Expression of Thyrotropin-Releasing Hormone by Human Melanoma and Nevi

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ABSTRACT

Purpose: Thyrotropin-releasing hormone (TRH) is a tripeptide hormone produced by the hypothalamus in response to hypothyroidism. RNA transcripts for the TRH prohormone have recently been described in melanoma cell lines. To expand these findings, we have examined cultured melanoma cells and melanocytes, human melanoma tumors, and nevi for the expression of TRH.

Experimental Design: Five melanoma cell lines were analyzed by reverse transcription-PCR/Southern blotting for preproTRH message. The same melanoma lines and two melanocyte lines were examined by immunocytochemistry for TRH protein expression and for growth response to exogenous TRH. Immunohistochemistry was used to test for TRH protein expression in sections of 19 melanomas, 33 dysplastic nevi, and 27 benign nevi.

Results: TRH message and protein were detected in all melanoma cell lines examined. Melanocytes were also found to express TRH protein. Four of the five melanoma cell lines but neither melanocyte line responded with a increase in proliferation to low concentrations of exogenous TRH. TRH immunoreactivity was observed in 12 of 19 melanomas (63%), 23 of 33 (69.7%) dysplastic nevi, and 14 of 27 (51.9%) benign nevi. Expression in dysplastic nevi was significantly greater than in benign nevi. Upon separate analysis of nevi from melanoma patients, the difference between dysplastic and benign nevi was even more significant. However, in healthy individuals, no difference between dysplastic and benign nevi was observed. Furthermore, dysplastic nevi from melanoma patients had a significantly higher percent-

Received 10/7/03; revised 4/7/04; accepted 4/15/04.
Grant support: NIH T32 CA72371 (to J. Ellerhorst).
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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INTRODUCTION

Melanoma is a malignancy diagnosed with increasing frequency in the United States population (1). Melanoma tumors are derived from melanocytes, which are pigmented cells of neural crest origin. Consequently, melanomas retain many morphological and functional features reminiscent of neurons and neuroendocrine cells. Melanomas expressing neuron-specific enolase, somatostatin receptors, chromogranin, and synaptophysin have been described, whereas reports exist in the literature of neuroendocrine tumors containing melanin pigment (2–7).

In a recent study examining the expression of various hormones of the hypothalamic-pituitary-thyroid axis by skin components, it was noted that thyrotropin releasing hormone (TRH) was produced by two of six melanoma cell lines examined (8). An earlier study had described TRH immunoreactivity in extracts of a human melanoma tumor (9). TRH is a tripeptide hormone produced by the hypothalamus in response to diminished circulating levels of the thyroid hormone triiodothyronine (T3) and to a number of other metabolic stimuli. The potential significance of TRH production and secretion by melanoma cells lies in the observation that, in vitro, TRH can bind and activate the melanocortin-1 receptor, which is routinely expressed by melanocytes and melanoma cells (10).

In the present study, we have attempted to confirm and expand the observations of TRH production by melanoma. We report that TRH expression is a common finding in human melanoma tissue and in cultured melanoma cells and melanocytes and that exogenous TRH stimulates melanoma cell growth. Furthermore, we show that this hormone is more likely to be expressed by dysplastic nevi than by benign nevi and that dysplastic nevi of melanoma patients have higher TRH expression than dysplastic nevi of healthy individuals, thus implicating TRH in the malignant transformation of melanocytes to melanomas.

MATERIALS AND METHODS

Cell Lines and Media. The melanoma cell line A375 was obtained from the American Type Culture Collection (Manassas, VA). The TXM18 melanoma line was kindly provided by Dr. Janet Price [M. D. Anderson Cancer Center (MDACC), Houston, TX], the MeWo cell line by Dr. David Menter (MDACC), and the WM35 and WM793 lines by Dr. Robert

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toxin (Calbiochem); 0.1 mM 3-isobutyl-1-methylxanthine (Calbiochem, San Diego, CA), 1 ng/ml basic fibroblast growth factor (Cambrex, Walkersville, MD), 5 μg/ml transferrin (Sigma); 10 nm cholera toxin (Calbiochem); 0.1 mM 3-isobutyl-1-methylxanthine (Calbiochem), 30 μg/ml bovine pituitary extract (Cambrex), and 5 μg/ml insulin (Calbiochem). TRH used in growth stimulation experiments was purchased from Sigma.

