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Active Transforming Growth Factor-β in Human Melanoma Cell Lines: No Evidence for Plasmin-Related Activation of Latent TGF-β

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Abstract Cultured human melanoma cells were found to secrete TGF-β mostly in latent biologically inactive form but in addition five of six melanoma cell lines studied produced in conditioned culture medium active TGF-β in the range from 370 to 610 pg per 10^6 cells per 24 h. A distinct characteristic of these melanoma cell lines is that they form active surface-bound plasmin by the activation of plasminogen with surface-bound tissue-type plasminogen activator. The present study was performed to assess the role of plasmin in the process of latent TGF-β activation in the melanoma cell lines. No direct correlation was found between cell-associated plasmin activity and the amount of active TGF-β present in the conditioned medium of individual cell lines. The melanoma cell lines exhibited diverse responses to exogenous active TGF-β1; three cell lines were growth-stimulated, two were growth-inhibited, and one had a very low sensitivity to the growth factor. The active TGF-β produced by the melanoma cells was found to inhibit the natural killer cell function of peripheral blood lymphocytes, suggesting that it may have an immunosuppressive effect and a role in the development of melanomas.

Key words: transforming growth factor-β, melanoma, activation

INTRODUCTION

Transforming growth factor-βs (TGF-β) comprise a highly conserved family of multifunctional cell-regulatory peptides considered to play significant roles in various pathological processes including carcinogenesis [Roberts and Sporn, 1990; Lyons and Moses, 1990; Lahm and Odartchenko, 1993]. Until now, five TGF-β isotypes have been identified. Three of these isoforms, TGF-β1, -β2, and -β3, with similar but not identical biological activities, have been found in mammalian cells [Massagué, 1990]. Earlier studies, analyzing TGF-β either at mRNA or protein level, have reported the presence of the growth factor in human melanoma cells [Derynck et al., 1987; Lizonova et al., 1990; Rodeck et al., 1991]. In a more detailed study it has been shown that cultured human melanoma cells express constitutively all three TGF-β isoforms [Albino et al., 1991].

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The action of TGF-β is mediated through binding to specific cell membrane receptors. TGF-βs bind to at least three different receptor-like molecules [Massagué, 1992]. TGF-β receptors are ubiquitously expressed on different cell types [Wakefield et al., 1987] including melanomas [Rodeck et al., 1994].

In general, TGF-β is produced and secreted in a biologically inactive high molecular weight form (L-TGF-β) by many types of cultured cells, both transformed and nontransformed [Pircher et al., 1986; Miyazono et al., 1988] but the physiological relevance of the TGF-β appears to rest on regulation of its activation [Laiho and Keski-Oja, 1989]. Activation of L-TGF-β in vitro can be achieved by a variety of physicochemical treatments such as transient acidification, alkalization, heating, or exposure to certain chaotropic agents, including SDS and urea [Pircher et al., 1986; Miyazono et al., 1988; Lyons et al., 1988; Brown et al., 1990]. Although acid activation has provided information regarding the production and secretion of TGF-β from a variety of cell types, it is unlikely to be a physiological mechanism. Several potential physiologic mecha-

nisms for activation of the latent form have been investigated. Cell-associated proteases were considered likely to be involved in the activation since the cDNA sequence indicated that the natural TGF-β homodimer is derived proteolytically from a longer precursor polypeptide which is secreted [Derynck et al., 1985]. Based on several studies, the broad spectrum serine protease, plasmin, and the aspartyl protease, cathepsin D, have been proposed to be physiological activators of L-TGF-β from human platelets [Lyons and Moses, 1990], in fibroblast-conditioned medium [Lyons et al., 1988], or from Chinese hamster ovary cells transfected with TGF-β cDNA [Lyons et al., 1990]. However, other reports have denied such a physiological role for plasmin [Miyazono and Heldin, 1989; Brown et al., 1990; Huber et al., 1991].

The activation of L-TGF-β by cultured cells has been reported in several cases. These include keratinocytes [Glick et al., 1989], monocytes/granulocytes [Grotendorst et al., 1989], endothelial cells cocultured with pericytes [Antonelli-Orlidge et al., 1989], breast cancer cells [Knabbe et al., 1987], and glioblastoma cells [Huber et al., 1991]. Here we present evidence that human melanoma cells also generate active TGF-β. We have shown previously that human melanoma cells are able to form active surface-bound plasmin by the activation of plasminogen with surface-bound tissue-type plasminogen activator [Bizik et al., 1990]. Therefore, the present study was carried out to analyze whether there is a relationship between the capacity of individual melanoma cell lines to generate surface-bound plasmin and their ability to activate L-TGF-β as estimated by the amounts of active TGF-β present in conditioned medium. The conditioned media from the melanoma cells growing in medium with plasminogen-depleted fetal calf serum or parallel cultures growing after addition of purified human plasminogen were assayed for active TGF-β and compared. The results provide no clear evidence for an involvement of plasmin in the activation of L-TGF-β in the melanoma cell cultures.

