

The participation of myofibroblasts in the capsular formation of human conventional and chromophobe renal cell carcinomas

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Summary. The presence of myofibroblasts has been elucidated in neoplastic capsules of various organs. In the present article, we examine the presence of myofibroblasts in the capsule of renal cell carcinoma (RCC) and discuss the origin of the myofibroblasts. Nineteen renal tumors (conventional RCC, n=17; chromophobe RCC, n=2) with evident and totally surrounded fibrous capsule were selected. Abundant myofibroblasts were immunohistochemically observed in the capsule of the RCCs. These findings were confirmed by electron and immunoelectron microscopic studies of three conventional RCCs. Type III and I collagens were predominant in the outer and inner layers of the RCC capsule, respectively. The cytoplasm of the tubular epithelial cells in the tissue surrounding the neoplastic capsule stained positively for transforming growth factor (TGF)- β 1. *In situ* hybridization detected type I collagen mRNA in myofibroblasts of the capsule. Myofibroblasts may participate in the capsular formation of conventional and chromophobe RCCs through the collagen production.

Key words: Human renal cell carcinomas, Capsule, Myofibroblast

Introduction

The presence of myofibroblasts or stromal cells exhibiting smooth muscle features has been detected in the capsule of neoplasms in several organs including the salivary gland, liver, thyroid gland, and pancreas (Ooi et al., 1997; Nakayama et al., 1999, 2002; Soma et al., 2001; Kuroda et al., 2004). The role and origin of myofibroblasts in renal fibrosis have been elucidated in

some human glomerular diseases and animal models (Diamond et al., 1995; Eddy et al., 1995; Muchaneta-Kubara and Nahas, 1997; Mizuno et al., 1998; Nakatsuji et al., 1998; Zeisberg et al., 2001). Although several investigators suggested that myofibroblasts in renal fibrosis associated with glomerular diseases originated in fibroblasts or pericytes, the actual origin of tubular epithelial cells has recently been elucidated (Fan et al., 1999; Yang and Liu, 2001, 2002; Cheng and Lovett, 2003). Additionally, we recently elucidated the participation of myofibroblastic cells in renomedullary interstitial cell tumors (Kuroda et al., 2002). Therefore, in the present study, we immunohistochemically and ultrastructurally examined the presence of myofibroblasts in the capsule of renal cell carcinomas (RCCs).

Materials and methods

Tissue specimens

Hematoxylin-eosin-stained preparations from all RCCs resected at Kochi Medical School and affiliated hospitals between 2000 and 2002 were histologically divided according to recent classifications (Kovacs et al., 1997; Störkel et al., 1997; Kuroda et al., 2003a,b). Among these, nineteen RCCs with an evident and totally surrounded fibrous capsule were selected for this study. Histologically, these tumors were subdivided into seventeen conventional RCCs and two chromophobe RCCs. The sex ratio (male:female) of the patients with these tumors was 17:2. The mean age and age range of the patients were 62.7 years and 34 to 80 years, respectively. The mean tumor size and size range were 3.1 cm and 1.5 to 6.0 cm, respectively.

Routine histological procedures and histochemistry

Paraffin-sections from all patients were stained with hematoxylin and eosin. Additionally, Azan and Elastica-

van-Gieson (EVG) stains were used to identify the fiber types in the capsule.

Immunohistochemistry and its interpretation

Immunohistochemistry was performed using a streptavidin-biotin immunoperoxidase technique. Antibodies employed in the present study are summarized in Table 1. Vascular smooth muscle cells were used as internal positive controls for alpha-smooth muscle actin (ASMA) and high molecular weight caldesmon (h-CD) immuno-staining.

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. The distribution of myofibroblasts in the capsule was evaluated.

