Immunohistochemical studies of basement membrane components in primary and metastatic lesions of early gastric cancer

Hiroyuki Takeda

The Second Department of Surgery (Chief: Prof. Tasuku Shoji), Department of Pathology (Director: Prof. Goro Asano), Nippon Medical School

Summary

The basement membrane components in the primary and metastatic lesions of 29 cases of early gastric cancer were histologically and immunohistochemically examined.

On silver-impregnated specimens, reticular fibers were much more abundant in the basement membrane regions and stromal tissues in the metastatic lesions than in the primary lesions. Immunohistochemically, the basement membrane components examined, i.e. laminin, type IV collagen and fibronectin were localized much more intensely in the basement membrane and more diffusely in the stroma in the differentiated adenocarcinomas than in the undifferentiated adenocarcinomas, having a closer relationship with the rate of proliferation of reticular fibers in the differentiated adenocarcinomas than in the undifferentiated ones. Electron microscopically, laminin was also detected in the endoplasmic reticulum of differentiated adenocarcinoma cells.

From these results it should be noted that the metastatic mechanism in the differentiated adenocarcinomas may be different from that in the undifferentiated adenocarcinomas of the stomach from the viewpoint of the localization pattern of basement membrane components.

Key words: immunohistochemistry, basement membrane, stroma, reticular fiber, metastasis, gastric cancer

Introduction

The basement membrane (or basal lamina, BM), which separates cells from the underlying connective tissue, consist largely of so called basement membrane components, such as type IV collagen, laminin and heparan sulfate proteoglycan. In addition to these components, there are varying amounts of fibronectin on the connective tissue face of the basement membrane. The basement membrane is now believed to guide the migration of neoplastic cells.

The cancer stroma (CS) is composed of cells of mesenchymal origin, stromal fibers, i.e. reticular, collagen and elastic fibers, and substrates such as proteoglycans, fibronectin and laminin. These components may modulate the cancer cell attachment, motion, differentiation and phenotypic expression. Among the fibers, the reticular fibers are immunohistochemically found in close association with the localization of basement membrane components. In malignant tumors, the degradation of the above basal membrane and stromal constituents, and the induction...
of their synthesis are very important events resulting from the interaction between neoplastic cells and host mesenchymal cells\(^7,8\). In order to clarify how cancer cells modify their extracellular environment in the primary and the metastatic lesions, the basement membrane and stroma of the different types of gastric cancers were studied by histological, immunohistochemical and immuno-electroscopical methods with special emphasis on the basement membrane components and reticular fibers.

**Materials and Methods**

Twenty nine stomachs resected from patients (16 male, 13 female, average age 56.3) with early gastric cancers with lymph node metastasis were obtained after gastrectomy. The 2.0 × 0.4 cm size step-wise parallel slices of stomachs and regional lymph nodes were fixed in a 10% formalin solution and embedded in paraffin by routine methods. The 2.5 to 8 μm paraffin sections were cut and stained with hematoxylin-eosin, Masson-trichrome, azan, van Gieson’s elastic stain and a silver-impregnation method modified by Watanabe\(^9\).

For immunohistochemical observation, an avidin-biotin-peroxidase complex method was performed on the sections using anti-laminin (LM) (CR Inc.), anti-type IV collagen (CIV) (Advance Inc.), anti-type III collagen (CIII) (Cappel Inc.), and anti-fibronectin (FN) (DAKO Inc.) polyclonal antibodies. The deparaffinized sections were treated as follows. Endogenous peroxidase was inhibited by treatment with 0.3% H\(_2\)O\(_2\) in 100% methanol. For enhancement, in the case of fibronectin antigen, the sections were pretreated with 0.1% protease and, in the case of laminin and type IV collagen, with 0.4% pepsin (Sigma P-7012) in 0.01 N hydrochloric acid for two hours at 37°C\(^10,12\). The reactions with the primary antibodies were performed at the following dilutions: anti-LM 1/100 (overnight), anti-CIV 1/500 (overnight), anti-FN 1/400 (one hour) and anti-type III collagen 1/100 (one hour). After incubation with the primary antibody, the sections were processed using a Vectastain ABC Kit (Vector Inc.). The peroxidase was developed with 0.01% H\(_2\)O\(_2\) and 0.05% 3′,3′-diaminobenzidine tetrahydrochloride in Tris buffered saline.

