Induction of Growth Factor RNA Expression in Human Malignant Melanoma: Markers of Transformation

Anthony P. Albino, Brigid M. Davis, and David M. Nanus

ABSTRACT

Alteration in the expression of growth factors is widely accepted as being one of several critical defects in the generation of the malignant cell. In the present study, 19 human metastatic melanoma cell lines were compared to 14 normal human foreskin melanocyte cell lines for the production of RNA transcripts specific for 11 different growth factors. Using the extremely sensitive technique of polymerase chain reaction to amplify growth factor-specific complementary DNAs, we analyzed the following: transforming growth factor (TGF) types \( \alpha, \beta_1, \beta_2 \), and \( \beta_3 \), acidic (a) fibroblast growth factor (FGF), basic (b) FGF, FGF-5, keratinocyte growth factor (KGF), HST, and platelet-derived growth factor (PDGF) types A and B. There were clear distinctions among the patterns of growth factor RNA expression by normal melanocytes and malignant melanoma cells. The prototypic melanocyte pattern of expression included TGF\( \beta_2 \), TGF\( \beta_3 \), and KGF. A subset of melanocyte cell lines also expressed PDGFA transcripts. In contrast, melanoma cells characteristically expressed RNA transcripts of TGF\( \alpha_1 \), TGF\( \beta_2 \), TGF\( \beta_3 \), TGF\( \alpha_3 \), bFGF, KGF, and PDGFA. Subsets of melanoma cell lines also expressed aFGF, FGF-5, and PDGF\( \beta \). The results presented indicated that TGF\( \beta_2 \), TGF\( \alpha_3 \), and bFGF may be particularly important in melanomagenesis and that these, as well as FGF-5, aFGF, and PDGFB, can be used as markers of transformation in this tumor type.

INTRODUCTION

Neoplastic transformation of human cells is consistently associated with quantitative or qualitative alterations in the expression of growth factors (1). These factors are thought to confer a growth advantage upon the malignant cell by acting, in part, as autocrine and paracrine mediators of cellular proliferation. Dissecting the types of biologically important growth factors produced by malignant and normal progenitor cells of a particular tumor type is the first step in elucidating whether particular growth factors have any role in the diverse array of phenotypic alterations observed in neoplastic cells. Malignant melanoma provides a valuable model for study of stage-specific events that accompany the progression of the melanocyte through the transformation process (2). To date, numerous studies have defined transformation-related alterations in (a) antigen expression (3, 4), (b) cell surface growth factor receptor expression (5), (c) production of growth factors (6, 7), (d) differentiation programs (8), (e) activation of oncopgenes (9), and (f) normal chromosomal structure (10). Despite the fact that specific roles for each of these alterations in the development or maintenance of melanoma cells has yet to be elucidated, they have been useful as markers of progression, differentiation, and transformation of the melanocyte (11). In order to more critically define genetic differences between the normal and malignant melanocyte that may have etiological importance, in the present study a series of cell lines established from human metastatic melanomas and normal human foreskin melanocytes was analyzed for the production of RNA transcripts specific to a spectrum of growth factors. The results of this analysis indicated clear distinctions among the transcription patterns of growth factors in normal melanocytes and metastatic melanoma cells.

MATERIALS AND METHODS

Cell Lines and Tumor Specimens. Cell lines established from human malignant melanomas (SK-MEL), human foreskin melanocytes, and human foreskin fibroblasts were derived and cultured as previously described (12). Purity of melanocyte cultures was determined by the expression of melanocytic specific cell surface antigens, cytoplasmic melanomatosomal proteins, and the presence of melanin (13). Melanoma and fibroblast cultures were maintained in Eagle's minimum essential medium supplemented with 2 mm glutamine, 1% nonessential amino acids, 100 units/ml streptomycin, 100 units/ml penicillin, and 7.5% fetal bovine serum. Melanocytes were maintained in an identical medium supplemented with TPA\( ^1 \) (10 ng/ml) and choloro toxin (10 \( ^{8} \) m) (Sigma Chemical Co., St. Louis, MO) (14). RNA was extracted from melanoma cells in culture from 8–40 passages and from melanocyte and fibroblast cell lines in culture for 40 passages. RNA extracted from a testicular germ cell tumor cell line, NTERA2 (15), was used as a positive control for HST transcription.

