Stromal Fibronectin Staining Pattern and Metastasizing Ability of Human Breast Carcinoma

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ABSTRACT

The peripheral stromal fibronectin (FN) staining patterns of invasive breast carcinomas (IBC) from 77 women were compared to the aggressivity of the tumors, which in each case had been determined through a complete clinical follow-up and autopsy investigation. Polyclonal, monospecific rabbit antibody to human FN was applied on formalin-fixed, paraffin-embedded tissue sections using the peroxidase-antiperoxidase staining technique. An FN-positive staining reaction was defined as a constant, diffuse, or pericellular demarcation of FN-positive fibers surrounding tumor cells at the invasive border. In lack of such a staining pattern, FN-negative staining was recorded. The FN-positive staining reaction was significantly associated with a low metastatic potential and appeared in a multivariate analysis to be an excellent prognostic factor, constant, diffuse, or pericellular demarcation of FN-positive fibers surrounding tumor cells at the invasive border. In lack of such a staining pattern, FN-negative staining was recorded. The FN-positive staining reaction was significantly associated with a low metastatic potential and appeared in a multivariate analysis to be an excellent prognostic factor.

INTRODUCTION

Several characteristics of IBC have been found to be important prognostic factors regarding metastatic potential and survival, e.g., clinical staging (1, 2), histological grading (3), receptor status (4, 5), and nuclear DNA changes (6). At the time of diagnosis, however, the prognosis still cannot be determined reliably as concerns the individual patient.

Recently, the insoluble, tissue-bound form of FN has attracted considerable attention regarding tissue dynamics (7-13). This widely distributed high molecular matrix glycoprotein is regarded as an important mediator of tissue modeling and organization by influencing growth (14) as well as adhesion (15), differentiation (16), and motility of cells (17). Accordingly, FN has been found to be especially abundant during states of rapid proliferation, such as the early stages of granulation tissue formation (18) and tissue repair (19) and, interestingly, in the florid stromal response characteristic of most IBC (20-24).

Malignant tumor cells are generally characterized by decreased adhesiveness (25) and may fail to lay down an extracellular FN matrix (26, 27). However, they are still capable of recognizing FN in vitro and adhere to FN produced by other cells (16, 28, 29). Recently, the soluble form of FN has been found to attach cultured metastatic melanoma cells (30) and suppress the invasive potential of such cells (31).

This study reports the immunohistochemical stromal distribution pattern of FN in 77 human IBC, and correlations are made to clinical stage, histological type, and metastatic potential of the tumors (32).

MATERIALS AND METHODS

The study included 86 consecutive, unselected autopsy cases of women with known IBC from Frederiksberg Hospital and Glostrup County Hospital, both in the Copenhagen area. The autopsies were performed from 1982 to 1984, during which period almost 65% of the total number of deaths in this part of Denmark occurred during the hospital stay. The autopsy rates in the two hospitals were 75% and 50%, respectively.

During their lifetimes, 78 of the 86 women had been treated by mastectomy and partial axillary lymph node dissection, in two cases for bilateral primary IBC, whereas eight had not received any surgical treatment for various reasons. Their IBCs were histologically verified at autopsy (32). The median age was 67 years at the time of first diagnosis of IBC (range, 38-92) and 74 at death (range, 44-96).

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The combination of post mortem autolysis and long term storage of sections in formalin before preparation (3-6 months) influenced FN antigenicity and caused insufficient staining. Also the majority of tissue specimens from distant metastases appeared to be unsuitable for immunohistochemistry due to post mortem autolysis.

Clinical Parameters. Clinical staging was performed according to the recommendations of the International Union Against Cancer (38). For detailed information concerning menopausal status, postoperative adjuvant treatment etc., see Nielsen et al. (32).

For immunoperoxidase staining for FN the peroxidase-antiperoxidase staining method (34) was used.

The production, purification, concentration and application of monospecific polyclonal rabbit antibody to human fibronectin has been described previously (39).

Evaluation of Staining Results. The primary, ipsilateral tumors and their respective lymph node metastases were for each patient evaluated without any knowledge of clinical data. Stromal staining, which was specifically registered along the invasive border, was defined as: (a) positive, if the tumor cells were surrounded by a constant, FN-positive, diffuse, or pericellular pattern and (b) negative, if this pattern was interrupted or lacking. The intensity of the diffuse staining was defined as the amount of collagen associated FN-positive strands per high power field: ++, many (coarse pattern); +, few (fine pattern); and −, none. If the diffuse and the pericellular types were both present, only the one which took up more than 50% of the tumor was registered (predominant type).

