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The appearance of myofibroblasts and the disappearance of CD34-positive stromal cells in the area adjacent to xanthogranulomatous foci of chronic cholecystitis

N. Kuroda[1], L. Guo[2], E. Miyazaki[1], T. Hamauzu[1], M. Toi[1], M. Hiroi[1] and H. Enzan[1]

[1]Department of Pathology, Program of Bioregulation and Genetics, Kochi Medical School, Kochi University, Kochi, Japan and
[2]Department of Pathology, Health Science Center, Peking University, Beijing, China

Summary. We investigated the distribution of myofibroblasts and CD34-positive stromal cells in normal gallbladder and its pathological conditions (cholecystitis, n=25) using immunohistochemistry and *in situ* hybridization. In the wall of normal gallbladder, myofibroblasts were generally absent from all layers, but many CD34-positive stromal cells were observed in the connective tissue layer. In chronic cholecystitis with mild perimuscular fibrosis, a small to moderate number of myofibroblasts appeared in the mucosal layer. In chronic cholecystitis with marked perimuscular fibrosis, a small to large number of myofibroblasts appeared predominantly in the connective tissue layer, whereas the number of CD34-positive stromal cells decreased at the same location, although the number of myofibroblasts increased. In chronic cholecystitis with xanthogranulomatous foci, a small to large number of myofibroblasts were observed in the periphery of the xanthogranulomatous reaction and adjacent area. In contrast, CD34-positive stromal cells were completely absent or were limited to the area just around the xanthogranulomatous reaction. Induction of collagen type I and III mRNA was predominantly observed in the cytoplasm of myofibroblasts associated with the marked fibrosis, which consisted primarily of mature collagen fibers, and in the cytoplasm of myofibroblasts around the xanthogranulomatous reaction, respectively. Finally, myofibroblasts were observed in all subtypes. The increased number of myofibroblasts was most prominent in the connective tissue layer of chronic cholecystitis with marked perimuscular fibrosis or in the area adjacent to xanthogranulomatous foci of chronic cholecystitis. Under these conditions, CD34-positive stromal cells tended to disappear from the connective tissue layer, which exhibited an increase in myofibroblasts.

Offprint requests to: Dr Naoto Kuroda, Department of Pathology, Program of Bioregulation and Genetics, Kochi Medical School, Kochi University, Kohasu, Oko-cho, Nankoku City, Kochi 783-8505, Japan. FAX: +81-88-880-2332. e-mail: nkuroda@med.kochi-u.ac.jp

Key words: Chronic cholecystitis, Myofibroblast, Xanthogranulomatous foci, CD34-positive stromal cell

Introduction

Myofibroblasts are representative stromal cells located in various normal organs including the digestive tract, pancreas and Fallopian tubes (Nakayama et al., 2000b; Barth et al., 2002a; Kuroda et al., 2004a,b). CD34 is a 110-kDa transmembrane cell-surface glycoprotein that was originally identified as a marker for human hematopoietic cells (Van de Rijn and Rouse, 1994). The presence of CD34-positive stromal cells, including vascular adventitial fibroblastic cells or dendritic interstitial cells, has been reported under some pathological conditions as well as in various normal organs (Yamazaki and Eyden, 1995, 1996a,b, 1997; Nakayama et al., 1999, 2000a, 2003, 2004; Papadas et al., 2001; Soma et al., 2001; Barth et al., 2002a-c; Kuroda et al., 2004a-c). However, the distribution of myofibroblasts and CD34-positive stromal cells in normal gallbladder or its pathological states has not been previously described. In order to understand the stromal reaction in gallbladder, we investigated the distribution of myofibroblasts and CD34-positive stromal cells using an immunohistochemical method and we focused primarily on cases with chronic cholecystitis. Additionally, the *in situ* hybridization technique was employed in order to identify collagen-producing cells in the stroma of chronic cholecystitis specimens.

