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Distribution and role of CD34-positive stromal cells and myofibroblasts in human normal testicular stroma

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Summary. CD34-positive stromal cells are distributed in various organs including breast, Fallopian tubes, thyroid gland, colon, pancreas, and uterine cervix. To elucidate the distribution of CD34-positive stromal cells, smooth muscle cells, and myofibroblasts in normal human testis, we examined 48 testes obtained by autopsy and operation, including five fetal, one neonatal, and 42 adult cases without evident testicular lesions, using a streptavidin-biotin immunoperoxidase technique. The expression of alpha-smooth muscle actin (ASMA), h-caldesmon, CD34, and CD31 were immunohisto-chemically examined in all cases. The tunica albuginea and the inner layer of seminiferous tubules in adult testis were predominantly composed of myofibroblasts. Smooth muscle cells were also scattered throughout these sites in some cases. CD34-positive stromal cells were abundant, and they formed a reticular network around the seminiferous tubules and Leydig cells as well as the outer layer of seminiferous tubules. Moreover, myofibroblasts and the CD34 reticular network were already present in the testicular stroma during fetal or neonatal development. Double immunostaining of fetal, neonatal and adult testes using ASMA and CD34 confirmed that myofibroblasts and CD34-positive stromal cells were present in the inner and outer layers of peritubular tissue, respectively. This distribution and cytological identification was also confirmed by an ultrastructural study of four cases. Finally, CD34-positive stromal cells and myofibroblasts are major components of human testicular stroma.

Key words: Human testis, CD34, Reticular network, Myofibroblast, Development

Introduction

CD34 has been discovered as a marker of human hematopoietic cells and this molecule is a 110kDa transmembrane cell surface glycoprotein. The gene of CD34 is located on chromosome 1 in the region 1q32. CD34-positive neoplastic cells were detected in various tumors, including leukemia, vascular tumors, dermatofibrosarcoma protuberans, solitary fibrous tumors and gastrointestinal stromal tumors (van de Rijn and Rouse, 1994). CD34-positive stromal cells have recently been identified in various normal organs including breast, thyroid gland, Fallopian tube, submandibular gland, colon, pancreas, meninges, palatine tonsil, and uterine cervix (Yamazaki and Eyden, 1995, 1996a,b, 1997; Nakayama et al., 2000; Cummings et al., 2001; Papadas et al., 2001; Barth et al., 2002a-c). These CD34-positive stromal cells are regarded as dendritic interstitial cells or fibrocytes, but their function remains unknown (van de Rijn and Rouse, 1994; Nakayama et al., 2000; Barth et al., 2002a-c). Contractile or myoid cells in the tunica albuginea and seminiferous tubules of human testis consist of smooth muscle cells, myofibroblasts or both cells (Ross and Long, 1966; Langford and Heller, 1973; De Kretser et al., 1975; Busts-Obregon, 1976; Furuya et al., 1977; Toyama, 1977; Johnson et al., 1986; Virtanen et al., 1986; Christl, 1990; Davidoff et al., 1990; Holstein et al., 1996; Arenas et al., 1997; Middendorff et al., 2002). To date, there are no studies on CD34-positive stromal cells in human testicular stroma. Additionally, there are few reports describing the relationship of CD34-positive stromal cells and myofibroblasts (Barth et al., 2002c). Therefore, in this study we examined human testis immunohisto-chemically and ultrastructurally to confirm the distribution and relationship of smooth muscle cells, myofibroblasts, and CD34-positive stromal cells in normal fetal and adult testis.

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Materials and methods

Tissue specimens

We selected five fetal (20, 22, 29, 31, and 32 weeks of gestation), one neonatal (7 months after birth), and thirty-four adult testes without evident testicular neoplastic lesions from the autopsy files of the Department of Pathology, Kochi Medical School. Additionally, normal testes from eight adult patients surgically resected for testicular tumors were selected.

The 42 adult patients ranged in age from 15 to 91 years (mean, 59.5 years old). Each specimen had been fixed in 10% formalin solution and the duration of fixation varied from two to five days. Paraffin-sections from all patients were stained with hematoxylin and eosin. Testicular tissues without autolysis of the seminiferous tubules or marked tubular atrophy were selected in the present study. Additionally, the testes from four patients obtained by autopsy were used for ultrastructural and immunocytochemical studies.

