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Cytokeratin-positive subserosal myofibroblasts in gastroduodenal ulcer; another type of myofibroblasts

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Summary. To investigate the distribution and origin of alpha-smooth muscle actin (ASMA)-positive stromal cells in the perforation of human gastroduodenal ulcers. Perforative lesions of 24 surgically resected gastroduodenal ulcers were examined immunohistochemically for ASMA, HCD, CD34, CD31, CAM5.2 and HMW-CK, and double staining of ASMA and CAM5.2 was also performed. In addition, to determine the cell source of collagen, *in situ* hybridization of collagen I mRNA was performed. In the normal gastroduodenal wall, the reticular network of CD34-positive stromal cells was identified in the muscularis mucosa, submucosa, muscular propria, and subserosa. In the subepithelial area, many myofibroblasts were observed, whereas no CD34-positive stromal cells were seen. In areas neighboring ulcerative lesions, no CD34-positive stromal cells were observed, but a significant number of myofibroblasts were present there. In the deep layer of ulceration, numerous fusiform or stellate stromal cells strongly positive for ASMA and CAM5.2 were observed in the subserosal area around the perforation. In the same site, many cells co-expressing ASMA and CAM5.2 were identified by double staining. In contrast, in the surface layer of ulceration, stromal cells expressing only ASMA were observed. The cytokeratin-positive subserosal myofibroblastic cell in human gastroduodenal ulcer is a novel type of myofibroblast.

Key words: Myofibroblast, Subserosal cell, Cytokeratin, Peptic ulcer

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Introduction

Myofibroblasts, which share biochemical and structural features with smooth muscle cells and fibroblasts, have been described in almost all human pathologies including tissue fibrosis (Powell et al., 1999a). In the normal gastric and duodenal walls, many myofibroblasts are distributed in the subepithelial area (Powell et al., 1999a,b). Some investigators have suggested that myofibroblasts in the stomach and intestine may be derived from the interstitial cell of Cajal or subepithelial myofibroblast, and that they perform a specific local function (Powell et al., 1999a). Nakayama et al. (2000, 2001, 2002) have observed abundant myofibroblasts in the stroma of gastric carcinomas, and have observed immature stromal cells expressing CD34 in the stroma of diffuse-type gastric cancer. On the other hand, the properties and functions of the subserosal cells in injury and regeneration are still controversial. Recent studies suggest that these cells, rather than fibroblasts, are multipotential mesenchymal cells. When proliferating in response to some forms of stimulation, these subserosal cells co-express low molecular weight cytokeratin including CAM5.2 as well as vimentin (Bolen et al., 1986, 1987). However, the relationship between myofibroblasts and immature or multipotential mesenchymal cells remains unclear. In the present study, we assayed for the presence of myofibroblasts in tissue surrounding ulceration lesions of the upper digestive tract and changes in subserosal cells around the perforation of peptic ulcer. We discuss the origin of myofibroblasts present in the lesion of peptic ulcer.

Materials and methods

We examined normal stomach and duodenum tissue (n=3) and 24 gastric (n=13) and duodenal (n=11) ulcers that were surgically resected due to perforation, from

1990 to 2004, using the surgical pathology files of the Department of Pathology, Kochi Medical School, Japan. The general histological appearance of normal and perforation tissues was assessed after routine hematoxylin and eosin staining.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin-biotin immunoperoxidase (SAB) method. Briefly, each specimen was evaluated using monoclonal antibodies against alpha-smooth muscle actin (ASMA), high-molecular-weight caldesmon (HCD), CD34, CD31, cytokeratin 8 (CAM5.2), and high-molecular-weight cytokeratin (HMW-CK) (Table 1). As internal positive controls of immunostaining, we used vascular smooth muscle cells (ASMA and HCD) and endothelial cells (CD34 and CD31). We classified ASMA+ and HCD-, CD34-, CD31-, CAM5.2- and HMW-CK- stromal cells as myofibroblasts (Nakayama et al., 2000, 2001, 2002). CD34+ and CD31-, ASMA-, HCD-, CAM5.2- and HMW-CK- stromal cells were considered to represent CD34+ stromal cells (Nakayama et al., 2002).

