Clinical Study on Plasma Fibronectin Level and Cellular Fibronectin Localization in Gastric Cancer

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ABSTRACT: Plasma fibronectin (pFN) in patients with gastric cancer was measured by ELISA method in the aspect of clinical significance. And also cellular fibronectin (cFN) was detected by means of immunochemical staining to clarify the localization of the basal membran and stroma.

pFN was a significantly reduced in patients with gastric cancer and hepatocellular carcinoma. In the postoperative course, a peak reduction was observed on day 1 and recovery was retarded in patients with gastric cancer. It was ascertained in this study that pFN indicated the patient's nutritional states and also reflected the function of the reticuloendothelial system. A few carcinomas showed discontinuous staining on the surface in some of papillary adenocarcinomas, well differentiated and moderately differentiated carcinomas and on the basement membrane of metastatic lymphnodes. It is certified that cFN localization correlated well with local invasion and node metastasis, and there was nothing to show cFN localization at the sites of deep invasion and involved lumen of lymph vessels.

INTRODUCTION

Fibronectin (FN) comprises of a large glycoprotein and is divided into the two, pFN and cFN respectively. pFN is present in plasma and body flude as being soluble protein. It plays a key role in blood coagulation, wound healing and phagocytic activity in the reticuloendothelial system as a non-specific opsonin. On the other hand, cFN is present in connective tissues and basement membranes and it contributes to connection with cells to cells and with cells to stromas and to cell extension, cell differentiation and cell formation.

A few reports are available for malignant diseases. In contrast, there is little available information on the clinical significance of pFN in gastric cancers. There are many devices for quantitative determination such as single radial immunoduffusion, quantitative immuno-electrophoresis (Laurell' method), immunoturbidimetric assay, laser nephelometric assay RIA and EIA and so on.

The purpose of this study is to clarify changes in pFN associated cFN localization at the site of basal membrane and stroma in gastric cancer in terms of cancer spreading and metastasis into the nodes.

MATERIAL AND METHOD

Fifty-one gastric cancer, 24 colon cancer and seven hepatoma patients were eligible for this study and 20 choleliathis patients with benign diseases also were subject to this study. The distribution of sex in this study was 54:48 of men to women. The ages ranged from 25 to 85 years old on the average of 61.3 years old. A control of twenty normal individual was
chosen. For pFN measurement, the blood samples were taken before surgery on day 1, 3, 7, 14 respectively and preserved at -80°C. pFN was measured by enzym-linked immunosorbent assay. Anti-rabbit antibody (MBL) was diluted to 1:1000 with phosphate buffer saline (PBS) in pH7.0 and incubated overnight at room temperature in Nunc-immunoplate II, Nunc. Inter Med), washed with 0.05% tween-20, adding 5% BSA and incubated in 0.01M PB at 37°C for two hours, washed and incubated at 37°C for one hour adding x100 diluted blood sample to standard FN (human, FN concentration 280μg/ml, Behring inst.), incubated in 100μl/well at 37°C for one hour. After washing, adding 0.4mg/ml, 30% H₂O₂ 4μl/ml to 100μl well of citrate buffer pH5.0, left to react for 30 min and to cease a reacitin using 50μl/well of 4N H₂SO₄. The pFN values were obtained by spectrophotometric measurement in comparison with standrad FN curve. Each sample was tested in triplicate and compensated for citrate dosis used at sampling. The Student's test was used for comparison.

Materials eligible for immunohistochemical study comprised of 76 gastric cancers (five papillary adenocarcinomas, 13 well differentiated tubular adenocarcinomas, 17 moderately differentiated adenocarcinomas, 27 poorly differentiated carcinomas, three medullar carcinoma, 11 signetting cell carcinomas and lymphnodes belonged to each carcinoma).

As staining method, ABC method (VECTA STAIN ABC KIT, VECTOR LAB) was used for this study. Paraffin-embedded preparat was dewaxed, indigested at 37°C for two hours and washed with PBS (pH7.6) for 15 min, inactivated peroxidase with 0.3% H₂O₂ methanol, washed for 20 min and inactivated with sheep sera for 20 min, subsequently incubated with anti-rabbit FN antibody (MBL) at room temperature for 60 min, washed with PBS for 15 min, incubated with Biothin-antibody for 60 min at room temperature. After washing with PBS, adding Avidin Biotin Peroxidase Complex (ABC), incubated for 60 min at room temperature and reacted with DAB-H₂O₂ (diaminobenzidine 20mg/0.05M Tris-HCL, pH7.6 100ml H₂O₂ 20μl) for 1-2 min at room temperature, washing with PBS. HE was used for nuclear staining.

