Malignant Melanoma

Interaction with Coagulation and Fibrinolysis Pathways In Situ

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Immunohistochemical techniques applied to fresh frozen sections of metastatic malignant melanoma tissue revealed abundant fibrinogen (or fibrin I) in perivascular areas throughout the tumor connective tissue stroma. Fibrin was readily detected in a focal distribution in the connective tissue around nodules of viable tumor. Staining for D-dimer of cross-linked fibrin (using an antibody that cross-reacted with fragment D of fibrinogen) coincided with staining for fibrin. Diffuse staining of tumor cell bodies was observed for Factor X, and Factor XIII ("a" subunit) was detected in scattered areas of connective tissue throughout the tumors. Factor VII was not detected, and only rare tumor cells stained for tissue factor. These results support the concept that a tumor cell–associated, thrombin-generating pathway exists *in situ* in malignant melanoma tissue that includes Factor X but neither tissue factor nor Factor VII. By contrast, tumor cell staining was observed rarely for urokinase and to a variable extent for tissue plasminogen activator. (Key words: Melanoma; Coagulation; Fibrinolysis; Fibrin; Cancer procoagulant) Am J Clin Pathol 1990;93:516–521

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MALIGNANT MELANOMA arises from melanin-producing cells and is characterized by its association with intermittent, intense exposure to ultraviolet solar radiation; its tendency to disseminate widely; and its relative resistance to conventional forms of treatment.[10,19] Although its cell of origin and relation to solar radiation set melanoma apart from certain other tumors, it is possible that the malignant behavior of melanoma cells may be expressed through mechanisms that it shares in common with other tumors. In this regard, interest has focused on the possible role of components of blood coagulation and fibrinolysis pathways in facilitating tumor invasion and metastatic dissemination. This interest is based on observations in certain experimental animal models of malignancy in which it has been shown that interruption of such pathways therapeutically blocks tumor dissemination. These concepts have been reviewed recently.[9,30] Although these findings are promising, they have also indicated that the coagulation–cancer interaction is not a simple one because various experimental models respond differently (and sometimes in diametrically opposing ways) to therapeutic intervention. Mechanisms clearly differ between tumor types.

Because these interactions may be relevant to dissemination of human malignancy, attempts have been made to elucidate pathways of interaction between cancer cells and coagulation/fibrinolysis pathways. Immunohistochemical techniques are capable of revealing components of these reaction pathways *in situ* within tumor tissue. These techniques have been used successfully to characterize the coagulation–cancer interaction *in situ* in several common malignancies.[27,28,31–33] Distinct differences have been observed that signal striking but not unexpected heterogeneity in mechanisms of interaction between these tumor types.

It has been suggested that both coagulation[8] and fibrinolysis[4,18] pathways may mediate progression of malignant melanoma in humans. However, few details are available on components of such reaction pathways that may exist in proximity to viable melanoma cells *in situ* that may mediate tumor progression. This study reports findings on the occurrence of components of coagulation and fibrinolysis pathways *in situ* in human melanoma tissue.

