Immunohistochemical Distribution of Heat Shock Protein 47 (HSP47) in Scirrhous Carcinoma of the Stomach

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Abstract. *Background: The aim of the present study was to examine the immunohistochemical distribution of the 47-kDa heat shock protein (HSP47) to enhance the understanding of the mechanisms involved in stromal fibrosis, which accompanies cancer infiltration in scirrhous carcinoma of the stomach. Materials and Methods: In vitro gastric cancer models were prepared by collagen gel cultures using three different human gastric cancer cell lines (KATO-III, MKN-74, MKN-45) and a human fibroblast cell line (TIG-101). Tumor tissues were obtained from ten patients with early gastric cancer (5 scirrhous carcinoma; 5 non-scirrhous carcinoma) and three patients with advanced scirrhous gastric cancer. The gels and the tissues were immunostained by a monoclonal antibody against human HSP47 and then examined by light and electron microscopy. Results: The staining intensity of the fibroblasts was stronger than that of cancer cells in both the culture models and patient tissues. Moreover, the number of fibroblasts in scirrhous gastric cancer was significantly greater than that in non-scirrhous gastric cancer ($p=0.0004$). In addition, the discharge of HSP47 was observed in the extracellular matrix as granular deposits of staining. Conclusion: These findings indicate that fibroblasts predominantly produce stromal collagen and may play an important role in the development of stromal change in scirrhous gastric cancer. Further studies are required to elucidate the significance of HSP47 in the development of new clinical approaches for treating scirrhous gastric cancer.*

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Key Words: Gastric cancer, scirrhous carcinoma, collagen, stroma, HSP47.

Scirrhous carcinoma of the stomach is characterized by diffuse cancer infiltration with abundant stromal collagen accumulation, not only in the primary lesion but also metastatic organs, resulting in an unfavorable prognosis for patients with this carcinoma due to lethal organ fibrosis (1, 2). This condition may be, partly, similar to that of other benign fibrotic diseases, *i.e.* liver cirrhosis, even though the change occurs secondarily due to cancer infiltration. The pathogenesis and biological nature of stromal fibrosis have been studied previously (3-10). However, the mechanisms of the particular changes that occur upon cancer infiltration, and cell-matrix interactions are poorly understood in scirrhous carcinoma of the stomach.

The 47-kDa heat shock protein (HSP47), which binds procollagen to correctly stabilize the triple helix formation, is a collagen-specific molecular chaperone found in the endoplasmic reticulum (ER), which assists in the maturation of collagen molecules (3, 4). Expression of HSP47 is known to be up-regulated in lesions of fibrotic diseases such as idiopathic pulmonary fibrosis, systemic sclerosis, renal scaring and keloids (5-8). Thus, HSP47 may play an important role in the establishment of organ fibrosis. However, HSP47 has not been used to study the stromal fibrosis that can occur upon carcinoma infiltration in scirrhous carcinoma of the stomach. The aim of the present study was to examine the immunohistochemical distribution of HSP47 in order to better understand the biological nature of scirrhous carcinoma of the stomach.

Materials and Methods

Collagen gel culture (in vivo models of gastric cancer). A normal human dermal fibroblast cell line (TIG-101) and three human cancer cell lines derived from carcinoma of the stomach, namely KATO-III (scirrhous type, signet-ring cell carcinoma), MKN-74 (non-scirrhous type, well-differentiated adenocarcinoma) and MKN-45 (non-scirrhous type, poorly-differentiated adenocarcinoma) (9), were

obtained from the Japanese Cancer Research Resource Bank. Collagen gel cultures were grown according to previously reported procedures (10), with minor modifications. Briefly, co-culture of $2.5x10^5$ cancer cells (KATO-III, MKN-74 or MKN-45) with $2.5x10^5$ fibroblasts (TIG-101) was set up at 4˚C in 2 ml RPMI medium containing 10% fetal calf serum (FCS) and 0.025% type I collagen (Koken Co., Japan), in a 3-cm plastic petri dish (Falcon Co., USA). The cells were cultured three-dimensionally and the collagen solution was gelated at 36˚C. The models were maintained according to standard procedures and cultivated for 14 days.

Preparation of materials from patients with gastric cancer. For light-microscopic examination, specimens of tumor tissues were obtained from ten patients who underwent resection for early gastric cancer at Kitasato East Hospital, Japan. All tumors were selected according to the following criteria: i) tumors invading the submucosal layer, because submucosal tumor regions are considered to be less heterogenous than advanced tumors, and ii) tumors without ulceration. Of these, five tumors were classified histologically as scirrhous (abundant stroma), and the other five as non-scirrhous carcinoma (scanty stroma), according το the Japanese Classification of Gastric Carcinoma (11). The regions of submucosal invasion were used for histological examination and the cell number of fibroblasts and cancer cells was also determined after immunostaining for HSP47. For counting cell numbers, three visual fields were examined for each tumor at original magnification x400 under light microscopy, and the cell number was calculated.