**Patient Material.** Sections of melanoma tissue and nevi from melanoma patients were obtained from the MDACC Melanoma Tumor Bank. Sections of nevi from healthy individuals were obtained from the Dermatology Clinic (Kiel, Germany). Pituitary adenoma tissue was procured as incidental surgical material from hypophysectomy. Procurement of all patient materials at MDACC was conducted in accordance with Health Insurance Portability and Accountability Act guidelines.

**Reverse Transcription-PCR and Southern Blotting.** Reverse transcription-PCR was carried out using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) and oligonucleotide primers prepared by Sigma Genosys (The Woodlands, TX). Total RNA was extracted from melanoma cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA from a pituitary adenoma was used as a positive control for both prepro-TRH and TRH receptor reverse transcription-PCR (11, 12). Reverse transcription to cDNA was carried out using 1 μg of RNA. Reverse transcription took place at 42°C for 30 min. For preproTRH message, the reverse transcription product was amplified as described in Ref. 11, using nested PCR with the following primers: forward 5′-CTCTTCTCCGGGAAA-CATC-3′; reverse 5′-CTGGCGGTTTTACAGCATCAG-3′; and nested 5′-CTCTTCTCCGGCAGCTCTTGT-3′. The nested primer was added after 20 cycles, followed by an additional 25 cycles. PCR conditions were 94°C 0.5 min, 56°C 1.5 min, and 72°C 1.5 min; magnesium chloride concentration was 1.0 mM. Amplification of TRH receptor cDNA was performed as previously described (12), using the following primers: forward 5′-TGTTCAATAACAGCCTTTACCATT-3′ and reverse 5′-CAGAATTACAACCACTGCGAGCAT-3′. PCR conditions were 94°C 0.5 min, 48°C 0.5 min, and 72°C 3.0 min for 40 cycles; magnesium chloride concentration was 1.0 mM.

For Southern blotting, PCR products were separated by agarose gel electrophoresis, transferred to a nylon membrane, and probed with a 32P-labeled oligonucleotide probe specific for nucleotides 509–583 of the preproTRH cDNA sequence (13) or for nucleotides 661–710 of the TRH receptor cDNA. Probed membranes were exposed to film at ~70°C for autoradiography.

**Immunohistochemistry.** Immunohistochemical (IHC) analysis of melanoma and nevi sections for TRH expression was performed using a commercially available polyclonal antibody to human TRH (ICN Pharmaceuticals, Aurora, OH). Briefly, tissue sections were deparaffinized in xylene and rehydrated in graded concentrations (100 to 85%) of ethanol. Sections were placed in Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) and microwaved intermittently for a total of 10 min to maintain boiling temperature. After cooling, the slides were placed in 3% H2O2 in cold methanol for 15 min to block endogenous peroxidase activity. This step was followed by permeabilization with 0.05% Triton X-100 (Sigma) in PBS for 15 min. An avidin-biotin-peroxidase complex kit (Vectorstain; Vector Laboratories) was then used for antigen detection. After a 30-min incubation with blocking serum, the primary antibody was applied at a dilution of 1:50 and incubated for 2 h at room temperature. The slides were then washed and incubated for 30 min with secondary biotinylated antibody, followed by a 30-min incubation with the avidin-biotin-peroxidase complex reagent. The immunolabeling was developed with the chromogen 3-amino-9-ethylcarbazole. Hematoxylin was applied as a counterstain.

Sections were evaluated by two dermatopathologists (V. Prieto and A. Diwan) and scored separately for percentage of immunopositive cells and intensity of immunoreactivity.

For immunocytochemistry, cell lines were grown on tissue culture chamber slides and fixed overnight in 1% paraformaldehyde. Immunostaining methods were similar to IHC, with the exception that deparaffinization and antigen retrieval were not required.

**Growth Stimulation Assay.** Cells were plated in 6-well plates at a density of 1 × 104 cells/well. On the following day, media containing the desired concentration of TRH was placed on the cells, TRH-containing media was replaced daily. Cells were counted after 3 days (melanoma lines) or 9 days (melanocytes) with a hemocytometer after trypan blue exclusion. All growth experiments were conducted at last twice.

**Statistical Analysis.** Differences in IHC scores between subsets of nevi were analyzed for statistical significance with the two-sided Wilcoxon rank-sum test at a significance level of 5%.

