American Journal of Pathology, Vol. 148, No. 6, June 1996

Melanoma-Associated Expression of Transforming Growth Factor-β Isoforms

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Melanocytic neoplasia is characterized by the aberrant overproduction of multiple cytokines in vitro. However, it is largely unknown how cytokine expression relates to melanoma progression in vivo. Transforming growth factor β (TGF-β) is a multifunctional cytokine commonly produced by cultured melanoma cells. The in situ expression of all three TGF-β isoforms (TGF-β1, -2, and -3) was determined immunohistochemically in melanocytes and in 51 melanocytic lesions using isoform-specific antibodies. Significant linear trends of expression were observed from melanocytes through nevi and primary and metastatic melanomas for all three isoforms. TGF-β1 was expressed by some melanocytes and almost uniformly by nevi and melanomas. TGF-β2 and -3 were not expressed in normal melanocytes but were expressed in nevi and early and advanced primary (radial and vertical growth phase) and metastatic melanomas in a tumor-progression-related manner. TGF-β2 was heterogeneously expressed in advanced primary and metastatic melanomas, whereas TGF-β3 was uniformly and highly expressed in these lesions. Thus, expression of TGF-β isoforms in melanocytes and melanocytic lesions is heterogeneous and related to tumor progression, and expression of TGF-β2 and of TGF-β3 commences at critical junctures during progression of nevi to primary melanomas. (Am J Pathol 1996, 148:1887–1894)

Transforming growth factor-β (TGF-β) is a 25-kd dimeric cytokine with pleiotrophic effects on a wide spectrum of target cells. Three highly conserved isoforms of TGF-β (TGF-β1, -2, and -3) encoded by separate genes have been isolated in humans; these isoforms share considerable sequence homology and exert similar effects when tested in biological systems.[1] Previous work has demonstrated that tumor cells often express TGF-β, raising the issue of whether tumor-derived TGF-β serves a role in tumor progression. Expression of TGF-β mRNA and protein has been described in cultured human tumor cell lines including breast carcinoma,[2,3] glioma,[4,5] and melanoma.[6,7] *In vitro*, all of the 20 human melanoma cell lines tested in our laboratory expressed TGF-β1, -2, and -3 mRNA and produced TGF-β activity as assessed by bioassay and enzyme-linked immunosorbent assay[6,7] (and unpublished results). *In vitro*, expression of TGF-β mRNA and protein is not restricted to melanoma cells, being seen also in normal melanocytes.[6] Albino et al[8] reported similar results with the exception that expression of TGF-β2 but not TGF-β1 and -β3 mRNA was restricted to cultured melanoma cells. In our studies, production of TGF-β protein was observed only in proliferating melanocytes; by contrast, melanocytes rendered quiescent by growth factor deprivation produced no or very little TGF-β.[7]

These results demonstrated that tissue culture conditions modulate TGF-β expression by melanocytic cells and raised the question of how production of the three TGF-β isoforms relates to tumor progression in the melanocytic system *in situ*. Tumor progression is the process by which malignant neoplasms may evolve from benign or low grade precursors by a series of stepwise, often genetic events that result in alterations of the lesional phenotype. In the melanocytic system, we have defined a series of tumor progression steps from melanocytes to nevi including banal and dysplastic nevi, which are regarded as benign neoplasms.[9] Primary melanomas in turn are divided into biologically early

Supported by National Institutes of Health grant CA25874.

Accepted for publication February 12, 1996.

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lesions, termed radial growth phase (RGP) because the clinical lesions evolve radially as a more or less circular skin plaque, and the biologically more advanced vertical growth phase (VGP), so called because a tumor nodule is formed that expands vertically into and out from the skin surface. These primary lesions have also been termed the microinvasive and the tumorigenic stages of tumor progression. The microinvasive RGP does not metastasize but has a high probability of progression to the tumorigenic VGP, from which metastatic melanoma may result.[10,11] We have previously reported antigenic differences among these tumor progression stages.[12] To address the relationship of TGF-β expression to melanocytic tumor progression, we have investigated the *in situ* expression of TGF-β proteins across nevi and primary and metastatic melanomas. We report tumor-progression-associated expression of all three TGF-β proteins by melanocytic lesions. We also demonstrate that distinct lesional steps are characterized by different patterns of expression of TGF-β1, TGF-β2, and TGF-β3.

