

Altered Metabolic and Adhesive Properties and Increased Tumorigenesis Associated with Increased Expression of Transforming Growth Factor β 1

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Abstract. Transforming growth factor- β (TGF- β) is a potent mediator of cell proliferation and extracellular matrix formation, depending on the cell type and the physiological conditions. TGF- β is usually secreted in a "latent" complex that needs activation before it can exert its effects. Several observations correlate increased expression of TGF- β 1 with tumorigenesis. To evaluate the physiological relevance of increased TGF- β 1 synthesis in tumor cells we established cell clones overexpressing TGF- β 1 and observed the resulting physiological changes in TGF- β overproducing cells in vitro and in vivo. As a model system we used the human E1A-transformed 293 tumor cells, which are insensitive to the direct growth modulatory effects of TGF- β . The selection of this cell line allows an assessment of physiological alterations independent of TGF- β induced proliferative changes.

The use of two TGF- β 1 expression vectors containing either the natural or a modified TGF- β 1 precursor cDNA permitted the establishment of separate 293 cell lines overexpressing latent or active TGF- β . Comparison of the resulting changes in glycolytic rate, adhesiveness and integrin and plasminogen activator expression established that, in vitro, both types of clones behaved similarly, indicating that expression of latent

TGF- β induces autocrine changes in the tumor cells and thus suggesting that some level of cell-associated activation occurs. TGF- β overexpression resulted in an increased metabolic rate due to enhanced glycolysis, a property long associated with tumor cells. This increased glycolysis was not associated with altered proliferation. Cells overexpressing TGF- β also displayed enhanced fibronectin mRNA and plasminogen activator synthesis and increased adhesiveness in vitro. They showed enhanced survival when plated sparsely on plastic in the absence of serum, and attached more readily to laminin. In addition, synthesis of several β 1 integrins, in particular the α 1/ β 1, α 2/ β 1, and α 3/ β 1, all of which recognize laminin, were enhanced. Finally, cells overexpressing active TGF- β , but not latent TGF- β , also showed increased tumorigenicity in nude mice. Thus, an increase in endogenous TGF- β synthesis confers several proliferation-independent phenotypic changes which may be of significance for the survival of the tumor cell inoculum or its subsequent growth, and for tumor formation and development. In the case of cells expressing active TGF- β , the release of active TGF- β into the vicinity of the tumor cells may also result in a more hospitable environment for tumor growth.

THE transforming growth factor- β (TGF- β)¹ family of structurally related growth and differentiation factors has been reported to display diverse biological activities in a wide range of experimental settings. The effects of TGF- β differ considerably, depending on cell type and physiological conditions. In vitro experiments have revealed that members of the TGF- β family are able to function as potent

regulators of cellular proliferation and differentiation, as well as immunosuppression. It is therefore assumed that TGF- β plays a crucial role in these different processes in vivo. In addition, TGF- β is known to modulate the expression of various proteins that contribute to extracellular matrix formation and to cell-matrix interaction. Such alterations are expected to exert some effects on phenotypic parameters of the cells, such as increased extracellular matrix deposition and cellular adhesiveness. Many cell types

1. *Abbreviation used in this paper:* TGF- β , transforming growth factor- β .

and tissues express mRNAs for one or more species of TGF- β , each encoded by genes with a distinctive complement of transcriptional and translational regulatory elements. Since most cells have functional TGF- β receptors, it is generally assumed that the biological activities of TGF- β are exerted in an autocrine or paracrine fashion (Roberts and Sporn, 1990).

TGF- β is a disulfide-linked homodimeric protein which is secreted as part of a complex consisting of two units of the large precursor segment of the TGF- β propolypeptide linked in a non-covalent association with the mature TGF- β dimer. This complex is "latent" in the sense that it does not bind to TGF- β receptors and therefore cannot exert any biological activities associated with TGF- β . The release under physiological conditions of active TGF- β from the latent complex may be a finely regulated event in which specific proteases are involved (Sato et al., 1990). Thus, localized proteolytic activation of the complex may be a critical determinant of the local concentration of the active form of TGF- β .

Several observations correlate increased expression of TGF- β with tumorigenesis. Viral transformation of normal rat kidney cells or NIH-3T3 fibroblasts was associated with a greater than 40-fold increase in TGF- β secretion (Anzano et al., 1985). In addition, transformation of 10T1/2 fibroblasts by radiation resulted in increased rates of TGF- β secretion, and rat tracheal epithelial cells transformed by carcinogens or irradiation contained elevated levels of TGF- β 1 mRNA (Ferriola et al., 1989; Schwarz et al., 1990). Moreover, analysis of TGF- β 1 mRNA levels in specimens of human tumors of various histological types demonstrated increased levels of expression in comparison with adjacent normal tissues (Derynck et al., 1987). Similarly, breast, hepatocellular, and renal cell carcinomas, as well as T-cell leukemia cells, displayed elevated levels of TGF- β 1 mRNA compared with their non-malignant counterparts (Gomella et al., 1989; Ito et al., 1991; Niitsu et al., 1988; Travers et al., 1988). Furthermore, TGF- β has recently been implicated as playing a critical role in the development of tumors in Rous sarcoma virus-infected chickens following wounding (Sieweke et al., 1990). Finally, metastatic fibrosarcomas derived by transfection of fibroblasts with any of four oncogenes were reported to release TGF- β in its active form at significantly higher levels than the corresponding non-transfected cells (Schwarz et al., 1990). Activated TGF- β has been detected in conditioned medium from several other tumor cell lines as well (Knabbe et al., 1987; Liu et al., 1988; Schwarz et al., 1990; Jennings et al., 1991; Constam et al., 1992).

These observations suggest that TGF- β synthesized by the tumor cells themselves may facilitate tumorigenesis and regulate tumor cell behavior. However, by their very nature, data obtained from such descriptive approaches are limited and correlative. Studies comparing TGF- β expression by surgically obtained tissue specimens are unavoidably complicated by cellular heterogeneity. Similarly, the multitude of cellular and genetic changes that follow viral transformation or oncogene transfection make assessment of the particular importance of TGF- β expression impossible.

We addressed these issues in designing this study to evaluate the importance of endogenous TGF- β synthesis for tumor cells by generating stable transfected tumor cell lines that produce elevated levels of TGF- β . We specifically wanted to

evaluate its importance for tumor cell behavior in the absence of a direct contribution by TGF- β to cellular proliferation, which might otherwise interfere with an evaluation of other TGF- β -mediated phenotypic changes, e.g., altered matrix production. In addition, many tumor cell lines have lost their sensitivity to the antiproliferative effects of TGF- β and it has been proposed that a loss of the autocrine antiproliferative effects of TGF- β may be a necessary step for progression to malignancy (Wakefield and Sporn, 1990), a possibility recently reinforced by the observation that carcinomas, but not papillomas have lost their responsiveness to the direct effects of TGF- β on proliferation (Haddow et al., 1991). We have therefore chosen the human embryonic kidney cell line 293, which has incorporated a functional E1A gene derived from adenovirus 5 (Graham et al., 1977). The formation of E1A protein complexes with the endogenous retinoblastoma gene product and/or several other nuclear proteins renders these cells insensitive to the TGF- β -induced modulation of proliferation, thus suggesting an involvement of the retinoblastoma gene product in TGF- β -induced growth inhibition (Pietenpol et al., 1990). From the parental 293 cells, we generated recombinant cell lines transfected with a TGF- β 1 expression vector, that secreted high levels of either latent TGF- β 1 complex or biologically active TGF- β 1.

