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Coexpression of Tumor-Associated α_2-Macroglobulin and Growth Factors in Human Melanoma Cell Lines

Alena Lizonová, Jozef Bízik, Marta Grófóva, and Antti Vaheri

Department of Viral Oncogenesis, Cancer Research Institute, 81232 Bratislava, Czechoslovakia (A.L., J.B., M.G.); Department of Virology, University of Helsinki, 00290 Helsinki, Finland (A.L., J.B., A.V.)

α_2-Macroglobulin (α_2M) is known as an inhibitor of various proteinases and to bind several of the growth factors. We previously demonstrated that clonal variation exists in the production of α_2M in a human melanoma and that this variation may be associated with growth stimulation. We have now analyzed six human melanoma cell lines for the simultaneous expression of TGF-α, TGF-β, PDGF-A chain, PDGF-B chain, and tumor-associated α_2M. In Northern blot analysis TGF-α was detected in four of the cell lines, TGF-β in all, PDGF-A chain in three, and PDGF-B chain in none of the cell lines. α_2M, detected by immunoblotting, varied significantly between the different melanoma cell lines and only one cell line was found to be negative. Evaluation of growth-promoting activity in conditioned media suggested that α_2-macroglobulin, secreted by these tumor cell lines, is a significant modulator of melanoma cell growth.

Key words: autocrine, mitogenic, platelet-derived growth factor, proteinase inhibitor, transforming growth factor

One goal of cancer research is to understand the molecular basis of the uncontrolled growth of neoplastic cells. In recent years much attention has been focused on the putative role of tumor-derived growth factors as effectors in neoplastic transformation [1]. The realization that transformed cells are able to grow and divide in the presence of fewer exogenous growth factors than normal cells, together with the demonstration that certain types of transformed cells secrete mitogenic polypeptides, suggested the autocrine secretion hypothesis [2,3]. Now, it is clear that there are many different endogenous polypeptide growth factors which can strongly influence the establishment of the

Abbreviations used: α_2M, α_2-macroglobulin; FCS, fetal calf serum; aFGF and bFGF, acidic and basic fibroblast growth factor, respectively; IGF, insulin-like growth factor; IL, interleukin; MEM, minimal essential medium; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; SDS, sodium dodecylsulfate; TA, tumor associated; TGF, transforming growth factor.

Jozef Bízik's present address is Department of Cell Biology, NYU Medical Center, New York, NY 10016.

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transformed character of different cells. The major types of tumor-derived endogenous growth factors include transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factor activities (IGF-1 and IGF-2), acidic and basic fibroblast growth factor (aFGF and bFGF), and bombesin [for review, see 4].

Consequently, the hypothesis of an autocrine mechanism of growth regulation has been extended to include the concept that transformation might also be correlated with the failure of cells to synthesize, express, or respond to specific negative growth factors which they release to control their own growth [3]. One candidate having properties of a negative growth factor is α_2-macroglobulin (α_2M). Its role in systemic circulation is thought to be mainly in limiting various reactions involving proteinase activation [5]. The observation that α_2M is capable of covalent binding to human growth hormone [6], nerve growth factor [7], PDGF [8,9], and TGF-β [10,11] suggests another important role for α_2M: regulation of cell growth. Recently α_2M was described as a binding protein of IL-1 [12], IL-6 [13], and bFGF [14].

We previously described detection and characterization of α_2M synthesized and secreted by human tumor cells [15,16]. Our recent results showing the presence of tumor-associated α_2M (TA-α_2M) in human melanomas in vivo [17] prompted us to investigate the role of TA-α_2M in carcinogenesis of human cells in more detail. We have now analyzed the growth properties of a panel of six human melanoma cell lines and have also tested growth-promoting activity present in their conditioned media. We set out to test the possibility that the growth properties of the cell lines could be associated with the expression of tumor-related growth factors and TA-α_2M.