Materials and Methods

Specimens

A total of 51 formalin-fixed, paraffin-embedded specimens were chosen from the patient files in the Section of Dermatopathology and the Division of Surgical Pathology, Hospital of the University of Pennsylvania. These included characteristic examples of 8 melanocytic nevi, 13 RGP, 13 VGP, and 17 metastatic melanoma lesions, classified according to standard criteria as previously published.[13] Sections were cut at 5 μm, mounted on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA), and air dried in a fume hood.

Immunohistochemistry

Goat antibodies raised to recombinant, Chinese-hamster-ovary-derived latency-associated peptide (LAP) of TGF-β1, purified native TGF-β2, and recombinant chicken TGF-β3 (all from R&D Systems, Minneapolis, MN) were used to detect the presence of the three isoforms on tissue sections. Specificity of the antibodies was confirmed using Western blots and recombinant TGF-β isoforms (see Figure 3). The assay was performed using manual capillary action technology on the MicroProbe staining system (Fisher), with modifications of previously described methods.[14] Briefly, the sections were rapidly dewaxed with a 3:1 mixture of Hemo-De (Fisher) and xylene, cleared with absolute ethanol, and rehydrated with a 1X Tris-based buffer, pH 7.4 (Immuno/DNA Buffer, Research Genetics, Huntsville, AL). Antigen retrieval was carried out by submerging the sections in a 1X solution of microwave buffer (ChemMate, BioTek, Santa Barbara, CA), and heating in a 60-Hz, 120-Amp standard microwave oven for two 5-minute cycles. Endogenous peroxidase was quenched by using a 1% solution of hydrogen peroxide in methanol for 4 minutes at room temperature. Nonspecific reactivity was blocked by incubation with a 0.6% solution of immunoglobulin-specific normal sera for 2 minutes at room temperature. The primary antibodies were diluted 1:25 in 1X Immuno/DNA buffer and applied to the tissues, and the slides were incubated for 10 minutes at 45°C. The antibody was detected with biotinylated anti-goat immunoglobulins (Vector Laboratories, Burlingame, CA), diluted 1:150, and incubated for 20 minutes at 45°C. The biotinylated complex was detected with a peroxidase-linked streptavidin (Boehringer Mannheim, Indianapolis, IN), 1000 U/ml diluted 1:1000 in 10X Immuno/DNA buffer, incubated for 10 minutes at 45°C. The signal was developed with a peroxidase chromogen system, amino-ethylcarbazol (Vector) for 10 minutes at 50°C. The sections were lightly counterstained with a 1:3 solution of aqueous hematoxylin (Biomeda, Fosler City, CA) in water for 10 seconds at room temperature, coverslipped with Crystal Mount (Biomeda), and heated to 70°C to harden the medium.

Slide Evaluation

Sections were evaluated by two observers at a two-headed microscope. Staining was evaluated as negative or 1+ to 4+ depending on signal intensity. The percentage of positive cells within each compartment of the specimen was assessed. Lesional compartments were defined as shown in Table 1 and Figure 1, namely melanocytes, dermal nevus cells, epidermal nevus cells, cells of the RGP and of the VGP of primary melanomas, and cells of metastatic melanoma deposits, classified according to our published criteria.[13] A given lesional compartment was recorded as positive with a given isoform when 5% or more of its cells reacted at an intensity of 1+ or greater.

Controls

A species-specific, immunoglobulin-isotype-matched antiserum (goat) was used as an independent negative control. Additionally, internal positive and negative controls within each stained section were eval-

Table 1. *Reactivity Of TGF-β Isoforms with Melanocytic Lesions*

	TGF-β1	TGF-β2	TGF-β3
Melanocytes	7/11 (63%)	0/16 (0%)	0/19 (0%)
Nevus-D	7/7 (100%)	1/7 (14%)	3/6 (50%)
Nevus-E	8/8 (100%)	3/8 (37%)	8/8 (100%)
RGP	11/13 (85%)	7/12 (58%)	9/10 (90%)
VGP	12/12 (100%)	10/13 (77%)	10/10 (100%)
Metastatic melanoma	15/15 (100%)	13/17 (76%)	15/15 (100%)
P	0.008	0.0000	0.0000

Results are presented as number of positive/number tested. Nevus-D, dermal nevus cells; Nevus-E, epidermal (junctional) nevus cells.
**P* for test of linear trend.

uated for each isoform to establish biological plausibility and to determine the adequacy of the materials.

