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Expression of transfected transforming growth factor α induces a motile fibroblast-like phenotype with extracellular matrix-degrading potential in a rat bladder carcinoma cell line

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Acquisition of cell motility is often correlated with the malignant progression of a transformed cell. To investigate some of the mechanisms involved in the development of a migratory state, we transfected the NBTII rat carcinoma cell line, which forms stationary epithelial clusters in culture, with the gene encoding human transforming growth factor alpha (TGFα).[1] Expression of TGFα in NBTII cells resulted in cells of motile and vimentin-positive phenotype with internalized desmosomal components, analogous to the treatment of cells with exogenous TGFα. The clones expressed a 5.2-kb TGFα message and synthesized an 18-kDa form of TGFα. Supernatants of TGFα-producing clones induced the internalization of desmosomal components, the production of vimentin, and increased motility in untransfected epithelial NBTII cells, indicating that the factor produced by the clones was in a biologically active form. TGFα-producing clones secreted significant levels of a 95-kDa gelatinolytic metalloproteinase, virtually absent in untransfected cell supernatants. In contrast, levels of inhibitors of metalloproteinases and of a plasminogen activator were similar in untransfected and TGFα-transfected NBTII cells. These results suggest that expression of TGFα in an epithelial tumor cell results in the development of a motile, fibroblast-like phenotype with matrix-degrading potential, which could result in a more aggressive tumor in vivo.

Introduction

A major problem in tumor cell biology is the nature of the process of invasion at the primary site and subsequent dissemination to distant secondary locations. Several processes have been implicated in these events, including the acquisition by the invasive or metastatic cells of enhanced cell motility and increased matrix-degrading capacity (reviewed by Liotta, 1984; Woolley, 1984). Tumor invasion can thus be viewed as the loss of control over the maintenance of a stationary phenotype, and several factors have been implicated in the induction of motility in tumor cells.

Purification of the factors involved in enhanced motility as well as screening of known growth factors for their capacity to induce migration of both tumor and normal cells has led to the identification of several proteins acting on a number of different cell types. Liotta *et al.* (1986) have purified autocrine motility factor, which is active on the producer cells but not on neutrophils, from melanoma cells (it is also produced by a number of *ras*-transformed 3T3 cell lines). Fibroblast-derived scatter factor has recently been purified (Gherardi *et al.,* 1989); this protein causes the separation of contiguous normal epithelial cells and enhances their motility. A similar scatter activity has recently been reported to be produced by human aortic smooth muscle cells. This factor also is able to induce the dissociation of carcinoma cells (Rosen *et al.,* 1990). Another fibroblast-derived factor, migration stimulating factor (Grey *et al.,* 1989), is active on the producer cells themselves and also is able to induce high rates of motility in adult fibroblasts. Among known growth factors that have been analyzed for mi-

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[1] Abbreviations: aFGF, acidic FGF; APMA, amino phenyl mercuric acetate; bFGF, basic FGF; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; TGFα, transforming growth factor alpha; SDS, sodium dodecyl sulfate; TIMP, tissue inhibitor of metalloproteinases.

gration-inducing potential, basic fibroblast growth factor (bFGF) has been implicated in endothelial cell motility, acting both as a paracrine agent (Presta *et al.,* 1986) and as an autocrine factor (Sato and Rifkin, 1988). Epidermal growth factor (EGF) and transforming growth factor alpha (TGFα) are known to induce migration in keratinocytes (Barrandon and Green, 1987). Insulin-like growth factors I and II (Stracke *et al.,* 1988) are chemotactic agents for melanoma cells, apparently acting through an independent signaling pathway to that of autocrine motility factor (Stracke *et al.,* 1989).

In previous studies we have developed a model system to study induction of a scattered motile phenotype in a rat bladder carcinoma cell line, NBTII. This cell line was shown to undergo an epithelial-to-fibroblast-like change under the influence of both soluble mediators (Boyer *et al.,* 1989) and collagen substrates (Tucker *et al.,* 1990). The complex events involved in this change include the breaking of intercellular contacts (desmosomes) and the scattering of cells from a group in monolayer culture or from a tumor-like aggregate in vitro. Subsequent studies showed that acidic fibroblast growth factor (aFGF) could induce this change in phenotype (Vallés *et al.,* 1990) and that aFGF and collagen substrates could act in consort to promote these effects still further (G.C. Tucker, unpublished observations). Because the fibroblast growth factors (FGFs) have recently been shown to bind to a tyrosine kinase receptor (Lee *et al.,* 1989), we have investigated whether another growth factor acting via a tyrosine kinase receptor, that is, TGFα, could induce the same phenotypic changes in NBTII cells. TGFα (reviewed by Derynck, 1988) is a 50-amino-acid peptide, originally identified as a factor with the ability to transform cells, but subsequently shown to affect a variety of cells in many ways, all of which, to date, overlap with the effects of EGF because of the interaction of TGFα with the EGF receptor. We show that exogenous TGFα has a potent effect on the NBTII cell line, inducing a fibroblast-like, motile phenotype. Crucially, although we and others have demonstrated the potent activity of motility-inducing factors in vitro, it has not as yet been possible to ascertain directly their roles in vivo. As a first step in analyzing the potential autocrine role of motility-inducing factors, we have transfected NBTII cells with a TGFα recombinant and analyzed the in vitro properties of the growth factor–producing clones.

Among the many genes with expressions induced by growth factors, the tissue metalloproteinases have attracted considerable attention (Chua *et al.,* 1985; Matrisian *et al.,* 1985; Edwards *et al.,* 1987). These proteinases are known to be involved in the degradation of extracellular matrix components, including collagens, proteoglycans, and basement membrane glycoproteins (Murphy and Reynolds, 1985; Murphy *et al.,* 1989b), and are thought to be involved in tumor cell invasion at primary and metastasic sites (Liotta, 1984). Therefore, we also examined the ability of the untransfected and transfected NBTII cells to produce such proteinases capable of degrading the extracellular matrix.

We report that TGFα-producing clones appear to secrete and respond to TGFα in an autocrine fashion, as assessed by both their fibroblast-like phenotype and their ability to migrate in vitro. These clones also produce a gelatinolytic activity, not normally synthesized by the untransfected NBTII cells.