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Materials and Methods

Studies were performed on fresh frozen tissue specimens of metastatic tumor obtained at autopsy from four cases of malignant melanoma. Staining procedures and controls for the avidin–biotin complex (ABC) technique[27,28,31–33] using reagents (Vectastain® Kits) from Vector Laboratories (Burlingame, CA) and for the immunofluorescence technique[34] have been described previously. Procedures used monospecific, purified rabbit antibodies to the following: recombinant human tissue factor (TF) (a gift of J. N. Wilcox and L. Paborsky, Genentech, Inc., South San Francisco, CA), Factor VII, Factor X, Factor V (the "a" subunit of Factor XIII), protein S, protein C, high molecular weight urokinase-[26] and low molecular weight urokinase-type plasminogen activator (u-PA),[24] tissue-type plasminogen activator (t-PA), and plasminogen activator inhibitor (PAI)-3.[25] The antibody to t-PA was prepared by immunizing rabbits with rt-PA (recombinant t-PA, Genentech, South San Francisco, CA) and isolating monospecific IgG as described for α-HMW-uPA.[26] Rabbit polyclonal antibodies were prepared from protein antigens that were more than 99% pure. Antibodies were purified from crude antiserum either by affinity chromatography on an antigen–sepharose column (for antibodies to Factor VII, Factor X, and protein C) or on a protein A–sepharose column (for antibodies to TF, Factor V, protein S, HMW-u-PA, LMW-u-PA, PAI-3). Antibodies to HMW-u-PA, LMW-u-PA, and PAI-3 were purified by sequential adsorption with the use of both of the above ligands. Antibody specificity was demonstrated on the basis of activity neutralization, Western immunoblot analysis, and immunoprecipitation studies using ^{125}I-labeled antigen or proteins labeled metabolically *in vivo* with the use of ^{35}S-methionine. The antibody to TF was capable of immunoprecipitating TF from cell extracts and stained a protein of about 42,000 daltons on Western blots. Antibodies to t-PA and u-PA did not cross-react with the alternative plasminogen activator.[24,26] Antibody to PAI-3 did not cross-react with other PAIs (PAI-1, PAI-2, protease nexin).[25] Monoclonal antibodies specific for epitopes on fibrin(ogen) or their degradation products included the following: antibody 1-8C6, that requires an intact 14 Arg-17 Gly bond in the Bβ chain of fibrinogen and therefore reacts with fibrinogen or fibrin I (des fibrinopeptide A-type fibrin) but not fibrin II (des-fibrinopeptide B-type fibrin)[14]; antibody T2G1 that reacts with the amino-terminal part of the Bβ chain only after removal by thrombin of fibrinopeptide B (FPB, Bβ 1–14) and thus with fibrin but not fibrinogen[13]; and antibody GC4 that reacts with fragment D of fibrinogen as well as D-dimer from cross-linked fibrin but not with either fibrinogen or fibrin.[12] Mouse monoclonal antibody to platelet surface glycoprotein II_bIII_a was obtained from Dr. Barry Coller. Goat antibody to PAI-1 and mouse monoclonal antibody to PAI-2 were obtained from American Diagnostica (New York, NY). Rabbit antibody to the human plasmin-α_2 antiplasmin complex neoantigen was obtained from Behring Diagnostics/Hoechst and used as described by others.[5]

Antibodies were tested on control and tumor tissues in concentrations that provided maximum staining intensity with minimum background staining. Controls consisted of omission from the procedure of the primary antibody and use of antibodies developed in the same species but with different or irrelevant specificities. Results of studies on melanoma tissues were compared with findings on normal control (placenta and liver) and neoplastic control (small cell carcinoma of the lung [SCCL], and colon cancer) tissues processed simultaneously.

Results

Antigen staining was detected by the dark brown reaction product obtained with the ABC procedure. This contrasted with the dark blue nuclei of cells and the pale pink appearance of unstained cells and stroma. The results obtained in melanoma are described together with the results of certain procedures performed on SCCL and colon cancer tissues processed simultaneously. Although results in the latter tumor types will be reported in detail separately,[27,28] these are described briefly here to illustrate the differences between tumor types and to emphasize certain essential positive controls. Although TF antigen was clearly demonstrable on the cell bodies of SCCL cells, TF was detected focally and on only rare cells in two cases of melanoma. Factor VII was readily detected in normal hepatocytes and was associated with SCCL cells, but no staining was observed in melanoma tissue. By contrast, more abundant staining of tumor cell bodies was observed for Factor X. These results are illustrated in Figure 1. Protein C and protein S antigens were readily detectable in normal liver and associated with SCCL tumor cells, but protein C was not detected in melanoma tissue, and only rare tumor cells stained for protein S in two cases. Antibody 1-8C6-reactive material, *i.e.*, fibrinogen (or fibrin I), was present in abundance particularly in perivascular areas throughout the tumor stroma in all cases. Fibrin (that is, T2G1-reactive material) was readily detected in a focal distribution in the connective tissue around nodules of viable tumor in all cases indicating that thrombin had formed and acted on fibrinogen in these areas. Antibody GC4 that reacts with the D-dimer site of cross-linked fibrin (as well as with fragment D of fibrinogen) stained the tumor connective tissue in a pattern that coincided with staining for fibrin. These findings are illustrated in Figure 2. Factor XIII ("a" subunit) was detectable in normal hepatocytes and in scattered areas of connective tissue throughout the tumors. HMW-u-PA was observed

