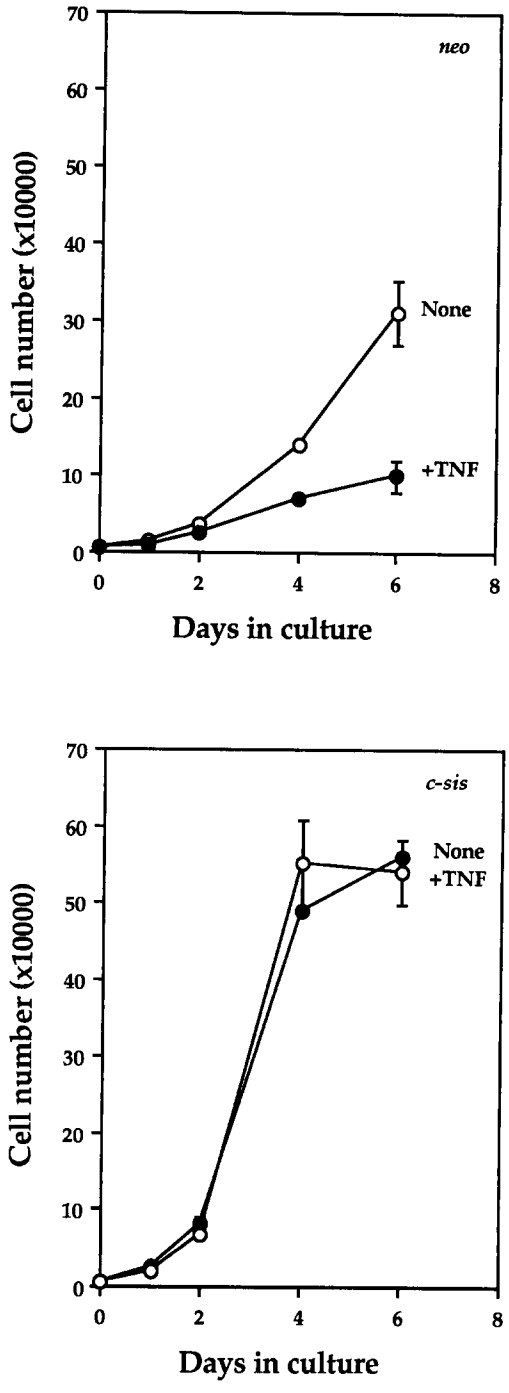


Fig. 2. Effect of TNF on the growth rate of *neo*- (upper panel) and *c-sis*- (lower panel) transfected NIH 3T3 cells. 5×10^3 cells/well in 1 ml were incubated in the presence or absence of TNF (20 ng/ml) in 24-well plates at 37°C. At indicated times, cell number was determined by the Trypan blue exclusion method as described in section 2. All determinations were made in duplicate.

3. Results

3.1. NIH-3T3 cells transfected with PDGF-B/c-sis are resistant to TNF

First we examined the antiproliferative effects of TNF by Crystal violet uptake on NIH3T3 cells transfected with either *neo* (control) or *c-sis*. The control cells were growth inhibited by TNF in a dose-dependent manner (Fig. 1), whereas *c-sis*-transfected cells were relatively resistant. At 20 ng/ml TNF, the growth inhibition of control- and *c-sis*-transfected cells was approximately 80 and 5%, respectively.

Because *c-sis*-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, whereas *c-sis*-transfected cells continued to proliferate in the presence of TNF at a rate identical to untreated cells. These results further confirm that *PDGF-B* transfection induces resistance to TNF. NIH-3T3 cells transfected with the *c-sis* gene were also examined for morphology. As shown in Fig. 3, cells transfected with *c-sis* differ in morphology from that of the *neo* control cells, the latter appearing more spindle-shaped as compared to cells transfected with *c-sis*.

