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Transfection of cells with basic fibroblast growth factor and Kaposi fibroblast growth factor genes induce resistance to and receptor modulation of tumor necrosis factor

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Abstract Tumor necrosis factor (TNF) has been shown to inhibit the growth of some cell types and stimulate the proliferation of others by a mechanism that is not understood. In the present study, we investigated the effect of transfection of NIH-3T3 cells with either the basic fibroblast growth factor gene (*bFGF*) or the kaposi FGF gene (*K-fgf*) on the growth-modulatory effects of TNF. Our results show that transformation of cells with either gene leads to resistance to the growth-inhibitory effects of TNF. The *K-fgf* gene was found to be a more potent inducer of cellular resistance than the *bFGF* gene. The cellular resistance correlated with the inhibition of TNF-induced activation of phospholipase A_2 and downmodulation of TNF receptors. Overall, our results indicate that both *K-fgf* and *bFGF* play an important role in suppression of antiproliferative effects of TNF.

Key words: Resistance; Tumor necrosis factor; Fibroblast growth factor; Proliferation; Tumor cell

1. Introduction

Tumor necrosis factor (TNF), a protein with a molecular mass of 17 kDa, originally described as a product of activated macrophages, has been shown to directly inhibit the growth of some tumor cells and stimulate the growth of others, whereas it has no affect on some tumor cells [1,2]. This difference has been assigned to manganese superoxide dismutase, glutathione, growth factors, and oncogenes [3,11].

Fibroblast growth factor (FGF) is a family of seven distinct heparin-binding polypeptides that control the growth of both normal and tumor cells. Among them, human basic FGF (bFGF) is a 155 amino acid protein that shares 50% amino acid sequence homology with products of kaposi FGF (kFGF). bFGF is an autocrine growth factor for a wide variety of tumors including melanoma, prostate, gliomas, and renal cell carcinoma [12–15]. Although, the role of the kFGF gene (*K-fgf*) in the genesis of tumors in humans has not yet been established, the transfection of NIH-3T3 cells with *K-fgf* induces tumorigenesis and metastasis [16]. Human bFGF differs from kFGF in that the former lacks the typical signal sequence for passage through the endoplasmic reticulum, whereas the latter encodes for a secreted protein with a molecular mass of approximately 22 kDa. Secretion of kFGF has been shown to be a prerequisite for its ability to transform cells [17].

In this report, we investigated the role of an oncogenes *K-fgf* and *bFGF* on the antiproliferative effects of TNF. Our results indicate that transfection of cells with either gene induces resistance to the anticellular effects of TNF and this is accompanied by a downmodulation of TNF receptors and TNF-induced phospholipase A_2 (PLA_2) activity.

2. Materials and methods

2.1. Materials

Highly purified, bacteria-derived recombinant human TNF with a specific activity of 50×10^6 units/mg was kindly provided by Genentech (South San Francisco, CA). Polyclonal antibodies to human bFGF were kindly provided by Dr. Denis Gospadarowicz of the University of California, San Francisco, CA.

2.2. Cells

Mouse NIH 3T3 cells transfected with the retroviral vector pZip-Neo SV(*X*) alone, pZip Neo SV(*X*) containing a human *bFGF* cDNA insert, or pZipNeo SV(*X*) containing *K-fgf/hst* cDNA insert as described [18] were kindly provided by Dr. Dan Rifkin of New York University Medical Center, New York, NY. Cells constitutively expressing the transfected vectors were selected by growth in the presence of G418 (500 μg/ml). As controls, cells transfected with the neomycin resistance vector alone were selected by the same procedure. These *bFGF*- and *K-fgf*-transfected cells which have been shown to acquire a transformed phenotype, express high levels of bFGF and kFGF, respectively [18,19]. All cell types were routinely grown in Dulbecco's minimum essential medium (DMEM) supplemented with glutamine (1%), gentamicin (50 μg/ml), and fetal bovine serum (FBS) (10%) in a humidified incubator in 5% CO_2 in air. Cells were tested for mycoplasma contamination using a DNA-based assay kit purchased from Gen-Probe (San Diego, CA).

2.3. Cell growth inhibition

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. Thereafter, the medium was removed and a serial dilution of human TNF was layered in 0.1 ml of the medium. After 72 h of incubation at 37°C, the medium was removed, and viable cells were monitored by crystal violet staining according to the procedure described [1]. 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. Cell growth assays

Cells (5×10^3/well) were plated in 1 ml of the medium (DMEM plus 10% FBS) in 24-well Falcon plates. After incubation overnight in a CO_2

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Abbreviations: TNF, tumor necrosis factor; bFGF, basic fibroblast growth factor; K-fgf, kaposi sarcoma growth factor/human stomach cancer/fibroblast growth factor-5; FBS, fetal bovine serum; DMEM, Dulbecco's minimum essential medium; PLA_2, phospholipase A_2.

