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TRANSFORMING GROWTH FACTOR-ß SUPPRESSES THE INVASIVENESS OF HUMAN FIBROSARCOMA CELLS IN VITRO BY INCREASING EXPRESSION OF TISSUE INHIBITOR OF METALLOPROTEASE

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SUMMARY: We have investigated the effects of TGF-ß on the ability of the human fibrosarcoma cell line, HT1080, to invade a reconstituted basement membrane (Matrigel) in vitro. Exposure of HT1080 cells to TGF-ß (1-10ng/ml) caused a dose-dependent inhibition of HT1080 cell invasion. Unexpectedly, TGF-ß (10ng/ml) significantly enhanced (10-fold) the mRNA expression of the 68-72kDa latent type IV collagenase. Zymogram analysis revealed a 7-fold increase in the 68-72kDa latent type IV collagenase concomitant with an increase in the activated form (62kDa). TGF-ß induced the 92kDa type IV collagenase to a lesser degree. HT1080 cells exposed to TGF-ß also produced more tissue inhibitor of metalloprotease (TIMP) at both the mRNA (10-fold) and protein levels (5-fold). Although TGF-ß induced both type IV collagenases and TIMP, the net collagenolytic activity in the conditioned media after invasion assay was reduced in the presence of TGF-ß. The data suggest that the inhibition of invasiveness is due, at least in part, to the increased TIMP expression. These data suggest that TGF-ß may play a role in tumor cell invasion by increasing the expression of TIMP.

Type IV collagenase is thought to play an important role in tumor invasion and metastasis because the activity of type IV collagenase correlates with the metastatic potential of malignant cells (1). There are two forms of type IV collagenase, a 68-72kDa and a 92-95kDa species (2-

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Abbreviations: TGF-ß, Transforming Growth Factor-ß1; TIMP, Tissue Inhibitor of Metalloprotease.

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4). Both enzymes are secreted in latent forms. TIMP and TIMP2, form complexes with the type IV collagenases (4-6). Several studies have shown that TIMP can inhibit the invasion or metastasis of tumor cells (7-9).

TGF-ß plays an important role in the control of extracellular matrix deposition and remodelling (10). However, the effects of TGF-ß on the invasion, type IV collagenase activity, and TIMP expression in tumor cells are unknown. Therefore, we investigated the effects of TGF-ß on the invasiveness of HT1080 cells, on the expression of type IV collagenases and TIMP at the mRNA and the protein levels.

MATERIALS AND METHODS

Cell Culture: HT1080 cells (American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% fetal bovine serum, and antibiotics (100units/ml penicillin and 100µg/ml streptomycin).
Incubation with TGF-ß: Confluent cultures of HT1080 cells in Costar 24-well plates (1.6cm) were washed twice with phosphate-buffered saline and incubated for 36hr in serum-free medium (DMEM supplemented with 0.1% bovine serum albumin, 5µg/ml transferrin, and 1µM selenium). The quiescent cultures were then incubated in the presence of bovine TGF-ß1 (0-20ng/ml) for 0-24hr in serum-free media. At appropriate time intervals, the cells were washed twice with serum-free medium and incubated for 5hr. The conditioned medium (300µl) was harvested.
Type IV Collagenase Analyses: To measure two forms of type IV collagenases, a gelatin-substrate zymogram was carried out as described in (11). To measure the net enzyme activity, the proteolytic degradation of type IV collagen by type IV collagenase was measured using a modified solid phase radioassay (11).
TIMP Analyses: The TIMP level in conditioned media was assayed by Western blotting. TIMP antibody was provided by Dr. H.G. Welgus (Jewish Hospital, St. Louis, MO). Western blotting was performed with immunoblotting system for rabbit polyclonal antibodies using streptoavidin-alkaline phosphatase (BRL Life Technologies Inc., Gaithersburg, MD).
RNA Isolation and Hybridization: Total RNA was prepared (12), electrophoresed (13), and transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH). cDNA probes specific for type IV collagenase (3) and TIMP (14) were labeled by random primed method using a DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed for 24hr at 42°C in 50 ml of 50% formamide, 5xSSC, 10x Denhardt's solution, 10% dextran sulfate, 0.05 M $NaPO_4$, pH 6.5, 1% glycine and 250µg/ml tRNA. Filters were washed with 0.1xSSC and 0.1% SDS, at 60°C, 30 min, twice and exposed to X-ray film at -70°C for 24hr.
Invasion Assay: HT1080 cells (2×10^5) were pretreated with TGF-ß (1-10ng/ml) for 24hr as decribed above, and then the invasion assay using Boyden chambers was performed in duplicate as described previously (15). The results are expressed as mean ± SD of three independent experiments.

