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SCANNING ELECTRON MICROSCOPIC STUDY OF THE THREE-DIMENSIONAL STRUCTURE OF THE COLLAGEN NETWORKS OF GASTRIC CANCER

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Abstract

The three-dimensional structure of the collagen networks in human gastric carcinoma was examined by scanning electron microscopy (SEM) after treatment with the cell-maceration method using a low temperature NaOH solution. Based on stromal content, the poorly differentiated adenocarcinoma can be divided into the medullary carcinoma area and the scirrhous carcinoma area. In the medullary carcinoma, the collagen sheath around the small tumor cell acinus formed spherical chambers (20-30 μm in diameter) with fenestrations (about 5 μm in diameter) connecting the chambers. The collagen sheath was composed of fine collagen fibrils (about 50 nm in diameter). In the scirrhous area, there was abundant fibrous stroma composed of thicker collagen fibrils (about 100 nm in diameter). Tiny tumor cell nests were sporadically seen in the fibrous stroma. These tumor nests were surrounded by collagen fibrils (about 50 nm in diameter). In the moderately differentiated tubular adenocarcinoma, the tumors were surrounded by spherical, ovoid or irregular shaped thick collagen sheaths (50-200 μm in diameter), which were composed of loosely packed 50 nm collagen fibrils. In well differentiated tubular adenocarcinoma, tumor glands were surrounded by spherical, ovoid or irregularly-shaped thick collagen sheaths (50-200 μm in diameter), composed of densely arranged fine collagen fibrils. In papillary carcinoma, the collagen sheaths were nipple-shaped. They were composed of very densely arranged fine collagen fibrils (about 50 nm in diameter).

Key Words: Gastric carcinoma, collagen, scanning electron microscopy.

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Introduction

Recently, Ohtani *et al.* (1988) visualized the three-dimensional structure of collagen fibrillar networks of various tissues by a cell-maceration / scanning electron microscopy (SEM) method employing a low temperature 2.5 N NaOH solution. Using this method, the three-dimensional structure of the collagen network of the various healthy organs were reported (Ohtani, 1987, 1988; Ohtani *et al.*, 1988; Sugimoto and Ogata, 1989). Recently, the collagen network of large intestinal tumors (Furuya and Ogata, 1993) was studied by this method. The collagen fiber in the stroma of gastric cancer, especially in scirrhous carcinoma, has already been studied by light and transmission electron microscopy (Minamoto *et al.*, 1988; Yamamoto *et al.*, 1984). However, these studies did not clearly visualize the three-dimensional structure of the collagen network in gastric cancer. To extend these studies we employed the maceration method to examine the three-dimensional organization of the collagen networks of various types of gastric cancer.

Materials and Methods

Tissue blocks from 32 human gastric cancers diagnosed as intestinal type [well differentiated tubular adenocarcinoma (5), moderately differentiated tubular adenocarcinoma (4), and papillary adenocarcinoma (3)], and diffuse type [poorly differentiated adenocarcinoma (20) which contained signet ring cell carcinoma] were obtained at surgery. They were fixed with 2% glutaraldehyde in cacodylate buffer (pH 7.4) and cut into 5 x 5 x 5 mm blocks. These blocks were treated by the following methods.

The cell-macerated specimens were prepared according to the method of Ohtani *et al.* (1988). The fixed blocks were washed in distilled water, then macerated in 10% (2.5 N) NaOH solution at 20° C for 7 days. After washing with distilled water for 2 days, the specimens were further fixed in 1.5% tannic acid solution for 8 hours and washed in running tap water for 2 hours.