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incubator at 37°C, the medium was removed, and TNF was layered in 1 ml of the fresh medium. After different days of incubation, the medium was decanted off, and cells were counted by hemocytometer. All determinations were made in triplicate.

For [^{3}H]thymidine incorporation assays, cells were cultured and treated with TNF exactly the same way as indicated above for 3 days. During the last 6 h, [^{3}H]thymidine (6.7 Ci/mmol; DuPont NEN Medical Products, Wilmington, DE) was added to each well (0.5 μCi/well). Thereafter, the culture medium was removed, the wells were washed twice with phosphate-buffered saline (PBS), and the cells were detached by the addition of a solution of trypsin (0.5%) with EDTA (5.3 mM). The cell suspension was then harvested with the aid of a PHD cell harvestor (Cambridge Technology Inc., Watertown, MA) and lysed by washing with distilled water. Radioactivity bound to the filter was measured in a liquid scintillation counter (Model 1600 TR; Packard Instruments Co., Meriden, CT).

2.5. Radioreceptor assay

Receptor binding assays were carried out essentially as described previously [20].

2.6. Arachidonic acid release assays

Arachidonic acid release assays were carried out according to the procedure previously described [21]. Briefly, cells (0.1×10^6/well) were plated in 12-well plates and labeled with [^{3}H]arachidonic acid (0.1 μCi/well) by incubation overnight at 37°C, washed four times at 10-min intervals, and then incubated with TNF for 20 h at 37°C. Thereafter, the supernatants were removed, cleared of debris by centrifugation, and the amount of radioactivity released was determined by liquid scintillation counting. The levels of total arachidonic acid incorporation in untreated cells were determined by solubilization of cells with 2% sodium dodecyl sulfate.

3. Results

We first investigated the antiproliferative effect of different concentrations of TNF on NIH-3T3 cells transfected with either neo (control) or *bFGF* or *K-fgf* genes (Fig. 1). The antiproliferative effects of TNF were examined by dye-uptake (upper panel) and the thymidine incorporation method (lower panel). These results show that exposure of control (neo) NIH 3T3 cells to 1.2 nM TNF for 72 h inhibited their growth by approximately 80%, whereas the growth of *bFGF* transfected cells was inhibited by only 40%. The growth of *K-fgf*-transfected cells under these conditions, however, was not significantly affected by TNF. Thus, these results suggest that *K-fgf* can induce a complete resistance to the antiproliferative effects of TNF, whereas only a partial resistance is observed with *bFGF*-transformed cells. The difference in the sensitivity to TNF between *K-fgf* and *bFGF*-transfected cells was more pronounced by the thymidine incorporation method than by the dye-uptake method.

Since cells transfected with *bFGF* and *K-fgf* grew at different rates than the control cells, we examined the effect of TNF on the growth rate of cells transfected with three different constructs. The rate of cell growth in these experiments was examined by the trypan blue exclusion method (Fig. 2). The results of these experiments indicate that the total growth of *bFGF*-and *K-fgf*-transfected cells on day 4 was approximately 3-fold and 5-fold higher than control cells, respectively. Growth of control cells could be almost completely inhibited by 1.2 nM TNF (upper panel), whereas growth of *bFGF*-transfected cells would be only partially (approximately 50%) (middle panel) and the growth of *K-fgf* transfected cells only minimally inhibited (lower panel). Thus, the results of these experiments are consistent with the conclusion that *K-fgf* is capable of blocking the antimitogenic effects of TNF. The resistance of transfected cells to TNF was maintained both in the presence and absence of serum (data not shown).