Preparation of RNA. Total RNA was extracted from logarithmically growing cells by the phenol/chloroform method as described previously (16). Extracted RNA was analyzed for integrity by agarose gel electrophoresis.

Preparation of cDNA. cDNA was generated from total RNA essentially as described in Ref. 17. Briefly, 1 \( \mu \)g of total RNA was incubated at 68°C for 5 min, chilled on ice, and reverse transcribed in a final volume of 20 \( \mu \)l containing: 50 \( mM \) Tris-HCl (pH 8.3), 75 \( mM \) KCl, 10 \( mM \) dithiothreitol, 75 ng of each dATP, dCTP, dGTP, and dTTP (Pharmacia, Piscataway, NJ), 500 ng random hexamer primers (Pharmacia), 12 units RNA Guard (Pharmacia), and 200 units Moloney reverse transcriptase (BRL Co., Gaithersburg, MD). The mixture was incubated at 37°C for 1 h, heated to 95°C for 10 min, and stored at \(-20°C\).

PCR Amplification. Amplification was performed as described previously (9, 18). Briefly, cDNA was heated to 95°C for 10 min and cooled on ice for 5 min. Then 1 \( \mu \)l was added to a 25-\( \mu \)l reaction mixture containing 2.5 \( \mu \)l 10 \( \times \) PCR reaction buffer \([0.5 \, M \, KCl, 0.1 \, M \, Tris\, (pH\, 8.0), 15 \, Mm\, MgCl\_2, 0.01 \, M\, gelatin\, (Fisher\, Scientific,\, Fair\, Lawn,\, NJ), 9.6 \, Mm\, each\, dATP,\, dCTP,\, dGTP,\, and\, dTTP\, (Pharmacia\, Inc.),\, 75\, ng\, of\, each\, priming\, oligomer,\, and\, 1.0\, unit\, Taq\, polymerase\, (Perkin-Elmer\, Cetus,\, Norwalk,\, CT)\, and\, H2O\). Reaction mixtures were prepared for multiple samples and aliquoted. A negative control consisting of a 25-\( \mu \)l aliquot without the addition of cDNA was included in each amplification. Twenty-five-\( \mu \)l mineral oil (Fisher Scientific) was layered over the aqueous phase to prevent evaporation. Amplification was performed using a DNA thermal cycler (Perkin-Elmer Cetus) for 20 cycles. A cycle profile consisted of 1 min at 95°C for denaturation, 1 min at 55–65°C.

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The abbreviations used are: TPA, 12-0-tetradecanoylphorbol-13-acetate; cDNA, complementary DNA; TGF\( \alpha_1 \) and TGF\( \beta_1 \), transforming growth factor, types \( \alpha \) and \( \beta \); aFGF and bFGF, fibroblast growth factor, types acidic and basic; KGF, keratinocyte growth factor; PDGFA and PDGFB, platelet-derived growth factor, types A and B; UVR, UV radiation.

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for annealing, and 1.5 min at 73°C for primer extension. Optimal annealing temperature was defined for each primer pair. Electrophoresis of 10 µl reaction mixture on a 1.4% agarose (IBI, New Haven, CT) gel containing ethidium bromide was performed to evaluate amplification and size of fragments generated.

PCR Oligonucleotide Primers. Oligonucleotide primers were synthesized using a DNA synthesizer 380A (Applied Biosystems, Foster City, CA). Gene sequences used to construct oligonucleotide primers were from published sources. All primer pairs used were designed to bracket cDNA sequences that in genomic DNA cross an intron-exon boundary. Table 1 details the specific oligonucleotide regions used. Primer sequences were as follows: β-actin, 5'-GTGGGGCGCCCCAGGCACCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ1, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ2, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ3, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ4, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ5, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ6, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ7, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ8, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ9, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ10, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ11, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ12, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ13, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ14, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ15, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ16, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ17, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ18, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ19, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ20, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ21, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ22, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ23, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ24, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ25, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ26, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ27, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ28, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ29, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20); TGFβ30, 5'-AAATGGATACGACCCACCCCA, 3'-GCTGCACTTGGGACCCAC (20). Restriction Endonuclease Digestion of PCR Products. Amplified fragments were purified with Centricron-100 microconcentrators (Amicon, Danvers, MA), lyophilized in a Speedvac concentrator (Savant Instruments Inc., Hicksville, NY), and redissolved in water. Restriction endonuclease digestion with the appropriate enzyme (5 units/µg) was performed at 37°C for 4 h. Digested fragments were fractionated on a 3% NuSieve/1% SeaKem agarose (FMC Bioproducts, Rockland, ME) gel.