Results

Effect of TGFα on NBTII cells

TGFα was added to cultured NBTII cells in order to investigate its effect on their epithelial phenotype. Previously it has been shown that, in NBTII cells, aFGF induces a reversible epithelial-to-fibroblast-like change, a major feature of which is the internalization of components of desmosomal junctions beginning at ~5 h after addition of the factor (Boyer *et al.,* 1989). The treated cells dissociate from one another, and cell motility is initiated at ~6 h, as observed with time-lapse videocinematography. Prolonged culture of NBTII cells in the presence of exogenous aFGF results in the appearance of vimentin intermediate filaments (Boyer *et al.,* 1989; Vallés *et al.,* 1990). TGFα caused a dose-dependent internalization of desmosomes in NBTII cells cultured for 2 d in the presence of the exogenously added growth factor (Figure 1A) with half-maximal activity obtained at a concentration of 1.5 ng/ml (250 pM). Vimentin staining was induced in 50% of NBTII cells cultured for an extended time period (5 d) in 20 ng/ml TGFα. A dose-dependent induction of motility was also observed in NBTII cells with TGFα (Figure 1B) with, in this case, half-maximal activity obtained with 2.5 ng/ml (415 pM), the motility of NBTII cells first being observed after a lag period of ~6 h after the addition of TGFα.

TGFα-transfected NBTII cells

Clones of NBTII cells transfected with TGFα, selected in G418, had a variety of morphologies

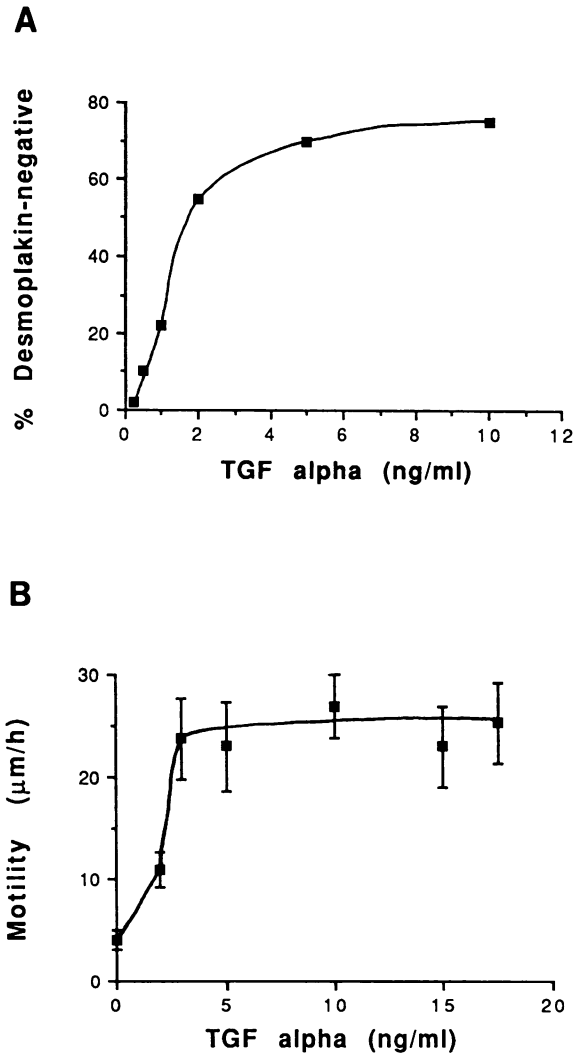

***Figure 1.* (A) Internalization of desmosomes in NBTII cells stimulated with TGFα.** NBTII cells were cultured on coverslips for 24 h before addition of TGFα at the concentrations indicated. After a further 24 h, cells were stained with a cocktail of monoclonal anti-demosplakin antibodies, and the number of cells lacking positively stained cell junctions was counted. Each point represents the average of two determinations from a representative experiment. (B) Induction of motility in NBTII cells by exogenous TGFα. NBTII cells were cultured for 48 h before addition of given concentrations of TGF-α. After time-lapse videocinematography, individual cell tracks were followed as described in Methods. Each point represents the mean ± SEM of 20 cells.

ranging from very closely apposed epithelial clusters to more fibroblast-like scattered cells. Thirteen clones with an initially fibroblast-type morphology were studied with respect to their motile behavior in nonselective medium and were found to have average speeds ranging from 11 to 81 μm/h (Table 1). Northern blot analysis was performed on the 6 of these 13 clones that maintained a fibroblast-like morphology through subsequent passages. A 5.2-kb message from all 6 clones hybridized to the TGFα probe, although with varying intensity (Figure 2). In contrast, messenger RNA from NBTII cells transfected with Neo alone did not hybridize to this probe (Figure 2).

TGFα-producing clones have internalized desmosomes

The three TGFα-producing clones that expressed a high quantity of TGFα mRNA, namely, Zip 11, Zip 17, and Zip 48, were selected for further study. The clones were studied with respect to desmosomal integrity and vimentin expression, shown in Table 2. In comparison with a clone transfected with the selective marker alone, the clones expressing TGFα had substantially diminished desmoplakin-positive junctions, and a significant proportion of the cells were synthesizing vimentin.

Conditioned media from TGFα-producing clones are biologically active

Twenty-four-hour conditioned media (Dulbecco's modified Eagle's medium [DMEM] + 10% fetal calf serum [FCS]) from TGFα clones were added to NBTII cells at the time of plating onto coverslips. After 7 d further culture, NBTII cells

Table 1. Motilities of TGF-α transfectants

TGFα-transfected clone	Motility (μ/h)
Zip 2	44 ± 4
Zip 3	36 ± 5
Zip 11	57 ± 3
Zip 16	49 ± 4
Zip 17	48 ± 7
Zip 34	13 ± 3
Zip 36	11 ± 3
Zip 48	72 ± 5
Zip 49	42 ± 7
Zip 77	81 ± 9
Zip 79	25 ± 5
Zip 82	22 ± 3
Zip 84	33 ± 5
Neo clone 1	0
Neo clone 3	3 ± 1
Neo clone 4	4 ± 1
Neo clone 5	0
Neo clone 4 + 10 ng TGFα	25 ± 3

The motilities of the transfectants were measured by time-lapse videocinematography as described in Methods. Each value represents the mean ± SE of 20 cells. The motility of four clones from a transfection with the selection marker Neo alone are given for comparison, as well as that of Neo clone 4 stimulated with TGF-α.

