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pp60$^{v\text{-}src}$ kinase overexpression leads to cellular resistance to the antiproliferative effects of tumor necrosis factor

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Abstract

While some tumor cells are sensitive to the antiproliferative effects of tumor necrosis factor (TNF), others are resistant. The molecular basis for cellular resistance to TNF is not completely understood. Previously we have shown that transfection of cells with an oncogene *HER2/neu/erb B2*, a receptor tyrosine kinase, leads to resistance to the anticellular effects of TNF [(1988) Proc. Natl. Acad. Sci. USA 85, 5102–5106]. In the present study, we demonstrate that the overexpression of another oncogenic tyrosine kinase, pp60$^{v\text{-}src}$ also induces resistance to TNF. In contrast to *HER2*, however, pp60$^{v\text{-}src}$ transfection of cells did not lead to down-modulation of TNF receptors but rather to decreased intracellular glutathione levels. The pp60$^{v\text{-}src}$-induced cellular resistance to TNF could be abrogated by interferon-γ. Thus, these results indicate that the resistance of certain tumors to TNF may also be due in part to the overexpression of *pp60$^{v\text{-}src}$* oncogene.

Key words: pp60v-src; TNF; Kinase; Cytokine; Resistance; Oncogene; Proliferation

1. Introduction

Tumor necrosis factor (TNF) a 17-kDa polypeptide produced by activated macrophages, is highly pleiotropic [1–3]. It inhibits the growth of different tumor cell types and stimulates the growth of certain normal cells in culture [3,4]. While several cell lines are highly sensitive to the antiproliferative effects of TNF, others are quite resistant. The molecular basis for this resistance is not understood but roles for manganese superoxide dismutase [5], growth factors [6], and glutathione [7] have been demonstrated. Interestingly, the expression of TNF itself by certain tumor cells has been shown to cause resistance to TNF [8].

The transforming gene of Rous sarcoma virus (RSV), *v-src*, encodes a 60-kDa phosphoprotein, pp60$^{v\text{-}src}$, which is a tyrosine-specific protein kinase [9]. Its cellular homolog pp60$^{v\text{-}src}$ is localized on the inner surface of the plasma membrane, as well as, other intracellular membranes and represents the prototype of nonreceptor tyrosine kinases. Several intracellular protein tyrosine kinases have been identified, that show a high degree of homology with the product of the protooncogene *c-src*. The receptors for several growth factors including epidermal growth factor, fibroblast growth factor, insulin-like growth factor and platelet-derived growth factor are also tyrosine kinase. These kinases are overexpressed in various tissues, including hematopoietic cells [9].

Previously, we have shown that tumor cell lines that overexpress human epidermal growth factor receptor-2 (*HER-2/erb B/neu*), a protein tyrosine kinase, are resistant to TNF and that transfection of cells with this oncogene also induces resistance to this cytokine [10]. In the present study, we investigated the effects of another tyrosine kinase, pp60$^{v\text{-}src}$, on the anticellular activity of TNF. We found that it also induced resistance to TNF. In constrast to HER2 kinase, however, resistance induced by *v-src* kinase was not due to modulation of TNF-receptors. Rather, cellular resistance to TNF correlated with the downmodulation of 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

pp60$^{v\text{-}src}$-transfected NIH3T3 cells and the neomycin-transfected control lines were the gift from Dr. Mark Smith from National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. 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; IFN, interferon; IL, interleukin; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; GSH, glutathione.

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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 72 h incubation at 37°C, the viable cells were monitored by Crystal violet staining according to the procedure as described [6]. 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 [11,12].

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 [13]. Briefly, 3×10^6 cells cultured 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.

2.6. Determination of pp60$^{v\text{-}src}$ tyrosine kinase activity

The activity of pp60$^{v\text{-}src}$ was assayed as described previously with minor modifications [14]. Briefly, enzyme was extracted from a pellet of 5×10^6 cells in a detergent medium. pp60$^{v\text{-}src}$ was immunoprecipitated with monoclonal antibody clone 327 and the kinase activity of the immunocomplex was determined in the absence (background control) and presence of 2 mg/ml poly E_4Y as the substrate. The phosphate incorporation into the substrate was determined by acid precipitation of the ^{32}P-labeled product onto 3MM filter paper. A unit of kinase activity was defined as pmol of phosphate incorporated into poly E_4Y per minute.