RESULTS

Analysis of PCR Products. Total RNA was extracted from 19 cultured human metastatic melanoma cell lines and 14 normal human melanocyte cell lines. RNA was also extracted from seven short-term cultures of normal human foreskin fibroblasts, which served as an independent control for these experiments. The RNAs were reverse transcribed into cDNA and then amplified using gene-specific primer pairs and polymerase chain reaction methodology. The location within each gene sequence of all oligonucleotides used for PCR and the expected size of each amplified DNA fragment are shown in Table 1. Three different methods were used to confirm the identity of the DNA product generated by sequence-specific primers: (a) PCR-amplified DNA was analyzed by electrophoresis in agarose and compared to fragment size predicted by the location of the primers used (see Table 1 and Fig. 1); (b) PCR-amplified DNA was first digested with restriction endonucleases and then fractionated by electrophoresis in agarose; the resultant fragments were compared to predicted sizes based on location of internal cleavage sites (method used for aFGF, FGF-5, HST, KGF, and PDGFB) (data not shown); (c) PCR-amplified DNA was sequenced and the results compared to the known DNA sequence of the growth factor gene (method used for β-actin, TGFα, HST, and PDGFA) (data not shown). Fig. 2 illustrates restriction endonuclease analysis of the 247-base pair amplified PCR products generated from TGFβ1, TGFβ2, and TGFβ3 cDNA. Despite the high sequence homology of these genes, differences in restriction endonuclease sites allowed the unambiguous detection of individual members of the TGFβ family after PCR amplification with specific primers.

Growth Factor Production in Melanocytes and Melanoma Cells. Table 2 summarizes the expression of RNA transcripts of a panel of growth factors by 19 metastatic melanoma cell lines and 14 melanocyte cell lines. All cell lines expressed RNA transcripts for β-actin, indicating the integrity of the cDNAs and their ability to serve as templates for amplification. Virtually all melanoma cell lines expressed RNA transcripts for TGFβ1 (18 of 19), TGFβ2 (18 of 19), TGFβ3 (19 of 19), TGFα (19 of 19), bFGF (19 of 19), KGF (18 of 19), and PDGFA (15 of 19). In addition, a subset of cultured melanomas expressed aFGF (3 of 19), FGF-5 (5 of 19), and PDGFB (2 of 19). No melanoma culture (0 of 19) expressed HST-specific RNA transcripts.

In contrast to melanoma cell lines, cultured melanocytes had a different pattern of expression for these growth factor RNA transcripts. The typical melanocyte pattern appeared to be expression of only TGFβ, (14 of 14), TGFβ, (14 of 14), and KGF (11 of 13). Cultured melanocytes had no detectable expression of TGFβ, (0 of 14), aFGF (0 of 13), bFGF (0 of 14), FGF-5 (0 of 13), PDGFB (0 of 13), or HST (0 of 13). A subset of melanocyte cell lines did, however, express PDGFA transcripts (5 of 14). In addition, because of the extreme sensitivity of the PCR reactions performed, TGFα transcripts were detectable in 3 of 13 cultured melanocyte cell lines. In dilution experiments, in which equal amounts of melanoma and melanocyte cDNAs were used, expression of TGFα was detectable in 3 of 13 cultured melanocyte cell lines and 14 melanoma cell lines. In dilution experiments, in which equal amounts of melanoma and melanocyte cDNAs were used, expression of TGFα was detectable in 3 of 13 cultured melanocyte cell lines and 14 melanoma cell lines.
were titrated before PCR amplification, the results suggested that there was at least 10-fold more TGFα transcripts in melanoma cell lines than in TGFα-positive melanocyte cultures (data not shown).

Since melanocyte cell lines were grown in the presence of TPA and cholera toxin, we examined melanoma cultures for induction or suppression of growth factor-specific RNAs by medium containing identical concentrations of TPA and cholera toxin (data not shown). Thus, the action of TPA and cholera toxin on melanocytes does not include the suppression of any of the growth factors examined.