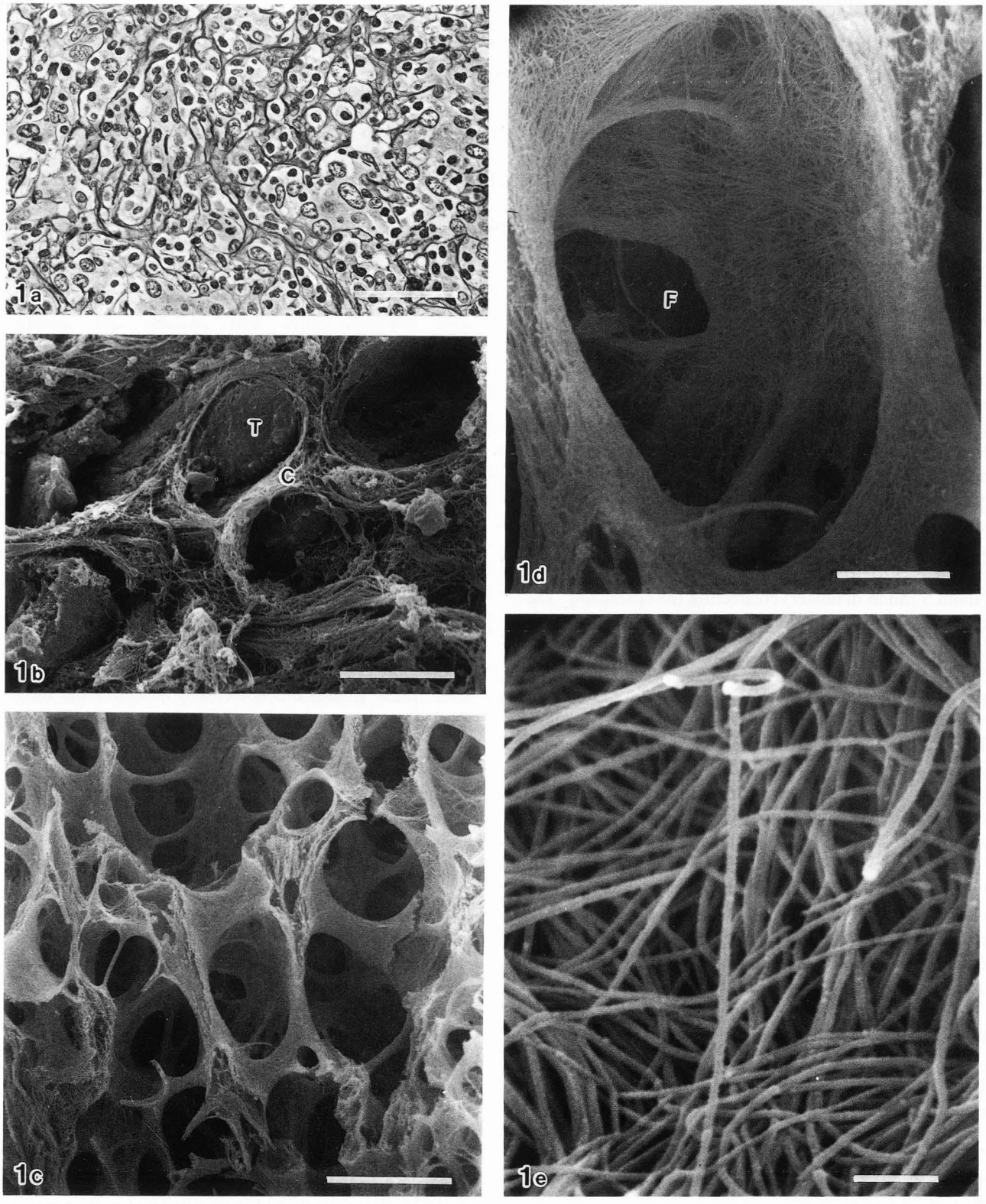
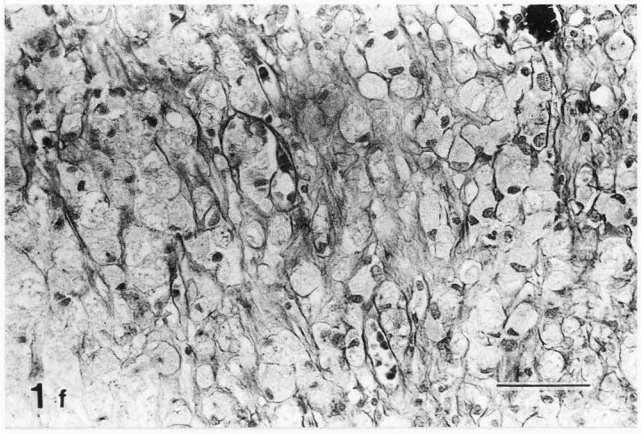
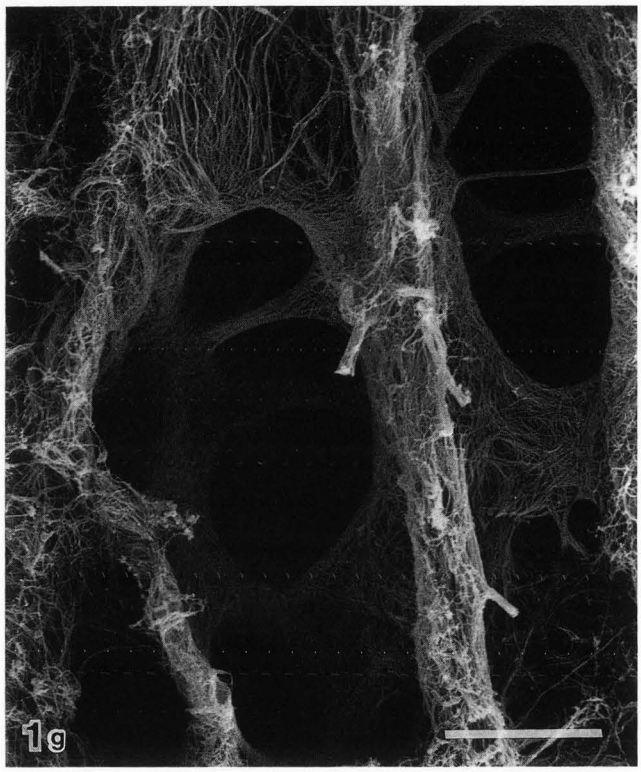