3. Results

3.1. NIH-3T3 cells transfected with pp60$^{v\text{-}src}$ overexpress tyrosine kinase activity and differ in morphology

As shown in Fig. 1, cells transfected with pp60$^{v\text{-}src}$

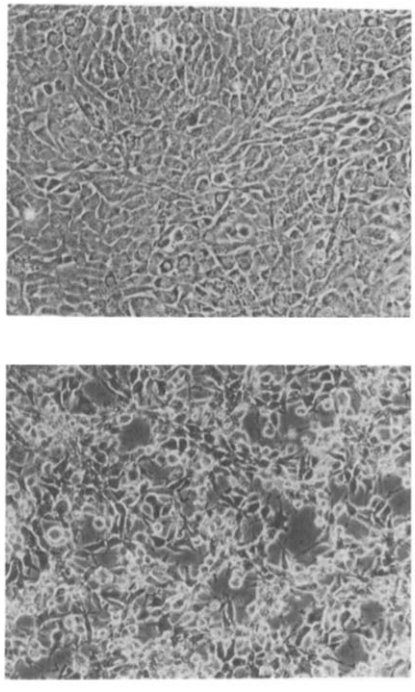
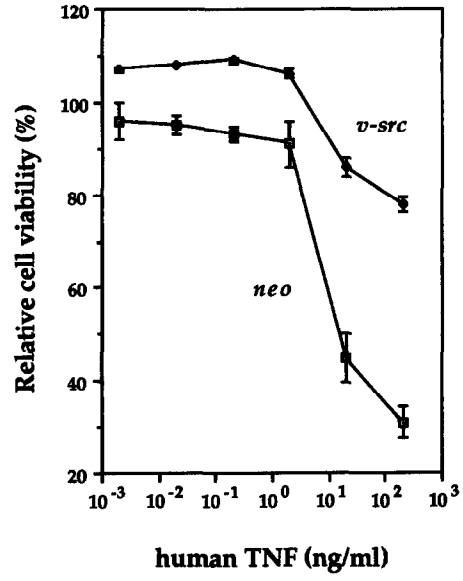


Fig. 1. Morphology of neo control and pp60$^{v\text{-}src}$-transfected NIH3T3 cells. Numbers in parenthesis indicate the kinase activity of pp60$^{v\text{-}src}$.



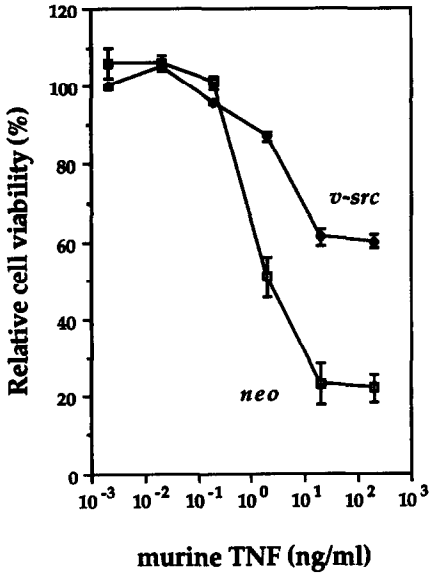


Fig. 2. Antiproliferative effects of different concentrations of TNF against neo and pp60$^{v\text{-}src}$-transfected NIH3T3 cells. 5×10^3 cells/well in 0.1 ml were incubated with varying concentrations of human TNF (upper panel) and murine TNF (lower panel) in 96-well plates at 37°C for 72 h. The relative cell viability was determined by staining with crystal violet as described in section 2. All determinations were in triplicate.

differ significantly in morphology from that of *neo* control. Both control and pp60$^{v\text{-}src}$-transfected cells were solubilized with detergent and tested for tyrosine kinase activity. pp60$^{v\text{-}src}$-transfected cells contained four times as much kinase activity as in control cells (2.4 vs. 0.6 units/10^6 cells).