Statistical analysis was carried out by Fischer’s exact test, the logrank test, and logistic regression analysis. For a life-table the Kaplan Meier plot was used.

RESULTS

Fibronectin Staining Reaction. Two different staining patterns, diffuse and pericellular, were observed. Some tumors displayed only one of these patterns but the majority showed a combination of the two. The diffuse staining appeared as a fine or coarse lattice of FN-positive strands separating the tumor elements (Fig. 1, A and B), whereas the pericellular staining formed a thin line around tumor islands giving the impression of a basement membrane-like demarcation (Fig. 1, C and D). However, immunostaining for the basement membrane glycoprotein laminin failed to show any positive reaction in these areas, and, consequently, the pattern was interpreted as condensed stromal FN around proliferating tumor islands. The blood vessels showed a consistent FN-positive staining reaction, which was located subendothelially corresponding to the basement membrane area (Fig. 2).

In areas of invasive margins, which were represented in at least one section from each tumor, 42 (55%) of the IBC showed a constant stromal staining reaction for FN (FN-positive staining) (Fig. 1). The other 35 IBC (45%) lacked this marginal, stromal FN stainability (FN-negative staining) (Fig. 2 and Table 1).

A desmoplastic reaction, which is defined as a cellular fibroblastic response, was characterized by numerous strongly FN-positive strands. 40 of the tumors (52%) displayed such a reaction, predominantly centrally (Fig. 3). Areas of dense, hyalinized connective tissue within the tumors had only a few FN-positive strands but were, in contrast to the cellular areas, often associated with dense elastic fibers.

The majority of the 40 tumors with a marked desmoplastic reaction (74%) also had an FN-positive stromal staining pattern at their invasive margins, as opposed to only 12 of the 37 tumors (32%) without this reaction ($P < 0.001$).

Local recurrences tended to display the same staining pattern as their primaries, whereas axillary lymph node metastases showed inconsistent staining patterns, which varied from one node to the other.

Histological Type. When the FN staining pattern was compared to histological type (Table 1), tumors with a well-differentiated growth pattern (Grade 1 duct carcinomas) were found to be FN-positive in a significantly higher proportion of cases than tumors with a more poorly differentiated growth pattern (Grade II and III duct carcinomas ($P < 0.01$)).

Clinical Staging. 29 tumors were associated with positive axillary lymph nodes at mastectomy (37.7%). Local recurrences occurred in 18 cases (node six, dermal/chestwall twelve) (23.4%), and distant metastases were found in 46 women (operative 27, autopsy 46) (59.7%). More tumors belonging to the low-risk group (stage I) had an FN-positive staining pattern when compared to Stage II-IV tumors, but the difference was not statistically significant ($P > 0.20$).

Extramammary Malignancies. Extramammary malignancies, which were diagnosed in 20 women during lifetime or at autopsy, were considered in order to exclude false-positive results regarding metastatic spread. Ten women had distant metastases, which in eight cases could be directly matched with their respective extramammary malignancies. However, this was not possible in the last two, which were interpreted as metastases from IBC.

Bilateral IBC. If the two women with bilateral IBC diagnosed during lifetime are included, as many as 26 women (17 with FN-positive and nine with FN-negative first tumors) had another primary IBC contralaterally. In 16 cases these tumors were associated with regional axillary lymph node metastases, and in 20 with distant metastases, which might have originated from either tumor (Table 2).

Survival. The time from mastectomy to death ranged from 0 to 38 years with a median survival time of 4 years and 4 months (Fig. 4). 31 women (39%) died without any sign of IBC, whereas 46 women (61%) died with disseminated IBC (Table 2).

Women who died without distant metastases, had a significantly increased frequency of IBC with an FN-positive staining pattern compared to women who died with disseminated IBC (27/31 or 87% compared to 15/46 or 33%, $P < 0.001$, Table 2). If women with bilateral IBC were excluded from the group of women with disseminated disease this difference became even more significant (27/31 or 87% compared to 3/26 or 12%, $P < 0.0005$, Table 2). No correlation was found between metastatic potential and subtype of FN staining pattern.

