Activation of human platelet-derived latent transforming growth factor- β 1 by human glioblastoma cells

Comparison with proteolytic and glycosidic enzymes

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Transforming growth factor- β (TGF- β), a regulator of cell growth and differentiation, is secreted by most cultured cells in latent form (L-TGF- β). Activation of L-TGF- β can be achieved by various physico-chemical treatments, including acidification, alkalization, heating and chaotropic agents. Proposed physiological activators include proteinases and glycosidases, which, however, only lead to limited activation (15–20 % of the total TGF- β activity after acidic activation). In the present study L-TGF- β 1 partially purified from human platelets was not activated by treatment with neuraminidase or the proteinases plasmin, endoproteinase Arg-C, elastase and chymotrypsin. The mechanism of activation of L-TGF- β was further assessed by using the human glioblastoma cell line 308, which releases biologically active TGF- β 2. Factor(s) secreted by 308 glioblastoma cells were found to be able to activate partially purified L-TGF- β 1 from human platelets. Our finding may prove to constitute a physiologically relevant mechanism for the activation of latent forms of TGF- β *in vivo*.

INTRODUCTION

Transforming growth factor- β (TGF- β) is a pleiotropic factor with important functions in regulation of cell growth, differentiation and development (for reviews see [1,2]). The majority of cultured cells studied to date have been shown to secrete TGF- β in an inactive latent form (L-TGF- β) that does not interact with specific TGF- β cell-surface receptors and is not recognized by antibodies to TGF- β . Three distinct glycosylated receptors for TGF- β have been identified [3,4]. The presence of specific TGF- β receptors on most cell types [5] and the ubiquity of the TGF- β molecule itself suggest that activation of L-TGF- β must be an integral component in the sequence of events leading to growth regulation by TGF- β .

Several different forms of L-TGF- β have been identified [6]. Platelet-derived L-TGF- β 1, a large latent complex ($\sim$ 235 kDa), is composed of three different subunits: the mature TGF- β 1 (25 kDa dimer) is non-covalently associated with the remainder of the processed precursor (75 kDa dimer), which in turn is disulphide-bonded to a novel 125–160 kDa TGF- β 1-binding protein (TGF- β 1-BP) with epidermal-growth-factor (EGF)-like repeats [7–10]. Although mature TGF- β 1 does not contain carbohydrate, TGF- β 1-BP is glycosylated [7], and the precursor remnant has three potential *N*-glycosylation sites, all of which carry carbohydrate [7].

TGF- β is present in serum complexed with the proteinase inhibitor α_2 -macroglobulin [11,12], which at least in some systems inactivates TGF- β by unknown mechanisms [13–15]. This complex is distinguished from the other latent forms in that it contains only mature, fully processed, TGF- β . It has been proposed that α_2 -macroglobulin in serum might function as a scavenging molecule for excess TGF- β [11,12].

Recombinant TGF- β 1 and TGF- β 2 expressed in mammalian cells were found to be synthesized in latent form [16–18]. L-TGF- β 1 obtained from recombinant sources is a small latent complex, composed of only mature TGF- β 1 and the *N*-terminal remnant

of the TGF- β 1 precursor, the latency-associated peptide (LAP) [6,16,19]. Site-directed mutagenesis of cysteine residues in the LAP has revealed that dimerization of the precursor pro-region may be crucial for latency [20]. Post-translational modifications of recombinant TGF- β 1 that take place before secretion include processing by acidic proteinases [21,22], glycosylation [21,23] and mannose 6-phosphorylation of the TGF- β 1 precursor [23, 24], as well as disulphide isomerization. In addition, both recombinant TGF- β 1 precursor and L-TGF- β 1 isolated from platelets have been reported to bind to the insulin-like growth factor (IGF)-II/mannose 6-phosphate receptor, which has been implicated in the targeting of lysosomal enzymes to the lysosomes [25]. However, all these processing events are clearly distinct from activation events that are required for the generation of biologically active TGF- β from the latent molecule.

Activation of L-TGF- β is achieved by a variety of physico-chemical treatments such as transient exposure to extremes of pH, heat or selected chaotropic agents, including SDS and urea [7,26,27]. Several potential physiological mechanisms for activation of the latent form have been investigated. The idea that acidic cellular environments, particularly in tumour tissues, could be conducive to activation of L-TGF- β has been proposed recently [28]. Furthermore, the broad-spectrum serine proteinases plasmin and cathepsin D have been proposed to be candidates for physiological activators of L-TGF- β from human platelets [29], in fibroblast-conditioned medium [30] or of recombinant L-TGF- β [29]. However, other reports clearly deny such a physiological role for plasmin [31,32]. The *N*-linked glycosylation of TGF- β 1-BP has been demonstrated to play a critical role in latency of platelet-derived TGF- β 1 [32]. Thus treatment of L-TGF- β 1 with endoglycosidase F or sialidase, or addition of sialic acid or mannose 6-phosphate, results in dose-dependent activation.