Using these cell lines, which had an unaltered proliferation rate, we asked several questions. First, we determined whether overexpression of biologically inactive, latent TGF- β resulted in any autocrine effects at all and in this context compared the physiological consequences of increased expression of latent versus active TGF- β . Second, we evaluated the behavior of these cell lines overexpressing latent or active TGF- β , *in vitro*, focusing on the most striking changes in glycolysis and adhesiveness. Finally, we examined the effect of overexpression of TGF- β on tumor formation *in vivo*.

Materials and Methods

Expression Plasmids for Latent and Active TGF- β 1

The parent plasmid used for expression of the TGF- β 1 cDNA was pRK5 (Graycar et al., 1989) (see Fig. 1). This plasmid incorporates a human cytomegalovirus promoter which controls the transcription of the cDNA and has a high efficiency of transcription in human 293 cells. The promoter is followed by a short intron containing an sp6 promoter which in turn is followed by a polylinker in which the cDNA will be incorporated. The polylinker site is followed by a sequence from the SV-40 late region containing the signals for polyadenylation and transcriptional termination.

Plasmid pRK5- β 1E is a derivative of pRK5 in which the human TGF- β 1 cDNA encoding the unmodified wild type TGF- β 1 precursor (Derynck et al., 1985) is inserted (see Fig. 1). The TGF- β 1 cDNA was engineered to obtain a high level of translation. The modifications of the cDNA, introduced by site-directed mutagenesis of the single-stranded cDNA in recombinant M13 derivatives (Zoller and Smith, 1984) were as follows. One mutation centered around the initiator ATG and converted the original sequence (Derynck et al., 1985) into TCTAGAAACAATGGCGCCC. This mutation resulted in the introduction of a unique restriction site for XbaI closely preceding the start codon. The XbaI site was subsequently used for incorporating the cDNA into pRK5, so that virtually nothing of the original long 5' untranslated sequence was retained in the expression vector. This mutagenesis also generated a sequence context for the initiator ATG which conformed well to the consensus sequence for eukaryotic mRNAs (Kozak, 1989). The sequence surrounding the ATG, incorporating an A-residue at the -3 position and a G at +4, is predicted to result in a high efficiency of translation. The second mutation removed the 86 bp CG-rich sequence immediately following the stop codon. Finally, a HindIII recognition site was introduced to immediately follow the Pst I site downstream from the

3' untranslated region (position 2740; Derynck et al., 1985). After these mutations were introduced, the cDNA was excised and introduced as an XbaI-HindIII fragment into the XbaI-HindIII-opened pRK5 plasmid.

Plasmid pRK5- β IEC2S2 was derived from pRK5- β IE (Fig. 1). Again using site-directed mutagenesis, codons for the two cysteine residues at positions 223 and 225 in the TGF- β 1 precursor were replaced by TCT and AGC codons for serine. As shown by Brunner et al. (1989), these mutations in the TGF- β 1 precursor cDNA result in expression of TGF- β 1 that is largely biologically active and thus apparently no longer in a latent complex.

Generation of Stable Transfected Clones

Subconfluent 293 cells were transfected with 30 μ g of pRK5, pRK5- β IE, and pRK5- β IEC2S2 plasmid DNA mixed with 1.5 μ g pRSV-Neo plasmid DNA by the calcium phosphate co-precipitation method (Gorman et al., 1983; Ausubel et al., 1991). Cells were maintained in RPMI with 5–10% FBS, and during exposure to precipitated DNA, cultures were incubated in F12/DME medium with 5% FBS. Media were supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were incubated with the DNA precipitate for 5 h and then exposed to 15% glycerol in PBS at room temperature for 90 s. Two days later, cells were split 1:20 and grown in the presence of G418 (600–800 μ g/ml) until colonies formed. Colonies were expanded and RNA was prepared (Chomczynski and Sacchi, 1987) for subsequent Northern analysis to detect TGF- β 1 mRNA. The transfected clonal cell lines with the highest plasmid-derived TGF- β 1 mRNA levels were selected for characterization. All selected colonies were tested for mycoplasma contamination and found to be negative.

mRNA Analysis

Northern analysis for the presence of specific mRNAs proceeded as follows: Total RNA was electrophoresed in 1% agarose, 5% formaldehyde gels, followed by electrotransfer to nylon membranes (Genescreen) and UV cross-linking. Membranes were probed with cDNA fragments radiolabeled by the random priming method (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's directions. The TGF- β 1 probe was the 1.9-kbp EcoRI-HindIII cDNA insert of plasmid pRK5- β IE. The fibronectin probe was the 1.0-kbp ClaI-BamHI fragment from plasmid pFH23 (obtained from Dr. Z. Werb, University of California, San Francisco, CA). All hybridizations were performed under high-stringency conditions. Unhybridized probe was removed by washing membranes with 0.1 \times SSC, 0.2% SDS at 60°C.

RNase protection analyses to detect and quantitate levels of TGF- β 1, - β 2, and - β 3 mRNA were performed as previously described (Arrick et al., 1990).

Quantitation of TGF- β Secretion

To prepare conditioned medium, cells were incubated for 24 h in serum-free F12/DME medium supplemented with transferrin (13.5 μ g/ml), BSA (300 μ g/ml), and Hepes (10 mM, pH 7.2). The conditioned medium was collected and assayed for TGF- β -induced inhibition of proliferation of CCL64 mink lung cells, with and without acid activation, as previously described (Arrick et al., 1990; Meager, 1991). The monolayers were then trypsinized and counted in a Coulter counter to allow normalization of the levels of secreted TGF- β against cell number.

Assay for Lactate Production

Cells harvested from routine cultures with detaching solution (125 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM EDTA, 50 mM Hepes, pH 7.4), were washed with serum-free medium, and replated in triplicate in serum-free medium at a density of 4×10^5 /ml on laminin-coated 24-well plates at 1 ml per well and incubated overnight at 37°C. Laminin-coated plates were used in order to obtain an adherent monolayer. Medium was then replaced with sodium phosphate-containing buffer supplemented with 20 mM glucose. After a 1-h incubation, lactate levels were measured as described (Resnick et al., 1986). In this assay, lactate-dependent reduction of nicotine adenine dinucleotide in the presence of lactate dehydrogenase was quantitated by measurement of OD at 339 nm. Cell counts obtained after trypsinization of the monolayers were used to normalize lactate determinations.

Cell Adherence on Defined Substrates

We evaluated the adherence and morphology of control and transfected cells

plated on plastic in the presence and absence of serum. Cell suspensions in serum-free DME medium were obtained as described above, and 5×10^4 cells (or 5×10^3 cells in some experiments) in 1 ml were plated per well in 24-well plates in the presence and absence of serum.

Experiments were also carried out on surfaces coated with laminin (from Engelbreth-Holms-Swarm tumor, Collaborative Research) or plasma fibronectin (Collaborative Research Inc., Lexington, MA) in the presence of serum-free medium containing 2% of Nutridoma (Boehringer Mannheim Biochemicals), a medium supplement that lacks the attachment factors present in serum. The wells were coated for 16 h at 4°C with laminin or fibronectin at 10 and 5 μ g/ml, respectively. Unoccupied sites on the substrates were blocked with 0.2% BSA in Ca^{2+} - and Mg^{2+} -free PBS (CMF-PBS) for 1 h before plating cells (Hall et al., 1990). Cells were plated in 24-well plates at a density of 4×10^4 cells/ml. Morphology was evaluated at 4 h and at 2 and 4 d.