3.2. PDGF-B expression in tumor cell lines correlates with sensitivity to TNF

The human epithelioid carcinoma cell line A-431 has been shown to express an elevated level of mRNA for PDGF-B, whereas the human breast adenocarcinoma cell line MCF-7 expresses no detectable levels of the message [20]. To correlate this with our observation on *c-sis*-transfected cells, we examined these cell lines for sensitivity to TNF. As shown in Fig. 4, the A-431 cell line was found to be almost as resistant to TNF as *c-sis*-transfected cells. In contrast, MCF-7 cells were as sensitive to the antiproliferative effects of TNF as the *neo* control.

3.3. Cellular resistance to TNF by c-sis transfection causes down-modulation of TNF receptors

We have previously shown that TNF resistance associated with transfection of cells with the HER2 oncogene or with

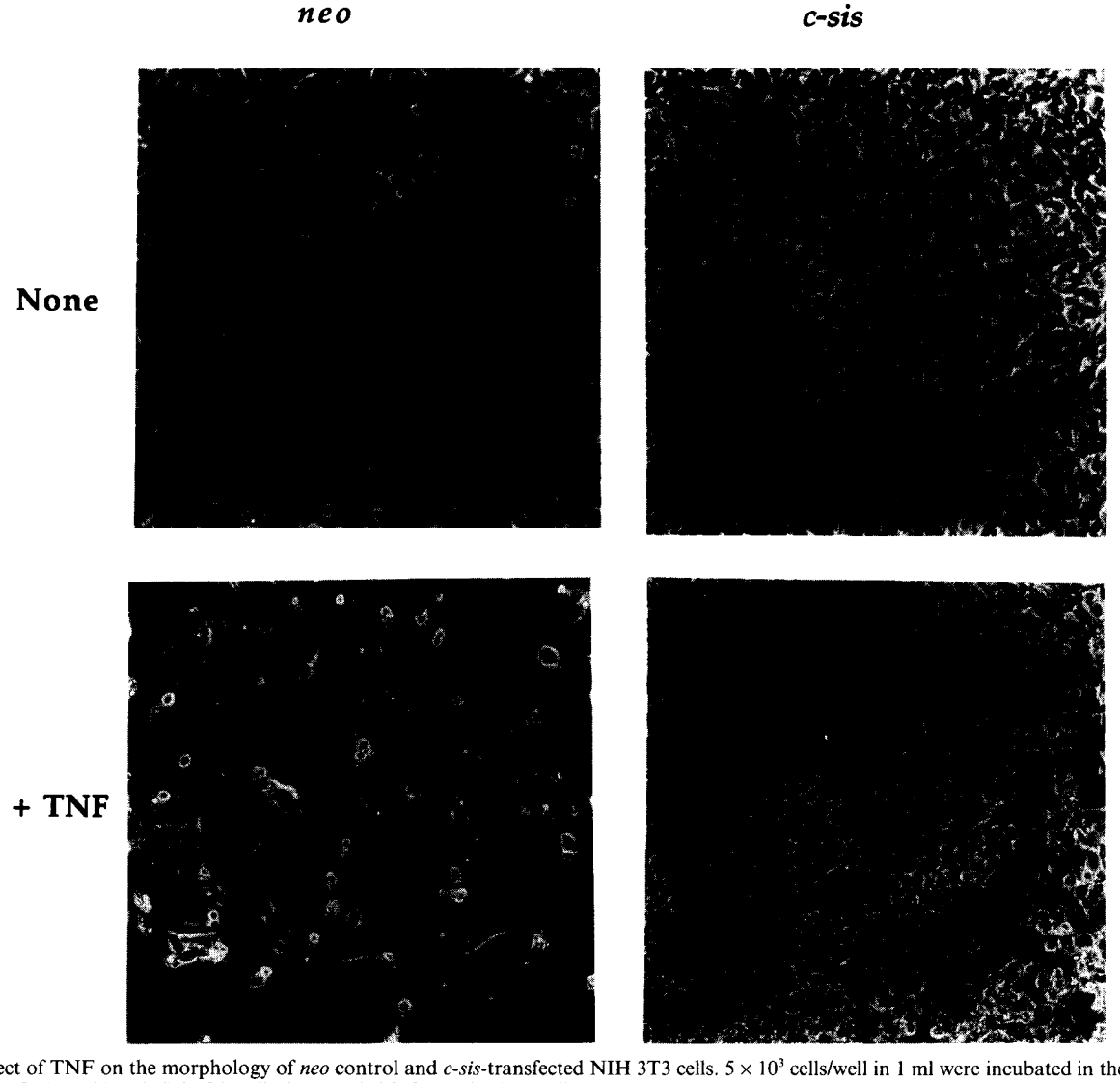


Fig. 3. Effect of TNF on the morphology of *neo* control and *c-sis*-transfected NIH 3T3 cells. 5×10^3 cells/well in 1 ml were incubated in the presence or absence of TNF (20 ng/ml) in 24-well plates at 37°C for 72 h, the medium was changed and the cells were photographed at ×40 magnification.

