ABSTRACT

A large body of experimental evidence suggests that plasminogen activators provide tumoral cells with efficient means to degrade extracellular matrix constituents and thereby facilitate their dissemination to distant sites. Melanocytic neoplasia encompass a spectrum of lesions exhibiting diverse clinical behavior that remain difficult to predict with current histopathological evaluations. Little information concerning the contribution of plasminogen activation in diagnostic specimens of human melanocytic tumors is presently available. We thus analyzed biopsy specimens of pigmented skin lesions by histological techniques that identify the cellular sites of synthesis of plasminogen activators and of their inhibitors and that localize the sites of plasminogen activators-catalyzed enzymatic activities. We found that urokinase-type plasminogen activators (uPA) and plasminogen activator inhibitor type 1 mRNAs accumulate in atypical nevocytes and in melanoma cells, but not in benignnevocytes. However, uPA-catalyzed proteolytic activity was detected exclusively in melanomas.

These observations suggest that up-regulation of the uPA gene is an early feature of melanocyte transformation and that unbalanced enzyme/inhibitor activity is associated with the malignant phenotype. By supporting a role for uPA in melanoma invasiveness, they provide a novel tool for the evaluation of atypia in nevi.

INTRODUCTION

Neoplastic tissues synthesize and secrete proteases which can degrade ECM constituents and thereby facilitate the migration of malignant cells through anatomical barriers (1-5). Among the diverse extracellular proteolytic enzymes produced by animal and human tumors, plasminogen activators (PAs) are considered to play a pivotal role in tissue invasion and metastasis formation (6). PAs are secreted serine proteases which catalyze the conversion of plasminogen into plasmin, a protease of broad specificity capable of degrading directly or indirectly most ECM proteins (7). Two types of PAs have been identified in mammals, uPA and tissue-type plasminogen activator (tPA); abundant experimental evidences support the contribution of uPA to tissue remodeling processes, in particular those associated with malignant cell invasiveness. Tissular extracts from human tumors of diverse histogenetic origins contain elevated amounts of uPA when compared to their nonmalignant counterparts (6, 7); however, correlations between enzyme production and metastasizing properties have not been found in all neoplasia. More recent investigations have suggested that such apparent discrepancy may be due to the complexity of the PA/plasmin system. For instance, localization studies have revealed that uPA is synthesized by the tumor cells themselves (8); while in others, stromal cells constitute the predominant source of enzyme (9), a situation similar to that described for other secreted proteases (10, 11). In addition, natural inhibitors of PAs (PAIs) have also been detected in tumoral tissues and their synthesis has been ascribed to tumor (8) as well as to stromal cells (12). Altogether, these findings suggest that the net proteolytic activity available to neoplastic cells likely results from a balance between proteases and antiproteases. Therefore, the various members of the PA/plasmin system must be considered for a proper assessment of plasmin-mediated proteolysis in tumor specimens.

Plagmented skin lesions are of particular importance because of their clinical implication and because of the opportunity they provide to explore early stages in tumorigenesis; we thus analyzed a series of pigmented skin lesions including benign nevi, atypical nevi, and melanomas, by a combination of histological zymographies and in situ hybridizations.

MATERIALS AND METHODS

Tissue Sampling and Histological Assessment. Nevi and melanomas were prospectively collected immediately after surgical removal and cut in two parts. One half was frozen in precooled methylbutyrate and stored at -70°C. The other half was fixed in 10% formalin and embedded in paraffin for histological examination.

All specimens were reviewed by a dermatopathologist and classified according to conventional histological criteria. Eleven lesions were classified as benign nevi; these lesions consisted of junctional (2), dermal (2), and compound (7) nevi. Eight lesions were classified as atypical nevi; they all exhibited the following signs of atypia, although to variable degrees: inversion of the nucleo-cytoplasmic ratio; increased mitotic index, along with architectural disorder; fibrosis of the dermal papillae; and fusion of the epidermal crests (13). Thirteen primary melanomas were analyzed: 6 were classified as superficial spreading melanomas, with Breslow indices of, respectively, 0.3, 0.47, 0.78, 0.83, and 0.9 mm; 6 were classified as nodular melanomas, with Breslow indices of, respectively, 1.7, 3.48, 5, 9, and 9.5 mm.