MATERIALS AND METHODS

Cell Cultures

The human melanoma cell lines M_1Do, M_2Ge, M_3Dau, M_4Be were originally provided by Dr. J.F. Doré, Centre Léon Bérard, Lyon, France [Jakubovich et al., 1985]. The Bowes cell line was a gift from Dr. D. Collen, Katholieke Universiteit, Leuven, Belgium. The HMB-2 cell line was kindly supplied by Dr. J. Svec, Cancer Research Institute, Bratislava, Slovakia [Siracky et al., 1982]. The mink lung epithelial Mv1Lu cells (CCL-64) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Stock cultures of cell lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 2 mM glutamine, 1% nonessential amino acids, 5% fetal calf serum (FCS), and antibiotics. All of these cell lines were monitored for mycoplasma contamination and were found to be negative.

Assay for TGF-β Activity

TGF-β activity was monitored as inhibition of growth of Mv1Lu mink lung epithelial cells using a [^{3}H]-thymidine incorporation assay as described earlier [Ikeda et al., 1987; Brown et al., 1990] with a slight modification. Mv1Lu cells were seeded in 24-well 2 cm^2 tissue culture plates at a density of 3×10^4 cells/cm^2 in MEM supplemented with plasminogen-depleted 1% FCS. After 24 h of incubation, the medium was replaced with fresh serum-free MEM for 4 h. After this period the medium was aspirated and the cells were incubated with the indicated samples for 24 h. During the last 4 h the cells were pulse-labeled with 0.5 μCi/ml of [^{3}H]-thymidine (80 μCi/mmol; Amersham, UK) and the incorporated radioactivity was measured. The percentage proliferation of Mv1Lu cells was calculated as follows: % proliferation of Mv1Lu cells = 100 × [^{3}H]-thymidine uptake in experimental sample/[^{3}H]-thymidine uptake in serum-free MEM. The lower detection limit of the bioassay was 30 pg of TGF-β/ml.

Lysine Thioesterase Activity

Parallel flasks of the analyzed melanoma cell lines cultured in the presence or absence of human plasminogen (Kabi-Vitrum, Stockholm, Sweden) were washed three times with phosphate-buffered saline (PBS). The cell-bound plasminogen/plasmin fraction was eluted with 10 mM tranexamic acid in PBS for 15 min. The eluate was cleared by centrifugation in a minifuge. Samples (50 μl) to be assayed for serine proteinase activity using Z-lysine thiobenzyl ester as a substrate [Stephens et al., 1987] were added to microtiter plate wells in triplicate, together with 200 μl of an assay solution containing 200 mM potassium phosphate (pH = 7.5), 200 mM KCl, 0.1% Triton X-100, 220 μM

Z-lysine thiobenzyl ester (Peninsula, Belmont, CA) and 220 μM 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma, St. Louis, MO). 50 μl aliquots of 10 mM tranexamic acid in PBS (with assay solution) were used as blanks. The samples were incubated for 3 h at 37°C, and the absorbance were recorded in a Labsystems Multiscan MCC/340 plate-reader at 405 nm.

DNA Synthesis Measurement in Melanoma Cell Cultures

DNA synthesis in semiconfluent cultures of melanoma cells was measured by incorporation of [^{3}H]-thymidine into trichloracetic acid (TCA)-insoluble material. The melanoma cell lines were seeded at 2.0–5.0 × 10^4 cells per 2 cm^2 well depending on growth characteristics [Lizonova et al., 1990] in 24-well plates in MEM containing 5% FCS and cultured for 48 h. The cells were rinsed twice and the medium replaced with fresh serum-free MEM, then 48 h later, different concentrations of active TGF-β1 (R&D System, Minneapolis, MN) were added for 24 h of incubation. The cells were exposed for the last 4 h to [^{3}H]-thymidine (1.0 μCi/ml), which was added directly to the control and treated wells. The cells were rinsed twice with PBS and then extracted with 10% TCA at +4°C for 60 min and solubilized with 1N NaOH, 0.5% SDS. The [^{3}H]-thymidine incorporation was measured with a Beckman scintillation counter.

