Growth Regulation of A431 Cells

MODULATION OF EXPRESSION OF TRANSFORMING GROWTH FACTOR-α mRNA AND 2',5'-OLIGOADENYLATE SYNTHETASE ACTIVITY

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Rakesh Kumar§ and John Mendelsohn±
From the §Laboratory of Receptor Biology, Memorial Sloan-Kettering Cancer Center and ±Cornell University Medical College, New York, New York 10021

To explore bidirectional regulatory interactions between interferons and autocrine polypeptide factors, we examined the modulation of expression of transforming growth factor-α and 2',5'-oligoadenylate synthetase activity in A431 epidermoid carcinoma cells after treatment with interferon-γ and transforming growth factor-α. Treatment of A431 cells with interferon-γ increased steady state levels of transforming growth factor-α mRNA by 4-fold and increased the levels of transforming growth factor-α in the culture medium. There were additive growth inhibitory effects upon coaddition of exogenous transforming growth factor-α and interferon-γ to the cultures. Addition of transforming growth factor-α to A431 cell cultures in the absence of interferon could stimulate the induction of 2',5'-oligoadenylate synthetase activity by more than 2-fold. These findings demonstrate that the induction of transforming growth factor-α in interferon-γ-treated A431 cells could act to regulate interferon-induced gene(s), e.g., 2',5'-oligoadenylate synthetase, suggesting interactions between a potential autocrine growth factor and the interferon system in the growth regulation of A431 cells.

Regulation of cell proliferation appears to be a complex process involving the coordinated action of cellular growth stimulatory and inhibitory factors in an integrated manner. The fundamental role of polypeptide growth factors in controlling the growth rate of normal and tumor cells has been well described (1, 2). Alterations in growth factor expression and function have been correlated with the malignant process; the products of many oncogenes are growth factors or receptors for growth factors (3), and growth factors are produced by and serve as natural ligands for receptors present on tumor cells (4). For example, TGF-α,1 a 50-amino acid polypeptide required for the growth of many epithelial cells (5, 6), is also expressed by a variety of tumor cell lines which also bear EGF receptors (7, 8) and thus has been implicated in malignant transformation of cells (9).

In contrast, the involvement of natural growth inhibitory factors in regulating cell proliferation is less well understood. Representative of this class of agents are the IFNs, which are regulatory secretory proteins that have a variety of biological activities on normal and transformed cells, including antiviral effects (10–13) and growth inhibitory effects (14–16). The biochemical mechanisms underlying these actions are poorly understood. In sensitive cells, all actions of IFNs are thought to be mediated by the products of specific sets of regulatory genes whose expression is increased by IFN (17, 18). These IFN-induced gene products, acting either singly or in conjunction with other regulatory mechanisms such as growth factors (19, 20), may alter cell growth. Among the many IFN-inducible genes, the 2,5-A synthetase pathway has been implicated in growth inhibition and differentiation of tumor cells (21, 22). 2,5-A synthetase catalyzes the synthesis of 2',5'-oligoadenylates from ATP. These are known to be potent inhibitors of protein synthesis (23), possibly through selective activation of 2',5'-oligoadenylate-dependent latent endonucleases which destroy mRNAs (12, 24).

In general, IFNs and growth factors influence proliferation in an opposite manner; IFNs antagonize growth factor-induced proliferative responses (25, 26), and growth factors counteract inhibitory effects of IFNs (27). To further explore these regulatory interactions we have chosen as a model the human epidermoid carcinoma line A431, which produces several potential autocrine polypeptides including TGF-α (7) and TGF-β (28) and is also responsive to the IFNs (16).

In the present study we examined the modulation of expression of TGF-α and 2,5-A synthetase in A431 cells after treatment with IFN-γ and TGF-α, respectively, to explore the bidirectional regulatory interactions between IFN and the autocrine polypeptide growth factor. We report the results of experiments seeking biochemical explanations for the observed interactions and discuss the implications of this phenomenon on growth regulation of A431 cells.

EXPERIMENTAL PROCEDURES

Interferon—Pure recombinant IFN-γ (8 × 10^6 units/mg) was a generous gift from G. Sen, Cleveland Clinic Foundation.

Cell Culture—A431 cells were grown in monolayer culture with modified Eagle’s medium: Ham’s F-12 (1:1) containing 5% fetal bovine serum.

Growth Assays—Cell proliferation was measured by the MTT-dye (Sigma) uptake method as previously described (29). Briefly, dye taken up by live cells was extracted in isopropyl alcohol: 1 N HCl (96%) for determination of optical density at a wavelength of 570 nm. Cell density was also measured by counting trypan blue dye-excluded cells.