Double immunostaining

Double staining for CAM5.2/ASMA, and CD34/ASMA was performed to investigate the relationship between subserosal cells and ASMA-positive myofibroblastic cells in each specimen. Sections were treated with 0.3% hydrogen peroxide/methanol for 10 min at room temperature (RT) and incubated overnight at 4°C with anti-CD34 or anti-CAM5.2 antibodies. Then, the sections were incubated with peroxidase-conjugated mouse IgG and rabbit IgG [Simple stain PO-MAX (multi), Nichirei, Japan] for 1h at RT and immersed in 0.2% DAB and 0.1% hydrogen peroxide in 0.05M Tris buffer (pH 8.0). After washing with PBS, both sections were incubated for 1h at RT with anti-ASMA antibody. Then, they were incubated with biotinylated rabbit anti-mouse IgG $F(ab')_2$ fragment (DAKOpatts, Denmark) for 1h, and then with alkaline phosphatase-conjugated streptavidin for 30 min at RT. To visualize the color reaction, sections were stained with Fast blue.

The staining number of ASMA, CD34 and CAM5.2 by stromal cells was evaluated as follows: -, none; +, <25%; ++, 25-50%; +++, >50% positive cells. The expression of CD31, HCD and HMW-CK was assessed as positive or negative without further quantification.

Probe of human collagen I

Total RNA was extracted from a normal renal capsule obtained from one autopsy case using RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, 1µg of total RNA from each specimen was reverse transcribed, to produce the cDNA for PCR. The upper primer was AGTGGTTACT ACTGGATTGACC, and the lower primer was TTGCCAGTCTCCTCATCC. For PCR, 2 µl of cDNA was used, and the reaction mixture also contained 0.2 mM of each primer, 400 µM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 0.5 unit Taq DNA polymerase (TaKaRa, Japan). First, the cDNA was denatured at 95°C for 3 min. Then, PCR was performed with 30 cycles of 94°C for 30 seconds, 58°C for 1.5 min, and 72°C for 1 minute. Next, the 352-bp fragment of the human collagen I gene produced by RT-PCR was subcloned into pGEM-T easy vector (Promega, USA). This ligation was then transformed and extracted using Miniprep kit (Qiagen, USA). The sequence was linearized by digestion with NheI or SphI. Sense and antisense digoxigenin-labeled cRNA probes of collagen I, produced by *in vitro* transcription assay with SP6 and T7 polymerase (Roche, Germany), were used for *in situ* hybridization.

In situ hybridization

Serial formalin-fixed paraffin-embedded sections (thickness, 3 µm) were used for *in situ* hybridization, as previously described (Zhang et al., 1994). Briefly, sections were permeabilized with 15mg/ml of proteinase K at 37°C for 30 min, and were then post-fixed in 4% paraformaldehyde/PBS at RT for 15 min. Prehybridization with Dig Easy Hyb buffer (Roche,

Table 1. Antibodies used for immunohistochemical analysis.

ANTIBODY (CLONE)	SPECIFICITY	SOURCE	WORKING DILUTION (ANTIGEN RETRIEVAL)
1A4	ASMA	DAKO, USA	1/50 (none)
h-CD	HCD	DAKO, USA	1/50 (microwave)
MY10	CD34	Becton-Dickinson, USA	1/25 (pronase)
JC/70A	CD31	DAKO, Denmark	1/25 (pronase)
CAM5.2	CK 8	Becton-Dickinson, USA	Ready for use (pronase)
34bE12	HMW-CK	Enzo Life Sciences, USA	1/50 (pronase)

ASMA, alpha-smooth muscle actin; HCD, high-molecular-weight caldesmon; HMW-CK, high-molecular-weight cytokeratin.

Germany) was performed at 65 for 2hr, followed by hybridization at 65°C for 10hr in Hyb buffer containing 350ng/ml of digoxigenin-labeled probe. Sections were then stringently washed with decreasing concentrations of standard saline citrate buffer at 60°C for 1.5hr, followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody for 2hr at RT, and color reaction with NBT/BCIP (Roche, Germany).