Preparation of Cell Conditioned Media and Heat Activation of Latent TGF-β

The melanoma cells were seeded at appropriate densities [Lizonova et al., 1990] and cultivated in MEM supplemented with plasminogen-depleted 5% FCS for 72 h. Then the cells were washed three times with serum-free MEM and incubated with fresh medium for 2 h. This medium was discarded and replaced by fresh medium. After a further 24 h incubation, the conditioned serum-free medium was harvested and centrifuged at 3000 × g for 15 min to sediment detached cells. Aliquots of media were adjusted by addition of serum-free MEM so as to represent the same number of cells per ml of conditioned medium for each cell line.

The thermal activation of L-TGF-β present in conditioned media was performed by incubating the aliquots of conditioned media over a range of temperatures between 37°C and 80°C, or at 80°C for 5 min [Brown et al., 1990]. The samples were chilled on ice and before the experiments were incubated at 37°C for 15 min. Commercial polypropylene tubes were used to prevent loss of the growth factor during incubation and thermal treatment.

RESULTS

Production of Active Versus Latent TGF-β by Human Melanoma Cell Lines

Our previous study [Lizonova et al., 1990] revealed that the human melanoma cell lines used in our panel uniformly expressed TGF-β mRNA. The TGF-β transcripts were identified using a mixture of two partially overlapping synthetic oligomers corresponding to regions of TGF-β1 and TGF-β2. Densitometric quantitation was performed to control for differences in mRNA expression of TGF-β in our set of six melanoma cell lines. This demonstrated that more TGF-β mRNA was expressed in Bowes and HMB-2 cells and relatively less in M_3Dau and M_2Ge cells.

In the paper by Albino et al. [1991], they detected the transcripts of TGF-β1, TGF-β2, and TGF-β3 after PCR amplification of the designated gene in all samples of 19 human melanoma cell lines involved in the analysis. Based on this study, where a statistically significant number of human melanoma cell lines was used, it is possible that melanoma cells express all three TGF-β isoforms. For this reason the designation TGF-β below refers to all three TGF-β isoforms, unless otherwise indicated.

While it has been widely reported in the literature that the majority of cultured cells secrete TGF-β in an inactive form, our preliminary analysis showed that the conditioned media of the melanoma cells contained active TGF-β assayed by the Mv1Lu growth inhibition assay. In order to test the conditioned media from melanoma cultures at optimal cell densities, the HMB-2 cells were grown at various cell densities and serum-free conditioned media were assayed (see Materials and Methods). As can be seen in Figure 1, biologically active TGF-β was detected in untreated samples of culture medium conditioned by 1.5–2.0 × 10^5 HMB-2 cells/ml for a period of 24 h. When the same sample of conditioned medium was heat treated at +80°C to activate completely the latent TGF-β, it was evident that the concentration of active TGF-β present in the sample was just subsaturating for this assay. Untreated conditioned medium from 7.0 × 10^5 HMB-2 cells/ml inhibited growth of Mv1Lu cells by 90%. The results also showed

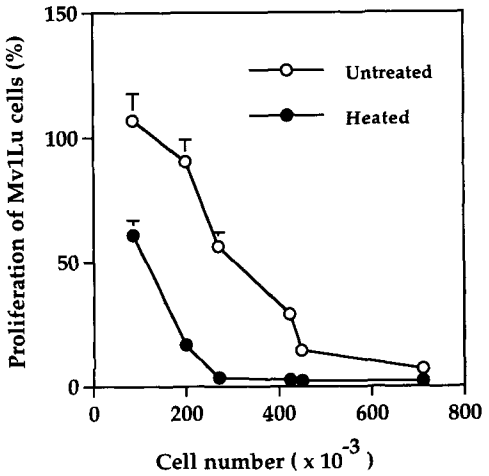

Fig. 1. Bioassay of conditioned media for active TGF-β from HMB-2 melanoma cell line. The cell line was cultured at various cell densities for 72 h in MEM containing plasminogen-depleted 5% FCS. The cell cultures were washed and cultured in serum-free MEM for 24 h. The TGF-β activity in the untreated samples (○) and heat-treated (80°C for 5 min) samples (●) was detected by the Mv1Lu cell growth inhibition assay. The Mv1Lu cells were cultured for 24 h in the presence of melanoma cell conditioned media harvested at the indicated cell densities. During the last 4 h the Mv1Lu cells were pulse-labeled with [^{3}H]-thymidine and incorporated radioactive label was determined. The background proliferation of Mv1Lu cells in serum-free MEM is taken as 100% and represents the reference point for growth factor induced changes. Data points are means ± S.D. of quadruplicate values.