For immunoelectron microscopy, specimens from three patients (2 differentiated type and 1 undifferentiated type) were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 6 hours at 4°C, and washed in graded saccharose solution (up to 20%) in phosphate buffered saline and quickly frozen in OCT compound at a temperature of −80°C. Frozen sections of 6 μm were then cut with a cryostat and mounted on albumin-coated slides. The sections were incubated with the same primary antibodies as those used for light microscopical observation at 4°C in a humidified chamber overnight. Then a 1/100 dilution of peroxidase-conjugated goat F\(_a\) anti-rabbit IgG (Cappel Lab. Inc.) was used as a secondary antibody. After the development of peroxidase, these sections were postfixed with 2% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon 812. Ultrathin sections were then cut and examined with an Akashi LEM-2000 electron microscope.

**Results**

1. **Histopathological findings**

Resected stomachs were histologically divided into differentiated, undifferentiated and mixed adenocarcinomas. The histological cell types in metastatic lesions coincided with those in the
primary lesions or the predominant cell types in the submucosal lesions of the mixed type (Table 1). On silver-impregnated specimens, reticular fibers were observed lineally in the region of the basement membrane of cancer glands and fibrously in the cancer stroma. The reticular fibers were much more abundant in the metastatic lesions of the differentiated adenocarcinomas (Fig. 1a, 2) than in the primary lesions (Fig. 1b). The collagen fibers of the undifferentiated adenocarcinomas were greatly proliferated both in the primary lesions (Table 2, Fig. 1c) and metastatic lymph nodes (Table 2, Fig. 1d).

2. Immunohistochemical findings

Laminin was continuously localized in the basement membrane in the primary lesions of the differentiated adenocarcinomas (Table 3, Fig. 3a). However, the localization of laminin was fragmentary or negative in the basement membrane in both primary and metastatic lesions of the undifferentiated adenocarcinomas (Fig. 3b). Type IV collagen deposits appeared continuously and were more prominent in the basement membrane in the primary lesions of the differentiated adenocarcinomas (Fig. 4a) than of the undifferentiated ones (Fig. 4b). However, the localization

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Primary lesion (Stomach)</th>
<th>Metastatic lesion (Lymph node)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyloric</td>
<td>Pyloric-Fundic</td>
</tr>
<tr>
<td>Diff. type</td>
<td>8(28%)</td>
<td>1(3%)</td>
</tr>
<tr>
<td>Mixed type (predominant type in submucosa)</td>
<td>3(10%)</td>
<td>2(7%)</td>
</tr>
<tr>
<td>Diff. type</td>
<td>2(7%)</td>
<td>1(3%)</td>
</tr>
<tr>
<td>Undiff. type</td>
<td>4(14%)</td>
<td>1(3%)</td>
</tr>
</tbody>
</table>

Diff. type: Differentiated type, Undiff. type: Undifferentiated type

<table>
<thead>
<tr>
<th>Staining intensities of reticular and collagen fibers in the stroma of primary and metastatic lesions in silver and azan stain</th>
</tr>
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<tbody>
<tr>
<td>BM</td>
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<tr>
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<tr>
<td>Diff. type</td>
</tr>
<tr>
<td>Undiff. type</td>
</tr>
<tr>
<td>Por. Sci.</td>
</tr>
<tr>
<td>Sig. Sci.</td>
</tr>
<tr>
<td>Por. Med.</td>
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</tbody>
</table>

BM: Basement membrane of cancer cells, CS: Cancer stroma, R: Reticular fiber, C: Collagen fiber
(-) to (++): gradings based on staining intensities, (-): negative, (+): mild, (++): moderate, (###): marked
Table 3  Immunohistochemical distribution of basement membrane components in primary and metastatic lesions

<table>
<thead>
<tr>
<th></th>
<th>Primary lesion (Stomach)</th>
<th>Metastatic lesion (Lymph node)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diff. type</td>
<td>Undiff. type</td>
</tr>
<tr>
<td>Laminin</td>
<td>BM</td>
<td>CS</td>
</tr>
<tr>
<td>Type IV collagen</td>
<td>(+)</td>
<td>(−)</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>(+)</td>
<td>(++)</td>
</tr>
<tr>
<td>Type III collagen</td>
<td>(−)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

BM: Basement membrane of cancer cells, CS: Cancer stroma, Diff. type: Differentiated type, Undiff. type: Undifferentiated type
Undiff. type represent Por. with scirrhous stroma

of laminin in the stroma of the metastatic lesions of the differentiated adenocarcinomas, was thicker, more irregular and more discontinuous (Fig. 5a) compared with the localization pattern of type IV collagen (Fig. 5b).

Electron microscopically, the laminin and type IV collagen were found in close proximity to the basement membrane of cancer glands and endothelial cells in the differentiated adenocarcinoma (Fig. 6 and 7). Additionally, laminin was localized in the rough endoplasmic reticulum of cancer cells (Fig. 6 inset). In the case of mesenchymal cells, it only appeared diffusely around their cell membrane and, in the stroma, it was also diffusely present.

