Expression of the Neuroectodermal Intermediate Filament Nestin in Human Melanomas

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Abstract

Nestin is a newly identified intermediate filament expressed in proliferating neuronal progenitor cells, but not in the adult brain. Nestin expression reappears in many tumors of the central nervous system and has in human glioblastomas been associated with a high degree of malignancy. Because melanocytes are of neuroectodermal origin, we studied nestin expression in benign and malignant cells of the melanocytic lineage using Northern blot and immunohistochemical analyses. Nestin mRNA was detected in 24 of 34 metastatic melanomas and in 1 of 4 benign nevi, whereas the protein was expressed in 10 of 15 primary melanomas, in 29 of 34 metastatic tumors, and in 3 of 4 nevi. Neither normal melanocytes nor any metastatic tumors which express nestin, particularly the metastatic melanomas, suggests that nestin may be a useful marker for such malignancies. Furthermore, although no significant correlation between nestin expression and tumor malignancy was observed, the protein was most abundantly expressed in the infiltrating part of the tumors, indicating a possible involvement of nestin in tumor invasion.

Introduction

During mammalian embryogenesis, a group of migrating cells known as the neural crest cells emerge from the dorsal aspect of the neural tube. Whereas the neural tube contains precursors of glial cells and neurons of the CNS, the neural crest gives rise to peripheral neurons, Schwann cells, and secretory cells of the peripheral neuroendocrine system, as well as to nonneuronal cells such as melanocytes, chondrocytes, and smooth myocytes (1). Melanocytes are located in the skin, eye, and other epithelial tissues and are the precursors of benign nevus cell tumors and of malignant melanomas (2, 3). Whereas the highly differentiated benign nevi have a low proliferation potential and retain melanin production, malignant melanomas show increased proliferation and may partly or fully have lost the ability to synthesize melanin, in parallel with loss of differentiation (3). Tumor aggressiveness is often associated with a "dedifferentiated" phenotype, a feature which may give the tumor cells some of the characteristics of their precursors (3).

Nestin is a newly identified gene, encoding a protein belonging to a sixth class of intermediate protein filaments (4, 5). The gene is predominantly expressed in neuroectodermal progenitor cells and in developing skeletal muscle (4–6) but is down-regulated in both muscle and the CNS upon cellular differentiation. However, nestin was expressed in a variety of CNS tumors and a rhabdomyosarcoma (8, 9). Since nestin was also expressed in the stem cells of the PNS (1) and a subset of Schwann cells appears to retain nestin expression into adulthood (1, 7), it was possible that nestin could be expressed also in tumors derived from the neural crest. In support of this, previous studies showed that immortalization of primary neuroectodermal progenitor cells with an activated oncogene produced cell lines that expressed nestin, whereas this was not the case when nonneuronal cells was used (10). Because melanocytes are derived from the neural crest and it is known that melanomas express several neuroectodermal marker proteins (11), the aim of the present work was to examine whether and to what extent nestin is expressed by cells of melanocytic origin.

Materials and Methods

Specimens. Fresh tumor tissue for Northern blot analysis was sampled from 34 patients with metastatic malignant melanoma. In addition, biopsied tissue of 4 benign nevi and 4 basal cell carcinomas of the skin were obtained. Immediately upon surgery, the tumor samples were frozen in liquid nitrogen and stored at −135°C. Formalin-fixed, paraffin-embedded tissue from the 34 metastases, from 15 of the corresponding primaries, and from all nevi and basal cell carcinomas was available for immunohistochemical analysis. Seventeen of the 34 melanomas were classified as superficial, 9 as nodular, and 5 belonged to other histological subgroups. In 3 cases the histology of the primary tumor was not known. Eighteen of the primary melanomas were localized to the truncus, 12 to the extremities, and 2 to head and neck, and 2 had unknown localization.