Results

Distribution of CD34+ stromal cells, myofibroblasts, and cytokeratin- stromal cells in the normal stomach and duodenum

As we reported, in the normal gastric and duodenal wall, the reticular network of CD34+ stromal cells was identified in the muscularis mucosa, submucosa, muscular propria, and subserosa (Nakayama et al., 2000, 2001, 2002). In the subepithelial area, many myofibroblasts were observed, but no CD34+ stromal cells were seen. Immediately beneath the serosal cells of gastroduodenum, subserosal cells showed negative immunoreaction of both CAM5.2 and HMW-CK.

Distribution of CD34+, ASMA+ or CAM5.2+ stromal cells in gastric and duodenal ulcers

No CD34+ stromal cells were seen within the stroma of the ulceration tissue of stomach and duodenum (Fig. 1a). However, many ASMA+ stromal cells were observed in the layer beneath the necrosis (Fig. 1b). These cells were negative for HCD, and we classified them as myofibroblasts. In the deep layer of ulceration, numerous fusiform or stellate-shaped stromal cells were strongly positive for CAM5.2 (Fig. 1c), but negative for HMW-CK, HCD and CD34. These cells were predominantly observed in the subserosal area around the perforation.

As internal positive controls of immunostaining, vascular smooth muscle cells showed positive staining of ASMA and HCD, and endothelial cells showed positive staining of CD34 and CD31.

Distribution of stromal cells by double immunostaining

In the surface layer of peptic ulcers, just beneath the necrosis (Fig. 2a), none of the stromal cells expressing ASMA also expressed CAM5.2 (Fig. 2b). However, in the deep layer adjacent to the perforation of gastric and duodenal ulcers, fusiform or stellate-shaped cells expressing CAM5.2 and ASMA were observed (Fig. 2c,d). We did not observe stromal cells co-expressing CD34 and ASMA in the normal gastroduodenal wall or around the perforation of the ulcerative lesion.

The distribution and staining number of ASMA+, CD34+ and CAM5.2+ stromal cells in normal gastroduodenum and perforated gastroduodenal ulcers are summarized in Table 2.

In situ hybridization of type I collagen

Myofibroblasts in the surface layer of peptic ulcer and fusiform or stellate-shaped cells in the deep layer exhibited strong signals of expression of type I collagen mRNA in the cytoplasm (Fig. 3). These signals tended to be more intense at sites with very dense collagen bundles.

Discussion

In the present study, we found two kinds of ASMA-positive stromal cells in the wall of gastric and duodenal ulcers. In the deep layer surrounding the perforation of peptic ulcer, ASMA-positive stromal cells co-express CAM5.2, whereas ASMA-positive stromal cells in the surface layer of peptic ulcer do not express CAM5.2.

It is important for pathologists to recognize the existence of CAM5.2 positive spindle or stellate cells in the deep layer of gastrointestinal ulcers to avoid misdiagnoses such as smooth muscle tumor, sarcomatoid carcinoma or other malignancies for biopsed and resected specimens.

Previously, Pitt and Habouri (1995) reported the existence of CAM5.2 positive spindle cells in the deep layer of chronic gastric ulcer. They found that these cells ultrastructurally had a myofibroblastic nature. Similar results were observed in the perforated ulcer samples from the upper digestive tract. In the present study, ASMA-positive stromal cells that co-expressed CAM5.2 in the deep layer around the perforation were negative for HCD. It is well known that HCD is a representative marker of smooth muscle cells (Sobue et al., 1981; Ueki et al., 1987). Therefore, these cells have no characteristics of smooth muscle cells. Additionally, by *in situ* hybridization analysis, we found that these

Table 2. Distribution of staining number of ASMA+, CD34+ and CAM5.2+ stromal cells in normal gastroduodenum and perforated gastroduodenal ulcer.

	CD34+ (fibroblast)	ASMA+/HCD- (myofibroblast)	CAM5.2+ (subserosal cell)
Normal			
Stomach (n=13)	+ (mm, sm, mp, ss)	+ (sp)	-
Duodenum (n=11)	+ (mm, sm, mp, ss)	+ (sp)	-
Perforation			
Stomach (n=13)			
surface layer	-	+~++	-
deep layer	-	++~+++*	++~+++*
Duodenum (n=11)			
surface layer	-	+~++	-
deep layer	-	++~+++*	++~+++*

mm: muscularis mucosa; sm, submucosa; mp, muscularis propria; ss, subserosa; sp, subepithelium. Evaluation of staining number of stromal cells was as follows: -, none; +, <25%; ++, 25-50%; +++, >50% positive stromal cells. *, Most of the proliferating stromal cells co-express ASMA and CAM5.2.