The activation of L-TGF- β by cultured cells has been reported in several cases. Monocytes activated by treatment with lipopolysaccharide and neutrophils appear to secrete TGF- β in a

Abbreviations used: Arg-C, endoproteinase Arg-C; EGF, epidermal growth factor; IGF, insulin-like growth factor; IL-2, interleukin-2; LAP, latency-associated peptide; Ova-7 T cells, ovalbumin-specific mouse T helper cells; PBS, phosphate-buffered saline; TGF- β , transforming growth factor- β ; L-TGF- β , latent TGF- β ; rhTGF- β , recombinant human TGF- β ; TGF- β 1-BP, TGF- β 1-binding protein.

fully active form, since acidification of the conditioned medium does not increase the amount of TGF- β activity [33]. A human erythroleukaemia cell line (HEL) is capable of activating added recombinant L-TGF- β 1 in a cell-associated manner [34]. When osteoclasts are activated by incubation with vitamin A or with bone particles, they liberate active TGF- β from the latent complex produced by bone organ cultures [35]. However, no factor or enzyme responsible for the activation of L-TGF- β could be demonstrated in conditioned medium from activated osteoclasts [35]. Finally, the production of an activated form of TGF- β by co-cultures of endothelial cells and pericytes or smooth-muscle cells [36,37] has been reported. The activation appears to be partially mediated by plasmin and requires close contact between the two cell types [38].

As much ambiguity about the susceptibility of latent forms of TGF- β to activation by a single enzyme remains, we have undertaken this study to measure effects of different enzymes on activation of platelet-derived L-TGF- β 1. However, there was no evidence for activation of L-TGF- β 1 from human platelets by plasmin and a number of other proteinases or neuraminidase. Consequently, the elucidation of physiological activation mechanisms may be derived from studies of cultured cells with the ability to secrete active TGF- β or to activate L-TGF- β from other sources. Indeed, when investigating human glioblastoma cells which secrete bioactive TGF- β 2 [39], it was found that these cells release factor(s) able to activate L-TGF- β 1 from human platelets.

MATERIALS AND METHODS

Materials

The polyclonal neutralizing antibody specific for TGF- β 1 was purchased from Oncomembrane Inc. (ams biotechnology, Lugano, Switzerland). The purified anti-TGF- β 2 IgG [39] was generously given by Sandoz Co., Basel, Switzerland. The non-immune rabbit control IgG was from Endogen Inc. (Boston, MA, U.S.A.). Highly purified recombinant human (rh) TGF- β 1 and - β 2 were kindly provided by Genentech Inc. (San Francisco, CA, U.S.A.) [40] and Sandoz Co. respectively. Clinically outdated platelet concentrates were obtained through the courtesy of the Swiss Red Cross (Zürich, Switzerland). All other chemicals used were of reagent grade and purchased from common commercial sources.

Methods

Cell culture media. Cells were cultured in an incubator in a humidified atmosphere containing 7% CO₂ at 37 °C. The following cell-culture media were used. (a) Ova-7 medium: Iscove's medium (Gibco, Paisley, Scotland, U.K.), supplemented with 1 mM-L-glutamine (Gibco), soybean phosphatidylcholine (50 mg/l; Nattermann Phospholipid G.m.b.H., Köln, Germany), human transferrin (100 mg/ml; Behringwerke A.G., Marburg, Germany), BSA (1 g/l; Fluka A.G., Buchs, Switzerland) and 2-mercaptoethanol (50 μ M; Fluka A.G.). (b) Glioblastoma medium: Dulbecco's modification of Eagle's medium (Flow Laboratories, Baar, Switzerland), supplemented with 2 mM-L-glutamine (Gibco) and 5% heat-inactivated (56 °C, 30 min) fetal-calf serum (Amimed, Basel, Switzerland).

Bioassay for quantification of TGF- β activity. H-2^b major histocompatibility complex class II-restricted ovalbumin-specific mouse T helper cells (Ova-7 T cells) were maintained in Ova-7 medium in the presence of interleukin-2 (IL-2; 20 units/ml; Amersham International, Amersham, Bucks., U.K.). For assessment of TGF- β growth-inhibitory activity, Ova-7 T cells [(0.5–1) $\times$ 10⁴ cells/well], cultured in 96-well flat-bottom Falcon

microtitre plates (Becton Dickinson, Lincoln Park, NJ, U.S.A.) for 72 h, were incubated with IL-2 (50 units/ml) and test samples in a final volume of 200 μ l. At 16 h before harvest, 1 μ Ci of [³H]thymidine (sp. radioactivity 5 Ci/mmol; Amersham International) was added per well. The results of the proliferation assays are given as percentage inhibition, calculated as 100 – [(c.p.m. in sample/c.p.m. in control) $\times$ 100]. Control values were obtained from Ova-7 cells stimulated with IL-2 (50 units/ml) to grow maximally and were between 200 000 and 300 000 c.p.m./well. A negative value for percentage inhibition indicates stimulatory activity compared with the control. All results are derived from mean values of triplicates, and s.d. was below 10%.

Glioblastoma cells and production of supernatant. The human glioblastoma cell line 308 used in this study has been described [41]. For production of supernatant, serum-free glioblastoma medium supplemented with 1 μ g of indomethacin (Sigma, St. Louis, MO, U.S.A.)/ml to prevent formation of prostaglandins was added to confluent cultures. After 3 days the spent culture media were collected and stored at –20 °C until further use.