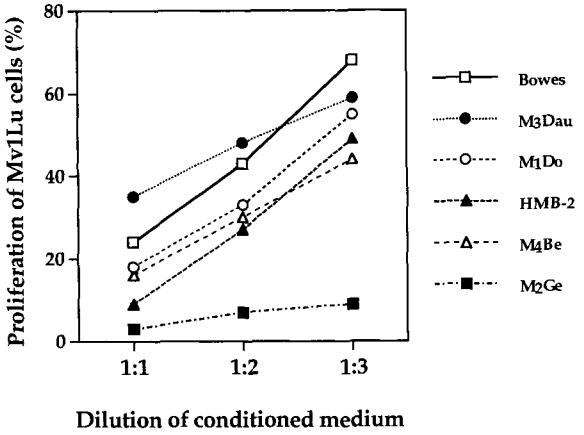

Fig. 2. Production of active form of TGF-β by the melanoma cell lines. The indicated melanoma cell lines were seeded at appropriate densities and were grown in MEM supplemented with plasminogen-depleted 5% FCS for 72 h. After this period, the cells were washed with serum-free MEM and incubated with fresh serum-free MEM for 24 h. The serum-free conditioned media were centrifuged and the aliquots were adjusted by addition of serum-free MEM so as to represent the same cell number of cells per ml of conditioned medium. The aliquots of undiluted (1:1) conditioned media (corresponding to 7.5 × 10^5 melanoma cells per ml) and the samples diluted 1:2 and 1:3 with serum-free MEM were tested for TGF-β activity by the Mv1Lu growth inhibition assay. Proliferation of Mv1Lu cells in the presence of various concentration of commercial active TGF-β1 was estimated in parallel. Data points are means of quadruplicate values.

that only 3.0 × 10^5 cells/ml produced a sufficient amount of heat activable TGF-β to cause complete growth inhibition of Mv1Lu cells. The amount of active TGF-β detected in HMB-2 conditioned media represented 10–15% of total secreted TGF-β.

The capacity of individual melanoma cell lines to produce and generate active TGF-β was further investigated. Based on well characterized growth patterns of the melanoma cell lines [Lizonova et al., 1990] the cells were seeded at appropriate densities in order to reach equal cell numbers per flask after 72 h of cultivation. After subsequent cultivation of cell cultures in serum-free medium for 24 h the conditioned media were analyzed by growth inhibition assay of Mv1Lu cells. Figure 2 illustrates that dilution of the conditioned medium from M_3Dau, Bowes, M_1Do, M_4Beu, and HMB-2 cells with serum-free medium 1:1 or 1:3, respectively, resulted in a linear concentration-dependent decrease of active TGF-β in the samples. The M_2Ge cell line was the only exception in the set of melanoma cell lines, as the M_2Ge cells expressed only very low levels of TGF-β message but the conditioned medium from this particular cell line exhibited the highest inhibitory activity for growth of Mv1Lu cells. Surprisingly the inhibitory activity present in the sample of M_2Ge conditioned medium diluted 1:3 was even stronger than the inhibitory activity in untreated conditioned medium from the HMB-2 cell line (which was the most positive for active TGF-β). The relative activities of active TGF-β produced by the melanoma cell lines were characteristic and consistent for each culture over many experiments. The amounts of active TGF-β produced by the melanoma cell lines were determined by calibration using commercially available TGF-β1 as a standard. Each sample was tested in several dilutions to obtain values in the linear range of the TGF-β1 standard curve. A distinct characteristic of the melanoma cell lines was that five of them produced considerable amounts of active TGF-β ranging from 370 pg/10^6 cells (M_3Dau) to 610 pg/10^6 cells (HMB-2) per 24 h.

Thermal Activation of L-TGF-β Present in Melanoma Cell Conditioned Media

Brown et al. [1990] have studied the pH and temperature at which different latent TGF-β complexes become active. It was demonstrated that although there is only limited sequence homology in the latency-associated peptides of TGF-β-1, -2, and -3, the latent growth factor complexes were remarkably similar in their activation profiles. Thermal activation of the latent TGF-β complexes proved to be an effective means of liberating mature TGF-β, consistently yielding slightly greater activity than acid activation.

In a separate experiment aliquots of the conditioned media were heat-treated at +80°C for 10 min in order to investigate the amounts of thermally activable TGF-β. Four cell lines, M_3Dau, Bowes, M_1Do, and M_4Be, in addition to HMB-2 cells (as shown in Fig. 1), produced significant amounts of L-TGF-β (i.e., activable TGF-β), comparable with HMB-2 cells production, which was converted into the active TGF-β form by thermal treatment (data not shown). In the process of thermal activation of L-TGF-β present along with active TGF-β in conditioned media the samples exhibited similar profiles of thermal activation as shown for HMB-2 cells in Figure 3. Conditioned medium from M_2Ge cells differed significantly as the inhibitory activity for Mv1Lu cells decreased drastically over the range of temperatures from +37°C to +60°C. It is evident from the results shown in Figure 3 that the conditioned medium became strongly growth-stimulatory for Mv1Lu cells. The analysis of complete L-TGF-β activation in the samples of conditioned media was also performed by acid treatment. The obtained values were nearly identical with those obtained after thermal activation but reproducibility of results was significantly higher by thermal activation (results not shown).