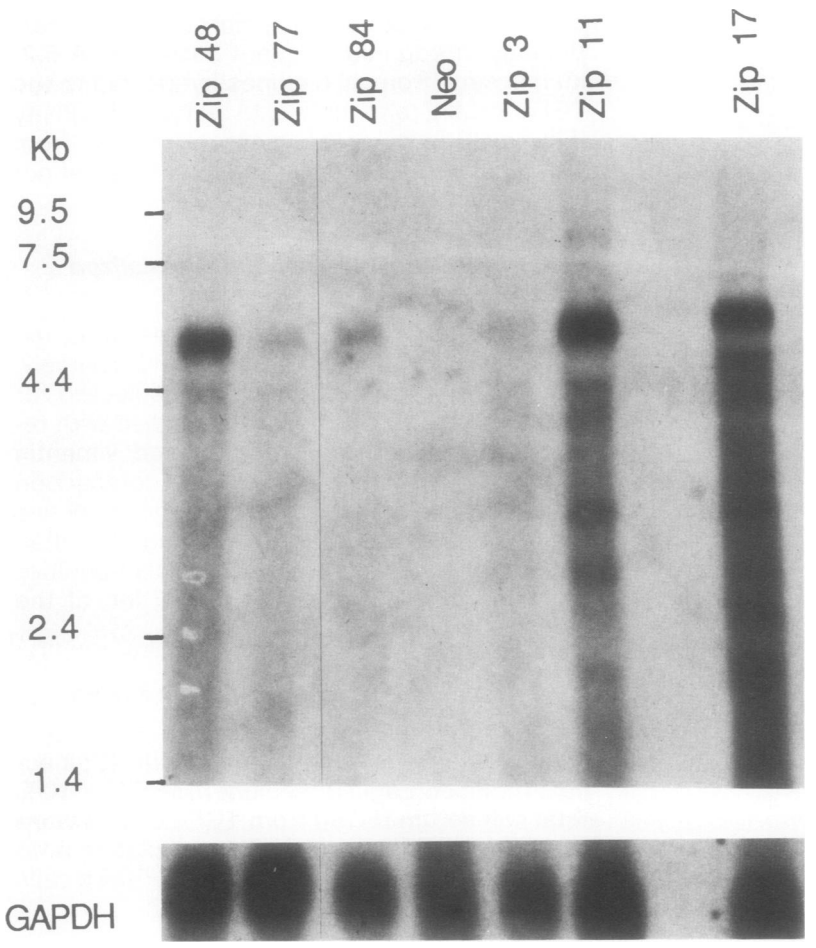

***Figure 2.* Northern blot analysis of total mRNA isolated from TGFα-producing clones, labeled Zip 48, 77, 84, 3, 11, and 17.** The track labeled Neo represents total mRNA extracted from a clone of NBTII cells transfected with the selection marker Neo alone. Size markers of an RNA ladder are indicated. Control hybridization with glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA is shown to confirm equal loading.

were double-labeled for desmoplakin and vimentin (Table 3 and Figure 3). These results indicated that the TGFα clones were secreting factor(s) with biological activity analogous to that of TGFα with respect to induction of desmosome internalization and expression of vimentin. The conditioned medium of clone Zip 48 was also tested for its ability to induce motility in untransfected NBTII cells. The medium was mixed 1:1 with fresh DMEM and 10% FCS and added to cells plated on glass coverslips 2 d previously. Analysis of a time-lapse video revealed that, after a lag period of 6 h, the NBTII cells began to move and reached an average

Table 2. Desmosome and vimentin immunoreactivity in TGFα-transfected NBTII cells

Cells	% Desmosome negative	% Vimentin positive
Untransfected NBTII cells	0	0
Neo clone 4	2	0
Zip 11	84	40
Zip 17	99	24
Zip 48	49	28

Cells were double-labeled with anti-desmoplakin and anti-vimentin antibodies as described in Methods. Cells with no desmoplakin-positive junctions were scored desmosome-negative.

Table 3. Effect of supernatants from TGFα-transfected cells on the phenotype of untransfected NBTII cells

Cell from which supernatant derived	% Desmosome negative	% Vimentin positive
Neo clone 4	2	0
Zip 11	97	78
Zip 17	98	80
Zip 48	99	78

NBTII cells were double-labeled with anti-desmoplakin and anti-vimentin antibodies as described in Methods. Cells with no desmoplakin-positive junctions were scored desmosome-negative.