Cell Extracts—Cells in monolayer culture were washed with chilled phosphate-buffered saline and extracted in lysis buffer (30 mM Hepes, pH 7.5, 5 mM magnesium acetate, 30 mM β-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride, 0.5% NonidetP-40, and 1% glyceral)
for 10 min (16). The cell lysates were centrifuged at 1000 \( \times g \) for 5 min, then for 10 min in an Eppendorf microcentrifuge, and supernatants were stored in aliquots at \(-70^\circ C\). Protein concentration was determined by the Bio-Rad microassay procedure with bovine albumin as a protein standard.

**Northern Blot Analysis**—Total cellular RNA was isolated as previously described (17) and analyzed by dot blot and Northern blot methods. The procedure for dot blot analysis and precautions to ensure a linear response were as previously described (30). For Northern blots, RNAs denatured in the presence of formaldehyde were fractionated on a 1% agarose formaldehyde gel. Transfer to a nitrocellulose fiber, hybridization, and washing were performed as previously described (12). The complementary DNA probe for TGF-\( \alpha \) mRNA was kindly provided by R. Derynck, Genentech, Inc. (31), and the 1.4-kilobase EcoRI fragment was used for hybridization experiments.

**Western Immunoblot Analysis of TGF-\( \alpha \)—**A431 cells were plated in complete medium (2 \( \times 10^6 \) cells/150-cm dish). After 24 h, the culture medium was changed to 6 ml of medium containing 0.5% fetal bovine serum. Cultures were treated with IFN-\( \gamma \) (500 units/ml) for 4 days. Using a procedure developed by J. Tam, Rockefeller University, 5 ml of conditioned medium (CM) was concentrated by adsorption onto a disposable C\( \alpha \) column (Seph-Pak, Waters Associates) which was prewashed within 5 ml of methanol and equilibrated with 10 ml of solution containing 5% acetonitrile and 0.045% trifluoroacetic acid. The sample was then eluted with 2.5 ml of 60% acetonitrile containing 0.035% trifluoroacetic acid into a polypropylene tube. The eluate was concentrated to complete dryness in a Speed-vat and reconstituted with 100 \( \mu l \) of distilled water. The concentrated extracts from CM were fractionated on a 1% agarose formaldehyde gel. Transfer to a nitrocellulose paper (Schleicher and Schull, BA-85) was performed as previously described (32). Electrophoretic transfer onto nitrocellulose paper (Schleicher and Schull, BA-85) was performed as previously described (33). Western immunoblot analysis was performed with a polyclonal goat anti-TGF-\( \alpha \) antibody (Biotope) according to the manufacturer’s directions, except that \( ^{125}\)I-labeled protein A (Du Pont-New England Nuclear) was used.

**2',5'-Oligoadenylate Synthetase Assay—**Enzyme activity was determined as previously described (12). The cellular extracts (25-40 \( \mu g \) of protein) were adsorbed on poly(rI)-poly(rC) agarose beads (Pharmacia LKB Biotechnology Inc.) and incubated for 16 h in 10 \( \mu l \) of reaction mixture containing 15 mM magnesium acetate, 25 mM KCl, 2.5 mM dithiothreitol, 5 mM ATP, and 10 \( \mu g \) of labeled ATP (Du Pont-New England Nuclear, >800 Ci/mmol). Bacterial alkaline phosphatase (Sigma) was directly added to the reaction mixture containing the beads followed by further incubation at 37 \( ^\circ C \) for 1 h. The reaction products were analyzed on an alumina column after diluting to 400 \( \mu l \) with 1 M glycine HCl, pH 2.0.

**Gel Electrophoresis and Immunoblotting of 2',5'-Oligoadenylate Synthetase**—25-40 \( \mu g \) of protein were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gels as previously described (32). Low molecular mass color markers (Amersham Corp.) were used as standards. Electrophoretic transfer onto nitrocellulose (BA-85, Schleicher and Schuell) was performed using methanol/glycine/Tris. For immunoblotting of the 2,5-A synthetase isoenzymes, we used antipeptide polyclonal antibody against 2,5-A synthetase (34), provided by J. Chehab, Weizmann Institute of Science, Israel, via J. Tam, Rockefeller University. Immune complexes were detected with \( ^{125}\)I-Protein A (Du Pont-New England Nuclear, 10 \( \mu g/\mu l \)).

### RESULTS

**Effect of IFN-\( \gamma \) on the Expression of TGF-\( \alpha 
**—The growth rate of A431 cells was very sensitive to the antiproliferative effects of IFN-\( \gamma \) in a dose- and time-dependent manner (16). This growth inhibition of A431 cells by IFN-\( \gamma \) was accompanied by morphologic changes which included increase in cell size, augmentation of multinucleate cells, and appearance of thin long processes.