For immuno-electron microscopic examination, fresh tumor tissues were prepared from three patients with type IV advanced scirrhous gastric cancer. The materials were obtained intra-operatively and small blocks of specimens were fixed in Zanboni's fixative immediately after surgical resection.

Immunohistochemical study. For light microscopic examination, these gels and tumors were fixed in Zanboni's fixative (4% paraformaldhyde, 0.2% picric acid, in 0.1 M phosphate-buffered saline [PBS]). Specimens of the material were sliced 6 μm thick after embedding in paraffin, and then incubated with a mouse monoclonal antibody to HSP47 (diluted at 1:250) (Stress Gen Co., USA). The avidin-biotin-peroxidase complex (ABC) method was used for immunostaining with the ABC kit (Nichirei Co., Japan).

The immunostaining for electron microscopy was performed using pre-embedding methods (12). Briefly, specimens fixed in Zanboni's fixative were embedded in OCT compound (Tissue Tec, Elkhart, IN, USA). Frozen sections (6 μm thick) were prepared and placed on glass microscope slides. The immunostaining was performed using similar procedures as for the light microscopic examination. The sections were post-fixed with 1% OSO_4 (Merck, Darmstadt, Germany) for an hour, embedded in epoxy resin (Nissin EM Co., Japan), and cut into 70 to 80-nm-thick sections and examined under an electron microscope.

Statistics. Statistical analysis was carried out using the Student's *t*-test, assuming a significance level of <5%.

Results

The immunohistochemical stainings for HSP47 in *in vitro* gastric cancer models are presented in Figure 1. In all the models, the cancer cells showed nuclear atypia and pleomorphism, while fibroblasts possessed small and oval nuclei and prominent cell processes that formed the bipolar shape. Cisterae of endoplasmic reticulum (ERs) were stained in both fibroblasts and cancer cells. However, the staining intensity of the fibroblasts was stronger than that of the cancer cells. ERs were distributed widely in cell cytoplasms, and were also observed even at the distal end of the prominent cell process of fibroblasts. In addition, staining was observed in the extracellular matrix (ECM), although HSP47 is a molecular chaperone that exists only in ER.

Table I. *Cell numbers obtained by light microscopic examination of gastric cancer tissues.*

	Non-scirrhous*	Scirrhous*	*P*
No. of fibroblasts**	25.6 (14.7- 47.6)	64.3 (46- 76.3)	0.0004
No. of cancer cells**	198.6 (141.7- 251.6)	40.1 (31- 54)	<0.0001

*n=5, average (range).
**Cells were counted in one visual field at original magnification x400. Three visual fields were examined for each tumor.

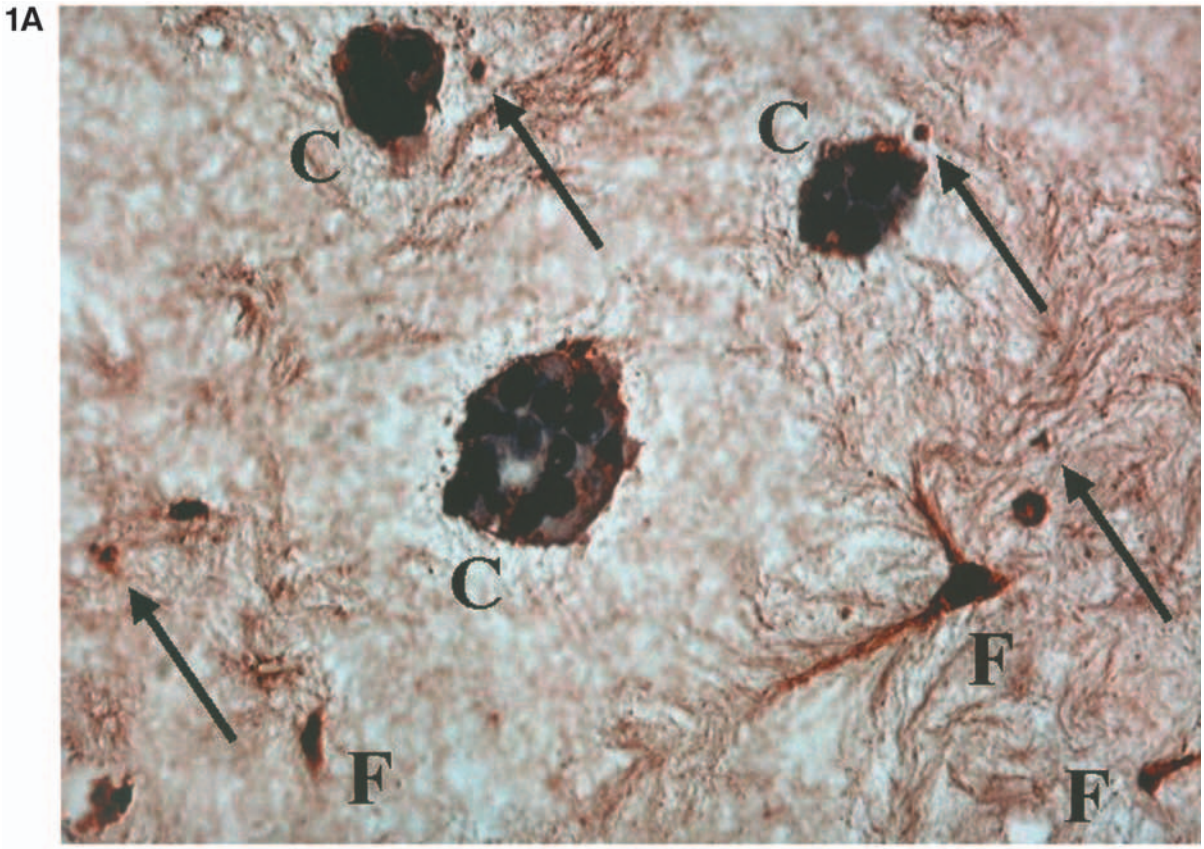
The immunostaining for HSP47 in gastric cancer tissues is presented in Figure 2. Staining was found not only in fibroblasts, but also in cancer cells (Figure 2-A, B), as seen in the *in vitro* models. However, the staining was not observed in normal epithelial cells (Figure 2-C). The intensity of fibroblast staining was stronger than that of cancer cells, and this property was not dependent on differences in the histological classification of gastric cancer.