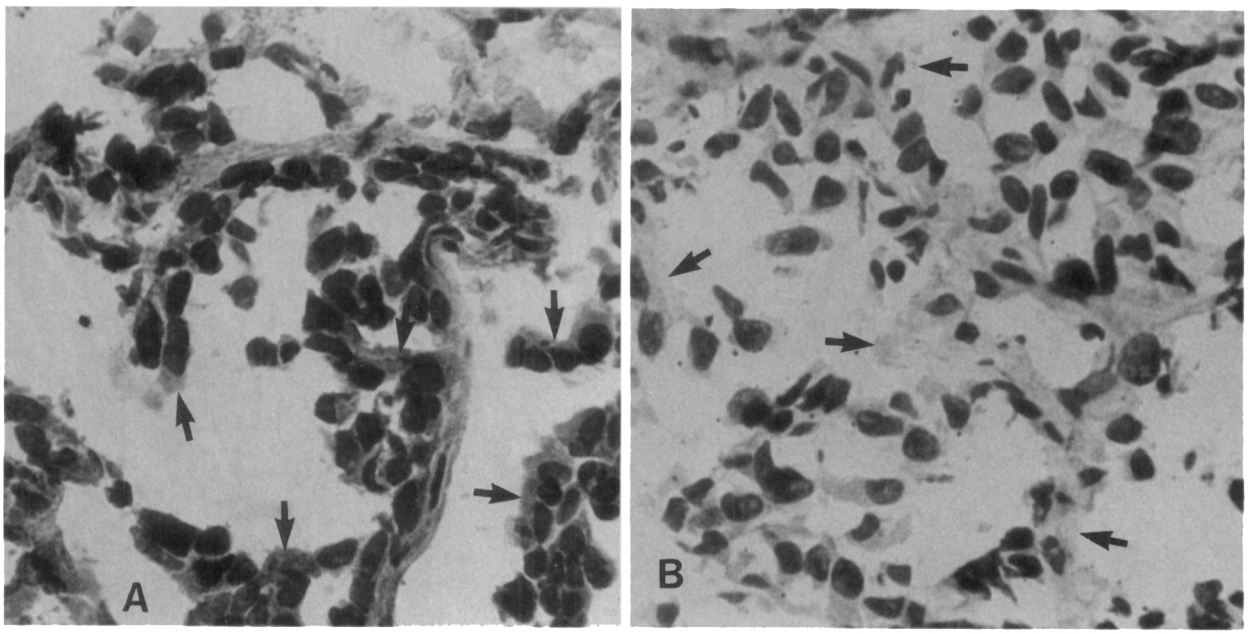

FIG. 1. Specific staining by the peroxidase technique for Factor X in fresh frozen sections of malignant melanoma tissue. *Arrows* show staining (that appeared dark brown in the original) of tumor cell bodies in specimens treated with antibody to Factor X (*A, left*) that were absent (the cell bodies appeared pale blue in color in the original) in control specimens (*B, right*). Adjacent connective tissue remained unstained. Hematoxylin counterstain (×250).

in rare tumor cells in one case. Staining for LMW-u-PA was observed diffusely on colon cancer cells but was seen in only occasional foci of apparently viable tumor cells in three of the four cases of melanoma. Strong staining for t-PA was detected in a variable number of tumor cells in three of the four cases. Antibody to PAI-1 stained antigen in syncytiotrophoblast and in a nodular pattern corresponding to fibrin deposits on the surfaces of placental villi in a focal distribution. PAI-1 was also detected by weak staining of vascular endothelial cells. PAI-2 was detected in the cytotrophoblast and syncytiotrophoblast of term placenta,[2] and PAI-3 was detected in a nodular pattern corresponding to fibrin deposits on the surface of placental villi. In contrast to these positive findings, no PAI-1 was detected in melanoma tissue, and equivocal (faint) staining of some tumor cells was observed for PAI-2 in a single case. PAI-3 antigen was detected only in areas of necrosis in two cases. No staining was observed with antibody to platelet surface glycoprotein II_bIII_a (which did stain platelets and megokaryocytes) or to the plasmin-α_2-antiplasmin complex neoantigen with the use of either the peroxidase or fluorescence procedures.

Discussion

Although the coagulation mechanism is known to be activated systemically in patients with malignant melanoma,[20,22] little information is currently available on the occurrence of components of coagulation and fibrinolysis pathways *in situ* in melanoma tissue that might mediate such activation. Donati and colleagues[8] described an activator of Factor X, termed cancer procoagulant (CP), in extracts of human melanoma tissue. This activator differed in its properties from tissue factor found in benign melanotic tissues and was observed to initiate thrombin formation in the absence of coagulation Factor VII. Levels of CP were found to be higher in metastases than in primary tumors. Kwaan and associates[17] claimed that fibrin existed in the connective tissue around melanoma tumor deposits, suggesting that coagulation was activated locally in melanoma. Although we did not have the opportunity to examine melanoma tissues directly for the existence of CP *in situ,* our observations of a paucity of TF and absence of Factor VII together with more abundant Factor X associated with viable tumor cells are consistent with a CP-initiated pathway of thrombin formation in melanoma tissue. Thrombin is indeed formed *in situ* in melanoma tissue because we succeeded in demonstrating not only fibrinogen, present throughout the perivascular tumor connective tissue, but also thrombin-specific cleavage sites on fibrinogen in a restricted distribution adjacent to nodules of viable tumor. Factor XIII ("a" subunit) detectable in the tumor connective tissue was likely derived from plasma and probably accounted for the staining with antibody GC4 that detects the D-dimer cross-linked site of fibrin. These results differ from observations on SCCL in which the existence of tumor cell–associated TF,[28] Factor VII, and Factor X[33] suggests that local thrombin