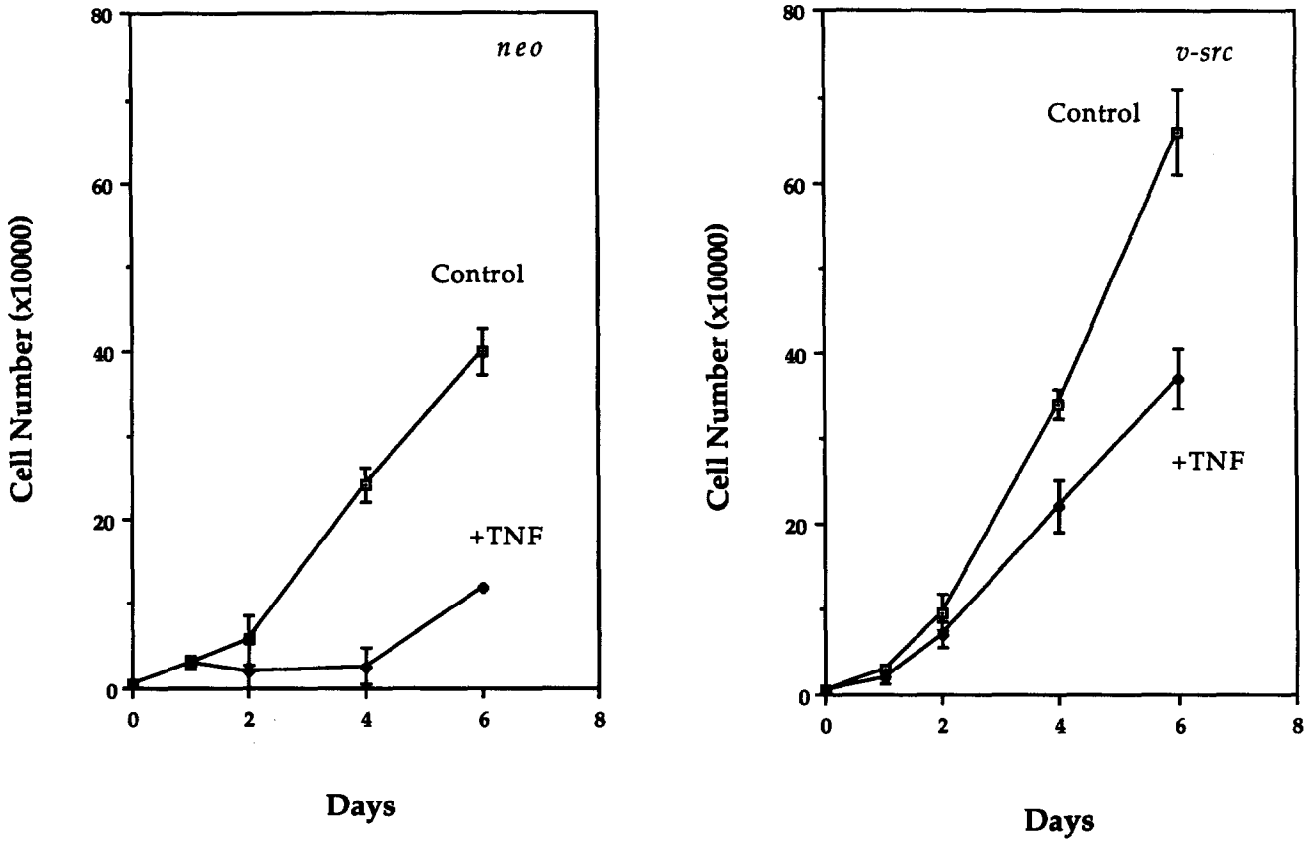


Fig. 3. The effect of hTNF on the growth rate of neo (left panel) and pp60$^{v\text{-}src}$ (right panel)-transfected NIH3T3 cells. 5×10^3 cells/well in 1 ml were incubated in the presence or absence of TNF (20 ng/ml) in 24-well plates at 37°C. At indicated times cell number was determined by Trypan blue exclusion method as described in section 2. All determinations were in duplicate.