Immunohistochemistry and its interpretation

Using a streptavidin-biotin immunoperoxidase technique, 3-μm sections of each specimen were evaluated for ASMA (1:50 dilution, 1A4, Dako Cytomation, Glostrup, Denmark), high molecular weight caldesmon (h-CD) (1:50 dilution, h-CD, Dako Cytomation, CA, USA), CD34 (1:20 dilution, MY10, Becton-Dickinson, San Jose, CA, USA), and CD31 (1:20 dilution, JC/70A, Dako Cytomation, Glostrup, Denmark). Microwave and pronase treatments were performed only for h-CD and CD31, respectively. Vascular smooth muscle cells and endothelial cells were used as internal positive controls for ASMA and h-CD, and CD34 and CD31 immunostaining, respectively.

As h-caldesmon is a novel smooth muscle actin-specific antibody and is not expressed in myofibroblasts (Ueki et al., 1987; Watanabe et al., 2000; Ceballos et al., 2000; Rush et al., 2001). 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, respectively. Furthermore, CD34-positive and CD31-negative stroma cells were classified as CD34-positive stromal cells. Thus, the distribution of smooth muscle cells, myofibroblasts and CD34-positive stromal cells in fetal and adult testis was evaluated.

Double immunostaining

Double immunostaining for CD34 and ASMA was performed on all specimens to identify the relationship between myofibroblasts and CD34-positive stromal cells. CD34 was labeled with peroxidase DAB, and ASMA was labeled with alkaline phosphatase fast blue.

Electron microscopy

The specimens extracted from four normal adult testes were 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 in an electron microscope (JEM 100S; JEOL Ltd, Tokyo, Japan).

Immunoelectron microscopy

Tissue samples obtained from three normal adult testes were fixed by immersion in a periodate-lysine-paraformaldehyde solution for 24 hours. Frozen sections (20 μm) were cut from the material after incubation in a mixed solution of phosphate-buffered saline (PBS) and sucrose. The tissue sections from the samples were subsequently incubated with anti-ASMA and anti-CD34 antibodies, 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 lead citrate were examined under an electron microscope.

Results

Distribution of stromal cells in normal fetal and neonatal testis

The results are summarized in Table 1. Except for in the vascular wall, no smooth muscle cells were identified in any of the six cases. No myofibroblasts were present in the 20-and 32-week-old fetal testes. However, myofibroblasts were observed in the tunica albuginea (Fig. 1a) and peritubular tissue in the 22-, 29- and 31-week-old fetal testes. At 7 months after birth, myofibroblasts were present in the tunica albuginea and peritubular tissue, but were absent between seminiferous tubules (Fig. 1b).

No CD34-positive stromal cells were observed in the 20-and 31-week-old fetal testes, although these cells appeared in the seminiferous tubules and around Leydig cells thereby forming a reticular network in the 22-, 29- and 32-week-old fetal testes (Fig. 1c). In the neonatal testis, CD34-positive cells were distributed predominantly in the seminiferous tubules and between tubules or around Leydig cells. Moreover, CD34-positive cells were focally present in the tunica albuginea. Using a double immunostaining, myofibroblasts and CD34-positive stromal cells seemed to be located in the inner and outer layers of peritubular tissue, respectively.

Distribution of stromal cells in normal adult testis

The stromal cell analysis results are also summarized in Table 1. Myofibroblasts were predominant in the tunica albuginea of 28 patients (Fig. 2a,b). However, smooth muscle cells were also identified among the myofibroblasts in the tunica albuginea of 14 patients (Fig. 2c). The intervention of smooth muscle cells in the tunica albuginea seemed to have no relationship to the ageing. ASMA-positive stromal cells were observed in the inner layer of the peritubular area. Among these cells, myofibroblasts, which were identified by ASMA-positive and h-CD-negative reactivity, were predominant (Fig. 2d,e), and smooth muscle cells, which were identified by h-CD-positivity, were scattered (Fig. 2e). CD34-positive cells were observed in the outer layer of the peritubular area (Fig. 2f). CD34-positive stromal cells were also

abundant between the seminiferous tubules and around the Leydig cells where they formed a reticular network (Fig. 2f). The reticular network of CD34-positive stromal cells was continuous between the peritubular tissue and intertubular stroma (Fig. 2f). Additionally, thin cytoplasmic processes of CD34-positive stromal cells were closely attached to macrophages of the stroma (Fig. 2g). The structural relationship between myofibroblasts and CD34-positive cells in the peritubular area was also confirmed by a double immunostaining (Fig. 3a). Additionally, peritubular stromal cells expressing both CD34 and ASMA were focally observed (Fig. 3b).

