Occurrence of Components of Fibrinolysis Pathways in Situ in Neoplastic and Nonneoplastic Human Breast Tissue

Vincenzo Costantini, Leo R. Zacharski, Vincent A. Memoli, Bohdan J. Kudryk, Sandra M. Rousseau, and David C. Stump

ABSTRACT

The occurrence and distribution of components of fibrinolysis pathways were determined using immunohistochemical techniques applied to 10 cases of primary carcinoma of the breast, normal breast tissue obtained from two patients undergoing reductive mammoplasty, and three cases of benign breast tumors. Tumor cells stained for urokinase- and tissue-type plasminogen activators, plasminogen activation inhibitor-1, plasminogen, and plasmin-antiplasmin complex neoantigen. The tumor connective tissue stained for fibrinogen and its D fragment plasmin digestion product. By contrast, only occasional nonneoplastic duct epithelial cells stained for urokinase- and tissue-type plasminogen activators and there was little or no staining for the other antigens tested. These results are consistent with the existence of local amplification of expression of enzymatically active plasminogen activators, and particularly of urokinase-type plasminogen activator, in situ in primary breast cancer tissue. These features distinguish malignant from benign breast tissue and may modulate neoplastic progression through an effect on tumor cell proliferation, invasion, and metastatic dissemination.

INTRODUCTION

Interest in defining mechanisms of activation of coagulation and fibrinolysis in cancer is based on evidence that these pathways may play an integral role in the pathogenesis of malignancy because of their ability to regulate tumor growth and dissemination (1). It is well known that accelerated fibrinolysis commonly accompanies malignancy because elevated plasma levels of peptide degradation products of fibrinogen/fibrin are commonly observed in patients with cancer (2, 3). Such systemic fibrinolytic activation accompanies activation of the coagulation mechanism (2). However, studies performed directly on tumor tissues have shown altered interactions of components of such tissues with pathways of fibrinolysis (4–6) and increased production of PAs* by explants of human tumor tissues (7). Studies such as these have suggested a role for the tumor itself in regulating fibrinolysis apart from primary activation of the coagulation mechanism within the circulation. However, such studies are not easily interpreted because of heterogeneity in findings among experimental systems. For example, tumor tissues have been claimed to express increased levels of both activators (4, 5) and inhibitors (6) of fibrinolysis. Furthermore, studies on whole tissues neither define with certainty which of the several cell types present within the tissues is responsible for the property described nor delineate possible differences between normal and neoplastic duct epithelial cells.

The potential significance of such questions arises from an increasing body of evidence that has implicated PAs as key agents in the pathogenesis of malignancy. These have been reviewed recently (8) and include involvement of PA (particularly u-PA) in expression of the transformed phenotype including cell proliferation, invasion, and metastasis. However, considerable variation also exists among model systems for these relationships (1), and little information exists concerning the relevance of PAs to human malignancy. Clarification of possible relationships requires definition of which participants of reaction pathways (e.g., relevant enzymes, inhibitors, substrates, reaction products) exist within tumor tissues in vivo; definition of the precise cellular location of these components; and clarification of whether these components are biologically active and, if so, where such reactivity occurs. For example, the activity and biological significance of an enzyme such as u-PA may be entirely different should this enzyme exist within the circulating blood; adjacent to viable cells, such as tumor cells or macrophages, within tissues; or within necrotic tissue.

Components of fibrinolysis pathways are known to exist within normal mammalian breast tissues (9–11), and abnormalities of these have been implicated in the pathogenesis of human breast cancer (10, 12–21). However, information available regarding the existence of such components in malignant versus nonmalignant breast tissue is incomplete (22). Thus, immunohistochemical procedures that are capable of resolving certain of these issues were used in the present study to define the occurrence and distribution of certain proteins relevant to fibrinolysis pathways in neoplastic and nonneoplastic breast tissue.