Materials and methods

Tissue specimens

Twenty-five gallbladder specimens, obtained between 1996 and 2004 by cholecystectomy, were selected from the surgical pathology files of the Department of Pathology, Kochi Medical School, Kochi

University and an affiliated hospital. In the present article, we focused on cases of chronic cholecystitis with mild perimuscular fibrosis (microscopical fibrosis) (n=5), chronic cholecystitis with marked perimusuclar fibrosis (macroscopical fibrosis) (n=4), and chronic cholecystitis with xanthogranulomatous foci (n=16). Normal specimens (n=5) from some cases with mild inflammation and without fibrosis were used for comparison with the pathological condition. The sex ratio (male: female) of the patients was 15:10. The mean age and age range of the patients were 67.8 years and 33 to 90 years, respectively. Specimens of gallbladder obtained by cholecystectomy were fixed in 10% neutral formalin and embedded in paraffin. Sections were cut (thickness, 3 μm) and routinely stained with hematoxylin-eosin.

Immunohistochemistry and its interpretation

Immunohistochemistry was performed by using a streptavidin-biotin immunoperoxidase technique. Antibodies used in this study are summarized in Table 1. Vascular smooth muscle cells and endothelial cells were used as internal positive controls for alpha-smooth muscle actin (ASMA) and high molecular weight caldesmon (h-CD), and CD34 and CD31 immunostains, respectively.

High molecular weight caldesmon (h-CD) is a well-developed smooth muscle actin-specific antibody and is not generally expressed in myofibroblasts (Ueki et al., 1987; Ceballos et al., 2000; Watanabe et al., 2000; Rush et al., 2001). Therefore, we classified stromal cells positive for both ASMA and h-CD as smooth muscle cells, and ASMA-positive and h-CD-negative cells as myofibroblasts. Additionally, we considered stromal cells positive for both CD34 and CD31 as vascular endothelial cells and CD34-positive and CD31-negative stromal cells as "true" CD34-positive stromal cells.

Preparation of human collagen type I and III probes

Total RNA was extracted from normal renal capsules obtained from one autopsy case for purification of a collagen type I probe and from renal cell carcinoma obtained from one surgically-resected case for purification of a collagen type III probe using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 1 mg of total RNA from each specimen was reverse-transcribed and cDNA was obtained for PCR. For human collagen I, the forward primer AGTGGTTACTACTGGATTGACC and the reverse primer TTGCCAGTCTCCTCATCC were used. For human collagen III, the forward primer TTGACCCTAACCAAGGATGC and the reverse primer CACCTTCATTTGACCCCATC were used. The PCR reaction mixture contained 2 ml of cDNA, 0.2 mM of each primer, 400 mM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). The mixture was denatured at 95 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 58 °C for 1.5 minutes, and 72 ºC for 1 minute. Subsequently, the 352-bp and 360-bp human collagen I and III fragments produced by RT-PCR were subcloned into pGEM-T easy vector (Promega, WI, USA). The ligation was transformed and the plasmids were extracted using the Midiprep kit (Qiagen, Hilden, Germany). The sequence was linearized by digestion with NheI or SphI. Sense and antisense cRNA probes for collagens I and III, produced by labeling with digoxigenin through in vitro transcription with SP6 and T7 polymerase (Roche, Tokyo, Japan), were used for *in situ* hybridization.

In situ hybridization

Formalin-fixed paraffin-embedded sections (3 μm) from three cases were used for *in situ* hybridization to detect collagen I and III production in the stroma of chronic cholecystitis with xanthogranulomatous foci, as previously indicated (Zhang et al., 1994). Briefly, sections were permeabilized with 15 mg/ml of proteinase K at 37 °C for 30 min and then post-fixed in 4% paraformaldehyde/PBS at room temperature for 10 min. Prehybridization with Dig Easy Hyb buffer (Roche, Germany) was performed at 58 °C for 2 hr followed by hybridization at 58 °C for 14 hr in Hyb buffer containing 1 mg/ml of digoxigenin-labeled probe. Sections were then stringently washed with decreasing concentrations of standard saline citrate buffer at 53 °C for 1.5 hr, incubated with peroxidase-conjugated anti-digoxigenin antibody and color-reacted with NBT/BCIP (Roche, Germany).