Ultrastructural findings

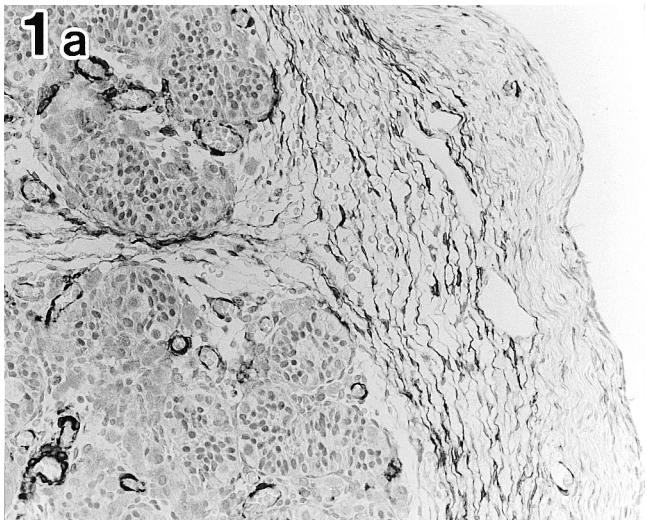
Three to five layers were observed in the peritubular tissue of human adult testis (Fig. 4a). Spindle cells of the inner layer of the peritubular tissue possessed a well-developed Golgi apparatus and rough endoplasmic reticulum and many peripheral myofilaments and dense bodies (Fig. 4b). However, the external lamina, attachment plaque, and plasmalemmal caveolae were absent from many cells, which was indicative of myofibroblasts rather than smooth muscle cells (Eyden, 2001). Spindle cells of the outer layer of the peritubular tissue contained abundant rough endoplasmic reticulum (Fig. 4c).

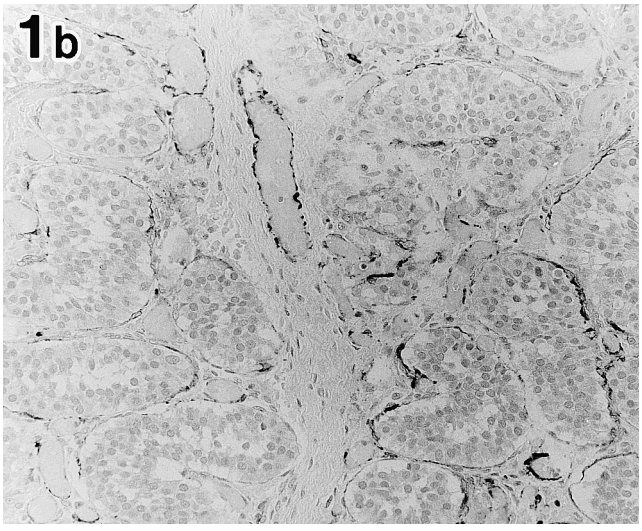
Immunoelectron microscopic findings

The cytoplasm of spindle and stellate-shaped cells in the adult tunica albuginea was positive for ASMA (Fig. 5a). The intracytoplasmic structures of spindle cells in the inner layer of the peritubular tissue also expressed ASMA (Fig. 5b). Additionally, spindle cells containing a thin cytoplasmic process in the outer layer of peritubular tissue and between seminiferous tubules expressed CD34. The expression of CD34 was predominantly distributed on the cell surface (Fig. 5c).

Discussion

To the best of our knowledge, this is the first report describing the distribution of CD34-positive stromal cells in human testis. In our study, CD34-positive stromal cells were chiefly distributed in the outer layer





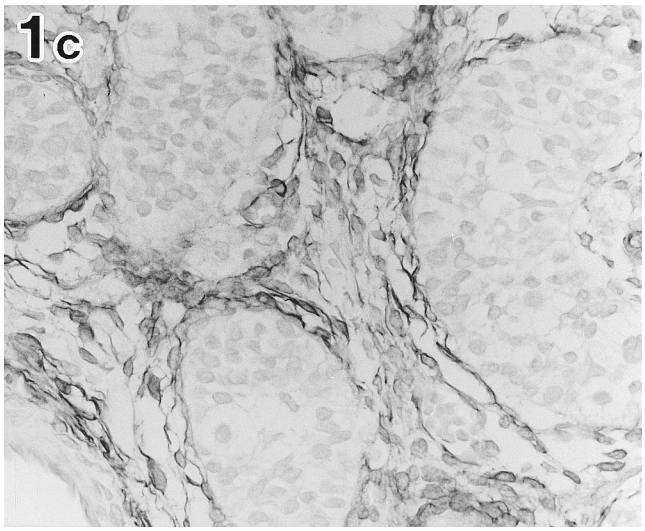


Fig. 1. Immunohistochemistry of alpha smooth muscle actin (ASMA) and CD34 in human fetal or neonatal testis. **a.** Gestational week 31. The expression of ASMA is observed in the tunica albuginea and septal stroma. However, ASMA-positive cells are absent in the stroma between seminiferous tubules. x 50. **b.** Seven months after birth. ASMA is absent between seminiferous tubules. x 50. **c.** Gestational week 32. CD34 is observed in the seminiferous tubules. Additionally, CD34-positive stromal cells are recognized between tubules. x 100