Northern Blot Analysis. Total RNA was isolated from the tissues by the guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (12) or by the guanidinium thiocyanate-CsCl method described by Maniatis et al. (13). Samples of 5 µg of total RNA were resolved by electrophoresis on 1% agarose-formaldehyde gels (13) and blotted in 10× standard saline-citrate (1 × 0.15 M sodium chloride-0.015 M sodium citrate), pH 7.0, onto Hybond-N° membranes according to the manufacturer’s manual (Amersham). The membranes were subsequently baked at 80°C for 2 h and UV-cross-linked. The blots were hybridized with a human nestin complementary DNA probe (5) labeled with 32P by the random primer technique (14), carried out in 0.5× sodium phosphate (pH 7.2), 7% SDS, and 1× EDTA at 65°C for 16 h as described by Church and Gilbert (15). After hybridization, the membranes were washed three times for 20 min in 40× sodium phosphate (pH 7.2) and 1% SDS. For multiple hybridizations, the bound probe was removed by incubating the filters twice for 5 min in 0.1× standard saline-citrate and 0.1% SDS at 95–100°C. To control for RNA integrity and amounts, the filters were rehybridized with a kinase-labeled (13) human specific oligonucleotide probe complementary to nucleotides 287 to 305 of 18S rRNA.

Immunohistochemistry. Sections from formalin-fixed, paraffin-embedded tissue were immunostained using the avidin-peroxidase complex method described by Hsu et al. (16). Brieﬂy, the sections were incubated for 18–22 h at room temperature with rabbit anti-nestin (No. 130) (6, 8, 9) diluted 1:200, followed by sequential incubations with biotin-labeled secondary antibody and avidin-biotin-peroxidase complex. The reaction was ﬁnally developed using 3-aminio-9-ethylcarbazole as chromogen. All series included positive controls. Negative controls included substitution of primary antiserum with normal rabbit serum diluted 1:200.

Results

The results are summarized in Table 1. Northern blot analysis showed that 24 of the 34 (71%) malignant melanoma metastases...
expressed nestin mRNA, with a considerable variation in expression level in different tumors (Fig. 1). The nestin transcript was detected in 1 of 4 benign nevi, but not in any of the 4 basal cell carcinomas examined. In all cases, the nestin mRNA was of the expected length, 6 kilobases (Fig. 1; Table 1).

Immunohistochemical analysis demonstrated that 10 of 15 (67%) primary melanomas, 29 of 34 (85%) metastases, and 3 of 4 (75%) benign nevi expressed detectable levels of nestin protein. Immunostaining was in all cases localized to the cytoplasm (Fig. 2). It is noteworthy that nestin-synthesizing cells were in several cases mainly found in the peripheral, infiltrating parts of the primary tumor (Fig. 2a). The fact that not all immunopositive tumors were detected in the Northern blot assay was probably a result of a lower sensitivity of the latter method. The fraction of immunopositive cells varied among the tumors, from less than 5% to nearly 100% found in a sample from a patient with a desmoplastic variant of malignant melanoma. Importantly, neither normal melanocytes adjacent to the nevi or the malignant tumors nor the basal cell carcinomas showed any immunoreactivity with the nestin antiserum (not shown).

In 5 of the 15 cases where corresponding pairs of primary and metastatic tumors from the same patient were examined, nestin protein immunoreactivity was observed only in the metastases. In one additional case, a higher fraction of cells was stained in the metastasis than in the primary tumor, whereas in one patient a few cells in the primary tumor were nestin positive although the metastasis lacked immunoreactivity. In the remaining 9 cases, the staining pattern was similar in both types of lesions.

Attempts were made to examine whether the level of nestin could be associated with histological subtype, localization, and thickness of the primary tumor and the relapse-free period from diagnosis of the disease to the appearance of distant metastases. As shown in Table 1, the fraction of nestin-positive tumors was higher in the metastatic (17 of 17) than in the primary (6 of 11) tumors of the superficial subtype of melanomas, whereas only 5 of 9 of metastatic nodular melanomas expressed nestin protein. No significant difference in the thickness of the primary tumor (2.5 versus 3.0 mm) or the relapse-free period (41 versus 33 months) was observed among nestin-expressing and non-expressing tumors (not shown).