Three women with FN-positive, unilateral IBC died with metastatic disease. One had, at 72, been mastectomized 5 years previously for a colloid type IBC, Stage 2. Another woman with a lobular type IBC, Stage 1, died only 11 months after mastectomy at the age of 60, and the third women died at age 87, 1 year and 3 months after mastectomy for a Grade I duct carcinoma, Stage 2. Among the 31 women without distant metastases four had FN-negative tumors. One had her first IBC diagnosed at autopsy, whereas two had had short follow-up intervals of 3 and 5 months, respectively. The fourth woman, who died at age 78, had been mastectomized 4 years previously and had had two local recurrences but never any lymph node metastases.

Multivariate Analysis. In order to test the value of the FN staining pattern as a prognostic factor at the time of diagnosis, the risk of dying from metastatic IBC was estimated in a multivariate analysis (logistic regression) including besides FN staining pattern, clinical stage, histological type (grade for ductal carcinomas) and lymph node status (Table 3). The FN staining pattern appeared to be a reliable prognostic factor of
Fig. 1. A–D, low power (bar, 50 μm) and high power (inset; bar, 15 μm) micrographs of four different FN-positive duct carcinomas showing a diffuse, fine lattice 
(A), a diffuse coarse lattice (B), and a pericellular reaction around small (C) and large (D) tumor islands. Arrows, invasive margins of the tumors. Immunoperoxidase 
staining for FN, counterstained with hematoxylin.
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Fig. 2. Micrograph (bar, 30 μm) of a moderately differentiated duct carcinoma showing an FN-negative staining at the invasive border. Note the many small vessels with a distinct, subendothelial staining reaction for FN (arrows). Immuno-peroxidase staining for FN, counterstained with hematoxylin.

Table 1 Stromal FN staining pattern (predominant type) of 77 clinically manifest primary invasive breast carcinomas related to histological type

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Positive</th>
<th>Staining intensity*</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>18</td>
<td>++8, +8, PC 2</td>
<td>4</td>
</tr>
<tr>
<td>Grade II</td>
<td>14</td>
<td>++5, +7, PC 2</td>
<td>21</td>
</tr>
<tr>
<td>Grade III</td>
<td>2</td>
<td>++2, +0, PC 0</td>
<td>2</td>
</tr>
<tr>
<td>Tubular/lobular</td>
<td>1</td>
<td>++0, +1, PC 0</td>
<td>0</td>
</tr>
<tr>
<td>Colloid</td>
<td>2</td>
<td>++1, +1, PC 0</td>
<td>1</td>
</tr>
<tr>
<td>Lobular</td>
<td>5</td>
<td>++1, +2, PC 2</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

*++, intense diffuse staining (coarse pattern); +, fine diffuse staining (fine pattern); PC, pericellular staining (basement membrane-like staining around tumor cells or tumor cell clusters).

metastatic spread, which to some extent was independent on other risk factors, as omission of this parameter alone resulted in a significant reduction in the predictability for the development of distant metastases. With the FN staining pattern included a similar reduction was only achieved, after all the other parameters had been left out from the calculations (Table 3).

If bilateral IBC was added to the model as an extra parameter the probability of predicting disseminated IBC from FN staining pattern was increased in a highly significant manner, which appeared to be dependent on the FN staining pattern. This is in accordance with the preponderance of FN positive tumors in women with a second IBC and the fact that the majority of women with bilateral disease (20 out of 26) had developed distant metastases.

DISCUSSION

Correlating stromal FN staining pattern to the prognosis of human IBC was possible in this study because a complete clinical follow-up and autopsy investigation including a meticulous examination of the second breast was carried out in each case. We found in accordance with others (40) that FN was most abundant in the peripheral invasive border of the tumors, and our study further revealed that a continuous, FN-positive staining pattern of the stroma surrounding tumor cells at this location was significantly correlated with a reduced metastatic potential (Table 2). Included in this relatively large group of FN-positive tumors (42 out of 77) were 15 associated with distant metastases, 12 of which occurred in women with a
contralateral primary IBC (80%). As this tumor might as well have been responsible for the dissemination, the survival for this group of women was likely to be higher than estimated (Table 2). The same was not true for the group of women with FN-negative tumors, where bilaterality occurred in only nine out of 31 cases of metastatic disease (29%). The prolonged survival of women with FN-positive tumors leaving more time to develop contralateral IBC may explain this discrepancy (Table 2, Fig. 4).