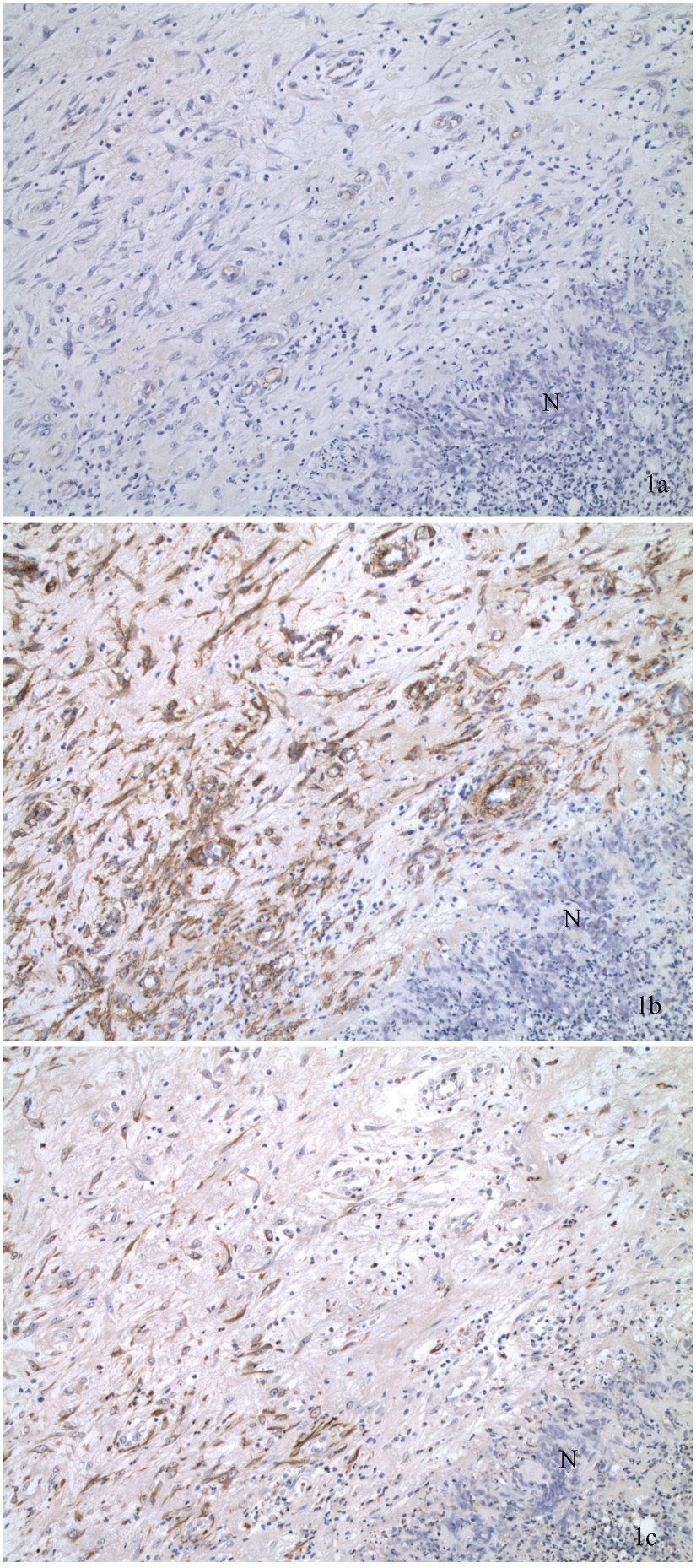

Fig. 1. Immunohistochemical staining for CD34, ASMA and CAM5.2 in peptic ulcerative lesions. **a.** No CD34+ stromal cells are observed within the ulcerative lesion. x 100. **b.** Many ASMA+ stromal cells are seen in the layer beneath the necrosis. x 100. **c.** In the deep layer of ulceration, numerous fusiform or stellate-shaped stromal cells exhibit strong immunoreaction for CAM5.2 in the cytoplasm. x 100. Fig. a-c show the same site; N: necrosis.