MATERIALS AND METHODS

Studies were performed on AMeX-fixed tissue (23) prepared from fresh surgically excised primary breast cancers from 10 patients (9 of which were infiltrating ductal carcinomas and 1 of which was an infiltrating lobular carcinoma). Of these, 8 cases were estrogen receptor positive and 2 estrogen receptor negative. No patient had received treatment for her malignancy. Comparisons were made with normal breast tissue obtained during reductive mammoplasty in 2 cases, from benign fibroadenomas in 2 cases, and from benign fibrocystic disease in 1 case. Staining procedures and controls for single- and double-labeling techniques using the avidin-biotin complex and indirect immunofluorescence techniques and methods of antibody preparation and characterization have been described elsewhere (24). Antigen staining was detected by the dark brown (peroxidase reaction with diaminobenzidine) or red (alkaline phosphatase reaction with Red Vector) reaction products obtained with the avidin-biotin complex procedure. This con-
trasted with the dark blue nuclei of cells and the pale pink appearance of unstained cells and stroma.

Monospecific, purified (by affinity or protein A-Sepharose chromatography) rabbit antibodies to low molecular weight u-PA (25), t-PA, plasminogen, and PAI-3 were used. The antibody to t-PA was prepared by immunizing rabbits with recombinant t-PA (Genentech, South San Francisco, CA) and isolating monospecific IgG as described previously (26). Rabbit polyclonal antibodies were prepared from protein antigens that were >99% pure. Antibodies to t-PA and u-PA did not cross-react with the alternative plasminogen activator (25, 26). Monoclonal antibodies specific for epitopes on fibrinogen or their degradation products included the following: antibody 1-8C6 that requires an intact 14 arginine-17 glycine 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) (27); antibody T2G1 that reacts with the amino-terminal part of the Bβ chain only following removal by thrombin of fibrinopeptide B (Bβ 1-14) and thus with fibrin but not fibrinogen (28); 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 (29). The reactivity of these antibodies in immunohistochemical procedures has been verified independently (30). Goat antibody to PAI-1 and mouse monoclonal antibody to PAI-2 were obtained from American Diagnostica (New York, NY). Rabbit antibody to α2-antiplasmin and human plasmin-α2-antiplasmin complex neoantigen (22) were obtained from Behring Diagnostics/Calbiochem (San Diego, CA).

Antibodies were tested on control and tumor tissues in concentrations that provided maximal staining intensity with minimal 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 breast cancer tissues were interpreted in association with staining procedures performed on normal control benign breast disease and other neoplastic tissues (e.g., small cell carcinoma of the lung, renal cell carcinoma, and colon cancer) tissues processed simultaneously. The fibrin-specific antibody, T2G1, revealed exposure of antigen adjacent to tumor cells in small cell carcinoma of the lung and real cell carcinoma and adjacent to tissue macrophages in lymphoproliferative disorders. (These positive findings indicate the utility of these techniques and will be reported in detail elsewhere.) We observed that antibody to PAI-1 stained antigen in placental syncytiotrophoblast in a nodular pattern corresponding to fibrin deposits on the surfaces of placental villi in a focal distribution. PAI-1 antibody also reacted with vascular endothelial cells. We also observed PAI-2 in the syncytiotrophoblast of term placenta (31) and PAI-3 was detected in a nodular pattern corresponding to fibrin deposits on the surface of placental villi.

RESULTS

Staining of tumor cell bodies was observed for u-PA, t-PA, plasminogen, α2-antiplasmin, plasmin-α2-antiplasmin complex neoantigen, and PAI-1. Tumor cell staining for these antigens was diffuse except for t-PA for which certain tumor nodules were positive, while others were negative. Findings for u-PA are shown in Fig. 1, for t-PA in Fig. 2, and for plasmin-α2-antiplasmin complex neoantigen in Fig. 3. Double-labeling techniques revealed coincident staining of malignant cells for plasmin-α2-antiplasmin complex and both u-PA and t-PA. Little or no staining was observed for PAI-2 or PAI-3. Diffuse staining of tumor connective tissue was observed for fibrinogen, but no staining was observed for fibrin. [The demonstration of tumor fibrinogen will be reported separately (24).] Antibody GC4, which reacted with the D plasmin digestion fragment of fibrinogen and also D-dimer cross-link sites on fibrin, stained material diffusely but in a patchy distribution throughout the tumor connective tissue stroma. This was interpreted as indicative of the presence of D fragment rather than D-dimer cross-link sites because of evidence for the presence of an active fibrinolytic pathway and because fibrin (T2G1-reactive material) could not be detected in these tissues.

