Interferon-Induced Enhancement of Transforming Growth Factor-α Expression in a Human Breast Cancer Cell Line (43512)

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Abstract. **Previous work from our laboratory has demonstrated that γ-interferon (IFN) inhibits growth of the human breast carcinoma cell line MDA 468, while enhancing expression of epidermal growth factor receptor (EGFR). Epidermal growth factor at high levels is known to inhibit growth of this cell line. Because MDA 468 cells produce low levels of transforming growth factor (TGF)-α (a ligand for epidermal growth factor receptor), we reasoned that IFN-induced cytotoxicity could be partially mediated by enhanced secretion of TGF-α. Therefore, we determined the ability of IFN to modulate the endogenous expression of TGF-α by MDA 468 cells. IFN-γ, at 500 units/ml, increased the levels of TGF-α in serum-free conditioned media of MDA 468 cells 3-fold as measured by radioimmunoassay. TGF-α mRNA was similarly increased approximately 3-fold after 5 days of IFN treatment as determined by dot blot and Northern analysis. IFN increased expression of TGF-α in conditioned media in a dose-dependent fashion. Increased secretion of TGF-α into conditioned media was not observed at Days 1 and 3. Similarly, increases in TGF-α mRNA were not observed at those time points. These results demonstrate that IFN-γ enhanced secretion of TGF-α by MDA 468 cells. Although exogenous TGF-α inhibited MDA 468 cell growth, the role that the enhanced endogenous production of TGF-α plays in the cessation of cell growth induced by IFN remains to be determined.** [P.S.E.B.M. 1993, Vol 202]

Interferons (IFN) are a family of secretory cellular proteins with a wide range of biological effects. In addition to their antiviral activity, IFN inhibit growth of both normal and transformed cells. Although the inhibitory action of IFN on cell proliferation has been recognized for many years, the mechanisms underlying this effect have not yet been identified (1).

IFN have been postulated to profoundly affect the synthesis of both growth factors and their receptors by tumor cells. Many studies have demonstrated that IFN can modulate (in either a positive or negative manner) growth factor receptor synthesis or affinity (2, 3). Changes in growth factor receptor physiology have been associated with reduced responsiveness to mitogens and inhibition of cell growth. IFN may also influence the secretion of autocrine growth factors by tumor and normal cells (4).

We have been studying the effect of IFN-γ on the growth of the human breast carcinoma cell line MDA 468. IFN-γ inhibits the growth of this cell line (5) and concomitantly increases expression of epidermal growth factor receptor (EGFR) protein and mRNA (6). However, we noted that IFN decreased the binding of ^{125}I-labeled EGF to its receptor without a change in receptor affinity. One possible explanation for these findings was that increased production of ligands for the EGFR such as transforming growth factor (TGF)-α or EGF might be blocking EGFR or inducing receptor internalization or degradation. Bjorge *et al.* (7) have demonstrated that unstimulated MDA 468 cells produce TGF-α. Thus, we postulated that IFN may enhance secretion of TGF-α. Enhanced expression of TGF-α could be partially responsible for IFN-induced growth inhibition, as it is known that cell lines such as MDA 468 with high numbers of EGFR are generally growth inhibited by EGF. In addition, exogenous TGF-α can enhance expression of EGFR in this cell line (8). Thus, increased autocrine production of TGF-α by IFN could possibly be responsible for both growth inhibition and enhanced expression of EGFR.

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The role of TGF-α in regulating the growth of human breast epithelial cells is emerging. Many human breast carcinoma cell lines (9) and primary carcinoma cells (10) express TGF-α mRNA and protein. Generally, TGF-α expression is correlated with stimulation of cell growth (10). The role of TGF-α as a possible growth inhibitor is less well understood. However, inhibition of growth of the squamous carcinoma A431 cell line, another line with high numbers of EGFR, is also associated with increased secretion of TGF-α (11). The possible role that TGF-α may play in inhibiting growth of human breast carcinoma cells has not been explored.

In the present study, we examined the effect of IFN-γ on the expression and secretion of TGF-α in MDA 468 cells. We found that IFN-γ enhanced secretion of TGF-α and the expression of TGF-α mRNA, but only at late time points.

Materials and Methods

Reagents. Human recombinant TGF-α was obtained from Bachem, Inc. (Torrance, CA). Human recombinant IFN-γ (2.5×10^7 units/mg of protein) was a gift from Genentech (San Francisco, CA). IFN was dissolved in phosphate-buffered saline at 1×10^7 units/ml and stored in aliquots at 4°C.