ASMA-positive stromal cells have the ability to produce collagen type I. Activated myofibroblasts have the ability to produce various collagens including collagens type I, III and IV (Schurch et al., 1998; Eyden, 2001). In the liver and pancreas, activated hepatic and pancreatic stellate cells express ASMA and produce collagen after acquisition of the "myofibroblastic" phenotype (Apte et al., 1999; Enzan et al., 1999). Given these findings, we speculate that these CAM5.2 positive stromal cells have a myofibroblastic nature in both morphological and functional aspects.

Regarding the origin of CAM5.2 positive stromal

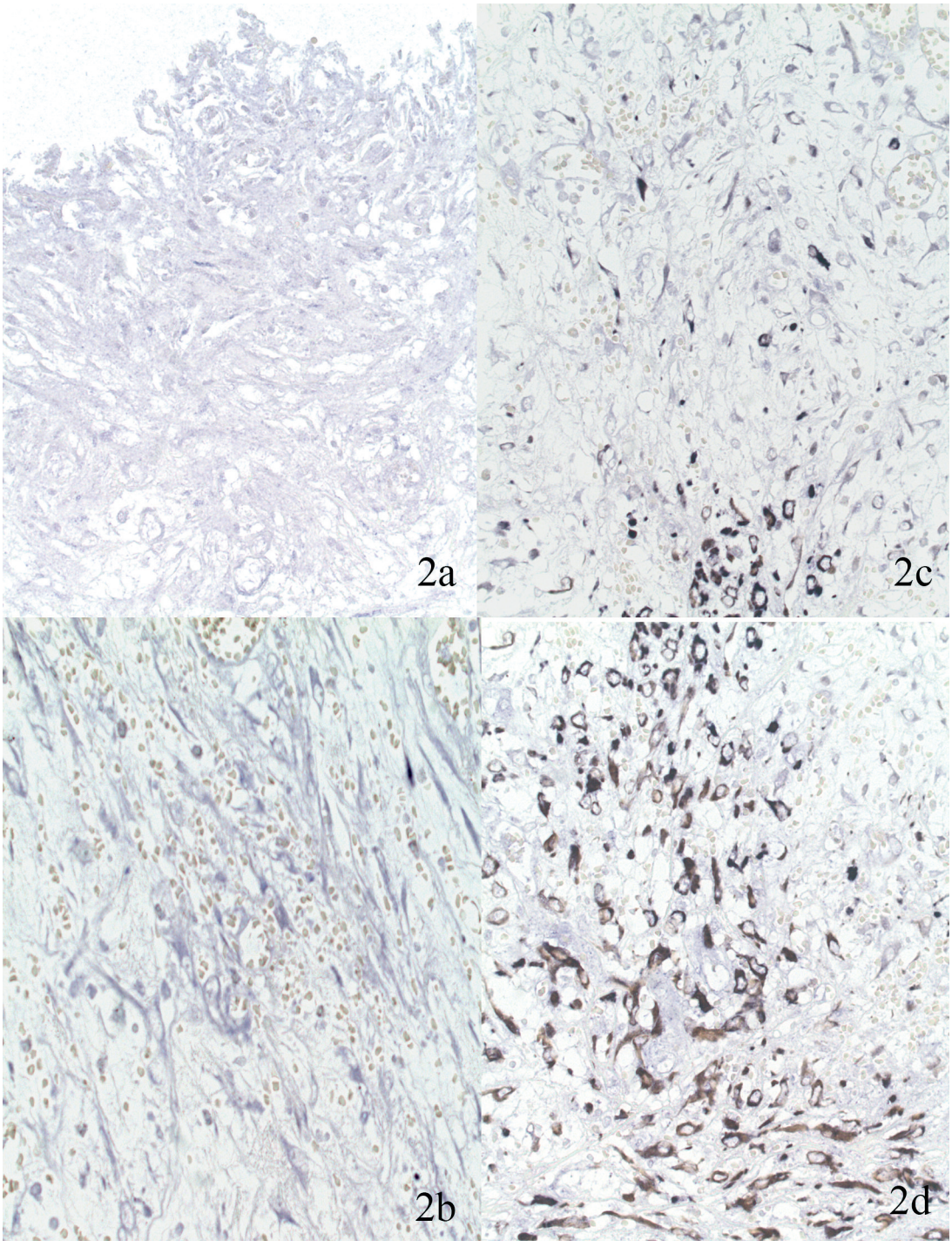

Fig. 2. Double staining of CAM5.2 (brown) and ASMA (blue) in the peptic ulcerative lesions. Beneath the necrosis **(a)**, stromal cells in the surface layer express ASMA only **(b)**. In the deep layer adjacent to the perforation of ulcers, fusiform or stellate-shaped cells co-expressing CAM5.2 and ASMA were observed **(c, d)**. x 200. Figures a, b, c and d show the typical findings in one section.

