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Co-expression of cytokeratin and vimentin filaments in rete testis and epididymis

An immunohistochemical study

Hans P. Dinges, Kurt Zatloukal, Christine Schmid, Sabine Mair, and Gerhard Wirnsberger

Institute of Pathology, University of Graz School of Medicine, Auenbruggerplatz 25, A-8036 Graz, Austria

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Summary. In 11 testes of different developmental stages (from 10-week-old embryos to adult) the cytokeratin and vimentin expression patterns of rete testis and epididymis were investigated immunohistochemically in formaldehyde-fixed paraffin-embedded material. In addition, immunofluorescence microscopy including double immunofluorescence was performed on frozen sections of 3 of these 11 cases. Rete testis and epididymis cells displayed a heterogeneous co-expression of cytokeratin and vimentin. In double immunohistochemistry, differences in distribution of keratin and vimentin intermediate filaments with predominance of cytokeratins in the apical cytoplasmic regions and of vimentin filaments in the basal portions of the cells were found. Cytokeratin expression preceded the appearance of vimentin: cytokeratin was already detectable in 10-week-old embryos, while weak vimentin immunoreactivity was first seen in 12-week-old embryos and became conspicuous in testes around the perinatal period. In testes of children up to 2 years of age the cytoplasmic distribution of cytokeratin and vimentin was more homogeneous. Predominance of the basal cell portions for vimentin and the apical regions for cytokeratin staining were less pronounced than in adult testes. In the proximal and distal parts of the epididymis a different intermediate filament expression pattern was found with a clear predominance of cytokeratin near the rete.

Key words: Immunohistochemistry – Testis – Epididymis – Cytoskeleton – Embryogenesis

Introduction

Co-expression of cytokeratin and vimentin filaments has been reported in a diversity of cultured epithelial and carcinoma cells (Franke et al. 1978, 1979b, c) as well as in salivary glands (Krepler et al. 1982; Caselitz et al. 1981), in ovary, in mesothelial cells (Miettinen et al. 1983; Czernobilsky et al. 1985; Benjamin et al. 1987), in parietal endodermal cells of mouse embryos (Franke et al. 1983; Lane et al. 1983; Lehtonen et al. 1983), in certain cells of the human amnion epithelium (Cremer et al. 1981; Regauer et al. 1985), in collecting duct epithelium in kidney (Holthöfer et al. 1984) and in tumours derived therefrom (Pitz et al. 1987). Furthermore, a co-expression of glial fibrillary acidic protein and vimentin has been found in astrocytomas (Herpers et al. 1986); co-existence of cytokeratin, vimentin and neurofilament proteins in human choroid plexus (Kasper et al. 1986) and in carcinoid tumours (Kimura et al. 1989); co-existence of cytokeratin, vimentin and desmin in smooth muscle cells of the umbilical cord (Kasper et al. 1988) and of glial fibrillary acidic protein, vimentin and cytokeratin in pleomorphic adenomas (Domagala et al. 1988). Co-expression of different intermediate filament proteins was found throughout the whole spectrum of lung tumours (Gatter et al. 1987).

Only few reports on co-expression of cytokeratin and vimentin intermediate filaments in the rete testis have been published (Ramaekers et al. 1985; Feitz et al. 1987). In contrast to these reports, Achtstätter et al. (1985) using double immunofluorescence microscopy found that cytokeratin but not vimentin filaments were present in rete testis. Cytokeratin polypeptides 7, 8, 18 and 19 (see Moll et al. 1982; Steinert et al. 1988) but no vimentin was detected after microdissection of rete epithelium, protein extraction and gel electrophoresis. In the seminiferous tubules of the testis Sertoli cells were stained by vimentin antibodies only (Franke et al. 1979a; von Vorstenbosch et al. 1984).

The findings of Achtstätter et al. (1985), who could not detect co-expression of cytokeratin and vimentin filaments in rete testis are also in contradiction to the report of Czernobilsky et al. (1985), who found co-expression in the rete ovarii. It is noteworthy in this context that both structures are of the same embryonal origin, they are remnants of the wolffian duct (mesonephric

Offprint requests to: H.P. Dinges

tubules). To resolve this discrepancy, we looked for cytokeratin and vimentin co-expression in the rete testis and in epididymis in fetal and adult testicular tissues using a series of poly- and monoclonal antibodies to cytokeratin and vimentin.

Materials and methods

Eleven human testes (autopsy cases; ages ranging between a 10-week-old embryo and adults) were selected (Table 1). The samples were fixed in 10% phosphate-buffered formaldehyde solution (pH 7.0) and embedded in paraffin. Three testes (numbers 9–11 of Table 1) were divided along the longitudinal axis into two parts. One half was fixed in formalin and embedded in paraffin; the other was frozen in methylpentane precooled with liquid nitrogen and stored in liquid nitrogen. The types, sources and dilutions of the antibodies used are listed in Tables 2 and 3.