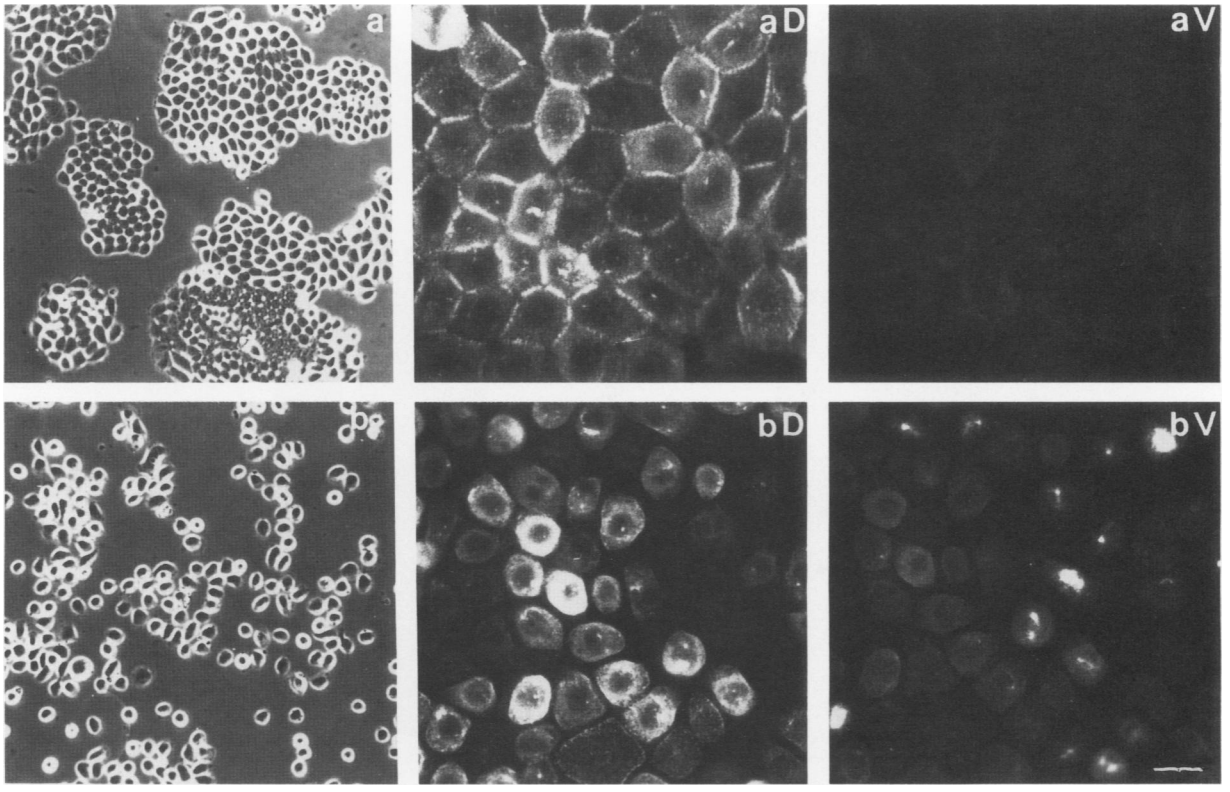

Figure 3. **Effect of supernatants from TGFα-producing transfectants on NBTII cells.** NBTII cells were cultured either in standard medium (a), or in the presence of the supernatant of clone Zip 48 for 5 d (b). (a and b) phase-contrast micrographs of the respective cells. Immunofluorescent staining with anti-desmoplakin antibodies revealed extensive desmosomal junctions in the untreated cells (aD), which were absent in the cells treated with the Zip 48 supernatant (bD). Staining with anti-vimentin revealed the absence of this intermediate filament in the untreated NBTII cells (aV), whereas it was present in the treated cells (bV). Bar represents 10 μm.

motility of 63 μm/h after 13 h. At this passage number clone Zip 48 itself was found to have a motility of 61 μm/h. It should be noted that the maximum motility obtained with exogenous TGFα did not exceed 30 μm/h (Figure 1B).

Immunoprecipitation of secreted TGFα

To characterize further the biological activity present in the supernatant of clone Zip 48, we performed immunoprecipitation of conditioned media with an anti-TGFα antibody, after ^{35}S-cysteine labeling of Zip 48 cells in comparison with NBTII cells transfected with the selection marker alone. As shown in Figure 4, the supernatant of Zip 48 contained a band running at 18 kDa (lane A), whereas no band migrating at this level was observed with the supernatant of Neo clone 4 (lane B).

Metalloproteinases are induced in TGFα-producing clones

The synthesis of metalloproteinases by NBTII cells was investigated by gelatin zymography. Serum-free conditioned medium from clones Zip 11, Zip 17, and Zip 48 was separated on gelatin gels, and these three clones were all shown to secrete a major gelatinolytic activity migrating at 95 kDa, as exemplified for clone Zip 48 (Figure 5A). Untransfected NBTII cells and cells transfected with Neo alone did not secrete this gelatinolytic activity (Figure 5A, lanes 1 and 2), although NBTII cells treated with exogenously added TGFα did produce this proteinase (results not shown). The gelatinolytic activity was shown to be a metalloproteinase by inhibition with 1,10 phenanthroline (Figure 5A, lane 4). A proportion of the activity was shown to undergo a 10-kDa drop in molecular mass after incubation with the mercurial activator amino phenyl mercuric acetate (APMA) (Figure 5B, lanes 3 and 4 compared with lanes 1 and 2), consistent with the activation observed for purified gelatinolytic activities (Murphy *et al.*, 1989c; Wilhelm *et al.*, 1989). Gelatinolytic activities running at 60–65 kDa were present in variable amounts in the supernatants of TGFα transfectants; these activ-

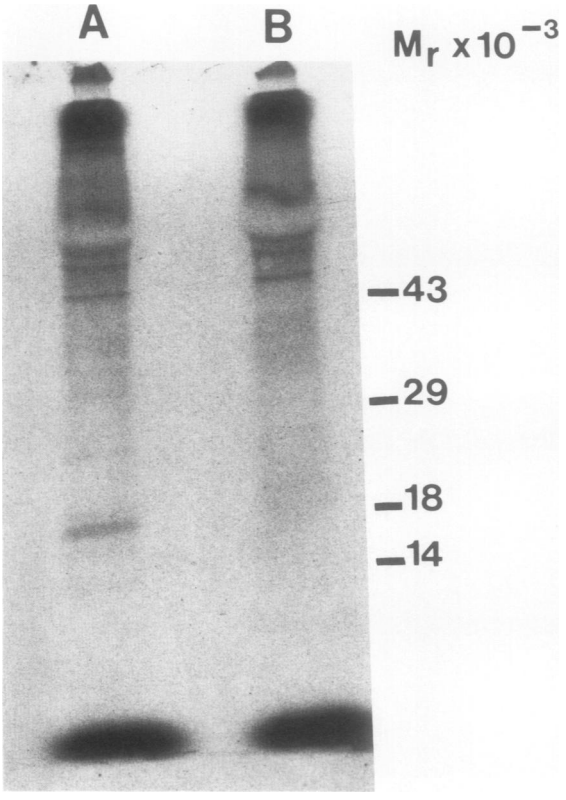

Figure 4. **Immunoprecipitation with anti-TGFα of medium from ^{35}S-cysteine labeled cells.** (A) TGFα-producing clone Zip 48. (B) Neo clone 4. Autoradiograph was exposed for 7 d at −70°C.

ities also underwent a drop in molecular weight after treatment with APMA (Figure 5B, lanes 3 and 4). Samples of conditioned medium from the TGFα-producing clones were subsequently assayed in a conventional quantitative biochemical assay by the use of radiolabeled gelatin. No activity was detectable with this method in either the presence or the absence of APMA.