Figure 1. Poorly differentiated adenocarcinoma with the medullary stroma. **(1a).** Light micrograph of a silver-impregnated specimen. Small acini of tumor cells were surrounded by thin collagen fibers stained black with silver. Bar = 50 μm. **(1b-1e).** Scanning electron micrographs of ultrasonicated specimens. **(1b)** The tumor cell (T) is surrounded by the collagen sheath (C). Bar = 10 μm. **Caption continued on the facing page 367.**

Figure 1 (caption continued from the bottom of the facing page). (**1c**). NaOH cell macerated specimen. Tumor cells and basal lamina are removed and collagen sheaths around tumor cell nests appear. The collagen sheaths are spherical or ovoid in shape, and the lumens are connected by fenestrations. Bar = 20 μm. (**1d**). Higher magnification of of an area in Figure 1c. The collagen sheath is formed with randomly arranged collagen fibrils. The chamber wall has a fenestration (F). Bar = 5 μm. (**1e**). High magnification of a part of Figure 1d. Collagen fibrils 50 nm in diameter are randomly arranged. Bar = 0.5 μm. (**1f, at right**). Light micrograph of the silver-impregnated specimen. Signet ring cells are surrounded by thin collagen fibers. Bar = 100 μm. (**1g at right**). Scanning electron micrograph of NaOH cell macerated specimen. The structure of collagen sheaths around signet ring cells is not different from that around non-signet ring cells. Bar = 10 μm.





They were post-fixed in 1% osmium tetroxide solution at 4°C for 2 hours (Murakami, 1974), washed in running water for 2 hours, and dehydrated through a graded series of ethanol.

The ultrasonicated specimens were prepared after the method of Highison and Low (1982). The fixed blocks were post-fixed with 2% osmium tetroxide solution in cacodylate buffer at 20°C for 48 hours, washed in running water, dehydrated in a graded series of ethanol, and treated at 26 kHz for 5-10 minutes in an ultrasonicator (UO 150 FS and UT-6, Kokusai Electric, Tokyo).

All specimens treated by the above methods were dried in a critical point drier (HPC-2, Hitachi, Tokyo) and mounted on metal stubs. Subsequently, they were coated with gold in an ion coater (IB-5, Eiko, Ibaragi) and observed under SEM (S-430, Hitachi, Tokyo) operated at an accelerating voltage of 15 kV.

Light microscope specimens were embedded in paraffin and stained with hematoxylin-eosin (H-E) or by the silver impregnation method.

Results

The NaOH cell maceration used in the present study proved very useful for disclosing the architecture of the collagen networks of different types of gastric cancer tissue. The results indicate that the tumor cells and the basal lamina were removed and the collagen network was exposed.

Diffuse type

Poorly differentiated adenocarcinoma: Poorly differentiated adenocarcinoma was divided into two areas based on stromal connective tissue, the area of the medullary carcinoma in which the connective tissue elements were not conspicuous, and the area of the scirrhous carcinoma with abundant connective tissue. The boundary between these two areas was not always distinct.

Area of the medullary carcinoma: In silver-impregnated specimens viewed by light microscopy, a few tumor cell nests were seen embedded in darkly stained collagen tissue sheaths (Fig. 1a). Ultrasonication removed most of the tumor cells. Figure 1b shows a few tumor cells still surrounded by the collagen tissue sheaths which formed chambers about 10 μm in diameter. In the NaOH macerated specimens, the collagen sheaths around tumor cells formed spherical or ovoid