3.2. *NIH-3T3 cells transfected with pp60$^{v\text{-}src}$ are resistant to TNF*

First we examined the antiproliferative effects of TNF by Crystal violet dye uptake on NIH3T3 cells transfected with either *neo* (control) or *pp60$^{v\text{-}src}$* genes. The control cells were growth inhibited by TNF in a dose-dependent manner (Fig. 2) whereas *v-src*-transfected cells were relatively resistant. At 200 ng/ml TNF, the growth inhibition of control and *v-src*-transfected cells was 75% and 20%, respectively (upper panel). Since hTNF binds only to the p60 form of the TNF receptor on murine cells, whereas murine TNF on murine cells binds to both the p60 and p80 receptor, we also examined the effect of murine TNF. The results also indicated that *v-src*-transfected cells were more resistant to murine TNF than control cells (Fig. 2; lower panel). At 20 ng/ml of murine TNF, the growth inhibition was approximately 80% for control and 35% for *v-src*-transfected cells.

Since *src*-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. 3, indicated that in the presence of TNF the growth of control cells was completely inhibited for up to 4 days, whereas *src*-transfected cells continued to proliferate at a rate only slightly slower than control cells. These results further confirm the role of pp60$^{v\text{-}src}$ kinase in induction of resistance to TNF.

3.3. *Cellular resistance to TNF by pp60$^{v\text{-}src}$ is not due to down-modulation of TNF receptors*

Previously we have shown that transfection of cells with HER2 oncogene leads to a resistance of cells to TNF that correlates with down modulation of TNF receptors [10]. Therefore, we examined the effect of pp60$^{v\text{-}src}$ transfection of cells on the TNF receptors. The results shown in Fig. 4 indicate that there was no statistically significant difference in the binding of TNF between control and *src*-transfected cells.

3.4. *Cellular resistance to TNF induced by pp60$^{v\text{-}src}$ correlates with a decrease in intracellular glutathione levels*

We have reported [15] that cell density-dependent cellular resistance to TNF correlates with the decrease in intracellular glutathione levels; therefore, we examined glutathione levels in control and *src*-transfected cells. There was about 40% less glutathione in the *src*-transfected cells than in the *neo* control (Fig. 5).

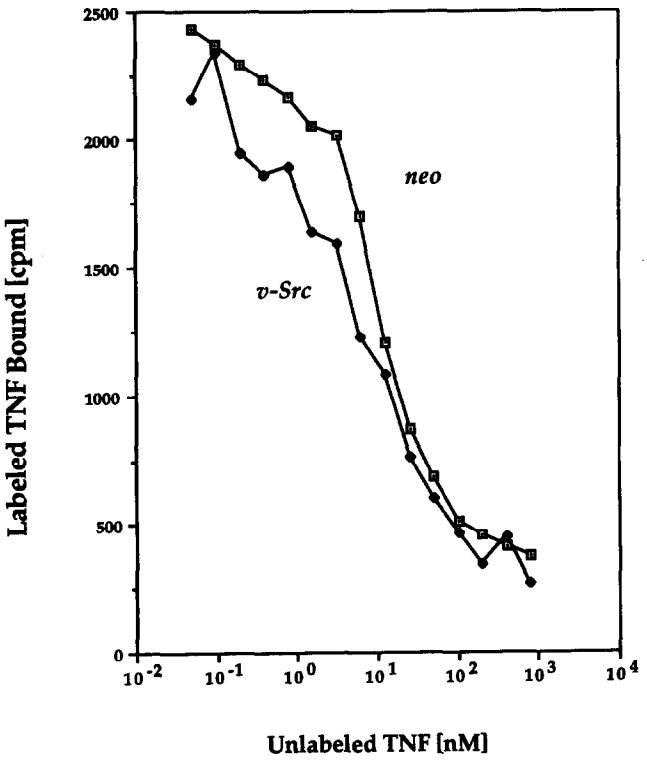


Fig. 4. Specific binding of labeled hTNF to control and pp60$^{v\text{-}src}$-transfected NIH3T3 cells. 1×10^6 cells (0.1 ml) in 96-well plates were incubated with different concentrations of labeled TNF either in the presence (nonspecific binding) or absence (total binding) of 100 nM unlabeled TNF for 1 h at 4°C. Thereafter cells were centrifuged and washed thrice, and cell-bound radioactivity counted. All determinations were made in triplicate.