TGFα-producing clones and untransfected NBTII cells secrete significant levels of metalloproteinase inhibitors

Because no gelatinase activity was detectable in the TGFα clones in the conventional radiolabeled gelatin assay, it seemed possible that high levels of the tissue inhibitor of metalloproteinases, TIMP, could be masking the gelatinase activity patently detectable on the gelatin zymogram. TIMP assays revealed the presence of significant levels of inhibitory activity in the medium of clones Zip 11 and Zip 48 (1.3 U/10^5 cells). Characterization of the inhibitory activity

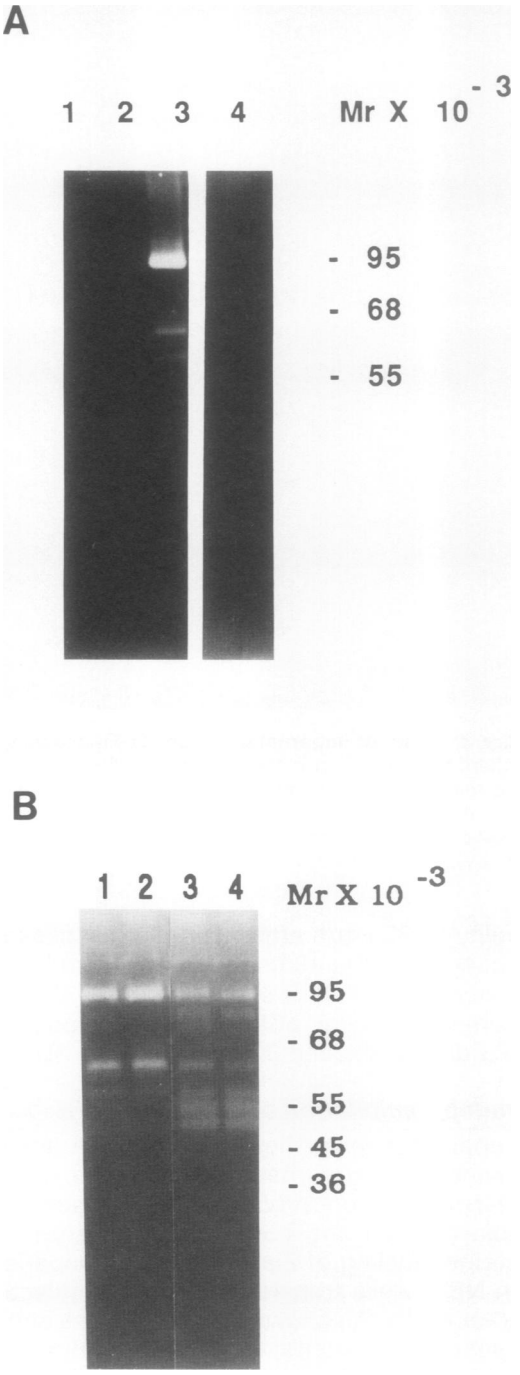

Figure 5. **A. Gelatin zymogram of media from (1) untransfected NBTII cells, (2) Neo-transfected NBTII cells, and (3 and 4) TGF-α transfectant, clone Zip 48.** The part of the gel containing lane 4 was incubated in the presence of 1,10 phenanthroline. (B) Treatment of gelatinolytic activities with APMA. Samples from two TGFα-producing clones, Zip 11 and Zip 48, were incubated alone (1 and 2) or in the presence of 2 mM APMA (3 and 4) before analysis in a gelatin zymogram.

produced by the TGFα-producing clones by reverse gelatin zymography revealed the presence of two inhibitory bands at 29 and 22 kDa (Figure 6A). No apparent differences in TIMP levels were observed among untransfected, Neo-transfected NBTII cells (Figure 6A, lanes 1 and 2), and TGFα-producing clones (Figure 6A, lane 3). It should be noted that the gelatinolytic activity observed in lanes 1 and 2, that is, in untransfected and Neo-transfected NBTII cells, represents a very low level of gelatinase when compared with that of the Zip 48 clone and is detectable only because these samples have been concentrated 10-fold.

Plasminogen activator levels are not modulated in TGFα-producing clones

Media from the same TGFα-producing clones, Zip 11 and Zip 48, were also separated on gels containing casein and plasminogen and were shown to contain a plasminogen activator (Figure 6B, lanes 3 and 4). Untransfected NBTII cells and cells transfected with the selection marker alone seemed to produce similar levels of this proteinase (Figure 6B, lanes 1 and 2). No degradation was observed when samples were separated on casein gels, which did not contain plasminogen (results not shown), indicating that the band observed was a plasminogen activator.

Discussion

The results reported in this paper demonstrate that the expression of TGFα in stationary epithelial NBTII cells induces a migratory, fibroblast-like phenotype, which also secretes a gelatinolytic metalloproteinase. TGFα was shown to induce a conversion in NBTII cells from an epithelial to a fibroblast-like state in a manner analogous to that previously shown with aFGF (Vallés *et al.,* 1990). The induction of desmosome internalization and motility followed a time scale similar to that shown for aFGF with comparable levels of the growth factors effective.

The TGFα-transfected clones expressed this growth factor at the message level, the 5.2-kb transcribed message being easily distinguishable from any endogenous rat TGFα mRNA, which would have hybridized at 4.5 kb (Lee *et al.,* 1985). The 18-kDa TGFα protein synthesized by the transfected cells might correspond to the 17- to 19-kDa precursor of TGFα, reported to be synthesized by retrovirally transformed rat cells (Ignotz *et al.,* 1986), and by rat hepatocellular carcinoma cells (Luetteke *et al.,* 1988), or to a heavily glycosylated form of the 6-kDa protein (Bringman *et al.,* 1987).

