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Type β transforming growth factor: A bifunctional regulator of cellular growth

(growth inhibitor/anchorage-independent growth/human tumor cells/epidermal growth factor)

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ABSTRACT **Type β transforming growth factor (TGF-β) is a two-chain polypeptide of 25,000 daltons isolated from many tissues, including bovine kidney, human placenta, and human platelets. It has been characterized by its ability to stimulate reversible transformation of nonneoplastic murine fibroblasts, as measured by the formation of colonies of these cells in soft agar (ED_{50} = 4 pM TGF-β for NRK fibroblasts). We now show that the response of cells to TGF-β is bifunctional, in that TGF-β inhibits the anchorage-dependent growth of NRK fibroblasts and of human tumor cells by increasing cell cycle time. Moreover, the anchorage-independent growth of many human melanoma, lung carcinoma, and breast carcinoma cell lines is inhibited by TGF-β at concentrations in the same range as those that stimulate colony formation of NRK fibroblasts (average ED_{50} = 10–30 pM TGF-β for inhibition). Whereas epidermal growth factor and TGF-β synergize to induce anchorage-independent growth of NRK fibroblasts, their effects on the growth of A-549 human lung carcinoma cells are antagonistic. The bifunctional response of cells to TGF-β is further demonstrated in Fischer rat 3T3 fibroblasts transfected with a cellular *myc* gene. In these cells TGF-β synergizes with platelet-derived growth factor to stimulate colony formation but inhibits the colony formation induced by epidermal growth factor. The data indicate that the effects of TGF-β on cells are not a function of the peptide itself, but rather of the total set of growth factors and their receptors that is operant in the cell at a given time.**

Transforming growth factors (TGFs) have been operationally defined as peptides that reversibly induce nonneoplastic cells to express the transformed phenotype, as measured by loss of density-dependent inhibition of growth and acquisition of anchorage-independent growth (1, 2). Two distinct sets of TGFs have recently been purified to homogeniety. Type α TGFs are single-chain peptides of 5700 daltons with three disulfide bridges in positions homologous to those of epidermal growth factor (EGF), with which they share sequence homology (3, 4). Type α TGFs and EGF both bind to the EGF receptor and have indistinguishable biological activities *in vitro* (2, 5); thus EGF belongs to the TGF-α family. Type β TGFs have a different primary sequence and consist of two apparently identical peptide chains, each of 12,500 daltons, crosslinked by disulfide bonds (6–8). Type β TGFs bind to a unique cell surface receptor distinct from the EGF receptor (9, 10).

Two nonneoplastic fibroblast indicator cell lines have been used for operational definition of TGF-β activity: normal rat kidney (NRK) cells require both TGF-β and either EGF or TGF-α to form colonies in soft agar medium (11, 12); mouse embryo AKR-2B cells require only TGF-β to grow under anchorage-independent assay conditions (13, 14). Although TGF-β stimulates the anchorage-independent growth of both of these cell lines, it is not mitogenic for either cell line assayed under conditions of anchorage-dependent growth (6, 8, 15). This observation is consistent with the recent finding by Moses, Holley, and their colleagues (15) that TGF-β and a growth inhibitor (16, 17) purified from conditioned medium of African green monkey kidney cells (BSC-1) are either identical or closely related.

In the present studies we show that TGF-β inhibits the anchorage-independent growth of many human tumor cell lines at concentrations in the same range as those that enhance the anchorage-independent growth of NRK or AKR-2B cells. As a further example of the bifunctional nature of the action of TGF-β, we show that in another cell line, namely *myc*-transfected Fischer rat 3T3 fibroblasts, TGF-β can function as either an inhibitor or an enhancer of anchorage-independent growth, depending on the particular set of growth factors operant in the cell together with TGF-β.

MATERIALS AND METHODS

Cell Culture. NRK cells, clone 49F, were maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 10% calf serum (GIBCO) supplemented with penicillin (50 units/ml) and streptomycin (50 μg/ml) in humidified 5% CO_2/95% air at 37°C. Unless specified, human tumor cell lines were obtained from the American Type Culture Collection and were grown under conditions identical to those for the NRK cells except that fetal bovine serum (GIBCO) was used. B16F1 (mouse) cells were a gift from I. J. Fidler, A-2058 and A-673 cells were from G. J. Todaro, A-375 cells were from C. Fryling, HT-1080 cells were from J. E. De Larco, and HT-29 and FMX-MetII cells were from A. C. Morgan and J. W. Pearson. Myc-1 cells were derived from Fischer rat 3T3 fibroblasts (18) by transfection with the second and third exons of a mouse cellular *myc* gene linked to the early simian virus 40 promoter (ref. 19; unpublished data). Transfected cells were maintained in the same media as NRK cells.