Growth Factor Production in Foreskin Fibroblasts. The expression of RNA transcripts of these same growth factors was examined in 7 human foreskin fibroblast cell lines. The pattern of expression was different from that observed in either melanoma cell lines or melanocytes. With the exception of PDGFB (0 of 7) and HST (0 of 7), all cultures expressed each of the growth factors analyzed: TGFβ1 (7 of 7), TGFβ2 (7 of 7), TGFβ3 (7 of 7), TGFα (6 of 7), aFGF (7 of 7), bFGF (7 of 7), FGF-5 (7 of 7), KGF (6 of 7), and PDGFA (7 of 7). The failure to detect HST transcripts in melanoma cells, melanocytes, or fibroblasts was due to the lack of transcription of this gene in these cells, and not to technical considerations, since HST-specific primers could detect HST transcripts in the germ cell tumor cell line, NTERA2 (data not shown).

DISCUSSION

In the present study, we compared the production by cultured human malignant melanomas and normal melanocytes of RNA transcripts for 11 different growth factors: TGFα, TGFβ1, TGFβ2, TGFβ3, aFGF, bFGF, FGF-5, KGF, HST, PDGFA, and PDGFB. Clear distinctions in the patterns of growth factor

Table 2 Expression of growth factor-specific RNA transcripts

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* Acknowledges detection of transcript after 50 amplification cycles.

ND, not determined.
expression in these cell types were evident. Virtually all foreskin melanocyte cultures expressed TGFβα, TGFββ, and KGF RNA transcripts, and 35% expressed PDGFA RNA transcripts. A strikingly different pattern of expression was detectable in melanoma cells. All melanoma lines expressed RNA transcripts for TGFβα, TGFββ, TGFβα, TGFα, aFGF, and KGF, while most also produced transcripts for PDGFA. A subset expressed aFGF, FGF-5, and PDGFB-specific RNAs. HST transcripts were not detected in any melanocyte or melanoma cell line. Foreskin fibroblast cultures expressed TGFβα, TGFββ, TGFβα, TGFα, aFGF, bFGF, KGF, FGF-5, and PDGFA but not PDGFB or HST.

**Transforming Growth Factors.** The TGFβ polypeptide family, which consists of at least 3 distinct members (TGFβ1, TGFβ2, and TGFβ3) (20–22), can both inhibit and stimulate cell proliferation and can influence differentiation (31). TGFβ3 is ubiquitously expressed in human tissues, while expression of TGFβ3 and TGFβ3 varies with cell type (20, 32). We showed that cultured normal melanocytes and malignant melanomas express TGFβ2 and TGFβ3, but that only melanoma cells synthesize TGFβ2 RNA transcripts. Since both normal and malignant melanocytes express TGFβ1 and TGFβ3, it is possible that these genes are involved more in the normal growth and differentiation of melanocytes than in melanoma development. In contrast, the transcription of TGFβ3 in all melanoma cell lines studied indicates that this gene is regulated independently of TGFβ1 and TGFβ3 and may serve a critical role during the development of the melanoma cell. TGFβ3 does have immunosuppressive effects (33), and it is speculated that induction of immunosuppression in hosts by TGFβ3-producing glioblastomas contributes to their growth by enabling these tumors to escape immune surveillance (34). TGFβ1, which shares a wide range of biological effects with TGFβ3 (35), has been shown to affect the regulation of expression of class II histocompatibility antigens in melanomas, and it is speculated that this regulation has an adverse effect on host immune response (36). Thus, by comparison with TGFβ1, TGFβ3 may similarly impact on the growth and maintenance of melanoma cells. While it remains to be determined whether three TGFβ mRNAs are actually translated into functional protein, the qualitative alteration in TGFβ2 transcription upon malignant transformation of the melanocyte may allow its use as a new marker of transformation in this tumor type. Whether the induction of TGFβ2 expression is associated with initial transforming events or with secondary events (e.g., those that affect growth, maintenance, or suppression of host immunity) is presently being analyzed.

