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Autocrine induction of tumor protease production and invasion by a metallothionein-regulated TGF-β_1 (Ser223, 225)

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An expression vector was constructed in which TGF-β_1 was placed under the control of the metallothionein promoter. Cys223 and Cys225 in the TGF-β_1 pro-peptide were converted to serines, mutations which result in dissociation of the pro-peptide and secretion of bioactive TGF-β_1 [Brunner,A.M., Marquardt,H., Malacko,A.R., Lioubin,M.N. and Purchio,A.F. (1989) *J. Biol. Chem.*, 264, 13660–13664]. A fibrosarcoma was transfected with this plasmid and a clone (17.18) was selected in which TGF-β_1 mRNA was able to be induced six-fold following zinc sulphate treatment. These cells increased the secretion of bioactive TGF-β_1 14-fold and exhibited a coincidental increase in *jun*-B mRNA expression, suggesting that secreted TGF-β_1 was acting to induce this early response gene by autocrine activation. Following zinc sulphate induction, the tumor cells became progressively more motile and able to invade collagen gels. In contrast to parental tumor not bearing the TGF-β_1 expression vector, zinc sulphate stimulation of clone 17.18 enhanced collagenase IV and procathepsin L mRNA levels and enhanced the secretion of many collagenolytic proteases into the medium. Since the action of TGF-β generally decreases proteolysis by suppression of protease transcription, we compared the response of normal parental fibroblasts to *ras*-transformed fibrosarcomas and confirmed that TGF-β could greatly enhance collagenase IV and procathepsin L mRNA levels while having little effect on non-transformed fibroblasts. These experiments indicate that induction of TGF-β secretion can enhance motility and protease production through autocrine activation, thus increasing the invasion potential of fibrosarcomas.

Key words: cathepsin L/collagenase/invasion/motility/TGF-β

Introduction

Transforming growth factor-betas (TGF-β) are 25 kDa homodimeric peptides (Roberts and Sporn, 1989) which are multifunctional regulators of mesenchymal, endothelial and epithelial cells (Roberts and Sporn, 1989; Barnard *et al.*, 1990; Massagué, 1990; Roberts *et al.*, 1990). TGF-β action is tightly regulated through transcriptional control (Kim *et al.*, 1989a,b) and production of the molecule in a latent form which cannot bind to its receptor (Lawrence *et al.*, 1985; Wakefield *et al.*, 1988). Cleavage of the mature TGF-β_1 dimer from the propeptide releases the native polypeptide and allows a productive receptor–ligand interaction (Lawrence *et al.*, 1985; Lyons *et al.*, 1988; Sato and Rifkin, 1989).

The role of TGF-β in tumor progression remains unclear. In most instances, normal cells are growth inhibited by TGF-β while tumor cells have lost the inhibitory response (Barnard *et al.*, 1990). It has been demonstrated that many, but not all, transformed cells secrete increased amounts and express high steady-state mRNA levels of TGF-β (Anzano *et al.*, 1985; Coffey *et al.*, 1987; Derynck *et al.*, 1987; Schwarz *et al.*, 1990). In those tumors that secrete increased levels of TGF-β it was postulated that TGF-β could promote tumor proliferation by increasing the growth of stromal elements through paracrine mechanisms (Roberts *et al.*, 1988; Roberts and Sporn, 1989). Combined with the loss of negative autocrine growth activity of TGF-β this could perpetuate uncontrolled proliferation (Sporn and Roberts, 1985).

We (Schwarz *et al.*, 1988, 1990) have proposed that tumors may use TGF-β in an autocrine manner which gives them an advantage over transformants that are not TGF-β secretors, thus ensuring their survival and progression. These autocrine effects may influence the invasion of cells through the ability of TGF-β to enhance cell motility (Postlethwaite *et al.*, 1987; Wahl *et al.*, 1987) and regulate protease activity (reviewed in Massagué, 1990). The action of TGF-β generally enhances cell adhesion and this is a result of a combination of increased matrix production and decreased proteolysis. TGF-β suppresses the expression of proteases (Laiho *et al.*, 1986; Matrisian *et al.*, 1986a; Edwards *et al.*, 1987; Mason *et al.*, 1987) while increasing protease inhibitors (Laiho *et al.*, 1986, 1987; Edwards *et al.*, 1987; Stetler-Stevenson *et al.*, 1990). Proteolytic modification is limited in time and strictly regulated at many levels in normal cells. Tumor cells, on the other hand, use proteolysis coupled with motility to achieve invasion and progression, but in circumstances which could be inappropriate for normal cells (Liotta, 1990). It has often been observed that highly malignant and invasive cells show increased protease activity when compared to normal and poorly invasive cells (Matrisian *et al.*, 1986a; Sloane *et al.*, 1986; Stetler-Stevenson, 1990). In other words, the strict regulation of proteolytic activity may be absent in aggressive tumor cells, thus suggesting a defective response to those regulatory factors which normally limit proteolysis, such as TGF-β.

To test these hypotheses, a tumor cell line was developed in which the secretion of active TGF-β_1 could be regulated under the control of a metallothionein promoter to assess whether TGF-β_1 can promote the invasive behavior of the tumor through an autocrine action. Cells overexpressing the TGF-β_1 gene secrete it with the propeptide which maintains the polypeptide in the latent form (Gentry *et al.*, 1988). To

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ensure secretion of active TGF-β_1, Cys223 and Cys225 in the propeptide were substituted with serine, a mutation that results in release of bioactive TGF-β_1 (Brunner *et al.*, 1989).