Table 1. Antibodies employed in the present study.

ANTIGEN	CLONE	DILUTION	SOURCE	TREATMENT
ASMA	1A4	1:50	Dako Cytomation, Glostrup, Denmark	-
h-Caldesmon	h-CD	1:50	Dako Cytomation, Glostrup, Denmark	microwave
CD34	MY10	1:20	Beckton-Dickinson, San Jose, CA, USA	-
CD31	JC/70A	1:20	Dako Cytomation, Glostrup, Denmark	pronase

ASMA, alpha-smooth muscle actin.

Results

Distribution of CD34-positive stromal cells and myofibroblasts in normal gallbladder and chronic cholecystitis

Immunohistochemical results are summarized in Table 2. Myofibroblasts were not generally observed in any layer of the normal gallbladder wall (Fig. 1a). However, many CD34-positive stromal cells were identified in the reticular network of the connective tissue layer (Fig. 1b). In chronic cholecystitis with mild perimuscular fibrosis, a small to moderate number of myofibroblasts was restricted to the mucosal layer (Fig. 2a). As was observed under normal conditions, many CD34-positve stromal cells were present in the connective tissue layer (Fig. 2b). In chronic cholecystitis with marked perimuscular fibrosis, few to many myofibroblasts were observed predominantly in the connective tissue layer (Fig. 2c). Additionally, a small number of myofibroblasts was present in the mucosal and muscular layers in some cases. In cases with Rokitansky-Aschoff sinus, a small to moderate number of myofibroblasts was observed around the sinus (Fig. 2d). In some cases, a reticular network of myofibroblasts was identified in the connective layer (Fig. 2e). The number of CD34-positive stromal cells decreased with the increasing abundance of myofibroblasts in the connective tissue layer (Fig. 2f). In chronic cholecystitis with xanthogranulomatous foci, a small to large number of myofibroblasts was observed in the periphery of the xanthogranulomatous reaction and adjacent areas (Fig. 3a). Additionally, myofibroblasts were seen in the center of the loose xanthogranulomatous region in some cases (Fig. 3b). In contrast, CD34-positive stromal cells were completely absent or were limited to the area around the xanthogranulomatous reaction (Fig. 3c).

In situ hybridization findings

The anti-sense probe detected collagen type I mRNA induction predominantly in the area with abundant mature collagen fibers, distant from the xantho-

Table 2. Distribution of stromal cells in the wall of normal gallbladder and various chronic cholecystitis.

	MYOFIBRO-BLASTS	CD34-POSITIVE STROMAL CELLS
Normal gallbladder		
mucosal layer	-	-
muscular layer	-	-
connective tissue layer	-	++~+++
Chronic cholecysitis		
with mild perimuscular fibrosis (n=5)		
mucosal layer	+~++	-
muscular layer	-	-
connective tissue layer	-	++~+++
with marked perimuscular fibrosis (n=4)		
mucosal layer	-~+	-
muscular layer	-~+	-
connective tissue layer	+~+++	+-~+
with xanthogranulomatous foci (n=16)		
mucosal layer	+~+++	-
muscular layer	+~+++	-
connective tissue layer	+~+++	-

-, negative; +-, trivial; +, a small number, positive; ++, a moderate number, positive; +++, a large number, positive.