The avidin-biotin complex (ABC) technique (Hsu et al. 1981) was used for demonstration of antigens with peroxidase as enzyme label and diaminobenzidine as substrate for the colour reaction. The incubation steps were performed as follow.

For cytokeratin detection 5-μm-thick paraffin sections were dewaxed in xylene, hydrated and then pretreated with protease to enhance sensitivity (protease type XXIV; Sigma, St. Louis, Mo, USA; 0.1% in Tris-HCl buffer, pH 7.5; for 20 min at 37° C). For the detection of vimentin the protease step was omitted. After incubation with primary antibodies (diluted in PBS containing 1% bovine serum albumin; for specifications of antibodies and dilutions see Table 3) the sections were washed twice in PBS to remove unbound primary antibodies and then incubated with biotinylated antibodies to rabbit, mouse or guinea pig immunoglobulins (dilution 1:100 in 20% human serum-PBS buffer, pH 7.6; Vector Laboratories, Burlingame, Calif., USA) for 30 min at room temperature. After washing in PBS the sections were treated with a preformed avidin-biotin-peroxidase complex (Vector Labs) for 30 min at room temperature. For development of the colour reaction the sections (after rinsing in PBS) were incubated for 5 min with a freshly prepared DAB solution [30 mg 3,3′-diaminobenzidine (DAB) hydrochloride; Serva, Heidelberg, FRG; dissolved in 10 ml 0.05 m Tris-HCl buffer, pH 7.4, containing 0.015% H_2O_2]. The sections were again washed in PBS and subsequently counterstained with haematoxylin, dehydrated in graded ethanol and coverslipped with Eukitt (Kindler, Freiburg, FRG). Tissues containing the respective antigens were included as positive controls. For negative controls, primary antibodies were replaced by normal serum.

For double immunohistochemistry vimentin was detected in the first sequence without protease treatment, using the alkaline phosphatase – antialkaline phosphatase (APAAP) technique with fast blue as substrate for the colour reaction. After completion of the first reaction, the tissue sections were treated with protease for the demonstration of cytokeratin by the ABC technique with amino-ethylcarbazole as substrate for the colour reaction. The incubation steps were performed as follows.

For the first sequence the primary (vimentin) antibody (see Table 3) was diluted with PBS containing 1% bovine serum albu-

Table 1. Summary of the results of single immunohistochemistry (SIH), double immunohistochemistry (DIH) and double immunofluorescence (DIF) in rete testis and epididymis

Case	Age		SIH					DIH		DIF		
			AE	Lu5	CK-D	PKK1	Vim	AE	Vim	CK	DP	Vim
1	Embryo	rete	+	+	+	−	−	ND[a]		ND		
	10 weeks	epid	0	0	0	0	0	0	0	ND		
2	Embryo	rete	+	+	+	−	−	+	−	ND		
	10 weeks	epid	0	0	0	0	0	0	0	ND		
3	Embryo	rete	+	+	+	−	+/−	ND[a]		ND		
	12 weeks	epid	0	0	0	0	0	0	0	ND		
4	Embryo	rete	+	+	+	−	+	ND[a]		ND		
	12 weeks	epid	0	0	0	0	0	0	0	ND		
5	Fetus	rete	+	+	+	−	+	+	+	ND		
	35 weeks	epid	+	+	+	+[b]	+	+	+	ND		
6	3 days	rete	+	+	+	−	+	+	+	ND		
		epid	+	+	+	−	+	+	+	ND		
7	5 days	rete	+	+	+	−	+	+	+	ND		
		epid	+	+	+	−	+	+	+	ND		
8	2 years	rete	+	+	+	+	+	+	+	ND		
		epid	+	+	+	+	+	+	+	ND		
9	4 months	rete			ND			+	+	+	+	+
		epid			ND			+	0	+	+	+
10	32 years	rete			ND			+	+	+	+	+
		epid			ND			+	+/−	+	+	+
11	62 years	rete			ND			+	+	+	+	+
		epid			ND			+	+/−	+	+	+

[a] No material was available for DIH

[b] Only few cells in epididymis displayed positivity

ND, not done; rete, rete testis; epid, epididymis; +, positive reaction; −, negative reaction; 0, no epididymis available; AE, antibody AE1/AE3; Lu5, antibody Lu5; CK-D, antibody CK-D; PKK1, antibody PKK1; Vim, antibody Vim po-1; CK, cytokeratin; DP, desmoplakin

min and the sections were incubated for 1 h. After washing in PBS an affinity-purified mouse anti-rabbit IgG (1:500; Dakopatts Glostrup, Denmark) was used as linking antibody. Then rabbit anti-mouse (pan)Ig (Dakopatts) was used as a second antibody in a dilution of 1:30 in PBS and incubated for 30 min in a humid chamber at room temperature. Prior to the addition of the APAAP complex (Dakopatts) the sections were washed twice in PBS. The APAAP complex (1:100 in PBS) was applied for 30 min and the colour reaction was developed with fast blue B salt [fast blue B working solution: 10 mg naphthol-AS-MX-phosphate (Serva) was dissolved in 0.5 ml *N,N*-dimethylformamide (Serva) and 50 ml Veronal acetate buffer (pH 9.2), then 50 mg fast blue B (C.I.37225;