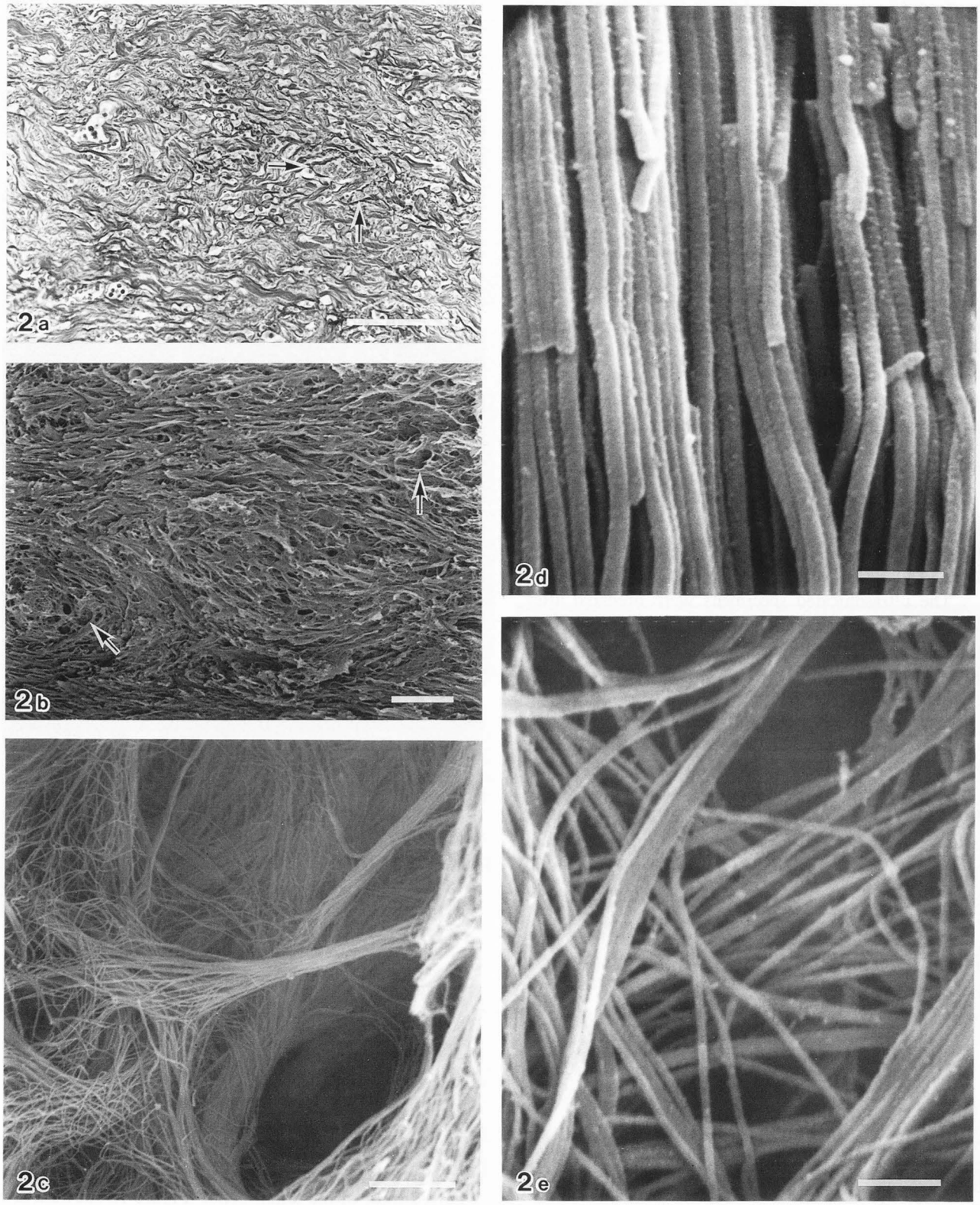


Figure 2. The portion of poorly differentiated adenocarcinoma with abundant collagen (scirrhous carcinoma). **(2a).** Light micrograph of the silver-impregnated specimen. Among conspicuous fibrosis tiny cancer cell nests (arrows) are seen. Bar = 100 μm. **(2b-2e).** Scanning electron micrographs. **Caption continued on the facing page 369.**

Figure 2 caption continued from the bottom of the facing page. (**2b**). Low magnification of cell macerated specimen. Dense collagen bundles are observed. Arrows show the collagen sheath around tiny cell nests. Bar = 100 μm. (**2c**). Collagen sheath around the small tumor cell nests. The sheaths are spherical in shape and composed of loose fine collagen fibrils. Bar = 3 μm. (**2d**). High magnification of the dense collagen bundle. The thick fibers about 100 nm in diameter run parallel to each other. Bar = 0.5 μm. (**2e**). Higher magnification of a part of Figure 2c. Solitary fine collagen fibrils about 50 nm in diameter run randomly. Bar = 0.5 μm.

10-20 μm chambers with walls 5-10 μm thick (Fig. 1c). The chamber walls had interconnecting fenestrations 5-10 μm in diameter (Fig. 1d). The total thickness and density of the collagen sheath varied among the lesions. At higher magnifications, the collagen sheath around the tumor cells was found to be composed of randomly oriented collagen fibrils about 50 nm in diameter (Fig. 1e). In some areas of poorly differentiated adenocarcinoma, predominantly signet ring cells were observed (Fig. 1f). In the NaOH macerated specimens, the three-dimensional structure of collagen sheaths around signet ring cells was not different from that around non-signet ring cells (Fig. 1g).

Area of the scirrhous gastric carcinoma: In silver-impregnated specimens, small scattered nests of tumor cells were seen sporadically among the well developed fibrous stromal tissue (Fig. 2a). In the macerated specimens, the very well organized collagen fibrils comprised most of the scirrhous carcinoma area (Fig. 2b). At higher magnification, the 100 nm collagen fibrils were seen in parallel array (Fig. 2d). Among these parallel collagen bundles, the collagen sheaths around the tumor cell nest were sporadically observed. The sheaths formed spherical chambers with fenestrations to other chambers (Fig. 2c). At higher magnification, the collagen sheath was composed of loosely and randomly arranged fine collagen fibrils about 50 nm in diameter (Fig. 2e).

Intestinal type

Moderately differentiated tubular adenocarcinoma: In silver-impregnated specimens, the tubular carcinoma glands were surrounded by collagen fibers (Fig. 3a). In the macerated specimens, the collagen sheath around the tumor cells appeared spherical and 100 μm in diameter (Fig. 3b). At moderate magnification, the collagen sheath was found to be loosely arranged (Fig. 3c). The component collagen fibrils were about 50 nm in diameter and occasionally tapered (Fig. 3d).