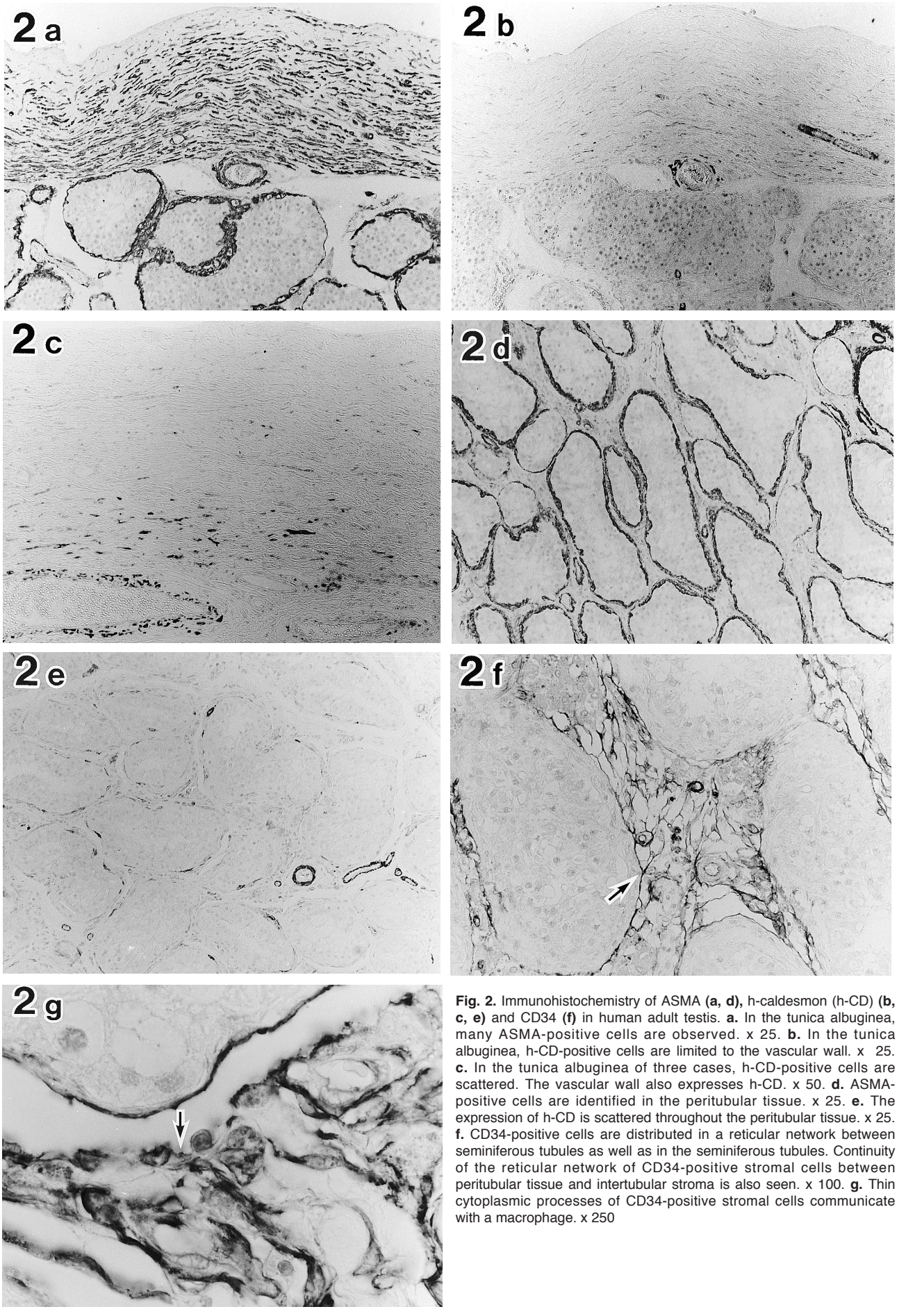


Fig. 2. Immunohistochemistry of ASMA **(a, d),** h-caldesmon (h-CD) **(b, c, e)** and CD34 **(f)** in human adult testis. **a.** In the tunica albuginea, many ASMA-positive cells are observed. x 25. **b.** In the tunica albuginea, h-CD-positive cells are limited to the vascular wall. x 25. **c.** In the tunica albuginea of three cases, h-CD-positive cells are scattered. The vascular wall also expresses h-CD. x 50. **d.** ASMA-positive cells are identified in the peritubular tissue. x 25. **e.** The expression of h-CD is scattered throughout the peritubular tissue. x 25. **f.** CD34-positive cells are distributed in a reticular network between seminiferous tubules as well as in the seminiferous tubules. Continuity of the reticular network of CD34-positive stromal cells between peritubular tissue and intertubular stroma is also seen. x 100. **g.** Thin cytoplasmic processes of CD34-positive stromal cells communicate with a macrophage. x 250