In primary lesions, fibronectin deposits were clearly seen in the basement membrane and stroma of the differentiated adenocarcinomas (Fig. 8), whereas they were faint in the undifferentiated ones (Fig. 9). In metastatic lesions, fibronectin was stained strongly and distributed diffusely in the stroma of differentiated adenocarcinomas (Fig. 10a), but weakly in the stroma of undifferentiated ones (Fig. 10b). Particularly, no fibronectin was localized in the stroma of signet ring cell carcinoma (Fig. 11a, b). Electron microscopically, fibronectin was localized in the basement membranes of cancer glands and endothelial cells (Fig. 12). Type III collagen deposits were demonstrated in the stroma of both types of carcinoma in the primary lesions and the deposits coincided well with the distribution of the reticular and collagen fibers on the silver-stained specimens. In metastatic lesions, the localization of type III collagen was seen around the blood vessels and along reticular fibers (Fig. 13).

Discussion

In this study, a large quantity of reticular fibers, demonstrated by argyrophilia, were seen in the basement membrane and stromal regions of metastatic lesions of differentiated gastric adenocarcinomas. Immunohistochemically, these fibers were found to include basement membrane components, i.e. laminin, type IV collagen and fibronectin. The difference in argyrophilia of reticular and collagen fibers is known to depend on the size of their fibrils and the relationship to the interfibrillar proteoglycans that bind them together13). The present case shows additionally that the reticular fibers in the lesions have a close association with basement membrane components. Recently, Kramer et al. have suggested the phenomenon in lymph nodes6).

The process of tumor metastasis consists of the destruction of surrounding stroma,
intravasation into the blood or lymphatic circulation system, and extravasation into the remote organs or regional lymph nodes\textsuperscript{7,8,14). In the first steps of metastasis, in order that extensive invasion may be permitted, migrating cancer cells must attach to vascular basement membrane components, and hydrolyse them\textsuperscript{15,16). Thereafter, cancer cells must bind to the component of stroma. Laminin contributes to this process. As is well known, laminin regulates a variety of biological phenomena including cell attachment, growth and migration\textsuperscript{17). Laminin facilitates the binding of cells to type IV collagen\textsuperscript{18). This study shows that in the primary and metastatic lesions of undifferentiated adenocarcinomas the immunohistochemical localization of laminin in the basement membrane and stroma was fragmentary or negative. As for this phenomenon, there is an interesting report showing that laminin receptors were uniformly polarized at the basal surface of the normal epithelium but distributed irregularly over the surface of invading undifferentiated cancer cells, and that the receptors were present in the cytoplasm of highly malignant and poorly differentiated cancer cells\textsuperscript{19).}

The author observed by electron microscopy, that laminin was in close proximity to the basement membrane of cancerous and endothelial cells and was also localized in the endoplasmic reticulum of the differentiated adenocarcinomas. The latter finding means that laminin is produced by cancer cells. Laurie and Leblond reported the localization of laminin in embryonal cells\textsuperscript{20,21). However no observation of the localization of laminin in the case of gastric cancer cells has been reported so far. The present study now confirms an active role of laminin in the cancer cell invasion and metastasis.

Also in this study, fibronectin was immunohistochemically localized in the basement membrane and stroma of the differentiated adenocarcinomas, but was not prominent around the undifferentiated ones. Fibronectin is an important component of the connective tissue matrix and basement membrane. Fibronectin mediates the adhesion of cells to collagen fibers and glycosaminoglycans. Ruoslahti reported that malignant cells synthesize an abnormal amount of fibronectin and fail to deposit an insoluble cell coat\textsuperscript{22). Van den Hooff mentioned that fibronectin promotes haptotactic motility or function as a primary scaffolding in malignant tumor growth\textsuperscript{23). It has also been reported that proteoglycans and fibronectin in the extracellular matrix and reticular fibers promoted tumor invasion by attachment to the tumor cells\textsuperscript{24-26). The present study made it clear that type IV collagen was also distributed in close proximity to the basement membrane of cancerous glandular and endothelial cells and that type III collagen was localized in the basement membrane and stroma of both types of carcinomas. Type III and IV collagen are also thought to be important in malignant tumor growth.

As described above, reticular fibers which include laminin, fibronectin, and type IV collagen, may contribute to cancer cell invasion and provide a mechanism for promoting tumor arrest\textsuperscript{27). Kramer observed that metastatic human tumor cells migrated in the matrix of the reconstituted basement membrane and move preferentially on preformed tracks\textsuperscript{28). These facts might be analogous to the differentiated adenocarcinomas being surrounded by the basement membrane and stroma.

The present findings are noteworthy in that the cytoplasmic localization of laminin was observed in the differentiated cancer cells and that the differentiated cancer stroma was associated with marked proliferation of reticular fibers which were rich in basement membrane
components. These findings are clinically very important, since differentiated tumors, early gastric cancers with lymph node metastasis, reveal many malignant features.