Effect of Recombinant TIMP on Invasion: HT1080 cells (2 x 10^5) were treated with human recombinant TIMP (0, 1, 5, 10, and 50 µg/ml) for 25min at 37°C, and assayed in duplicate for invasiveness. The results are presented as the mean of two experiments and expressed as per cent of control.

RESULTS AND DISCUSSION

TGF-ß Suppresses the Invasiveness and the Type IV Collagenolytic Activity of HT1080 Cells

We have found that HT1080 cells pre-incubated with TGF-ß (1–10ng/ml) for 24 hr have a reduced capacity to invade Matrigel in a dose-dependent manner (Table1). At 10ng/ml of TGF-ß, a 40% inhibition of HT1080 cell invasion was observed. This result is consistent with the previous report by Mignatti et al. (16) that TGF-ß (1-10ng/ml) inhibits bovine capillary endothelial cell invasion through amniotic membrane in a dose-dependent manner. They suggested that both the plasminogen activator-plasmin system and collagenases (type IV and interstitial collagenase) participate in the invasion process (16).

Analysis of type IV collagenolytic activity in the media collected from the upper compartment of the Boyden chamber revealed a significant reduction in the degradation of labeled type IV collagen in cells pretreated with TGF-ß (Table1). Since invasion of Matrigel depends on cell adhesion, we tested the attachment of TGF-ß–treated cells to Matrigel-coated dishes, and found no significant differences in adhesion and in spreading whether or not the cells were exposed to TGF-ß (data not shown). We also tested the effects of TGF-ß on the expression of interstitial collagenase and stromelysin in HT1080 cells. We did not find any significant changes in

Table 1. Effects of TGF-ß on the invasiveness of HT1080 cells and the net type IV collagenase activity

TGF-ß (ng/ml)	Invasion ($\mu m^2 x 10^{-3}$)	Type IV collagenase (cpm x 10^{-3})
0	31 ± 2.3[a]	2.6 ± 0.46
1	26 ± 1.7[b]	0.83 ± 0.84[b]
5	21 ± 2.4[c]	0.53 ± 0.41[c]
10	19 ± 2.1[c]	0.37 ± 0.39[c]

[a]Mean ± SD.
[b]$P<0.05$ versus control.
[c]$P<0.01$ versus control.
The invasiveness and type IV collagenase activity were measured as described in Materials and Methods.

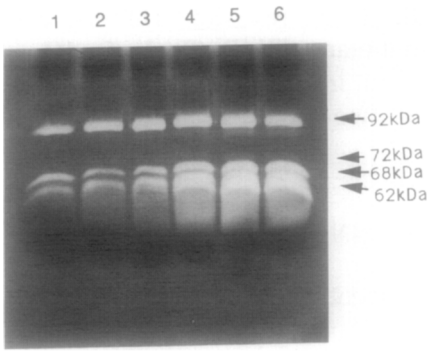

Fig. 1. Effect of TGF-ß on secreted type IV collagenase by HT1080 cells. Aliquots (75µl) of serum free conditioned medium from cells incubated for 0-24 hr in the presence of 10ng/ml TGF-ß were analyzed by gelatin zymogram. Lane 1, 0 hr; lane 2, 3 hr; lane 3, 6 hr; lane 4, 12 hr; lane 5, 18 hr; and lane 6, 24 hr.

mRNA levels (data not shown). Thus, it is likely that TGF-ß reduces the invasive activity of HT1080 cells through an effect on their capacity to degrade type IV collagen.