**RESULT**

a) Clinical evaluation of pFN

In normal individual, pFN averaged 398.1±71.2μg/ml. Meanwhile, it was 390.1±76.6μg/ml, in gastric cancer patients, 385.6±101.6μg/ml in colon cancer patients. There was not significantly different between patients without and with malignant diseases. On the other hand, it was lower in patients with hepatoma than in healthy persons with significant difference (p<0.01). In patients with cholelithiasis, pFN was somewhat increased without significant difference.

According to the general rule of gastric cancer study, pFN values in accordance with the disease stage were shown in Fig. I, stage I was 42.1±84.5μg/ml, stage II 393.6±38.5μg/ml, stage III 379±65.4μg/ml, stage IV 342.3±59.1μg/ml. In advances in disease stages, pFN values were reduced and there was significantly different.
Table 1. Preoperative pFN levels in patients and normal controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Mean±S. D. (μg/ml)</th>
<th>Levels of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>51</td>
<td>390.0±76.6 NS</td>
<td></td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>24</td>
<td>385.6±101.6 NS</td>
<td></td>
</tr>
<tr>
<td>Hepatoma</td>
<td>7</td>
<td>275.1±51.5 P&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Benign disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholelithiasis</td>
<td>20</td>
<td>426.6±78.3 NS</td>
<td></td>
</tr>
<tr>
<td>Normal controls</td>
<td>20</td>
<td>398.1±71.2</td>
<td></td>
</tr>
</tbody>
</table>

On the other hand, pFN in patients with colon cancer showed 421.6±114.3 μg/ml in Dukes A, 425.0±102.3 μg/ml in Dukes B, 341.2±69.8 μg/ml in Dukes C in accordance with Dukes' classification. There was a significant difference (p<0.05) between Dukes A and C as shown in Fig. 2. pFN values were reduced in cases of Borrmann IV type, serosal invasion (ssr-sei) in the depth of cancer infiltration and signet ring cell carcinoma of histologic types without statistically significant difference as shown in Fig. 3.

Fig. 4 showed changes in pFN values in the postoperative course according to the disease stage of gastric cancer. These were significantly reduced on day 1, followed by recovery to the prior level after on day 3. Delay in recovery from reduced pFN was observed in stage IV patients with gastric cancer. The correlation with pFN values and the other clinical data were evaluated between the two groups of more than 400 μg/ml of pFN and less. As shown in Table 2, a significant disparity (p<0.01–0.05) was
Table 2. Comparison of pFN levels and other hematologic parameters

<table>
<thead>
<tr>
<th></th>
<th>PFN401 &lt; (n=32)</th>
<th>PFN400 &gt; (n=56)</th>
<th>t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Fibronectin</td>
<td>489.3±56.3</td>
<td>329.4±53.1</td>
<td>-</td>
</tr>
<tr>
<td>WAC</td>
<td>6220±1900</td>
<td>5850±2230</td>
<td>n. s.</td>
</tr>
<tr>
<td>Lymph. C.</td>
<td>2140±740</td>
<td>1650±480</td>
<td>1%</td>
</tr>
<tr>
<td>Monocyte</td>
<td>483±233</td>
<td>386±164</td>
<td>5%</td>
</tr>
<tr>
<td>Platelet (×10⁴)</td>
<td>24.9±8.1</td>
<td>22.9±9.9</td>
<td>n. s.</td>
</tr>
<tr>
<td>Total Protein</td>
<td>6.7±0.4</td>
<td>6.6±0.6</td>
<td>n. s.</td>
</tr>
<tr>
<td>Albumin</td>
<td>3.9±0.3</td>
<td>3.7±0.4</td>
<td>5%</td>
</tr>
<tr>
<td>GOT</td>
<td>35.5±60.8</td>
<td>22.7±23.1</td>
<td>n. s.</td>
</tr>
<tr>
<td>GPT</td>
<td>52.1±126.2</td>
<td>22.1±26.3</td>
<td>n. s.</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>304±82</td>
<td>301±10.0</td>
<td>n. s.</td>
</tr>
</tbody>
</table>

Fig. 4. Postoperative changes of pFN levels in patients with gastric cancer according to staging of the General Rules for the Gastric Cancer Study

noted between pFN values and lymphocyte and monocyte counts, and albumin levels.

b) Immunohistochemical study on cFN

The epithelium in normal gastric tissues was not immunohistologically stained. However, continuous staining of cFN was observed in the basement membrane of epithelial cells which formed glandular ducts (Fig. 5). Striated FN staining was observed in the basement membrane of glandular ducts in the field of dysplastic epithelium. (Fig. 6).

