Fibrinogen Deposition without Thrombin Generation in Primary Human Breast Cancer Tissue

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ABSTRACT

The occurrence and distribution of components of coagulation pathways in situ 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 patients with benign breast tumors. Tumor cells stained for factor X and thrombomodulin but not for tissue factor, factor V, factor VII, or factor XIII. Rare nonneoplastic duct epithelial cells stained for thrombomodulin, but these tissues did not otherwise stain for any of these antigens. Macrophages within the tumor stroma stained for tissue factor, factor VII, and factor XIII but not for factor V or factor X. These features of macrophages were the same in malignant and nonmalignant breast tissue. Fibrinogen was present in abundance throughout the connective tissue in breast cancer but not in nonmalignant tissues. By contrast, no staining was observed using fibrin-specific antibodies. These results suggest that an intact coagulation pathway does not exist in breast cancer tissue and that thrombin capable of transforming fibrinogen to fibrin is not generated in significant amounts in this tumor type. While fibrin is not a feature of the connective tissue stroma in breast cancer, it is conceivable that the abundant fibrinogen present in the tumor connective tissue (and factor XIII present in connective tissue macrophages) might contribute to the structural integrity of breast tumor tissues.

INTRODUCTION

Carcinoma that arises from the epithelial cells lining the ducts of the mammary gland will develop in approximately 1 of 14 American women (1). Endocrine, dietary, and hereditary factors as well as ionizing radiation have been incriminated in the development of breast cancer, but the details of the pathogenesis of this common malignancy are largely unknown (1). While a measure of success in controlling breast cancer has been realized with surgical excision, chemotherapy, hormonal therapy, and radiation therapy, this tumor tends to disseminate relatively early in its course in many women and such treatments are of limited efficacy (1). There is ample motivation to obtain new information concerning the pathogenesis of this disorder.

Attention has been directed to mechanisms of coagulation activation in cancer because of interest in understanding the nature of the coagulopathy commonly associated with malignancy and also because such activation may play an integral role in tumor cell proliferation, invasion, and metastasis (2). The latter concept has been tested in controlled clinical trials of treatment designed to interrupt such pathways (3–6). These trials have met with success in certain tumor types but not in others (7, 8).

In an attempt to sort out mechanisms of coagulation activation in cancer, immunohistochemical techniques have been applied to several human tumor types in an attempt to determine the occurrence and cellular distribution of components of pathways of thrombin formation and fibrinolysis in situ (9–15). The goal of these studies has been to determine whether the cellular elements of tumors (e.g., the tumor cells themselves versus stromal cells such as macrophages) are associated with reactants that might contribute to such pathways.

Data available so far suggest that coagulation activation in cancer may arise by at least two different mechanisms. “Direct” activation may result from assembly of coagulation pathway components on tumor cells themselves that leads to thrombin generation and conversion of fibrinogen to fibrin adjacent to viable tumor nodules. Direct activation may occur in SCCL because both coagulation pathway intermediates and fibrin have been discovered in situ in this tumor type by means of immunohistochemical techniques (9–11). By contrast, “indirect” activation may result when a tumor produces a soluble substance that triggers the production of coagulation initiators on host cells (e.g., macrophages or endothelial cells) at sites distant from the tumor. Evidence suggests that an indirect mechanism of coagulation activation may exist in acute myelogenous leukemia (16). Other mechanisms of coagulation activation in cancer may also exist.

It is well known that systemic activation of coagulation, manifested by changes in peripheral blood tests of coagulation or by thromboembolism, occurs commonly in breast cancer (17–20). Evidence has also been presented suggesting that fibrin may be formed within breast cancer tissue (21). However, doubt remained because reagents available earlier were not capable of distinguishing with certainty between fibrinogen and fibrin. Furthermore, an intact coagulation pathway has not been demonstrated in human breast cancer. The present study was undertaken in an attempt to resolve these uncertainties.

MATERIALS AND METHODS

Studies were performed on AMeX-fixed tissue (22) prepared from fresh surgically excised primary breast cancers from 10 cases (9 of which were infiltrating ductal carcinomas and one of which was an infiltrating lobular carcinoma). No patient had received treatment for her disease. Comparisons were made with normal breast tissue obtained during reductive mammoplasty surgery from two cases, from benign fibroadenomas in two cases, and from benign fibrocystic disease in one.
case. Staining procedures and controls for the avidin-biotin complex technique (9–15) using reagents (Vectastain Kits; Vector Laboratories, Burlingame, CA) and for the indirect immunofluorescence technique (23) have been described previously. Antibody 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 contrasted with the dark blue nuclei of cells and the pale pink appearance of unstained cells and stroma.

The double-labeling technique was used to visualize simultaneously two different antigens in the same section. For demonstration of two antigens on the same cell, an immunoenzymatic step that gave a colored reaction product was applied to unstained sections, and this step was followed by the indirect immunofluorescence technique (24). When both first and second primary antibodies were raised in the same species, the specificity for binding of the fluorescein-conjugated secondary antibody applied to the second primary antibody is preserved because the peroxidase-staining procedure applied to the first primary antibody prevents its binding to the second secondary antibody (25).