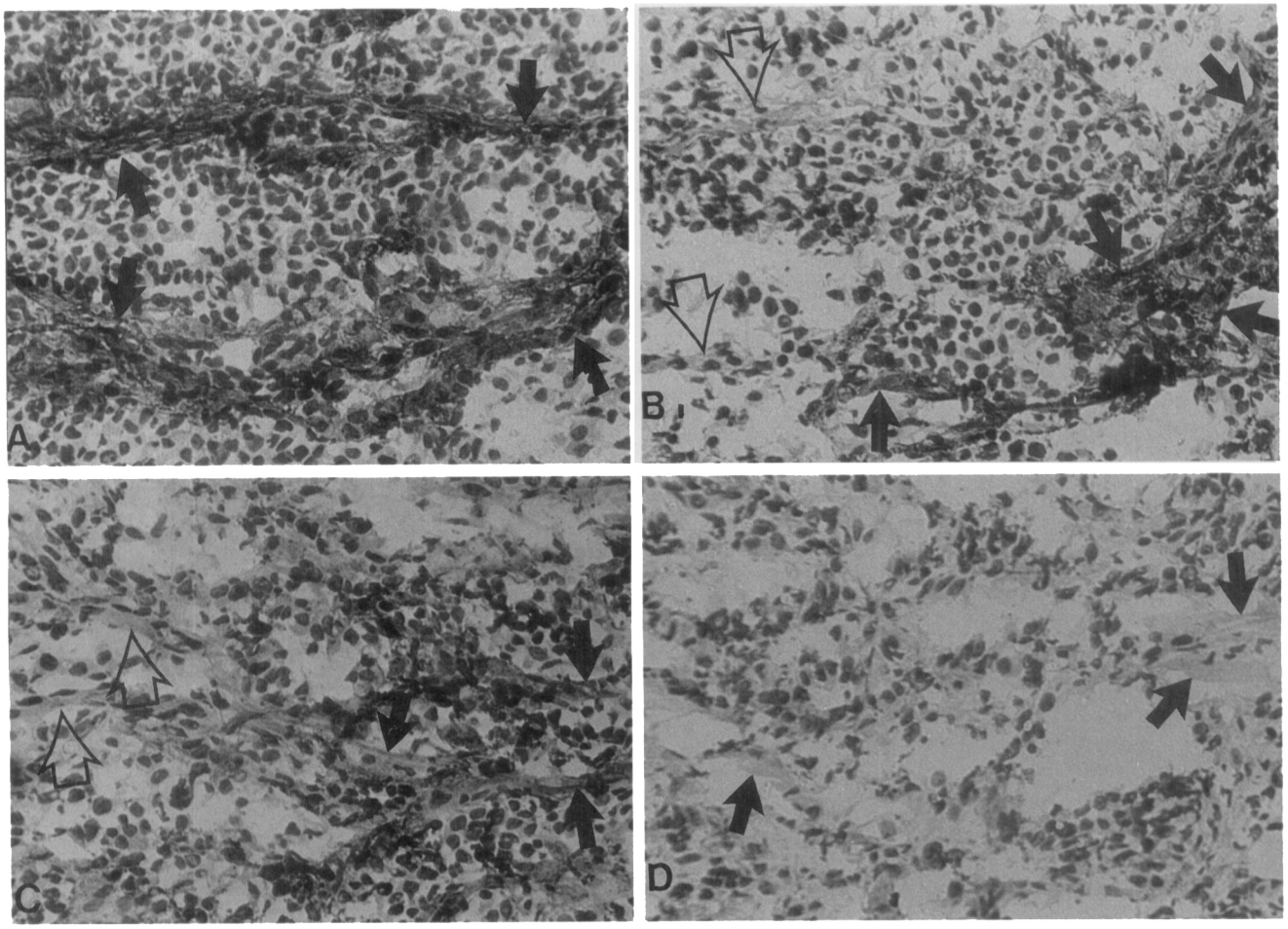

FIG. 2. *A* (*upper, left*). Specific staining by the peroxidase technique for fibrinogen (or fibrin I) in frozen sections of malignant melanoma tissue. *Closed arrows* show diffuse connective tissue staining (that appeared dark brown in the original). Hematoxylin counterstain (×100). *B* (*upper, right*). Specific staining for fibrin in an area similar to that illustrated in *A*. Note more restricted distribution of connective tissue staining (*closed arrows*) and adjacent area of unstained connective tissue (*open arrows*) that appeared pale pink in the original. Hematoxylin counterstain (×100). *C* (*lower, left*). Specific staining for the D-dimer cross-link site of fibrin in an area similar to that illustrated in *A* and *B*. Note staining similar to that of fibrin (*closed arrows*) and adjacent area of unstained connective tissue (*open arrows*). Hematoxylin counterstain (×100). *D* (*lower, right*). Absence of staining (pale pink appearance in the original) of connective tissue (*closed arrows*) in the negative control in which the primary antibody was omitted from the procedure. Hematoxylin counterstain (×100).

formation[28,31] occurs because of an intact TF-initiated pathway of coagulation.

It is well known that cultured human melanoma cells are capable of producing t-PA.[7] However, Leong and associates[18] observed t-PA production by only 4 of 13 human melanoma cell lines tested. We observed staining for t-PA in a variable number of cells in three of the four cases of melanoma studied. Our results, together with those of Leong and associates,[18] suggest that t-PA expression by cultured melanoma cells may relate to outgrowth of a clone of cells having this property from original tumors that contained cells having mixed properties. It has been suggested that production of fibrinolytic activators by human melanoma cells may be related to the aggressiveness of the malignancy. For example, in the study of Leong and associates,[18] high levels of t-PA production by cultured melanoma cells were associated with increased invasiveness in an *in vitro* model of invasion. Bruggen and associates[4] found that plasminogen activator release from cultured melanoma cells was correlated quantitatively with tumorgenicity in nude mice. These authors suggested that the tumorgenicity might be attributable to the plasminogen activator produced. However, the relevance of these models to human disease is questionable. Markus and colleagues[21] found that plasminogen activator secretion by explants of melanoma tissue, as well as total extractable plasminogen activator (which was primarily u-PA rather than t-PA), was relatively low in melanoma tissue compared with certain other human tumor types. Furthermore, levels of fibrinolytic inhibitors are elevated both in the plasma of patients with melanoma[1] and in melanoma tissue.[15,16] Kwaan and colleagues[16,17] described