Activation of Plasminogen on the Surface of the Melanoma Cells

According to studies on cultured cells, human tumor cells of different histological origin differ in the mechanism of pericellular plasminogen activation. Most types of human adherent tumor cells use urokinase to generate cell-bound plasmin [Vaheri et al., 1992]. Human melanoma [Hoal-Van Helden et al., 1986; Bizik et al., 1990] and neuroblastoma cells [Neuman et al., 1989]

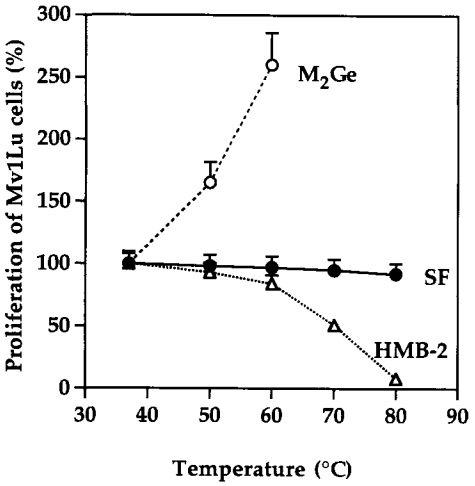

Fig. 3. Profiles of thermal activation of latent TGF-β present in conditioned media of HMB-2 and M_2Ge cell lines. *Samples of* serum-free conditioned media from HMB-2 and M_2Ge cell lines corresponding to 3.0×10^5 cells/ml were heat-treated for 5 min at the indicated temperatures and active TGF-β was determined by the growth inhibition assay of Mv1Lu cells. Simultaneously heat-treated samples of serum-free MEM (SF) were used as controls. The reference point (100%) for individual cell lines represents the value of [^{3}H]-thymidine uptake by Mv1Lu cells in the presence of untreated sample of the conditioned medium or serum-free MEM incubated at +37°C for 10 min. Data points are means ± S.D. of quadruplicate values.

form an exception among adherent human tumor cells in that they synthesize and secrete predominantly tPA. The melanoma cell lines used in our study constitutively secrete tPA and binds it to cell surface [Bizik et al., 1993]. After addition of purified human plasminogen to melanoma cell cultures growing in a medium with plasminogen-depleted FCS, plasmin activity could be recovered as a bound fraction from the cell layer. Parallel flasks of the melanoma cells were cultivated in MEM containing 5% plasminogen-depleted FCS for 48 h and then the cells were treated with serum-free MEM for 24 h. Human plasminogen (5 μg/ml) was added to one set of cell cultures for the 24 h. The conditioned media were collected and analyzed for active TGF-β by the growth inhibition assay. Plasmin generated on the melanoma cell surface during 24 h was recovered by 1 mM tranexamic acid from the cell layers. The eluates from plasminogen-treated cells and control cells were assayed for serine proteinase activity using Z-lysine thiobenzyl ester as substrate. Figure 4 shows that Bowes and M_2Ge cell lines were the most effective, M_1Do and M_4Be moderately effective, and M_3Dau and HMB-2 the least effective in generat-

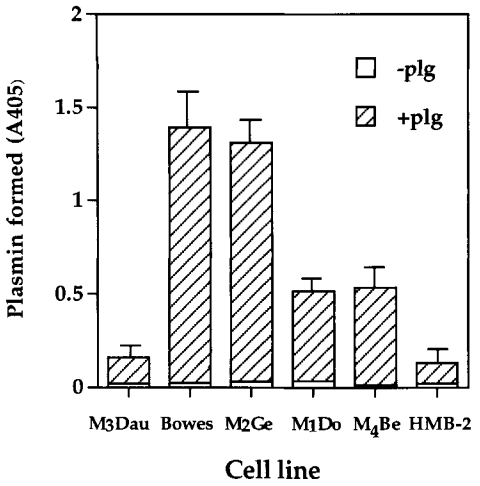

Fig. 4. Activation of plasminogen on the surface of human melanoma cells. The melanoma cell lines were grown in MEM supplemented with plasminogen-depleted 5% FCS for 72 h. Then the cells were washed with serum-free MEM and incubated with fresh aliquots of serum-free MEM for 24 h. Parallel sets of cells were incubated for 24 h with human plasminogen (5 μg/ml) in serum-free MEM. The conditioned media were harvested and the TGF-β activity was measured in the parallel samples by the Mv1Lu cell growth inhibition assay (see Fig. 5). The melanoma cell-bound plasmin was eluted from the cell surface with 1 mM tranexamic acid and the activity of released plasmin was measured by its thioesterase activity. Data points are means ± S.D. of quadruplicate values.