Cell Culture. The MDA 468 cell line (kindly provided by Dr. Ron Buick, Ontario Cancer Institute, Ontario Canada), was derived from a human breast carcinoma (12). It was routinely cultured in 50% Dulbecco's modified Eagle's medium- 50% F12 medium (Gibco, Gaithersburg, MD) supplemented with 10% calf serum (Sigma, St. Louis, MO). Cells were subcultured twice weekly by trypsinization. Cells were used within 20 passages of the original stock. MDA-MB 231 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in L-15 media with 10% fetal bovine serum.

RIA for TGF-α. Conditioned media were prepared as described previously (13). Briefly, cells were grown to confluence in 175-cm^2 tissue culture flasks in serum containing medium with or without IFN, washed three times with serum-free Dulbecco's modified Eagle's medium over a period of 6 hr, and cultured for an additional 48 hr in 30 ml of serum-free minimal essential medium supplemented with transferrin (2 μg/ml) (Collaborative Research, Bedford, MA) and fibronectin (2 μg/ml) (BTI, Cambridge, MA). The conditioned media were then collected and processed as described previously. Conditioned media were treated with pepstatin, leupeptin, and aprotinin (all at 1 μg/ml) and dialyzed against 0.05 *N* acetic acid. Conditioned media were lyophilized and resuspended in 5 ml of 0.4 *N* HCl. TGF-α was concentrated on a Sep-Pak C_{18} Waters column (Millipore, Bedford, MA) after acidification with 0.4 *N* HCl. Samples were eluted in 5 ml of methanol and 0.2% trifluoroacetic acid. Samples were then dried under nitrogen and stored. The TGF-α concentration was determined using a solid phase radioimmunoassay kit obtained from BTI. The sensitivity of this radioimmunoassay is approximately 0.25 ng of human TGF-α per tube. All points were performed in duplicate.

RNA Extraction and Analysis. Total RNA was isolated from MDA 468 cells with guanidinium thiocyanate as described (14) and quantitated by absorbance at 260 nm. Poly(A) + mRNA was isolated using an oligodeoxythymidine spin column (Stratagene, La Jolla, CA). For Northern analysis, 2.5 μg of mRNA (or 20 μg of total RNA) were denatured and analyzed by electrophoresis on 1% agarose-formaldehyde gels for 5 hr at 70 V, and then transferred to nitrocellulose paper by capillary blotting. Hybridizations were performed at 42°C in the presence of 50% (v/v) deionized formamide with a cDNA clone for TGF-α provided by Dr. R. Derynck (Genentech). The purified 1.4-kb insert was labeled with [α-^{32}P]dCTP using random priming (Boeringher Manheim, Indianapolis, IN). At the end of the hybridization, the blots were washed three times as described previously (15) with the final wash of 0.1% sodium dodecyl sulfate, 2× standard saline citrate (1 × standard saline citrate = 0.15 *M* sodium citrate) at 50°C. Hybridization signals on the blot were quantitated using a Betascope analyzer (Betagen, Cambridge, MA), which directly measures disintegrations per minute in individual dots or bands. After boiling off the bound probe, blots were rehybridized with a cDNA probe for the housekeeping gene GAPDH (American Type Culture Collection) to normalize data for the amount of mRNA present on the blot.

Statistical Analysis. The two-tailed Student's *t* test was used on unpaired samples to compare control with experimental groups. Data are expressed as mean ± SE. Statistical significance was established at the 5% level.

Results

Growth Inhibition by TGF-α. Although EGF inhibition of growth of MDA 468 cells has been documented (12), the effect of TGF-α on this cell line is not known. We determined the ability of TGF-α to modulate the growth of MDA 468 cells. As expected, TGF-α inhibited MDA 468 cell growth in a dose-dependent manner at concentrations greater than 0.1 ng/ml (Fig. 1).

Effect of IFN-γ on Secretion of TGF-α. We examined conditioned media from control and IFN-treated cells for the presence of TGF-α by radioimmunoassay. Either control or IFN (500 units/ml, 5 days)-treated cells were placed in serum-free media as described in the Materials and Methods. Conditioned media were then harvested and processed as described. TGF-α was significantly ($P < 0.05$) increased from 0.94 ± 0.2 ng/10^8 control cells to 2.9 ± 0.5 ng/10^8 IFN-treated cells (three experiments).