MATERIALS AND METHODS

Cell Lines

The human melanoma cell lines M_3Dau, M_1Do, M_4Beu, and M_2Ge were originally provided by Dr. J.F. Doré (Centre Léon Bérard, Lyon, France) [18]. The HMB-2 cell line was kindly supplied by Dr. J. Svec (Cancer Research Institute, Bratislava, Czechoslovakia) [19,20]. The Bowes melanoma cell line and the human foreskin fibroblast cell strain AG 1523 were obtained from the American Type Culture Collection and the Human Mutant Cell Repository Institute for Medical Research (Camden, N.J.), respectively. All these cell lines were monitored for mycoplasma contamination and found to be negative. The cultures were grown in Eagle's minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS) and antibiotics and were harvested by brief exposure to 0.25% trypsin/0.02% EDTA and subcultured weekly.

Growth Curves

Cells were plated on tissue culture multi-well plates with 24 flat-bottom wells (Linbro) in 5% FCS containing MEM. One day later the medium was aspirated, cells were rinsed twice with serum-free medium, and then fresh serum-free medium or medium containing 5% FCS was added and not renewed for 10 days. At daily intervals, medium was aspirated from aliquots of the cultures, cells briefly trypsinized, diluted in PBS, and immediately counted in triplicate. Prior to counting, the cells were examined by light microscopy for morphological appearance and viability. As assessed by morphological criteria and dye exclusion, attached cells were >95% viable.

Preparation of Conditioned Medium and Assay for Mitogenic Activity

The melanoma cell lines were grown in MEM supplemented with 5% FCS. After 3 days the cells were washed twice with serum-free medium and incubated with fresh serum-free medium for 24 h. This medium was discarded to avoid contamination by serum proteins and replaced by fresh medium. After 72 h of incubation the conditioned serum-free medium was harvested and centrifuged at 3000*g* for 10 min to sediment detached cells. To remove cellular debris, the medium was passed through a 0.22 μm pore size nitrocellulose filter (Millipore) and then tested for mitogenic activity. Aliquots of the media were adjusted by serum-free MEM to the same number of cells for each cell line. Mitogenic activity was determined by measuring [^{3}H]thymidine incorporation in serum-free cultures of human foreskin fibroblasts (AG 1523). The cells were plated in MEM with 5% FCS on flat-bottom 12 well plates (Costar). After 4 days of incubation, the medium was removed and the cultures were incubated for 96 h in serum-free MEM. These cells were confluent and quiescent in the G_1/G_0 phase of the cell cycle [21]. Conditioned medium (1.5 ml) was added along with [^{3}H]thymidine (Amersham; specific activity, 47 Ci/mmol) to a final concentration of 0.05 μCi/ml. After 48 h of incubation the cultures were extracted with 10% trichloroacetic acid, washed in running tap water, and lysed with 0.3 M NaOH and 1% SDS in water. Radioactivity was determined in a liquid scintillation counter Packard Tricarb 4640. The activity of the test samples was compared to that obtained with aliquots of [^{3}H]thymidine in serum-free MEM.

RNA Hybridization and DNA Probes

Total cellular RNA was extracted by the urea-LiCl method [22]; poly(A)$^+$RNA was selected by oligo(dT)-cellulose (Type 77F, Pharmacia), electrophoresed in 1% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose (Schleicher-Schuell), and hybridized to [^{32}P]labeled DNA probes according to established procedures [23]. When the hybridization was completed the filters were washed under nonstringent (1 × NaCl/citrate, 0.5% SDS, 65°C) and stringent (0.1 × NaCl/citrate, 0.5% SDS, 65°C) conditions. Filters were exposed to Kodak X-Omat AR film at −75°C. DNA probes were labeled by nick translation [23] using a nick-translation kit (Amersham) or random priming. The PDGF-A probe consisted of the 1.3 kb human cDNA, the PDGF-B probe of the 2.7 kb human cDNA, and TGF-α of 925 bp of human cDNA. These probes were inserted in PUC-13 vector and cut out with EcoRI. TGF-β transcripts were identified using a mixture of two partially overlapping synthetic oligomers corresponding to a region of TGF-β1 and TGF-β2. The DNA probes used in this study were a generous gift from Dr. Christer Betsholtz (Uppsala, Sweden).