TGFα is a 50-amino acid polypeptide that shares high structural homology in the receptor-binding domain with epidermal growth factor and can bind and activate the epidermal growth factor receptor (37), thus stimulating proliferation by an autocrine-mediated pathway. TGFα is expressed by a range of malignant human tissues (32, 38) and by some normal adult tissues (39). Our results showed that the majority of melanocyte cell lines lacked expression of TGFα RNA. This confirms the findings of a recent study that showed that TGFα protein is only detectable in foreskin melanocytes after treatment with UVR (40). However, because of the extreme sensitivity of the PCR method, low level transcription of TGFα was detected in a few melanocyte cultures, suggesting that induction by UVR is quantitative as well. In contrast, virtually all cultures of metastatic melanomas produced large amounts of TGFα transcripts (at least 10-fold more transcripts than TGFα-positive melanocytes). Thus, the expression of TGFα may be useful as a marker of melanocyte transformation. While a specific role for TGFα in melanogenesis remains obscure, TGFα is speculated to be an autocrine growth factor (32, 38), because it stimulates the growth of melanomas (40, 41) and melanocytes (40) in vitro. Moreover, the induction of TGFα by UVR may be linked to the promotion of preneoplastic or neoplastic cells (40), and the presence of a restriction fragment length polymorphism in the TGFα gene of melanoma patients may be a risk factor (42).

**Fibroblast Growth Factors.** The FGF family is a diverse group of polypeptide growth factors characterized by structural and sequence homology, heparin-binding characteristics, and the ability to promote angiogenesis and provide mitogenic stimuli for a wide range of mesoderm- and ectoderm-derived cell types (43). The FGF family includes bFGF, aFGF, FGF-5, HST/K-FGF, FGF-6, Int-2, and KGF (43). bFGF, a cell-associated polypeptide, is found in a wide range of cell types and tissues (44). It can mediate neoplastic growth and the development of metastasis by acting as an autocrine mediator of cell proliferation or as a transforming oncogene (44). The induction of bFGF transcription in all melanoma cell lines examined suggests that it plays a fundamental role in the development of the malignant phenotype. This interpretation is strengthened by the observations that normal melanocytes require exogenous bFGF to proliferate in culture (45) and that the growth of melanoma cells in culture can be inhibited by suppressing endogenous bFGF synthesis with antisense oligonucleotides (46). Although the spontaneous production of bFGF by transformed melanocytes may allow unrestrained proliferation, it is unlikely to be an initiating event, since transfection of bFGF cDNA into murine melanocytes abrogates proliferation dependence on exogenous bFGF but does not induce tumorigenicity (47).

KGF is a potent mitogen for keratinocytes but not fibroblasts (27, 48); unlike bFGF, it is efficiently secreted. KGF may be necessary for renewal of cell populations that line the skin (27). We show here that KGF transcripts were found in dermal fibroblasts, epidermal melanocytes, and malignant melanoma cells. Whether the expression of KGF by melanocytes has any role in epithelial cell proliferation in the epidermis remains unknown. However, since both normal and malignant melanocytes express KGF, it may be more related to the normal growth and differentiation of melanocytes and may not have any fundamental role in the development of melanoma.

Overexpression of normal FGF-5 and HST, which were originally isolated from human neoplasias as oncogenes capable of transforming rodent cells (26, 49), can cause transformation of mammalian cells (28, 50). FGF-5 transcripts were not detected in any melanocyte culture, whereas approximately 25% of cultured melanomas did express FGF-5 transcripts. Whether FGF-5 has a significant biological role in a subset of melanomas remains an open question, but the expression of FGF-5 only by malignant melanoma cells indicates that the transcription of this gene may be a useful marker of transformation. Transcription of the HST gene, which may contribute to the development of breast cancer (51), appeared to be more tightly regulated in cells of melanocytic lineage, since no melanocyte or melanoma culture expressed HST RNA transcripts. Thus, HST is unlikely to play a role in the biology of melanoma.

Similar to bFGF, aFGF can act as an autocrine mediator of cell proliferation and transform cells when overexpressed (52). A subset (15%) of melanoma cultures synthesized aFGF transcripts. While the biological impact of aFGF is as yet unknown, one conjecture is that aFGF is more relevant to late stages in
tumor progression. This speculation is based on the fact that there were qualitative differences in aFGF expression among tumor progression stages in human melanocytic nevi and melanomas. Cancer Res., 49: 5091–5096, 1989.


46. Becker, D., Meier, C. B., and Herlyn, M. Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor. EMBO J., 8: 3655–3691, 1989.


Induction of Growth Factor RNA Expression in Human Malignant Melanoma: Markers of Transformation

Anthony P. Albino, Brigid M. Davis and David M. Nanus


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