Table 2. Antibody (AB) preparations, antigen(s) detected, commercial source, animals in which the antibody was created, antibody dilutions and fluorochrome labelling (immunofluorescence microscopy)

AB	Animal	Dilution	Antigen detected	Source
A. Primary antibodies				
AE1/AE3[a]	Mouse	1:2	Cytokeratin, broad spectrum	Hybritech Liege, Belgium
CK-18[a]	Mouse	1:5	Cytokeratin 18	Boehringer Ingelheim, FRG
CK-D[b]	Guinea pig	1:50	Cytokeratins, broad spectrum	Denk et al. (1981)
VIM po-1[b]	Rabbit	neat	Vimentin (from calf lens)	Medac Hamburg, FRG
Vim mo-1[a] clone V9	Mouse	1:10	Vimentin	Dakopatts Glostrup, Denmark
Vim po-2[b]	Rabbit	1:50	Vimentin (from human aorta)	Denk et al. (1981)
Vim mo-2[b]	Mouse	1:20	Vimentin (from pig kidney)	Labsystems Oy Helsinki, Finland
Vim mo-3[b]	Mouse	1:300	Vimentin (from human fibroblasts)	Enzo New York, USA
DP[a]	Mouse	1:10	Desmoplakins I+II	Boehringer Ingelheim, FRG

Antibody	Animal	Dilution	Label	Source
B. Secondary antibodies				
a-mouse IgG+IgM	Goat	1:20	FITC	Medac
a-mouse IgG+IgM	Goat	1:20	Texasred	Medac
a-rabbit Ig	Swine	1:15	FITC	Dakopatts
a-guinea pig Ig	Rabbit	1:15	FITC	Dakopatts

[a] Monoclonal antibody
[b] Polyclonal antibody

Table 3. Antibody (AB) preparations, antigen(s) detected, commercial source, animals in which the antibody was created and antibody dilutions (enzyme immunohistochemistry)

AB	Animal	Dilution	Antigen detected	Source
AE1/AE3[a]	Mouse	1:20	Cytokeratin, broad spectrum	Hybritech Liege, Belgium
Lu5[a]	Mouse	1:500	Cytokeratin broad spectrum	Hoffmann/Roche Basel, Switzerland
CK-D[b]	Guinea pig	1:500	Cytokeratins, broad spectrum	Denk et al. (1981)
PKK1[a]	Mouse	1:200	Cytokeratins of 44, 46, 52, 54, kDa	Labsystems Oy Helsinki, Finland
Vim po-1[b]	Rabbit	1:10	Vimentin (from a calf lens)	Medac Hamburg, FRG

[a] Monoclonal Antibody
[b] Polyclonal antibody

Serva) and 10 mg levamisol (Sigma, St. Louis, Mo., USA) were added. Before use, the solution was adjusted to pH 9.2–9.8 and filtered. The sections were incubated for 15–30 min at room temperature].

In protease treatment, after the substrate reaction with fast blue the sections were washed twice in PBS (10 min) and then treated with pronase as described for single immunohistochemistry.

For the second sequence (demonstration of cytokeratins with the antibody AE1 + AE3) the ABC technique as described for single immunohistochemistry was used, except that the colour reaction was performed with aminoethylcarbazole as substrate instead of DAB [1 mM 3-amino 9-ethylcarbazole (Sigma) in 50 mM acetate buffer, pH 5.0, with 0.015% H_2O_2].

Controls consisted of replacement of the first primary antibody, the second primary antibody, or both primary antibodies by non-immune serum; and omission of the complete first sequence or omission of the complete second sequence.

Single immunofluorescence and double immunofluorescence microscopy was performed using the primary and secondary antibodies listed in Table 2 essentially as described elsewhere (Zatlouk-al et al. 1990). The following combinations were made in double immunofluorescence in the sequence cited (for description and specification of the antibodies see Table 2): (i) AE1/AE3 and Vim po-1; (ii) DP and CK-D; (iii) Vim mo-1 and CK-D. The monoclonal antibody was applied in the first and the polyclonal antibody in the second sequence.

Results

The results of the intermediate filament expression in rete testis and epididymis cells are summarized in Table 1.