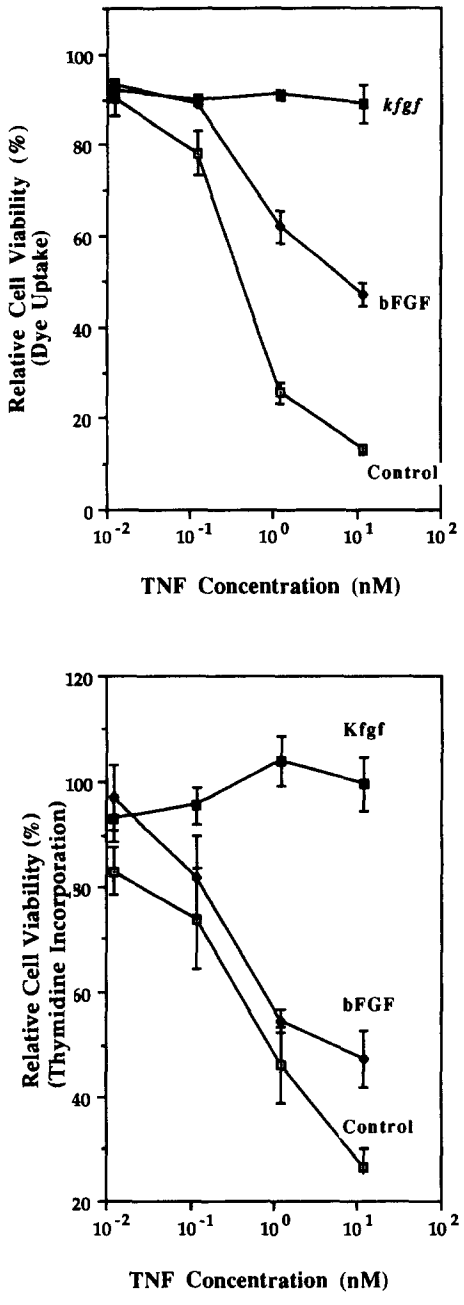

Fig. 1. Antiproliferative effects of different concentrations of TNF against *neo*-, *bFGF*- and *K-fgf*-transfected murine NIH-3T3 cells. 5×10^3 cells (0.1 ml) in 96-well plates were incubated with TNF at 37°C for 72 h, and then the relative cell viability was determined by staining with crystal violet (upper panel) or by thymidine incorporation (lower panel). The relative cell viability was determined as indicated in section 2. All determinations were made in triplicate.

To investigate the mechanism by which *K-fgf* and *bFGF* induce resistance to TNF, we examined the presence of TNF receptors on all three different cell types (Fig. 3). The specific binding of TNF to *bFGF*- and *K-fgf*-transfected cells was about 20% that of the control cells. The amount of binding in *K-fgf*-transfected cells was similar to that in *bFGF*-transfected cells.

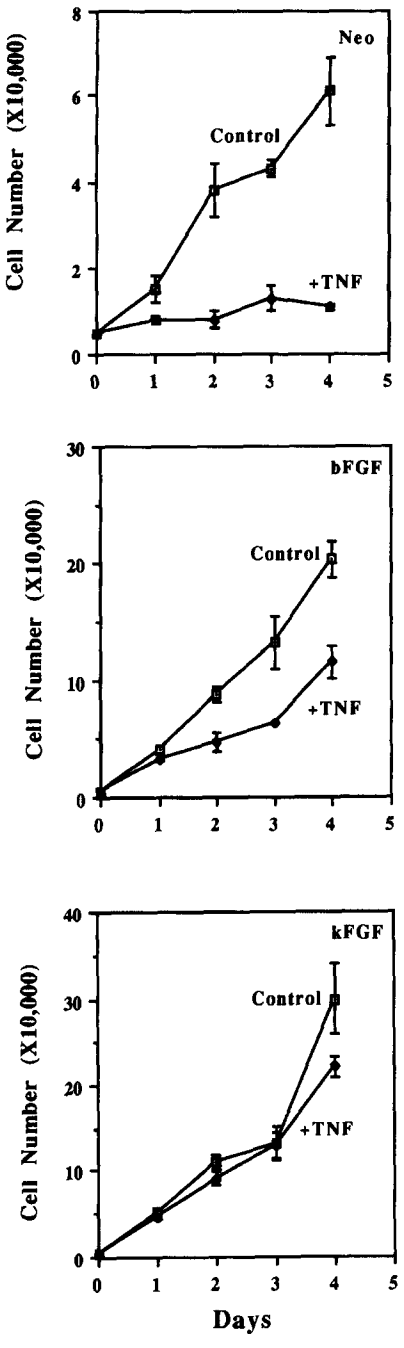

Fig. 2. The effect of TNF on the growth rate of *neo* (upper panel), *bFGF* (middle panel) and *K-fgf* (lower panel) -transfected NIH-3T3 cells. 5×10^3 cells (1 ml) in 24-well plates were incubated for different durations in the presence or absence of TNF (1.2 nM) at 37°C. Cell number was determined as described in section 2. All determinations were made in triplicate.

There was no significant change in receptor affinity; however, total receptor density had decreased after transfection of cells with bFGF and *K-fgf*.