Growth Factors. TGF-β, homogeneous by analysis on sodium dodecyl sulfate/polyacrylamide gels as well as by amino acid analysis, was purified from human platelets (6). EGF was purified from male mouse submaxillary glands (20). Purified platelet-derived growth factor (PDGF), kindly provided by G. R. Grotendorst, was prepared from the acid/ethanol extract of human platelets (6) by successive chromatography steps on Bio-Gel P-60 (Bio-Rad), CM-cellulose, and phenyl-Sepharose (Pharmacia).

Soft-Agar Assay. Colony formation in 0.3% agar was assayed as previously described (20). Assays were carried out

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; NRK, normal rat kidney; TGF, transforming growth factor.

in either 10% or 20% calf serum or fetal calf serum as stated. Most assays were scored after 7 days of incubation; however, certain of the human tumor cell lines were incubated for periods up to 14 days. Colonies greater than 62 μm in diameter were counted by using an Omnicon Image Analysis system programmed to count a 5-cm^2 area of a 35-mm-diameter plate used for the assay.

Growth Curves. Cells were plated in 16-mm-diameter 24-well multi-dishes at the density and serum concentration indicated, using 1 ml of medium per well. Growth factors were added in 10 μl of 4 mM HCl per ml of medium 6–10 hr after seeding the cells. The amount of 4 mM HCl was controlled in all wells. Cells were counted in a Coulter Counter.

RESULTS

Effects of TGF-β on Anchorage-Independent Growth of Tumor Cell Lines. Two possible corollaries of the definition of TGF-β as a peptide that confers the transformed phenotype on cells are (*i*) that synthesis and release of TGF-β by transformed cells that have TGF-β receptors (autocrine secretion) might play a role in the maintenance of the transformed phenotype (21) and (*ii*) that addition of TGF-β to transformed cells in culture might exaggerate their transformed phenotype. In sharp contrast to these expectations, initial experiments with a human lung carcinoma cell line, A-549, demonstrated that addition of picomolar concentrations of TGF-β causes nearly complete inhibition of the growth of the cells in soft agar (Fig. 1*A*). A-549 cells are extremely sensitive to inhibition by TGF-β; colony formation was inhibited 50% by 0.3–0.5 pM TGF-β, which is 1/10th the concentration effective in stimulating 50% maximal colony formation by NRK cells (2–4 pM) (Fig. 1*A Inset*). Furthermore, whereas colony formation by NRK cells requires both EGF and TGF-β (refs. 2 and 6–8; Fig. 1*A Inset*), the effects of EGF and TGF-β on colony formation by A-549 cells are antagonistic. As shown in Fig. 1*A*, nearly 5 times as much TGF-β is required to reduce colony formation to the same level in the presence of 0.2 nM EGF as in the absence of added EGF. Conversely, as shown in Fig. 1*B*, the addition of EGF can partially overcome the inhibition of colony formation caused by treatment of the cells with TGF-β; at higher concentrations of TGF-β (10 pM), EGF can no longer reverse the inhibitory action of TGF-β. These inhibitory effects of TGF-β are observed regardless of whether calf serum or fetal bovine serum is used in the assay. However, colony formation by A-549 cells is affected by the concentrations of serum; the number of colonies of A-549 cells assayed in 30% fetal bovine serum is only 40% that of the cells assayed in 10% fetal bovine serum, but the ED_{50} for TGF-β inhibition is not affected.