Preparation of human collagen I probe

Total RNA was extracted from normal renal capsule obtained from one autopsy case using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 1 µg of total RNA from each specimen was reversely transcribed and the cDNA was obtained for PCR. For human collagen I, the forward primer AGTGGTTACTACTGGATTGACC and the reverse primer TTGCCAGTCTCCTCATCC 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 MgCl₂, and 0.5 units 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 °C for 1 minute. The 352 bp fragment of human collagen I produced by RT-PCR was then subcloned into pGEM-T easy vector (Promega, WI, USA). The ligation was transformed and extracted using the Midiprep kit (Qiagen, Hilden, Germany). The sequence was linearized by digestion with NheI or SphI. Sense and antisense cRNA probes of collagen I 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 (5 µm) were used for *in situ* hybridization to detect collagen I expression in the RCC capsules, as previously indicated (Ramm et al., 1998). Briefly, sections were permeabilized with 15 mg/ml of proteinase K at 37 °C for 30 min and then post-fixed in 4% para-formaldehyde/PBS at room temperature for 10 min. Prehybridization with Dig Easy Hyb buffer (Roche) was performed at 58 °C for 2 hr followed by hybridization at 58 °C for 14 hr in Hyb buffer containing 350 ng/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).

Electron microscopy

Three samples extracted from the capsule of surgically resected RCCs were immediately fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide. After processing and embedding in epoxy resin, ultrathin sections stained with uranyl acetate were examined under an electron microscope (JEM 100S; JEOL Ltd, Tokyo, Japan).

Immunoelectron microscopy

Tissue samples obtained from three RCCs, as described above, were fixed by immersion in a periodate-lysine-paraformaldehyde solution for 24 hr. Frozen sections (20 µm) were cut from the material after incubation in a mixed solution of phosphate-buffered saline and sucrose. The tissue sections from the samples were subsequently incubated with anti-ASMA and analyzed immunohistochemically using the procedures described above with the addition of prefixation in 0.5% glutaraldehyde. The sections were processed and embedded in epoxy resin. Ultrathin sections stained with

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
Type I collagen	I-8H5	1:50	Daiichi Fine Chemical, Toyama, Japan	microwave
Type III collagen	III-53	1:50	Daiichi Fine Chemical, Toyama, Japan	microwave
Type IV collagen	PHM-12+CIV22	1:50	NeoMarkers, CA, USA	microwave
TGF-β1	9016,2	1:50	R&D systems. Inc., MN, USA	pronase

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lead citrate were examined under an electron microscope.

Results

Routine histological features

The neoplastic capsule consisted of identical components, irrespective of the two histological subtypes (conventional and chromophobe RCCs). The capsule of some RCCs contained entrapped rounded or distorted tubules.

Histochemical and immunohistochemical findings

Histochemical and immunohistochemical results are summarized in Tables 2 and 3. Azan stain identified collagen fibers in the capsule of all RCCs. When the capsule was thin, coarse bundles of collagen fibers were observed. On the other hand, when the capsule was thick, a double-layered structure was observed. Specifically, dense and coarse bundles of collagen fibers were identified in the inner and outer layers of the capsule, respectively (Fig. 1). However, EVG staining did not identify elastic fibers in the neoplastic capsule of any tumors.

No significant differences were detected between the two histological types in the distribution of myofibroblasts and collagen subtypes. In conventional and chromophobe RCCs, the capsule contained a moderate to large number of myofibroblasts (Fig. 2a, b). Additionally, abundant myofibroblasts were observed in the renal interstitium or perivascular space adjacent to the neoplastic capsule (Fig. 2c, d). Epithelial cells of the

entrapped tubules in the neoplastic capsule appeared to focally stain with ASMA. Irrespective of histological subtypes, moderate to dense deposition of collagen type III was observed. The deposition of type I collagen was light to dense in almost all renal tumors. Type I and III collagens were predominantly located in the inner and outer layers of the capsule, respectively (Fig. 3a, b). The amount of collagen fibers or hyalinization appeared to be associated with the amount of type I collagen. The deposition of type IV collagen was generally light in almost all RCCs. Additionally, disruption of collagen type IV was observed in the basement membrane of the distorted or elongated tubular epithelial cells in the neoplastic capsule (Fig. 3c). Tubular epithelial cells in the surrounding tissue of the capsule were positive for transforming growth factor (TGF)- β 1 in the cytoplasm (Fig. 4). However, no cells positive for TGF- β 1 were observed in tubules away from the capsule.