**RESULTS**

**Cultured Melanoma Cells and Melanocytes Express TRH.** We first examined melanoma cell lines for the presence of message for the prohormone preproTRH. Total RNA was extracted from five melanoma cell lines: WM35, WM793, A375, MeWo, and TXM18. The RNA was reverse transcribed to cDNA, and amplified with preproTRH specific primers. PCR products were subsequently examined by Southern blotting. As shown in Fig. 1, all cell lines examined express the gene. Using similar methods, we analyzed these cell lines for expression of TRH receptors, whereby TRH might function in an autocrine fashion. Only one of the five lines, TXM18, was weakly positive by Southern blotting after prolonged exposure to film (data not shown).
shown), suggesting that TRH receptor expression by melanoma cells is uncommon and that an autocrine pathway would require an alternative receptor.

To establish the presence of TRH protein, cells from the same melanoma lines, as well as from two primary melanocyte cultures, were grown on tissue culture slides and examined for TRH expression by immunocytochemistry. All melanoma and melanocyte cell lines expressed the peptide hormone (Fig. 2).

**TRH at Low Concentrations Stimulates the Proliferation of Melanoma Cells but not Melanocytes.**

To examine the effect of TRH on growth, exogenous TRH was added to the media of cultured melanoma cells and melanocytes at concentrations ranging from 0 to 1000 pg/ml. To account for the possibility of additional TRH being secreted into the media by the cells, fresh media were placed on the cells daily. In four of the five melanoma cell lines tested, a consistent growth stimulatory effect was observed at TRH concentrations of 10–15 pg/ml (27.5–41.3 pM). No effect was seen at lower concentrations, and variable effects resulted from higher concentrations (data not shown). In contrast, TRH at 10 and 100 pg/ml failed to stimulate proliferation of normal melanocytes (FMC3C and FMC7C) and actually appeared to provide some degree of growth inhibition. Representative experiments are shown in Fig. 3.

**Expression of Immunoreactive TRH by Human Melanomas, Dysplastic Nevi, and Benign Nevi.**

The findings of TRH peptide in cultured melanoma cells and melanocytes led us to predict that the peptide would also be found in human melanoma tissue and nevi. Nevi, as with melanomas, are composed of proliferating melanocytes. The majority of nevi are benign, with the exception of dysplastic nevi, which are considered by many authors to be premalignant and potential precursors of melanoma. We hypothesized that if TRH contributes to the malignant phenotype of melanomas, the hormone should be expressed by dysplastic nevi to a greater extent than benign nevi. Expression of TRH in these various melanocyte-derived tissues was addressed using IHC to analyze sections of 19 primary melanomas, 33 dysplastic nevi, and 27 benign nevi. Samples of dysplastic and benign nevi were obtained both from patients with a history of melanoma and from healthy individuals (i.e., patients without a prior diagnosis of melanoma). TRH immunoreactivity was scored separately for percentage of positive cells and for intensity of immunostaining.

Results of the IHC analysis are shown in Table 1 and Fig. 4. The peptide hormone was detected in 12 of 19 (63.2%) primary melanomas, 23 of 33 (69.7%) dysplastic nevi, and 14 of 27 (51.9%) benign nevi. TRH expression was focal and, in the melanomas, was more likely to be seen in the superficial portions of the tumors (Fig. 4A). Expression was not detected in melanocytes present in areas of normal skin. Intense TRH immunoreactivity was also noted in melanophages present in TRH-positive samples (Fig. 4D, arrows). On the basis of the scoring for percentage of positive cells, there was a trend for greater expression in melanomas versus benign nevi (P = 0.154) and a significantly increased expression in dysplastic nevi versus benign nevi (P = 0.038). In contrast, melanomas and dysplastic nevi did not differ in this regard (P = 0.711). Samples were also scored and compared based on intensity of immunoreactivity, using the categories of “mild,” “moderate,” and “marked.” The majority of samples expressing TRH fell into the mild category, and no differences were noted between the three histological groups (data not shown).

A second analysis was similarly performed to discern differences in TRH expression between nevi from individuals with

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**Fig. 2** Expression of TRH by cultured melanoma cells and melanocytes. Expression of TRH by the melanoma cell line TXM18 (A) and cultured melanocytes (C) is shown, along with isotype controls (B and D). Original magnification, ×40.
and without a history of melanoma (Table 2). Among the melanoma patients, the pattern of higher TRH expression in dysplastic versus benign nevi was maintained with even greater significance ($P = 0.013$). However, in the group of healthy individuals without melanoma, no difference between benign and dysplastic nevi was detected ($P = 0.606$). Furthermore, scores for dysplastic nevi from melanoma patients were significantly higher than those for dysplastic nevi from healthy individuals ($P = 0.009$).