Immunoblotting

Immunoblotting analysis was performed as described previously [16]. Aliquots of conditioned media prepared for mitogenic activity assay were immediately frozen and lyophilized. Then the samples were dissolved in 1.25 M Tris-HCl pH 6.8 containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.005% bromphenol blue, heated for 2 min in boiling water, and separated using vertical polyacrylamide slab gels with a 3.5% acrylamide spacer gel [24]. After separation, the proteins were transferred electrophoretically to nitrocellulose sheets (Schleicher-Schuell) according to established procedures [25]. For immunological staining of the transferred proteins, rabbit antiserum to

TA-α_2M [16] was used at a dilution of 1:1,000, and, after binding of peroxidase-conjugated swine anti-rabbit IgG (DAKO, Copenhagen, Denmark), the bands were visualized using 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as substrate.

RESULTS

Growth of Human Melanoma Cell Lines in Serum-Free Versus Serum-Supplemented Media

In order to evaluate the dependence on and responsiveness to growth factors, the growth of the six melanoma cell lines in the presence and absence of 5% FCS in the culture medium was studied. As shown in Figure 1, of the cell lines, Bowes and M_1Do exhibited the most active proliferation, both in serum-supplemented as well as serum-free medium. M_3Dau cells grew almost equally well in the presence and absence of serum in the culture medium. The cell lines M_4Beu and HMB-2 grew well in the presence of serum but not without it. Among the cell lines tested, the M_2Ge line showed the lowest proliferative capacity under both conditions. Growth capacity in serum-free culture medium of a given cell line in comparison to that in serum-supplemented conditions can be considered to be attributed to the synthesis and secretion of endogenous growth factors working in an autocrine manner.

Blot-Hybridization Analysis of PDGF-A, PDGF-B, TGF-α, and TGF-β mRNAs in the Melanoma Cell Lines

To evaluate the role of growth factors in growth properties of melanoma cells, we analyzed the expression of TGF-α, TGF-β, and PDGF in the six cell lines. Simultaneously we have analyzed the cell lines for secretion of TA-α_2M as a potential negative modulator of autocrine and paracrine growth-stimulating activity. The level of PDGF-A chain mRNA level was determined by Northern hybridization using a 1.3 kb human cDNA probe. Relatively abundant PDGF-A transcripts, sized 1.9, 2.3, and 2.8 kb [26], were found in M_3Dau, M_1Do, and HMB-2 cells, whereas such transcripts were not detected in the melanoma lines Bowes, M_2Ge, and M_4Beu (Fig. 2). None of the cell lines analyzed expressed PDGF-B chain transcripts, a result obtained in repeated experiments using a 2.7 kb human cDNA probe at different hybridization stringencies (Fig. 2). This result is in agreement with a previous report on three different human melanoma-derived cell lines: None were found to produce PDGF-B chain (*c-sis* transcripts) [27]. Also, according to a more recent study, PDGF-B chain was detected only in one of five human melanoma cell lines [28]. TGF-α mRNA of about 4.5–4.8 kb was detected in M_3Dau, M_1Do, M_4Beu, and HMB-2 cell lines but not in the Bowes and M_2Ge cell lines, as determined using a 925 bp human cDNA probe (Fig. 3). TGF-β mRNA, sized 2.5 kb, was found to be present in all six cell lines. Blot analysis with a synthetic oligonucleotide probe, composed of two oligomers of TGF-β1 and TGF-β2, demonstrated that TGF-β mRNA was present in all six cell lines; the highest levels were found in the Bowes and HMB-2 cell lines (Fig. 3).