Detection of Integrins by Immunoprecipitations

Antibodies used to detect integrins were obtained as follows: rat mAbs against the human integrin subunits β 1 (A1B2), α 5 (BIIG2; Werb et al., 1989; Hall et al., 1990), and α 6 (JIB5; Damsky et al., 1992) were produced as described. Other antibodies were obtained as follows: mouse monoclonal anti- α 2 (PIH5) and α 3 (PIB5) were gifts of Drs. E. Wayner (Fred Hutchinson Cancer Center, Seattle, WA) and W. Carter (Fred Hutchinson Cancer Center) (Wayner and Carter 1987); mouse monoclonal anti- α 1 (TS2/7; Hemler et al., 1984) was purchased as ACT-T-SET from T-Cell Sciences, (Cambridge, MA); mouse anti- α V (LM142) was a gift of Dr. David Cheresch (Scripps Institute, La Jolla, CA) (Cheresch and Harper, 1987). Unconjugated rabbit anti-mouse IgG was purchased from Jackson Immunoresearch Lab Inc. (West Grove, PA). Protein A-conjugated agarose beads were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells were either metabolically labeled or surface labeled. For metabolic labeling, cells were harvested using trypsin-EDTA, plated at a density of 2×10^6 cells per T75 flask in DME medium with 10% FBS and radiolabeled for 24 h with 50 μ Ci/ml ^3H -glucosamine. The cells were then harvested with 2 mM EDTA in CMF-PBS, washed in PBS containing Ca^{2+} and Mg^{2+} , and lysed in 25 mM Tris buffer, pH 7.2, containing 150 mM NaCl, 0.5% NP-40, and 1 mM PMSF. Alternatively, cells were harvested with EDTA as described and surface-labeled with ^{125}I -iodine using the lactoperoxidase/glucose oxidase method. After being washed in PBS containing 50 mM Na-iodide and again in Ca^{2+} - and Mg^{2+} -containing PBS, these cells were lysed as described for metabolically labeled cells. Lysates were centrifuged at 12,000 g for 10 min. All procedures were carried out at 4°C. The supernatant was precleared with Sepharose 4B beads, and aliquots containing equal protein amounts (determined using the Lowry method; Ausubel et al., 1991) were incubated for 2 h or overnight with one of the following subunit-specific monoclonal anti-integrin antibodies: A1B2 (anti- β 1), TS2/7 (anti- α 1), PIH5 (anti- α 2), PIB5 (anti- α 3), BIIG2 (anti- α 5), JIB5 (anti- α 6), or LM142 (anti- α V). The antibodies were present in excess such that addition of another aliquot of primary antibody, following the first round of precipitation, did not bring down any additional material. The samples were next incubated with a 1:100 dilution of rabbit anti-rat or anti-mouse IgG for 1 h, and then with 100 μ l packed protein A-agarose beads for 2 h. The beads with bound immune complexes were washed sequentially with 10 mM Tris-acetate, pH 8.0, 0.5 mM Ca^{2+} , and 0.5% NP-40 (TNC), TNC with 1 M NaCl, TNC with 0.1% SDS, and again with TNC. The proteins bound to the washed beads were analyzed by SDS-polyacrylamide electrophoresis in 7% polyacrylamide gels under non-reducing conditions, followed by autoradiography.

Detection of Proteases by Substrate Gel Electrophoresis

To detect expression of extracellular matrix-degrading metalloproteinases and plasminogen activators, cell extracts and conditioned media were analyzed on 10% polyacrylamide-SDS gels copolymerized with either 1 mg/ml gelatin (for the detection of metalloproteinases) or 13 μ g/ml plasminogen and 1 mg/ml casein (for the detection of plasminogen activators) as described previously (Unemori and Werb, 1986; Fisher and Werb, 1991). Before this analysis, the conditioned media were concentrated by dialysis using Centriprep concentrator tubes with a 10-kD exclusion filter (Amicon, Beverly, MA). To determine whether the substratum influenced proteinase expression, cells were plated on fibronectin- or laminin-coated plates in serum-free, Nutridoma-containing medium, or on plastic in serum-containing medium as described above.

Assessment of Invasiveness by a Matrigel Invasion Assay

Cells were harvested and 6×10^4 cells in 200 μ l of serum-free, Nutridoma-containing medium were added to the upper section of Costar Transwell filter culture chambers partitioned by a Nucleopore filter (growth surface area: 0.28 cm^2 ; Costar, Cambridge, MA) that had been coated with a thin layer of Matrigel (Collaborative Research Inc., Lexington, MA), the reconstituted basement membrane-like extract of the EHS tumor (Librach et al., 1991). After 24 or 48 h, the cultures were fixed with 2% glutaraldehyde and processed for scanning electron microscopy by standard methods (Librach et al., 1991). Filters were mounted such that either the top or the underside of the filter could be viewed. The presence of cells on the underside of the filter constituted evidence of invasive activity.

Tumor Formation in Nude Mice

Subconfluent cultures were detached with detaching solution (125 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM EDTA, 50 mM Hepes pH 7.4). Cells were resuspended in F12:DME medium at a density of $3 \times 10^6/\text{ml}$ and kept on ice until injection. Female nude mice between 4- and 6-wk old (Charles River) were injected subcutaneously with 0.2 ml of the cell suspension. Some mice were injected with cell suspensions at two different sites. After 4 wk, mice were killed by cervical dislocation and the sizes of the subcutaneous tumor masses were measured in three dimensions (x, y, and z). From animals with a palpable tumor mass, we removed the lung, liver, kidneys, spleen, femur, and tumor, fixed the tissues in 10% formalin, and evaluated the organs and tissues macroscopically and histologically for the appearance of metastases. The microscopical examination of histological sections of the tissues was done by a veterinary pathologist. The volumes of the tumors were calculated as $0.5 \times$ the product of the measurements in three dimensions (Tomayko and Reynolds, 1989).

Results

Characterization of the Parent 293 Cells

In this study we evaluated the effects of TGF- β overexpression in 293 cells, a transformed and tumorigenic human kidney fibroblast cell line with an incorporated, functional E1A gene from adenovirus 5 (Graham et al., 1977). Analysis of the TGF- β expression pattern in the untransfected cells revealed that they synthesized only TGF- β mRNA of the expected size of 2.5 kb. No traces of TGF- β 2 or - β 3 mRNA were detected by RNase protection assay (data not shown). Measurements of the TGF- β levels using a TGF- β bioassay indicated that 0.3–0.4 ng/ 10^6 cells was secreted per 24 h. The requirement for acid activation to detect the TGF- β indicated that most, if not all, of the TGF- β was secreted in a latent form.

The presence of TGF- β receptors in the nontransfected 293 cells was assayed using specific cross-linking of cell surface proteins in the presence of ^{125}I -TGF- β 1 essentially as described by Frolik et al. (1984). Analysis of the cross-linking pattern revealed the presence of all three known species of TGF- β cell surface receptors or binding proteins, i.e., the type I 65-kD receptor, the type II 85-kD receptor and the large type III proteoglycan (data not shown). Thus, the parental 293 cells synthesize and secrete low levels of TGF- β 1 and have TGF- β receptors, suggesting that changes in endogenous TGF- β synthesis could affect their cellular physiology in an autocrine fashion.