The number of fibroblasts and cancer cells counted under light microscopic examination is presented in Table I. The number of fibroblasts in scirrhous gastric cancer (average 64.3 cells/one visual field at x400) was significantly higher than that in non-scirrhous gastric cancer (average 25.6 cells/one visual field at x400) ($p=0.0004$). However, the number of cancer cells in scirrhous gastric cancer (average 40.1 cells/one visual field at x400) was significantly lower than that in non-scirrhous gastric cancer (average 198.1 cells/one visual field at x400) ($p=0.0004$).

Immuno-electron microscopic examination for HSP47 in gastric cancer tissues is presented in Figure 3. The staining was observed not only in ERs, but also in the ECM. Cytoplasmic remnants stained for HSP47 were found in the ECM, whereas membrane structures were not observed.

Discussion

Previous studies have shown that an excessive amount of collagen is accumulated through three different processes and their combination in scirrhous gastric carcinoma: i) primary production by tumor cells, that is, so-called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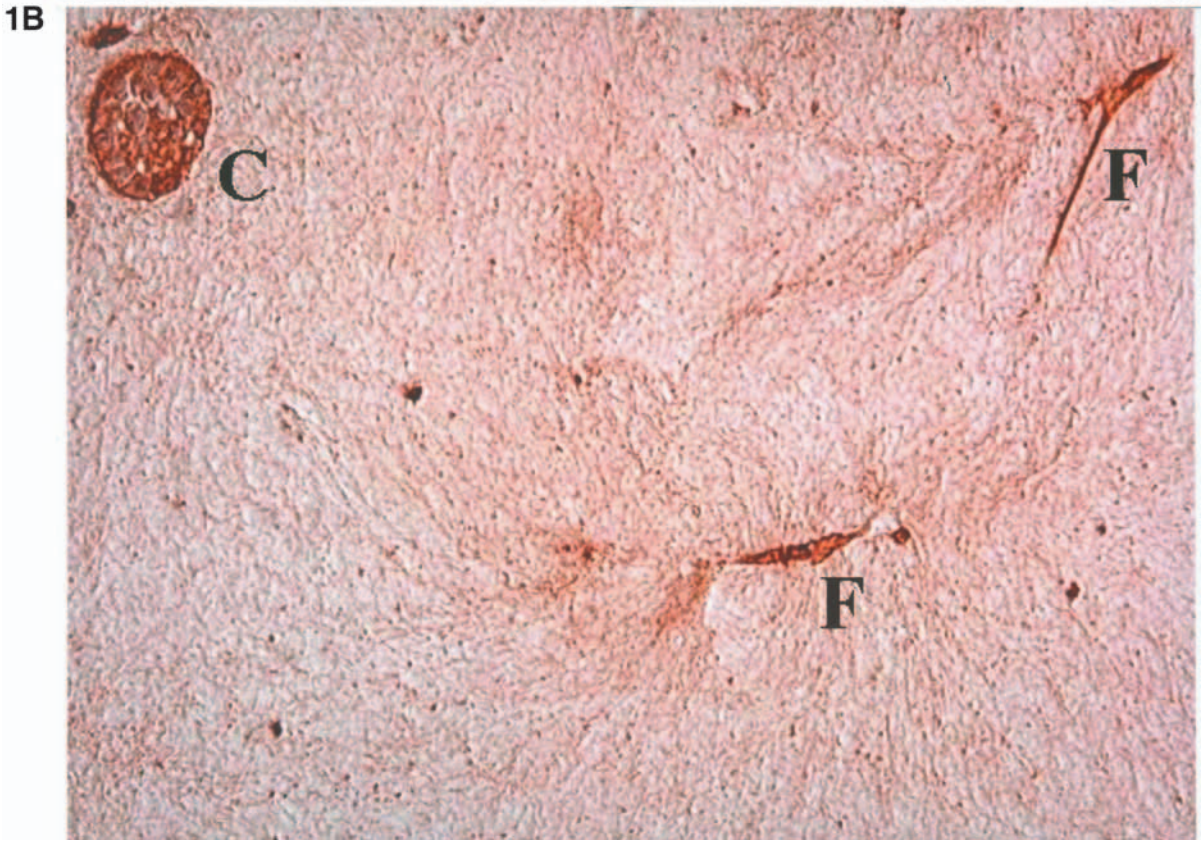
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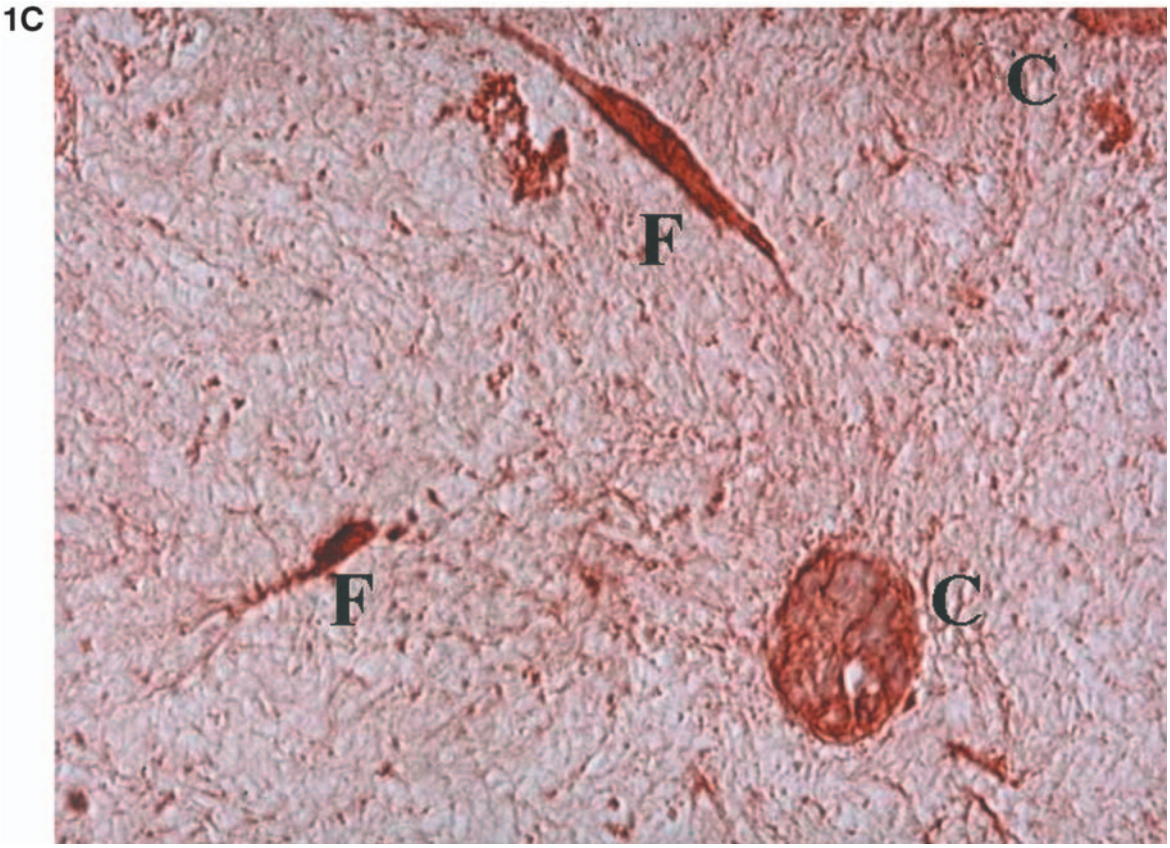
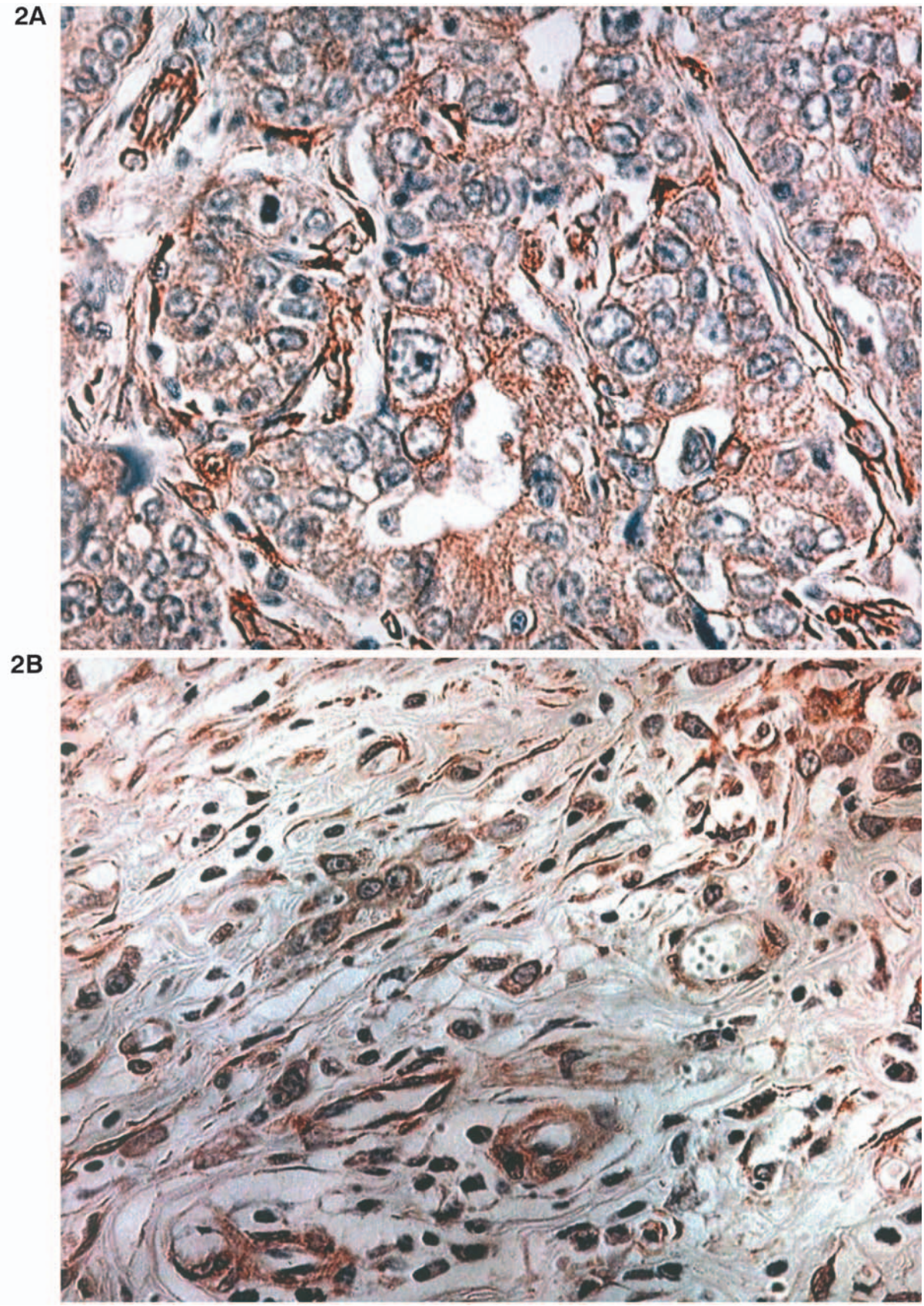