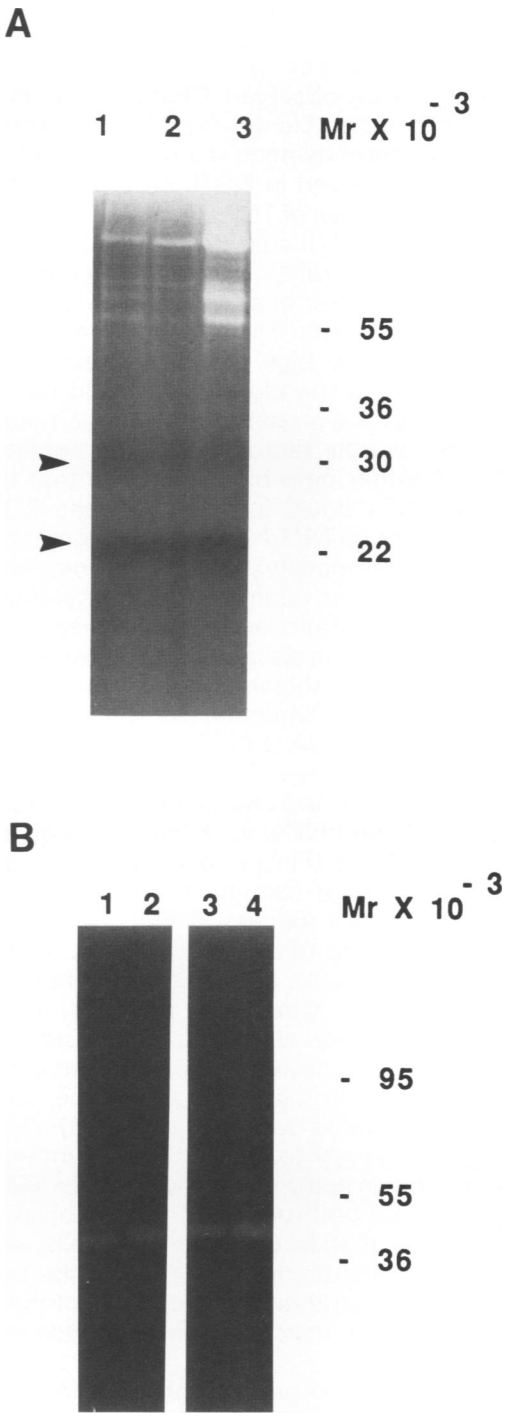

Figure 6. **A. Reverse zymography to detect TIMP activities.** Samples were concentrated 10-fold before electrophoresis. Arrowheads indicate zones of inhibitory activity in (1) untransfected NBTII cells, (2) Neo clone 4, and (3) TGFα-producing clone Zip 48. Note that gelatinolytic activities in the samples are also visible toward the top of the gel. (B) Plasminogen activator gel. Samples were separated on a casein gel containing plasminogen. Media from (1) untransfected NBTII cells; (2) Neo clone 4; (3) TGF-α transfectant, clone Zip 11; and (4) clone Zip 48.

TGFα-synthesizing clones were constitutively motile, but no direct correlation could be made between the extent of TGFα expression and the degree of motility observed. Characterization of TGFα-synthesizing clones revealed that these cells had apparently acquired motilities higher than those observed in NBTII cells stimulated with a maximal dose of TGFα (e.g., 81 compared with 35 μm/h). NBTII cells have been observed to move at comparably high rates when plated on collagens (Tucker *et al.,* 1990). The fact that conditioned medium from clone Zip 48 could induce an equally high rate of movement in NBTII cells as in the clone itself could be accounted for by the presence in the supernatant of another soluble factor that could enhance motility. Another possibility would be that the secreted TGFα could interact with the EGF/TGFα receptor on NBTII cells in a novel manner to induce higher motility. It should be noted that addition of greater quantities of the synthetic growth factor to stationary untransfected NBTII cells did not result in elevated motility compared with the maximum shown in Figure 1B. The motilities of other cell types that have been transfected with the cDNA encoding TGFα have not been studied. However, TGFα has been shown to confer transformed characteristics on Rat-1 fibroblasts (Rosenthal *et al.,* 1986), although not on 3T3 fibroblasts (Finzi *et al.,* 1987). Here we have transfected a cell line that was derived from a chemically induced tumor, that is tumorigenic in nude mice (Tucker *et al.,* 1990), and that is, therefore, already transformed.

The presence of vimentin in the TGFα-transfected clones indicated that the autocrine expression of this growth factor resulted in the generation of a fibroblast-like phenotype, analogous to the events occurring when TGFα was added exogenously to NBTII cells. Of interest is the fact that in some cases the cells of a TGFα clone could be both positive for desmoplakin, albeit weaker than in untransfected cells, and positive for vimentin, suggesting that the regulation of the distribution or synthesis of these proteins by TGFα may occur through independent pathways.

It was of great interest to observe the production of gelatinolytic activities by the transfected NBTII cells, which, to our knowledge, is the first demonstration of the induction of metalloproteinases by a transfected growth factor gene. However, the elegant studies of Tsuboi *et al.,* (1990) have recently demonstrated that high endogenous synthesis of bFGF in cloned endothelial cells resulted in the induction of a 95-kDa gelatinolytic activity as well as a more motile phenotype. The major 95-kDa gelatinase produced by the TGFα transfectants may correspond to the similarly sized gelatinases (also known as type IV collagenases) purified from polymorphonuclear leukocytes (Murphy *et al.,* 1989c) and from SV40-transformed fibroblasts (Wilhelm *et al.,* 1989) and observed in the media of stimulated endothelial cells (Herron *et al.,* 1986), as well as to mouse colon carcinoma cells, where high levels of gelatinolytic activity are associated with metastatic cells (Yamagata *et al.,* 1988, 1989). The 60-kDa band observed in the supernatant of the TGFα transfectants may correspond to the activity of the same molecular weight secreted by mouse colon carcinoma cells (Yamagata *et al.,* 1988). It is possible that the synthesis of gelatinolytic activities by the TGFα transfectants could confer on those cells an increased invasive potential, because a gelatinase purified from other sources has potent activity on the basement membrane constituent (type IV collagen), as well as on type V collagen and denatured interstitial collagens (Murphy *et al.,* 1989c), and could lead to enhanced invasion of the host stroma. In the future, when a cross-hybridizing probe becomes available, it will be of interest to investigate whether the gelatinase activity was induced at the message level in the transfected cells.