Generation of Stable Cell Lines Overexpressing Latent or Active TGF- β

We constructed two mammalian expression plasmids for TGF- β 1 from the parental expression vector pRK5 as de-

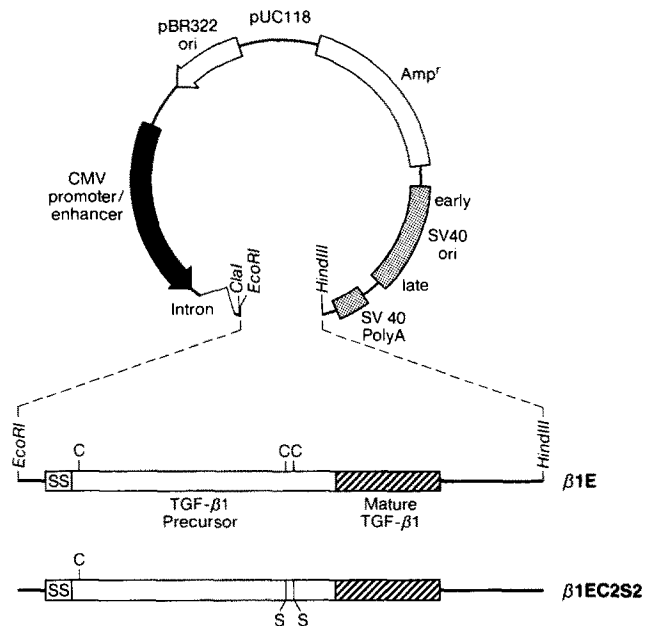


Figure 1. Schematic diagram of the TGF- β 1 expression plasmids pRK5- β 1E and pRK5- β 1EC2S2. The cytomegalovirus promoter segment with the direction of transcription is shown as a heavy black arrow, and the segments derived from SV-40 are shown as shaded boxes. The two TGF- β 1 cDNA transcription units, corresponding to either plasmid, are shown with their flanking restriction sites. SS, signal sequence.

scribed in Materials and Methods and diagrammed in Fig. 1. Plasmid pRK5- β 1E incorporates a human TGF- β 1 cDNA (Derynck et al., 1985), the expression of which should yield an unmodified human TGF- β 1 precursor protein, and thus TGF- β in its latent or biologically inactive complex. The second plasmid, pRK5- β 1EC2S2, is identical to pRK5- β 1E except for the replacement of the codons for two cysteines at positions 223 and 225 in the TGF- β 1 precursor with serine codons, a mutation that results in secretion of TGF- β largely in its biologically active form (Brunner et al., 1989).

Transfection of the individual TGF- β 1 expression plasmids along with the pRSV-Neo plasmid containing the neomycin-resistance transcription unit, followed by selection for G418-resistant clones, resulted in the generation of stable transfected 293 cell clones. Northern hybridization indicated that several of the G418-resistant cell lines expressed elevated levels of TGF- β 1 mRNA (Fig. 2), the size of which agreed with the size predicted from the design of the expression plasmids. Two clonal cell lines transfected with pRK5- β 1E (B6 and B9) and two transfected with plasmid pRK5- β 1EC2S2 (C15 and C19) were selected for further analysis. As controls, two G418-resistant clones cotransfected with the parent pRK5 lacking any cDNA sequence (R1 and R6) were characterized in parallel with the TGF- β -overexpressing cell lines. RNase protection assays indicated that the neomycin-resistant control clones expressed TGF- β 1 mRNA at levels equivalent to untransfected 293 cells and that the TGF- β -overexpressing B6 and C19 clones had about 100-fold higher levels of TGF- β 1 mRNA (not shown).

We measured secretion of TGF- β by the selected 293 clones by assaying the serum-free conditioned medium for TGF- β bioactivity in a cell growth inhibition assay (Table I).

R1 R6 B6 B9 C15 C19

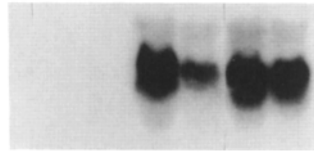


Figure 2. Expression of mRNA encoding TGF- β 1 by transfected cell clones, determined by Northern hybridization. Cells were transfected with parental plasmid pRK5 lacking TGF- β cDNA (R), with pRK5- β 1E (B), or with pRK5- β 1E

C2S2 (C). Each lane contained 18 μ g total RNA prepared from transfected cell clones as indicated. Equivalent RNA loading and transfer was confirmed by subsequent probing with a glyceraldehyde-6-phosphate dehydrogenase cDNA. The TGF- β 1 mRNA expressed from the transfected expression plasmid is about 2-kb long on the basis of migration in the gel. The low endogenous TGF- β 1 mRNA levels are not detectable using the short duration of autoradiography of this experiment and are more easily assessed by RNase protection assay.

The assay was done with and without acid activation, which quantitatively converts latent TGF- β to its biologically active form. The control transfected clones, like the parental 293 cells, secreted low levels of TGF- β in the latent form. In contrast, the transfected clones transfected with pRK5- β 1E or pRK5- β 1EC2S2 secreted TGF- β levels 100 to 300 times higher than the control clones. Their relative TGF- β levels were in agreement with the relative TGF- β 1 mRNA levels. As expected, \sim 99% or more of the TGF- β secreted by the clones transfected with pRK5- β 1E was in the latent form. We do not know whether the very low levels of active TGF- β in the conditioned medium of these clones are a result of technical manipulations, temperature- or protease-induced activation, or activation in the bioassay system. In contrast, 50% of the TGF- β released by the stable clones transfected with the pRK5- β 1EC2S2 plasmid, with its mutated precursor cDNA, was in a biologically active form. Remarkably, the total TGF- β expression from this plasmid was generally much higher than with the same expression vector containing the unmodified TGF- β precursor cDNA (Arrick, B., and R. Derynck, unpublished data).

Analysis of the TGF- β receptors by cross-linking, using 125 I-TGF- β as ligand, indicated that all clones had the three TGF- β receptor types at their cell surface. Some differences in receptor levels between the different clones were apparent, suggesting clonal heterogeneity (data not shown).

Table I. Secretion of TGF- β by Transfected 293 Cell Clones

Cell clone	TGF- β bioactivity (ng/10 ⁶ cells)		Percent active without acidification
	Without acid	With acid	
R1	ND	0.42	—
R6	ND	0.41	—
B6	0.49	95	0.5
B9	0.60	55	1.1
C15	58	110	52
C19	69	125	56

Serum-free conditioned medium was collected after a 24-h incubation and assayed for TGF- β bioactivity by inhibition of [3 H]TdR uptake by mink lung cells CCL64 with and without prior acid activation. TGF- β bioactivity in the conditioned medium of the two control clones was not detectable (ND) without acid activation. Values reported are means from triplicate determinations. Average coefficient of variance was 7.6%.

Table II. Lactate Production by Transfected 293 Cell Clones

Cell clone	Lactate (mmol/10 ⁶ cells/h)
R1	37.3 \pm 0.73
R6	39.0 \pm 1.3
B6	59.3 \pm 4.1
B9	53.6 \pm 0.74
C15	54.1 \pm 1.4
C19	50.1 \pm 0.79

Cells were incubated overnight in laminin-coated plates under serum-free conditions, followed by a 1-h incubation in a phosphate- and glucose-containing buffer. Lactate production was determined by quantitative conversion to pyruvate by lactate dehydrogenase, with stoichiometric formation of NADH (Resnick et al., 1986). Data are normalized to cell number and presented as means \pm SD for triplicate determinations.

Increased Glycolysis in TGF- β Overexpressing Cells

Increased aerobic glycolysis by tumors has been noted for over half a century. More recently it was shown that TGF- β was able to stimulate glycolysis in normal rat fibroblasts mitogenically stimulated by TGF- β (Boerner et al., 1985). During the course of our experiments, we noted a more pronounced acidification of the medium by several of the clones overexpressing TGF- β , even though, as expected, there were no differences in proliferation rate between the overexpressing clones and the control clones. We therefore assessed the glycolytic activity of our transfected clones by measuring lactate production. We found that the pRK5- β 1E- and pRK5- β 1EC2S2-transfected clones demonstrated a greater rate of lactate production than the control clones (Table II). This increase in lactate production was about equal to that observed upon exposure of rat kidney fibroblasts to exogenous TGF- β (Boerner et al., 1985).

It is important to observe that there was no major difference in lactate production between the pRK5- β 1E clones B6 and B9, and the pRK5- β 1EC2S2 clones C15 and C19. Thus, at least in this respect, the clones producing latent TGF- β behaved similarly to those secreting active TGF- β . This suggests that the clones producing latent TGF- β have the ability to activate it and are responsive to it.