Immunoblotting Analysis of TA-α_2M

Synthesis and secretion of TA-α_2M has previously been documented for several human sarcoma and melanoma cell lines [15,16]. We have proposed that TA-α_2M may serve an important role as a modulator of mitogenic activity in human melanoma cells

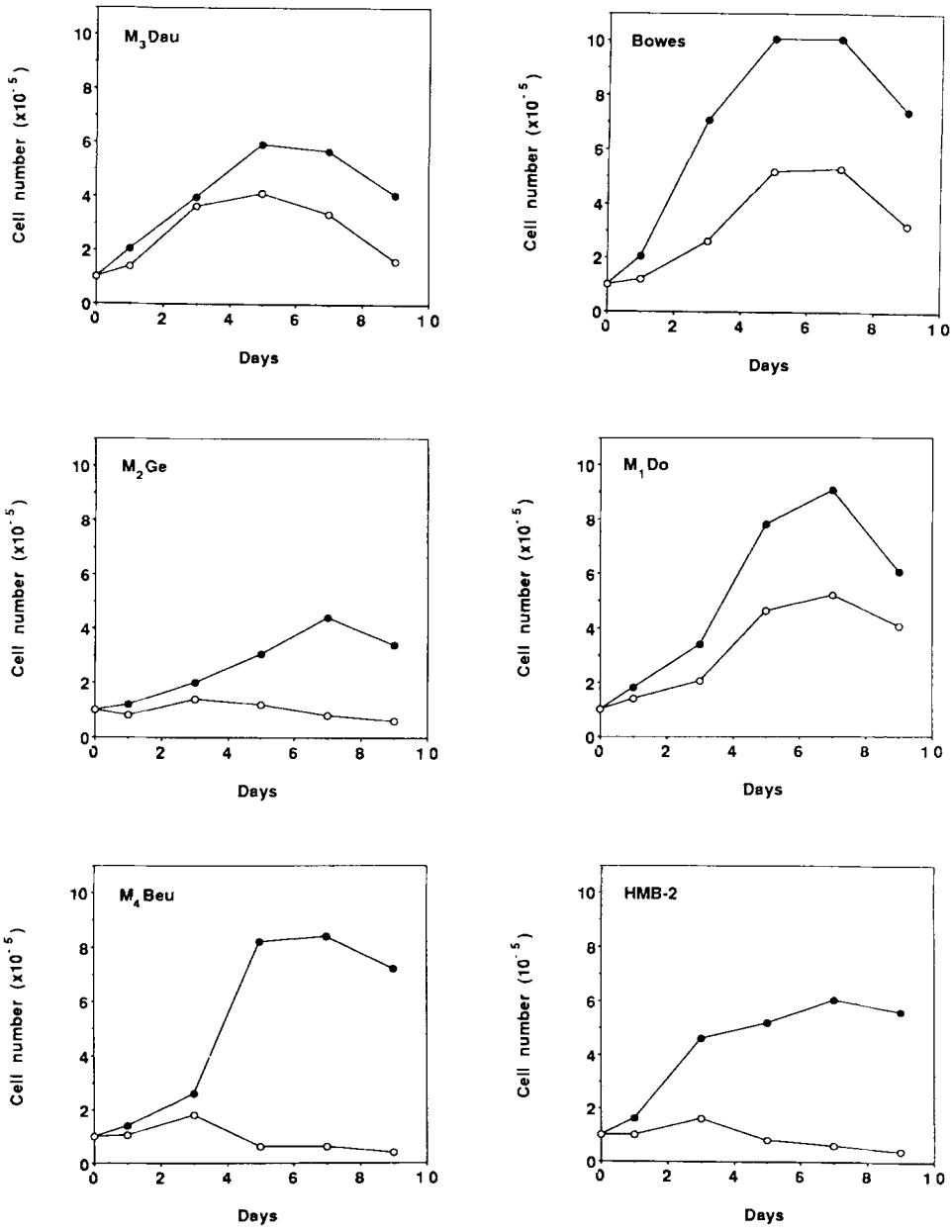

Fig. 1. Proliferation of melanoma cell lines in serum-free versus serum-containing medium. Cells were seeded at the indicated density (10^5 cells per 2 cm^2 well) on multi-well plates in medium containing 5% FCS. One day later culture media were removed, the cell layers were rinsed with serum-free medium, and fresh serum-free medium or medium containing 5% FCS was added. Cell numbers were determined daily. The results represent mean cell counts of triplicate wells in serum-containing medium (●) and serum-free medium (○).