Procedures used monospecific, purified rabbit antibodies to the following: recombinant human TF, factor V, factor VII, factor X, the "a" subunit of factor XIII, protein C, and protein S. Rabbit polyclonal antibodies were prepared from protein antigens that were >99% pure. Antibodies were purified from crude antiserum by affinity chromatography either on an antigen-Sepharose column (for antibodies to factor VII and factor X) or on a protein A-Sepharose column (for antibody to TF). Antibody specificity was demonstrated on the basis of activity neutralization, Western immunoblot analysis, and immunoprecipitation studies using 125I-labeled antigen or proteins labeled metabolically in vivo using [35S]methionine. The antibody to TF apoprotein was capable of immunoprecipitating TF apoprotein from cell extracts and stained a protein of M, about 44,000 on Western blots. Monoclonal antibodies specific for epitopes on fibrin(ogen) or their degradation products included the following: antibody 1–8C6 that requires an intact I4 arginine-17 glycine bond in the Bβ chain of fibrinogen and therefore reacts with fibrinogen or fibrin (des-fibrinopeptide A-type fibrin) but not fibrin II (des-fibrinopeptide B-type fibrin) (26); antibody T2G1 that reacts with the amino-terminal part of the Bβ chain only following removal by thrombin of fibrinopeptide B (Bβ1–14) and thus with fibrin but not fibrinogen (27); and antibody GC4 that reacts with fragment D of fibrinogen as well as a-dimer from cross-linked fibrin but not with either fibrinogen or fibrin (28). The reactivity of these antibodies in immunohistochemical procedures has been verified independently (29).

Mouse monoclonal antibodies to the thrombin cleavage sites on the Aα and Bβ chains of fibrinogen and a goat antibody to rabbit TM that cross-reacted with human TM were obtained from American Diagnostica (New York, NY). In our hands, the anti-TM antibody recognized TM in the syncytiotrophoblast of human placenta and in the endothelium of small blood vessels. The macrophtage-specific monoclonal antibody EBM-11 and rabbit polyclonal antibody to antithrombin III were obtained from Dako Corp. (Santa Barbara, CA). We have shown previously that reactivity of these antibodies is similar in fresh frozen and AMeX-fixed tissue (9–15).

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 (i.e., normal breast, placenta, and liver), benign breast disease, and other neoplastic tissues (e.g., SCCL, renal cell carcinoma, and colon cancer) processed simultaneously. Particularly, the fibrin-specific antibody T2G1 stained material adjacent to viable tumor cells in SCCL (11) and renal cell carcinoma (13) and adjacent to tissue macrophtages in lymphoproliferative disorders. These tissues served as positive controls for this antibody and the results in these tumor types will be reported separately.

RESULTS

Tumor cell bodies stained for factor X to a variable extent in 5 of the 10 cases of breast cancer, but no tumor cell staining was detected for TF, factor V, factor VII, or factor XIII "a" subunit. Results for factor X are illustrated in Fig. 1. Focal staining of breast cancer cells (and occasional nonneoplastic duct epithelial cells) for TM was also observed. Otherwise, no staining was observed for any of these antigens on epithelial cells from normal tissues or benign breast disease. Double-labeling procedures revealed that macrophages scattered throughout the connective tissue in malignant, benign, and normal breast tissues stained for TF, factor VII, and factor XIII "a" subunit. Scattered fibroblasts present in the connective tissue also stained for factor XIII "a" subunit (Fig. 2). There was no macrophage staining for factor V or factor X. These features of macrophages were the same for malignant and nonmalignant tissues. Macrophage staining for factor VII in a case of benign fibroadenoma is illustrated in Fig. 3.

Patchy but diffuse staining of the tumor connective tissue was observed for factor V, and more uniform, diffuse strong staining of the connective tissue was observed for fibrinogen using the fibrinogen-specific monoclonal antibody 1–8C6 (26). The appearance of the fibrinogen is illustrated in Fig. 4.
staining for factor V or fibrinogen was observed in the connective tissue of nonmalignant disease. In addition, no connective tissue staining was observed using fibrin-specific antibodies in either malignant or nonmalignant tissues. Staining with antibody GC4 that reacts with fragment D of fibrinogen as well as D-dimer of cross-linked fibrin was observed in a patchy distribution throughout the connective tissue. This was interpreted as consistent with the existence of plasmin-degraded fibrinogen rather than cross-linked fibrin because fibrin itself could not be detected. Of course, other interpretations for these findings exist because fibrinogen may be degraded by enzymes unrelated to the coagulation mechanism. (Interactions of breast cancer tissues with pathways of fibrinolysis will be reported separately.) Little or no staining of these tissues was observed for protein C, protein S, or antithrombin III. Apart from the sporadic findings for factor X, results of these immunohistochemical procedures were consistent among cases.