of the peritubular area, surrounding the layer of myofibroblasts. Additionally, the reticular network of CD34-positive stromal cells between seminiferous tubules and around Leydig cells was already complete during the fetal and neonatal periods, irrespective of the formation of myofibroblasts. Thus, stromal cells in the outer layer of seminiferous tubules and those between tubules or around Leydig cells may have a common origin, as was indicated by the continuity of the reticular network. Additionally, the thin cytoplasmic processes of

Table 1. Distribution of stromal cells in normal fetal and adult testis.

	TUNICA ALBUGINEA	SEMINIFEROUS TUBULES	BETWEEN TUBULES OR AROUND LEYDIG CELLS
Smooth muscle cells			
20W	-	-	-
22W	-	-	-
29W	-	-	-
31W	-	-	-
32W	-	-	-
7M after birth	-	-	-
Adult	-~+	- (focal, +)	-
Myofibroblasts			
20W	-	-	-
22W	++	+	-
29W	++	+	-
31W	++	+	-
32W	-	-	-
7M after birth	+	+	-
Adult	++~+++	++ (inner layer)	-
CD34-positive stromal cells			
20W	-	-	-
22W	-	+	++
29W	-	+	++
31W	-	-	-
32W	-	++	++
7M after birth	+	++	++
Adult	-	+ (outer layer)	++~+++

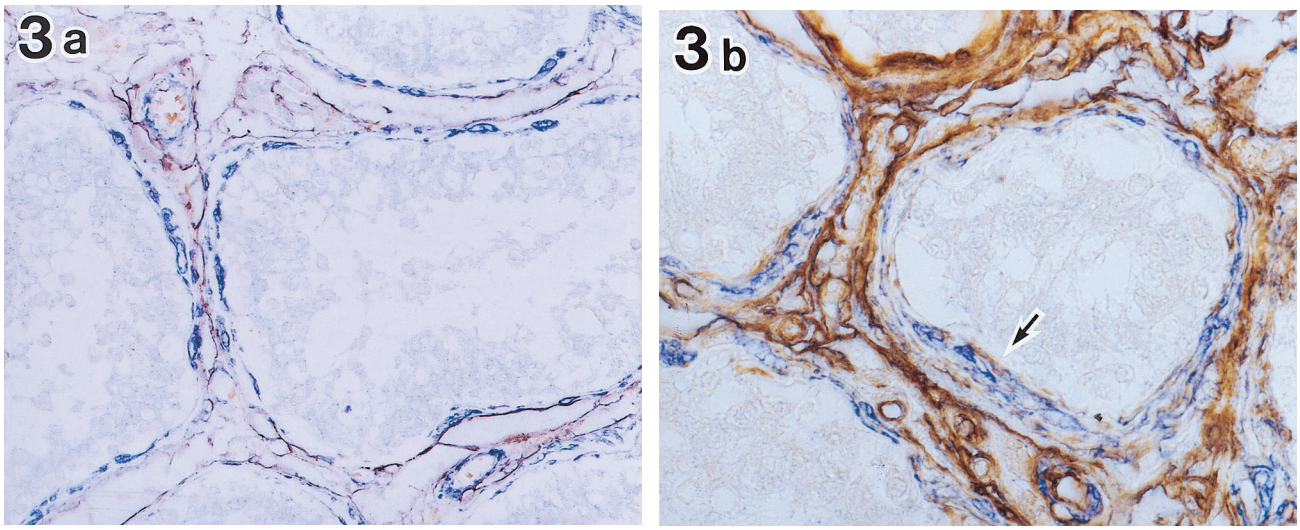


Fig. 3. Double immunostaining of ASMA and CD34 in adult testis. **a.** Myofibroblasts positive for ASMA (purple) and CD34-positive stromal cells (brown) are observed in the inner and outer layers of the seminiferous tubules, respectively. x 50. **b.** Peritubular stromal cells positive for both ASMA and CD34 antigens (arrow) are observed. x 100