In the rete testis cytokeratin filaments are demonstrable in 10-week-old embryos with the monoclonal antibodies AE1/AE3, Lu5 and the polyclonal (pancytokeratin anti CK-D) antibody (Figs. 1a, 2a, 3a). PKK1 showed no reaction with the rete cells in embryonal and fetal testes, but after birth a positive reaction could be noted in single rete cells and small cell groups. No vimentin reactivity was found in the rete cells of embryos of 10 weeks and only a weak vimentin positivity was detected in 12-week-old embryos. In a 35-week-old fetus and in testicles of the perinatal, infantile and adult life period vimentin was consistently expressed as demonstrable with a series of mono- and polyclonal antibodies. The intensity of the reaction and the overall distribution pattern of the different types of intermediate filaments showed differences. The cytokeratin expression was usually stronger than that of vimentin. With regard to the

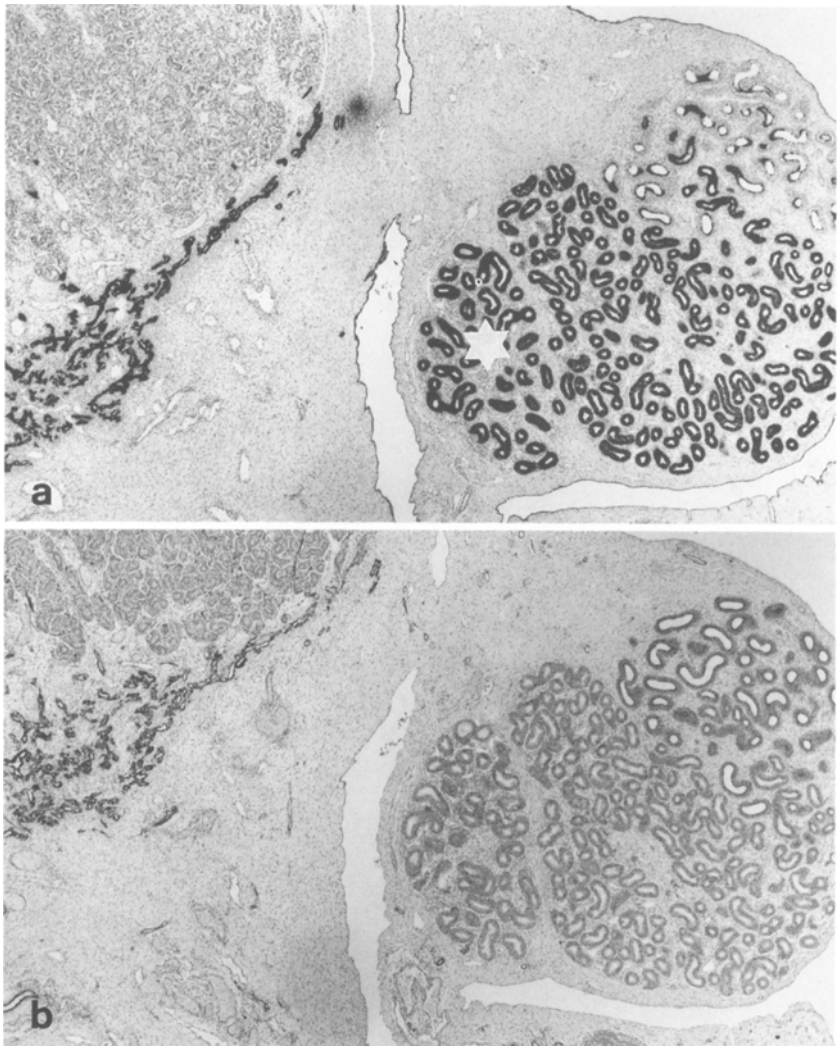

Fig. 1a, b. Case 5 (fetus, 35 weeks of gestation). Rete testis and epididymis. *a* Cytokeratin (AE1/AE3). The strongly cytokeratin positive rete is sharply demarcated from the testis (*upper left*). Near the rete the epididymis (*asterisk*) also strongly displays cytokeratin reactivity; at the periphery of the epididymis (*upper right*) only some groups of cells express cytokeratin. **b** Vimentin (Vim po-1). The rete epithelium displays also strong vimentin reactivity. At the periphery of the epididymis (*upper right*) all cells strongly express vimentin (compare with Fig. 1a). Single immunohistochemistry. **a, b** ABC technique, ×20, hematoxylin counterstain

distribution pattern, in adult testes vimentin was preferentially located at the cell bases, whereas cytokeratin was concentrated at the apical regions (Figs. 2, 4, 5). During embryonal and fetal life and up to 2 years after birth this heterogeneous distribution was less distinct and a rather uniform cytoplasmic co-distribution of cytokeratin and vimentin was observed. In this stage, the rete was mostly made up of solid strands. Desmoplakin immunoreactivity, which was investigated in single immunofluorescence and double immunofluorescence microscopy, was located preferentially at the apical cell borders in the rete epithelium in close association with cytokeratin filaments (Fig. 3a, b).

Tissue samples containing epididymis were only available from the late fetal period upwards (see Table 1). In all cases studied the epithelium of the epididymis expressed both intermediate filament proteins. The cytokeratin and vimentin distribution pattern showed consistent topographical differences. In the neighbourhood of the rete testis the epithelium of the ductuli efferentes displayed a more pronounced cytokeratin reactivity than in the more distal regions. In contrast, the vimentin distribution showed a reversed pattern and was more pronounced in the distal parts of the epididymis (ductus epididymidis) than in those close to the rete (Fig. 1a, b). The intracellular distribution of the intermediate filaments was similar to that in the rete cells with a tendency to a concentration of cytokeratin in the apical cell portions and of vimentin in the basal parts of the cells (Figs. 4–6). In ductus deferens cytokeratin clearly dominated. Desmoplakin-reactive spots were more concentrated in the apical regions of epithelial cells, particularly in those areas in which cytokeratin was also expressed.