It could be noted that tumor cells alter the environmental stroma through their growth and metastasis depending on their own grade of differentiation. In conclusion, the author suggests that the metastatic mechanism in the differentiated adenocarcinomas is different from that in the undifferentiated adenocarcinomas of the stomach in the localization pattern of basement membrane components.

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References

Plate (1)

Fig. 1

Differentiated adenocarcinomas

Undifferentiated adenocarcinomas

Fig. 2
Plate (2)

Fig. 3

Fig. 4

Fig. 5
Plate (5)

Fig. 12

Fig. 13
Legends

Fig. 1 Light micrograph showing the distribution of reticular and collagen fibers in primary and metastatic lesions of gastric cancers. (×200)

a. Reticular fibers in the basement membrane and stroma of the metastatic lesion of a differentiated adenocarcinoma. Prominent proliferation of reticular fibers are noted. Silver stain (×200)
b. Collagen fibers in the stroma of the primary lesion of a differentiated adenocarcinoma. Silver stain (×200)
c. Collagen fibers in the stroma of the metastatic lesion of an undifferentiated adenocarcinoma (Poorly differentiated adenocarcinoma). Diffuse proliferation of collagen fibers are seen. Silver stain (×200)
d. Collagen fibers in the stroma of the primary lesion of an undifferentiated adenocarcinoma (Poorly differentiated adenocarcinoma). Diffuse proliferation of collagen fibers are observed. Silver stain (×200)

Fig. 2 Schematic illustration showing the basement membrane (BM) and stroma (CS) of the gastric cancers.

Fig. 3 Immunohistochemical photographs showing the presence of laminin.

a. Basement membrane of the primary lesion (stomach) of a differentiated adenocarcinoma. Laminin is continuously localized along the basement membranes. (×200)
b. A localization of laminin in a primary lesion of an undifferentiated adenocarcinoma. Laminin is fragmentary or negative in the basement membranes and stroma. (×200)

Fig. 4 Immunohistochemical photographs of type IV collagen.

a. Continuous localization of type IV collagen along the basement membrane of a differentiated adenocarcinoma in the stomach. (×200)
b. Faint localization of type IV collagen in the primary lesion of an undifferentiated adenocarcinoma. (×200)

Fig. 5 Immunohistochemical photograph of laminin (a) and type IV collagen (b).

a. Diffuse localization of laminin around the cancer glands in the metastatic lesion of a differentiated adenocarcinoma. (×200)
b. Irregular localization of type IV collagen in the basement membrane and cancer stroma in a metastatic lesion. (×200)

Fig. 6 Electron microphotograph of laminin.

a. A localization of laminin in the basement membrane (BM, arrow) of cancer cells (C) in the metastatic lesion of a differentiated adenocarcinoma and associated with diffuse stromal localization between cancer cells and mesenchymal cells (MES). (×6,400)
b. A localization of laminin in the endoplasmic reticulum (ER) of cancer cells in a differentiated adenocarcinoma. Enzyme immunohistochemistry by indirect method. (×6,400)

Fig. 7 Electron micrograph of type IV collagen.

Type IV collagen is noted in the basement membrane (BM) of cancer cells in differentiated adenocarcinomas and capillary endothelium (END). Enzyme immunohistochemistry by indirect method. (×7,500)

Fig. 8 Immunohistochemical photograph showing the localization of fibronectin that is noted in the basement membrane and cancer stroma in the primary lesion of differentiated adenocarcinomas. (×200)

Fig. 9 Immunohistochemical photograph showing the faint and irregular localization of fibronectin that is noted in the primary lesion of undifferentiated adenocarcinomas (Por.). (×200)

Fig. 10 Immunohistochemical photograph showing the localization of fibronectin.

a. Diffuse localization of fibronectin around the cancer glands in the metastatic lesion of a differentiated adenocarcinoma in lymph node. (×200)
b. Faint localization of fibronectin in the metastatic lesion of an undifferentiated adenocarcinoma in lymph node. (×200)

Fig. 11 Immunohistochemical photograph showing fibronectin that is not localized in the cancer stroma of a signet ring cell carcinoma of the stomach (a) or lymph node (b). (×200)

Fig. 12 Electron micrograph of fibronectin that is noted in the basement membrane (BM) of cancer cells in differentiated adenocarcinomas and capillary endothelium (END). Enzyme immunohistochemistry by indirect method. (×6,400)

Fig. 13 Immunohistochemical photograph showing the linear and fragmental localizations of type III collagen (a), type IV collagen (b), laminin (c), and fibronectin (d) that are noted along the reticular fibers in normal lymph nodes. (×200)

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