Figure 1. *Immunohistochemical staining of HSP47 in three-dimensional co-cultures (C: cancer cell, F: fibroblast). Staining of endoplasmic reticulum for HSP47 was observed in cancer cells (KATO-III; 1-A, MKN-74; 1-B, MKN-45; 1-C) and fibroblasts (TIG-101). Endoplasmic reticula were stained in both fibroblasts and cancer cells. However, the staining intensity of fibroblasts was stronger than that of cancer cells(original magnification x400). Staining was also found in the extracellular matrix (arrows).*

collagen-producing carcinoma (13-15, 21), ii) consequential production by fibroblasts associated with cancer infiltration (16-18, 21), and iii) disproportional amounts of collagenase and its inhibitor (19, 20). However, a definitive conclusion has not been drawn regarding excess collagen. The present study showed the following new information with respect to collagen production in scirrhous cancer tissues. First, although both fibroblasts and cancer cells can produce collagen as reported previously, the intensity of fibroblast staining for HSP47 was much greater than that of cancer cells, not only in *in vitro* models but also in tumor tissues. Second, the number of cancer cells in scirrhous gastric cancer was significantly lower than that in non-scirrhous gastric cancer, but the number of fibroblasts was significantly higher than that in non-scirrhous cancer tissues. These findings suggest that fibroblasts are the predominant producers of stromal collagen and may play an important role in the development of particular stromal changes in scirrhous gastric cancer.