Results

Transfection of C1 fibrosarcoma with pPK9A and screening for clones with inducible TGF-β_1 expression

The plasmid pPK9A (Figure 1) was co-transfected with the hygromycin resistance gene (*PY3*) into the H-*ras* transformed 10T½ fibroblast cell line C1 described in Methods. Approximately 50 discrete hygromycin resistant colonies were picked and subsequently screened by Northern blotting for TGF-β_1 expression in the presence or absence of 100 μM $ZnSO_4$ for 24 h. Northern blotting of these 50 clones was done using the *Bgl*II insert of the TGF-β_1 cDNA derived from pPK9A. From this initial screen, we selected six clones which showed a significant increase in *TGF-β_1* mRNA expression over the uninduced state. Only one (clone 17.18) had sufficiently low basal levels of *TGF-β_1* mRNA to be useful for further study. This was subcloned and chosen for further study. Figure 2a shows that there was a 6-fold increase of *TGF-β_1* mRNA expression in clone 17.18 when induced with $ZnSO_4$ over a period of 24 h. This was greater than mRNA levels detected in the C3 fibrosarcoma, a highly malignant tumor also derived from 10T½ fibroblasts (Egan *et al.*, 1987). *TGF-β_1* mRNA levels were slightly higher in uninduced clone 17.18 than in the parental C1.

In a time course analysis, it was observed that *TGF-β_1* mRNA levels were increased 2 h post-induction with $ZnSO_4$ and expression continued to increase to 5-fold above controls at the 8 h time interval after which no further increase was seen (Figure 2b). *jun*-B mRNA expression was observed at 2 h after $ZnSO_4$ treatment and was coincidental with increased *TGF-β_1* mRNA levels (Figure 2b). *jun*-B expression could also be induced by stimulation of these cells with porcine TGF-β_1 (data not shown). Since both *TGF-β_1* and *jun*-B mRNA are induced after 2 h post-induction with $ZnSO_4$, the expression of these genes at earlier time points was examined to determine which mRNA was induced first. In this time course analysis, it was observed that *TGF-β_1* mRNA levels were increased 30 min post-induction with $ZnSO_4$ while *jun*-B mRNA expression was not increased until 45 min post-induction (Figure 2c). No change in either *TGF-β_1* or *jun*-B mRNA was observed after $ZnSO_4$ stimulation of the parental C1 tumor line.

The amount of TGF-β_1 being secreted into the medium was next determined by the CCL-64 growth inhibition assay. When induced, clone 17.18 fibroblast cells secreted 5500 ng/10^6 cells/24 h, 14-fold more than in the uninduced state (Table I). Furthermore, following $ZnSO_4$ treatment,

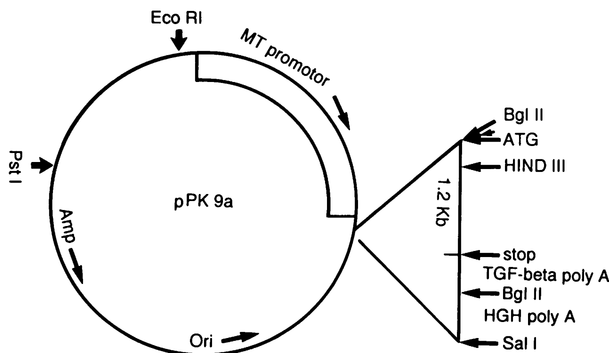

Fig. 1. Construction of metallothionein–TGF-β_1 expression vector. Porcine TGF-β_1 was digested with *Bgl*II and inserted into the *Bgl*II site of the pEV142 expression vector containing a metallothionein promoter at the 5′ end and a growth hormone poly A site at the 3′ end. The construct was subjected to site-directed mutagenesis to change Cys223 and Cys225 to serines. This plasmid is pPK9a.