Adhesive and Invasive Properties

During cell culture propagation of the transfected cells, we noted that clones overexpressing TGF- β required longer exposure to trypsin-EDTA than the control clones. To evaluate this further, we studied the adhesive properties of these cells to plastic, laminin, and fibronectin.

In one set of experiments we plated and incubated the cells (5×10^4 /ml) in the presence or absence of serum in plastic culture wells. When cultured on plastic in serum-free conditions, the control clones proliferated as non-adherent clumps of cells. In contrast, the TGF- β -overproducing clones B6, B9, C15, and C19 adhered to the plastic, spread out, and proliferated into a monolayer. Again, there were no apparent differences between clones overproducing latent or active TGF- β (Fig. 3). However, when plated in serum-free medium at very low density (5×10^3 /ml), only the C15 and C19 clones, which expressed active TGF- β 1 attached, survived, and proliferated (as assessed by phase-contrast mi-

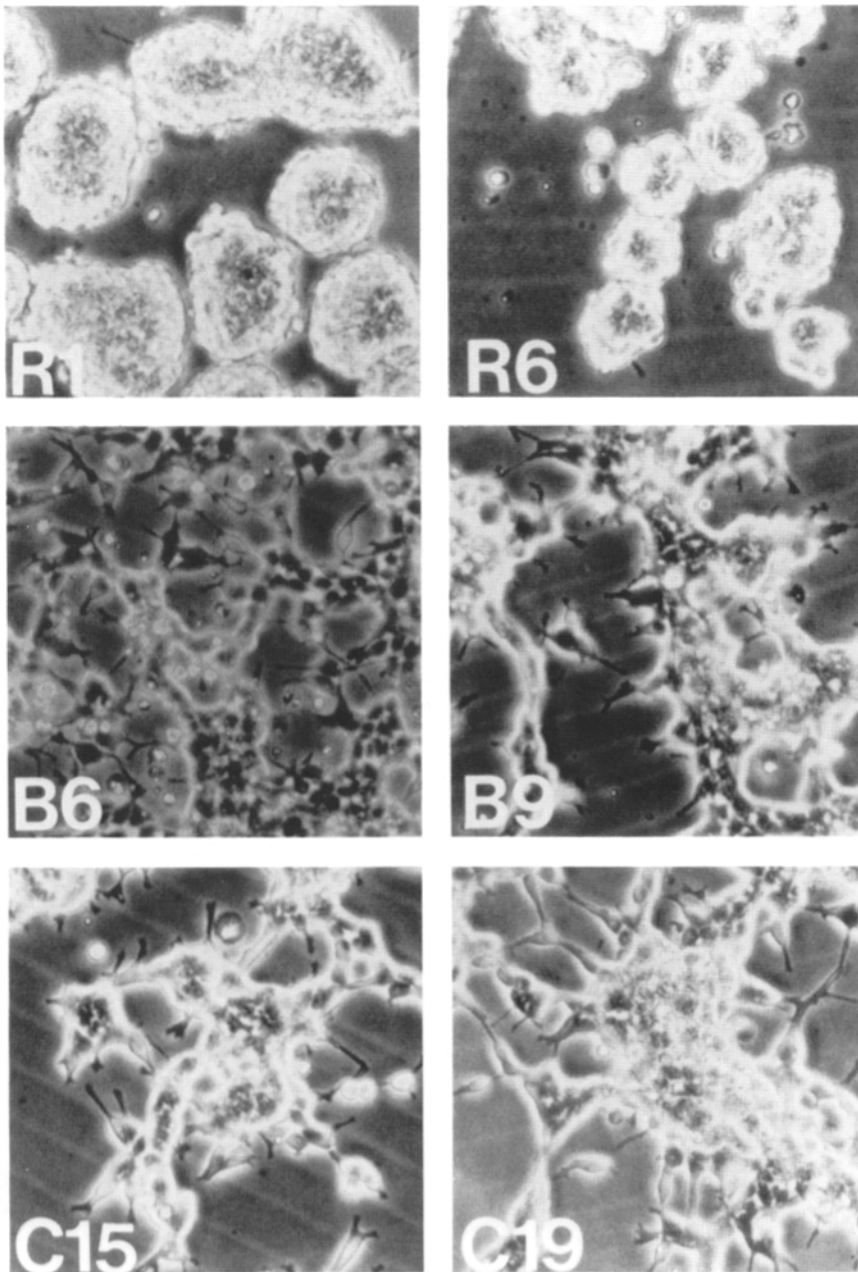


Figure 3. Adhesiveness of cell clones overexpressing TGF- β in serum-free medium. R6, B6, and C15 cells were plated on uncoated plates for 5 d before photomicrography. Cells were photographed at a 200 \times magnification.

croscopy and trypan blue exclusion; data not shown), indicating the importance of the increased adhesiveness for cell survival. In the presence of serum, we observed no differences in adherence or morphology between the control clones and the TGF- β -overproducing clones.

We also evaluated the adhesive behavior of the R6, B6, and C15 clones on laminin and fibronectin in serum-free medium containing Nutridoma, a commercially available additive that facilitates cell growth under serum-free conditions but lacks the attachment factors present in serum. When plated on fibronectin under these conditions, all clones tested attached well and displayed similar morphology (Fig. 4). However, there were differences when the cells were seeded on laminin-coated plates. The R6 control cells formed cell clumps that did not attach during the first 4 h of culture. At 2 D, the clumps of R6 cells were still only loosely attached.

By four days the clumps were more firmly attached, but the cells had not spread (Fig. 4). This inability to attach and spread on laminin was reminiscent of what occurred when these cells were plated on plastic in serum-free medium. In contrast, the B6 and C15 clones readily attached to the laminin-coated plates in the presence of Nutridoma (Fig. 4). At early time points, the B6 line was able to spread more extensively on laminin than the C15 line (Fig. 4), although neither cell line spread as extensively on laminin as they did on fibronectin. Both cell lines proliferated under these conditions.

Finally, we evaluated the invasiveness of several transfected clones using an *in vitro* invasion assay (Librach et al., 1991). Cells were seeded on Nucleopore filters coated with a thin layer of Matrigel, a commercially available basement membrane preparation. The ability of the cells to invade