TGF-ß Enhances the Production of Type IV Collagenases

The conditioned media of HT1080 cells exposed to TGF-ß were analyzed for gelatinase activity using the zymogram technique. HT1080 cells secreted into the medium two major type IV collagenase: a 68-72kDa and a 92kDa species (Fig.1, lane 1). These bands correspond to the previously reported size of the two isoforms of type IV collagenase (3,4,17). Exposure of HT1080 cells to increasing amounts of TGF-ß (1-20ng/ml) for 24hr resulted in a dose-dependent increase in gelatinolytic activity in the 68-72kDa doublet (data not shown). Maximal activity was observed with 10ng/ml of TGF-ß. Therefore, a time course study was performed using 10ng/ml TGF-ß. As shown in Fig. 1, TGF-ß induced the 68-72kDa latent type IV collagenase after 6 hr with maximum induction (7-fold) after 18 hr. Concomitant with the increase in the 68-72kDa band, we observed the appearance of a gelatinolytic band of 62kDa which corresponds to the reported size of the active form of the 68-72kDa type IV collagenase (17). In contrast, TGF-ß induced the 92kDa type IV collagenase to a lesser degree.

To examine whether 92kDa or 68-72kDa type IV collagenase is a precursor form, we treated conditioned media with 1mM aminophenylmercuric acetate (17). This treatment induced molecular weight reductions of both forms (92kDa to 85kDa and 68-72kDa to 62kDa)(data not shown).

To date no physiological activation mechanism of latent type IV collagenase has been identified (17,18). Since TGF-ß has been shown to

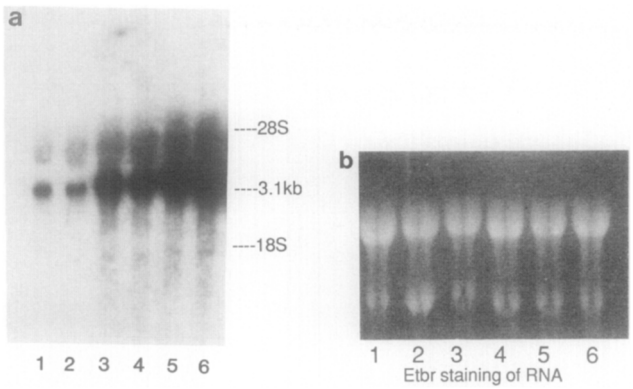

Fig. 2. Effect of TGF-ß on the expression of 72kDa type IV collagenase mRNA. Confluent, quiescent HT1080 cells were incubated for 0-24 hr in the presence of 10ng/ml TGF-ß. a, Lane 1, 0 hr; lane 2, 3 hr; lane 3, 6 hr; lane 4, 12 hr; lane 5, 18 hr; and lane 6, 24 hr. At the indicated times total mRNA was prepared. Ten µg of total RNA was loaded on the gel for Northern blotting. b, Ethidium bromide staining of mRNA.

decrease activity of the plasminogen activator (10), activation of the 68-72kDa type IV collagenase may not be stimulated by the action of the plasminogen activator cascade. Further, proteases such as plasmin are not effective activators of latent form of type IV collagenases (18). HT1080 cells express TGF-ß (10). Taken together, the data suggest that TGF-ß might be involved in activation of latent type IV collagenase.

In a previous study which assayed conditioned media of normal human fibroblasts using gelatin substrate gels, a 1.6-1.8-fold induction of a 72kDa gelatinase was observed in the media after a 2 day exposure to TGF-ß (2). mRNA transcription which is important in the study of the regulation of protein synthesis was not analyzed. The relatively slow (48hr) and low (1.6-1.8-fold) induction of this enzyme by TGF-ß in the normal fibroblasts, as compared to the HT1080 cells, may be related to differences between normal and malignant cells.

We next examined the effect of TGF-ß on the expression of the 72kDa type IV collagenase mRNA using a specific human cDNA probe (3). Northern blot analysis using total RNA isolated from quiescent controls and from TGF-ß treated cells stimulated for 3, 6, 12, 18, and 24 hr showed that the 72kDa type IV collagenase mRNA levels increased about 10-fold in response to TGF-ß in a time-dependent manner (Fig. 2). 92kDa type IV collagenase mRNA levels slightly increased in response to TGF-ß (data not shown).