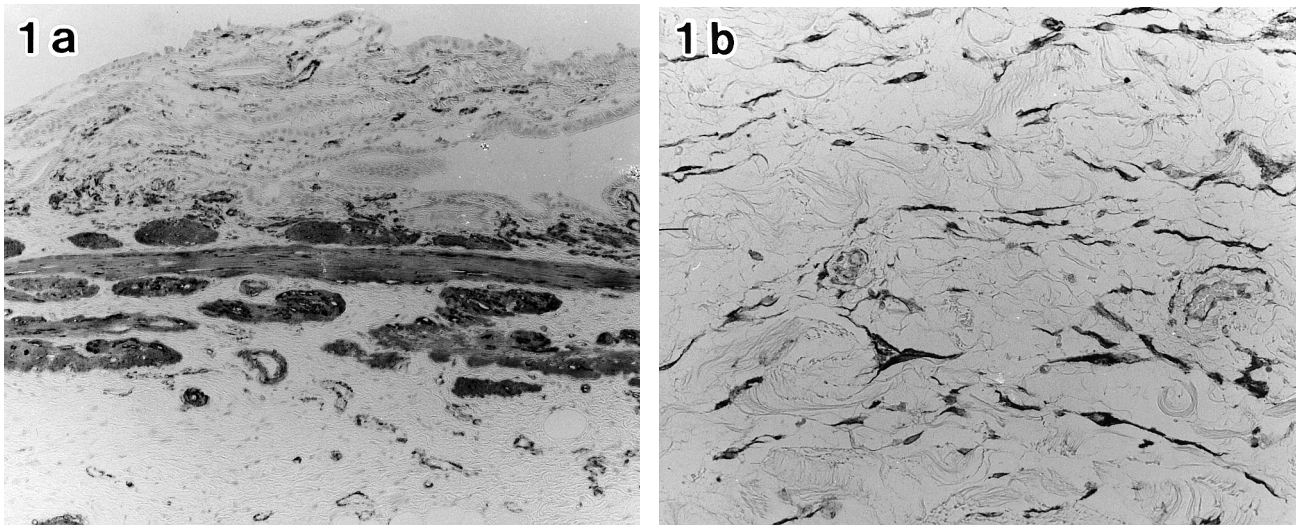


Fig. 1. Immunohistochemical observations of normal gallbladder wall. **a.** Myofibroblasts are absent in all layers. x 25. **b.** Many CD34-positive stromal cells are observed in the connective tissue layer with a reticular network. x 50