DISCUSSION

The pathogenesis of the coagulopathy of malignancy is complex and incompletely understood. However, emerging data suggest that such activation may result in certain tumor types from a direct interaction between tumor cells themselves and coagulation factor intermediates such that thrombin is generated in situ adjacent to tumor deposits (9-13). In other tumor types, the tumor appears to activate coagulation indirectly through production of a soluble mediator (cytokine) that induces procoagulant activity on host cells such as macrophages or endothelial cells (16).

While systemic activation of coagulation in breast cancer has been abundantly documented (17-20), the relative contribution of tumor cells versus tumor products, treatment (19, 20), and host organ dysfunction (30) have not been clearly distinguished. In fact, fibrinopeptide A and fibrin degradation product levels (indicative of increased systemic formation of thrombin and plasmin, respectively) have been found in carefully conducted studies to be elevated to a similar extent in both benign and malignant breast disease (17, 31). Thus, coagulation activation in breast cancer may not be a feature that is specific for the malignant cell. It is conceivable that coagulation activation in both benign and malignant breast disease may be cytokine mediated. Results presented in the present study suggest that conditions that exist in situ in primary human breast cancer may not be conducive to local thrombin production. While
factor X was detected on tumor cell bodies in some (but not all) cases, the tumor cells did not manifest TF, factor VII, or factor V. Stromal macrophages manifested TF and factor VII but not factor V or factor X, and these features of macrophages were similar in benign and malignant tissues. The tumor cells also stained for thrombomodulin. Furthermore, monoclonal antibodies capable of distinguishing fibrinogen from thrombin-cleaved fibrinogen (fibrin) have revealed exposure of thrombin-specific cleavage sites on fibrinogen in various tissues (29), including SCCL (10, 11) and renal cell carcinoma (13), but such cleavage sites were not demonstrable in breast cancer. In this regard breast cancer resembles colon cancer (14) and mesothelioma (15). The limited information available suggests a correspondence between the existence of an intact coagulation pathway and evidence for local thrombin formation in situ in the tumor types examined thus far (9–15). The lack of an intact coagulation pathway associated with either tumor cells or stromal macrophages, the existence of tumor cell-associated TM, and the absence of exposure of thrombin cleavage sites on the fibrinogen that is otherwise present in abundance in the connective tissue adjacent to deposits of viable breast cancer is consistent with the concept that thrombin is not formed in significant quantities in primary human breast cancer tissue. These observations do not support the concept that local fibrin formation either enhances the vascularization of breast cancer tissue (32) or serves as a barrier that prevents invasion of the tumor by host inflammatory cells (33, 34). The antigen detected by others in breast cancer tissue by means of polyclonal antibody to fibrinogen may, in fact, have been fibrinogen rather than fibrin (34, 35) as confirmed in the present study. Reports of variable procoagulant activity of cultured breast cancer cells (36, 37) should be interpreted with caution based on the present data.

Although fibrin was not detected in the breast cancer stroma, it is possible that the fibrinogen present may have a role in extracellular matrix organization in this disease. It has been shown that factor XIII (that we have detected here associated with stromal macrophages and fibroblasts in breast cancer tissue) is capable of inducing gelation of fibrinogen and of fibrinogen combined with fibronectin in the absence of thrombin and without release of either fibrinopeptide A or B (38, 39). The potential ability of such stable gels to support cell adhesion has been discussed (40).

The significance of breast carcinoma tumor cell staining for factor X is speculative. Extracts of breast cancer tissue have been shown to possess an activator of factor X termed cancer procoagulant (41). It is conceivable that factor X derived from the plasma may have become bound to such a substance present within the tumor cells or that the tumor cell might have synthesized factor X. However, it does not appear that this factor X participates in a thrombin-generating pathway.

An additional and unexpected finding in the present studies was the observation of TM associated with breast cancer cells. TM is known to occur in the syncytiotrophoblast of placenta and vascular endothelium (42) and has been described in choriocarcinoma (43) and in tumors of vascular origin (44). We detected TM in tissue macrophages in a case of large cell lymphocytic lymphoma but we are unaware of reports of this substance in other tumor types. It is conceivable that this TM might be capable of regulating the functional state of any thrombin formed to further limit in situ procoagulant activity of breast cancer tissue, but the significance of TM in this disease remains to be determined.

The present findings may be of importance for planning therapeutic trials. Initiation of thrombin formation in situ by SCCL tumor cells has been thought to contribute to growth regulation of this tumor because interruption of thrombin generation by warfarin therapy ameliorates the course of this disease (3, 4). Warfarin has been administered to patients with breast cancer in pilot studies (45, 46), but this drug has not been proven to be effective in this tumor type. Based upon the present findings, warfarin therapy might not be expected to exert an effect by limiting local thrombin formation in situ, but it is conceivable that this drug could influence cell behavior by modifying the activity of other vitamin K-dependent proteins that might exist in these cells. Alternatively, warfarin may influence the properties of embolic tumor cells within the circulation.

REFERENCES


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