Plasmid Constructions and in Vitro Transcriptions. The uPA antisense and sense probes were prepared from pSP64-hUK (14) and pSP65-hUK, respectively. The tPA antisense and sense probes were prepared from pBSKS-hPAI(7). The PAI-1 antisense and sense probes were prepared from pBSKS-hPAI-1 (16). The PAI-2 antisense probe was prepared from pDB4707 (17). RNA probes were transcribed in vitro with linearized plasmids in the presence of 12.5 mM [α-32P]UTP (400 Ci/mmol; Amersham International, Amersham, United Kingdom), [α-33P]UTP (2-3000 Ci/mmol; DuPont-NEN Products, Germany), or 30 mM [3H]UTP and 30 mM [3H]CTP (40 and 20 Ci/mmol, respectively; Amersham International). 3H-Labeled probes were reduced to an average size of 50-100 nucleotides by mild alkaline hydrolysis as described previously (18).

In situ Hybridizations. In situ hybridizations were carried out on 5-μm cryostat tissue sections as described previously (8, 18, 19). Localization of mRNAs were performed by hybridization of 32P, 33P, or 3H-labeled cRNA probes to tissues sections and revealed either by film autoradiography for macroscopic localization or by emulsion autoradiography for microscopic localization.

Immunohistochemistry. Immunohistochemistry was performed on 10-μm cryostat tissue sections with the avidin-biotin-horseradish peroxidase system from Dakopatts using mouse monoclonal anti-human CD68 (Dako A/S, Denmark), mouse monoclonal anti-human von Willebrand factor (Dako A/S), and goat polyclonal anti-human tPA (American Diagnostica). The primary antibody was detected either with biotinylated rabbit anti-goat or biotinylated sheep anti-mouse antibodies.

Enzymatic Assays. Histological zymographies were performed on 10-μm cryostat tissue sections as described elsewhere (19). Sections were overlaid with a mixture containing 0.75 ml of phosphate-buffered saline (with 0.9 mm
Ca\(^{2+}\) and 1 mM Mg\(^{2+}\), 2% nonfat dry milk, 0.9% agar, and 40 µg/mL of purified human plasminogen in PBS. After a heating at 50°C, 80 or 130 µL of this mixture were applied over prewarmed cryostat tissue sections and evenly spread under 22- by 22- or 24- by 32-mm glass coverslips. The slides were incubated in a humid chamber at 37°C for 3-6 h. The same procedure was performed applying a mixture without plasminogen, containing 1 mM amiloride (20), a uPA inhibitor, or 0.2 mg/mL of anti-human tPA goat immunoglobulins (American Diagnostica, Inc., Greenwich, CT). Photographs were taken using dark-ground illumination.

For SDS-PAGE zymographies, tissular protein extracts were prepared and analyzed as described elsewhere (21). After separation of total proteins on a 10% SDS-PAGE, zymographies were performed on a casein underlay containing the same mixture as mentioned above for histological zymographies.

**RESULTS**

**Benign Nevi.** In 11 of 11 specimens analyzed, histological zymograms performed on consecutive cryostat tissue sections revealed plasminogen-dependent zones of lysis that predominated in the dermis (Fig. 1, upper left). These caseinolytic areas were not affected when amiloride (Fig. 1, upper middle) was added to the substrate, while they were abolished in the presence of anti-tPA antibodies (not shown), indicating that the observed catalytic activity was due to tPA.

For SDS-PAGE zymographies, tissular protein extracts were prepared and analyzed as described elsewhere (21). After separation of total proteins on a 10% SDS-PAGE, zymographies were performed on a casein underlay containing the same mixture as mentioned above for histological zymographies.