Well differentiated tubular adenocarcinoma: In silver-impregnated specimens, the tumor nests were surrounded by abundant collagen fibers (Fig. 4a). In macerated specimens, examined at lower magnification, the tumor glands were surrounded by spherical collagen sheaths (Fig. 4b). Medium magnification revealed the collagen sheath as spherical or polygonal chambers with a few fenestrations (Fig. 4c). Higher magnification observation showed that the collagen sheath was formed by densely packed collagen fibrils about 50 nm in diameter (Fig. 4d). These collagen fibrils were packed denser than those seen in moderately differentiated adenocarcinoma.

Papillary adenocarcinoma: In silver-impregnated specimens, the papillary adenocarcinoma tumor had nipple-like growth of carcinoma cells on a connective tissue base (Fig. 5a). In the macerated specimens, the appearance of the collagen sheath around the tumor cells was also papillary in shape (Fig. 5b). At medium magnification, the surface of the collagen sheath was smooth (Fig. 5c). At higher magnification, the sheath was composed of densely and irregularly arranged collagen fibrils about 50 nm in diameter (Fig. 5d).

Discussion

The cell maceration method (Ohtani *et al.*, 1988) removed the cell and basal lamina by maceration with NaOH and exposed the collagen networks. Application of this method to gastric cancer tissues successfully exposed the collagen tissues under cancer cell nests.

In the well and moderately differentiated adenocarcinoma, large tumor cell nests were surrounded by the collagen sheaths, while in poorly differentiated adenocarcinoma, the small cell nests, usually composed of one or a few tumor cells, were surrounded by the collagen sheaths. At higher magnification, these collagen sheaths were found to be composed of solitary fine collagen fibrils, and there was a tendency for the collagen sheath of the poorly differentiated carcinoma to contain loosely arranged collagen fibrils. As the differentiation progressed, the collagen fibrils became more densely and regularly arranged, and in the papillary carcinoma, the density was the highest.

In well differentiated adenocarcinoma, type III collagen was found to be distributed around the tumor cells (Yamamoto *et al.*, 1984). Our findings showed that the tumor cells were surrounded mainly by collagen fibrils 50 nm in diameter, forming the collagen sheath.

The scirrhous type of gastric carcinoma is a special kind of tumor characterized morphologically by extensive fibrosis and clinically by a poor prognosis. In the scirrhous carcinoma, very well developed collagen bundles were seen. Immunohistochemically, dense fibrous