Because the growth inhibitory properties of TNF have been linked to the activation of PLA_2 [21], we examined the effect of FGF transfection on the ability of TNF to induce the arachidonic acid release by activation of PLA_2. Approximately 35% of the total arachidonic acid incorporated by the control (neo) cells was released in response to TNF within 20 h. Under these conditions, only a 10% release of the arachidonic acid was observed with *K-fgf*-transfected cells, thus suggesting its relative resistance to TNF. With *bFGF*-transfected cells, however, the amount of arachidonic acid released was not significantly different from that of the control cells. The difference between *K-fgf*- and *bFGF*-transfected cells with respect to arachidonic acid release may be caused by the difference in the kinetics of production or their differential sensitivity as indicated by other methods.

We also examined the morphological changes in *neo*-, *bFGF*- and *K-fgf*-transfected cells before and after treatment of cells with TNF (Fig. 4). There was a difference in the morphology of the three cell types untreated with TNF. The difference in the sensitivity between these cells to TNF is also quite apparent.

4. Discussion

In the present studies, we demonstrate that growth factors modulate the cell growth inhibitory property of TNF. The transfection of cells with either *K-fgf* or *bFGF* genes induced resistance to the antiproliferative effects of TNF. The resistance was almost complete for cells carrying the *K-fgf* gene and par-

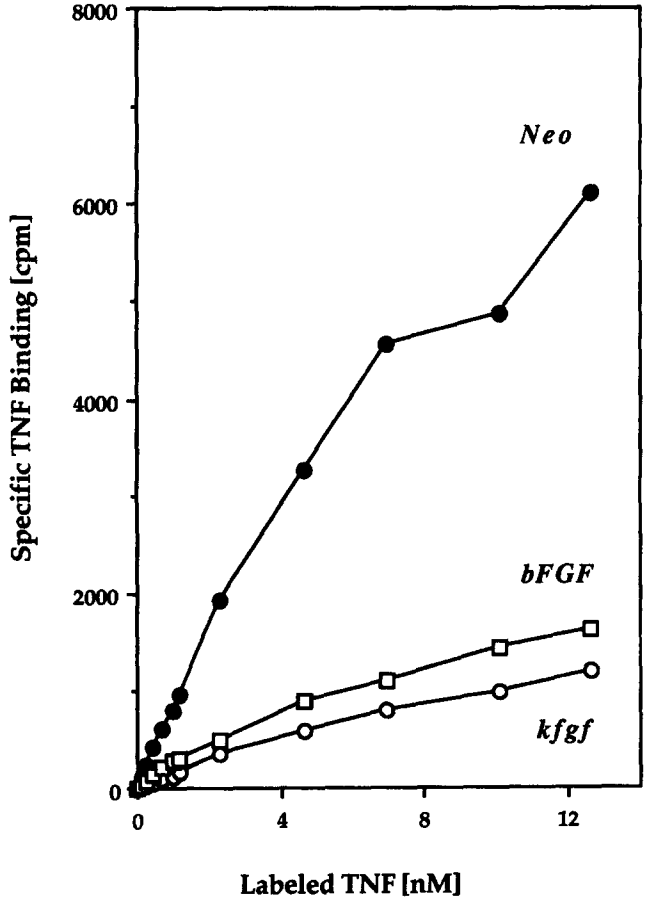

Fig. 3. Specific Binding of labeled TNF to *neo*-, *K-fgf*- and *bFGF*-transfected NIH-3T3 cells. 1×10^6 cells (0.1 ml) in 96-well plates were incubated with different concentrations of labeled TNF either in the presence (non-specific binding) or absence (total binding) of 100 nM unlabeled TNF for 1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