Proteinases and partial proteolytic digestion. Plasmin (EC 3.4.21.7) was purchased from Sigma (product no. P 4895) or from Boehringer Mannheim (Schweiz) A.G. (Rotkreuz, Switzerland; cat. no. 602 361). Plasmin from Boehringer Mannheim was delivered as a suspension in 3.2 M-(NH₄)₂SO₄ solution which has proved to be incompatible in the Ova-7 T cell proliferation assay. Therefore, the enzyme was transferred to phosphate-buffered saline (PBS; 140 mM-NaCl/10 mM-Na₂HPO₄/3 mM-KH₂PO₄) by using a Centricon 10 micro-concentrator (Amicon Schweiz, Lausanne, Switzerland). Endoproteinase Arg-C (EC 3.4.21.40; cat. no. 269 590), chymotrypsin (EC 3.4.21.1; cat. no. 103 306) and elastase (EC 3.4.21.36; cat. no. 1027 891) all were from Boehringer Mannheim (Schweiz) A.G. Before use, all proteinases were tested for proteolytic activity in a spectrophotometric assay as explained below. For partial proteolytic digestion, samples were incubated with the indicated concentrations of enzymes. The incubations were performed at 37 °C for 2 h in PBS (pH 8.5). Proteinase activity was then quenched by addition of Ova-7 culture medium, which contains 0.1% BSA, and samples were subjected to assessment of growth-inhibitory activity either directly or after acidification.

Treatment with neuraminidase. Samples were incubated with the indicated concentrations of neuraminidase type V (EC 3.2.1.18) from Sigma (product no. N 2876) at 37 °C for 18 h in PBS (pH 6.0). Subsequently they were diluted with Ova-7 culture medium, and growth-inhibitory activity was determined either directly or after acidification.

Partial purification of latent TGF- β 1 from human platelets. Standard platelet concentrates were processed as described previously [27]. Briefly, platelets were washed with PBS, added to PBS supplemented with 1 mM-phenylmethanesulphonyl fluoride (Fluka A.G.), 1 μ g of leupeptin hemisulphate salt (Fluka A.G.)/ml, 1 μ g of pepstatin A (Fluka A.G.)/ml and 3 μ g of aprotinin (Sigma)/ml and sonicated immediately, then left for 2 h at 4 °C. Cell debris was removed by centrifugation (1 h, 4 °C, 100 000 g) and the supernatant was considered as the neutral extract. The neutral extract was freeze-dried, rehydrated in a small volume of water, centrifuged to remove insoluble material and applied to a Sephadex G-200 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden) equilibrated with PBS (pH 7.4) [35]. The fractions containing L-TGF- β 1 were collected and further purified on a Mono Q HR 5/5 column (Pharmacia Fine Chemicals AB), equilibrated with PBS (pH 7.4). Bound material was eluted with a linear gradient of 0–0.6 M-NaCl in the same buffer. The fractions containing L-TGF- β 1 were pooled and rechromatographed on the same column by using a linear gradient of 0.12–0.3 M-NaCl in PBS (pH 7.4). Three fractions

Fig. 1. Activation of platelet L-TGF- β 1 by transient acidification (a) and highly specific neutralization of the growth-inhibitory activity of native and acid-activated platelet L-TGF- β 1 by various antibodies (b)

(a) The growth-inhibitory activity of L-TGF- β 1 was assessed either directly ($\circ$) or after acidification ($\bullet$) at various final dilutions as indicated. Results are expressed as percentage suppression of the IL-2-induced proliferative response of Ova-7 T cells cultured in the presence of a medium control (200 000–300 000 c.p.m./well). (b) Latent TGF- β 1 was used either non-acidified (1:400 diluted) or after acidification (1:1600 diluted). Growth-inhibitory activity was determined either directly ($\square$) or after incubation with specific neutralizing antibodies directed against TGF- β 1 ($\boxtimes$) (80 μ g/ml) and TGF- β 2 ($\boxdot$) (80 μ g/ml), or with non-immune rabbit control IgG ($\boxplus$) (80 μ g/ml). In order to show the high specificity of the antibodies used, rhTGF- β 1 (10 pM) and rhTGF- β 2 (10 pM) were subjected to the same treatment. Results are expressed as described above.

Fig. 2. Treatment of platelet L-TGF- β 1 and rhTGF- β 1 or glioblastoma supernatant and rhTGF- β 2 with plasmin

Samples were incubated either without ($\circ$) or with ($\square$) plasmin (2 units/ml, Sigma) for 2 h at 37 °C. Growth-inhibitory activity was then determined in neutral samples ($\circ$, $\square$) or after acidification ($\bullet$, $\blacksquare$) at various final dilutions or concentrations as indicated. Titration curves are shown for L-TGF- β 1 (a) and rhTGF- β 1 (b), or glioblastoma supernatant (c) and rhTGF- β 2 (d). Results are expressed as described in Fig. 1.

containing most of the L-TGF- β 1 were pooled, and this pool will be referred to below as partially purified L-TGF- β 1.

Preparation of neutral and acidified samples. Before acidification, samples were diluted with Ova-7 culture medium (containing 0.1 % BSA). Then they were transiently acidified by addition of 1 M- or 2 M-HCl to a final concentration of 70 mM (final sample pH 2.0) for 1 h at room temperature. The samples were re-neutralized by addition of 1 M- or 2 M-NaOH to a final concentration of 70 mM and 1 M-Hepes buffer (pH 7.0) to a final concentration of 25 mM. Neutral samples were treated likewise, but no acid or base was added.