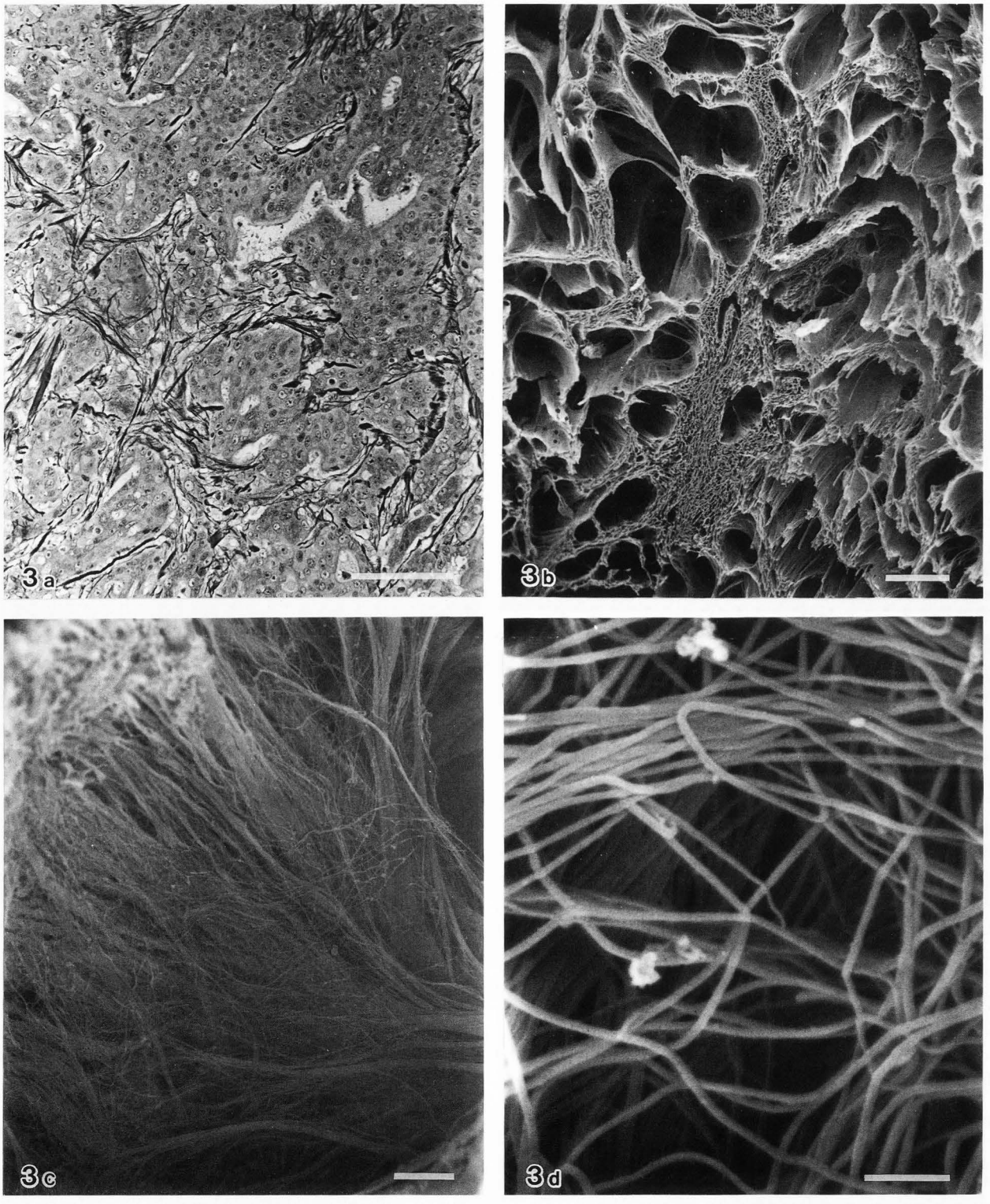


Figure 3. Moderately differentiated tubular adenocarcinoma. (**3a**). Silver-impregnated specimen. Tumor cell nests are surrounded by collagen fibers. Bar = 100 μm. (**3b**). Macerated specimen. The tumor nests are surrounded by irregularly shaped collagen sheaths. Bar = 100 μm. (**3c**). Medium magnification. The collagen sheath is composed of solitary collagen fibrils that are loosely arranged. The collagen sheath has a fenestration (F). Bar = 10 μm. (**3d**). Solitary collagen fibrils are loosely arranged and run randomly. Bar = 0.5 μm.