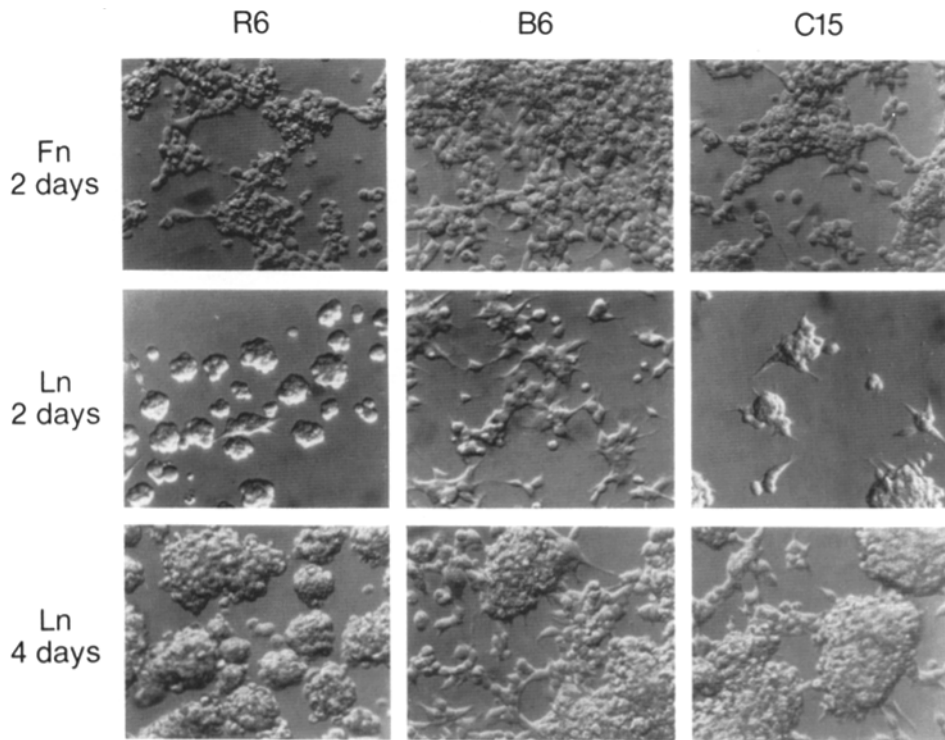


Figure 4. Adhesiveness of TGF- β -overexpressing cell clones in Nutridoma-containing medium. R6, B6, and C15 cells, cultured in DME medium containing Nutridoma, were plated on fibronectin (Fn)- or laminin (Ln)-coated culture wells. All three lines adhered and spread on fibronectin. Note that control R6 cells did not attach efficiently to laminin and did not spread. In contrast, TGF- β -overexpressing B6 and C15 cells attached readily to laminin and spread more efficiently. Cells were photographed at a 50 \times magnification.

through the Matrigel and through the pores of the filters over a 48-h period was evaluated by scanning electron microscopy of both sides of each filter (data not shown). No evidence of invasion was detected for the R6 and C15 lines in three separate assays. A very low level of invasion by the B6 line was sometimes detected, but this was far less than that displayed by several other cell types tested (Demeure et al., 1991; Librach et al., 1991; Walter, P., and C. H. Damsky, unpublished experiments).

Expression of Fibronectin, Integrins, and Proteinases

Exogenous TGF- β has previously been shown to induce increased expression of the 9.1-kb fibronectin mRNA in human fibroblasts (Ignatz et al., 1987; Ishikawa et al., 1990). We therefore determined the effects of the endogenous TGF- β overexpression on the expression of fibronectin mRNA. The three transfected clones with the highest TGF- β expression levels, B6, C15, and C19, had a greatly increased level of fibronectin mRNA in comparison with the control clones (Fig. 5). Clone B9 displayed only a moderate increase of fibronectin mRNA, consistent with an intermediate TGF- β expression level. These data may suggest that a certain

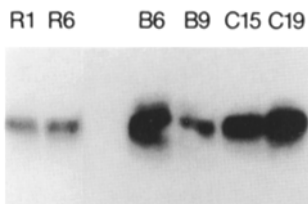


Figure 5. Expression of fibronectin mRNA by transfected cell clones, determined by Northern hybridization. Each lane contained 18 μ g of total RNA prepare from transfected 293 cell clones as indicated. Estimated from the migration in the gel, the fibronectin mRNA is about 9-kb long. Equivalent RNA loading and transfer was confirmed by subsequent probing with a glyceraldehyde-6-phosphate dehydrogenase cDNA.

threshold of endogenous TGF- β level must be reached before fibronectin gene expression is stimulated.

The control and TGF- β -overexpressing cells were also evaluated for changes in their levels of expression of several members of the integrin superfamily of extracellular matrix receptors (reviewed in Albeda and Buck, 1989). Cells that had been either metabolically or surface labeled were lysed and immunoprecipitated with subunit-specific mAbs and the results were similar for both labeling protocols. Experiments were carried out under conditions of antibody excess so that all antigen was precipitated in a single round of immunoprecipitation, enabling levels of individual integrins present in each clone to be compared. All clones examined expressed all of the tested integrins at some level ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$, αV). However, the B6, C15, and C19 lines showed elevated $\beta 1$ integrin levels in comparison with the control R6 clone. Detailed analysis of the R6, B6, and C15 lines (Fig. 6) showed that levels of $\alpha 2/\beta 1$ (a collagen and laminin receptor) and $\alpha 3/\beta 1$ (a fibronectin, laminin, and collagen receptor) were particularly strongly affected.

To determine whether increased TGF- β expression influenced proteinase production by 293 cells, we analyzed the metalloproteinase and plasminogen activator levels in conditioned medium and cell extracts of the control R6 and TGF- β -overexpressing clones B6 and C15. Experiments were carried out using cells plated in Nutridoma-containing serum-free medium on plastic or laminin- or fibronectin-coated wells. This was done to determine whether substrate affected protease expression. A gelatin-degrading metalloproteinase with an apparent molecular mass of 68 kD was present at similar low levels in the extracts and media of all three clones (not shown). All three clones also produced a casein/plasminogen-degrading activity with the apparent molecular mass of pro-urokinase type plasminogen activator (52 kD) (Fig. 7). The C15 and the B6 cells, whether cultured

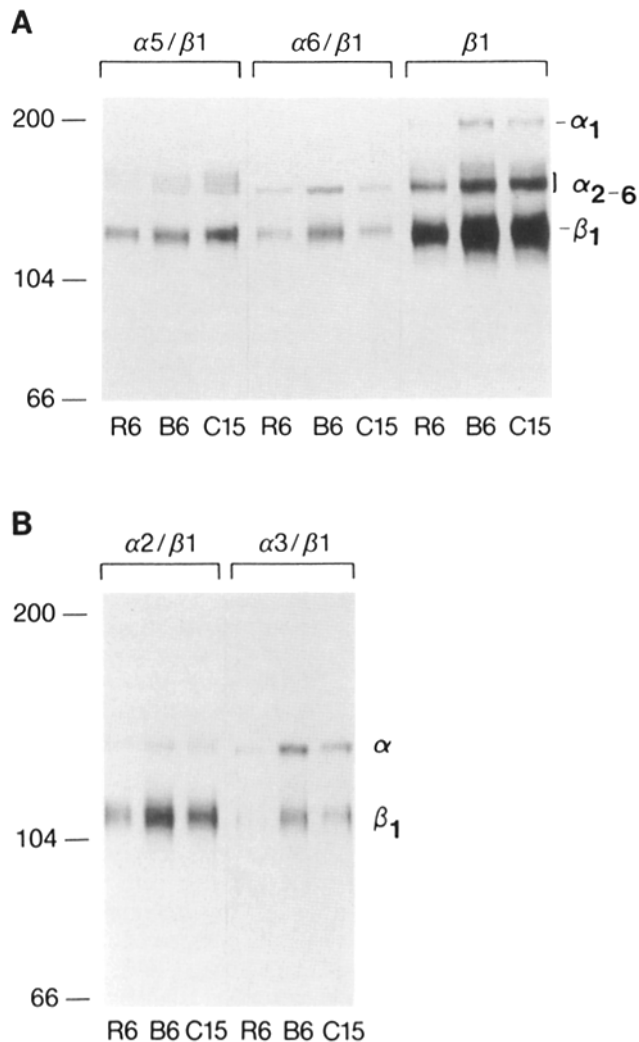


Figure 6. Expression of integrins by the TGF- β -overexpressing cell clones. R6, B6, and C15 were metabolically labeled with ^3H -glucosamine for 24 h, extracted, and immunoprecipitated with mAbs specific for members of the $\beta 1$ integrin family as described in Materials and Methods. Precipitates were analyzed by SDS-PAGE under nonreducing conditions in 7.5% polyacrylamide gels. When compared to the R6 line, the B6 line displayed some elevation of all integrin levels. The C15 line showed the greatest elevation in the level of the $\alpha 5 / \beta 1$ fibronectin receptor. Similar results were obtained when cells were surface labeled with ^{125}I .

on fibronectin or laminin, produced higher levels of this protease than the R6 clone. However, on plastic, all lines produced similar levels. The low levels of metalloproteinases produced by all clones, and the apparent absence of activated forms of either metalloproteinases or plasminogen activators are consistent with the lack of invasiveness of these cells in the Matrigel invasion assay.