It was intriguing that gelatinolytic activity could not be detected in the conventional radiolabeled gelatin assay, although the same samples had significant levels of the proteinase in zymograms. This was probably due to the presence of TIMP in the samples: on activation, progelatinase can become bound to TIMP in a manner analogous to that following the activation of procollagenase, and it has been previously shown that high TIMP levels need to be overcome for collagen or gelatin degradation to occur in either an assay (Herron *et al.,* 1986) or a cell-substrate situation (Gavrilović *et al.,* 1987). In our experiments the gelatinolytic activity could only be visualized when physically separated from TIMP-like activity on a gelatin gel, as reported previously for endothelial cells (Herron *et al.,* 1986). The two bands of inhibitory activity seen in the reverse zymograms are likely to correspond to TIMP 1 (29 kDa) and TIMP 2 (22 kDa). TIMP 1, initially isolated from connective tissue fluids (Cawston *et al.,* 1981; Murphy *et al.,* 1981), is a very well-characterized inhibitor of the tissue metalloproteinases collagenase, gelatinase, and stromelysin (reviewed by Cawston, 1986). TIMP 2 was originally observed in the medium of stimulated rabbit endothelial cells (Herron *et al.,* 1986) and has since been

purified (DeClerck *et al.*, 1989) and cloned (Stetler-Stevenson *et al.*, 1989). Although TIMP 1 and 2 levels were apparently unaltered in the TGFα-transfected cells compared with control NBTII cells, it is possible that the gelatinase activity induced in these transfected cells could confer a more invasive phenotype if, for example, the level of activation of the gelatinase was enhanced or if TIMP levels were reduced by other, as yet uncharacterized, agents.

Secreted plasminogen activator levels were apparently similar in untransfected and TGFα-producing cells, and the activity observed had an apparent mass of 43kDa, similar to that previously reported for urokinase-type plasminogen activator. Of relevance to these results are those of Dubeau *et al.* (1988), who demonstrated the synthesis of urokinase-type plasminogen activator, which was not up-regulated by addition of EGF, in unstimulated EJ bladder carcinoma cells. Our data are also in keeping with the recent report of Tsuboi *et al.* (1990), who showed that, although more motile endothelial cells had elevated gelatinase levels, their plasminogen activator levels remained constant.

The results obtained in this study indicate that TGFα expressed in NBTII cells can induce a highly motile phenotype, which, combined with the elevated levels of gelatinase produced by these cells, could contribute to a more invasive phenotype in vivo. It will be of great interest to determine whether the TGFα-producing clones generated in our study will indeed be more invasive and metastatic compared with the parent cell line, when injected into nude mice or syngeneic rats. It should be noted that Finzi *et al.* (1988) observed that, although the expression of TGFα in benign papilloma cells resulted in the generation of larger tumors than those produced by the parental cells, no effect on neoplastic progression was apparent. However, it is not known whether the transfected papilloma cells expressed an increased motile phenotype, and, therefore, a correlation with progression might not be expected.

In conclusion, we have shown that NBTII cells can respond to TGFα by undergoing an epithelial-to-fibroblast-like conversion. Transfection of NBTII cells with the cDNA encoding TGFα resulted in the generation of clones expressing the motile, fibroblast-like phenotype constitutively and in the induction of gelatinase synthesis.

Materials and methods

Human synthetic TGFα (6 kDa) was purchased from Bachem (Bubendorf, Switzerland). The expression vector pZipTGFα (Finzi *et al.*, 1987) was kindly supplied by Drs. Stuart Aaronson and Rik Derynck. This vector contains the entire coding sequence of human TGFα cDNA, under the control of MoMuLV LTR sequences and the Neo gene, which confers resistance to neomycin. Geneticin (G418) was from GIBCO BRL (Cergy Pontoise, France). Sheep anti-TGFα, raised against the 50-amino-acid mature TGFα molecule, was purchased from Triton Biosciences (Alameda, CA). Tissue culture reagents were as previously described (Boyer *et al.*, 1989; Tucker *et al.*, 1990; Vallés *et al.*, 1990). All chemicals were reagent grade.

NBTII cells

The NBTII cell line was established from a rat bladder carcinoma chemically induced by Toyoshima *et al.* (1971) and forms clusters of tightly apposed epithelial cells when grown in monolayer culture in DMEM supplemented with 10% FCS (Boyer *et al.*, 1989).

Immunofluorescent staining of NBTII cells

To determine the status of desmosomal junctions in cells treated with synthetic TGFα or in TGFα-transfected NBTII cells, we stained components of such junctions with specific antisera. It has been shown previously that certain proteins of desmosomal contacts are internalized after treatment with aFGF (Boyer *et al.*, 1989; Vallés *et al.*, 1990). Staining for the desmosomal component desmoplakin and for the intermediate filament vimentin was carried out as previously described (Boyer *et al.*, 1989). Briefly, NBTII cells or TGFα-producing clones were grown on 13-mm-diam glass coverslips, fixed in methanol (5 min, −20°C), and permeabilized with ice-cold acetone (1 min), then washed in phosphate-buffered saline and incubated for 45 min with a monoclonal antibody to desmoplakin (kindly provided by Dr. W. Franke, Heidelberg, FRG) followed by a Texas red–coupled goat anti-mouse IgG (Immunotech, Marseille, France). For double-labeling for vimentin, cells were then incubated with a monoclonal anti-vimentin antibody (Amersham, Les Ullis, France), followed by a goat anti-mouse fluorescein antibody (Nordic, The Netherlands) with thorough washing after each incubation. Cells were mounted in Moviol (Hoechst, Frankfurt, FRG) and viewed with a Leitz (Weltzar, FRG) microscope equipped with epifluorescence. Photographs were taken on T-Max film (Eastman Kodak, Rochester, NY).

Time-lapse videocinematography

The motilities of NBTII cells in the presence or absence of exogenously added TGFα and of TGFα-producing clones were assessed in time-lapse videocinematography as described in detail previously (Tucker *et al.*, 1990). In brief, cells were passaged to 25-mm-diam glass coverslips at a density of 20 000 cells/cm^2. After 2 d culture, cells were filmed over 13 h in a Sykes-Moore chamber (Bellco Glass, Vineland, NJ). After acceleration of videos, cell migration pathways were traced from a television monitor screen, and the total distance migrated was measured with a map measurer. The tracks of individual cells were measured over a constant time period and the pathways of at least 20 randomly selected cells were averaged for each film. Motility was expressed as micrometers per hour.