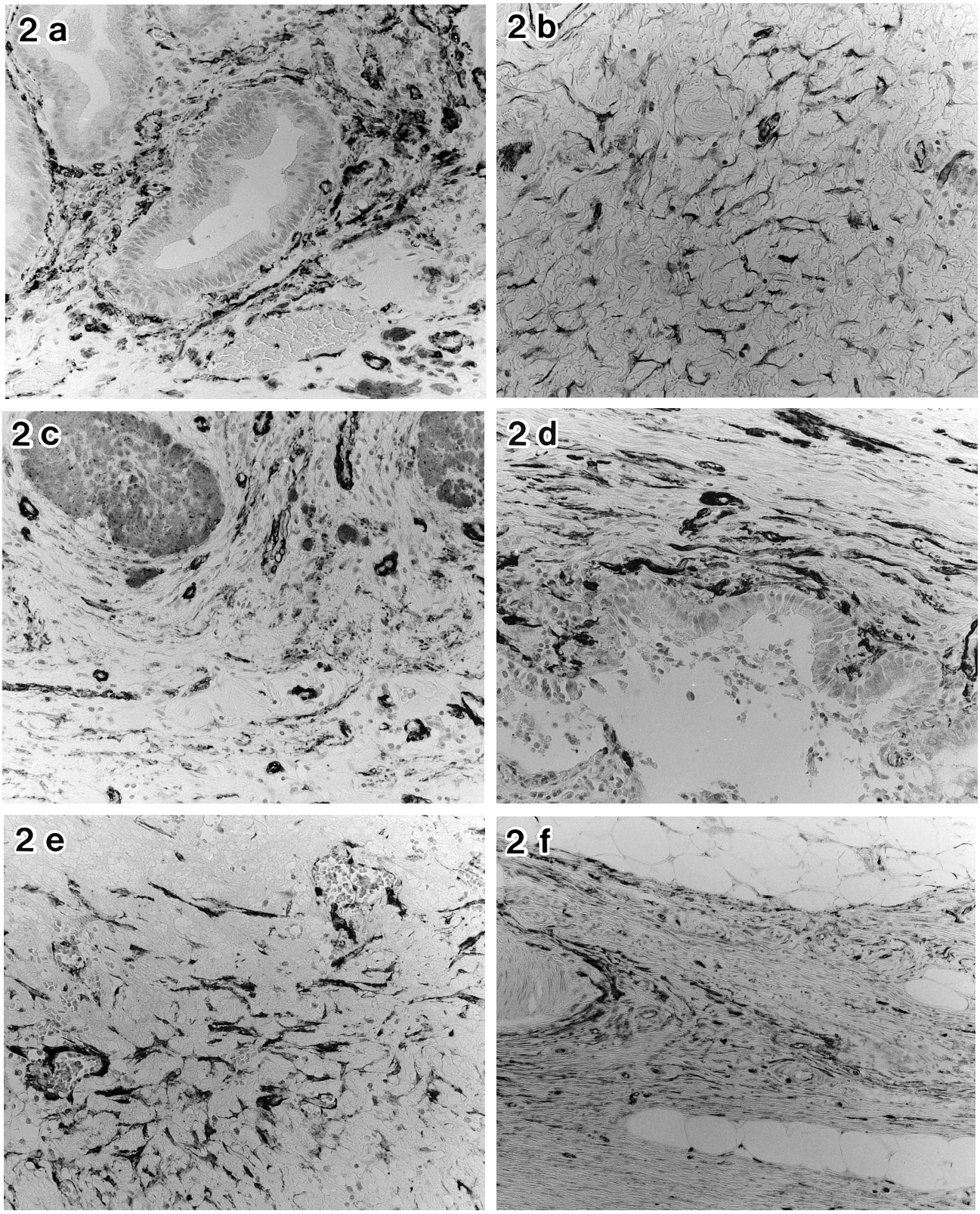
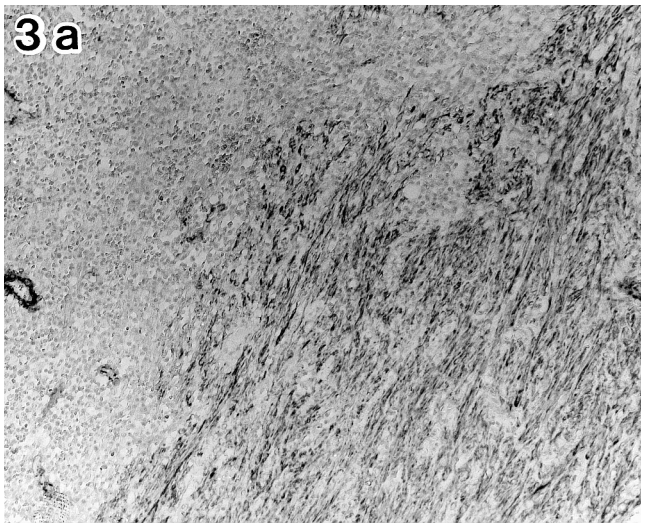
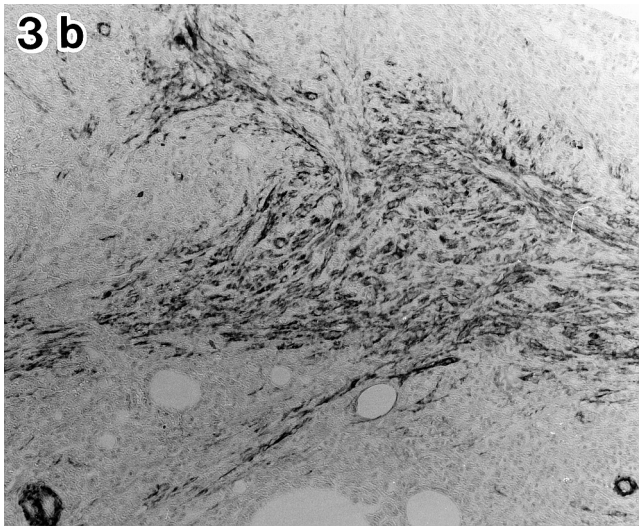