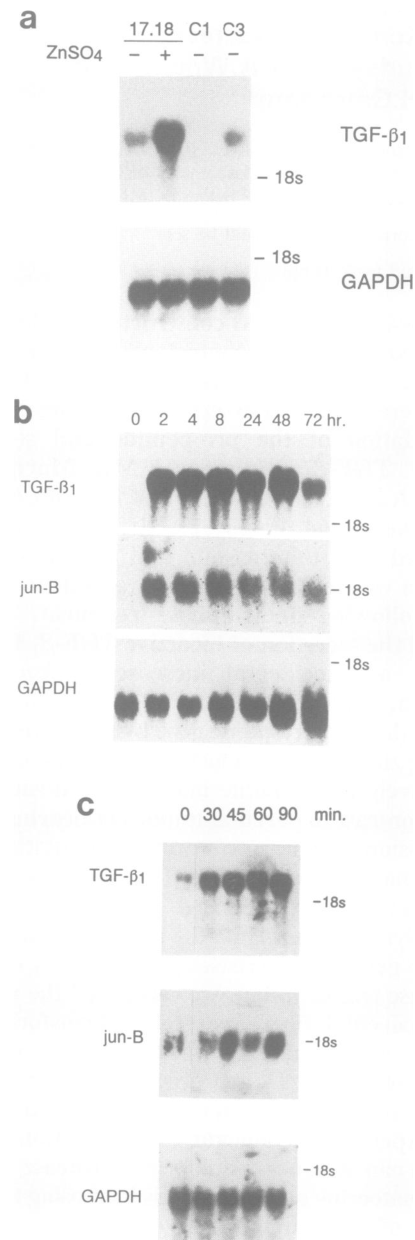

Fig. 2. (a) Overexpression of TGF-β_1 in clone 17.18 after $ZnSO_4$ treatment. TGF-β_1 mRNA levels of clone 17.18 after 24 h treatment in $ZnSO_4$ (+) is compared to uninduced controls (−). The parental Cl tumor and a highly aggressive tumor C3 which overexpresses TGF-β_1 constitutively, are presented for comparison. GAPDH loading controls are shown in the lower panel. (b) Kinetics of TGF-β_1 induction following $ZnSO_4$ treatment of clone 17.18. Both TGF-β and jun-B are significantly increased by 2 h of incubation. GAPDH loading controls are shown in the lower panel. (c) TGF-β_1 induction following $ZnSO_4$ treatment of clone 17.18 occurs prior to *jun*-B induction. *TGF-β_1* and *jun*-B mRNA levels are elevated at 30 and 45 min, respectively, post-induction of clone 17.18 with $ZnSO_4$. GADPH loading controls are shown in the lower panel.

a very high proportion of the TGF-β (67%) was active in neutral conditioned medium and did not require acid activation. The isoform of TGF-β released by these cells was over 95% TGF-β_1 on the basis of neutralization studies with specific anti-TGF-β_1 and anti-TGF-β_2 antiserum (data not shown). The kinetics of secretion of mature TGF-β_1 between 2–24 h after induction was also determined. As is seen in Table I, TGF-β_1 increased 2.2-fold within 2 h of $ZnSO_4$ addition to the cells. This rate of secretion steadily increased and at 24 h was 2530 ng/10^6 cells/24 h or nine-fold higher than uninduced controls.

Induction of TGF-β_1 secretion stimulates motility and invasion of clone 17.18

A significant change in cell phenotype that contributes to invasion is the initiation of motility (Liotta and Schiffman, 1988; Weidner *et al.*, 1990). TGF-β is a potent stimulator of chemotaxis for dermal fibroblasts (Postlethwaite *et al.*, 1987) and monocytes (Wahl *et al.*, 1987). To analyze the locomotive properties of clone 17.18, motility rates were determined by timelapse analysis of random locomotion. It was observed that following $ZnSO_4$ stimulation, clone 17.18 cells became progressively more motile. At 4–6 h a rate of 22.2 ± 3.6 μm/min was calculated and continued to increase to 51.6 ± 6.0 μm/min or four-fold higher than uninduced controls at the termination of the experiment at 26 h (Figure 3). Cells in the uninduced state maintained a motility rate between 9.6 and 13.2 μm/min.

Having observed that when clone 17.18 is induced to secrete TGF-β_1 cells are highly motile, cells were then analyzed for their invasive potential. Since collagen I is a major constituent of the extracellular matrix *in vivo*, an invasion assay using collagen I was utilized to observe tumor cell invasion *in vitro* (Schor *et al.*, 1982; Shields *et al.*, 1984). Invasion of the collagen matrix by clone 17.18 was observed only in the induced state and was time-dependent. As shown in Figure 4, 4 h after $ZnSO_4$ induction of TGF-β_1, the cells were penetrating the collagen matrix at a rate of up to 6.66 μm/min. By 6 h the invasion rate had increased to 10.00 μm/min and at 24 h had reached a maximum rate of 13.33 μm/min or four-fold greater than controls. When not induced for TGF-β_1 secretion, clone 17.18 was virtually non-invasive with only a few cells reaching rates of 3.33 μm/min.

Table I. Bioactive TGF-β_1 secretion by $ZnSO_4$ stimulated[a] clone 17.18

Treatment	Time no. (h)	TGF-β_1 (ng/10^6 cells/24 h) Neutral (fold)[b] medium	Acidified (fold) medium
Control	24	246 –	465 –
$ZnSO_4$	24	3540 (14.4)	5271 (11.3)
Control	0	282 –	ND
$ZnSO_4$	2	612 (2.2)	ND
$ZnSO_4$	4	1398 (5.0)	ND
$ZnSO_4$	8	1387 (4.9)	ND
$ZnSO_4$	24	2530 (9.0)	ND

ND = Not done.
[a]100 μM $ZnSO_4 \cdot 7H_2O$ was used throughout all experiments.
[b]Fold increase over untreated controls.