Antibody neutralization. For neutralization of TGF- β activity, samples were preincubated in assay medium for 1 h at room temperature on a rocking platform with the indicated specific neutralizing antibodies at the given concentrations. These pre-incubations were then tested for remaining TGF- β activity in the Ova-7 T cell proliferation assay at the final dilutions as indicated. Controls were preincubated in assay medium without antibodies and treated likewise.

Spectrophotometric assay for proteinases. DL-N- α -Benzoyl-L-arginine 4-nitroanilide hydrochloride (Bachem A.G., Bubendorf, Switzerland) was used as substrate for plasmin and Arg-C [42],

Table 1. Treatment of platelet-derived L-TGF- β 1 with proteinases

Platelet-derived L-TGF- β 1 and glioblastoma supernatant were incubated with the given concentrations of proteolytic enzymes as described in the Materials and methods section. No enzymes were added to control incubations. rhTGF- β 1 and rhTGF- β 2 were used to illustrate the extent of proteolytic degradation. Growth-inhibitory activity was assessed in neutral and acidified samples as indicated. Results are expressed as activation index, calculated from titration curves by determining the reciprocal dilution necessary for 50% suppression of the IL-2-induced Ova-7 T cell proliferation. The activation index of control incubations was arbitrarily set to 1; values less than 1 indicate diminished growth-inhibiting activity compared with control incubations.

Proteinase	Latent TGF- β 1		rhTGF- β 1
	Non-acidified	Acidified	
Control	1.00	3.83	1.00
Arg-C (4.4 units/ml)	1.23	5.86	0.76
Control	1.00	7.04	1.00
Elastase (0.05 unit/ml)	1.31	5.37	0.75
Control	1.00	7.82	1.00
Chymotrypsin (0.01 unit/ml)	1.20	6.32	0.68

Proteinase	Glioblastoma supernatant		rhTGF- β 2
	Non-acidified	Acidified	
Control	1.00	3.25	1.00
Arg-C (4.4 units/ml)	1.05	2.95	0.72
Control	1.00	2.82	1.00
Elastase (0.1 unit/ml)	0.79	2.62	0.61
Control	1.00	2.17	1.00
Chymotrypsin (0.1 unit/ml)	0.91	2.81	0.50

and hydrolysis of the substrate was monitored by an increase in A_{405} . *N*-Benzoyltyrosine *p*-nitroanilide (Bachem A.G.) was used as substrate for chymotrypsin [43], and hydrolysis of the substrate was monitored by an increase in A_{385} . *N*-Succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Bachem A.G.) was used as substrate for elastase [44], and hydrolysis of the substrate was monitored by an increase in A_{405} .

RESULTS

Our previous observation that glioblastoma cells secrete biologically active TGF- β 2 [39] prompted us to investigate potential physiological activators of L-TGF- β . In a first set of experiments several proteinases and neuraminidase were tested for their capacity to activate partially purified platelet-derived L-TGF- β 1. Additionally, glioblastoma cells, cellular extracts and glioblastoma-derived cell-free supernatant were used to look for factors capable of activating L-TGF- β 1.

Effects of acidification and neutralizing antibodies on the growth-inhibitory activity of L-TGF- β 1

Fig. 1(a) demonstrates that native L-TGF- β 1 partially purified from human platelets suppresses the growth of IL-2-stimulated Ova-7 T cells. This growth-inhibitory action can be potentiated more than 10-fold by transient acidification to pH 2.0. As evidenced in Fig. 1(b), the growth-inhibitory action of native as well as acid-activated platelet L-TGF- β 1 on Ova-7 T cells is neutralized by an antibody specific for TGF- β 1, but not by an anti-TGF- β 2 antibody or non-immune rabbit control IgG. With regard to neutralization, identical results are obtained when rhTGF- β 1 is subjected to the same treatment. The growth-inhibitory action of rhTGF- β 2 is neutralized by an antibody specific for TGF- β 2, but not by an anti-TGF- β 1 antibody or non-immune rabbit control IgG. Hence the neutralizing antibodies used in this study are highly specific; especially, the anti-TGF- β 2 antibody does not display any cross-reactivity with TGF- β 1.

Effects of partial proteolytic digestion and treatment with neuraminidase on the growth-inhibitory activity of L-TGF- β 1

Although limited action of proteinases and glycosidases has been reported to result in the partial activation of native L-TGF- β 1 [30,32], controversy about this point has arisen recently.

Fig. 3. Treatment of platelet L-TGF- β 1 and glioblastoma supernatant with neuraminidase

Samples were incubated either without (○) or with (□) neuraminidase (2 units/ml) for 18 h at 37 °C. Growth-inhibitory activity was then determined in neutral samples (○, □) or after acidification (●, ■) at various final dilutions as indicated. Titration curves are shown for L-TGF- β 1 (a) and glioblastoma supernatant (b). Results are expressed as described in Fig. 1.

Fig. 4. Treatment of rhTGF- β 1 and rhTGF- β 2 with neuraminidase

Samples were incubated either without (○) or with (□) neuraminidase (2 units/ml) for 18 h at 37 °C. Growth-inhibitory activity at various final concentrations is shown for rhTGF- β 1 (a) and rhTGF- β 2 (b). Results are expressed as described in Fig. 1.