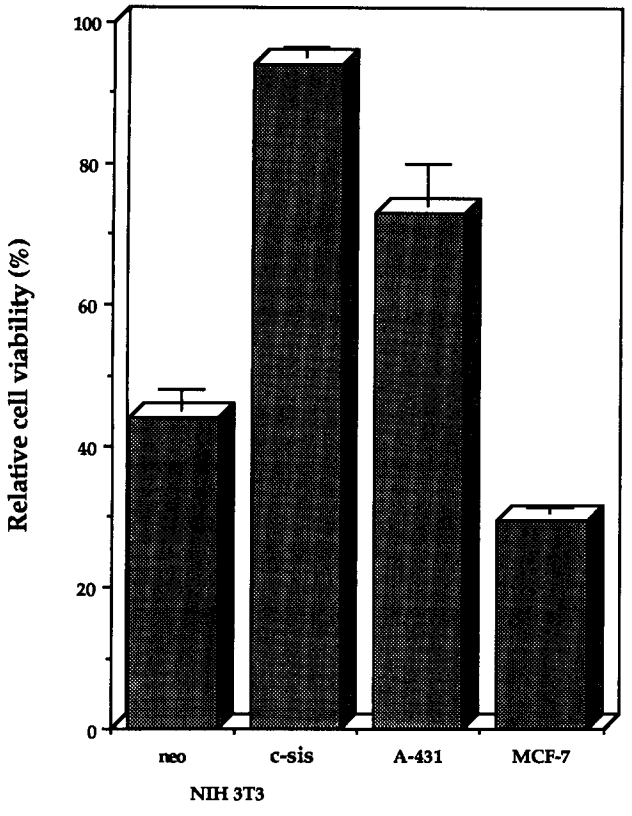


Fig. 4. Effect of TNF on tumor cell lines which over-express *PDGF-B/c-sis*. 5×10^3 cells/well in 1 ml were incubated in the presence or absence of TNF (20 ng/ml) in 24-well plates at 37°C for 72 h and then cell viability was determined by Trypan blue exclusion as described in section 2. All determinations were in duplicate.

TGF-α correlates with down-modulation of TNF receptors [10,17]. Therefore, we decided to examine the effect of *c-sis* transfection of cells on the TNF receptors. The results shown in Fig. 5 indicate that there was a significant decrease in the binding of TNF to *c-sis*-transfected cells as compared to the *neo* control. Scatchard analysis revealed that the decrease was mainly due to receptor number rather than receptor affinity.

3.4. *Cellular resistance to TNF induced by* c-sis *correlates with an increase in intracellular glutathione levels*