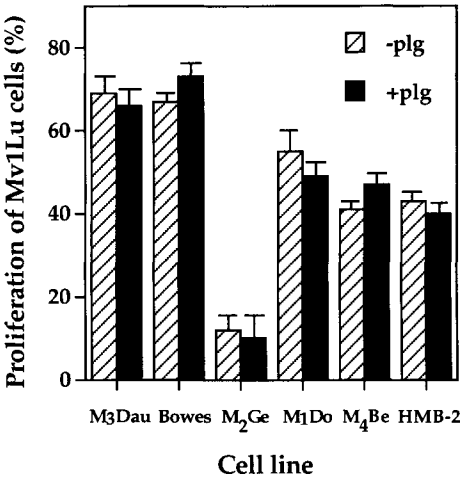

Fig. 5. Active TGF-β in conditioned media from melanoma cells cultured in the presence or absence of human plasminogen. Serum-free conditioned media from parallel cell cultures for each cell line (see Fig. 4) were centrifuged and their final volumes were adjusted by serum-free MEM to the same ratio of cells per ml of conditioned medium. Aliquots of conditioned media corresponding to 3.0×10^5 cells per ml were tested for TGF-β activity by the Mv1Lu growth inhibition assay. Data points are means ± S.D. of quadruplicate values.

ing plasmin on their cell surface. The difference between Bowes and HMB-2 cells was nearly 10-fold. The control eluates from cell cultures without addition of plasminogen exhibited only background level of proteinase activity. Simultaneously the conditioned media were assayed for the presence of active TGF-β. Figure 5 shows characteristic values for individual cell lines as was outlined in Figure 2.

Conditioned media from three cell lines (M_3Dau, M_1Do, and HMB-2) contained higher concentrations of active TGF-β after plasminogen treatment although the increase never exceeded 10% as compared with the respective controls. Conditioned media from plasminogen-treated Bowes and M_4Be cells showed a slight decrease in active TGF-β as compared with controls. The strong inhibition of Mv1Lu cell growth by conditioned medium from M_2Ge cells seems to be unrelated to TGF-β activity (see Fig. 3) and the results are not consistent with the suggested involvement of plasmin in the process of L-TGF-β activation.

Various proteases including plasmin have been previously shown to be mitogenic for different cells. As we have used a growth inhibition assay to quantitate content of active TGF-β in conditioned media it was necessary to test growth of Mv1Lu cells in the presence of plasminogen. It is evident, as shown in Figure 6, that human plasminogen is growth-stimulatory for Mv1Lu cells. We chose for use 5 μg plasminogen/ml of culture medium as this amount did not stimulate growth of Mv1Lu cells by more than 5%. We obtained the same pattern of growth stimulation when the plasminogen was activated by tPA and then added to indicator cells (results not shown). We used commercially available standard preparations of plasminogen in the whole series of experiments.

Effect of Exogenous TGF-β1 on DNA Synthesis of Melanoma Cells

To assay for the growth-regulatory effect of exogenous active TGF-β1, the melanoma cells were seeded at appropriate densities in MEM containing 5% FCS and grown for 48 h then kept quiescent in serum-free MEM for 48 h and finally exposed to active TGF-β1 (0.1–5.0 ng/ml). As shown in Figure 7, the effects of the TGF-β1 on proliferation of 5 melanoma cell lines were dose-dependent. M_3Dau, Bowes, and HMB-2 cells demonstrated positive proliferative

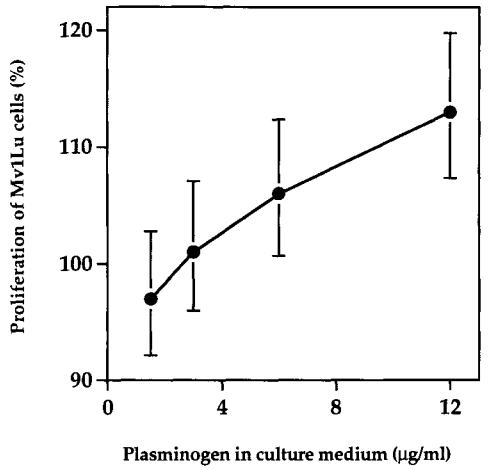

Fig. 6. Effect of human plasminogen on proliferation of Mv1Lu cells. Mv1Lu cells were cultured in MEM supplemented with plasminogen-depleted 1% FCS for 24 h and then treated the same way as in the assay for TGF-β activity (see Materials and Methods). Indicated concentrations of human plasminogen were added in quadruplicate wells for each concentration and stimulation of DNA synthesis was estimated by [^{3}H]-thymidine incorporation. The background proliferation of Mv1Lu cells in serum-free MEM was taken as 100%. Data points are means ± S.D. of quadruplicate values.