On the other hand, it remains unclear why HSP47 should be discharged to the ECM from the ER. HSP47 binds procollagen to form a folding triple helix formation. HSP47 is part of the quality control system for procollagen, and prevents the secretion of procollagen in an abnormal conformation (4). So, HSP47 exists primarily in the cisternae ER. However, it has been reported that expression of HSP47 and its antigen were found in the sera of patients with collagen diseases (22). It is known that a part of the cell process detaches from the cytoplasm during cell migration and forms cytoplasmic remnants containing ERs. The intracellular organelles are then discharged from the cell to the ECM by way of auto lysis of the cytoplasmic remnants. Here, specific staining of HSP47 was found even at the distal end of prominent cell processes in fibroblasts, and HSP47 was observed in the ECM, indicating that the discharge of HSP47 may be caused mainly by destruction of cytoplasmic remnants during the process of cell migration. These findings suggest that serum HSP47 may be used as a possible target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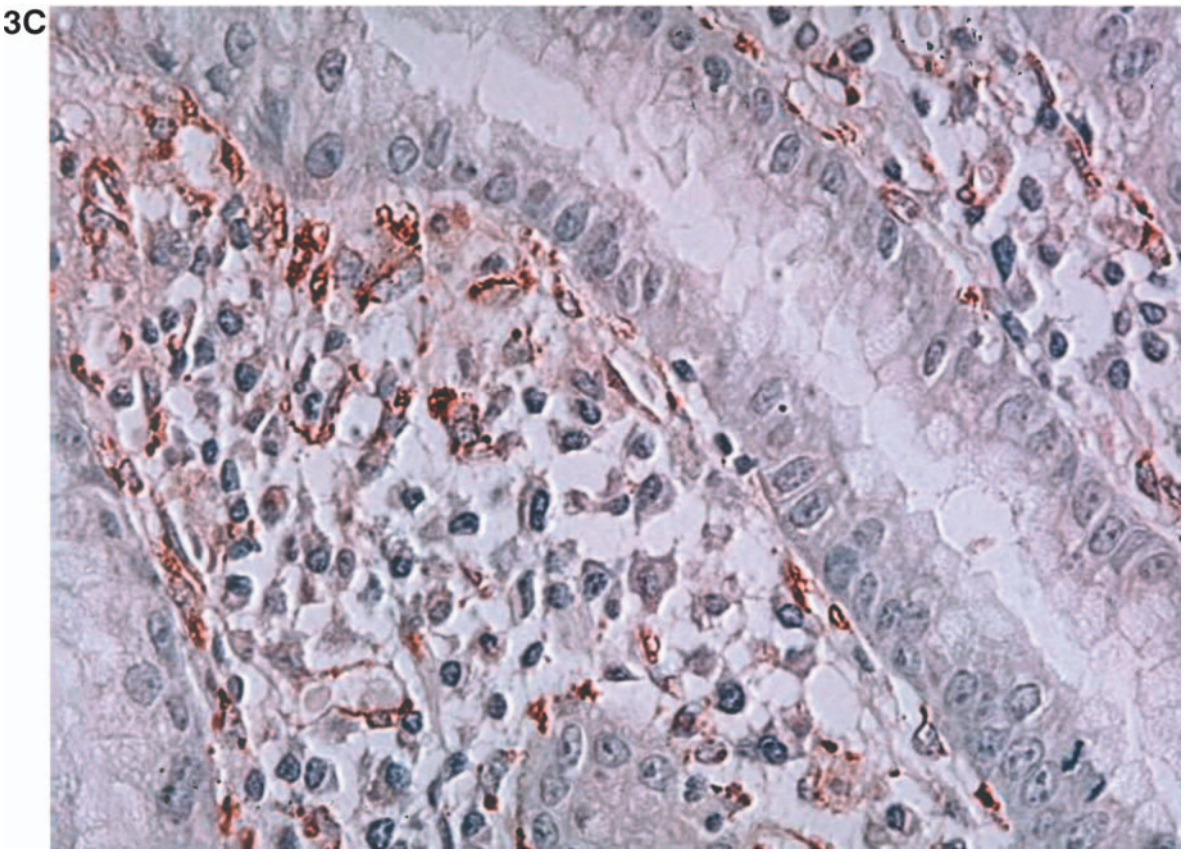

Figure 2. *Immunohistochemical staining in gastric cancer tissues for HSP47. Staining was found not only in fibroblasts, but also in cancer cells (Figure 2-A, B), as seen in the co-culture models. However, staining was not observed in the normal epithelial cells (Figure 2-C). The staining intensity of fibroblasts was stronger than that of cancer cells (medullary type; 2-A., scirrhous type; 2-B.). Normal epithelial cells did not stain for HSP47 (2-C).*

for tumor identification in patients with scirrhous gastric cancer, although further studies are necessary to clarify the significance of the serum HSP47 level in this cancer.

Previous reports have shown that HSP47 is related to other benign diseases, which form extensive stromal fibrosis (7-9, 22-25). Recent reports have also demonstrated that some fibrotic diseases, including kelloid, rheumatoid arthritis and peritoneal fibrosis, were improved in response to HSP47 inhibition in animal models (23-25). Collagen is not only a constitutional ingredient of the ECM, but it also plays a major role in carcinomic invasion (26, 27), indicating that growth of scirrhous gastric cancer may occur by the interaction of cancer cells, fibroblasts and the ECM. Thus, the down-regulation of HSP47, which regulates collagen synthesis, may be a key protein for the development of new therapeutic approaches for treatment of patients with scirrhous gastric cancer.

In conclusion, the findings of the present study indicated that fibroblasts are the predominant producers of stromal collagen and may play an important role in the development of particular stromal changes in scirrhous gastric cancer. Further studies are required to elucidate the clinical significance of HSP47 in the development of new diagnostic and therapeutic approaches to the treatment of scirrhous gastric cancer.

Acknowledgements

We thank the technicians at the Electron Microscopy Center of Kitasato University, Japan, for their help with this study.