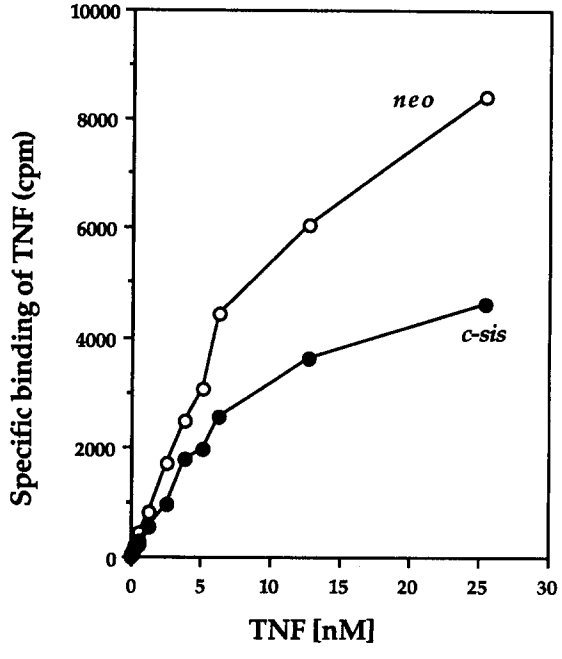
We have reported that cell density-dependent [21] cellular resistance to TNF and that induced by $pp60^{v\text{-}src}$ [9] correlates with the decrease in intracellular glutathione levels; therefore, we examined glutathione levels in control and *c-sis*-transfected cells. There was approximately a 50% decrease in the glutathione contents of the *c-sis*-transfected cells compared to the *neo* controls (Fig. 6).

3.5. *Cellular resistance to TNF induced by* c-sis *cannot be reversed by IFN-γ*

We and others have shown that IFNs can potentiate the antiproliferative effects of TNF [22–26]. We therefore examined the possibility that *PDGF-B*-induced cellular resistance to TNF could be overcome by IFN-γ. The results indicate that IFN-γ had no effect on the anticellular effects of TNF on *c-sis*-transfected cells, but it did potentiate the effect on control cells (data not shown).

4. Discussion

We report here that transfection of NIH 3T3 cells with *c-sis* leads to resistance to the antiproliferative effects of TNF. The induction of resistance was accompanied by the modulation of TNF receptors and with a decrease in intracellular glutathione levels. Furthermore, the cellular resistance to TNF could not be overcome by treatment of cells with IFN-γ.



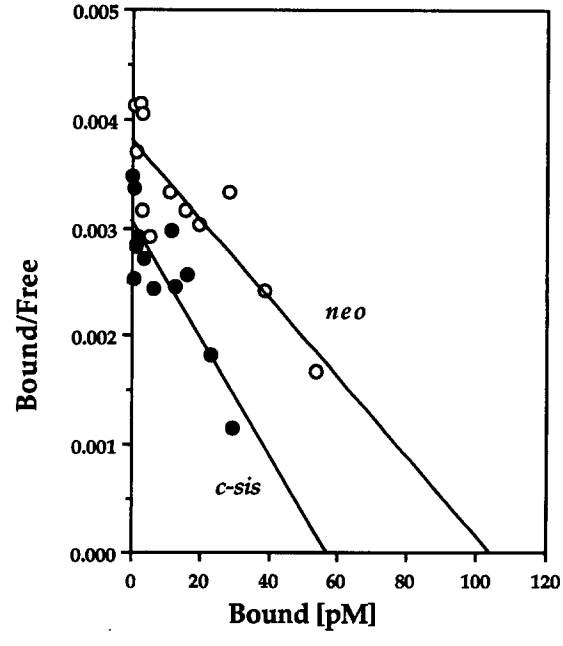


Fig. 5. Specific binding of labeled human TNF to *neo*- and *c-sis*-transfected NIH 3T3 cells. 1×10^6 cells in 0.1 ml in 96-well plates were incubated with different concentrations of labeled TNF either in the presence (non-specific binding) or absence (total binding) of 100 nM of unlabeled TNF for 1 h at 4°C. Cells were then centrifuged and washed three times, and cell-bound radioactivity counted. All determinations were made in triplicate.