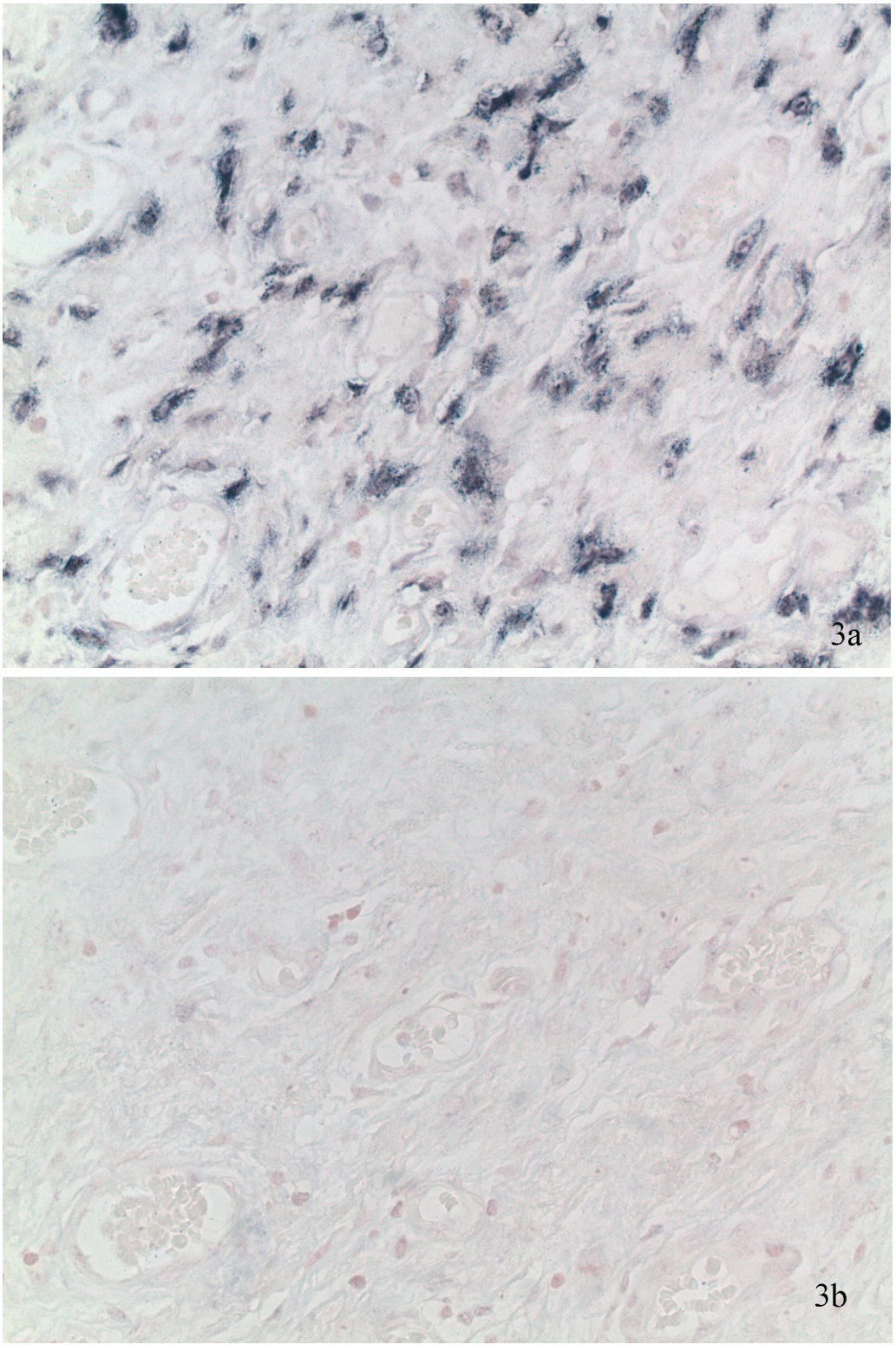

Fig. 3. *In situ* hybridization of type I collagen in the subserosal area of the perforation of peptic ulcer. **a.** With the antisense probe, fusiform or stellate-shaped cells (myofibroblastic cells) express signals of collagen I mRNA in the cytoplasm. x 200. **b.** No specific signal is detected using sense probe in a serial section. x 200