Another possibility, which should be considered regarding discrepancies between FN staining pattern and metastatic potential is the fact that although only slight variations were found in the FN staining pattern from one section to the other within the same tumor, the number of tissue samples available for immunohistochemical evaluation was restricted, and foci of no peripheral FN staining might have been missed. Finally, the study is based on a consecutive autopsy material, and there is no guarantee that our women have been typical representatives of breast cancer patients in general at the time of diagnosis.

FN has during the past 10–15 years been the subject of considerable interest. This matrix glycoprotein, which appears to be an important mediator of tissue construction, modulation, and differentiation (15–17) has along with other matrix components such as collagen (41, 42), proteoglycans (43), and the basement membrane glycoprotein laminin (28, 35, 44) been found to show a reduced or altered expression in malignantly transformed cells (27, 28) and tissues (20, 22, 45). In accordance with these reports we were not able to identify any intact basement membrane around infiltrating tumor cells as illustrated by the immunoperoxidase staining for laminin. However, the connective tissue stroma of most of our breast carcinomas (52%) displayed FN richly, as has also been reported by others (20–22, 40), and frequently these FN-positive fibers were observed around tumor cell clusters (Fig. 1). Lagace et al. (40) has described a similar FN staining pattern around tumor islands, which, like ours, failed to stain for laminin. They assumed that it originates from the rapid growth of tumor cells and the subsequent compression of surrounding stroma (40). A particularly dense network of FN-positive fibers was present in cellular, proliferating areas, typical of the so-called desmoplastic response (Fig. 3). The exact mechanism by which this FN-rich reaction is induced in IBC is obscure. Some authors believe that the breast cancer cells produce their own collagen stroma, as prolyl hydroxylase, a key enzyme in collagen biosynthesis, and collagen has been found within malignant epithelial cells of collagen-rich IBC and not in the fibroblasts of these lesions (41). Others have shown that the matrix of human breast cancer cells is mitogenic for fibroblasts and these researchers consider the stromal changes as a host response to invasive neoplasms (24).

In a multivariate analysis including other known risk factors, which can be determined at the time of diagnosis, we found that the stromal staining pattern of invasive borders was the single most valuable parameter regarding metastatic potential of the tumor (Table 3). The mechanism behind this reaction is unknown but undoubtedly complex. Possibly, FN represents, just as in granulation tissue and during embryonic development, a stromal, perhaps immunologically triggered response to morphogenetic movement, which is lost in highly metastatic tumors.

Previous investigations on tissues have concluded that even though FN is present within the cytoplasm of some breast...
cancer cells (22, 45, 46), it does not seem to be secreted into the surroundings as filamentous material, as it does in normal breast epithelium (47–49).

However, it is now generally accepted that cell surface receptors for FN are not lost following malignant transformation. Transformed rat kidney cells will not migrate in Boyden chamber assays in response to FN (50), whereas some epithelial tumor cells show enhanced migration towards filters coated with FN (51).

The patchy or complete lack of stromal FN along the invasive border was convincing in view of the strongly FN-positive vessels in the area (Fig. 2), and unlikely to represent fixation, processing, or staining artifacts (34). We observed this incomplete stromal FN staining pattern in the majority of metastasizing IBC (Table 2). Whether a reduced release of FN to the stroma from fibroblasts or tumor cells is responsible or proteolytic degradation of FN triggered by the tumor cells is the cause is a matter of controversy. It is well known that IBC cells produce various tissue degradating proteolytic enzymes, such as collagenases (52, 53), plasminogen activator, which converts plasminogen to FN-degrading plasmin (54, 55) and a thiol-proteinase (56) all of which are capable of degrading FN as well as other tissue constituents in vitro. In fact, in one study it was found that the amount of collagenase released by the tumor cells could be correlated to the aggressive behavior of the tumor (57). However, more information is needed concerning the extracellular milieu of tumors in order to predict if these enzymes are activated in vivo. It has been shown that activation of plasminogen as an isolated event is not sufficient to cause the absence of FN from cell surfaces (15).

Besides FN, several other constituents of supporting tissues such as laminin, collagens, proteoglycans, and entactin have been suspected to play a role in tumor growth (8, 58). FN is, however, one of the best characterized and most widely studied components. Even though the mechanisms behind its occurrence in IBC still remains to be fully clarified, the stromal FN staining pattern of invasive borders has proven to be an excellent, and in this study the best, indicator of the propensity for metastatic spread.

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