To explore the possible relationship between the expression of autocrine growth factors and IFN-\( \gamma \)-mediated growth inhibition of A431 cells, we analyzed the effect of IFN-\( \gamma \) on the expression of TGF-\( \alpha \) mRNA, which may play an autocrine role in regulating growth of these cells (6, 7). As shown in Fig. 1A, the kinetics of expression of TGF-\( \alpha \) mRNA, assayed by dot blot hybridization, indicated that treatment of A431 cells with IFN-\( \gamma \) increased (2-fold) levels of TGF-\( \alpha \) mRNA for 4 days, followed by a return to basal levels by day 7. Other experiments demonstrated that TGF-\( \alpha \) mRNA was higher on day 4 than on day 2 after initiating IFN-\( \gamma \)-treatment. IFN-\( \alpha \), which had no antiproliferative effect on A431 cells (16), did not alter the expression of TGF-\( \alpha \) mRNA. Northern blot analysis of cytoplasmic RNA isolated from IFN-\( \gamma \)-treated cells demonstrated a 4-fold elevation of steady state levels of TGF-\( \alpha \) mRNA (Fig. 1B). This up-regulation of TGF-\( \alpha \) mRNA by IFN-\( \gamma \) was seen to be selective, as the human glycerolaldehyde-3-phosphate dehydrogenase gene remained unaffected (Fig. 1C).

In order to determine whether the increase in steady state levels of TGF-\( \alpha \) mRNA in IFN-\( \gamma \)-treated A431 cells is accompanied by increased secretion of the protein, Western blot analysis of concentrated medium was performed. Culture medium containing 0.5% bovine serum was conditioned for 4 days in cultures of A431 cells with or without IFN-\( \gamma \) treatment. As shown in Fig. 2 CM contained two high molecular mass TGF-\( \alpha \) forms of 27-30 and 55-60 kDa, with no detectable 6-kDa mature form. When equal volumes of CM were immunoblotted there was no significant increase in TGF-\( \alpha \) in cultures incubated with IFN-\( \gamma \) (lane 3). However, differences in the amount of secreted TGF-\( \alpha \) forms were detected when the volumes of CM concentrated for immunoblot analysis were corrected for 47% inhibition in cell counts in the IFN-
γ-treated cultures, so that TGF-α production per cell was assayed (lanes 4 and 5). These results indicate that IFN-γ may increase the accumulation of high molecular weight TGF-α forms on a per cell basis by 1.7-fold. The amount of TGF-α detected in the cultures is governed by the balance between IFN-γ-treated cultures, so that TGF-α production per cell was increased via EGF receptors on the cell surface. A number of reports have described comparably high molecular weight species of TGF-α in conditioned medium: 15–48 kDa in human rhabdomyosarcoma (35), 30 and 68 kDa in mammary carcinoma cells (36), and 24, 40, and 43 kDa in retrovirus-transformed cells (37). It remains to be resolved whether the multiple TGF-α precursor species observed are the result of glycosylation or other factors.

Since TGF-α actions are mediated through the EGF receptor (38, 39) and Zoon et al. (19) have reported that IFN-γ causes a decrease in EGF receptors in bovine kidney cells, we investigated the effect of IFN-γ on the expression of EGF receptors. Reprobing of the same blots with cDNA for the EGF receptor revealed that the IFN-γ-induced increase in the expression of TGF-α mRNA was accompanied by less than a 2-fold increase in EGF receptor mRNA. However, there was no change in binding sites for [125I]-EGF on the cell surface of IFN-γ-treated cells, as determined by Scatchard analysis of binding studies. (19) The differences between our results and previous findings (19) could be due to the different cell lines used in these two studies or differences between secretion and measurements of TGF-α.

**Interactions between IFN-γ and TGF-α**—Our results have raised the possibility of involvement of increased expression of TGF-α mRNA in the action of IFN-γ, a negative growth signal in A431 cells. When epithelial tumor cell lines with elevated EGF receptors such as A431 cells produce TGF-α (7), it appears that an optimal concentration of EGF or TGF-α may enhance proliferation (40), but higher concentrations can inhibit growth (41–43). As expected, addition of exogenous TGF-α was antiproliferative for A431 cells (Fig. 3). When IFN-γ and TGF-α were added together, the antiproliferative effects were additive in a dose-dependent manner (Fig. 3), providing biological evidence of cooperative interactions between these regulatory polypeptides in A431 cells.