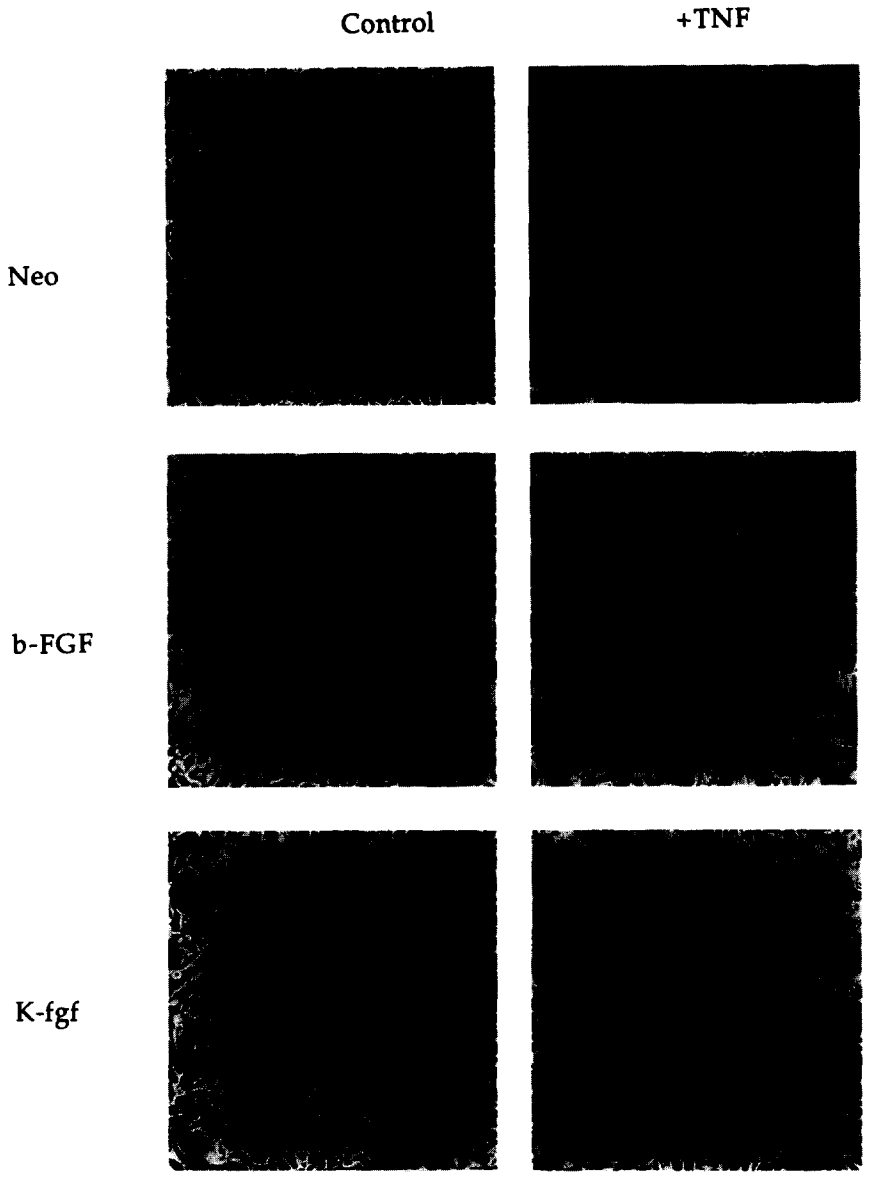

Fig. 4. Morphological effects of TNF on the *neo-*, *K-fgf-* and *b-FGF*-transfected NIH-3T3 cells. Cells were exposed to TNF (1.2 nM) for 72 h and then photographed under the Nikon TMS microscope (× 100) on T_{max} 100 Kodak film, exposed at 100 ASA.

tial for the *bFGF* gene. Our results also show that induction of resistance by both the growth factors downmodulated TNF receptors and PLA_2 activity.

How transfection of cells with *bFGF* and *K-fgf* genes induces resistance to TNF and causes receptor modulation is not clear. Because the number of bFGF receptors/cell has been shown to be inversely proportional to the intracellular contents of bFGF, it suggests an autocrine downregulation of the receptors [18,22]. It is possible that downmodulation of bFGF receptors is linked to that of TNF receptors in some manner. Alternatively, binding of bFGF to its receptors elicits tyrosine phosphorylation of several cellular substrates including the bFGF's own receptors [23], thus suggesting that FGF receptors belong to the tyrosine kinase receptor gene family. Previously, we have shown that transfection of cells with other tyrosine kinase receptor or receptor activating ligands also cause resistance to TNF and is accompanied by downmodulation of the TNF receptor [8–11] analogous to bFGF and *K-fgf*. How activation of a growth factor tyrosine kinase receptor induces resistance and receptor modulation is not clear. Since the ligand-induced transphosphorylation between different FGF receptors has been demonstrated [23], it is possible that TNF receptor or receptor-associated protein undergoes transphosphorylation in response to the activation of the growth factor receptor tyrosine kinase, which in turn leads to downregulation of TNF receptor and finally to induction of resistance to the cytokine. This hypothesis is supported by evidence that the activation of protein kinase C by phorbol esters downmodulates whereas inhibition of protein kinase C by staurosporin upmodulates the TNF receptors [24,25].

In the past, we have reported several glioma cell lines that are resistant to the antiproliferative effects of TNF [26]. Since glioma cells are known to express bFGF [13], it is possible that the TNF-resistance of these cells is in part caused by the over-

expression of the FGF. Overall, our results indicate that expression of these growth factors by tumor cells protects them from the host defense system. Our results also imply that blocking the synthesis or action of these growth factors by their antibodies or with antisense oligodeoxynucleotides directed against it may have potential in overcoming the resistance to immune system cytokines such as TNF in these tumor cells.

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