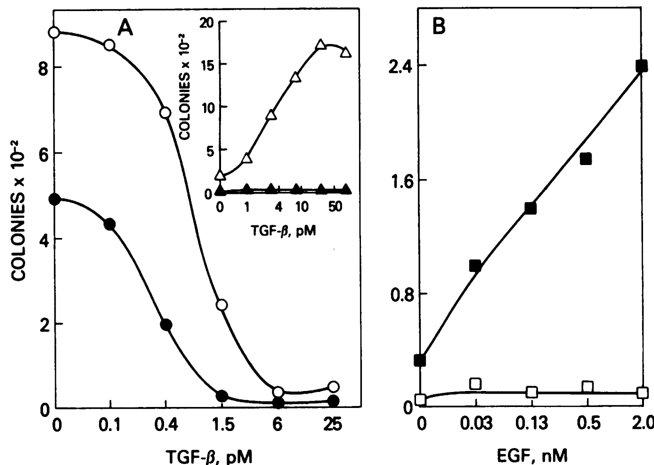

FIG. 1. Antagonistic and synergistic effects of EGF and TGF-β on the anchorage-independent growth of cells. Human lung carcinoma A-549 cells or NRK cells were assayed for colony formation in soft agar medium in 10% fetal calf serum or calf serum, respectively, as previously described (20). Colonies greater than 62 μm in diameter were scored. (*A*) Dose–response curve of the inhibition by TGF-β of the colony formation of A-549 cells in the presence (○) or absence (●) of 0.2 nM EGF. (*Inset*) Dose–response curve of the stimulation by TGF-β of the colony formation of NRK cells in the presence (△) or absence (▲) of 0.8 nM EGF. (*B*) Dose–response curve of the antagonism between EGF and TGF-β on the anchorage-independent growth of A-549 cells grown in the presence of either 2 pM (■) or 10 pM (□) TGF-β. The A-549 cells formed 800–1000 colonies when treated with 0.03–2 nM EGF in the absence of added TGF-β (not shown).

To explore whether the inhibitory action of TGF-β on A-549 cells was specific to that cell line, the effects of TGF-β on the anchorage-independent growth of a wide variety of human tumor cell lines—including melanomas, carcinomas of the lung, breast, and colon, and sarcomas—were determined. As shown in Table 1, the anchorage-independent growth of 9 of 12 cell lines was inhibited by TGF-β; it had no effect on the growth of 3 cell lines, 2 of which (HT-29 and FMX-MetII) were highly malignant cells selected for their ability to grow in ascites fluid of nude mice. In many cell lines only 10–30 pM TGF-β (0.25–0.75 ng/ml) was required for 50% inhibition. Complete inhibition of colony formation was seen only in two cell lines, A-549 and the murine melanoma B16F1. More typically, a plateau of 50–60% inhibition of the colony-forming response was reached between 50 and 100 pM TGF-β (Table 1). Similar effects of TGF-β were observed on the anchorage-dependent growth of these same cells (data not shown).

Bifunctional Effects of TGF-β on the Anchorage-Independent Growth of Fibroblasts Transfected with the *myc* Oncogene. The opposite effects of TGF-β on colony formation by NRK cells and various tumor cell lines suggested that the differences might result from inherent differences in response patterns of nonneoplastic versus neoplastic cells, or of fibroblastic versus epithelial cells. However, experiments with a cell line constructed by transfection of a cellular *myc* gene into Fischer rat fibroblasts (Myc-1 cells) demonstrate that a bifunctional response pattern to TGF-β can be observed in a single cell line, depending on the set of other growth factors in which TGF-β is operant. Thus, treatment of Myc-1 cells with TGF-β alone induced the formation of only a few colonies (Fig. 2). In contrast, the introduction of a *myc* gene under regulation of the simian virus promoter has caused these rat fibroblasts to become extremely sensitive to EGF, and EGF alone can induce a maximal colony-forming response at concentrations above 0.3 nM. However, when both TGF-β and EGF are present in the assay, TGF-β inhibits the EGF-induced colony formation in a dose-dependent manner (Fig. 2*A*), similar to the result shown for the A-549 cells (Fig. 1*A*). On the other hand, when the cells are assayed in the presence of PDGF, which by itself does not promote anchorage-independent growth, TGF-β markedly enhances the colony formation (Fig. 2*B*). The concentrations of TGF-β that either inhibit (ED_{50} approximately 10 pM) or stimulate (ED_{50} approximately 20 pM) colony formation by Myc-1 cells in the presence of either EGF or PDGF, respectively, are nearly identical. Thus, the opposite effects of TGF-β appear to be the result of the cellular integration of all the signals generated by the total set of growth factors operant in the cell at a particular time.