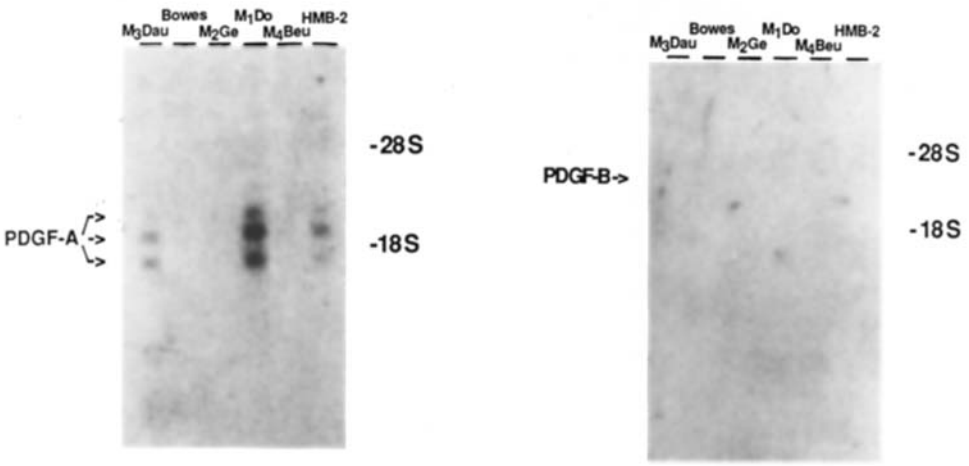

Fig. 2. Northern blots of melanoma mRNAs hybridized to PDGF-A chain (**left**) or PDGF-B chain (**right**) nick-translated cDNA probes. Ten micrograms of poly(A)$^+$RNA from each melanoma cell line was size-fractionated on agarose gels, blotted to nitrocellulose membrane filters, and hybridized to PDGF-A chain cDNA and subsequently rehybridized with PDGF-B chain cDNA. Hybridization (10^6 cpm/ml) was performed in 50% formaldehyde–5 ×SSC–5 mM EDTA, 0.5% SDS, 1×Denhardt's–250 μg/ml denatured salmon sperm DNA at 42°C overnight. The filter was then washed in 1 ×SSC, 0.5% SDS at 65°C and consequently in 0.1 ×SSC, 0.5% SDS at 65°C. Ribosomal RNAs were separated on the same gel and were used as markers.

[29]. We therefore compared the growth properties of the six melanoma cell lines with respect to production of TA-α_2M. The results of immunoblotting analysis, shown in Figure 4, demonstrate that the cell lines differ in secretion of TA-α_2M. TA-α_2M secretion was higher in the cell lines M_3Dau, M_4Beu, and HMB-2 than in M_1Do and M_2Ge cells. Bowes cells were negative for production of TA-α_2M.