In situ hybridization findings

Expression of type I collagen mRNA was observed in many myofibroblasts in the capsule of conventional RCCs using an antisense probe. Higher levels of mRNA expression were evident in the inner layer than in the outer layer of the capsule (Fig. 5a). However, no signals were identified anywhere using a sense probe (Fig. 5b).

Ultrastructural findings

Stromal cell-intervening collagen fibers in the capsule possessed a well-developed Golgi apparatus and rough endoplasmic reticulum in the cytoplasm, and many myofilaments and dense bodies were also

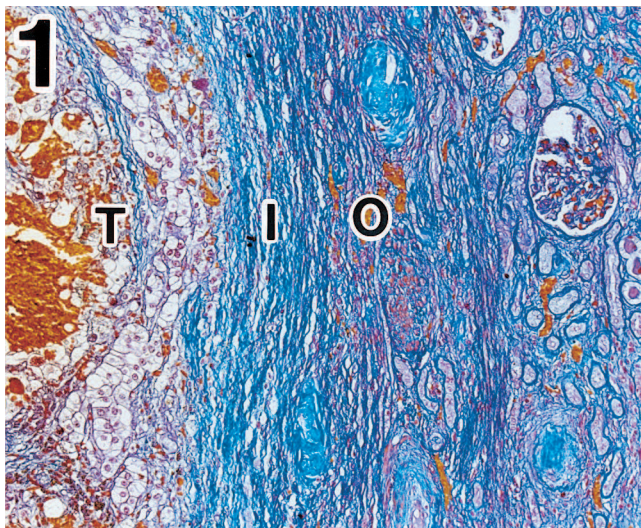


Fig. 1. An abundance of collagen fibers is observed in the neoplastic capsule of conventional RCC. T: tumor; I: inner layer of the capsule; O: outer layer of the capsule. Azan stain, x 25

Table 2. The distribution of myofibroblasts in the capsule and expression of TGF- β 1 in surrounding tubules.

HISTOLOGY (number)	CAPSULE Myofibroblasts	SURROUNDING TUBULES TGF- β 1
Conventional RCC (n=17)	+++++	+~++
Chromophobe RCC (n=2)	+++++	+~++

Table 3. Histochemical and immunohistochemical results of the capsule in two subtypes of renal cell carcinomas.

CAPSULE	AZAN	TYPE I COLLAGEN	TYPE III COLLAGEN
Thin	coarse	~+~	+++++
Thick			
inner layer	dense	+++++	~+
outer layer	coarse	~+~	+++++

observed (Fig. 6) (Schürch et al., 1998; Eyden, 2001).

Immunoelectron microscopic findings

The cytoplasmic structures of stromal cells in the capsule showed a positive reaction for ASMA (Fig. 7).

Discussion

To date, the participation of myofibroblasts in renal fibrosis has been elucidated in various studies of human and experimental glomerulonephritis (Diamond et al., 1995; Eddy et al., 1995; Muchaneta-Kubara and Nahas, 1997; Mizuno et al., 1998; Nakatsuji et al., 1998; Fang et al., 1999; Yang and Liu, 2001, 2002; Zeisberg et al., 2001; Cheng and Lovett, 2003). Therefore, we theorized

that capsular formation of RCCs may occur in a similar fashion to renal interstitial fibrosis associated with glomerular diseases. In the present study, we immunohistochemically and ultrastructurally elucidated the presence of myofibroblasts in the capsule of conventional and chromophobe RCCs. In renal fibrosis, myofibroblasts are derived from interstitial fibroblasts, pericytes or tubular epithelial cells (Fan et al., 1999; Yang and Liu, 2001, 2002; Zeisberg et al., 2001; Cheng and Lovett, 2003). In the present study, we identified the disruption of a basement membrane component, collagen type IV, in distorted tubular epithelial cells in the neoplastic capsule. Therefore, these findings suggest the observation that the early process of epithelial-myofibroblastic transdifferentiation occurs in the capsule