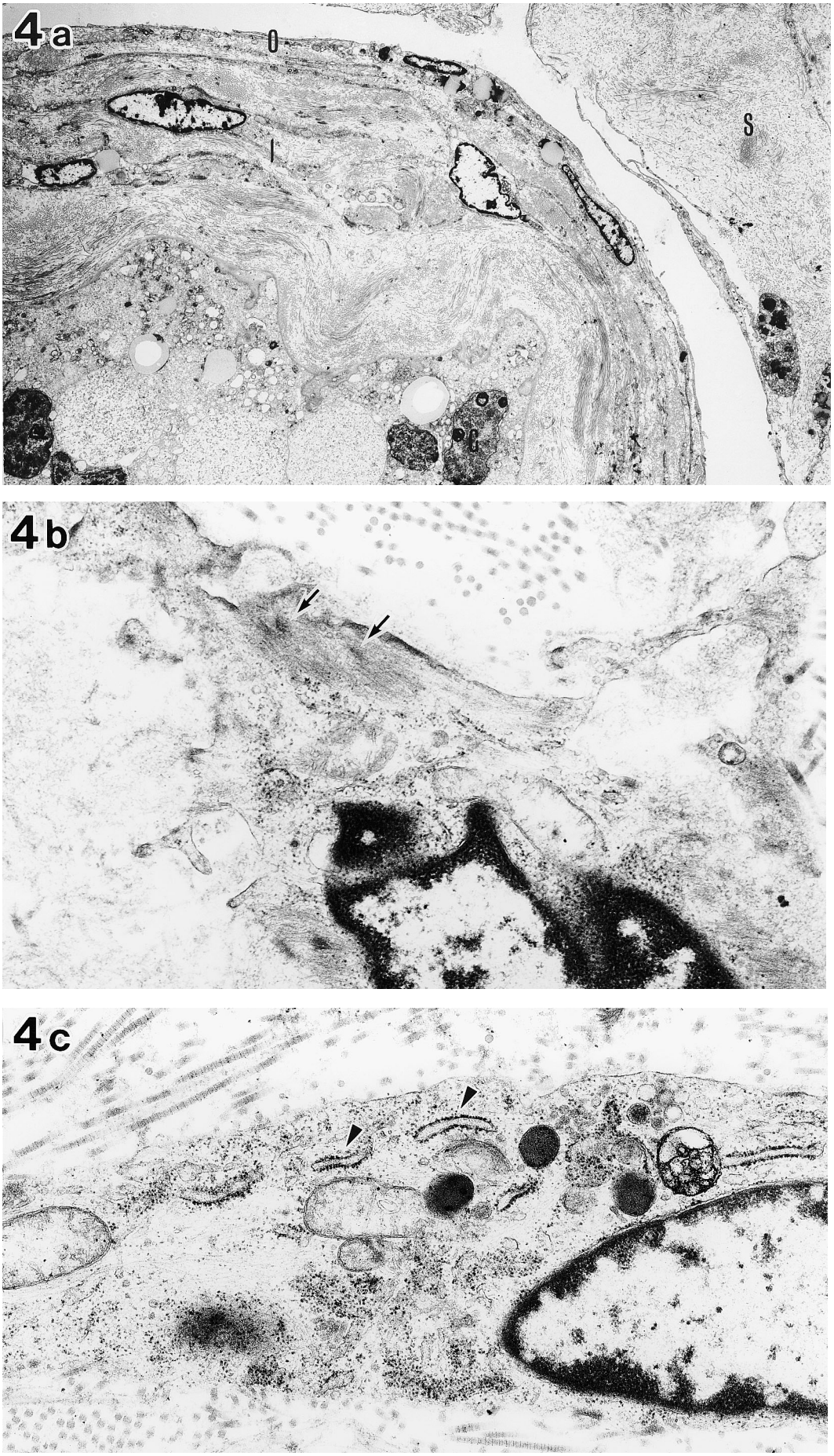
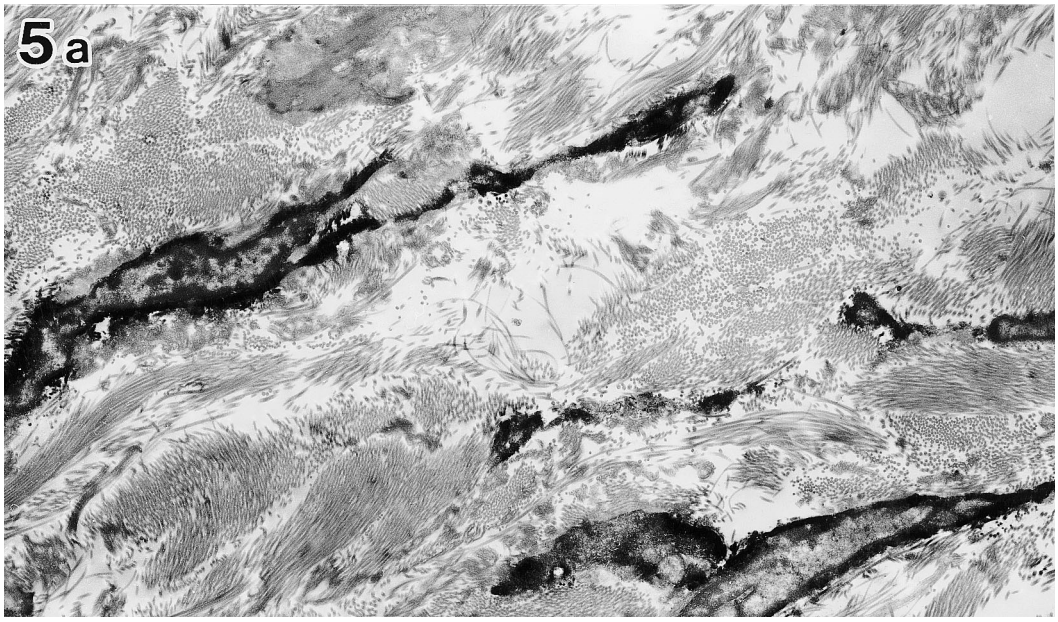