Statistical Methods

Data groups were analyzed by lesional compartments, comparing expression of the three TGF-β isoforms across compartments and comparing expression within the compartments for each isoform.

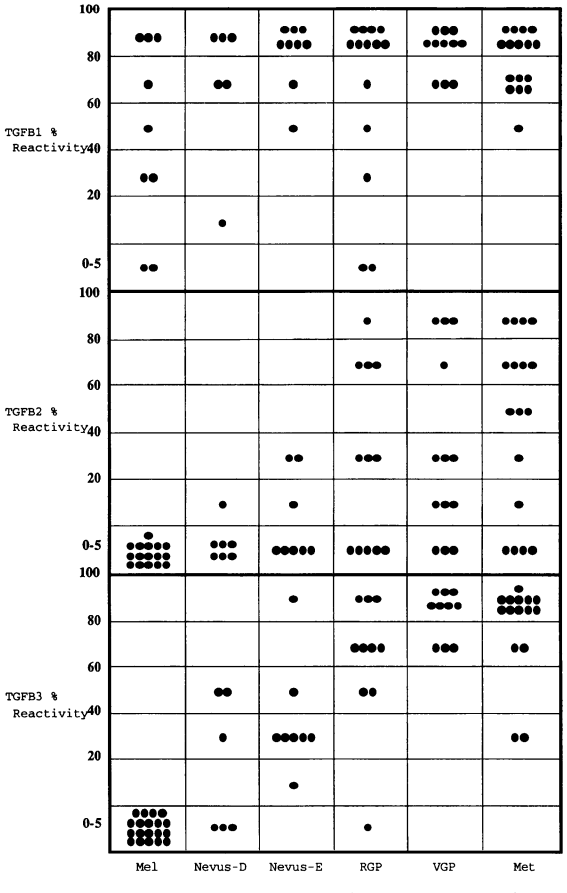

Figure 1. *Three panels show distribution of percent reactivity of TGF-β isoforms in melanocytic lesional compartments (for abbreviations see Table 1). Each dot indicates a single compartment, categorized according to the percentage of cells in that compartment that were reactive with the particular isoform.*

For the statistical analyses, the percentage of reactive cells was categorized into three groups: 0 to 20%, 20 to 60%, and 60 to 100%. The comparisons were done using exact computational methods based on Fisher's statistic, and the overall significance levels were adjusted for multiple hypothesis testing. For each isoform, the same tests were used to determine differences in melanoma progression among the different lesions. Individual lesions were also compared for differences in expression among the three TGF-β isoforms. All analyses were carried out using STATXACT from CYTEL Software Corp., Cambridge, MA.

Results

The number and percentage of positive cases are presented by lesional type for each isoform in Table 1, which is based on qualitative categorization of a lesional compartment as positive when greater than 5% of its lesional cells expressed the antigen. The distribution of the percentage of reactive cells by lesional compartments is presented graphically for each TGF-β isoform in Figure 1. The results of significance tests for quantitative differences of expression among lesional compartments based on the percentage of reactive cells are presented in Table 2 and for differences among the isoforms in Table 3.

As shown in Table 1 and Figure 1, TGF-β1 was expressed by some melanocytes and almost uniformly expressed by nevi and primary and metastatic melanomas. Although there was a significant linear trend overall (Table 1), there were no differences among the various categories of melanocytic neoplasms across tumor progression (Figure 1 and Table 2). In contrast, TGF-β2 and -β3 were not expressed by normal melanocytes, and there was an overall significant trend of expression of these isoforms across tumor progression from melanocytes to nevi, early and advanced primary (RGP and VGP), and metastatic melanomas (Table 1). Quantitative estimates of expression revealed statistically signifi-