3.5. *Cellular resistance to TNF induced by pp60$^{v\text{-}src}$ can be reversed by interferon-γ*

We and others have shown that IFNs can potentiate the antiproliferative effects of TNF [16–21]. Therefore, we examined the possibility that pp60$^{v\text{-}src}$-induced cellular resistance to TNF could be overcome by IFN-γ. IFN-γ had little effect on the anticellular effects of TNF on control cells, but under similar conditions, the pp60$^{v\text{-}src}$-transfected cells became sensitive when treated with TNF and IFN-γ together (Fig. 6). Thus IFN-γ could modulate the oncogene-induced resistance of cells to TNF.

4. Discussion

We report here that transfection of NIH-3T3 cells with pp60$^{v\text{-}src}$ leads to resistance to the antiproliferative effects of TNF. The induction of resistance was not due to the modulation of TNF receptors; rather it correlated with a decrease in intracellular glutathione levels. Furthermore, the cellular resistance to TNF could be overcome in part by IFN-γ.

Why some tumor cells are sensitive while others are resistant to TNF is not understood. Previously, we have shown that tumor cells overexpressing *HER2* are resistant to TNF [10]. We now report that pp60$^{v\text{-}src}$ also induces TNF resistance, but not through modulation of TNF receptors as noted in case of *HER2*. pp60$^{v\text{-}src}$ is expressed in several different types of tumor, including neuroblastomas, colon carcinoma, small cell lung carcinoma, bladder carcinoma, breast carcinoma and various other tumor cell lines [22–29]. Furthermore, when myeloid cell lines such as U937 and HL-60 are induced to differentiate by TNF and IFN-γ, the expression of pp60$^{v\text{-}src}$ is enhanced [30]. Human tumor cell lines A-549 (lung adenocarcinoma), HT29 (colon carcinoma) and SK-OV-3 (ovarian carcinoma) which overexpress *c-src*, are known to be resistant to TNF [3,4].

How pp60$^{v\text{-}src}$ induces resistance to TNF is not clear. It has been shown, however, that TNF causes the growth inhibition and decrease of pp60$^{v\text{-}src}$ kinase activity of colon carcinoma cell lines in a dose-dependent manner [31]. No decrease in the kinase activity was observed in cell lines resistant to TNF. Recently we have shown that protein tyrosine phosphatases play an important role in TNF-mediated antiproliferative effects [32]. Since pp60$^{v\text{-}src}$ is a protein tyrosine kinase, it is possible that its effects on cellular resistance are mediated through inactivation of the protein tyrosine phosphatase pathway. The