TGF-β Increases the Cell-Cycle Time of Cells in Monolayer Culture. As shown in Fig. 3, addition of TGF-β to sparse cultures of A-549 cells resulted in a dose-dependent increase in the apparent doubling time of the cells. The cells treated with TGF-β are fully viable and continue to divide, but at a slower rate than the untreated cells; 60 pM TGF-β increases the doubling time of the cells from 21 to 36 hr (Fig. 3 *Inset*).

Table 1. Effects of TGF-β on anchorage-independent growth of human tumor cells

Type of tumor	Cell line	Colonies per plate (no TGF-β)	Effect of TGF-β	ED_{50} of TGF-β, pM	Maximal inhibition with 100 pM TGF-β, %	TGF-β (max), pM
Lung carcinoma	A-549	1500	I	0.4	93	4
Lung carcinoma	Calu-6	1900	I	>100	40	30
Melanoma	A-375	860	I	8	53	11
Melanoma	SK-MEL-28	1150	I	11	60	50
Melanoma	SK-MEL-5	670	I	9	66	33
Melanoma	A-2058	1800	I	8	50	30
Melanoma (murine)	B16F1	770	I	10	94	100
Breast carcinoma	MCF-7*	500	I	4	74	100
Fibrosarcoma	HT-1080	1100	I	50	60	100
Melanoma	FMX-MetII	2300	NE	>100	0	—
Colon carcinoma	HT-29	2700	NE	>100	0	—
Rhabdomyosarcoma	A-673	1300	NE	>100	0	—

Single-cell suspensions (4×10^3 per 35-mm Petri dish for all cell lines except HT-1080, A-2058, and A-673, which were seeded at 14, 12, and 6 $\times 10^3$ cells, respectively) of tumor cells were plated in 0.3% agar in medium containing serum. For cell lines assayed in the presence of fetal bovine serum, the following concentrations were used: HT-29 and FMX-MetII cells, 20%; A-549 and A-2058 cells, 10%, and HT-1080 cells, 5%. All other cell lines were assayed in the presence of 20% calf serum, with the exception of A-673 cells, for which 2% calf serum was used. ED_{50} for inhibition of colony formation by TGF-β represents the concentration of TGF-β that reduces the number of colonies (>62-μm diameter) to 50% the value in the absence of TGF-β, as determined from a dose-response curve of six different concentrations of TGF-β in the range 0.4–100 pM. Maximal inhibition of colony formation represents the highest level of inhibition observed in the presence of 100 pM TGF-β, and TGF-β (max) is the lowest concentration of TGF-β that resulted in inhibition to the maximal extent. All assays were performed in duplicate. I, inhibition; NE, no effect.

*Colonies greater than 30-μm diameter were scored.

Similar results have been obtained with NRK cells (data not shown).

The inhibitory effects of TGF-β on anchorage-dependent growth of both the neoplastic A-549 cells and the nonneoplastic NRK cells are reduced only slightly as serum concentrations are increased. In 1% fetal calf serum, TGF-β (40 pM) increased the doubling time of A-549 cells from 17 to 25 hr, and in 5% serum, from 14 to 20 hr. Similarly, in 2% calf serum, TGF-β (20 pM) increased the doubling time of NRK cells from 31 to 46 hr, and in 7% serum from 18 to 27 hr.

Interactions of TGF-β and EGF in NRK Cells Can Be Synergistic or Antagonistic. In NRK cells, both EGF and TGF-β are required for anchorage-independent growth, and their effects are synergistic (Fig. 1*A Inset*). It has been shown previously that the colony-forming response of these cells can be controlled by the concentration of either EGF or TGF-β (11). However, when the cells are grown under anchorage-dependent conditions in monolayer culture, the effects of EGF and TGF-β are antagonistic. As shown in Fig. 4*A*, EGF (4–100 pM) markedly stimulates the growth of the NRK cells in 2% serum, and the stimulation can be significantly blocked by the addition of 20 pM TGF-β. Conversely, when the cells are grown in 7% serum (Fig. 4*B*), TGF-β (4–100 pM) inhibits the growth of the cells, and the inhibition can be partially overcome by the addition of 20 pM EGF. At higher EGF concentrations, inhibition by TGF-β is totally over-