Staining of normal and fibroadenomatous breast tissue for these antigens differed from staining of neoplastic tissue. Occasional staining of nonneoplastic duct epithelial cells and luminal secretions was observed for u-PA. Strong staining for t-PA also existed in scattered duct epithelial cells (predominantly on the luminal aspect of the cells), in amorphous secretions within the lumen of the ducts, in myoepithelial cells at the base of the epithelium, and in the endothelium of adjacent blood vessels. The microvascular endothelium also stained for t-PA in breast cancer tissue. These findings are shown in Fig. 4. Little or no staining of nonneoplastic breast tissue was observed for PAI-1, plasminogen, α2-antiplasmin, plasmin-α2-antiplasmin complex neoantigen, fibrinogen, fibrin, or D fragment/D-dimer cross-link sites.

Fig. 1. Specific staining by the peroxidase technique (that gave a brown reactions product) for u-PA on tumor cells (arrows) in primary breast cancer tissue (a). Staining was absent (cell bodies appeared pale blue) in preparations handled identically but from which the primary antibody was omitted (b). Hematoxylin counterstain; original magnification, × 250.
Systemic activation of fibrinolysis secondary to activation of coagulation is clearly present in patients with breast cancer (2, 3). However, there is reason to doubt that the elevated plasma levels of fibrinopeptide A, indicative of thrombin cleavage of fibrinogen, and of Bβ 15–42, indicative of plasmin cleavage of fibrin, reflect reactions occurring in breast cancer tissue. Thus, these proteolytic cleavage products were found to be elevated to an equivalent extent in the circulation of both patients with benign and patients with malignant breast disease (2), and we have found neither an intact coagulation pathway nor evidence for thrombin transformation of fibrinogen to fibrin in primary breast cancer tissue (24). Therefore, we postulated that such systemic activation of coagulation may reflect production by the abnormal (either benign or malignant) breast tissue of a soluble mediator that activates procoagulant activity of host cells at sites distant from the tumor (24).

Findings reported in the present study suggest that distinct differences exist between neoplastic and nonneoplastic breast tissue in the occurrence and distribution of components of fibrinolysis reaction pathways. Plasminogen, u-PA, and t-PA have been found in nonmalignant breast tissues and secretions (9, 11, 32). Findings reported here demonstrate that scattered nonneoplastic duct epithelial cells and secretions stained for both u-PA and t-PA (Figs. 2 and 3). The existence of PAs in normal breast tissue may be related to the growth and maintenance of duct structures under physiological stimuli and pathologic conditions. No staining of nonneoplastic tissue was observed for PAI-1. By contrast, strong staining was observed for breast cancer cells for u-PA, which was present diffusely in the neoplastic cells throughout all specimens (Fig. 1), and for t-PA, which was present in the cells of some but not all tumor nodules (Fig. 2). The latter observation suggested that the distribution of t-PA may have been clonal. Malignant (but not nonmalignant) breast epithelial cells also stained for PAI-1, plasminogen, β2-antiplasmin, and plasmin-antiplasmin complex neoantigen (Fig. 3). Plasmin-antiplasmin complex stained coincidently with both u-PA and t-PA in double-labeling studies. Antibody GC4 staining of the tumor connective tissue was interpreted as indicative of the presence of the D plasmin digestion fragment of fibrinogen. These results suggest that expression of u-PA, t-PA, and PAI-1 is greatly increased in neoplastic as opposed to nonneoplastic breast epithelial cells. They further suggest that u-PA or t-PA possess the ability to activate plasmin beyond the capacity of PAI-1 to inhibit these enzymes. Such plasminogen activation is more likely to be due to u-PA than to t-PA because u-PA is known to activate plasminogen in association with cell surfaces (8, 33). By contrast, the activity of t-PA is strongly dependent on the presence of fibrin (34) which was not detected in these tissues. Findings reported here could not be related to estrogen receptor status in the cases studied. In addition, we have not had an opportunity to compare primary versus metastatic disease sites.