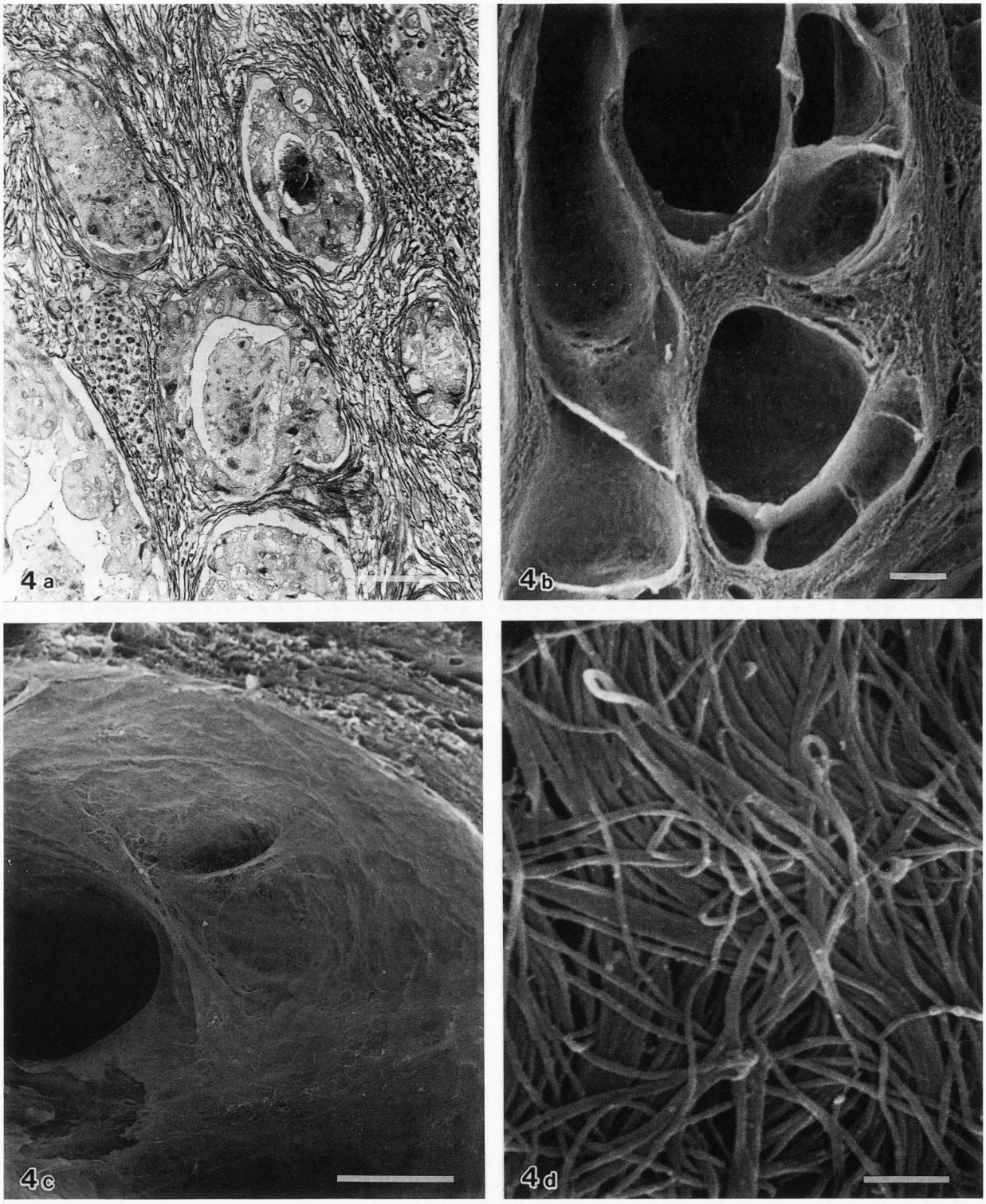


Figure 4. Well differentiated tubular adenocarcinoma. (**4a**). Silver-impregnated specimen. Tumor cell nests are embedded among well developed collagen tissues. Bar = 100 μm. (**4b**). Cell macerated specimen. The tumor nests are surrounded by hemispherical collagen sheaths. Bar = 100 μm. (**4c**). Medium magnification. Macerated specimen of the collagen sheath adjacent to Figure 4b. Note a relatively smooth surface with a fenestration. Bar = 50 μm. (**4d**). Higher magnification of a part of Figure 4c. The collagen sheath is composed of collagen fibrils about 50 nm in diameter, that are denser than those of moderately differentiated adenocarcinoma. Bar = 0.5 μm.

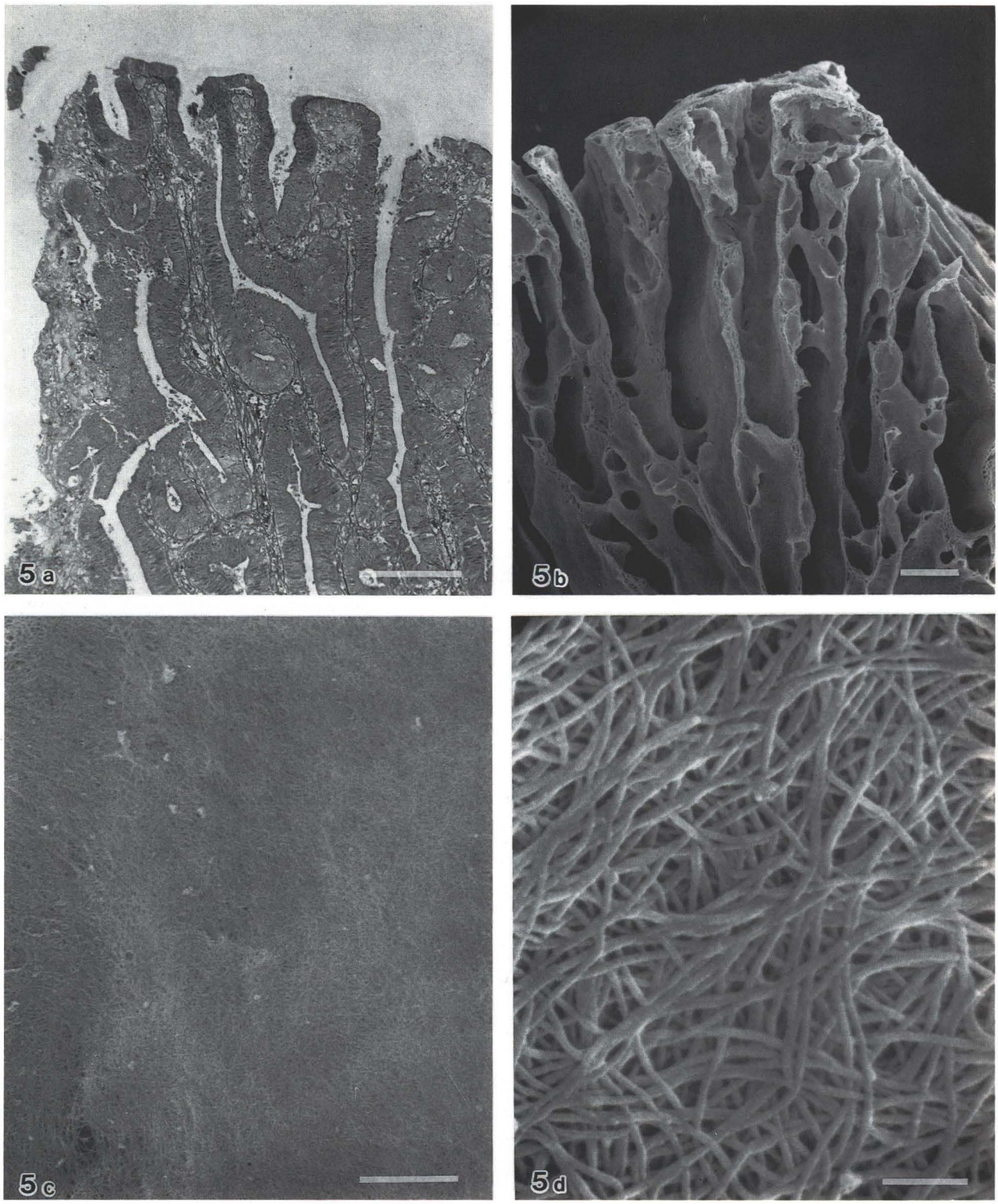


Figure 5. Papillary adenocarcinoma. (**5a**). Silver-impregnated specimen. Bar = 200 μm. (**5b**). The appearance of the collagen sheaths around the tumor cells is papillary. Bar = 200 μm. (**5c**). Higher magnification of the area outlined in Figure 5b. The surface of the collagen sheath around the tumor cells is smooth and dense. Bar = 5 μm. (**5d**). Higher magnification of the area outlined in Figure 5c. The collagen sheath is composed of densely and regularly arranged collagen fibrils about 50 nm in diameter. Bar = 0.5 μm.