Tumor Formation in Nude Mice

Finally, we evaluated the ability of our transfected 293 cell clones to form tumors when subcutaneously injected into nude mice. Because the parent 293 cells are tumorigenic in this assay and we thus anticipated at best a quantitative modulation of tumor formation rather than an all-or-none effect, we concentrated on the difference between the clones

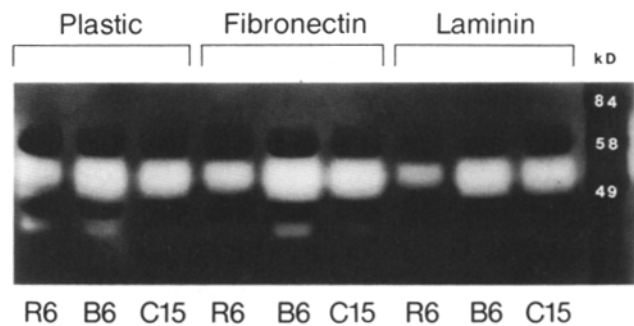


Figure 7. Detection of plasminogen activator in conditioned medium of the transfected cell clones by plasminogen/casein substrate gel zymography. Cell clones R6, B6, and C15 were plated on plastic, fibronectin, or laminin. The prominent lysis zone in each well migrates with the mobility of single chain urokinase-type plasminogen activator at 52 kD. The C15 and B6 line produced increased levels of this proteinase. There was no evidence of activated two chain urokinase. The gel was a 10% polyacrylamide gel.

presenting the two extremes, i.e., the control clones and clones overproducing active TGF- β . Several experiments using different cell numbers as inocula were used and the tumor incidence and size were measured at regular intervals (Table III). As usually occurs in this type of experiment, the incidence of tumor formation differed among individual cell clones and tumor volumes differed among animals injected with cells from the same clone. This presumably reflects variability in tumor cell survival and tumor growth during the early stages of the experiment. Despite this heterogeneity, major differences between the TGF- β -overproducing clones and the control clones were evident. For example, in experiments using single injections of an inoculum of 6×10^5 cells, 9 of 12 mice injected with cells producing active TGF- β (C15 and C19) developed subcutaneous tumors at the site of injection, compared with only 2 of 12 mice injected with the control clones. The clones that secreted latent TGF- β (B6 and B9) were generally less tumorigenic than the C15 and C19 clones and more closely resembled the controls. This suggested that active TGF- β secreted in the environment of the injected cells may have been an important contributor to tumor initiation. We also injected several mice with cells from a control clone at one site and cells overexpressing active TGF- β at a separate site. The overall incidence of tumor formation by these different cell clones did not differ significantly from that observed in mice receiving one injection at a single site (Table III).

Besides this difference in tumor incidence and presumably related to it, there were also differences in time of appearance. This was apparent in two separate experiments in which mice were injected with greater numbers of cells. For example, when mice were injected with 4×10^6 cells, all animals developed tumors at the site of injection. However, tumors generated from the active TGF- β overproducing cells were detected earlier and grew to large size more rapidly (Table IIIB).

None of the tumor-bearing mice were found to have metastatic lesions in any of the examined tissues. Histologically, there were no significant or consistent differences among the tumors, although in one of the two experiments in which a histological evaluation was carried out on many

Table III. Tumor Formation in Nude Mice

A. Cell clone	Incidence	Tumor volumes (mm ³)	
Single injection			
R1	1/6	71	
R6	1/6	24	
C15	5/6	177, 327, 349, 874, 1360	
C19	4/6	10, 46, 91, 110	
R8	2/5	149, 649	
R4	1/5	451	
B9	1/5	29	
R6	2/6	213, 1073	
B6	2/6	25, 51	
Double injection			
R1 (shoulder)	2/4	255, 2230	
C15 (flank)	4/4	475, 622, 1340, 2260	
R6 (shoulder)	0/6		
C19 (flank)	3/6	69, 73, 77	
B. Average tumor volume (mm³)			
Cell clone	Day 17	Day 20	Day 25
R1	5.5 (± 8)	69 (± 79)	312 (± 213)
C19	223 (± 160)	357 (± 162)	578 (± 195)

In A, mice received 6×10^5 cells in a volume of 0.2 ml subcutaneously per injection. Tumor measurements were made after killing the mice 4 wk later. In B, mice received 4×10^6 cells in 0.2 ml subcutaneously. Tumor measurements were made on the indicated days post inoculation. All mice developed tumors, and average tumor volumes for five mice (with SD) are provided.

tumors, those derived from clones overproducing active TGF- β had a more fibrotic appearance. Desmoplastic host response at the tumor site was variable and generally mild, and local invasion, when evident, was minimal. The lack of invasiveness and metastasis of the transfected cells in vivo, despite their increased tumor-forming ability, is consistent with their lack of invasiveness in the in vitro Matrigel assay and their low levels of metalloproteinase production.

Discussion

Several studies have shown that tumor cells display increased synthesis of TGF- β in comparison to their normal counterparts, suggesting that secreting higher levels of TGF- β may advantage tumor cells in some way. These observations led us to evaluate the physiological consequences of increased expression of TGF- β in tumor cells. However, such studies are greatly complicated by the diversity of biological activities that the different forms of TGF- β display. This complexity is particularly apparent when it comes to the effects of endogenous TGF- β synthesis on neoplastic transformation, tumor growth and metastatic spread. For example, it is possible that the antiproliferative effects of TGF- β on some tumor cells in vitro are counteracted in vivo by its potent immunosuppressive effect and/or by its induction of growth factor production by the surrounding host cells. To avoid some of these pitfalls, we chose for our study the tumor cell line 293 which is known to express a functional E1A gene

product and is therefore expected not to be affected in its proliferation by TGF- β . Accordingly, transfected cells overexpressing TGF- β did not display an altered proliferation rate. This allowed us to rule out proliferation-related physiological changes and examine potential changes that might otherwise have been obscured by an altered proliferation rate. In addition, by developing clones of this cell line that expressed either latent or active TGF- β 1, we addressed the issue of whether the frequently observed increased expression of latent, and thus "biologically inactive", TGF- β by tumor cells has any physiological relevance, and, if so, whether these effects differ from those resulting from increased expression of active TGF- β . We evaluated the effects of increased endogenous TGF- β synthesis on the metabolism and adhesive properties of these tumor cells in vitro and on tumor formation in vivo.

The first important finding emerging from this study is that overproducers of both latent or active TGF- β behaved similarly in the in vitro assays performed. This leads us to conclude that the 293 cells overproducing latent TGF- β either directly secrete low levels of active TGF- β or have the ability to convert the secreted latent form into active TGF- β which then induces physiological changes in an autocrine way. In our experiments, the resulting low levels of TGF- β were sufficient to elicit a phenotype in vitro similar to that of cells overproducing active TGF- β . Previous studies have shown that latent TGF- β complex can be activated by acid treatment and by exposure to glycosidases and proteases, such as plasmin and cathepsin (Lyons et al., 1988). Proteolytic activation is probably the most common mechanism and is responsible for activation of TGF- β in co-cultures of endothelial cells and smooth muscle cells (Sato et al., 1990). Since many tumor cells, including 293 cells, secrete proteases such as plasminogen activators and cathepsins, it is likely that these cells also have the ability to activate latent TGF- β . Thus, our results imply that even when they secrete most or all TGF- β in its latent form, tumor cells may still undergo autocrine, TGF- β -induced physiological alterations. It is then possible that the TGF- β activation process may occur in a cell-associated manner, as has been observed in co-cultures between endothelial and smooth muscle cells (Sato et al., 1991; Dennis and Rifkin, 1991).