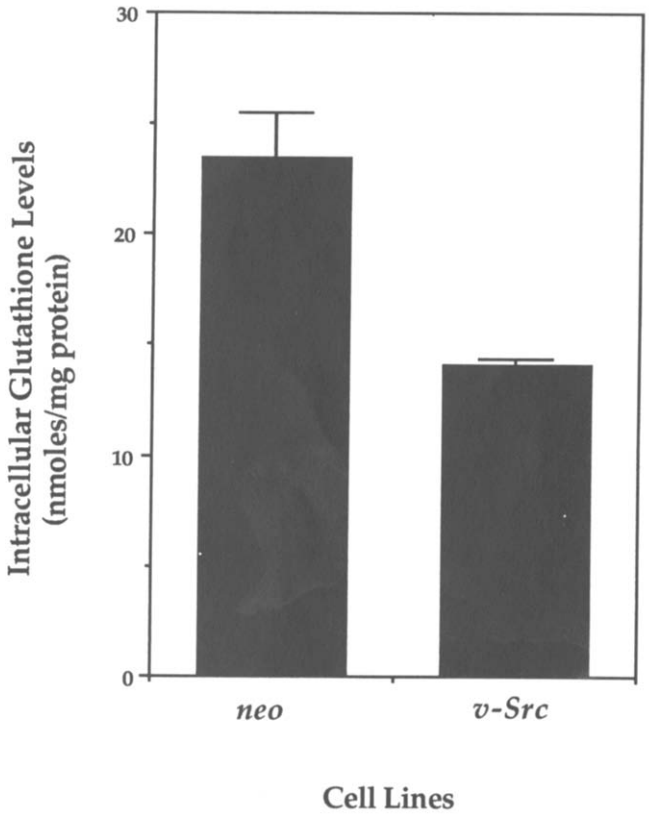


Fig. 5. Intracellular glutathione levels in control and pp60$^{v\text{-}src}$-transfected NIH3T3 cells. Bars represent the glutathione levels (nmol per mg of protein) in *neo* and *v-src*-transfected NIH3T3 cells.

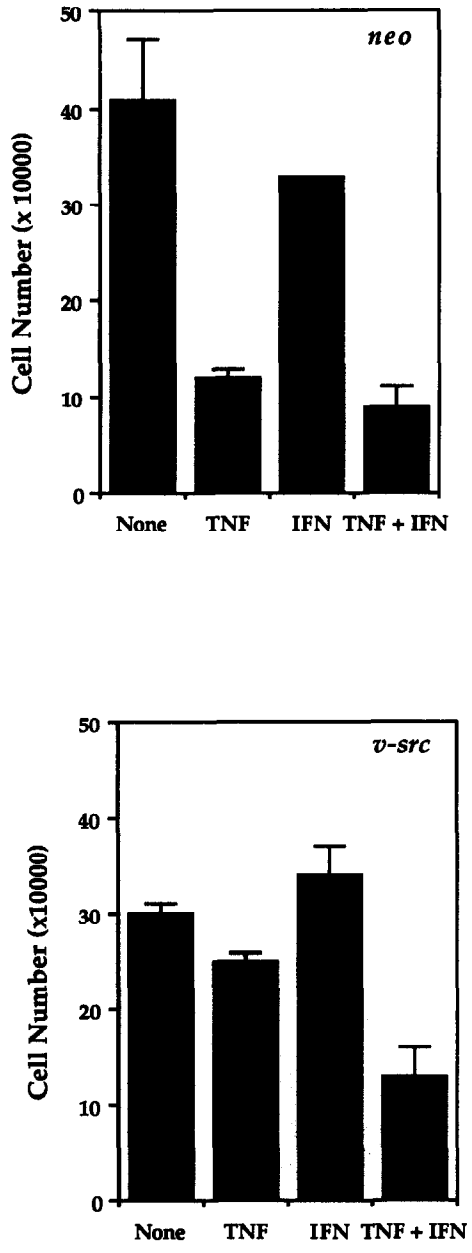


Fig. 6. The effect of hTNF and murine IFN-γ on the of control (upper panel) and pp60$^{v\text{-}src}$ (lower panel) transfected murine NIH3T3 cells. 5×10^3 cells/well in 1.0 ml were incubated with hTNF (20 ng/ml), mIFN-g (500 U/ml), and two together, in a 24-well plate at 37°C for 72 h. The cell number was determined by the Trypan blue exclusion method. All determinations were made in duplicate.

effect of tyrosine phosphatases on the activation of c-src kinase has been demonstrated [10,33]. We also found pp60$^{v\text{-}src}$-mediated cellular resistance leads to downmodulation of intracellular glutathione levels. This is consistent with our recent report showing an association between cell-density-dependent resistance to TNF and a decrease in intracellular GSH levels [15]. Although it is not clear how pp60$^{v\text{-}src}$ decreases GSH levels, pp60$^{v\text{-}src}$ also appears to be related to cellular resistance to TNF.

Since tumorigenesis is a result of altered expression of multiple oncogenes, it is possible that different oncogenes in different tumor cells contribute to the induction of resistance to TNF. Besides *HER2* and pp60$^{v\text{-}src}$, the role of other oncogenes in induction of resistance to TNF, however, remains to be determined.

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