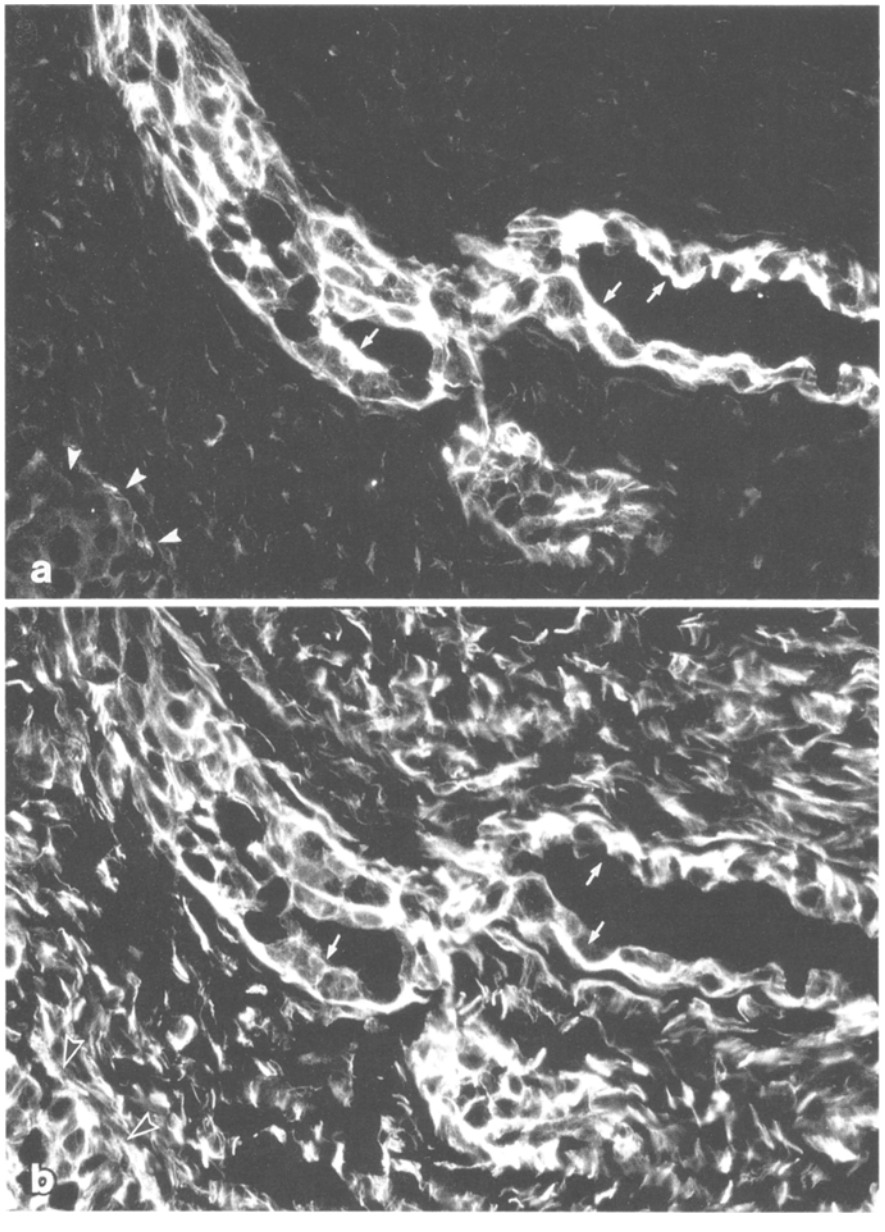

Fig. 2a, b. Case 8. Double immunofluorescence microscopy using AE1/AE3 and Vim po-1 antibodies in rete testis. **a** Demonstration of cytokeratin (AE1/AE3); secondary antibody labelled with Texas red. The rete testis is lined by strongly cytokeratin-positive epithelium. The apical portions of the epithelium (*arrows*) show a stronger reaction for cytokeratin than for vimentin (heterogeneous co-expression of cytokeratin and vimentin; compare with Fig. 2b). Note the absence of any reaction in the interstitial tissue. **b** Demonstration of vimentin (Vim po-1); secondary antibody labelled with FITC. Note strong positivity of the rete epithelium for vimentin. Preferentially the basal cell portions (*arrows*) react with the vimentin antibody. Note also the strong positivity of the mesenchymal cells. In the left lower corner (*arrowheads* in **a** and **b**) a seminiferous ductule with only vimentin-positive cells (Sertoli cells) is shown. **a, b,** ×400