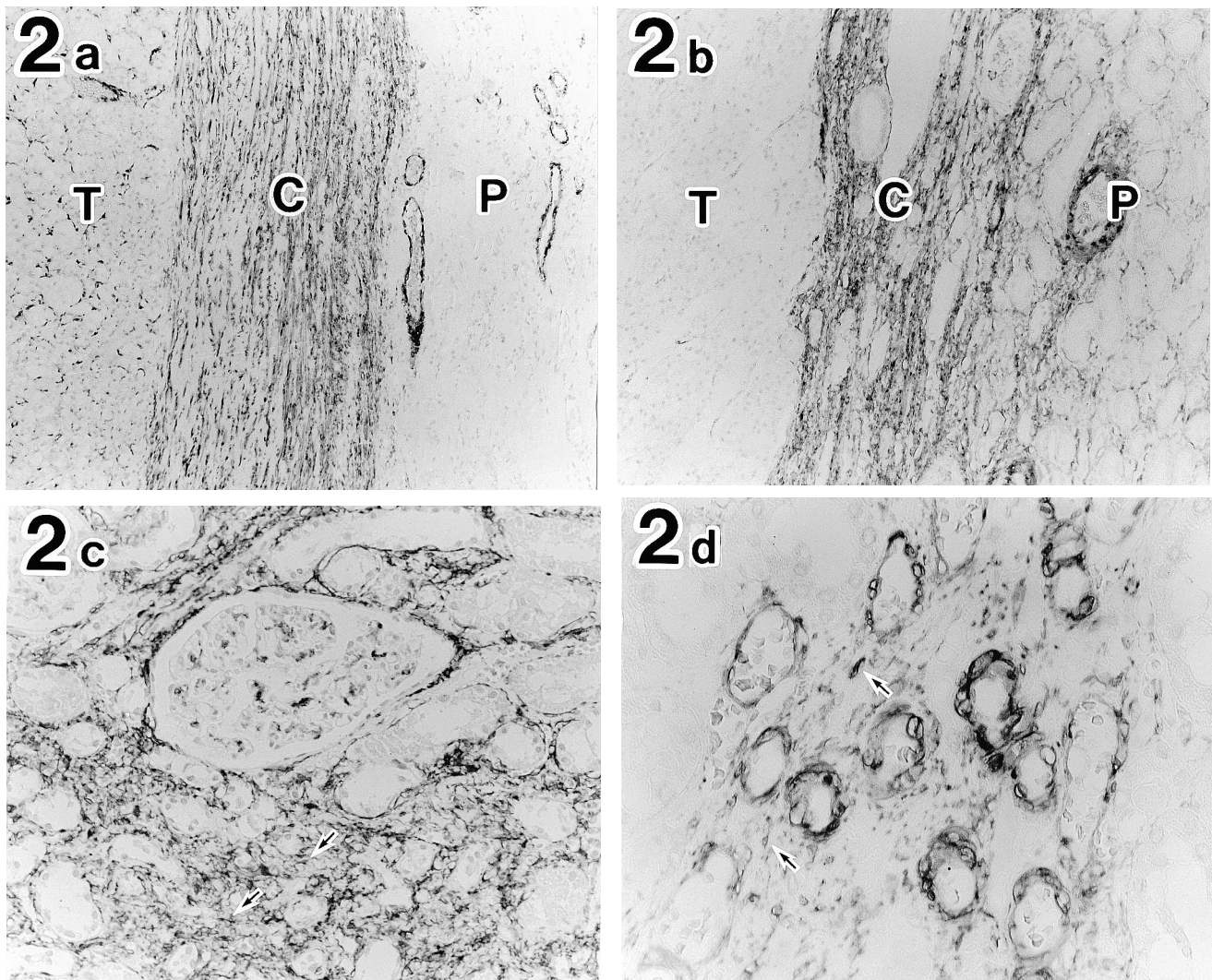


Fig. 2. ASMA immunohistochemistry. **a.** Conventional RCC. Many myofibroblasts are observed in the capsule. x 25. **b.** Chromophobe RCC. Many myofibroblasts are observed in the capsule. x 25. **c, d.** Many myofibroblasts (arrows) are observed in the renal interstitium (**c**) or perivascular space (**d**) adjacent to the neoplastic capsule. **c,** x 50; **d,** x 100. T: tumor; C: capsule; P: renal parenchyma.

of RCCs, as Fan et al. (1999) and Yang et al. (2001) showed in their studies. Likewise, interstitial fibroblasts and pericytes appeared to transform into myofibroblasts, based on the distribution of myofibroblasts around the capsule.

On the other hand, tubular epithelial cells positive for TGF- β 1 were restricted to the surrounding area of the tumor. Therefore, it is possible that TGF- β 1 may play a role on the capsular formation of conventional and chromophobe RCCs. Type III and I collagens were relatively abundant in the outer and inner layers of the renal tumor capsule, respectively. Lagace et al. (1985)

showed that type I collagen was most prominent in the central sclerotic zone and type III collagen was present in the peripheral area corresponding to the “young” edematous stroma. Therefore, our observations of the capsule of RCCs appear to correspond with their results. Myofibroblasts in and/or around the capsule secrete the extracellular matrix, such as type I collagen, as we confirmed by *in situ* hybridization in the present study.

In conclusion, we demonstrated that myofibroblasts participated in capsular formation in human conventional and chromophobe RCCs through the collagen production.