According to histologic types, discontinuous and irregular cFN staining was noted at the site of the basement membrane in spite of no staining on the surface of the epithelium in cancer cells and cytoplasm (Fig. 7). In identical patient, there was nothing to be stained in deeply invasive lesions and glandular organization (Fig. 8). The surface of the epithelium in well differentiated carcinoma was discontinuously and irregularly stained but it was very faint. In contrast, cFN was diffusely stained in stromas surrounded by glandular structure (Fig. 9). A strong line bordering cFN staining stromas around glandular structure was present in the deep invasive lesions in spite of no staining of glandular basement membrane as well as the sites involved by carcinoma, cFN was densely stained in stromas surrounded by the tumor but there was not stained in cancer cells as well as basement membrane (Fig. 11). With respect to metastatic nodes, irregular and weak cFN staining was seen in the glandular basement membrane and it was identical to cFN localization on the surface of tumor cells (Fig. 12). Even in poorly differentiated carcinoma, the modes of cFN staining of metastatic nodes were the same as those on the surface of tumor cells.

cFN localizations by ABC method were summarized in Table 3. Neither the surface of tumor cells nor cytoplasm posed cFN localization regardless of histologic types. There were many cases with an expression of cFN of glandular basement membrane on the surface of the tumor cells in patients with papillary adenocarcinoma, well and moderately differentiated, tubular adenocarcinoma.
Fig. 5. cFN staining in normal mucosa (ABC method x 400)

Fig. 6. cFN staining in atypical epithelium (ABC method x 200)

Fig. 7. cFN staining on the surface of papillary adenocarcinoma (ABC method x 200)

Fig. 8. cFN staining in deeply invasive site of papillary adenocarcinoma (ABC method x 200)

Fig. 9. cFN staining on the surface of well differentiated tubular adenocarcinoma (ABC method x 100)

Fig. 10. cFN staining in deeply invasive site of well differentiated tubular carcinoma
Positive rates of cFN staining were higher in papillary adenocarcinoma and lower in moderately differentiated carcinoma. On the other hand, even though cFN staining on the surface of tumor cells were positive, cFN localization was not seen in glandular basement membrane where cancer infiltration was deeply extending. Furthermore, cFN staining was negative in glandular basement membrane on the surface of tumor cells and deeply invasive site in poorly differentiated, signet ring cell and medullary carcinomas. Nevertheless, the intensity of cFN staining was attributable to connective tissue volume although it was positive in all histologic types.

Next, cFN positive rates in glandular basement membrane of metastatic nodes were compared between primary tumor foci and metastatic nodes (Table 4).

With advancing in node metastasis, the positive membrane were reduced in evaluation of the patients with n1 (+) in six, n2 (+) in six
and n3 (+) in five. In addition, cFN staining positive rates in neighbouring node with metastasis also revealed a reduction with progression of metastasis.

DISCUSSION

FN has been introduced as a plasma protein in 1984 and it called cold insoluble globulin. Thereafter similar protein was detected on the surface of fibroblasts and it was named large external transformation sensitive (LETS) protein because of disappearance on the surface of the membrane with advances in malignancy. Later it was recognized that both were the same. Mosher called it fibronectin. It is assumed that pFN contributes to blood coagulation, wound healing, excretion of foreign body and debridement with the function of the reticuloendothelial system. It is accepted that pFN reduced in case of burn, sepsis, DIC, surgical insult. Kikuchi reported that pFN levels reduced in acute fulminating phase of crohn disease and ulcerative colitis. In contrast, those increased in remitting phase. On the other hand, pFN levels in malignant diseases were investigated. Choate clarified that pFN levels increased in colon cancer and breast cancer irrespective of metastasis. The same reports were published by Blumenstock in lung cancer, by Stathakis in pancreas cancer with obstructive jaundice. Nevertheless, Nishimura reported not significant difference in pFN levels between patients with benign and malignant diseases.