**Atypical Nevi.** As observed in benign nevi, histological zymograms performed on cryostat tissue sections of atypical nevi revealed circumscribed zones of plasminogen-dependent caseinolysis that predominated in the dermis (Fig. 2, upper left). In 8 of 8 samples examined, areas of caseinolysis were similar in the presence or absence of amiloride (Fig. 2, upper left) but were suppressed by anti-uPA antibodies (Fig. 2, upper right), indicating that tPA was responsible for the observed proteolysis. Immunohistochemistry with anti-tPA and anti-factor von Willebrand antibodies assigned tPA expression to endothelial cells. Macrosopic mRNA localization studies revealed in 6 of 8 atypical nevi an accumulation of uPA mRNA in nevocytes (Fig. 2, lower left). To identify the sites of uPA and tPA synthesis at a cellular level, adjacent tissue sections were hybridized with \(^{33}P\)- or \(^{3}H\)-labeled cRNA probes: uPA mRNA was exclusively found in foci of nevocytes displaying morphological signs of atypia (Fig. 3, A, B); while tPA mRNA was predominantly localized in endothelial cells (not shown). To explore the apparent discrepancy between the presence of uPA mRNA and the absence of uPA-mediated catalytic activity, we searched for the concomitant expression of PAIs. Hybridization of tissue sections to PAI-1 and PAI-2 \(^{33}P\)-cRNA probes demonstrated the presence of PAI-1 mRNA in 5 of 8 specimens analyzed and of PAI-2 mRNA. PAI-2 mRNA was present in the epidermis in all specimens in atypical nevi, as described previously in other skin tumors (8), as well as in benign nevi (not shown). Microscopic observations of tissue sections hybridized with \(^{33}P\)- or \(^{3}H\)-labeled cRNAs confirmed the accumulation of PAI-1 mRNA in foci of atypical nevocytes (Fig. 3, C, D). The melanocytic nature of uPA- and PAI-1-producing cells was supported by immunohistochemistry for CD68 performed on adjacent cryostat tissue section: it showed only a few inflammatory cells, which could not account for the
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amount of uPA- and PAI-1-labeled cells. In addition, the presence of melanin in some of the uPA and PAI-1 mRNA-containing cells supported the nevocytic nature of these cells. Although our investigations were performed on serial tissue sections, we cannot ascertain whether PAI-1-producing cells are the same cells that produce uPA. These findings demonstrated that a substantial proportion of atypical nevi differed from benign nevi in their capacity to synthesize uPA; in addition, they showed that the very same atypical nevi produce PAI-1 simultaneously, which may damp the activity of secreted enzyme, as suggested by the lack of detectable uPA-catalyzed proteolytic activity in these lesions.

Melanomas. In contrast to benign and atypical nevi SSM displayed both types of plasminogen-dependent caseinolytic activity (Fig. 4, upper left): (a) in all specimens analyzed (6 of 6), zones of caseinolytic activity were noticed over tumoral tissues; they were inhibited by the addition of amiloride in the substrate but were not modified by anti-tPA antibodies, indicating that the catalytic activity associated with the neoplastic tissues was mediated by uPA; (b) areas of caseinolytic activity were observed on adjacent nonmalignant tissues that were not abolished by amiloride (Fig. 4, upper middle) but by anti-tPA antibodies (not shown), indicating that the latter catalytic activity was due to tPA. Endothelial cells were found to express tPA immunoreactivity (not shown), corroborating our observations on benign and atypical nevi. Macroscopic and microscopic mRNA localization studies documented the expression of uPA mRNA in some melanoma cells (Fig. 4, lower left). As in atypical nevi, the melanocytic nature of uPA-producing cells was supported by immunohistochemical CD68 determination on adjacent tissue sections, revealing