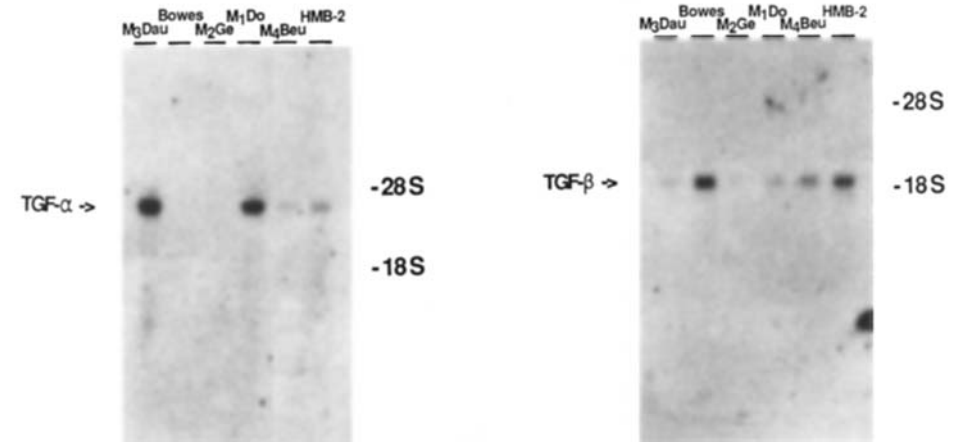

Fig. 3. Northern blots of melanoma poly(A)$^+$RNA hybridized to TGF-α probe (**left**) and TGF-β probe (**right**). Poly(A)$^+$RNA (10 μg) was added to each line. The TGF-α probe had been ^{32}P-labeled by nick-translation and the TGF-β probe labeled by random priming. Hybridization was performed as in Figure 2.

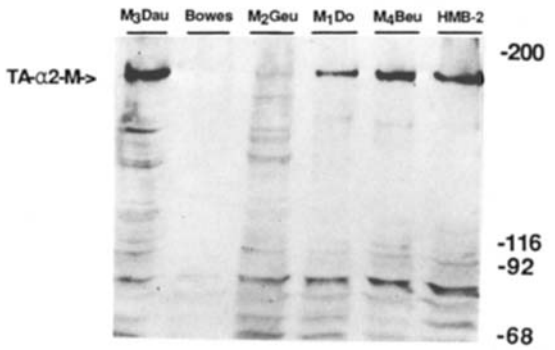

Fig. 4. Immunoblotting of melanoma cell conditioned media with rabbit antiserum raised to tumor-associated α_2-macroglobulin. One millimeter aliquots of conditioned medium were immediately frozen and lyophilized. Samples were dissolved in sample buffer and run in SDS-PAGE (5%–15% acrylamide). After separation the proteins were transferred electrophoretically to nitrocellulose and stained with antiserum to TA-α_2M. Staining was visualized by incubation with peroxidase-conjugated secondary antiserum followed by enzyme substrate. The arrow indicates the position of TA-α_2M.

Effect of Melanoma Cell Conditioned Media on DNA Replication of Fibroblasts

In order to investigate the simultaneous influence of autostimulatory mitogens and TA-α_2M, the conditioned media were tested for mitogenic activity using AG 1523 human foreskin fibroblasts, having a high amount of PDGF and TGFs receptors [21,30], as indicator cells. Conditioned medium of Bowes cells with the highest level of TGF-β mRNA and no production of TA-α_2M exerted significantly higher mitogenic activity than the other cell lines (Fig. 5). Conditioned medium from M_3Dau cells, expressing TGF-α, TGF-β, PDGF-A, and relatively high secretion of TA-α_2M, also had a strong mitogenic effect on the fibroblasts. Our recent experiments have shown that the TA-α_2M secreted by this cell line is rapidly autodegraded [Bízik, J., unpublished observation]. The cell lines M_1Do, M_4Beu, and HMB-2 showed moderate growth-promoting activity consistent with the levels of expression of the growth factors and of TA-α_2M observed. M_2Ge cells exerted only minimal mitogenic activity.