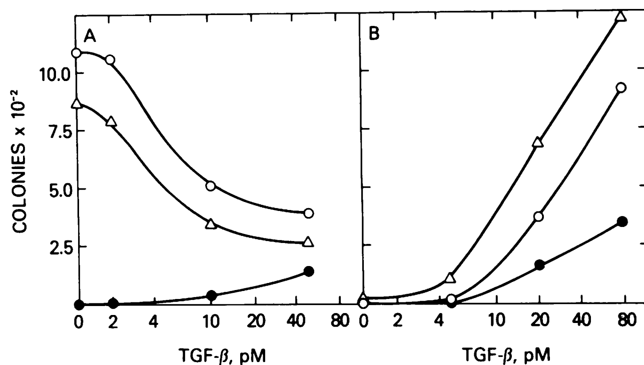

FIG. 2. Antagonistic and synergistic effects of TGF-β on the anchorage-independent growth of Myc-1 cells assayed in the presence of either EGF or PDGF. Fischer rat 3T3 cells transfected with the *myc* gene were assayed for formation of colonies greater than 62 μm in diameter in soft agar in 10% calf serum as described (20). (*A*) Dose–response curve of TGF-β acting on Myc-1 cells either alone (●) or in the presence of either 0.4 nM EGF (○) or 0.08 nM EGF (△). (*B*) Dose–response curve of TGF-β acting on Myc-1 cells either alone (●) or in the presence of either 0.36 nM PDGF (△) or 0.09 nM PDGF (○).

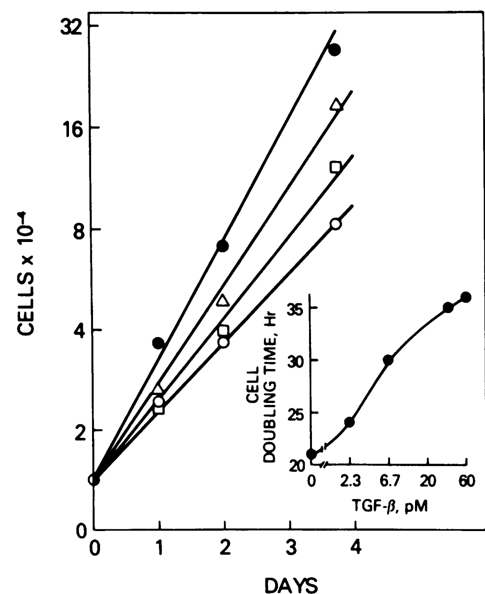

FIG. 3. Effects of TGF-β on the cell cycle time of human lung carcinoma A-549 cells. A-549 cells were grown in monolayer culture in 2% fetal calf serum at an initial cell density of 1.5×10^4 cells per ml. Cells were grown in the presence of serum alone (●) or with added TGF-β at concentrations of 2.3 pM (△), 6.7 pM (□), and 60 pM (○). Cell counts were determined at the times indicated. (*Inset*) Cell doubling time was calculated from the slope of the growth curves in the presence or absence of TGF-β and plotted versus concentration of TGF-β added.

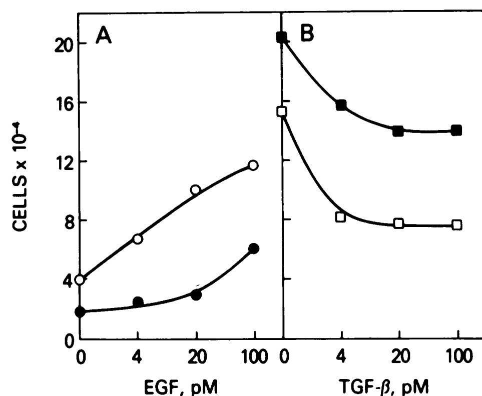

FIG. 4. Antagonistic interactions between EGF and TGF-β on the anchorage-dependent growth of NRK cells. NRK cells were seeded in monolayer culture at an initial cell density of 1×10^4 cells per ml and counted 4 days later. (A) Inhibition by TGF-β (20 pM) of the EGF-dependent growth of NRK cells. NRK cells were seeded in 2% calf serum and various concentrations of EGF in the presence (●) or absence (○) of 20 pM TGF-β. (B) Reversal by EGF (20 pM) of the inhibition of growth of NRK cells caused by TGF-β. NRK cells were seeded in 7% calf serum and various concentrations of TGF-β in the presence (■) or absence (□) of 20 pM EGF. After 5 days, saturation densities were 20×10^4 cells per ml for cells treated with 20 pM EGF alone, and 25×10^4 cells per ml for cells treated with both EGF (20 pM) and TGF-β (20 pM).

come (data not shown). Interestingly, the cultures treated with both EGF and TGF-β eventually reach a higher cell density than those treated with EGF alone, in keeping with the previously observed loss of density-dependent inhibition of growth of the cells treated with both type α and β TGFs (2, 11).