Fig. 5. Incubation of platelet L-TGF- β 1 with glioblastoma cells in culture

Glioblastoma cells were seeded at a density of 0.5×10^6 cells/well in a Linbro plate in a volume of 2 ml of glioblastoma medium containing indomethacin (1 μ g/ml). After 3 days, supernatants were removed and cells were washed twice with serum-free glioblastoma medium containing indomethacin. Subsequently, L-TGF- β 1 was diluted 10-fold with serum-free glioblastoma medium and added to the glioblastoma cells (final volume 400 μ l/well) in the presence of indomethacin (1 μ g/ml) and BSA (0.5 mg/ml). After various periods of time (0 min, 6 h, 24 h, 48 h), supernatants were removed and centrifuged to clear them from cells and cell debris. Neutral (○) or acidified (●) supernatants were then incubated with a neutralizing antibody specific for TGF- β 2 at a concentration of 80–160 μ g/ml. Growth-inhibitory activity was subsequently assessed by titration on Ova-7 T cells. Alternatively, L-TGF- β 1 was diluted 10-fold and incubated in a 96-well Falcon flat-bottom microtitre plate under the same conditions as described above but in the absence of glioblastoma cells. Neutral (○) or acidified (●) samples were further processed as given above. Efficient neutralization of TGF- β 2 present in supernatants from glioblastoma cells was verified by adding a specific anti-TGF- β 2 antibody to non-acidified (△) or acidified (▲) control glioblastoma supernatants. Results are expressed as described in Fig. 1.

Therefore, human platelet-derived L-TGF- β 1 was tested for susceptibility to such action. As shown in Fig. 2, partial proteolytic digestion of L-TGF- β 1 with plasmin (Sigma) does not liberate active TGF- β 1 from its latent complex, but even somewhat decreases activity of L-TGF- β 1 (Fig. 2a) as well as rhTGF- β 1 (Fig. 2b). Comparable results are obtained when using crude glioblastoma supernatant as a source of TGF- β 2 (Fig. 2c) or rhTGF- β 2 (Fig. 2d). Pretreatment of platelet-derived L-TGF- β 1 with plasmin does not affect subsequent activation of L-TGF- β 1 by transient acidification (Fig. 2a). The same series of experiments, when performed with plasmin purchased from Boehringer Mannheim and used at a concentration of up to 6 units/ml, revealed similar results (not shown).

Several other serine proteinases were used in a further series of experiments. Similarly to plasmin, the endoproteinase Arg-C hydrolyses peptide and ester bonds at the carboxylic side of arginine residues in a highly specific manner. Chymotrypsin and elastase were chosen for their specificity for cleavage at the carboxylic side of aromatic and uncharged non-aromatic amino acids respectively. All these proteolytic enzymes at high concentrations drastically degraded TGF- β obtained from cell extracts of platelets or from supernatant of glioblastoma cells. The highest possible concentration of enzymes at which no substantial degradation should occur was determined by incubating rhTGF- β 1 or rhTGF- β 2 with increasing amounts of enzymes. Therefore, different concentrations of proteolytic

Table 2. Incubation of platelet-derived L-TGF- β 1 with glioblastoma cell extracts

Glioblastoma cells (20×10^6 /ml in PBS) were sonicated for 40 s on ice and then kept on ice for an additional 1 h. Cell extracts were either taken directly (referred to as crude extract) or as the supernatant after high-speed centrifugation (15 min, 16000 g, 4 °C; referred to as membrane-free extract). Latent TGF- β 1 was incubated with an equal volume of cell extracts for the indicated periods of time at 37 °C. Samples were centrifuged to remove particles and assessed for growth-inhibitory activity in the Ova-7 T cell proliferation assay at final dilutions of 1:200. As controls, L-TGF- β 1 was incubated with an equal volume of PBS as described above, and neutral or acidified samples were subsequently tested at final dilutions of 1:200. In order to evaluate possible non-specific inhibition, the cell extracts were incubated with an equal volume of PBS and subjected to the same procedure as mentioned above. Results are expressed as described in Fig. 1.

Time of incubation	Conditions	Inhibition (%)
1 h	L-TGF- β 1, neutral	9.0
	L-TGF- β 1, acidified	86.2
	L-TGF- β 1 plus crude extract	8.5
	L-TGF- β 1 plus membrane-free extract	14.5
	Crude extract alone	3.0
	Membrane-free extract alone	-2.0
2 h	L-TGF- β 1, neutral	10.0
	L-TGF- β 1, acidified	85.0
	L-TGF- β 1 plus crude extract	-12.5
	L-TGF- β 1 plus membrane-free extract	10.0
	Crude extract alone	10.0
	Membrane-free extract alone	6.5
4 h	L-TGF- β 1, neutral	21.0
	L-TGF- β 1, acidified	82.0
	L-TGF- β 1 plus crude extract	2.0
	L-TGF- β 1 plus membrane-free extract	3.0
	Crude extract alone	2.7
	Membrane-free extract alone	7.5

enzymes used in the experiments for partial digestion also reflect distinct sensitivity of rhTGF- β 1 and rhTGF- β 2 towards proteolytic degradation (Table 1). No considerable activation of

human platelet-derived L-TGF- β 1 and TGF- β 2 in glioblastoma supernatant was observed for all the enzymes examined (Table 1). Moreover, total TGF- β activity determined after transient acidification, as well as the growth-inhibitory activity of rhTGF- β 1 and rhTGF- β 2, were decreased in most cases as compared with control incubations. Lower concentrations of proteinases also did not activate L-TGF- β 1 (or glioblastoma-derived TGF- β 2), and gave no, or minor, decrease in growth-inhibitory activity of rhTGF- β 1 and rhTGF- β 2 (results not shown).