Table 2. *Differences in TGF-β Reactivity of Melanocytic Lesions*

	TGF-β1	TGF-β2	TGF-β3
Mel *v* Nevus-D	NS	NS	NS
Mel *v* Nevus-E	0.0007	NS	<0.001
Mel *v* RGP	NS	0.0011	<0.001
Mel *v* VGP	0.0002	0.0011	<0.001
Mel *v* Met	<0.0001	<0.0001	<0.001
Nevus-D *v* Nevus-E	NS	NS	NS
Nevus-D *v* RGP	NS	NS	NS
Nevus-D *v* VGP	NS	NS	0.0001
Nevus-D *v* Met	NS	0.007	0.0002
Nevus-E *v* RGP	NS	NS	NS
Nevus-E *v* VGP	NS	NS	0.0003
Nevus-E *v* Met	NS	NS	0.0010
RGP *v* VGP	NS	NS	NS
RGP *v* Met	NS	NS	NS
VGP *v* Met	NS	NS	NS

Columns show probability of differences between indicated lesional categories for three TGF-β isoforms. After correction for multiple tests, a P value of 0.003 is consistent with an overall type I error of 0.05. Mel, melanocytes; *v*, *versus*; Nevus-D, dermal nevus cells; Nevus-E, epidermal (junctional) nevus cells; Met, metastatic melanoma; NS, not significant at the $P < 0.003$ level.

cant differences of expression of all three isoforms between melanocytes and primary or metastatic melanomas (Figure 1 and Table 2). Expression of TGF-β2 increased progressively from melanocytes to nevi, RGP and VGP primary, and metastatic melanoma. Expression of TGF-β3 increased significantly from nevi to VGP and metastatic melanoma. The expression of TGF-β3 was uniformly high in these latter stages of advanced melanoma progression, which in contrast showed heterogeneous expression of TGF-β2 (Figure 1). In the TGF-β2 studies, metastatic melanoma lesions in general exhibited a higher percentage of reactive cells than VGP or RGP, but the intensity of the reactivity was generally moderate. The primary melanomas were more heterogeneous, but the strongest reactivity of individual cells or groups of cells was seen in them (Figure 2). Reactivity for TGF-β1 and -β3, when present, was in general uniformly strong. A recent report described a correlation between the expression of TGF-β2 mRNA and tumor thickness in primary melanomas in tissue.[15] We found no strong correlation between tumor thickness and expression of TGF-β protein for any isoform as determined by regression analysis. The regression equation for TGF-β2 showed a slight negative correlation with thickness ($y = 4.6 - 0.05x$ where y = thickness in millimeters, and x = TGF-β2 expression in % of cells, $P = 0.035$).

Comparing the expression of the various isoforms with each other, as shown in Figure 1 and in Table 3, the expression of TGB-β1 differed significantly from that of TGF-β3 in melanocytes and from that of TGF-β2 or -β3 in nevi. The expression of TGF-β1 and of TGF-β2 differed significantly in advanced primary and metastatic melanomas. Similarly, the expression of TGF-β2 and of TGF-β3 differed in advanced primary and in metastatic melanomas, and also in the epidermal compartment of nevi.

Figure 2 shows representative tissue sections of skin with normal melanocytes, dysplastic nevi, RGP, and metastatic melanomas stained with antibodies to TGF-β3. The specificity of the antibodies was confirmed by Western blots with recombinant TGF-β isoforms (Figure 3).

Discussion

This study demonstrates that TGF-β expression is a tumor-progression-associated phenomenon in human melanoma. Human melanoma cells *in situ* produced all three known human TGF-β isoforms whereas normal melanocytes expressed low levels of TGF-β1 and no TGF-β2 and -β3 proteins at levels detectable by the immunohistochemical techniques employed in this study. In previous studies, cultured melanocytes have been found to express TGF-β as assessed by Northern blot analysis,[6] reverse transcriptase polymerase chain reaction analysis,[8] and determination of TGF-β protein[7] at levels comparable to cultured melanoma cells. The present study suggests that TGF-β production previously observed in cultured melanocytes was in large measure induced by culture conditions. In support of this idea, we noted earlier[7] that, *in vitro*, TGF-β production by melanocytes depended on the presence of multiple cytokines in the culture medium and was not observed when melanocytes were kept in protein-free, chemically defined media.