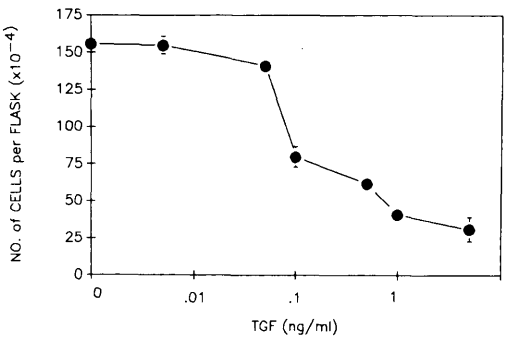

Figure 1. Effect of TGF-α on growth of MDA 468 cells. MDA 468 cells were seeded into T25 flasks at 5×10^4 cells/ml (1.0×10^5 cells/flask) in media with 10% serum in the presence of the indicated concentrations of TGF-α. Cells were harvested at Day 5 and cell number per flask was determined in a hemocytometer. Each point represents the mean ± SE of two experiments, with duplicates at each point. Standard errors not indicated on the graph were less than 10% of the mean.

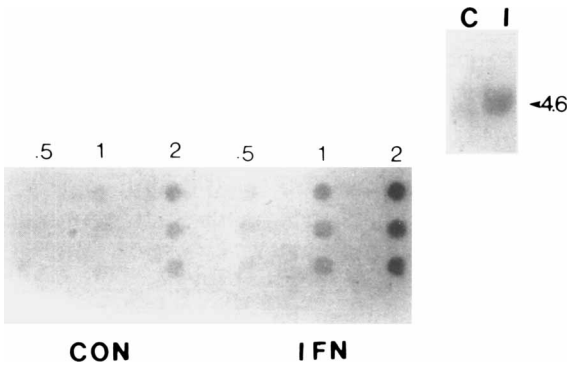

Figure 2. Effect of IFN on TGF-α mRNA levels in MDA 468 cells. MDA 468 cells were incubated for 5 days in the presence of IFN (500 units/ml). Control cells were untreated. Cells were harvested on Day 5 and total RNA was extracted and RNA dotted on nitrocellulose filters at the concentrations (in μg) indicated. The filters were hybridized to a cDNA probe for TGF-α as described. The inset shows results of Northern analysis of mRNA from control and IFN-treated cells. Each blot is representative of two trials.

To determine whether enhanced secretion of TGF-α by IFN was unique to MDA 468 cells, we exposed MDA-MB 231 cells to IFN-γ (500 units/ml) for 5 days. This human breast carcinoma cell line also displays high numbers of EGF receptors, is growth-stimulated by EGF, and is growth-inhibited by IFN-γ (5). IFN-γ (500 units/ml, 5 days) also increased TGF-α secretion in this cell line 2.4-fold to 5.7 ng/10^8 cells (two experiments).

We next examined the effect of IFN-γ treatment on expression of TGF-α mRNA (Fig. 2). Cells were incubated for 5 days in the presence or absence of IFN-γ (500 units/ml) and total mRNA harvested. Dot blot analysis revealed that the expression of TGF-α mRNA was increased approximately 2-fold by IFN treatment (776 dpm hybridized to 2 μg of RNA from IFN-treated cells, and 335 dpm to 2 μg of RNA from control cells). Reprobing the blot with a GAPDH probe revealed no significant differences in mRNA on the blot. Northern analysis revealed the presence of the expected 4.6-kb species of TGF-α mRNA (15). Expression of TGF-α mRNA was increased approximately 2-fold in the Northern analysis when normalized for the amounts of RNA present on the blot, as 857 dpm per band were observed in RNA from control cells and 2507 dpm were observed in the RNA band from IFN-treated cells. Reprobing the blot with a GAPDH probe revealed 6039 dpm in control cells and 9169 dpm in IFN-treated cells.

We also examined the effect of different concentrations of IFN-γ on the secretion of TGF-α. Cells were incubated with IFN-γ at 10, 100, and 500 units/ml for 5 days. Treatment with 10 units/ml of IFN-γ increased TGF-α levels 1.5-fold, 100 units/ml 2.1-fold, and 500 units/ml 2.8-fold (Fig. 3). The fact that TGF-α secretion increased with increasing concentrations of IFN paralleled data indicating IFN inhibited cell growth in a dose-dependent manner, with 500 units/ml being the most inhibitory dose (5).