Enhanced protease production in clone 17.18 following stimulation of TGF-β_1 secretion

The ability to permeate through the extracellular matrix has been associated with enhanced protease production (Garbisa *et al.*, 1987; Ostrowski *et al.*, 1988). To determine whether increased TGF-β_1 production was associated with enhanced protease activity, we assayed the conditioned medium of clone 17.18 using gelatin gel electrophoresis (Heussen and Dowdle, 1980; Bernhard *et al.*, 1990). When conditioned medium was analyzed on these gels, $ZnSO_4$-stimulated

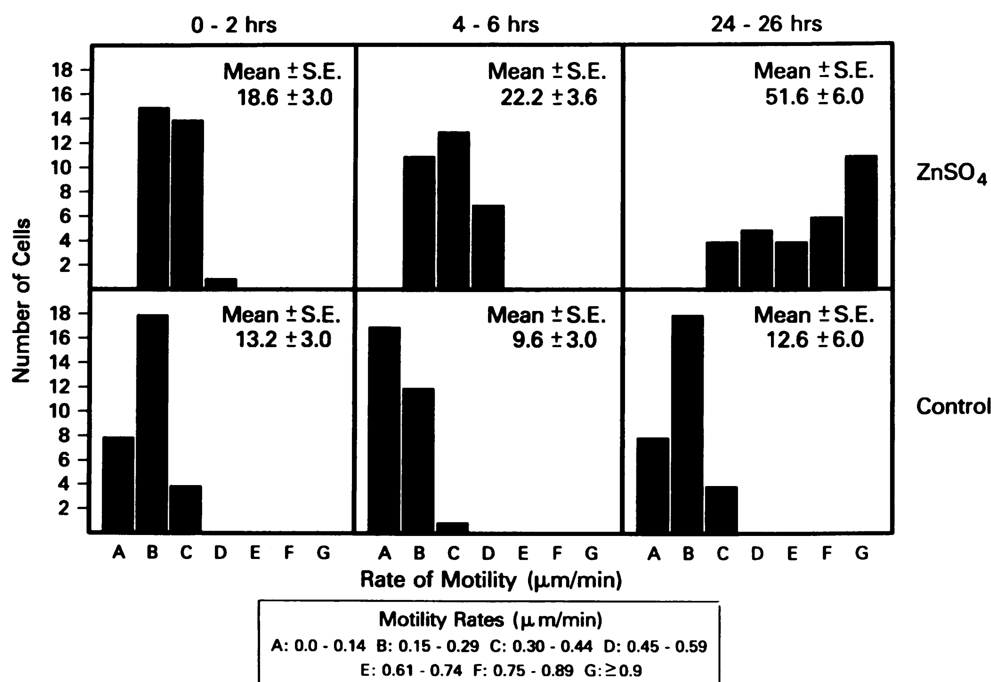

Fig. 3. Motility rate of clone 17.18 following $ZnSO_4$ stimulation of TGF-β secretion. Approximately 30 individual cells were tracked over a 26 h period in 100 μM $ZnSO_4$ by digital image analysis and compared to unstimulated controls. $ZnSO_4$ treatment of the parental C1 tumor was without effect (not shown).

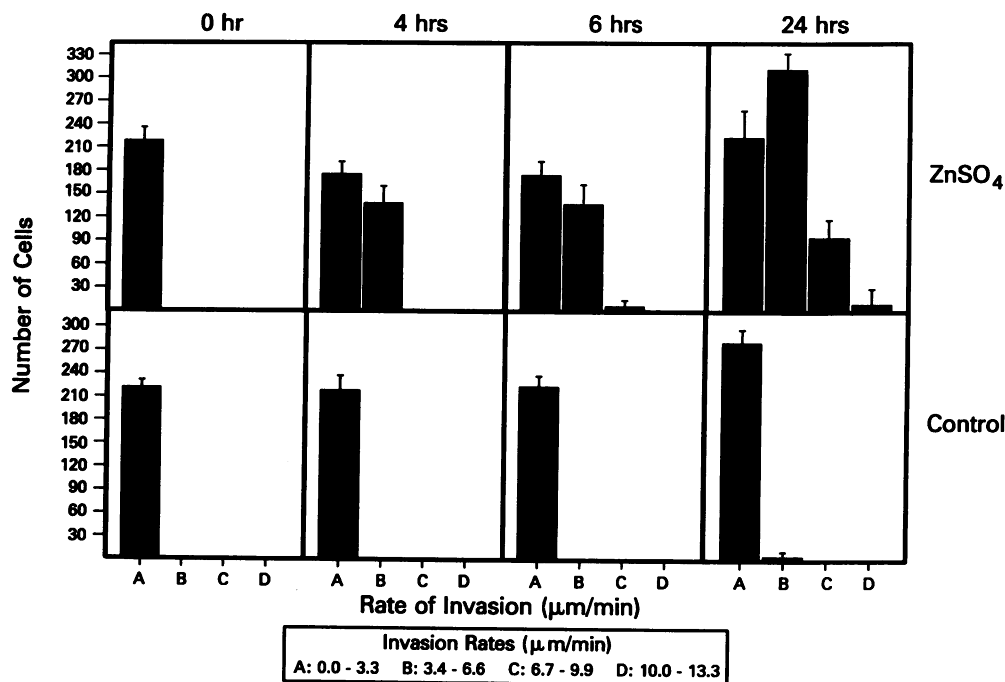

Fig. 4. Invasion of Vitrogen collagen gel by clone 17.18. Penetration rate of collagen matrix over 24 h was analyzed for >300 cells at increasing time intervals following incubation in $ZnSO_4$.

clone 17.18 exhibited striking increases in the activity of many proteases possessing gelatinolytic activity in bands varying from M_r 12 kDa to 105 kDa (Figure 5). The proteases which showed the greatest increase in gelatinolytic activity when TGF-β_1 production was induced had a molecular weight of ~M_r 92 kDa, 44 kDa and 38 kDa. Other zones of clearing that were enhanced were detected at M_r 105 kDa, 29 kDa, several bands between 20 and 25 kDa, and a single band at ~12 kDa. The parental C1 control was unaffected by the identical $ZnSO_4$ treatment but did possess some bands of gelatinolytic activity which were similar to the uninduced clone 17.18 (Figure 5). When clone 17.18 was stimulated for 24 h with porcine TGF-β_1 added to the culture medium, cells released gelatinolytic proteases into the conditioned medium similar to that seen after $ZnSO_4$ stimulation (data not shown).