In this series, pFN levels revealed not significant difference between healthy and diseased subjects in spite of a decrease in hepatoma. There was disagreement among various authors concerning pFN levels in benign and malignant diseases. Choate investigated that disparity of pFN levels did not correspond to progression of diseases but was contributary for patient's condition. The validity of a tumor marker of pFN levels might be impaired on the basis of a result that pFN levels were in associated with patient's condition and nutrition. Takeuchi insisted that FN levels in urine increased with progression of the tumors and measurement of urin FN were of great benefit to know the progression of malignant diseases.

In this study, it was confirmed that pFN levels were influenced by nutritional condition in patients and function of the reticuloendothelial system. As for changes in pFN levels in the postoperative course, Matsuda reported that pFN levels varied in accordance with surgical stress, a transient decrease in pFN levels was observed in early postoperative period due to enhanced availability and consumption around the wound. Gaupera clarified that pFN levels reached to a peak reduction immediately after surgery, followed by gradual increase, still maintaining lower levels even in 120 hours later by using immunoturbidimetric assay in analysis of 72 patients. He explained that a decrease in pFN levels was based on FN sequestration. Nishimura also reported that there was a significant decrease in pFN levels on day 1 and 3 and complete recovery on day 7. The results in this series were consistent with their conclusions.

The use of ELISA technique was first attempted to measure FN in 1971 by Engvall. Recent studies suggest the advantage of this technique is in a specific reaction with a small amount of antigen and antibody without the use of radioactive isotope with high sensitivity equivalent to that in RIA method. Zerlauth clarified that the reasons for high levels obtained by ELISA and EIA methods are that FN is excessively sensitive to response to proteolysis and much more responds to small fragments rather than antigenecity, due in part to the use of pFN polyclonal antibody, not monoclonal antibody.

Many investigators have been studied on the relationship between cFN levels and neoplastic and/or metastatic diseases. Vaheri reported a possible fact that FN disappeared from the surface of cells transformed to malignancy. Smith also reported that FN production of culture cells from parent cells of human cancer cells was compared among culture cells. As a result, he clarified that FN production of cultured metastatic cells was inferior to that of non-metastatic cells. In comparative study in
FN distribution between metastatic and non-metastatic breast cancer cells, Labat-Robert reported positive FN staining sites in non-metastatic cells distributed in the line bordering cells and basement membrane in contrast with negative staining in metastatic cells. In fact, many reports evidenced negative cFN staining was characteristic of metastatic cells with high potential.

Stenman showed FN staining in solid tumors by using immunofluorescence method and they compared the modes of FN staining between carcinomas and sarcomas. It was clarified that in sarcomas FN staining showed a network of FN staining in connection with stromas, meanwhile in carcinomas staining in cancer cells and/or around cells was not. In their conclusions, the findings of FN staining are of great benefit to differentiate between the two.

On the other hand, Christensen reported stronger cFN staining in cytoplasm of malignant cells according to poorly differentiated intensity and he clarified a difference in cFN localization in accordance with histologic types. Nagai reported that FN localization was seen around cells in poorly differentiated and undifferentiated lung cancers. On the contrary, it was only in stroma in well differentiated lung cancers. In contrast, Tagiridar showed FN localization inside and outside hepatoma cells by using biotin fluoresceinated-avidin method. It is concluded from the above reports that diversity of FN localization is in association with primary organs and histologic types.

Burtin cited that FN antigen diffusely located in peritumorous stroma, in contrast in the basement membrane it is difficult to express FN antigen because of dense staining in peritumorous connective tissues. Forster studied on distribution of FN and laminin by means of PAP method in 50 patients with rectal cancer. He concluded that FN distributed in the basement membrane of glandular carcinoma cells although FN and laminin distributions were noted in stromas. On the other hand, Maeda reported that FN antigens in gastric cancer cells were present in the basement membrane and stroma regardless of cell differentiation by using immunofluorescence method. On the basis of a result that cFN failed to stain in gastric cancer cells irrespective of histologic types in this series, it is suggestive that the deeper cancer infiltration, the more would cFN staining become reduced.

As reported by many investigators, there was close correlation with the amount of connective tissues and the intensity of cFN staining. It is reasoned that either host response or cFN production might be facilitated in accordance with progression of the tumor. In this study, it is emphasized that node metastasis correlates well with cFN staining in the basement membrane.

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