To examine the kinetics of the changes in secretion of TGF-α, cells were treated with IFN-γ (500 units/ml) for 1, 3, or 5 days and conditioned media were harvested and processed as described. TGF-α levels were assayed by radioimmunoassay. Results (Fig. 4) indicate that TGF-α levels significantly increased 3.3 ± 0.5-fold only after 5 days of IFN treatment. There were no significant differences between TGF-α levels in conditioned media of IFN-γ-treated or control cells at Days 1 and 3. This finding parallels our previous data indicating that both IFN-induced inhibition of cell growth, and decreased binding of EGF occurred initially after 4–5 days of IFN treatment (5).

Northern blot analysis of mRNA isolated from cells treated with IFN-γ (500 units/ml) for 1 or 3 days demonstrated no changes in the steady state levels of TGF-α mRNA from IFN-treated cells as compared with controls. Two separate blots, hybridized on different occasions, were analyzed in the Betascope. At Day

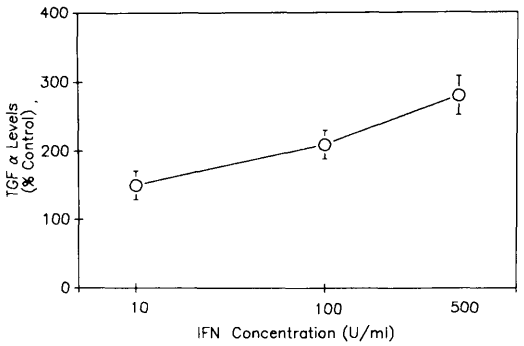

Figure 3. Effect of different concentrations of IFN on TGF-α levels. MDA 468 cells were treated with IFN at the indicated concentrations and conditioned media were prepared after 5 days of treatment. TGF-α levels were measured in conditioned media by radioimmunoassay, as described. Results are expressed as the percentage of control. Control levels ranged from 0.8 to 1.3 ng/10^8 cells. Each point represents mean ± SE of two experiments, with two replicates per point (four total).

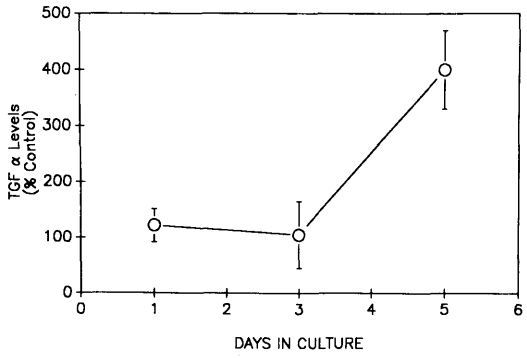

Figure 4. Secretion of TGF-α by IFN-treated and control MDA 468 cells as a function of time. MDA 468 cells were treated with IFN for 1, 3, and 5 days, as described and placed in serum-free media, and conditioned media were harvested at the various time points indicated. TGF-α levels were measured in conditioned media by radioimmunoassay as described and calculated as ng/10^8 cells. Results are expressed as percentage of control. Control levels ranged from 0.8 to 1.45 ng/10^8 cells in different experiments. Each point represents mean ± SE of three experiments, with two replicates per point.

1, approximately 1869 dpm were bound by RNA derived from control cells, and 1546 dpm bound to RNA from IFN-treated cells. TGF-α mRNA levels were also equal in control and Day 3 IFN-treated cells (626 dpm vs 539 dpm). When RNA from control cells harvested at Days 1, 3, and 5 were electrophoresed on the same blot, approximately equal amounts TGF-α mRNA were present (5048 dpm for Day 1, 4257 dpm for Day 3, and 5048 dpm for Day 5). Variations in the levels of TGF-α in control cells with time were not observed. Thus, the relative increases in TGF-α mRNA observed in interferon-treated cells were not due merely to the fact that levels of TGF-α mRNA in control cells were decreasing.

Discussion

This investigation demonstrates that IFN-γ increased steady state levels of TGF-α mRNA and production of TGF-α protein by MDA 468 cells. In addition, exogenous TGF-α inhibited growth of this cell line. However, the role that increased endogenous production of TGF-α plays in mediating IFN-induced cytotoxicity remains to be elucidated.