It was shown previously that the carbohydrate structures in TGF- β 1-BP are crucial to its latency [32]. As illustrated in Fig. 3, no activating effect was observed when platelet L-TGF- β 1 or glioblastoma supernatant was incubated with neuraminidase (2 units/ml). Control incubations of rhTGF- β 1 and rhTGF- β 2 with neuraminidase led to the surprising finding that neuraminidase markedly increased the growth-inhibitory activity of rhTGF- β 1 (Fig. 4a), whereas no such effect could be detected with rhTGF- β 2 (Fig. 4b).

Investigation of mechanisms for activation of L-TGF- β 1 mediated by glioblastoma cells

Glioblastoma cells secrete preferentially biologically active TGF- β 2 into their supernatant [39]. Since the glioblastoma-cell-derived TGF- β 2 activity can be completely neutralized by a specific anti-TGF- β 2 antibody, these cells should be useful to search for physiological factors involved in activation of L-TGF- β 1. In order to evaluate the activation of L-TGF- β 1 by cultured glioblastoma cells, L-TGF- β 1 was layered on to a confluent culture of glioblastoma cells, and culture supernatant was taken after different exposure times. All samples were pretreated with neutralizing anti-TGF- β 2 antibody to eliminate TGF- β 2 activity released by the glioblastoma cells, thus specifically allowing detection of only the platelet-derived TGF- β 1 in the subsequent bioassay. As Fig. 5 reveals, no activation of L-TGF- β 1 occurred within 48 h. Surprisingly, after 6 h growth-inhibitory activity in neutral supernatants was markedly decreased. However, total TGF- β 1 activity in acid-activated supernatants remained constant, indicating that L-TGF- β 1 was not simply lost from the supernatant by non-specific absorption or degradation during the incubation. Control incubation of L-TGF- β 1 in the absence of glioblastoma cells caused a smaller loss of growth-inhibitory activity in neutral samples at low dilutions. At 24 h and 48 h total TGF- β 1 activity in acidified samples from control

Fig. 6. Incubation of platelet L-TGF- β 1 with glioblastoma supernatant leads to activation

Latent TGF- β 1 was incubated with an equal volume of glioblastoma supernatant for 6 h at 37 °C in the presence of BSA (0.5 mg/ml). Neutral ($\square$) or acidified ($\blacksquare$) samples were then incubated with a neutralizing antibody specific for TGF- β 2 at a concentration of 40 μ g/ml, and growth-inhibitory activity was subsequently determined by titration on Ova-7 T cells. Alternatively, L-TGF- β 1 was incubated with medium used for the production of glioblastoma supernatant and further processed exactly as described above ($\circ$, $\bullet$). Efficient neutralization of TGF- β 2 present in glioblastoma supernatant was ensured by incubating it with an equal volume of PBS and further processing as above (Δ , $\blacktriangle$). Results are expressed as described in Fig. 1.

incubations somewhat vanished, possibly owing to non-specific absorption. When supernatants from control wells with glioblastoma cells alone were analysed for their content of TGF- β 2 in a parallel experiment, it was recognized that the glioblastoma cells secreted increasing amounts of TGF- β 2 in the course of the incubation (results not shown). Therefore, as expected, the active TGF- β 2 present in acidified supernatant from glioblastoma cells after 48 h could not be completely neutralized when assayed at a final dilution of 1:1600, owing to the exhausted capacity of the anti-TGF- β 2 antibody.

In a further series of experiments, cell extracts prepared by sonication of glioblastoma cells were incubated for up to 4 h with L-TGF- β 1, and TGF- β 1 activity was subsequently measured in the Ova-7 T cell proliferation assay. As seen in Table 2, a crude extract as well as an extract devoid of membrane particles were tested and found to exert no stimulatory effect on the growth-inhibiting activity of platelet L-TGF- β 1.

In further experiments, cell-free glioblastoma supernatant was tested for its activating capacity for human platelet-derived L-TGF- β 1. The results of a representative experiment are illustrated in Fig. 6. It is important to note that the TGF- β 2 activity derived from the glioblastoma cells themselves was completely and specifically neutralized before determination of platelet-derived TGF- β 1 activity in the Ova-7 assay. Incubation of platelet L-TGF- β 1 with a glioblastoma-cell-derived supernatant results in an almost 3-fold increase in native platelet-derived TGF- β 1 activity by 6 h, whereas a control incubation clearly shows no such activation of L-TGF- β 1. This argues in favour of glioblastoma cells secreting factor(s) into their supernatant which have the capacity to activate platelet-derived L-TGF- β 1. Likewise, pretreatment of platelet L-TGF- β 1 with glioblastoma cell supernatant for 6 h and subsequent acidification also results in an almost 3-fold increase in TGF- β 1 activity as compared with a control incubation without glioblastoma-cell supernatant followed by acidification. Therefore the results also demonstrate that TGF- β 1 activity of the platelet L-TGF- β 1 sample after transient acidification does not necessarily represent maximum biologically available TGF- β 1, since also the acid-activated L-TGF- β 1 was potentiated by pretreatment with glioblastoma supernatant.