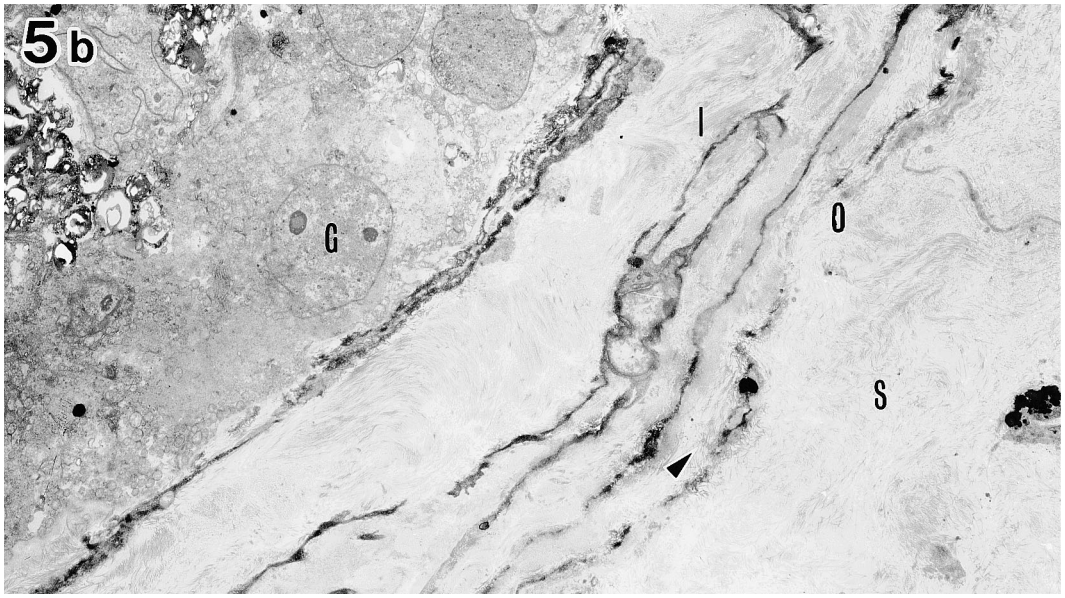