Autocrine production of TGF-α has been demonstrated to be an important modulator of growth of normal and malignant breast epithelia. However, TGF-α is usually stimulates the growth of such cells. Bates *et al.* (16) found that estrogen treatment of ER+ breast cancer cell lines such as MCF7 enhances the autocrine production of TGF-α. Transfection of a spontaneously immortalized human mammary cell line with Ha-*ras* causes increased expression of TGF-α mRNA, which suggests that TGF may be an intermediary in the transformation of human mammary epithelial cells by activated cHa-*ras* genes (17). Endogenous production of TGF-α has also been implicated in the growth control of normal breast epithelium. Increased TGF-α mRNA expression coincides with the conversion of normal breast organoid cells to a proliferative state *in vitro* (18). Withdrawal of EGF from the medium, which leads to cytostasis, decreases TGF-α expression. An autoregulatory loop was suggested to be operative in proliferating breast epithelial cells (19).

The fact that increased TGF-α expression is associated with growth inhibition by IFN is, therefore, somewhat surprising. However, findings such as ours indicating that IFN-induced growth inhibition is accompanied by increased TGF-α production are not without precedent. Nickoloff and Mitra (4) found that IFN-γ inhibits growth of normal keratinocytes, decreases binding of EGF to its receptor, and increases TGF-α production. Similarly, Kumar and Mendelsohn (11) have demonstrated that treatment of squamous carcinoma A431 cells with IFN-γ also results in a late induction of TGF-α. EGFR mRNA is increased 2-fold, but binding of ^{125}I-EGF to the cell surface of IFN-γ-treated cells is unchanged. However, our study is the first to demonstrate such a phenomenon in a human breast cancer cell line.

We were able to detect TGF-α in conditioned media without the use of blocking antibodies to the EGFR. In contrast, Bjorge *et al.* (7) were able to detect immunoreactive TGF-α accumulating in cell culture medium only in the presence of an antibody that blocks EGFR. The antibody was necessary due to the high numbers of EGF receptors on which TGF-α is bound and internalized. One discrepancy between their work and ours is that our line displays approximately 5×10^5 EGF binding sites per cell, whereas their line exhibits 2×10^6 sites per cell. It is possible that in our study, the addition of EGFR blocking antibodies would have further enhanced the yield of immunoreactive TGF-α found in conditioned media.

The fact that TGF-α protein was increased in conditioned media could have been due to either increased synthesis and secretion, or decreased destruction of TGF-α. IFN-γ treatment may preferentially enhance secretion or slow binding and internalization of TGF-α. Previous studies in our laboratory (5) have shown no differences in the EGF internalization rates of control and IFN-treated cells; therefore, it is likely that IFN may not affect TGF-α internalization rates. The fact that TGF-α protein and mRNA levels were enhanced in IFN-treated cells suggests that TGF-α production is increased. The experiments reported here do not take into account changes in cell-associated TGF-α that may modulate cell growth (20). Attempts in our laboratory to demonstrate changes in membrane-bound TGF-α by immunofluorescence and quantitative image analyses are ongoing.

In our study, prolonged (5 day) exposure of MDA 468 cells to IFN was required to cause significant changes in TGF-α production. Changes in TGF-α levels were observed concomitant with inhibition of cell

growth (5). Because the effects of IFN-γ on TGF-α secretion were noted only after a long exposure, it is likely that IFN did not directly affect TGF-α expression. IFN may change expression of more proximal mediators of TGF-α gene expression (21). Finally, enhanced expression of TGF-α may be a consequence, rather than a cause, of the growth inhibition. The fact that MDA 231 cells, which are growth stimulated by EGF, also produce more TGF-α in response to IFN-γ suggests that IFN-treated cells may produce TGF-α to compensate for growth arrest.

As expected, exogenous TGF-α inhibited MDA 468 cell growth. It has been demonstrated previously that EGF usually inhibits growth of epithelial tumor cell lines with high numbers of EGF receptors (2). However, the concentrations of exogenous TGF-α that were inhibitory in this study were approximately 10-fold greater than the concentrations present in conditioned media of IFN-treated cells (approximately 0.009 ng/ml). Therefore, the increases in TGF-α in conditioned media alone may not have been enough to account for the cytostatic effects of IFN (approximately 60% at Day 5) (5). Our studies did not measure changes in cell surface TGF-α, which might have influenced cell growth in a juxtacrine fashion (20).

In summary, our studies have indicated that TGF-α mRNA expression and protein secretion in the human breast carcinoma cell line MDA 468 is increased by IFN-γ treatment. The fact that TGF-α inhibits growth of this cell line poses the intriguing possibility that part of IFN's inhibitory effect may be mediated by TGF-α. However, further work is needed to prove a definitive link between the increased secretion of TGF-α observed and IFN-γ-induced cytotoxicity.

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