Fig. 3. Cellular localization of uPA and PAI-1 mRNAs in an atypical nevus (A-D) and in a nodular melanoma (E-H). Tissue sections were hybridized to [3H]cRNAs and exposed for 12 weeks or to [32P]cRNAs (A, B) and exposed for 4 weeks. B, D, F, and H, light field micrographs; A, C, E, and G, corresponding dark-field micrographs. Notice in a junctional nest, the accumulation of uPA (A, B) and PAI-1 (C, D) mRNAs in nevocytes (arrowheads), recognizable by their melanin granules. X 400. Insets, high magnification (X 600) of nevocytes containing uPA and PAI-1 mRNA. Some tumoral cells (arrowheads) that contain abundant uPA (E, F) or PAI-1 (G, H) mRNA are recognizable by their melanin granules. G, X 400; H, X 600.
that SSM contained very few inflammatory cells that did not correspond to the uPA mRNA-labeled cells (not shown). In addition, their melanin content supported the melanocytic nature of these cells. The proportion of cells containing uPA mRNA in SSM exceeded that observed in atypical nevi, while only occasional melanoma cells were found to contain PAI-1 mRNA, in a proportion similar to that noticed in atypical nevi. In addition, cells containing uPA mRNA were found at the invasive edge of the tumor and beyond the front of PAI-1 mRNA-containing cells. These findings demonstrated that in SSM malignant cells synthesize uPA mRNA and that uPA mRNA accumulation is consistently coupled to detectable uPA-mediated catalytic activity.

In 6 of 6 nodular melanomas analyzed, two types of plasminogen-dependent caseinolytic activity were observed over tumoral tissues; zones of amiloride-sensitive and zones of amiloride-resistant caseinolytic activities were noticed, indicating that both uPA and tPA enzymes were associated with nodular melanomas (Fig. 5, upper left and upper right). In addition, the respective enzymatic activities showed distinct and nonoverlapping distributions: amiloride-sensitive, i.e., uPA-mediated, catalytic activity was predominantly localized at the borders of malignant nodules (Fig. 5, upper left); whereas amiloride-resistant catalytic activity (Fig. 5, upper right), i.e., tPA mediated, was mainly detected in the central zones of tumoral nodules. Macroscopic mRNA localization confirmed the accumulation of both uPA and tPA mRNAs in 6 of 6 nodular melanomas and their distribution in distinct and nonoverlapping areas of the tumors (Fig. 5, middle left and middle right). High levels of uPA mRNA were consistently detected in the peripheral zones of neoplastic nodules corresponding to areas displaying uPA-mediated catalytic activity, while high amounts of tPA mRNA were not systematically coupled to detectable tPA-mediated catalytic activity. Total protein analysis by SDS-PAGE and zymography of the denaturing gel displayed the presence of high molecular weight complexes between tPA and PAI-1, indicating that tPA is bound to PAI-1 in melanomas (not shown). In addition, high amounts of PAI-1 mRNA were found in most regions of tumoral tissues (Fig. 5, lower left). Cellular mRNA localization demonstrated that uPA mRNA was contained in melanoma cells, some of them recognizable by their melanin granules (Fig. 3, E, F), whereas tPA mRNA was expressed by melanoma and endothelial cells residing in the central areas of tumoral nodules (not shown). Immunohistochemical localizations of CD68 and von Willebrand factor confirmed that uPA, PAI-1, and tPA mRNAs were predominantly contained in tumoral cells rather than in endothelial or inflammatory cells. Although PAI-1 mRNA (Fig. 3, G, H) was found in most melanoma cells, it is of interest to note that in situ hybridizations performed on consecutive tissue sections indicated that a proportion of uPA mRNA containing melanoma cells were located beyond the front of malignant cells containing high amounts of PAI-1 mRNA, whereas most melanoma cells expressing tPA mRNA appeared to contain simultaneously
DISCUSSION