DISCUSSION

Cultured human glioblastoma cells have been shown to secrete an immunosuppressive factor which inhibits the lectin-induced response of mouse thymocytes [45], the generation of cytotoxic T cells [40,45], as well as the IL-2-induced growth of IL-2-dependent T-cell lines [13,45]. This factor, initially termed glioblastoma-derived T-cell suppressor factor ('G-TsF'), has been purified and cloned from a human glioblastoma cell line and was found to be TGF- β 2 [46–48]. Most interestingly, further investigations have revealed that cultured human glioblastoma cells preferentially produce a biologically active form of TGF- β 2 [39]. In contrast, most other cells release TGF- β in latent, biologically inactive, form. Human platelets, the most abundant source of human TGF- β 1, also store this factor in latent form.

Therefore the present study was undertaken to elucidate possible mechanisms by which human platelet-derived L-TGF- β 1 is activated under physiological conditions, and whether glioblastoma cells dispose of a mechanism effective for the activation of L-TGF- β . Plasmin as well as other proteolytic enzymes such as Arg-C, elastase and chymotrypsin failed to exert effects on the growth-inhibiting action of L-TGF- β 1 on Ova-7 T cells. The same was true when neuraminidase was tested for its potential to activate L-TGF- β 1. However, cultured human glioblastoma cells secrete factor(s) which have the capacity to

activate platelet-derived L-TGF- β 1. The results presented herein give evidence that the activation of L-TGF- β 1 does not result from the action of the above-mentioned enzymes, but probably is achieved by factor(s) not yet characterized that are present in glioblastoma cell-free supernatant.

Lymphocytes have receptors for active TGF- β , and active TGF- β prevents IL-2-driven proliferation of T cells [13,49]. As shown herein, active TGF- β suppresses the growth of an ovalbumin-specific mouse T helper-cell clone. We have confirmed previous reports [26,27] that transient acidification activates partially purified L-TGF- β 1 from human platelets by a factor of 10–20-fold. Moreover, native L-TGF- β 1 also has intrinsic growth-inhibiting activity in this assay system. This finding is analogous to some other reports, since native L-TGF- β 1 purified to homogeneity from human platelets was recently reported to inhibit cell growth when added at high concentrations, but to increase activity 200-fold after transient exposure to 1 M-HCl when tested on pig aortic endothelial cells [32]. In addition, a 200-fold excess of native L-TGF- β 1 over acidified L-TGF- β 1 decreased binding of 125 I-labelled TGF- β 1 to normal rat kidney fibroblasts (NRK cells) to the same extent [32]. Similar results were obtained with recombinant human L-TGF- β 1 on mink lung epithelial (CCL-64) cells [34]. The range of activation *in vitro* of L-TGF- β by acid treatment may depend on the specific assay system used for the detection of TGF- β . The growth-inhibitory activity of L-TGF- β in conditioned medium from African green-monkey kidney epithelial (BSC-1) cells (consisting mostly of TGF- β 2) can be stimulated 8–10-fold after acidification [50]. Limited activation of L-TGF- β from chick-embryo fibroblasts [26] and from fibroblast-conditioned medium [30] at pH 4–5 has been reported previously. However, in our experiments, and according to two other reports [7,31], no evidence of activation of platelet L-TGF- β 1 under these mildly acidic conditions was found (results not shown).

Very much controversy about the susceptibility of L-TGF- β from different sources to activation by plasmin and other proteolytic enzymes exists. Possibly the use of different assay systems to measure the effects of plasmin on activation of L-TGF- β may lead to the contradictory results (e.g. bioassay versus radioreceptor assay). Whereas L-TGF- β 1 from human platelets has been shown not to be activated by plasmin or cathepsin D [32], it has been reported that incubation of platelet L-TGF- β 1 and fibroblast L-TGF- β 1 with increasing amounts of plasmin produced an increase in TGF- β competing activity [29,30]. In addition, four distinguishable forms of bone-matrix-derived L-TGF- β were described, only two of which are activated up to 30% by plasmin [51]. A mechanism for the activation of recombinant L-TGF- β 1 by plasmin [29] was proposed that includes cleavage of the N-terminal region of the TGF- β 1 precursor, thereby destabilizing the latent complex and releasing active TGF- β 1. However, in all previous studies, plasmin activates only a relatively small proportion of available L-TGF- β , even in the presence of relatively high levels of enzyme activity. As shown here, several proteinases, including plasmin, at adequate concentrations did not liberate active TGF- β from the latent complex; instead they even partially decreased TGF- β activity. Similar results were obtained with recombinant L-TGF- β 1 after exposure to comparable amounts of plasmin [31]. The results of the present work further support the evidence that plasmin is unlikely to represent the physiological mechanism by which cells activate L-TGF- β *in vivo*, especially in the light of the fact that levels of plasmin that liberate some active TGF- β also cause substantial degradation thereof.