References

1 Kikuchi S, Hiki Y, Tsutsumi O, Kobayashi N, Tsukamoto H, Shimao H, Sakakibara Y and Kakita A: Surgical outcome of curative resection in patients with Borrman type IV gastric carcinoma with particular reference to the extent of lymph node metastasis. Hepatogastroenterology *47*: 890-892, 2000.

2 Kitamura K, Beppu R and Anai H: Clinicopathological study of patient with Borrman type IV gastric carcinoma. J Surg Oncol *58*: 112-127, 1995.

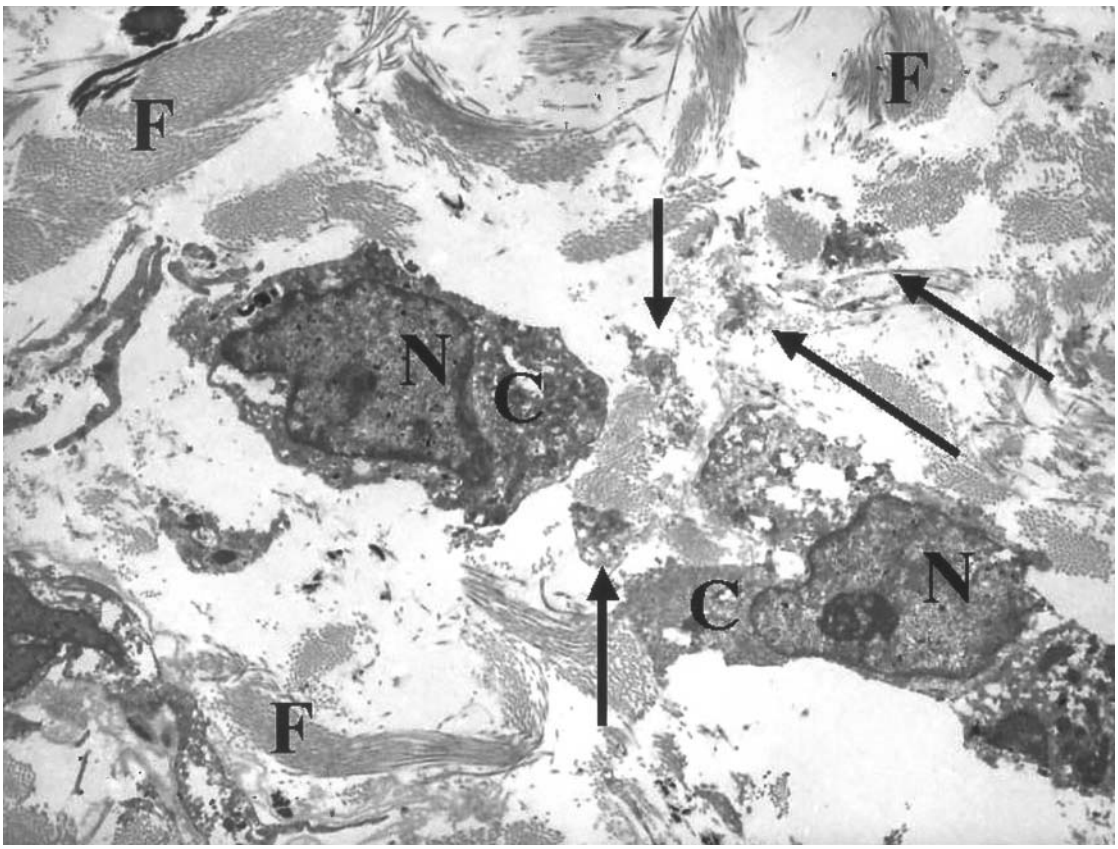

Figure 3. *Immunohistochemical staining in gastric cancer tissues for HSP47 (N: nucleus C: cytoplasm F: collagen fiber). The staining was observed not only in endoplasm reticula but also in the extracellular matrix. Cytoplasmic remnants that stained for HSP47 were found in the extracellular matrix (original magnification x15, 000, arrows). Membrane structures were not observed.*