The response of Myc-1 cells to TGF-β and PDGF is also bifunctional. We have shown that PDGF and TGF-β have synergistic effects on the anchorage-independent growth of the cells (Fig. 2A). However, when assayed in monolayer culture in 2% serum, PDGF (200 pM) has a marked mitogenic stimulus on cell growth, and the addition of TGF-β (100 pM) blocks that effect (data not shown).

DISCUSSION

In this paper we present evidence that the action of TGF-β on cells is bifunctional: in certain instances TGF-β stimulates anchorage-independent growth; in others it acts to inhibit that growth; and it can act either synergistically or antagonistically with other growth factors such as EGF, PDGF, or other factors present in the serum used in these assays. The bifunctional response of cells to TGF-β cannot be ascribed to differences between nonneoplastic and neoplastic cell types or to differences between fibroblastic and epithelial cell types. Rather, a bifunctional response to TGF-β has been shown to occur in a single cell type (Myc-1 rat fibroblasts) under almost identical growth conditions and TGF-β concentrations, the response being a function of the entire set of growth factors and their receptors operant in the cell in combination with the TGF-β.

Recent work from the laboratories of Moses and Holley has established that TGF-β and the epithelial cell growth inhibitor isolated from conditioned medium of African green monkey kidney cells (16, 17) are either identical or belong to the same family of peptides (15). This association was made on the basis of the interchangeability of the two peptides in assays of the promotion of the anchorage-independent growth of AKR-2B cells and in assays of the inhibition of growth of cells in monolayer culture. In addition, the growth inhibitor competed with TGF-β for binding to cell surface receptors (15).

TGF-β is therefore one of the first peptide "chalones" (22) or growth regulators (23–25) that has been purified to homogeneity, and its amino acid composition and partial amino acid sequence have been determined (6–8). It has been found in many neoplastic and nonneoplastic tissues and cells examined (26) and has been totally purified from bovine kidney (8), human placenta (7), and human platelets (6). With the evidence presented here and by Moses, Holley, and colleagues (15), it must now be considered whether TGF-β might be an active component in various growth inhibitor preparations reported in the literature. The widespread tissue distribution of TGF-β (26); its high stability to denaturation by heat, acid, or organic solvents; its highly potent activity (significant inhibition of cell growth at picomolar concentrations, less than 1 ng/ml); as well as its hydrophobic nature, which causes it to associate with other peptides during purification (27), all make this a likely possibility.

It is now critical to determine whether TGF-β might have practical therapeutic applications for control of growth of cancer cells. Several years ago, we proposed an "autocrine hypothesis" that malignant transformation may result from the inappropriate cellular expression of *positive* growth factors (21). This hypothesis states that specific peptide growth factors are produced by transformed cells themselves, and that transformed cells have their own functional receptors for these peptides. A number of laboratories have obtained substantial evidence supporting this hypothesis (28–31), particularly with the findings that oncogenes may code for growth factors (32, 33) or their receptors (34).

The autocrine hypothesis may now be extended to *negative* peptide growth factors (such as TGF-β) to include the concept that transformation may also result from the failure to express or respond to specific growth inhibitory substances that may be released by cells to regulate their orderly growth. This general concept has been enunciated in many forms over the years (22–25, 35), but firm experimental evidence in its support, involving definitive isolation and structural characterization of specific peptides with a high level of potency, has been singularly lacking until the discovery (15) that TGF-β can suppress the growth of the very same monkey kidney cells that produce this peptide (16, 17). We are now proposing that the failure to express autocrine physiological TGF-β activity may result in uncontrolled growth of certain cells that require this peptide as a normal growth-regulatory substance. There are several mechanisms that might contribute to such a deficit, including a mutation or loss of the structural gene for TGF-β itself, a loss of positive transcriptional or translational controls for expression of TGF-β, or a defect in the specific high affinity cellular receptor for TGF-β. These mechanisms might be germane to the genesis of cancer.

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