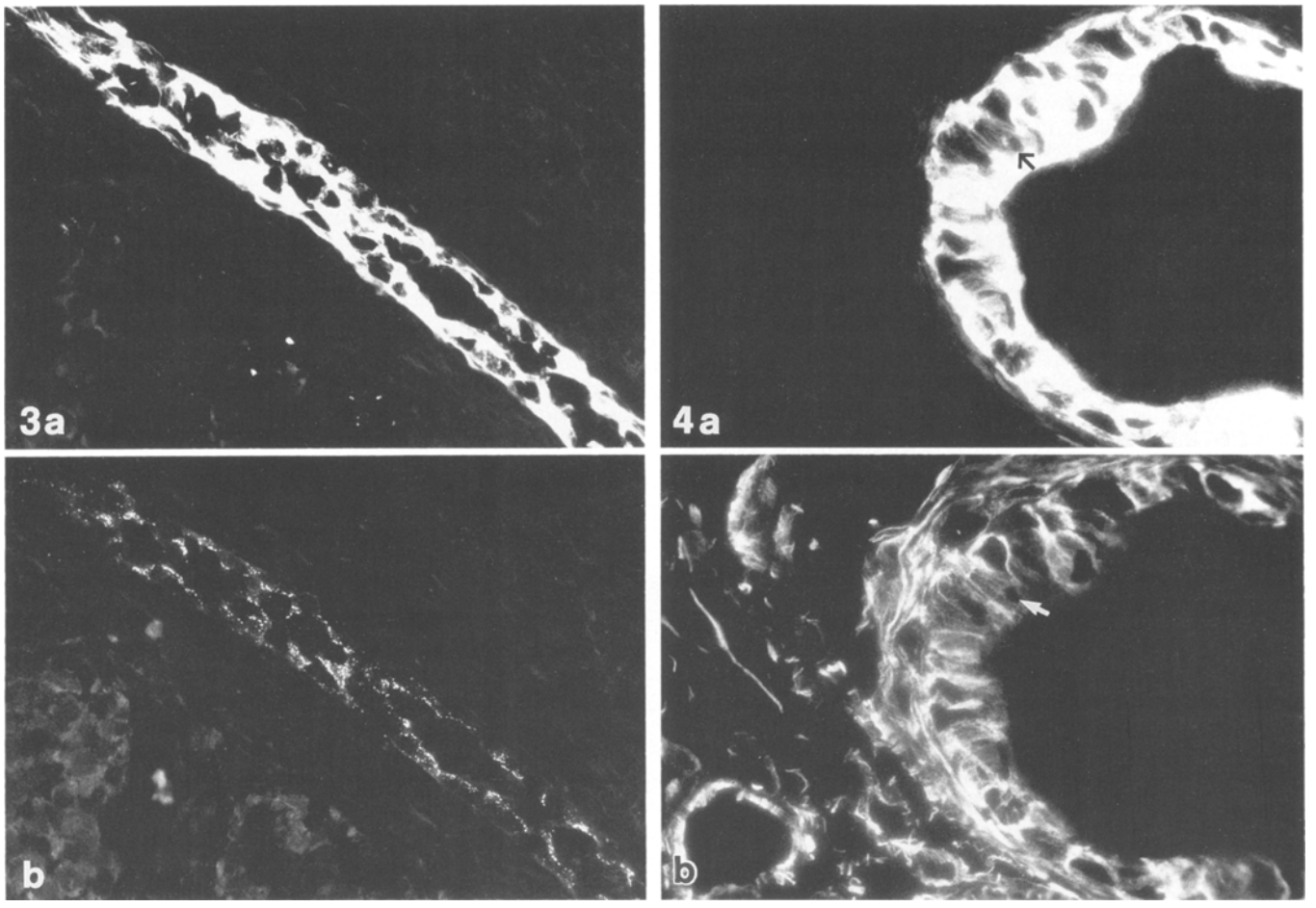

Fig. 3a, b. Case 8. Double immunofluorescence microscopy using AE1/AE3 and desmoplakin antibodies in rete testis. **a** Demonstration of cytokeratin (AE1/AE3); secondary antibody labelled with FITC. A single rete tubule is lined by epithelium with strong positivity for cytokeratin. **b** Demonstration of desmoplakins; secondary antibody labelled with Texas red. Preferentially the apical portions of the epithelium (where desmosomes are arranged in higher concentration) display a strong positivity for desmoplakins. **a, b,** × 400

Fig. 4a, b. Case 8. Double immunofluorescence microscopy using AE1/AE3 and Vim po-1 antibodies in epididymis. **a** Demonstration of cytokeratin (AE1/AE3); secondary antibody labelled with FITC. The epithelium of the ductuli efferentes displays strong and uniform cytokeratin reactivity. Note the filamentous reaction pattern (*black arrow*). **b** Demonstration of vimentin (Vim po-1); secondary antibody labelled with Texas red. Strong positivity for vimentin preferentially at the basal portions of the epididymal epithelium. Note the filamentous reaction pattern (*white arrow*) and the difference with respect to the distribution of keratin and vimentin filaments within a given cell (compare **a** with **b**). Connective tissue cells also intensely react with vimentin antibodies. **a, b,** × 630