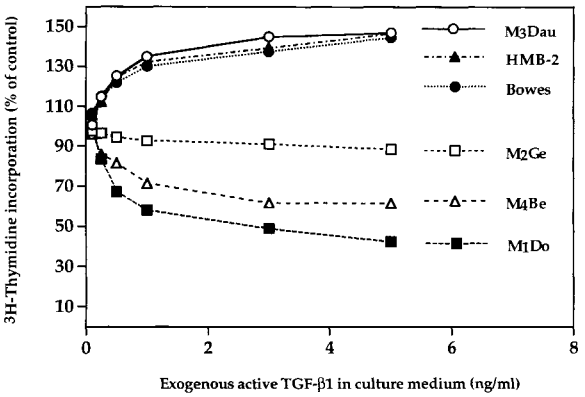

Fig. 7. Stimulation of DNA synthesis by exogenous TGF-β1 in melanoma cells. Semiconfluent cell cultures were rinsed and cultured in serum-free MEM for 48 h. Then aliquots of fresh serum-free MEM containing indicated amounts of active TGF-β1 were added to the wells for 24 h. The cells were exposed for the last 4 h of the period to [^{3}H]-thymidine. TCA-precipitated counts were quantitated in quadruplicate wells for each concentration point. [^{3}H]-thymidine uptake by the respective cell lines in serum-free MEM with no addition of active TGF-β1 represents 100%. Data points are means of quadruplicate values.

response to TGF-β1. At the maximum dosage (5 ng TGF-β1/ml) a stimulation by 40–50% was detected in the cell lines. In contrast, at the same concentration M_1Do and M_4Beu cells were growth-inhibited by 38% and 58%, respectively. For M_2Ge cells, TGF-β1 was only weakly inhibitory. Thus, application of exogenous active TGF-β1, at concentrations which were approximately 10-fold higher than the average concentrations of endogenous active TGF-β detected in the respective conditioned media, can have positive, negative, or indeed neutral influences on melanoma cell growth, depending on the particular cell line tested. Data are summarized in Table I.

Because active TGF-β was shown before to inhibit NK cell function [Roberts and Sporn, 1990] we investigated whether active TGF-β present in melanoma cells conditioned media can inhibit the cytolytic function of this population of immune competent cells. The results indicated that the conditioned media of HMB-2, Bowes, M_4Be, M_1Do, and M_3Da cells contained suppressive agent(s) for cytolytic activity of effector cells (data not shown). The inhibition characteristics for particular melanoma cell lines exhibited inhibitory effect which was in accordance with relative amounts of active TGF-β in the respective conditioned media (see Fig. 2).

DISCUSSION

In human melanoma cells plasminogen activation represents the most significant extracellular proteolytic system, and it seems to correlate with progression level of the tumor cells [Vaheri et al., 1992; De Vries et al., 1994]. We have previously shown [Bizik et al., 1990; Bizik et al., 1993] that human melanoma cells activate plasminogen on their cell surfaces and proteolytically active plasmin retains bound to cell surface. In the view of several contradictory results on the significance of plasmin in activation of L-TGF-β [Miyazono and Heldin, 1989; Brown et al., 1990; Huber et al., 1991] we have decided to take advantage of a well-characterized panel of human melanoma cell lines and assess the role of plasmin in the process. It is widely believed that L-TGF-β is activated on the cell surface and that spatial orientation of the enzymes participating in the process may be critical for the activation. Our results presented here indicate that 1) TGF-β produced by the melanoma cells was mostly in its latent form, 2) active TGF-β represents 10–15% of total secreted TGF-β, 3) the active form was generated even in cell cultures propagated for several passages in plasminogen-depleted FCS, 4) when purified human plasminogen was involved in the cultures and consequently converted to active plasmin no significant increase in the amount of active

TABLE I. Comparison of Generation of Active TGF-β, Plasminogen Activation, and Responsiveness to Exogenous Active TGF-β in Respective Melanoma Cell Lines

Cell line	TGF-β mRNA[a]	Generation of active TGF-β[b]	Activation of plasminogen[d]	Responsiveness to exogenous active TGF-β[e]
M_3Dau	+	+	+	+
Bowes	+++++	+	+++	+
M_2Ge	+	+++[c]	+++	−
M_1Do	++	++	++	−
M_4Be	+++	++	++	−
HMB-2	++++	++	+	+