3 Nagata K: Hsp47: a collagen specific molecular chaperone. Trends Biochem Sci *21*: 22-26, 1996.
4 Nagata K: Therapeutic strategy for fibrotic diseases by regulating the expression of collagen-specific molecular chaperone HSP47. Nippon Yakurigaku Zasshi *121(1)*: 4-14, 2003 review (in Japanese).
5 Iwashiota T, Kadota J, Naito S, Kaida H, Ishimathu H, Miyazaki M, Ozono Y and Kohno S: Involvement of collagen-binding heat shock protein 47 and procollagen type I synthesis in idiopathic pulmonary fibrosis: contribution of type II pneumocytes to fibrosis. Hum Pathol *31*: 1498-1505, 2000.
6 Kuroda K, Tsukifuji R and Shinkai H: Increased expression of heat-shock protein 47 is associated with overproduction of type I procollagen in systemic sclerosis skin fibrosis. J Invest Dermatol *111*: 1023-1028, 1998.
7 Razzaque MS Ahsan N and Taguchi T: Heat shock protein 47 in renal scaring. Nephron *86*: 339-341, 2000.
8 Naitoh M, Hosokawa N, Kubota H, Tanaka T, Shirane H, Sawada M, Nishimura Y and Nagata K: Upregulation of HSP47 and collagen type III in the dermal fibrotic disease, keloid. Biophy Res Commun *280*: 1316-1322, 2001.
9 Motoyama T, Hojo H and Watanabe H: Comparison of seven cell lines derived from human gastric carcinomas. Acta Pathol Jpn *36*: 65-83, 1986.
10 Kikuchi S, Sato T, Arai Y, Tsutsumi O, Hiki Y and Kakita A: An *in vitro* model of scirrhous carcinoma of the stomach using stomach fibroblasts derived from gastric cancer patients. Hepatogastroenterology *47*: 1256-1259, 2000.
11 Japanese Research Society for Gastric Cancer. Japanese Classification of Gastric Carcinoma. First English edition. Kanehara, Tokyo: Japanese Gastric Cancer Association, pp. 1-71, 1995.
12 Adachi E, Hayashi T and Hashimoto PH: Type V collagen in splenic reticular fibrosis of the Macaque monkey. Acta Anatomica *129*: 169-175, 1987.
13 Takeuchi T: Studies on fibro genesis in the tissue of scirrhous lesion of the stomach with special reference to collagen biochemistry. Stomach Intestine *11*: 1321-1326, 1976 (in Japanese with English abstract).
14 Sakakibara K, Suzuki T, Motoyama T, Watanabe H and Nagai Y: Biosynthesis of an interstitial type of collagen by cloned human gastric carcinoma cells. Cancer Res *42*: 2019-2027, 1982.

15 Niitsu Y, Ito N, Kohda K, Owada M, Morita K, Sato S, Watanabe N, Kohgo Y and Urushizaki I: Immunohistochemical identification of type I procollagen in tumor cells of scirrhous adenocarcinoma of the stomach. Br J Cancer *57*: 79-82, 1988.
16 Yoshi T: Concept and histogenesis of scirrhous carcinoma of stomach. Stomach Intestine *11*: 1297-1305, 1976 (in Japanese with English abstract).
17 Naito Y, Kino I, Horiuchi K and Fujimoto D: Promotion of collagen production by human fibroblasts with gastric cancer cells *in vitro*. Virchows Arch Cell Pathol *46*: 145-154, 1984.
18 Yamamoto M, Sumiyoshi H, Nakagami K, Taniyama K and Tahara E: Distribution of collagen type I and III and basal lamina in human gastric carcinoma: an immunohistochemical and electron microscopic study. Virchows Arch *403*: 313-322, 1984.
19 Matsui H, Kubochi K, Okazaki I, Yoshino K, Ichibiki K and Kitajima M: Collagen biosynthesis in gastric cancer: immunohistochemical analysis of prolyl 4-hydroxylase. J Surg Oncol *70*: 239-246, 1999.
20 Matsui H, Kubochi K, Shimada A and Hasumi A: Desmoplastic response in scirrhous gastric carcinoma: imbalance between collagen synthesis and degradation. Anticancer Res *20*: 4733-4738, 2000.
21 Kurita A, Kikuchi S and Kakita A: Immunohistochemical study of collagen synthesis in an *in vitro* model of scirrhous carcinoma of the stomach. Hepatogastroenterology *49*: 1235-1238, 2002.
22 Yokota S, Kubota H, Matsuoka Y, Naitoh M, Hirata D, Minota S, Takahashi H, Fujii N and Nagata K: Prevalence of HSP47 antigen and autoantibodies to HSP47 in the sera of patients with mixed connective tissue disease. Biochem Biophys Res Comm *303*: 413-418, 2003.
23 Hattori T, Kawaki H, Kubota S, Yutani Y, Benoit de Crombrugghe B, von der Mark K and Takigawa M: Downregulation of a rheumatoid arthritis-related antigen (RA-A47) by ra-a47 antisense oligonucleotides induces inflammatory factors in chondrocytes. J Cell Physiol *197*: 94-102, 2003.
24 Nishino T, Miyazaki M, Abe K, Furusu A, Mishima Y, Harada T, Ozono Y, Koji T and Kohno S: Antisense oligonucleotides against collagen-binding stress protein HSP47 suppress peritoneal fibrosis in rats. Kidney Int *64*: 887-896, 2003.
25 Ohba S, Wang ZL, Baba TT, Nemoto TK and Inokuchi T: Antisense oligonucleotide against 47-kDa heat shock protein (Hsp47) inhibits wound-induced enhancement of collagen production. Arch Oral Biol *48*: 627-633, 2003.
26 Matsui H and Hasumi A: Stromal changes in linitis plastica (scirrhous gastric carcinoma). Connect Tissue *32*: 345-320, 2000.
27 Liotta L and Kohn E: The microenvironment of the tumor host interface. Nature *411*: 375-379, 2001.

Received August 16, 2005
Accepted October 6, 2005