**Induction of 2,5-A Synthetase by TGF-α**—Analysis of the kinetics of induction of 2,5-A synthetase revealed a sustained rise and persistence of elevated enzyme activity until day 4 after IFN-γ treatment of A431 cells (18). This observation and the growth experiments prompted us to hypothesize that the prolonged induction of 2,5-A synthetase activity could result from interaction(s) with autocrine cellular growth factors such as TGF-α, whose expression was up-regulated after IFN-γ treatment of A431 cells (this work). As shown in Fig.

**Fig. 2. Western immunoblot analysis of secreted TGF-α protein.** Culture medium was conditioned for 4 days by A431 cells grown without or with IFN-γ. Unconditioned culture medium was used as a control. The culture media were concentrated and analyzed by Western immunoblotting as described under "Experimental Procedures." Lanes: 0, unconditioned medium; C, control CM; I, IFN-γ-treated CM; STD, recombinant TGF-α (2 μg). Volumes of concentrated and reconstituted media used were: lanes 1–3, 18 μl; lane 5, 14.7 μl. Molecular weight markers used: bovine serum albumin (M, 68,000), ovalbumin (M, 46,000), carbonic anhydrase (M, 30,000), and lysozyme (M, 14,000).

**Fig. 3. Growth inhibition of A431 cells by IFN-γ and TGF-α.** Cells were seeded at low density (7,000 cells/0.5-ml well in 24-well plates) in 5% fetal bovine serum-Dulbecco's modified Eagle's medium/F-12. Twenty-four hours after seeding (day 0), cultures were supplemented with IFN-γ (500 units/ml) and/or TGF-α (10 ng/ml, panel A; 1.0 ng/ml, panel B). At the indicated times, cell density was determined by the MTT dye uptake method as previously described (29). All cultures received a medium change on day 3. Values are the mean of triplicate samples, and the figure is a representative of four separate experiments.

**Fig. 4. Induction of 2,5-A synthetase activity by TGF-α in A431 cells.** A, effect of increasing concentrations of TGF-α on 2,5-A synthetase activity. Subconfluent cultures were treated with various doses of TGF-α for 20 h. Detergent extracts were made and enzyme activities were measured as previously described (12). Amounts of 2,5-A synthetase are expressed as nanomoles of AMP polymerized per mg of protein, with the assumption that the products were all 2,5-A. Panel A (A), time course of induction of 2,5-A synthetase activity by TGF-α. Subconfluent cultures were treated with TGF-α (100 ng/ml) for the indicated durations. Detergent extracts were made and enzyme activities were determined. C, specificity of induction of 2,5-A synthetase by TGF-α. Subconfluent cultures were treated with TGF-α (100 ng/ml) either alone or in the presence of anti-EGF receptor antibody (αAb) (no. 528, 2 μg/ml) for 18 h. Detergent extracts were prepared and enzyme activities were determined. These experiments (A, B, and C) were repeated three times.
Our results indicate that treatment of A431 cells with IFN-γ is accompanied by increased steady state levels of TGF-α mRNA and secreted TGF-α forms. There were additive inhibitory effects when A431 cells are exposed to IFN-γ and TGF-α. These data provide evidence for the possible involvement of an autocrine growth factor in the action of IFN-γ. It remains to be seen whether induction of TGF-α by IFN-γ is a phenomenon restricted to A431 cells or a general mechanism of action of IFN-γ in cell lines capable of producing TGF-α and/or being stimulated by TGF-α. The finding that TGF-α can induce 2,5-A synthetase activity is interesting, as it indicates that the induction of TGF-α in IFN-γ-treated A431 cells could activate a pathway ordinarily associated with IFN actions such as 2,5-A synthetase, implying regulatory interactions between IFN-γ and TGF-α at the biochemical level. It is possible that IFN-γ-induced late expression of TGF-α, which peaks on day 4, may account in part for the prolonged induction of 2,5-A synthetase activity observed in IFN-γ-treated A431 cells (16). It is also possible that the high basal levels of 2,5-A synthetase observed in A431 cells could be due to the autocrine actions of TGF-α. For cells stimulated by TGF-α, induction of 2,5-A synthetase may mediate an inhibitory feedback regulation by this growth factor, a mechanism that seems to be functionally operating for another growth factor (46, 47). These observations suggest a hypothesis that modulation of expression of potential autocrine growth factors by IFNs and their subsequent action or interaction via common biochemical pathways may play a role in the regulation of cell proliferation.

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REFERENCES

TGF-α and 2,5-A Synthetase in Growth Regulation


Growth regulation of A431 cells. Modulation of expression of transforming growth factor-alpha mRNA and 2',5'-oligoadenylate synthetase activity.

R Kumar and J Mendelsohn