Transfection of NBTII cells with TGFα expression vector

NBTII cells were grown in DMEM and 10% FCS supplemented with penicillin and streptomycin and, before trans-

fection, cloned by limiting dilution. A clone with epithelial morphology was chosen for transfection in which 100% of cells displayed junctions positive for desmoplakin and 100% of cells were negative when labeled with anti-vimentin. Cells (10^6) were transfected with 17.5 μg pZip TGFα as a calcium phosphate precipitate according to Graham and Van der Eb (1973). After incubation at 37°C for 8 h, the DNA-containing medium was removed, and 3 ml of 15% glycerol in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, pH 7.05, was added for 15 min. The glycerol was then removed and the shocked cells washed before culture in DMEM and 10% FCS for 16 h to allow recovery. Cells were then trypsinized; and each dish was divided into four and cultured in DMEM, 10% FCS, and 400 μg/ml G418 (selective medium). Neomycin-resistant clones were first visible after 6 d and were subsequently transferred to 24-well plates in selective medium after 9–12 d. After three further passages in selective medium, expanded independent clones were cultured in DMEM and 10% FCS (standard medium). From passage 4, cells of some clones were passaged to coverslips for time-lapse videocinematography. Control epithelial NBTII cells were transfected with the pAG60 recombinant vector containing the Neo gene conferring resistance to neomycin (Colbere-Garapin *et al.*, 1981).

Northern blot analysis

Six of the clones that expressed a motile phenotype were analyzed further by Northern blotting to detect the presence of exogenous TGFα message. Total RNAs were prepared according to Favarolo *et al.* (1980) in the presence of 10 mM vanadyl ribonucleoside complexes. Ten to 15 μg of total RNA samples were electrophoresed into a formaldehyde-1.2% agarose gel and blotted onto Hybond (Amersham). Filters were hybridized with cDNA probes and ^{32}P-labeled with a random-priming kit (Amersham) for 16 h at 42°C in 50% formamide (Fluka, Buchs, Switzerland), 2× SSC (0.15 M NaCl, 15 mM sodium citrate ph 7) 5× Denhardt (Maniatis *et al.*, 1982), 0.1 % sodium dodecyl sulfate (SDS), and 100 μg/ml sonicated salmon sperm DNA. Filters were washed twice with 2× SSC, 0.1% SDS for 15 min at room temperature and twice with 0.1× SSC, 0.1% SDS at 55°C for 30 min. The human TGFα probe consisted of the cDNA *Bam*HI insert of pZipTGFα comprising the complete 595-bp coding sequence. The size of the detected message was estimated by comparison with a 0.24- to 9.5-kb RNA ladder (GIBCO BRL). Plasmid containing the rat cDNA coding for glyceraldehyde phosphate dehydrogenase was used as an internal control of expression (Fort *et al.*, 1985).

Immunoprecipitation of TGFα

NBTII cells transfected with TGFα or with Neo alone were incubated for 30 min in cysteine-free DMEM and then in the same medium in the presence of 120 μCi ^{35}S-cysteine (600 mCi/ml) for 16 h at 37°C. Supernatants were then centrifuged and incubated with a polyclonal sheep anti-TGFα antibody for 4 h at 4°C. Protein A sepharose (preincubated for 30 min with FCS) was then added and, after 30 min incubation on ice, samples were centrifuged for 5 min. The protein A pellet was then washed extensively and finally boiled in the presence of 40 μl of 2× reducing sample buffer (Laemmli, U.K. and Favre, M., 1973). Samples were separated on 10–20% gradient SDS polyacrylamide gels, which were treated with En^{3}Hance (Amersham), dried, and exposed to X-ray film at −70°C for 1–3 wk.

Gelatin substrate gels

Gelatin substrate gels were performed as previously described (Murphy *et al.*, 1989a). Twenty-four-hour, serum-free, DMEM-conditioned media from TGFα-producing clones, untransfected NBTII cells, and NBTII cells transfected with Neo alone were incubated in the presence of nonreducing Laemmli sample buffer for 20 min at 37°C. During this incubation with SDS any metalloproteinases present in the supernatant undergo a change in conformation that enables them to become active without a change in size (Birkedal-Hansen and Taylor, 1982). Samples were then separated at 4°C on 9% SDS polyacrylamide gels containing 0.5 mg/ml gelatin (Sigma Chimie SARL, L'Isle d'Abeau, France). Gels were then incubated in two changes of 2.5% Triton X-100 for 30 min at room temperature and rinsed with water before overnight incubation at 37°C in assay buffer (100 mM tris(hydroxymethyl)aminomethane HCl, pH 7.4, 30 mM $CaCl_2$). Gels were then stained in 1% Coomassie Brilliant Blue G and destained to visualize unstained areas where enzymes had cleaved the substrate. In some cases samples were loaded in duplicate and the gel divided and incubated with or without the metalloproteinase inhibitor, 1,10 phenanthroline (2 mM). Some samples were subjected to activation by the mercurial compound, APMA, by incubation in 2 mM APMA for 2 h at 25°C before mixing with sample buffer. Reverse gelatin zymography was performed to detect inhibitors of gelatinases and other metalloproteinases (Herron *et al.*, 1986). Samples, concentrated 10-fold by centrifugation in a Millipore (Bedford, MA) Centricon filter (cut-off 10 000), were separated on gelatin gels as described above, but, after incubation in Triton X-100, gels were incubated for 1 h in a mixture of activated metalloproteinases from rabbit skin cultures in order to partially degrade the gelatin (Cawston *et al.*, 1981). Gels were then incubated overnight as before, and, after staining and destaining, areas of inhibition of enzymatic degradation of the gelatin in the gel were revealed as densely staining areas.

Plasminogen activator gels

The presence of plasminogen activators in supernatants of TGFα-producing clones was investigated by separation of the samples on plasminogen-containing casein gels according to the method of Heussen and Dowdle (1980). SDS polyacrylamide gels (9%) containing 1 mg/ml casein and 20 μg/ml porcine plasminogen (Sigma Chimie SARL) were run as described above, incubated in Triton X-100, and then incubated overnight at 37°C in 0.1 M glycine, pH 8.3. Gels were stained and destained as described before. Control gels containing casein but no plasminogen were run in parallel.

Gelatinase and TIMP assays

Samples were assayed for gelatinase activity in a quantitative gelatinolytic assay using ^{14}C-labeled denatured type I collagen (Murphy *et al.*, 1985). The presence of TIMP activity was measured by the inhibition of degradation of radiolabeled collagen by activated rabbit skin metalloproteinases (Murphy *et al.*, 1981).

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