DISCUSSION

As mentioned above (for references, see Introduction), several growth factors have been shown to form complexes with α_2M. In our previous studies [29], analyzing cell clones established from the HMB-2 human melanoma cell line, we found a correlation between the level of TA-α_2M in culture medium and the population doubling time of these clones. The growth rate of the clones correlated with the level of α_2M in culture medium ($r^2 = 0.69$, $P < 0.01$). Northern hybridization indicated quantitative variation in the TA-α_2M mRNA expressed by the clones that was comparable to the quantity of TA-α_2M in the respective culture media. Consequently, clones with lower TA-α_2M production had proportionally shorter population doubling time than the clones with intermediate or high production. When exogenous α_2M was added, inhibition of mitogenic activity present in the culture medium was observed. On the other hand, IgG antibodies to TA-α_2M promoted the mitogenic activity. In this previous study [29] the

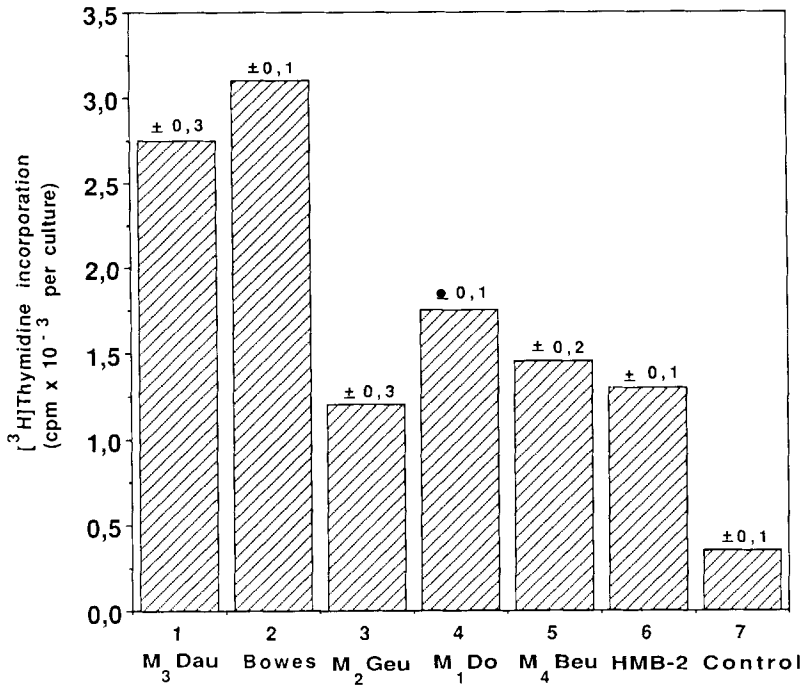

Fig. 5. Effect of serum-free conditioned media from melanoma cell lines on [^{3}H]thymidine incorporation of quiescent AG 1523 fibroblasts. Confluent monolayers of AG 1523 cells in 12-well plates were rinsed with serum-free MEM and cells were incubated in this medium for 4 days. After this period the cells were incubated with melanoma conditioned media (estimated for 0.5×10^6 cells/0.5 ml/24 h) together with [^{3}H]thymidine (0.05 μCi/ml). Incorporation of radioactivity was measured after 48 h incubation at +37°C. Values represent the mean ± SE of triplicate wells.

intensity of PDGF A-chain mRNA signal was very similar for all cell clones analyzed. The present experiments were designed in order to investigate in more detail the relationship of TA-α_2M to different tumor-derived growth factors.

Of the six melanoma cell lines, only one, Bowes, did not produce α_2M. Of the four growth factors, three (PDGF-A, TGF-α, and TGF-β) were expressed in different quantities and combinations by these cell lines. Two of these three growth factors, PDGF-A and TGF-b, have been shown to be complexed by α_2M, a process leading to a decrease in growth factor activity [8–11]. On the basis of these results we propose that 1) TGF-α promotes melanoma cell growth and this activity does not seem to be modulated by TA-α_2M, as seen in the high growth rate of M_3Dau and M_1Do cell lines (Table I); 2) melanoma cell lines (M_4Beu, HMB-2) producing TGF-β as their major growth factor are regulated for their growth by simultaneous expression of high levels of TA-α_2M; and 3) the high level of expression of TGF-β and lack of production of TA-α_2M by Bowes cells may provide a growth advantage for these cells. It has been well established that tumor cells produce many different polypeptide growth factors [for review, see 4] and α_2M is able to complex with at least seven different growth factors.