Discussion

Using double immunohistochemistry on paraffin sections and double indirect immunofluorescence on cryocut sections we demonstrated co-expression of cytokeratin and vimentin intermediate filaments in the same cells in the rete testis and also in the epididymis. In the rete testis nearly all cells displayed co-expression, whereas in the epididymis a more diversified co-expression could be found in different parts of the organ with a predominance of cytokeratin near the rete. Whereas cytokeratin was strongly expressed in cells of the rete anlage of embryos, vimentin positivity was only weak in the late embryonal stage but became clearly positive in the late fetal period. The distribution pattern of cytokeratin and vimentin also differed in different developmental stages of the rete testis: during the embryonal and fetal periods co-distribution of both intermediate filament proteins could be found in sections of paraffin-embedded tissue. In the rete epithelium of adult men the distribution pattern of the intermediate filament proteins was clearly different, which could be best shown in double immunofluorescence, where the filamentous structure of these proteins was well preserved.

In a study on the intermediate filament constituents of testicular tumours, Ramaekers et al. (1985) also found a co-expression of cytokeratin and vimentin in rete testis epithelial cells. This observation, however, is in contrast to the findings of Achtstätter et al. (1985) who detected cytokeratins exclusively in rete testis by immunofluorescence and gel electrophoretic techniques. Miettinen et al. (1985) also found cytokeratins as the only intermediate filament proteins in rete testis using frozen and ethanol-fixed paraffin-embedded material. However, Czerno-