immunohistochemical staining of melanoma cells for alpha$_2$-antiplasmin and alpha$_1$-antitrypsin and suggested that fibrinolytic activators known to be present in melanoma cells may be inactive because of binding to these inhibitors within (and possibly produced by) the melanoma cells. Our finding of a net accumulation of fibrin in melanoma tissue is consistent with the concept that a relative deficit in fibrinolytic activity exists *in situ* in melanoma.

Human melanoma cells in culture are known to express platelet surface glycoprotein II_bIII_a.[3,11] It has been proposed that this glycoprotein may mediate cell attachment to fibrinogen and other proteins bound to the surface of the culture vessel[11] and may also mediate platelet aggregation induced by these tumor cells that may contribute to more aggressive behavior of the tumor.[3] Although we were unable to demonstrate II_bIII_a antigen *in situ* in melanoma tissue, it is conceivable that binding sites for the monoclonal antibody to II_bIII_a used in this study were occupied and not detectable by our techniques.

The results of the present study raise several questions. For example, the findings reported here were obtained in studies on metastatic tumor deposits. It is not known whether similar mechanisms exist in primary tumors, especially early in their development. However, the demonstration of thrombin generation *in situ* suggests the possibility that local coagulation activation may contribute to growth control of malignant melanoma. Experimental models have been developed recently that provide an ideal setting for testing the relationship between coagulation activation and progression of human melanoma.[23,35] However, the relative importance of this mechanism, if any, can only be determined by controlled clinical trials of drugs known to interfere with thrombin formation. It has been shown in two prospective randomized clinical trials that warfarin anticoagulation prolongs survival and increases response rates in patients with SCCL.[6,29] SCCL is known to have an intact coagulation pathway assembled locally in association with viable tumor cells[33,34] that likely accounts for generation of thrombin at the host–tumor interface in this tumor.[28,31] Therefore, the beneficial effects of warfarin therapy in SCCL may be attributable to the ability of this drug to limit tumor cell–directed thrombin formation. Our results suggest that a similar mechanism may exist in malignant melanoma, but the effects of therapeutic intervention in this disease remain to be determined.

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