cells, there is at least one possibility of subserosal cells. Pitt and Haboubi (1995) suggested that multipotential subserosal cells which may differentiate into surface serosa become myofibroblastic. On the other hand, Yang et al. (2003), Yanez-Mo et al. (2003) and Jimenez-Heffernan et al. (2004) suggested that these cells arise from local conversion of mesothelial cells undergoing epithelial-to-mesenchymal transition during serosal inflammation and wound healing peritoneal dialysis. Furthermore, Yang et al. (2003) reported that TGF-ß induced morphological and functional reformation in differentiated human mesothelial cells. Jimenez-Heffernan et al. (2004) suggested that the loss of CD34 expression in peritoneal fibroblasts correlated with the degree of peritoneal fibrosis and myofibroblasts seemed to derive from both activation of resident fibroblasts and local conversion of mesothelial cells. Thus, it is still debatable whether myofibroblasts around the serosal area originate from serosal cells or subserosal cells. Mutasaers et al. (2000) suggested that regenerating mesothelium more likely originated from the surrounding uninjured mesothelial cell population in murine model of testicular mesothelial healing. However, normal surface mesothelium expresses low- and high-molecular weight cytokeratins while the underlying, fibroblast-like submesothelial cells express only vimentin when at rest. When proliferating, these submesothelial cells co-express low-molecular-weight cytokeratin as well as vimentin (Bolen et al., 2002). In the present study, ASMA-positive stellate-shaped or spindle cells in the subserosal area of peptic ulcer showed no reaction with HMW-CK. Consistent with the present findings, the existence of subserosal stromal cells co-expressing ASMA and CAM5.2 in the gastric wall has also been suggested by Daum et al. (2004) as the progenitor cell of fibrous pseudotumors of the gastrointestinal tract. In their study, tumor cells with the features of myofibroblasts are proposed to result from a proliferation of multipotential subserosal cells rather than ordinary myofibroblasts or fibroblasts. Therefore, it seems to be more likely that the stromal cells co-expressing CAM5.2 and ASMA in the subserosal area of peptic ulcer originate from subserosal cells rather than serosal cells.

Also, we speculate that in the surface layer of peptic ulcer, myofibroblasts that do not express CAM5.2 originate from non-subserosal cells such as subepithelial myofibroblasts or CD34+ stromal cells. Barth et al. (2002) suggested that myofibroblasts and CD34+ stromal cells have a common origin in the stroma of the uterine cervix, and found that mesenchymal cells exhibit up-regulation of ASMA and down-regulation of CD34 in the malignant stroma. Although in the present study we found no stromal cells co-expressing CD34 and ASMA, it is possible that such a mechanism also occurs around the perforation of peptic ulcers. Therefore, given these previous findings and our present results, we speculate that myofibroblasts may originate from fibroblasts, probably expressing CD34 antigen, in the surface layer and subserosal cells in the deep layer. Further study is needed to elucidate the relationship between CD34+ stromal cells and myofibroblasts, and the characteristics of cells expressing both ASMA and CAM5.2 in the subserosal area.

In conclusion, there are different kinds of ASMA positive stromal cells in the perforation of peptic ulcer. They may originate from non-subserosal cells (e.g., CD34 positive stromal cells or subepithelial myofibroblasts) and subserosal cells, in the superficial and deep layers of ulceration lesion, respectively. In the deep layer of the perforation of peptic ulcer, activated subserosal cells are another myofibroblastic phenotype. They proliferate, undergo "myofibroblastic" transformation, and acquire the ability to produce collagen fibers. This phenomenon may play an important role in tissue repair in the perforation of peptic ulcer.

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