Fig. 4. Ultrastructural findings of the peritubular tissue in human adult testis. **a.** Several layers composed of spindle cells are observed in the peritubular tissue. G: germ cell; I: inner layer; O: outer layer; S: intertubular stroma. x 1,000. **b.** Some peripheral myofilaments and dense bodies (arrows) are observed in the inner layer cells. x 10,000. **c.** The outer layer cells contain abundant rough endoplasmic reticulum (arrowheads). x 10,000





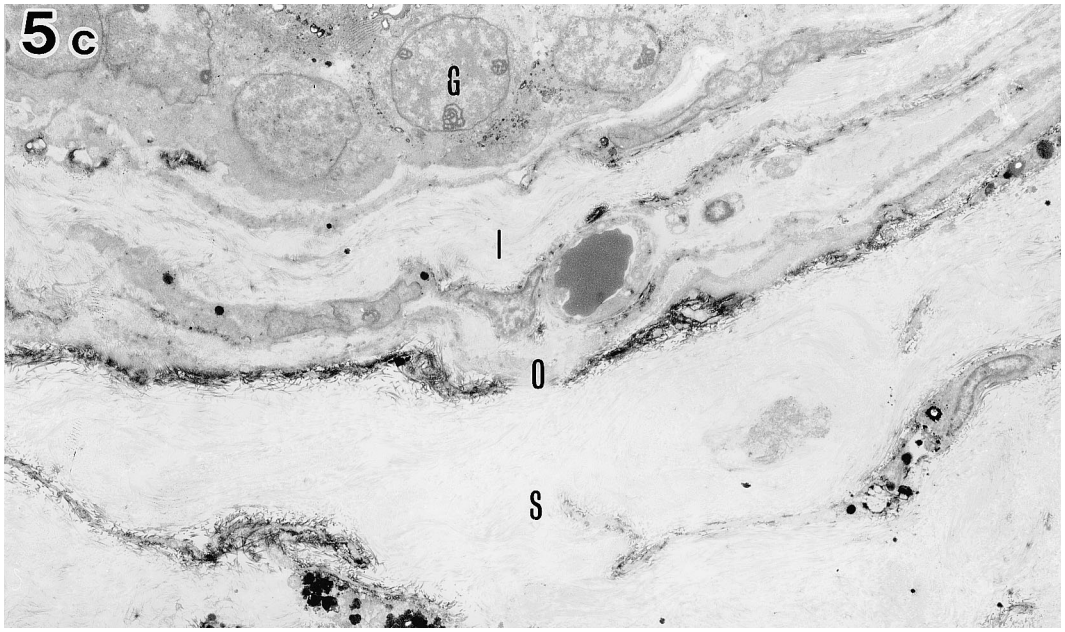


Fig. 5. Immunoelectron microscopic findings in human adult testis. **a.** Spindle cells in the tunica albuginea show a positive reaction for ASMA. x 1,000. **b.** Inner layer cells of the peritubular tissue are positive for ASMA. x 1,000. **c.** The outer layer cells of the peritubular tissue are positive for CD34. G: germ cell; I: inner layer; O: outer layer; S: intertubular stroma. x 1,000

CD34-positive stromal cells were closely connected to the cell membrane of stromal macrophages. Therefore, we suggest that CD34-positive stromal cells may play a role in immune surveillance in the testicular stroma, as Yamazaki and Eyden (1996a,b) suggested for other organs. Using a double immunostaining, peritubular cells expressing both CD34 and ASMA antigens were focally observed. Therefore, we suggest that CD34-positive stromal cells and myofibroblasts may have the same origin.

On the other hand, contractile or myoid cells have been previously identified in human testis. In the present study, ASMA-positive stromal cells were predominantly distributed in the tunica albuginea and the inner layer of seminiferous tubules. Smooth muscle cells or contractile cells have been identified in the tunica albuginea of various animals such as horse, pig, and sheep (Chacon-Arellano and Woolley, 1980). In contrast, several investigators have reported that myofibroblasts are abundant in the rat tunica albuginea (Gorgas and Bock, 1974; Santamaria et al., 1990). Langford and Heller (1973) suggested that smooth muscle cells are present in human tunica albuginea. Middendorff (2002) reported that myofibroblasts and smooth muscle cells are predominantly present in outer and inner layers of the tunica albuginea of human testis, respectively. Arenas (1997) reported that the total number of desmin-positive myoid cells was significantly increased in ageing men and that the distribution of myoid cells changed as ageing advanced. In the present study, we confirmed that myofibroblasts were predominant in human tunica albuginea. Additionally, the level of smooth muscle cell intervention among myofibroblasts in human tunica albuginea appears to vary from case to case, irrespective of ageing. Previous reports have shown that myoid or contractile cells are also present in human seminiferous tubules. Whether these cells in human seminiferous tubules are smooth muscle cells or myofibroblasts was debatable (Ross and Long, 1966; Bustos-Obregon, 1975; De Kretser et al., 1975; Furuya et al., 1977; Toyama, 1977; Johnson et al., 1986; Virtanen et al., 1986; Christl, 1990). However, Davidoff et al. (1990) have unequivocally demonstrated that the contractile peritubular cells of human seminiferous tubules are myofibroblasts. In the present study, we confirmed immunohistochemically that the majority of myoid cells in human seminiferous tubules were myofibroblasts rather than smooth muscle cells. Additionally, electron microscopy was used to confirm the myofibroblastic status of some of these cells. Preservation was suboptimal owing to the autopsy nature of the material, but the cells regarded as myofibroblasts by their ASMA positivity lacked the lamina of smooth muscle cells. We suggest that myofibroblasts in human tunica albuginea and seminiferous tubules may play an important role in sperm transport.

Finally, we elucidated for the first time that CD34-positive stromal cells were distributed in the outer layer of seminiferous tubules and between tubules or around Leydig cells in a reticular network. Moreover, this network of CD34-positive stromal cells is established during the fetal or neonatal periods. Myofibroblasts are abundant in the tunica albuginea and the inner layer of seminiferous tubules of normal human fetal and adult testis. Further studies will be required to elucidate the role of CD34-positive stromal cells and myofibroblasts in human testis.

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