TGF-ß stimulates TIMP Production

Since the increase in type IV collagenase production in HT1080 cells exposed to TGF-ß was associated with a reduced invasiveness and reduced

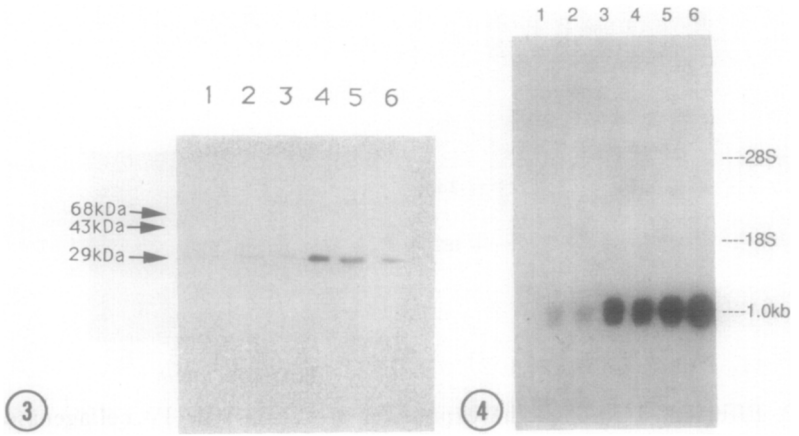

Fig. 3. TIMP level by Western blot analysis. Aliquots (20µl) of serum-free conditioned medium from HT1080 cells incubated for 0-24 hr in the presence of 10ng/ml TGF-ß were analyzed by Western blot using anti-TIMP antibody. Lane 1, 0 hr; lane 2, 3 hr; lane 3, 6 hr; lane 4, 12hr; lane 5, 18 hr; and lane 6, 24 hr.

Fig. 4. Effect of TGF-ß on the expression of TIMP mRNA. Confluent, quiescent HT1080 cells were incubated for 0-24 hr in the presence of 10ng/ml TGF-ß. The nitrocellulose filter shown in Fig. 2b was stripped and reprobed. Lane 1, 0 hr; lane 2, 3 hr; lane 3, 6 hr; lane 4, 12 hr; lane 5, 18 hr; and lane 6, 24 hr.

type IV collagenolytic activity, we measured the effect of TGF-ß on TIMP production. We measured TIMP level by Western blot analysis using specific anti-TIMP antibody. TGF-ß increased the TIMP level after 3 hr and reached a maximum (5-fold) after 12-24 hr (Fig. 3). Northern blot analysis using total RNA isolated from quiescent controls and from TGF-ß treated cells stimulated for 3, 6, 12, 18, and 24 hr showed a 7-fold increase at 6 hr with maximum induction (10-fold) after 18-24 hr (Fig. 4). Recent studies have shown that TIMP or TIMP2 forms a complex with the 92kDa and 68-72kDa type IV collagenase (4-6). Since TGF-ß induced TIMP, 68-72kDa and 92kDa type IV collagenases, and reduced net type IV collagenolytic activity by HT1080 cells, TIMP likely suppresses the enzymatic activity of the 68-72kDa and 92kDa type IV collagenases.

Recombinant TIMP Inhibits HT1080 Cell Invasion

To confirm the direct involvement of TIMP in the invasiveness of HT1080 cells, we tested the effect of human recombinant TIMP on HT1080 cell invasion. Human recombinant TIMP concentrations at 1, 5, 10, and 50µg/ml inhibited HT1080 cell invasion by 27, 36, 44, and 59%, respectively, compared to untreated cells.

In the present study, we showed two new findings. First, TGF-ß suppressed the invasiveness of HT1080 cells by reducing the net type IV

collagenase activity, although TGF-ß induced both type IV collagenases and TIMP. Second, as we already presented preliminary data (19), TGF-ß induced the 62kDa active type IV collagenase and might be involved in the activation of the 68-72kDa latent type IV collagenase. Brown et al. have recently reported that TGF-ß increases 72kDa type IV collagenase and induces 62kDa active form in HT1080 cells (20). Their data is consistent with ours. During the preparation of this manuscript, a report was published by Welch et al.. They have shown that TGF-ß increases the invasive and metastatic potential in mammary carcinoma cells (21). The result is in contrast to ours. They suggest that TGF-ß increases invasion and metastasis by increasing type IV collagenase and heparanase. Although they have not studied the effects of TGF-ß on TIMP expression, the difference is likely due to the different cell types.

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