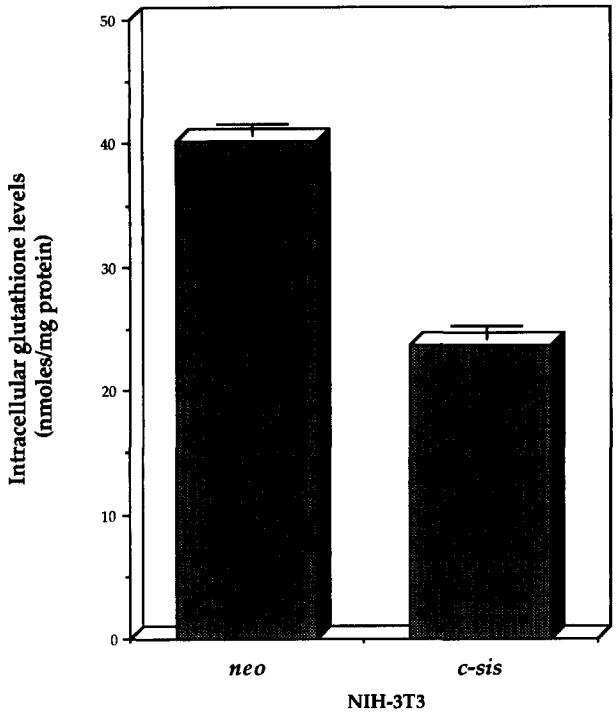


Fig. 6. Intracellular glutathione levels in *neo*- and *c-sis*-transfected NIH 3T3 cells. Bars represent the glutathione levels (nmol/mg protein) in *neo*- and *c-sis*-transfected NIH 3T3 cells. All determinations were made in triplicate.

The molecular basis for the sensitivity of certain tumor cell types to TNF and resistance of others is not understood. It appears that the expression of certain growth factors and genes could play an important role in this process. Previously, we showed that tumor cells that over-express tyrosine protein kinase *HER-2* or pp60$^{v\text{-}src}$ or with growth factor TGF-α are resistant to TNF [9,10,17]. We now report that *PDGF-B/c-sis* expression also induces TNF resistance, and this may occur through modulation of TNF receptors, as noted in the case of *HER-2*. *c-sis* mRNA is over-expressed in several different types of tumors and tumor cell lines, including glioblastoma, fibrosarcoma, stomach carcinoma, pancreatic carcinoma, prostate carcinoma, and breast carcinoma (for references see [13]). Human tumor cell line A-431 (epithelioid carcinoma), which is known to over-express *PDGF-B* [20] was also found to be resistant to TNF, but MCF-7 (breast carcinoma), which does not express this gene, was found to be sensitive.

The mechanism by which *PDGF-B/c-sis* could induce resistance to TNF is not clear. It has been shown, however, that TNF, but not IFN-γ, drastically up-regulates the mRNA levels for *PDGF-B* chain in a dose-dependent manner in a number of pancreatic tumor cell lines [20]. PDGF is known to display mitogenic effects through interaction with its receptor, which leads to autophosphorylation. Like TGF-α, the PDGF receptor is also a tyrosine kinase. Our previous results indicated that cells transfected with tyrosine kinases such as *HER-2* or pp60$^{v\text{-}src}$ become resistant to the anticellular effects of TNF [9,10]. It is therefore possible that the activation of tyrosine kinase by the PDGF-B chain is responsible for protection of cells from TNF. Recently we have shown that protein tyrosine phosphatases play an important role in TNF-mediated antiproliferative effects [27]. Because the proliferative effects of the PDGF-B chain homodimer are mediated through protein phosphorylation whereas the antiproliferative effects of TNF may require dephosphorylation, it is possible that the effect of *c-sis* on cellular resistance is due to the inactivation of the protein tyrosine phosphatase pathway. We also found that *c-sis*-mediated cellular resistance leads to a decrease in intracellular glutathione levels. This is consistent with our recent report showing an association between cell density-dependent resistance to TNF and to a decrease in intracellular GSH levels [21]. Since *c-sis*-transfected cells grow at a rate faster than control cells, TNF resistance may also be due to higher cell density. This is, however, less likely since subconfluent cultures were used for our studies. A decrease in glutathione was also observed in cells transfected with pp60$^{v\text{-}src}$ [9]. These results, however, are inconsistent with those showing tumor cells that develop resistance to chemotherapeutic agents, which leads to elevation of intracellular GSH levels [19,28]. As tumorigenesis is a result of 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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