Guided by the recognized contribution of extracellular proteolytic systems to ECM remodeling, greater attention has been devoted during the past decade to study the role of proteolytic enzymes in invasive cell phenotypes. For instance, uPA is a protease produced by cells endowed with marked invasive properties such as trophoblastic cells, monocytes-macrophages and malignant cells. This enzyme is thought to provide cells with efficient means to degrade ECM proteins and thereby facilitate their migration through normal tissues (7). A large body of experimental evidence supports the participation of the PA/plasmin system in the biology of melanomas. Melanoma cell lines have been shown to produce uPA and/or tPA (22–25) as membrane bound (24, 25) and/or as secreted enzymes (22, 23). Additional studies have documented that both enzymes can catalyze degradation of ECM constituents (26, 27), and correlations between uPA production and metastatic properties have been drawn for animal and human melanoma cell lines (28, 29). However, little information concerning PA-catalyzed extracellular proteolysis in diagnostic specimens of human melanoma is presently available (30).

Our results, summarized in Table 1 represent a systematic determination of PAs and PAIs on a series of human pigmented skin lesions. The uPA mRNA accumulated in neovocytes from atypical nevi and in melanoma cells, while it remained undetectable in morphologically benign melanocytosis. These observations indicate that up-regulation of uPA gene expression allowed a discrimination between atypical and benign neovocytes and, conversely, that it identifies atypical neovocytes which share differentiation features with melanoma cells. A similar pattern of differential expression was noticed for PAI-1 mRNA, which was found in atypical neovocytes and in melanoma cells, but not in benign neovocytes. It should be noted that our observations differ from those obtained in an analogous exploration that has found uPA expression only in melanomas (30). This apparent discrepancy could be attributed to methodological differences, since for in situ hybridizations we used cRNA probes and determined microscopic localization with 3H-labeled probes; in our hands, this approach is more sensitive and provides a better cellular resolution than that achieved by using 35S-labeled complementary DNA probes. However, our findings are in agreement with the morphological and functional features that relate atypical nevi to melanomas and is consistent with the hypothesis that some atypical nevi represent precursor lesions of melanomas. Whether this may provide a new tool for the evaluation of atypia in melanocytic nevi in practice remains to be further evaluated. Furthermore, our findings reveal functional differences between atypical neovocytes and melanoma cells; it demonstrates that uPA mRNA accumulation in atypical neovocytes is not coupled to detectable uPA-catalyzed proteolytic activity, whereas in melanoma cells increased uPA mRNA levels are accompanied by a concomitant increase in uPA activity. Although the methodological approaches used in the present study did not allow precise quantifications, our results raised the possibility that differences in the net uPA-catalyzed proteolytic activity observed between atypical nevi and melanomas may result from variations in the relative amounts of protease (uPA) and antiprotease (PAI-1) produced by these respective lesions. Variations in the amount and the distribution pattern of PAI-1 could account for such differences. However, the participation of other inhibitors to the control of plasmin-mediated proteolysis, such as α2-antiplasmin and protease-nexin I, is not excluded. Finally, we show here that nodular melanomas display striking regional heterogeneity with regard to PA production: distinct subpopulations of malignant cells were found to produce either uPA or tPA; while most of them synthesized PAI-1. Such a mosaicism is compatible with the well recognized intra- and intertumoral heterogeneity observed by immunophenotyping of melanoma specimens (31, 32) and could account for the relative variability in terms of PA and PAI production observed among cell lines derived from common parental origins.

Extracellular proteolysis is a tightly controlled process which results from a balance between proteases and their inhibitors. Plasminogen activation is thought to catalyze ECM degradation during a variety of physiological and pathological processes. Recent work suggests that unbalanced production of PAs and PAIs is responsible for the disturbance in the net proteolytic activity mediated by PAs during pathological processes. Our exploration of pigmented lesions provided novel evidence for alterations in plasmin-catalyzed proteolysis system during neoplasia. We have documented progressive changes in gene expression that lead to the overproduction of uPA-mediated proteolysis in invasive melanocytic tumors. Although the contribution of uPA to melanoma invasiveness remains to be established, our observations strengthen the concept that unrestrained uPA catalytic activity may account for differences between physiological and pathological invasive cell phenotypes.

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Plasminogen Activation in Melanocytic Neoplasia
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