Fig. 2. Immunohistochemical results. **a, b.** Chronic cholecystitis with mild perimuscular fibrosis. **a.** Myofibroblasts are localized in the mucosal layer. x 50. **b.** CD34-positve stromal cells are present in the connective tissue layer. x 50. **c-f.** Chronic cholecystitis with marked perimuscular fibrosis. **c.** Myofibroblasts are predominantly distributed in the connective tissue layer 50. **(d)** A small to moderate number of myofibroblasts are seen around the Rokitansky-Aschoff sinus. x 50. **e.** The reticular network of myofibroblasts in the connective layer is identified in some cases. x 50. **f.** The reticular network of CD34-positive stromal cells is disrupted by the intervention of myofibroblasts and collagen fibers in the connective tissue layer. x 25





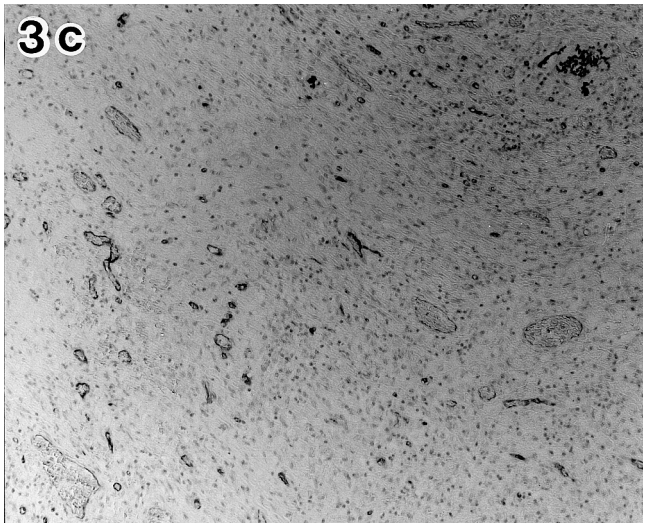
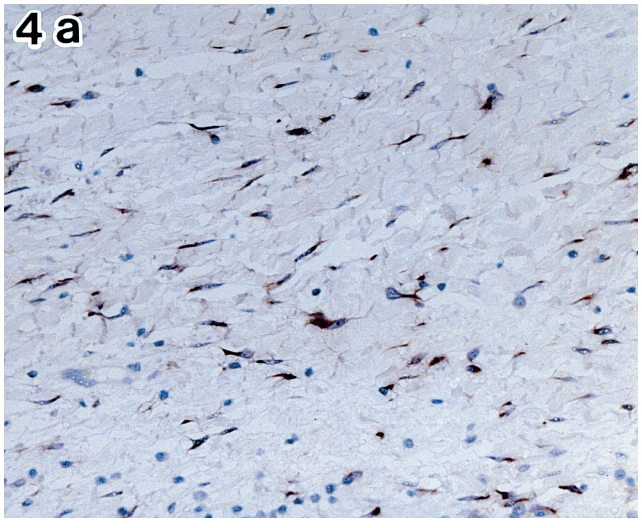


Fig. 3. Immunohistochemical analysis of chronic cholecystitis with xanthogranulomatous foci. **a.** Many myofibroblasts are observed in the periphery of the xanthogranulomatous reaction and the adjacent area. x 25. **b.** Myofibroblasts are also seen in the center of the loose xanthogranulomatous reaction area. x 25. **c.** No CD34-positive stromal cells are seen in or around the xanthogranulomatous reaction. x 25