Two protease bands of M_r 92 kDa and ~29 kDa were similar to that reported for collagenase IV and cathepsin L (Mason *et al.*, 1987; Wilhelm *et al.*, 1989). We examined the expression of these genes following $ZnSO_4$ stimulation and found a 4-fold increase in procathepsin L and a 2.7-fold increase in collagenase IV mRNA levels after 24 h (Figure 6a). The level of expression was comparable with or greater than that detected in the highly invasive C3 line. Increased collagenase IV mRNA is first detected at 24 h and procathepsin L is observed at 8 h after $ZnSO_4$ (Figure 6b), well after the induction of TGF-β_1 and *jun*-B (Figure 2b).

To determine whether the direct addition of TGF-β to 10T½ derived cell lines could induce these genes, we compared the parental 10T½ to the C3 fibrosarcoma. Following incubation of 10T½ cells in 10 nM TGF-β_1, the expression of both genes remained the same or lower than unstimulated controls, while C3 cells were induced to express procathepsin L and collagenase IV at high levels within 1 h of treatment (Figure 7). Furthermore, C1 cells treated with 10 nM TGF-β_1 responded in a fashion similar to that seen with C3 cells although the magnitude of expression was less (data not shown).

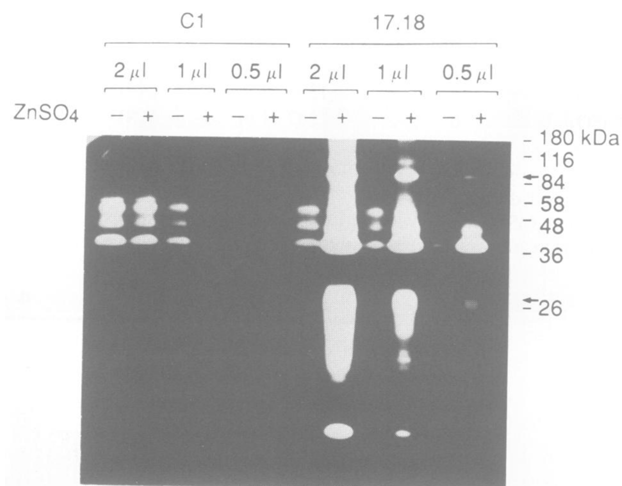

Fig. 5. Gelatinolytic activity of clone 17.18. Gelatin gel electrophoresis was carried out on aliquots of serum free conditioned medium from clone 17.18 and the C1 parental tumor after 24 h incubation in $ZnSO_4$ (+) or control medium (−). Molecular weight markers are indicated on the right. The most prominent gelatinase activity following $ZnSO_4$ was noted at 92 kDa (arrow), 44 kDa, and 38 kDa. Other bands of clearing which were increased by TGF-β include 105 kDa, 29 kDa (arrow), several bands between 20 and 25 kDa and a 12 kDa band at the bottom of the gel.

Discussion

The ability of tumor cells to invade is a significant and potentially lethal attribute. Recent observations suggest that factors from the environment may modulate the invasive potential of tumor cells (Liotta *et al.*, 1991). One such factor, TGF-β_1, has been shown to elicit behaviorial changes in a

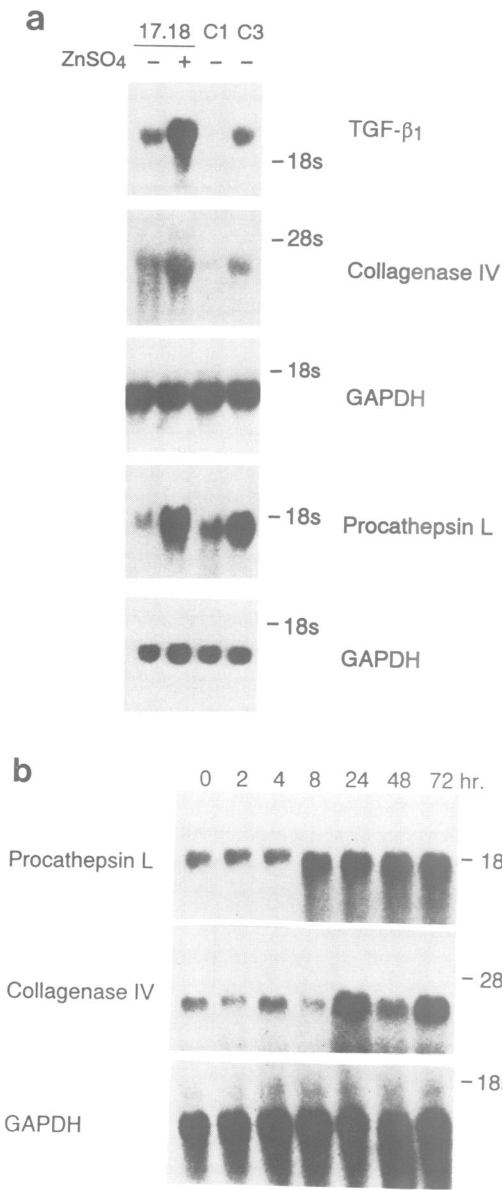

Fig. 6. (**a**) Increased collagenase IV and procathepsin L mRNA following $ZnSO_4$ stimulation of TGF-β_1 secretion. mRNA of collagenase IV and procathepsin L levels were either at the same level or exceeded that of the C3 fibrosarcoma. GAPDH loading controls are also shown. (**b**) Kinetics of protease expression following $ZnSO_4$ stimulation of clone 17.18. Increased procathepsin L was seen at 8 h while collagenase IV was not significantly elevated until 24 h. GAPDH loading controls are shown in the lower panel.