Table 3. *Differential Reactivity Of TGF-β Isoforms in Melanocytic Lesions*

Comparison	Melanocytes	Nevus-D	Nevus-E	RGP	VGP	Metastatic melanoma
TGF-β1 *v* -β2	0.008	0.0006	0.0002	NS	0.0030	0.0096
TGF-β1 *v* -β3	0.0031	NS	0.0014	NS	NS	NS
TGF-β2 *v* -β3	NS	NS	NS	NS	0.0025	NS

*Rows show probability of differences between indicated pairs of TGF-β isoforms by lesional category. After correction for multiple tests, a P value of 0.017 is consistent with an overall type I error of 0.05. Nevus-D, dermal nevus cells; Nevus-E, epidermal (junctional) nevus cells.

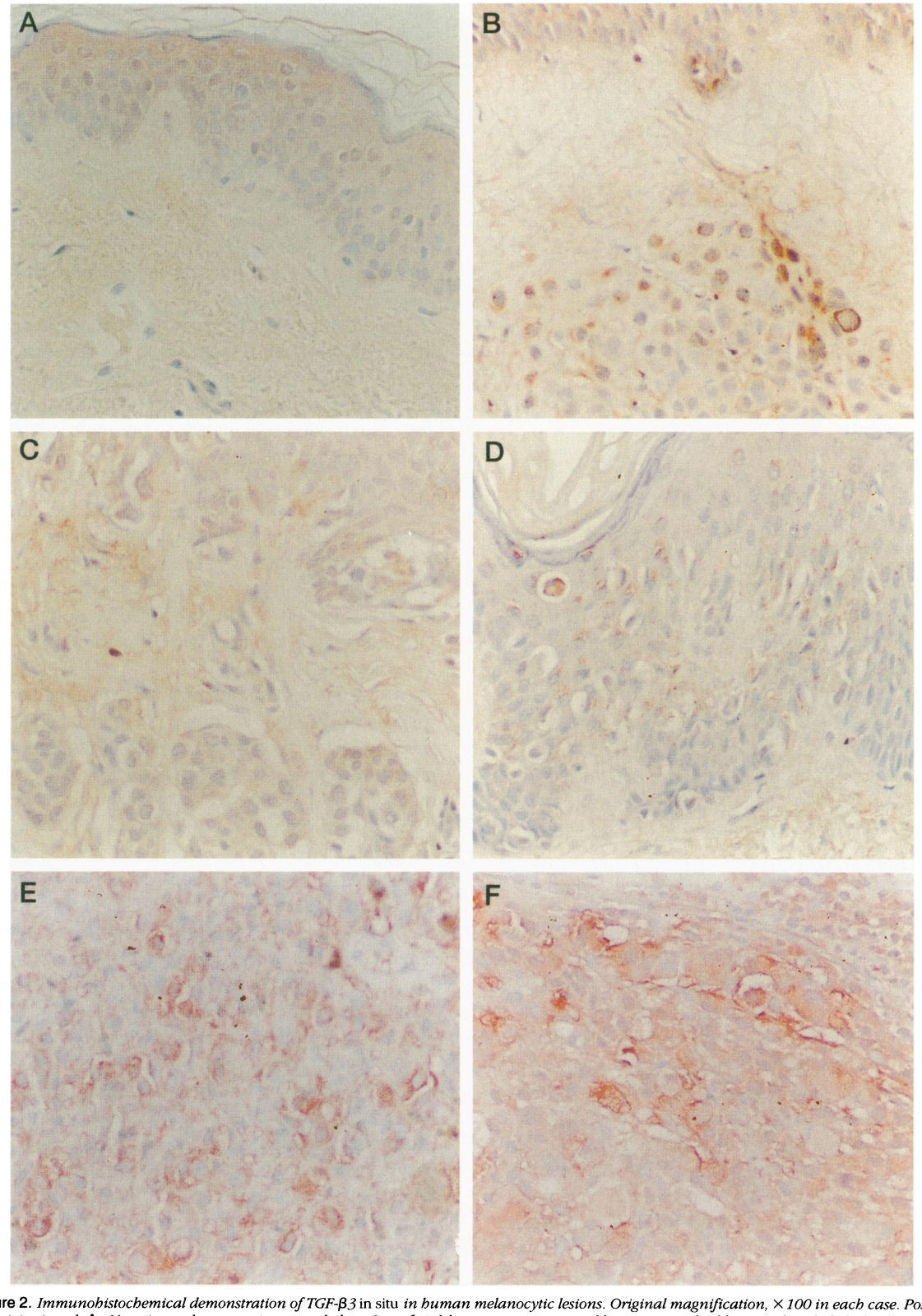

Figure 2. *Immunohistochemical demonstration of TGF-β3* in situ *in human melanocytic lesions. Original magnification, ×100 in each case. Positive reactivity is red.* **A:** *Negative melanocytes in normal skin. Superficial keratinocytes are weakly reactive. Fibroblasts and endothelial cells provide negative controls in this and other sections.* **B:** *Heterogeneously and weakly reactive dermal nevus cells (bottom half) and weakly reactive epidermis above. Note brown melanin pigment in some nevus cells.* **C:** *Weak 1+ reactivity in a few cells of the junctional component of a compound dysplastic nevus. The dermal nevus cells are negative.* **D:** *3+ reactivity in pagetoid cells of radial growth phase superficial spreading melanoma,* in situ *in this field.* **E:** *Homogeneous 4+ reactivity in vertical growth phase of a superficial spreading melanoma.* **F:** *Homogeneous 4+ reactivity in a metastatic melanoma. Note reactivity of mononuclear cells (histiocytes and lymphocytes) in upper right.*

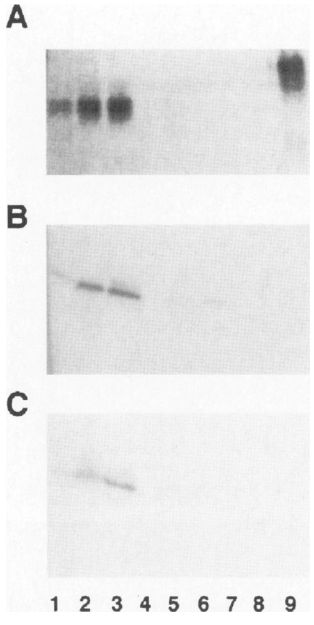

Figure 3. *Specificity of TGF-β antibodies used in this study. Binding of the anti-latency-associated peptide 9LAP/TGF-β1 antibody AB-246-NA* (A), *the anti-TGF-β2 antibody AB-112-NA* (B), *and the anti-TGF-β3 antibody AB-24-NA* (C) *was determined by Western blot analyses under nonreducing conditions. Cross-reactivity of each antibody with different TGF-β isoforms was determined by using recombinant preparations of these at the concentrations indicated below.