**Discussion**

The presence in tumor cells of various intermediate filaments has been used widely in diagnosis because of their tissue-specific expression and the distinct cytoplasmic localization of the encoded protein (17). Expression of the intermediate filament nestin in neuroectodermal progenitor cells (6) prompted us to examine whether nestin was expressed by cells of melanocytic origin and to what extent such expression might be correlated to the malignancy of the tumors. It was found that nestin mRNA was expressed in 24 of 34 (71%) malignant melanoma metastases and in 1 of 4 (25%) benign nevi and that the nestin protein was present in 10 of 15 (67%) primary melanomas, in 29 of 34 (85%) metastatic malignant melanomas, and in 3 of 4 (75%) nevi. In contrast, none of 4 basal cell carcinomas showed detectable levels of nestin mRNA and/or protein.

These data indicate that nestin is expressed in melanocytic tumors, but not in nonneoplastic melanocytes. This is in keeping with data from normal development showing that PNS stem cells express nestin (1), while in the adult PNS expression is restricted to a subpopulation of Schwann cells (1, 7). We did not detect nestin protein in Schwann cells of human skin, a finding which makes it unlikely that Schwannian differentiation is the reason for the presence of nestin in the nonmalignant nevus cells. The predominantly early expression of nestin in cells of the neuroectoderm and its reappearance in tumors of melanocytic origin is thus clearly similar to the situation in the CNS (4, 5, 8, 9) and in skeletal muscle (7, 8). It appears that the nestin gene is expressed in tumors or cell lines derived from cell types where nestin is normally expressed during development. The lack of detectable levels of expression in the basal cell carcinoma and in carcinomas metastasizing to the brain (8) further corroborates this view. Moreover, induced immortalization of epidermal and fibroblast primary cells did not produce nestin-expressing cell lines, while immortalization of neuroectodermal and myogenic cells did (6, 10).
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Given the reexpression of nestin in melanocytic tumors it could be asked whether the nestin expression pattern might correlate with specific characteristics of such tumors, similar to the correlation between nestin expression and malignancy previously observed in CNS tumors (8). A slightly higher proportion of immunopositive cells was found among the malignant melanomas, compared to the benign nevi, but the limited size of the material does not permit any firm conclusions. However, the fact that the level of nestin expression was higher in the metastases from superficial spreading melanomas than in the primary tumors and in the infiltrating compared to noninfiltrating tumor regions may suggest a correlation between nestin expression and metastatic capacity. On the other hand, since nestin expression did not correlate with tumor thickness, melanin content, or progression of the disease, a direct relationship between nestin expression and malignancy is not likely.

Little is yet known about the biological function of the nestin protein, but it has been suggested that intermediate filaments are involved in migration, proliferation, or structural remodeling of cells during development. It is interesting to observe that nestin is expressed in proliferating progenitor cells of the CNS, PNS, and muscle and that nestin expression precedes expression of other types of intermediate filaments, e.g., neurofilaments, GFAP, and desmin in the CNS and muscle (6). This may be because a nestin cytoplasmic network is more compatible with the structural dynamics of a dividing cell. Alternatively, the nestin network may serve as a framework for other intermediate filaments expressed later in the development (6). In any case, it is possible that nestin expression is advantageous for dividing migratory cells and that the higher level of nestin in the infiltrating parts of the neoplastic cells may suggest a role for nestin in the tumor invasion process.

In conclusion, our data show that nestin expression is confined to melanocytic tumors and is not present in surrounding, nonneoplastic melanocytic tissue. Moreover, there is a tendency that malignant melanomas more often express nestin in the advanced stages of the disease. Although no general association of nestin expression and disease parameters could be demonstrated, nestin expression might be useful as a supplement to other melanoma markers. The relationship between melanocytic tumors and their malignant counterparts in the CNS, here demonstrated by the expression of nestin, indicates that further studies of embryonic links between their progenitor cells are warranted.

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References

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