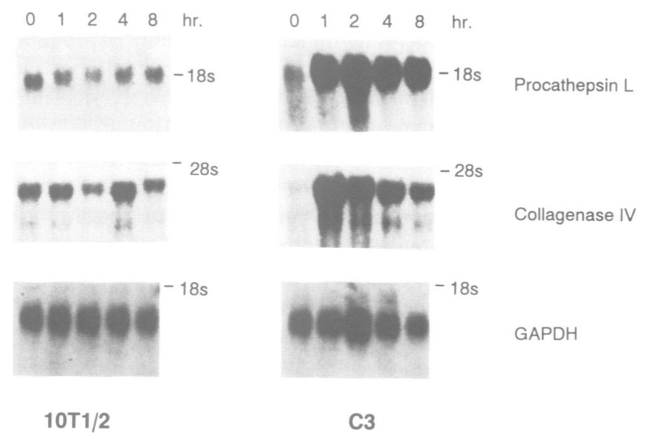

Fig. 7. Differential induction of collagenase IV and procathepsin L by porcine TGF-β_1 in C3 fibrosarcoma and parental 10T½ fibroblasts. The C3 tumor and non-transformed parental 10T½ were incubated with 10 nM TGF-β_1 and RNA extracted for Northern blotting at the indicated time intervals. GAPDH loading controls are shown in the lower panels.

cell and context dependent manner (reviewed in Massagué, 1990; Barnard *et al.*, 1990; Nathan and Sporn, 1991). The observation that many malignant cells express and secrete high levels of TGF-β which has the ability to modulate, among other phenotypes, motility and protease production suggests that it may be involved in regulating the invasive potential of tumor cells. In this study, we have demonstrated that enhanced secretion of mature TGF-β_1 by a *ras*-transformed fibrosarcoma line promoted its motility and invasion of collagen gels and significantly increased the secretion of a variety of collagenolytic proteases.

Clone 17.18 was selected from the C1 fibrosarcoma (Egan *et al.*, 1987), a relatively unaggressive *ras*-transformed fibroblast, following transfection with the plasmid pPK9A which contained a porcine TGF-β_1 regulated by the metallothionein promoter. The *TGF-β_1* cDNA was mutated so that Cys223 and Cys225 were modified to serines. Brunner *et al.* (1989) have found that this mutation does not alter TGF-β_1 production but does yield a high proportion of mature 24 kDa dimer which is bioactive without acid activation. Analysis of TGF-β_1 secreted by clone 17.18 indicated that >50% TGF-β_1 was in the active form in neutral conditioned medium and when stimulated with $ZnSO_4$, TGF-β_1 increased 9- to 14-fold and >65% was in the active form. Secretion of TGF-β_1 closely followed mRNA levels indicating that the mutated gene was regulated through the stimulation of the metallothionein promoter. Paralleling the increase in TGF-β_1 expression, an increase in *jun*-B mRNA levels was observed. It has been found that *jun*-B expression is an early genomic response in cells sensitive to TGF-β_1 stimulation (Pertovaara *et al.*, 1989). The rapid *jun*-B response in clone 17.18 to $ZnSO_4$ immediately after TGF-β_1 stimulation suggests that induction is occurring through autocrine action of the secreted bioactive TGF-β_1 homodimer. It is also possible that the effect of TGF-β_1 on clone 17.18 could be occurring through an intracrine pathway, however, whether this occurs and to what extent is not immediately apparent.

Using *in vitro* assay systems, we were able to observe that when clone 17.18 was induced for TGF-β_1 production it was more invasive than in the uninduced state. The most likely explanation is that the enhanced invasive potential was due to a combination of increased motility (Figure 3) and gelatinolytic activity. Elevated levels of the collagenase type IV and procathepsin L mRNA were detected along with a number of collagenolytic proteases in the conditioned medium of induced cells. Welch *et al.* (1990) have recently shown that treatment of mammary adenocarcinoma cells with TGF-β_1 promoted invasion and increased production of 92 and 64 kDa gelatinolytic enzymes. We have identified a 92 kDa collagenase IV (Stetler-Stevenson, 1990) but not a 64 kDa band. Some of the proteases showing gelatinolytic activity at greatly elevated levels in clone 17.18 were of lower molecular weight and were only observed when TGF-

β_1 expression was induced. The identity of these proteases is unknown and is currently under investigation. Expression was detected 8–24 h after $ZnSO_4$ treatment, well after the appearance of *TGF-β_1* transcript and protein secretion into the medium. Direct stimulation of clone 17.18 with porcine TGF-β_1 for 24 h also produced similar increases in protease production. $ZnSO_4$ stimulation of the C1 and C3 cell lines, respectively, did not induce an increase in motility or invasion over unstimulated controls. In total, these experiments strongly support the interpretation that $ZnSO_4$ stimulation of TGF-β_1 secretion was activating protease production in an autocrine manner.