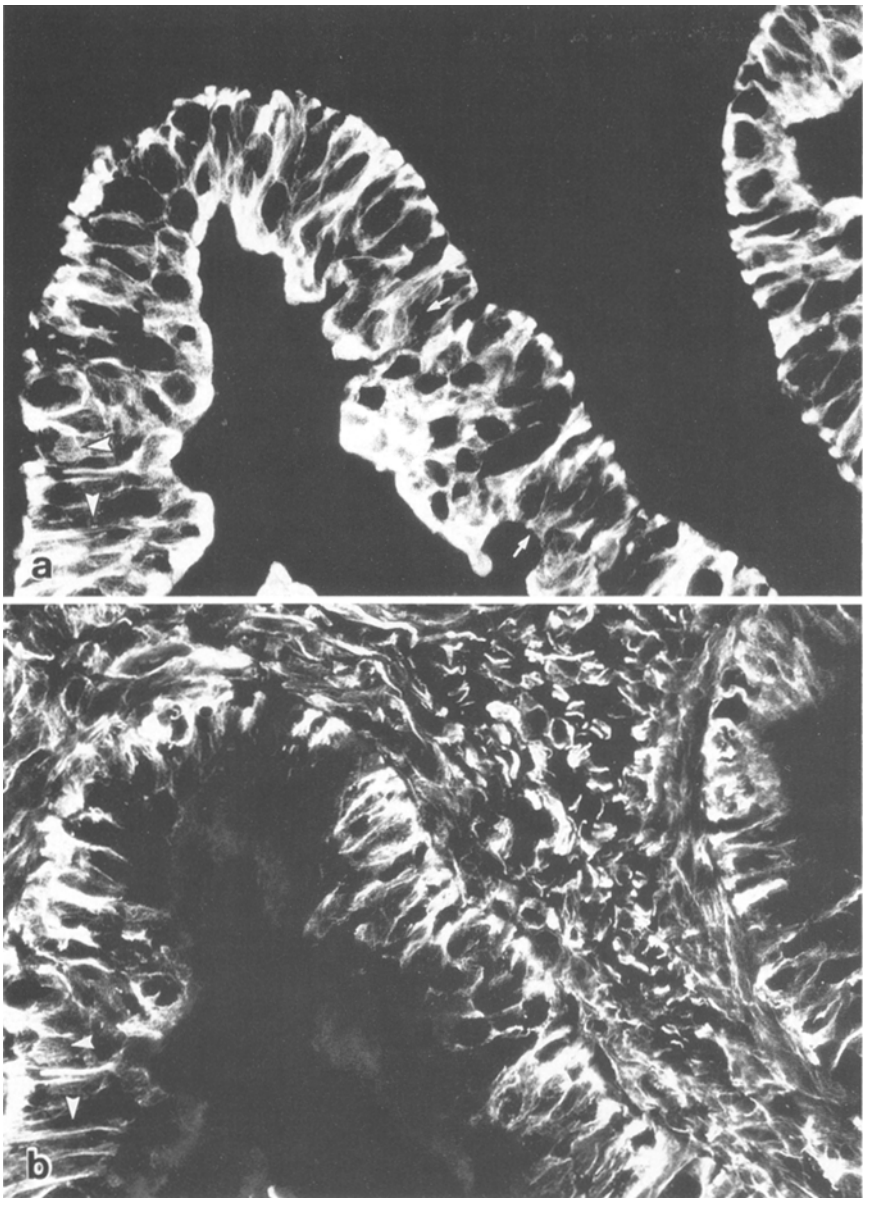

Fig. 5a, b. Case 10. Double immunofluorescence microscopy using AE1/AE3 and Vim po-1 antibodies in epididymis. **a** Demonstration of cytokeratin (AE1/AE3); secondary antibody labelled with FITC. The epithelial lining of an epididymal tubule displays a uniform strong positivity for cytokeratin. Many cells show a filamentous reaction pattern (*arrows and arrowheads*). **b** Demonstration of vimentin (Vim po-1); secondary antibody labelled with Texas red. Epithelial lining of epididymal tubule with strong positivity for vimentin, preferentially at the basal portions of the cells. *Arrowheads* in **a** and **b** indicate identical cells. **a, b**, ×630

bilsky et al. (1985) reported co-expression of cytokeratin and vimentin in the rete ovarii cells which is consistent with our findings of co-expression in the epithelial cells of the rete testis and in epididymis. However, the distribution of both types of intermediate filaments within the cells was different: in our studies cytokeratin (and desmoplakin) staining was concentrated near the luminal portions of the cells, whereas vimentin expression preferred the basal regions of the cells. Presence of cytokeratin and vimentin in rete testis and ovarii is not surprising since rete testis and rete ovarii are analogous structures, both representing remnants of the mesonephric tubules (Byskov 1978).

The co-expression of cytokeratin and vimentin in rete cells has some implications for the diagnostic work in histopathology: tumours derived directly from the rete testis – such as the very rare carcinoma of the rete – should co-express cytokeratin and vimentin. Due to the fact that tumours derived from mesonephric tubules, from sex cord stroma (Miettinen et al. 1983) and from the multipotent müllerian duct epithelium show a co-expression of cytokeratin and vimentin, histogenetic distinctions between tumours derived therefrom cannot be made on the intermediate filament composition of the tumour cells.

With single immunohistochemistry and double immunohistochemistry on paraffin sections the immunopositive structures were more easily characterized with respect to their structure of origin. Consequently, a clear-cut distinction could be made between ductuli efferentes and ductus epididymidis.

As already noted by Czernobilsky et al. (1985), the distribution pattern of cytokeratins differed from that of vimentin. This is in line with the assumption that

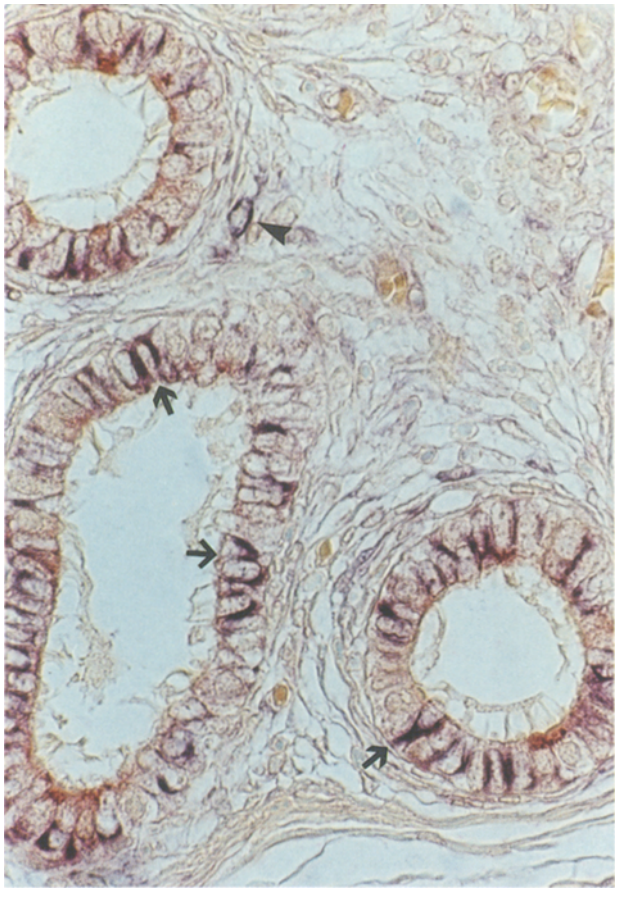

Fig. 6. Case 9. Double staining with AE1/AE3 (brown reaction product) and VIM po-1 (blue reaction product) in epididymis. The cytoplasm of the cells of ductuli efferentes displays a blue (vimentin positive) to dark brown-violet colour (co-expression of vimentin and cytokeratin; *arrows*). Cytokeratin reactivity (brown colour) is restricted preferentially to the apical portions of the cells. Within the connective tissue vimentin-positive cells are present (e.g. *arrowhead*). × 400

in rete cells cytokeratin and vimentin occur in separate intermediate filaments (Czernobilsky et al. 1985).

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