* A: *Reactivity of the anti-LAP antibody with 10, 50, and 100 ng of recombinant LAP* (lanes 1 *to* 3, *respectively*), *no sample* (lane 4), *50 ng of recombinant human TGF-β1* (lane 5), *50 ng of recombinant human TGF-β2* (lane 6), *50 ng of recombinant human TGF-β3* (lane 7), *50 ng of recombinant amphibian TGF-β5* (lane 8), *and 50 ng of recombinant human latent TGF-β1* (lane 9). B: *Reactivity of the TGF-β2-specific antibody with 1, 5, and 10 ng of TGF-β2* (lanes 1 *to* 3, *respectively*), *no sample* (lane 4), *50 ng of recombinant human TGF-β1* (lane 5), *50 ng of TGF-β3* (lane 6), *50 ng of TGF-β5* (lane 7), *50 ng of LAP* (lane 8), *and 50 ng of latent TGF-β1* (lane 9). C: *Reactivity of the TGF-β3-specific antibody with 1, 5, and 10 ng of TGF-β3* (lanes 1 *to* 3, *respectively*), *no sample* (lane 4), *50 ng of recombinant human TGF-β1* (lane 5), *50 ng of TGF-β2* (lane 6), *50 ng of TGF-β5* (lane 7), *50 ng of LAP* (lane 8), *and 50 ng of latent TGF-β1* (lane 9).

An earlier study focused on the melanoma-associated expression of TGF-β2 mRNA as determined by *in situ* hybridization.[15] In agreement with this earlier study, we found increased expression of TGF-β2 protein at a critical juncture in melanoma progression, RGP melanoma, when compared with normal melanocytes. However, in contrast to this study, we observed a weak negative rather than a positive correlation between depth of tumor invasion into the dermis and expression of the TGF-β2 isoform. It remains to be determined whether this difference is due to discrepancies in mRNA and protein expression or to the relatively small sample numbers in both studies. It is also possible that TGF-β2 expression may correlate with prognosis independently of thickness, and this possibility will be explored in our future studies.