In comparison with their parental controls, cells overexpressing latent or active TGF- β underwent several changes in their metabolic and adhesive properties in vitro. One major change was their increased anaerobic metabolism. It has long been known that tumor cells have an increased rate of glycolysis and our results suggest that increased TGF- β synthesis, or its activation, by the tumor cells may contribute significantly to this alteration in cellular metabolism. Since the transfected 293 cells did not proliferate more rapidly, presumably as a result of the functional E1A gene product (Pietenpol et al., 1990), our results indicate both that the increased glycolysis is independent from the proliferation rate and that the TGF- β -induced cellular signalling mechanisms that regulate glycolysis and proliferation are distinct.

The other major phenotypic change observed in TGF- β -overproducing cells was increased substratum adhesiveness. The neomycin-resistant control clones, like the untransfected parental cell line, were unable to adhere to plastic in the absence of serum. Instead, they proliferated in non-adherent clumps. In contrast, cells overexpressing latent or active TGF- β adhered to the plastic, spread and proliferated

in a monolayer. Of even greater potential significance in view of our *in vivo* results, was the observation that only the C15 and C19 clones overproducing active TGF- β , and not those overproducing latent TGF- β , adhered and survived at low cell inoculum. In parallel experiments using different culture conditions, the control cell line R6 was unable to attach rapidly or to spread on laminin when cultured in serum-free medium in the presence of Nutridoma, whereas the TGF- β overproducing clones B6 and C15 attached readily and spread on laminin. Thus, at least under these conditions, expression of TGF- β strongly influenced the ability of 293 cells to adhere to the substratum. Such differences in adhesive properties could be of importance *in vivo* in regulating the ability of small numbers of cells to initiate tumor formation.

Consistent with the changes in adhesive behavior were the enhanced levels of expression of fibronectin mRNA and of several β 1 integrin complexes. This observation is consistent with a large body of information documenting the ability of TGF- β to enhance extracellular matrix production (Masagué et al., 1990; Barnard et al., 1990). Production of a broad range of integrins was increased in the B6 as well as the C15 clones overproducing TGF- β . This may favor opportunistic adhesive behavior which could enhance survival *in vivo*. The observed increased expression of α 3/ β 1, a promiscuous receptor that recognizes laminin, collagen, and fibronectin, may be of particular importance, as it has been reported that enhanced expression of α 3/ β 1 or elevated ratios of α 3/ β 1 to α 5/ β 1 accompany oncogenic transformation in several cell lines (Plantefaber and Hynes, 1989). Taken together these data support the idea that acquisition of an optimal adhesion phenotype is permissive for a more rapid and successful colonization by tumor cells of their immediate environment.

Having stably transfected clones that produced increased amounts of TGF- β gave us the unique opportunity to determine the consequences of TGF- β overexpression *in vivo*, because local concentration gradients of tumor-derived factors and their effect on nearby host cells could be reproduced. Two published studies have used stable clone transfected with the TGF- β 1 cDNA and have demonstrated significant *in vivo* suppression of cytolytic T cell and natural killer cell activities (Torre-Amione et al., 1990; Wallick et al., 1990). In the present study, we found that at a low tumor cell inoculum, 293 cells that overexpressed active TGF- β produced subcutaneous tumors more readily than control 293 cells. This may reflect some degree of immunosuppression or a growth advantage for these cells when few in number. This increase in tumorigenicity may also be related to our observation that only the clones overexpressing active TGF- β survived when plated in serum-free medium at very low density. In addition, the increased ability of cells overexpressing TGF- β to produce and interact with extracellular matrix could create a more permissive environment for tumor cell proliferation and tumor formation. This explanation would be consistent with recent results, documenting an increase in tumor formation when tumor cells were injected together with the basement membrane analog Matrigel (Fridman et al., 1991). The greater incidence of tumor formation with the C15 and C19 clones was apparently not the result of a systemic effect from the secreted TGF- β since tumor formation from control clones injected into the same animals was not increased. Although enhanced production of TGF- β by 293

cells may generate a particular adhesion phenotype that is favorable for their anchorage and permissive for their survival *in vivo*, such an adhesion phenotype is clearly not sufficient for successful tumor initiation. Only the clones producing active TGF- β had enhanced rates of tumor formation when injected into nude mice at low inoculum levels, suggesting that the active TGF- β may have profound effects on the immediate environment of the tumor cells, e.g., by inducing enhanced matrix formation or growth factor production by the neighboring cells, or increased proliferation by host fibroblasts. In this context it is important to note that histological evaluation of some, but not all, tumors derived from cells overproducing active TGF- β showed increased matrix formation and interstitial fibroblast proliferation in comparison with tumors derived from the control clones. Neither the parental cell line, nor any of the TGF- β -overexpressing clones were metastatic in this animal model. Consistent with these observations, the transfected clones did not produce higher levels of metalloproteinases and did not display activated forms of metalloproteinases or plasminogen activators.

The observed increased tumorigenicity by 293 cells overexpressing active TGF- β is not necessarily inconsistent with some recent results indicating that a colon carcinoma cell line transfected with an antisense mRNA expression vector for TGF- β 1 caused a higher tumor incidence in nude mice (Wu et al., 1992) or that a rat prostate cell line transfected with a latent TGF- β expression vector demonstrated enhanced tumor growth (Steiner and Barrack, 1992). These recent studies were performed in cell lines that were sensitive to the growth-regulatory effects of TGF- β , whereas we intentionally focused on a tumor cell line which is insensitive to the direct growth modulatory activities of TGF- β and have evaluated the tumorigenicity of clones with similar rates of proliferation, thus allowing a more direct evaluation of, e.g., the physiological consequences of alterations in cell-matrix interactions. It is thus possible that the inhibitory effects of TGF- β on cell proliferation could counteract altered adhesive interactions of the cells with the environment in determining tumor cell proliferation *in vivo*. Thus, the advantage of an increased endogenous TGF- β synthesis for tumorigenicity may depend on the balance of these two parameters, and on the degree of local TGF- β -induced immunosuppression, and thus may vary depending on the tumor type or cell line. On the other hand, it is also important to realize that many tumor cells have lost their sensitivity to the growth inhibitory activities of TGF- β , as is the case with the 293 cells, possibly as a consequence of functional inactivation of the Rb gene product (Pietenpol et al., 1990) and possibly also as a step in the progression of tumor cells of full malignancy (Wakefield and Sporn, 1990).

In conclusion, we have evaluated the role of endogenous TGF- β overexpression on several physiological parameters of tumor cells and tumor formation, using an E1A transformed tumor cell line. We found that cells overexpressing latent or active TGF- β , compared with control transfected cells, displayed similar physiological changes *in vitro* without a change in proliferation rate. The major effects of the increased TGF- β synthesis, evaluated in this study, were increased glycolysis and enhanced cell-matrix adhesiveness. In addition, increased release of active TGF- β resulted in higher incidence of tumor formation. These findings may

have relevance to pathological tumor formation since, at least in several systems, it has been documented that tumor cells release higher levels of TGF- β compared to their non-malignant controls. In addition, several tumor cell lines have been shown to release significant levels of already activated TGF- β (Knabbe et al., 1987; Schwarz et al., 1990; Constam et al., 1992). The cell line used in our study was minimally invasive and non-metastatic. As such, it does not provide an adequate assessment of the potential role of TGF- β in tumor cell metastatic behavior. Similar experiments with metastatic tumor cell lines are in progress to address this issue.

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