A striking feature of the response of the H-*ras* transformed fibroblasts examined in this study is the induction of collagenase IV and procathepsin L gene expression by TGF-β_1. Expression of collagenases (Edwards *et al.*, 1987), transin/stromelysin (Matrisian *et al.*, 1986b) and cathepsin L (Mason *et al.*, 1987) are suppressed by TGF-β_1 in normal fibroblasts. The increased protease production in response to TGF-β_1 in the 10T½ derived transformants indicates that H-*ras* has uncoupled TGF-β_1 signalling and these cells are now utilizing a novel pathway for stimulation of protease gene expression.

Following transformation, proteases are often constitutively expressed at high levels. The observation that TGF-β_1 stimulated a *ras*-transformed fibrosarcoma to express proteases at high levels (Figure 7) and that malignant fibrosarcomas secrete high levels of TGF-β (Schwartz *et al.*, 1990), raises the possibility that these tumors may be maintaining increased protease production through TGF-β autocrine effects. This is further supported by the observation that clone 17.18 enhanced protease production following stimulation of TGF-β_1 secretion through an autocrine pathway. Since enhanced protease production has been implicated in tumor invasion and metastasis (Stetler-Stevenson, 1990; McDonnell *et al.*, 1990), we postulate that the invasive phenotype may be regulated through a TGF-β_1 autocrine activation pathway.

Materials and methods

Cell lines

The properties of the cell lines (10T½, CIRAS-1 and CIRAS-3) used in this study have been described previously (Egan *et al.*, 1987). In brief, CIRAS-1 (C1) and CIRAS-3 (C3) were obtained when parental 10T½ fibroblasts were transfected with plasmid pA9 which contains the 6.6 kb fragment of T24-H-*ras* and *neo*^{R} gene. All cell lines were maintained at 37°C on 150 mm plastic tissue plates (Falcon) in culture medium containing α-minimal essential medium (α-MEM; Flow Laboratories, Mississauga, Ontario) supplemented with antibiotics and 10% (vol/vol) fetal calf serum (FCS; Gibco, Grand Island, NY). A serum-free medium called defined medium (DM) was also used which contained a 4.0 μg/ml transferrin and 2.0 μg/ml insulin in α-MEM.

Vector construction, transfection of cells and establishment of cell lines

Porcine TGF-β_1 (pTGF-β33) open reading frame (from ATG to poly A site) was amplified using oligonucleotides by PCR. The 5′ (PC1) primer was designed to have *Bgl*II and *Kpn*I restriction sites at the end and also the sequence around the ATG translational start codon were modified to meet the Kozak's consensus sequence. The 3′ (PC2) primer also had *Kpn*I and *Bgl*II restriction sites at the end. The resulting amplified product was digested with *Bgl*II and inserted at the *Bgl*II site of the expression vector, pEV 142, which has a metallothionein promoter at the 5′ end of the *Bgl*II site and the growth hormone poly A site at the 3′ end. Following transformation into an *Escherichia coli* strain (DH5), positive recombinants were determined by restriction digests and subsequently confirmed by dideoxysequencing using Sequenase. The clone which had the insert in the coding orientation with respect to the metallothionein promoter was subjected to site-directed mutagenesis to modify Cys223 and Cys225 to serines in the pro-region of TGF-β_1 in order to allow for the secretion of active TGF-β_1. This plasmid was designated pPK9A.

The C1 tumor cell line was chosen as the candidate for transfection since it was previously reported that it was of low metastatic potential (Egan *et al.*, 1987). Furthermore, C1 secreted relatively low amounts of TGF-β_1 (<200 pg/ml) and predominantly in the latent form (Schwarz *et al.*, 1990). pPK9A was stably cotransfected with the hygromycin resistance gene (PY3) into C1 using Lipofectin Reagent (BRL, Gaithersburg, MD) (Felgner *et al.*, 1987). Hygromycin-resistant colonies were screened for overexpression of the TGF-β_1 transcript in the presence of 100 μM zinc sulphate ($ZnSO_4 \cdot 7H_2O$).

Preparation of conditioned medium

Cell lines were cultured in 150 mm tissue culture plates (Falcon) with α-MEM–10% FCS, were washed once with serum free medium and then cultured in DM for 24 h at 37°C with or without 100 μM $ZnSO_4 \cdot 7H_2O$. This conditioned medium was then removed, centrifuged to remove cell debris, placed in siliconized polypropylene tubes with 1 μg/ml aprotinin, 2.5 μg/ml leupeptin and 0.5 μg/ml pepstatin A and then frozen at −80°C as described by Danielpour *et al.* (1988).