To investigate the possibility that sialic acid residues present in the latent complex of TGF- β 1 may be involved in complex formation and thus important for latency, we tested

neuraminidase/sialidase, which specifically removes sialic acid residues [52], for activating effects on L-TGF- β 1. Neuraminidase/sialidase up to a concentration of 2 units/ml had no relevant effect on the growth-inhibiting activity of human platelet L-TGF- β 1. It has been reported that treatment with a low concentration of neuraminidase/sialidase (1 unit/ml) activates platelet L-TGF- β 1 significantly only if the treated L-TGF- β 1 was added at high concentration to the target cells [32]. The activating effect of neuraminidase/sialidase on recombinant L-TGF- β 1 is also reported to be limited [31]. The increase in activity of highly purified rhTGF- β 1 after treatment with neuraminidase/sialidase as reported here (Fig. 4) needs further investigation.

Incubation of L-TGF- β 1 with glioblastoma cells did not increase the growth-inhibiting activity of L-TGF- β 1 on Ova-7 cells. Consequently, it is unlikely that the activation of L-TGF- β 1 is mediated by a membrane-bound process or, alternatively, TGF- β 1 liberated from the latent complex may not be released into the supernatant. It has been shown that cultured human erythroleukaemia (HEL) cells can activate recombinant L-TGF- β 1, but do not release active TGF- β 1 in the culture supernatant [34]. HEL cells therefore activate L-TGF- β 1 in a cell-associated manner, and subsequently respond to TGF- β 1 by decreased growth rate. Since most cultured glioblastoma cell lines are not growth-inhibited by TGF- β (S. Bodmer, J. Heid, U. Werner & A. Fontana, unpublished work), we cannot rule out that also these cells activate L-TGF- β 1 in a strictly cell-associated manner. In addition, activated osteoclasts can activate the bone-derived L-TGF- β complex, probably by the local acidic environment produced by them [35].

Conversion of L-TGF- β into biologically active TGF- β was also reported in co-cultures of endothelial cells and pericytes or smooth-muscle cells [36,37]. Activation appeared to require cell-cell contact or the very close apposition of the two different cell types. The activation reaction also seemed to require plasmin, as inclusion of inhibitors of plasmin in the heterotypic culture medium abrogated the formation of active TGF- β [36]. In addition, it has been described that the generation of TGF- β in these co-cultures stimulated the production of the proteinase inhibitor plasminogen activator inhibitor-1 (PAI-1) [38]. The increased expression of PAI-1 subsequently blocked the activation of the proteinase required for conversion of L-TGF- β into TGF- β . Thus the activation of L-TGF- β in this kind of cell co-culture appears to be a self-regulating system. Recently, mixed lymphoid cell cultures have been shown to produce endogenously L-TGF- β capable of regulating lymphocyte activities *in vitro* [53]. The mechanism by which these cells convert L-TGF- β into its apparently active form remains unclear.

As indicated by the lack of activation by incubation of platelet L-TGF- β 1 with cellular extracts prepared from glioblastoma cells, activation of L-TGF- β 1 is not attributable to a process which is localized intracellularly. It must be considered, however, that we cannot exclude the destruction of a putative activating mechanism in the course of preparation of cell extracts. Specifically, the spatial orientation of enzymes may be critical for activation, and sonication is a relatively harsh treatment probably incompatible with preservation of enzyme activity.

The cellular mechanism by which glioblastoma cells produce TGF- β 2 in biologically active form still awaits elucidation. However, as shown in the present report, glioblastoma cells secrete factor(s) which are also able to activate platelet-derived L-TGF- β 1. Therefore the mechanism of activation of latent forms of TGF- β probably is similar for both TGF- β 1 and TGF- β 2. The similarity in the properties of the latent complexes of the different TGF- β subtypes has been established as far as structure and activation are concerned [31]. It remains to be clarified

whether the activation of latent forms of TGF- β is mediated by a multi-step process or a single entity. Interestingly, pretreatment of platelet L-TGF- β 1 with glioblastoma supernatant and subsequent activation by transient acidification leads to a more pronounced increase of TGF- β activity than does control acidification alone. In this regard, it is important to note that activation *in vitro* of recombinant L-TGF- β 1 as well as of platelet L-TGF- β 1 by transient acidification is at least partially reversible, in a time- and concentration-dependent manner [6,8]. In addition, the rate of re-association of the latent complex was reported to be dependent on sample preparation and mode of activation [31]. Consequently, TGF- β activity in samples activated by transient acidification does not necessarily represent maximum biologically available TGF- β . This may explain the apparent differences in TGF- β 1 activity in acid-activated samples. Finally, the factor(s) in glioblastoma-cell supernatant responsible for the activation of L-TGF- β 1 are not plasmin-related, and remain to be identified.

Production of active TGF- β as well as the activation of circulating or locally produced L-TGF- β by a tumour cell may have several implications. Impaired cell-mediated immunity has been described in patients with glioblastoma [45], and glioblastomas are characterized by high malignancy. The production of active TGF- β and the activation of L-TGF- β may contribute to decreased immune surveillance for tumour development and neovascularization of the tumour tissue. In conclusion, the regulation of production of active TGF- β and of activation of L-TGF- β is most crucial for normal and tumour cell growth. It is probable that the study of systems such as glioblastoma tumour cells leads to the understanding of physiologically relevant mechanisms for activation of L-TGF- β .

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