It is instructive to consider available information concerning the possible role of PAs in the pathogenesis of breast cancer. Explants of breast cancer tissue secrete u-PA into the medium (7). The concentrations of both u-PA (10, 12, 14, 16, 20, 21) and u-PA mRNA (13), but not t-PA (10, 17, 18), are increased in extracts of breast cancer tissues in comparison to nonneoplastic breast tissue. This u-PA is associated primarily with the microsomal fraction of tissue homogenates (15). Our results confirm those of Burtin et al. (22) and indicate that this u-PA is a component of the tumor cells themselves rather than of some other cell type (such as endothelial cells or macrophages) present within the breast cancer tissue. Higher levels of u-PA have been associated with more advanced tumor stage, a greater number of positive lymph nodes, greater proliferative and invasive activity, and more rapid recurrence of disease and metastasis (16). Recently, Janicke et al. (20) demonstrated that higher u-PA antigen levels in homogenates of resected breast tumors were associated with several variables indicative of poor prognosis including negative hormone receptors, vascular invasion, and lymph node involvement. Strong statistical correlations existed between higher u-PA antigen levels and early recurrence of disease. Multivariate analysis revealed that u-PA content had a far stronger predictive value for recurrence than did hormone receptor status and node involvement. They concluded that u-PA antigen appeared to be an independent prognostic factor in breast cancer. By contrast, higher t-PA levels...
were associated with a longer disease-free survival which was independent of stage, grade of the malignancy, or lymph node involvement (15, 17, 18). Based on such observations, it has been postulated that u-PA is a marker for more aggressive breast cancer because it may mediate certain reactions that impart neoplastic properties to breast cancer cells (1, 8, 13, 16, 19, 21), while t-PA may be a marker for a more favorable outcome (15, 17, 18).

It is customary to search for the existence of ERs in breast cancer cells because the existence of ERs signals a more favorable (protracted) disease course (35) and indicates that treatment with estrogen antagonists may ameliorate the course of the disease (36). ER positivity has been associated with lower u-PA levels (20) and higher t-PA levels (15) in breast tumor homogenates. In cultured breast cancer cells, estrogen stimulated production of both u-PA and t-PA but only in ER-positive cells (37, 38). However, ER-negative cells had considerably higher unstimulated u-PA production than ER-positive cells (37, 38).

Recent evidence has provided insight into possible mechanisms of genetic control of u-PA production in breast cancer and other tumor types. Expression of the ras oncogene is commonly abnormal in breast cancer (39–41) and is associated with an adverse prognosis (39). Transfection of the ras gene into ras-negative cells leads to amplification of u-PA production (42–44) and increased invasiveness (43) of these cells. Such observations suggest a possible link between the oncogenic constitution of breast cancer cells and u-PA that might mediate the neoplastic phenotype. These findings have several implications. First, because u-PA has properties like those of growth factors (8, 45–47), it is possible that estrogen stimulation of tumor growth may be mediated by stimulation of u-PA production. Second, production of PAs and PAI by cells are known to be coordinated (48). Thus, while higher levels of u-PA in breast cancer tissue predict worse outcome (16, 20), it is conceivable that measurement of PAI along with u-PA (and t-PA) may provide additional prognostic information. Third, it is possible that the more protracted course of ER-positive cases may be due to the fact that in these tumors u-PA production is under at least some degree of physiological (hormonal) regulatory control, while in ER-negative cases a more virulent course may be related to increased u-PA production that is autonomous and not under hormonal control.

Proof of a role for u-PA in the progression of breast cancer requires studies of effects of intervention aimed at inhibition of these pathways. It has been shown that estrogen antagonists, which are known to ameliorate the course of breast cancer (36), are capable of inhibiting u-PA expression in human breast cancer cells (49). We postulate that further studies of intervention based on these insights may clarify mechanisms of growth control in breast cancer such that new and more effective treatment can be designed.

REFERENCES


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