Rate of secretion of TGF-β_1

CCL-64 mink lung epithelial cells were maintained in either MEM or Dulbecco's Modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine plasma (FBP, Gibco). Subconfluent cells were used in the TGF-β growth-inhibition assay as previously described (Danielpour *et al.*, 1988). Cells were trypsinized and washed with MEM in 0.2% FBP and resuspended in MEM, 0.2% FBP, 10 mM HEPES (pH 7.4), penicillin (25 units/ml) and streptomycin (25 μg/ml) and seeded as 5×10^5 cells/0.5 ml in 24-well Costar dishes (Flow Laboratories, Mississauga, Ontario). Aliquots of acidified or neutral conditioned medium (Danielpour *et al.*, 1988) were added 3 h later. After 22 h the cells were pulsed with 0.25 μCi (5 Ci/ml) of 5′-[^{125}I]UdR (ICN, Edmonton, Alberta) for 2–3 h at 37°C then harvested as described previously (Schwarz *et al.*, 1990). The amount of TGF-β in conditioned medium was estimated by comparison to a standard growth inhibition curve using porcine TGF-β_1. The species of TGF-β found in conditioned medium was determined using specific anti-TGF-β_1 and anti-TGF-β_2 antibody (Danielpour *et al.*, 1988).

Preparation of RNA

Cells were plated onto 150 mm tissue culture plates containing 25 ml α-MEM with 10% FCS. At 60–75% confluence TGF-β_1 secretion was induced by 100 μM $ZnSO_4 \cdot 7H_2O$. Following 24 h incubation, cells were washed with Hanks medium and 3.0 ml of phosphate-buffered 0.1% trypsin–2 mM EDTA was added. Cells were pelleted and the RNA extracted by the guanidinium-isothiocyanate method as described by Chomczynski and Sacchi (1987), and 40 μg was fractionated on 1.0% agarose gels in the presence of 18.0% (v/v) formaldehyde and transferred to nylon (Nytran). The RNA was hybridized at 42°C for 18 h to ^{32}P-labelled cDNA (Klenow extension or Nick translation). cDNA used as probes were the following: 1.5 kbp *Bgl*II–*TGF-β_1* fragment from the plasmid pPK9A; 660 bp *Eco*RI–*Pvu*II procathepsin L fragment from the plasmid pW7 (a gift from Dr D.Edwards, University of Calgary, Calgary, Alberta), 1.8 kbp *Eco*RI–*jun*B fragment from the plasmid p465.20 (a gift from Dr D.Nathans, Johns Hopkins University, Baltimore, MD), and a 1.1 kbp *Eco*RI–collagenase IV fragment from the plasmid pH3a (a gift from Dr. W.Stetler-Stevenson, NIH, Bethesda, MD). Autoradiography was performed at −70°C with X-Omat AR film (Eastman Kodak Co., Rochester, NY). Quantification was performed on a Biorad Model 620 video-densitometer.

Cell motility analysis

An image analysis system capable of quantifying cell motility as nuclear displacement from a sequence of digitalized or 'timelapsed' images was used (Image I, Universal Imaging Corporation, Westchester, PA). Aliquots of 5×10^3 cells were placed onto a 25 cm^2 tissue culture flask (Falcon) and cultured as described above with or without 100 μM $ZnSO_4 \cdot 7H_2O$. Cells were tracked every 10 min for 24 h and a minimum of 30 cells were examined in each experiment.

In vitro tumor cell invasion assay

To quantify tumor cell invasion we used an *in vitro* assay with Vitrogen purified collagen (Collagen Corporation, Palo Alto, CA) as a matrix. Use of collagen matrices to observe normal and tumor cell invasion has been described before (Schor *et al.*, 1982; Shields *et al.*, 1984). Briefly, Vitrogen 100 solution was made aseptically as per manufacturer's instructions and 5 ml of solution was placed in a 25 cm^2 tissue culture flask and allowed

to solidify at 37°C. An aliquot of 10^4 cells, in a total volume of 5 ml of growth medium with or without 100 μM $ZnSO_4 \cdot 7H_2O$, was layered gently on solidified collagen matrix. The flask was then incubated at 37°C and at specific time intervals these cells were manually tracked at 5 μM intervals as they descended through the gel using an inverted microscope. The number of cells within each level was recorded and converted to an invasion rate expressed as μM/h.

Substrate gel electrophoresis to assay for protease activity

Proteolytic activity was examined using the protocol described by Bernhard *et al.* (1990). To summarize, conditioned medium collected as mentioned above was mixed 4:1 (v:v) with sample buffer (10% SDS, 0.312 M Tris−HCl, pH 6.8), 0.1% bromphenol blue (without reducing agents) and warmed to 37°C for 5 min. Equal aliquots of each sample were loaded into wells of a 5% stacking gel and resolved by electrophoresis at a constant current of 10 mM at ambient temperature. The 10% resolving gel contained type I gelatin (Sigma, St. Louis, MO) at a final concentration of 1 mg/ml. Following electrophoresis, gels were incubated in buffer composed to 0.05 M Tris−HCl (pH 7.4) and 2% Triton X-100 (Sigma) and rinsed in buffer containing 0.05 M Tris−HCl (pH 7.4) at room temperature. Gels were then incubated for 24 h in substrate buffer containing 0.05 M Tris−HCl (pH 7.4) 0.005 M $CaCl_2$, 1% Triton X-100 and 0.02% sodium azide at 37°C. Gels were stained with 0.1% Coomassie Blue R-250 in acetic acid:methanol:water (5:50:45; v:v:v) and destained in a solution of 5% acetic acid and 10% methanol. Prestained molecular weight markers (Sigma) were resolved on the same gel. Gelatinase activity appeared as zones of clearing due to gelatin degradation after staining with Coomassie Blue.

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