**DISCUSSION**

This study was conducted to confirm the expression of TRH by human melanoma cells. Furthermore, we wished to expand the data from prior studies to include the spectrum of human melanocyte-derived tumors, including benign and dysplastic nevi. We report the presence of TRH protein in 63% of human melanomas. Furthermore, we find that TRH protein is highly expressed by dysplastic nevi and to a lesser degree by benign nevi, the differences in expression being statistically significant. A finding of potential clinical importance is the increased TRH expression by dysplastic nevi of melanoma patients when compared with dysplastic nevi of individuals who have not been diagnosed with this malignancy. Dysplastic nevi are considered to be premalignant; however, <20% of patients with dysplastic nevi develop melanoma (14). The degree of histological atypia is currently the only means to identify individuals at greatest risk. Our data, if confirmed, would suggest that patients with TRH-positive dysplastic nevi are at high risk for developing melanoma, thus providing new prognostic information that might be used in management decisions. A prospective longitudinal study of dysplastic nevi patients with no melanoma history, designed to determine the correlation of TRH expression with the eventual development of melanoma, would be of considerable clinical interest.

The immunostaining experiments demonstrate that TRH is expressed by melanoma cells and cultured, proliferating, neonatal melanocytes but not by quiescent adult melanocytes.

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Fig. 3 Growth stimulation of melanoma cells but not melanocytes by TRH. Cells were grown in the presence of TRH at the concentrations indicated. A375, WM793, TXM18, MeWo, and WM35 are melanoma cell lines. FMC3C and FMC7C are primary melanocyte cultures. Error bars represent one SD from the mean of duplicate samples. – TRH 0 pg/ml; □, TRH 10 pg/ml; ■, TRH 100 pg/ml.
in normal skin. These findings support our hypothesis that TRH expression plays an important role in the proliferation of cells of this lineage and that melanomas may recall this mechanism that is normally limited to very early melanocytes. Our findings from the in vitro experiments of cells grown in the presence of exogenous TRH suggest that TRH in the extracellular environment provides a growth stimulus to melanoma cells and raise the question of how this stimulus might be conferred. One possibility is that TRH is secreted by melanoma cells and, subsequently, stimulates growth in an autocrine fashion by binding to a melanoma surface receptor. The absence of transcripts for specific TRH receptors in four of five melanoma cell lines suggests that a secondary receptor must be used for this purpose. Of note, it has been reported that TRH binds and activates melanocortin-1 receptor in vitro (10). This receptor, which is routinely expressed by melanocytes and melanoma cells, is normally bound by α-MSH, which provides a growth stimulus to the cells. If melanoma-derived TRH can activate melanocortin-1 receptor in vivo, this would provide a hypothetical mechanism whereby TRH might function as an autocrine growth factor. It is interesting that neither melanocyte line responded to exogenous TRH with an increase in growth rate. The explanation may lie in the presence and density of the receptors used by the peptide or differences in signal transduction after receptor ligation. The elucidation of the proposed autocrine loop and the differential sensitivities of melanoma cells and melanocytes are currently areas of active research in our laboratory.

We have recently reported a high prevalence of hypothyroidism among patients with uveal and cutaneous melanoma (15, 16). Our hypothesis of TRH as an autocrine growth factor for melanoma provides a model for the association between these two diseases. We propose that melanoma cells, similar to the neuroendocrine cells of the hypothalamus, are able to detect the state of hypothyroidism (i.e., low circulating thyroid hormone levels) and respond by producing TRH. This would provide additional TRH-induced growth stimulation to developing melanomas in hypothyroid individuals, resulting in their overrepresentation in the melanoma population.

In conclusion, TRH expression is a frequent finding in human melanomas and in dysplastic nevi of melanoma patients. This peptide can also stimulate melanoma cell proliferation at low concentrations. Elucidation of the biological function and regulation of TRH in melanoma cells may lead to a better understanding of the pathogenesis of this malignancy and provide new avenues for therapy-directed research.

ACKNOWLEDGMENTS

We thank Dr. Ian McCutcheon (MDACC) for assistance in procurement of human pituitary tissue and Dr. Tim Walch (MDACC) for technical advice.

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