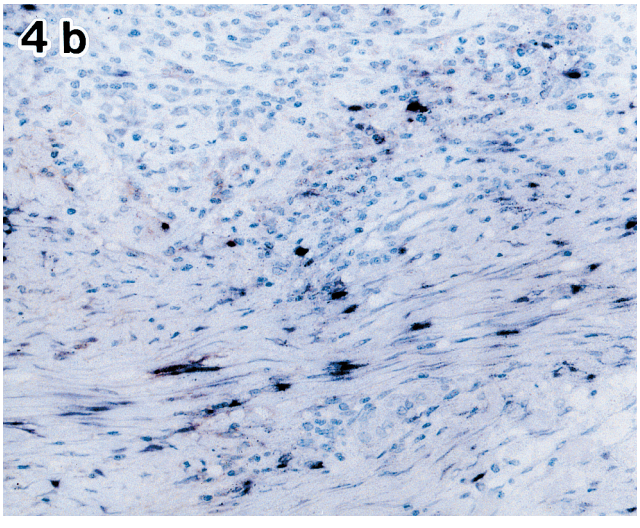


Fig. 4. Results of *in situ* hybridization analysis of collagen types I and III. **a.** Anti-sense probe analysis identified collagen type I mRNA signals in the cytoplasm of myofibroblasts intervening between dense collagen fibers, distant from the xanthogranulomatous foci. x 50. **b.** Collagen type III mRNA analyzed using anti-sense probe. Signals are found in the cytoplasm of myofibroblasts just around the xanthogranulomatous foci. x 50

granulomatous area (Fig. 4a). Nevertheless, collagen type III mRNA induction was detected only around the xanthogranulomatous area using the anti-sense probe (Fig. 4b). However, no collagen type III mRNA signals were identified in the area with marked fibrosis. The sense probes did not detect collagens type I and III anywhere in the specimens.

Discussion

In the present study, we report for the first time the structure of the reticular network of the CD34 antigen in the connective tissue layer of normal gallbladder. To date, the reticular network of CD34 antigen has been reported in normal Fallopian tube, salivary gland and testis (Yamazaki and Eyden, 1996a,b; Kuroda et al., 2004b,c).

In chronic cholecystitis with mild perimuscular fibrosis, a small to moderate number of myofibroblasts appeared in the mucosal layer. These myofibroblasts may be derived from fibroblasts in the mucosal layer, which are originally negative for CD34. On the other hand, myofibroblasts may be present in every layer of the gallbladder wall depending on the degree of inflammation and fibrosis in chronic cholecystitis. Of all the gallbladder wall layers, the most prominent increase in the number of myofibroblasts in chronic cholecystitis with marked perimuscular fibrosis was observed in the connective tissue layer. As the number of myofibroblasts in the connective tissue layer increased, the number of CD34-positive stromal cells at the same site tended to decrease.

In the present study, CD34-positive stromal cells completely disappeared from the xanthogranulomatous reaction and adjacent area in cases with chronic cholecystitis with xanthogranulomatous foci. In contrast, the number of myofibroblasts increased in the area neighboring the xanthogranulomatous foci, even if this reaction occurred in all layers. Additionally, a reticular network of myofibroblasts was detected in the connective tissue layer in some cases. These findings suggest that myofibroblasts in the connective tissue layer may originate from CD34-positive stromal cells present at that location. As Serini and Gabbiani (1999) suggested, the accumulation of myofibroblasts around the xanthogranulomatous reaction may be due to the action of cytokines such as TGF-ß, which are secreted by macrophages.

We found that collagens type I and III were predominantly produced in the cytoplasm of myofibroblasts located in the area of marked fibrosis, chiefly consisting of mature collagen fibers, and in the cytoplasm of myofibroblasts around the xanthogranulomatous reaction, respectively. Lagace et al. (1985) previously reported that in invasive breast carcinoma, collagens type I and III are abundant in the central sclerotic zone and young edematous stroma, respectively. Therefore, our results suggest that xanthogranulomatous foci consisting of collagen type III are areas of stromal development.

In conclusion, myofibroblasts are observed in various chronic cholecystitis subtypes. The increase in the number of myofibroblasts is most prominent in the connective tissue layer of chronic cholecystitis with marked perimuscular fibrosis or in the area adjacent to xanthogranulomatous foci of chronic cholecystitis. Under these conditions, CD34-positive stromal cells tend to disappear from the connective tissue layer in conjunction with an increase in myofibroblasts.

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