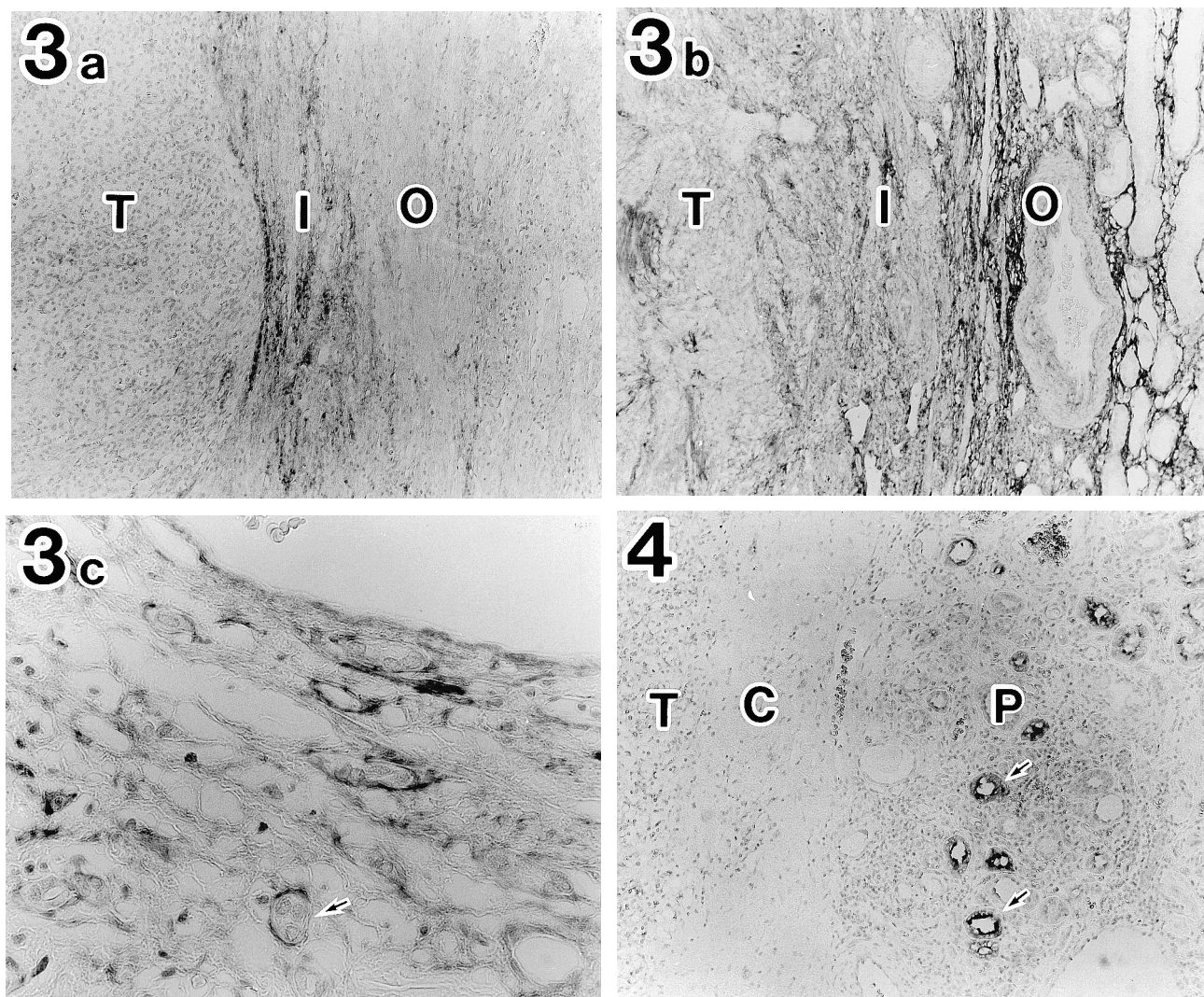


Fig. 3. Immunohistochemical analysis of type I and IV collagens in the capsule of conventional RCC. **a, b.** The deposition of type I (**a**) and III (**b**) collagens is observed in the inner and outer layers, respectively. x 25. **c.** A disruption (arrow) of collagen type IV is observed in the basement membrane of distorted or elongated tubular epithelial cells. x 100. T: tumor; I: inner layer of the capsule; O: outer layer of the capsule.

Fig. 4. Immunohistochemical analysis of TGF- β 1 in conventional RCC. The cytoplasm of tubular epithelia adjacent to the neoplastic capsule is reactive for TGF- β 1 (arrows). x 25. T: tumor; C: capsule; P: renal parenchyma.