[a]mRNA levels are given in arbitrary units, (+) to (+++++) estimated from Northern blots [data adopted from previous publication; Lizonova et al., 1990].
[b]Values of active TGF-β estimated on inhibition of growth of Mv1Lu cells by melanoma conditioned media at 1:2 dilution and data are expressed as following; inhibition in the range 100–80% (+++), 80–60% (++), 60–40% (+).
[c]Inhibitory activity present in M_2Ge conditioned medium is not due to active TGF-β, see Figure 3.
[d]Amount of generated active plasmin corresponds to its lysine thioesterase activity measured as absorbance at 405 nm; absorbance in the range 0.0–0.5 (+), 0.5–1.0 (++), 1.0–1.5 (+++).
[e]Responsiveness of melanoma cell lines to exogenous active TGF-β is based on evaluation of DNA synthesis as compared to control; data exhibited as stimulation (+) or inhibition (−).

TGF-β was detected. In general, our results clearly showed that the human melanoma cells have the capacity to convert L-TGF-β to its active form but do not confirm the previous finding of some laboratories concerning the importance of plasmin for L-TGF-β activation.

Surprisingly, the HMB-2 cell line produced the highest amounts of active TGF-β. We have demonstrated previously [Bizik et al., 1986; Bizik et al., 1989] that this particular cell line secretes significant amount (300 ng/10^6 cells/24 h) of alpha-2-macroglobulin (α_2M). There are numerous data on the involvement of α_2M in complexing of TGF-β and in this way modulating TGF-β activity [O'Connor-McCourt and Wakefield, 1987; Danielpour and Sporn, 1990]. In spite of this complexation there is a pool of unbound active TGF-β in conditioned medium of HMB-2 cells. Moreover, α_2M is a proteinase inhibitor with capacity to inhibit a wide spectrum of endoproteases including plasmin [Borth, 1992]. Based on our data it can be proposed that the proteolytic system which is involved in activation of L-TGF-β is not inhibited by α_2M. Because the number of possible L-TGF-β activating proteases is here highly restricted we assume that the HMB-2 cell line will be a valuable cell system in vitro to identify the proteolytic system resp. protease which activates L-TGF-β.

TGF-β was shown to play a fundamental role in the growth of both normal and neoplastic cells acting as a multifunctional growth regulator [Roberts and Sporn, 1990]. Previously it has been discovered that exogenous TGF-β inhibits growth of some melanoma cell lines [Roberts et al., 1985]. However more recent findings indicate that melanoma derived TGF-β may act as a bidirectional growth regulator [Rodeck, 1994]. Recent report also demonstrated the development of TGF-β resistance during human melanoma progression [MacDougall et al., 1993]. In our study three melanoma cell lines were mitogenically stimulated and the another three were inhibited by exogenous active TGF-β1. The first, these findings confirmed data of Rodeck et al. [1994] that TGF-β can function as bidirectional growth regulator for the histological type of tumor. The second, the melanoma cells, have capacity to respond to the exogenous growth factor so it means that TGF-β receptors on the cell surface are not fully occupied by endogenous active TGF-β. This fact outline a possibility that melanoma cells during progression can be still regulated by neighboring normal cells producing active TGF-β. The cell lines analyzed in our panel were established from metastatic melanomas [Jakubovich et al., 1985; Siracky et al., 1982] and it is evident that the melanoma cells from the late stage of progression respond to active TGF-β differently.

In an attempt to better understand the mechanism of active TGF-β action in melanoma cell progression, the NK activity of peripheral blood lymphocytes was examined in the presence of melanoma-produced TGF-β. The inhibitory activity present in respective conditioned media was in accordance with the amount of active TGF-β as estimated by Mv1Lu growth inhibition assay. Expression of the three TGF-β isoforms has been detected in human melanoma

cells, whereas normal melanocytes express TGF-β1 and -β3 but not TGF-β2 [Rodeck et al., 1991; Albino et al., 1991]. In human glioblastomas it was confirmed that TGF-β2 does have immunosuppressive effects [Bodmer et al., 1989]. Taken together, literature data and our results on modulation of NK activity by endogenous active TGF-β lead as to speculate that induction of immunosuppression in hosts by active TGF-β and more specifically by the TGF-β2 isoform producing melanoma cells may contribute to their progression by enabling these tumors to escape of immune surveillance.

The most striking finding in our study was that L-TGF-β is activated in cell culture in the presence of concomitant α_2M secretion by the same tumor cells. We have detected previously α_2M in biopsies from melanoma patients. There was a tendency for unfavorable prognosis in these α_2M-positive melanoma cases [Vaheri et al., 1992]. This can be interpreted that α_2M keeps the tumor cells dormant [Bizik et al., 1989; Lizonova et al., 1990] but they still produce active TGF-β which protects them from the host immune system.

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