Our results on the M_3Dau and M_1Do cell lines suggest that high expression of TGF-α can substantially potentiate cell growth in spite of simultaneous production of TA-α_2M. It should be noted that, unlike several other growth factors, at least so far,

TABLE I. Relative Amounts of PDGF-A and -B Chains, TGF-α and TGF-β mRNAs, and Tumor-Associated α_2M in Human Melanoma Cell Lines With Different Growth Properties

Cell lines	PDGF-A[a]	PDGF-B	TGF-α	TGF-β	TA-α_2M[b]	Growth rate in serum-free culture medium (%)[c]
M_3Dau	+	−	+++++	+	+++++	60.0
Bowes	−	−	−	+++++	−	45.0
M_2Ge	−	−	−	+	+	12.5
M_1Do	+++	−	++++	++	++	56.3
M_4Beu	−	−	+	+++	+++++	9.4
HMB-2	+	−	++	++++	+++++	9.1

[a]mRNA levels are given in arbitrary units, − to +++++, estimated from Northern blots.
[b]TA-α_2M levels were determined from immunoblotting staining.
[c]The growth rates were calculated from growth curves where maximal cell number in serum-supplemented medium was considered to be 100%.

TGF-α has not been demonstrated to be bound by α_2M. Previously, TGF-α mRNA has been detected in many cell lines derived from solid tumors, including melanomas [31,32]. Based on several studies it has been concluded that TGF-α expression may correlate with a higher degree of mitotic activity of these cells and contribute to tumor development [33].

Exceptionally high mitogenic activity present in the conditioned medium, as well as very high growth capacity, was demonstrated for the Bowes cell line. This cell line expressed no TA-α_2M, but a high amount of TGF-β mRNA. TGF-β mRNA is expressed in nearly all cell types studied [31,34]. Most cells, however, secrete TGF-β in a biologically inactive form [35,36] which is unable to bind to the TGF-β receptor [37]. The latent form can be activated by strong acid or alkali treatment [38] or by some proteinases such as plasmin [39,40], elastase, trombin, and cathepsin D [Dr. R.M. Lyons, personal communication]. Some of these enzymes were reported to be secreted by certain human tumor cells [41,42]. As mentioned above, TGF-β can be inactivated by its covalent binding to α_2M [10,11]. In the process of proteolytic activation of TGF-β and regulation of its action the TA-α_2M may have dual function. One of the roles of TA-α_2M may be a direct entrapment of TGF-β and the second indirect role an inhibition of proteinases which activate latent TGF-β. These roles are supported by our observations on Bowes cells which do not produce TA-α_2M. When we tested the TGF-β activity in serum-free conditioned medium from Bowes cells we have found active TGF-β [A. Lizonova, unpublished observation] that induced soft agar growth of indicator cells, as it was previously described [43].

To characterize the autocrine growth-promoting mechanisms an attempt has been made in an independent study to establish conditions for the Bowes cell line to grow in a basal serum-free culture medium. Recently, Bowes cells were adapted to grow permanently in completely serum-free Eagle's MEM [Dr. E.L. Wilson, New York University, Medical Center, personal communication]. This suggests that the lack of TA-α_2M production may provide a special growth advantage for the cell line. As human melanoma cells were reported to produce a large variety of different growth factors [4,44], it is reasonable to take into account other growth factors which were not analyzed in our study. Another potent growth factor, bFGF, was rather weak and uniformly expressed in the melanoma cells (data not shown). Moreover, regulation of the mitogenic

activity of melanoma cells could be mediated by the recently characterized melanoma growth-autostimulatory activity [45]. Growth-promoting activity of Bowes melanoma cells and its possible regulation by TA-α_2M is the subject of our current studies.

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