tissue of the scirrhous carcinoma was determined to be mainly composed of type I and III collagen (Minamoto *et al.*, 1988; Yamamoto *et al.*, 1984). Recently, Keene *et al.* (1987) indicated that fibrils of the skin, tendon and amnion are copolymers of at least types I and III collagen, and there is a tendency that the diameter of the collagen fibrils becomes larger as the ratio of type I to type III increases (Fleischmajer *et al.*, 1981; Romanic *et al.*, 1991). Therefore, it is assumed that large diameter collagen fibrils, about 100 nm in diameter, in the scirrhous stroma are predominantly composed of type I collagen, while smaller collagen fibrils, about 50 nm in diameter, mainly observed in the collagen sheath around the tumor cells, are predominantly composed of type III collagen. Collagen synthesis is active in granulation tissue as wound closure proceeds. The initially produced collagen is type III. With time, the collagen becomes more rigid (type I) and the myofibroblasts gradually disappear (Seemayer *et al.*, 1979; Gabbiani *et al.*, 1976). In the scirrhous stroma, thick collagen bundles composed of collagen fibrils about 100 nm in diameter were dominant, and the collagen fibrils ran parallel to each other. Therefore, it may be assumed that the parallel thick collagen fibrils are mature and stable.

In the poorly differentiated adenocarcinoma, one or a few tumor cells were surrounded by collagen sheaths. Collagen formation around the tumor cells is called desmoplastic reaction. Some authors believe that the tumor cells are confined by these collagen sheath and their growth is inhibited (Seemayer *et al.*, 1979). If this hypothesis is true, the scirrhous carcinoma, in which tumor cells are surrounded by dense collagen tissue, would be expected to have a better prognosis than it does. However, present observations showed that the collagen sheaths around the tumor cells have the fenestrations through which the chambers are connected. Therefore, it is reasonable to assume that tumor cells are not confined by the sheath, because the fenestrations enable metabolites to reach the tumor cells. The fenestrations also provide channels by means of which tumor cells are able to spread.

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Discussion with Reviewers

O. Ohtani: Some papers, e.g., Liotta *et al.* [Liotta LA, Thorgeirsson UP, Garbisa S (1982). Role of collagenase in tumor cell invasion. Cancer Metastasis Rev. **1**, 277-288] report that gastric cancer cells themselves produce collagen which may serve for scaffolds for cancer cells to metastasize. Do you have data to suggest that cancer cells produce collagen?
Authors: It has been reported that the basal lamina was elaborated by cooprration between cancer cells and fibroblasts (Yamotomot *et al.*, 1984) and that myofibroblasts produced type I, type III and type V collagen (Minamoto *et al.*, 1988). In our study, type IV collagen and laminin were macerated by NaOH solution. Therefore, our findings would not show the collagen that carcinoma cells produce. The ultrasonicated specimens might show type IV collagen and laminin around carcinoma cells. But we could not correlate the membranous structure around carcinoma cells to type IV collagen and laminin.

S. Siew: Are you able to diagnose the degree of differentiation of the tumors by means of the arrangement of the collagen fibrils?
Authors: Even in an area of the carcinoma, there existed one lesion in which collagen network was dense and the other lesion in which collagen network was loose. But, generally, as the differentiation of the carcinoma became higher, the density of the collagen network around the carcinoma cells became more dense. Therefore, it may be possible to diagnose the degree of differentiation of the tumors using this method.

W.H. Wilborn: Do you believe that the new information you have obtained can be used to improve methods for treating gastric cancer?
Authors: No.

W.H. Wilborn: Please comment on the significance of the tapered fibrils you noted.
Authors: The tapered fibrils can be one of the phenomena that correlate with invasion of cancer cells, or they may be artifact. We believe that such collagen fibrils were altered by cancer cells. The collagen fibrils we presented using the NaOH maceration method were composed of type I, type III, and type V collagen; these collagen fibrils are produced by myofibroblasts. The tapered collagen fibrils we presented surround the tumor cells. Therefore, it is reasonable to think that cancer cells influence the collagen fibrils.

T. Ushiki: What is the meaning of the sentence "the collagen becomes more rigid (type I)" in **Discussion**? Do you mean that the type I-rich collagen fibrils are structurally stronger than type III-rich fibrils?
Authors: The sentence "the collagen becomes more rigid (type I)" was from Seemayer *et al.* (1979) who reported that the tissue with type I collagen needed plasticity. We do not mean that type I-rich collagen fibril is structurally stronger than type III-rich collagen fibril. The collagen fiber of scirrhous stroma is mature and stable.