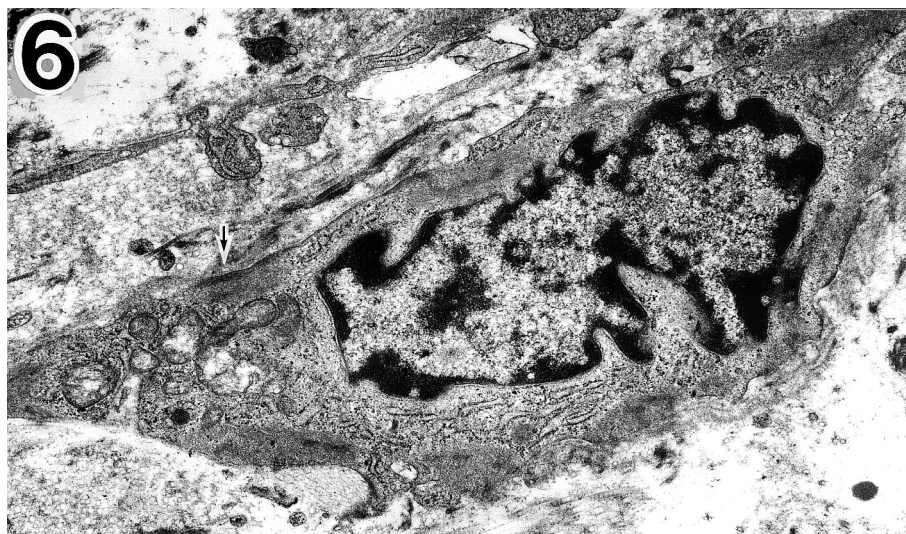
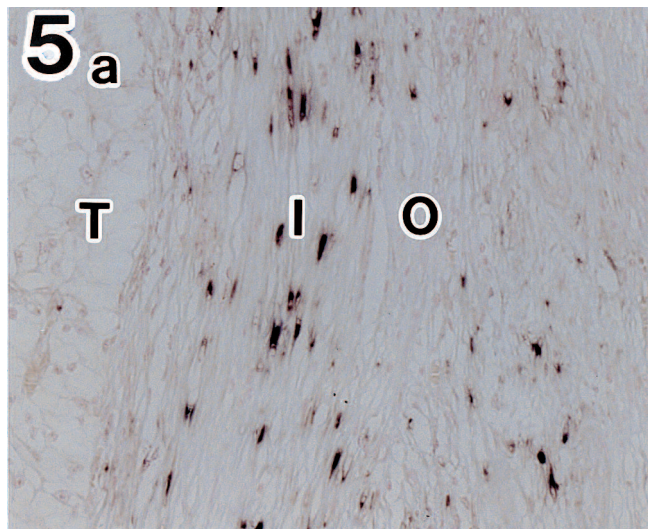


Fig. 5. Results of *in situ* hybridization for type I collagen in the capsule of conventional RCC. **a.** Results from an antisense probe. The expression of type I collagen mRNA is observed in the cytoplasm of myofibroblasts. x 50. **b.** Results from a sense probe. No signals are seen anywhere. x 50. T: tumor; I: inner layer of the capsule; O: outer layer of the capsule.

Fig. 6. Ultrastructural observations of stromal cells in the capsule of conventional RCC. The stromal cells contain rough endoplasmic reticulum and myofilaments (arrow). x 4,000

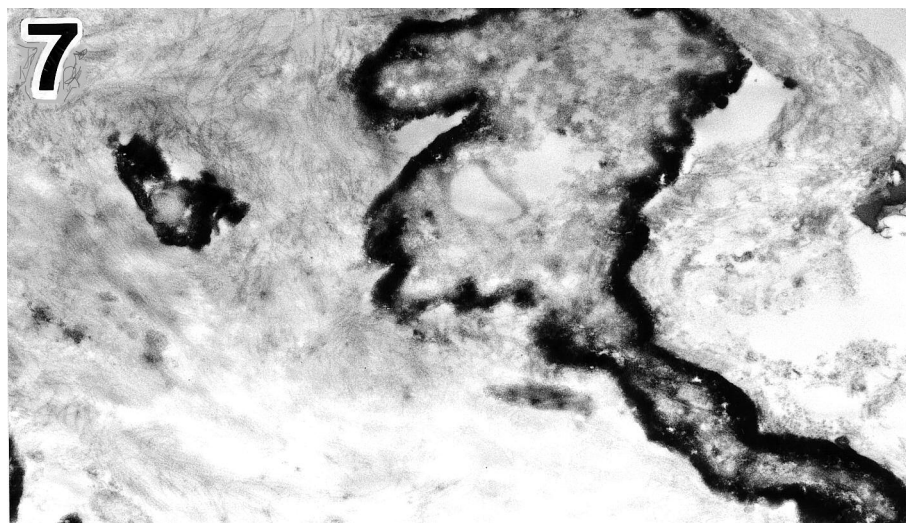


Fig. 7. Immunoelectron microscopic findings of ASMA in stromal cells of the capsule of conventional RCC. The peripheral filamentous structures are immunoreactive for ASMA. x 3,000

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