Tumor-progression-related expression of TGF-β3 has not been previously reported. Here we describe increased expression of this isoform in the progression from melanocytes to nevi and again from nevi to VGP primary melanoma, the stage of tumor progression at which competence for metastasis may be acquired. Expression of TGF-β3 was absent in melanocytes but was seen in 100% of junctional nevi. The expression was heterogeneous in these nevi but tended to be more intense and was significantly more homogeneous in VGP primary and in metastatic melanoma cells (Figure 1), suggesting that this isoform may be an important attribute of the fully malignant phenotype *in situ*.

The present study suggests that expression of the three different TGF-β isoforms in melanocytic cells occurs independently from each other. This conclusion is supported by the finding that the expression of TGB-β1 differed significantly from that of TGF-β3 in melanocytes and from TGF-β2 or -β3 in nevi and by the finding that expression of TGF-β2 and TGF-β3 differed significantly in advanced primary and in metastatic melanomas. Finally, we identified several primary and metastatic lesions that expressed only one or two TGF-β isoforms but not all three. Differential expression of the three TGF-β isoforms has been described in embryonic development[16,17] and in human breast cancer.[18,19]

Our results support the possibility that melanoma-associated TGF-β contributes to the initiation and/or progression of melanocytic lesions. We[6] and others[20] have demonstrated that TGF-β inhibits proliferation of most melanoma cell lines, particularly those derived from early primary lesions. A few cell lines derived from advanced metastatic lesions are either resistant to TGF-β-dependent growth inhibition[7] or are stimulated by exogenous TGF-β.[20] As shown here, production of TGF-β commences at early stages of melanoma progression, ie, either melanocytic nevi (TGF-β1) or RGP melanoma (TGF-β2 and -β3) in which TGF-β can be expected to inhibit cell growth. The production of potentially growth-inhibitory TGF-β by nevus cells and early melanoma cells *in vivo* suggests that endogenous TGF-β may serve important paracrine roles in the development and progression of pigmented lesions that in melanoma may outweigh the growth-inhibitory effects observed *in vitro*. This hypothesis is supported by a recent study using Meth A sarcoma cells engineered to overexpress TGF-β1.[21] Although *in vitro* proliferation of Meth A cells was profoundly inhibited by overexpressed TGF-β1, the cells formed tumors more rapidly and grew faster *in vivo* when compared with parental cells. Additional support for this hypothesis comes from studies using MDA-231 human breast cancer cells in which suppression of TGF-β effects in

mice by treatment with neutralizing antibodies to TGF-β has been shown to reduce tumor growth.[22,23] The tumorigenic effect of TGF-β in this system was associated with suppression of natural killer cell activity in tumor-bearing mice suggesting that TGF-β-dependent immunosuppression may facilitate tumor growth *in vivo*. Additional indirect evidence in support of the immunosuppressive role of tumor-derived TGF-β comes from studies in human glioma. An activity that inhibits proliferation of T cells and lymphokine-activated killer cells was isolated from conditioned media of glioma cells[24,25] and cyst fluid recovered from the tumor bed of sub-totally resected glioblastomas[26]; this activity has been shown to be TGF-β2. The study by Ruffini et al[26] demonstrated that, in glioma, as in melanoma, TGF-β production is a tumor-associated phenomenon *in vivo*.

Interestingly, we observed expression of melanoma-associated TGF-β2 in the early stages of melanoma as well as in later stages of tumor progression, which often lack a brisk immune response.[10] It remains to be investigated which of the multiple effects of TGF-β isoforms that relate to modulation of the immune response, tissue remodeling, and invasive properties account for the overall role of tumor-derived TGF-β in melanoma. Our results are consistent with the hypothesis that the different TGF-β isoforms may serve distinct functions in promoting melanoma development. Taken together with its tumor-progression-related expression, the heterogeneous expression of TGF-β2 in advanced primary and metastatic melanomas suggests that expression levels of this cytokine might correlate with prognosis in these lesions whereas the uniform expression of TGF-β3 in advanced primary and in metastatic melanomas suggests that this cytokine may be relevant in fundamental mechanisms of the neoplastic phenotype in melanoma.

Acknowledgement

The results of the Western blot analyses were provided by Dr. M. S. Kissil from R&D Systems, Minneapolis, MN.

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