Virchows Archiv A Pathol Anat (1992) 421:163–169

Virchows Archiv A
Pathological Anatomy and Histopathology

Testicular sex cord-stromal lesions: immunohistochemical analysis of cytokeratin, vimentin and steroidogenic enzymes

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Received December 13, 1991 / Received after revision April 6, 1992 / Accepted April 7, 1992

Summary. We have studied immunolocalization of all steroidogenic enzyme involved in sex steroids biosynthesis, P-450 side chain cleavage (P-450scc), 3β hydroxy steroid dehydrogenase (3β-HSD), P-450 17α hydroxylase ($P\text{-}450_{17\alpha}$) and P-450 aromatase (P-450arom) and that of vimentin and cytokeratin in 14 cases of testicular sex cord-stromal tumours (6 Leydig cell tumours, 5 Sertoli cell tumours, 2 fibromas and 1 granulosa cell tumour) as well as 4 cases of hyperplasia (2 Leydig and 2 Sertoli). Leydig cell tumour expressed all four steroidogenic enzymes examined, indicating that this tumour can synthesize oestrogen from cholesterol. In 2 cases of Sertoli cell tumour, the tumour cells with clear cytoplasm and without Reinke's crystals expressed $P\text{-}450_{ssc}$, 3β-HSD and $P\text{-}450_{17\alpha}$, suggesting the capability of androgen production in these tumour cells. Fibromas and granulosa cell tumour were negative for the enzymes examined. In immunohistochemistry of intermediate filaments, Leydig cell tumours demonstrated only vimentin. Sertoli cells in hyperplasia and non-neoplastic testis expressed only vimentin but Sertoli cell tumours expressed both cytokeratin and vimentin. Cytokeratin immunoreactivity was correlated with morphological epithelial differentiation in Sertoli cell tumour. These findings in testicular Sertoli cell tumour are considered to represent the multiple differentiation capacity of this neoplasm. Immunohistochemical study of steroidogenic enzymes and intermediate filaments provided new insight into neoplastic steroidogenesis and the differentiation capacity of testicular sex cord-stromal neoplasms.

Key words: Testis – Sex cord-stromal tumors – Immunohistochemistry – Steroidogenesis – Intermediate filaments

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Introduction

Gonadal stromal tumours are rare, with a reported incidence of 2–4% of all testicular tumours in the general population (Mustofi and Price 1987). The majority of these tumours are largely composed of Leydig cell tumours; pure Sertoli and granulosa cell tumours are relatively rare but admixture of these cell types is frequently observed. A majority of testicular sex cord-stromal tumours of the testis are known to be associated with sex-steroid abnormalities (Hatakeyama 1990). These tumours are believed to arise from the specialized testicular stroma which supports and interacts with germ cells but controversies exist as to the exact origin of these testicular tumours (Evans and Glick 1977; Greco et al. 1984; Miettinen et al. 1986; Düe et al. 1990), especially with relation to epithelial differentiation (Miettinen et al. 1986). Therefore, in order to obtain a better understanding of neoplastic steroidogenesis, it is necessary to correlate it with complicated morphological features, so to determine which tumour cells produce what kinds of steroid hormones. In addition, it is important to examine the expression of markers for cellular differentiation in these neoplasms in order to understand their differentiation capacity.

The rarity of these neoplasms has precluded systematic study of the pathological, immunohistochemical and hormonal study of testicular sex cord-stromal tumours. Therefore, in this report, we have studied the immunohistochemical localization of all stereoidogenic enzymes involved in testicular sex steroid biosynthesis for the purpose of localizing the sites of specific steroid hormone production in 14 cases of the tumour and 4 cases of hyperplasia (2 Sertoli and 2 Leydig) contributed by different institutions in Japan. In addition, immunolocalization of cytokeratin and vimentin was performed in order to examine the differentiation capacity of the tumour. This is the first reported immunohistochemical study of a series of testicular sex cord-stromal lesions.

Table 1. Summary of clinical and histopathological data and immunohistochemical findings

Case no.	Histological diagnosis	Age	Initial symptoms	Size (cm)	Adjacent testis		Immunohistochemistry					
					Leydig	Sperm	scc	3β	17α	aro	CK	vim
1	Leydig cell tumour	4	Mass	5	↓	↓	+	+	+	+	−	−
2	Leydig cell tumour	77	Mass	2	↓	↓	+	+	+	+	−	+
3	Leydig cell tumour	51	Mass	4×3×2.5	N	↓	+	+	+	+	−	+
4	Leydig cell tumour	77	Mass	6×4×4	N.A.	N.A.	+	+	+	+	−	−
5	Leydig cell tumour	14	Mass	N.A.	↓	Sertoli only	+	+	+	+	−	+
6	Leydig cell tumour	62	Mass	N.A.	N	↓	+	+	+	+	−	+
7	Sertoli cell tumour	53	Mass	6×3.5×1	↓	↓	+	−	−	−	+	+
8	Sertoli cell tumour	17	Mass	8	N	↓	−	−	−	−	+ (f)	+ (f)
9	Sertoli cell tumour	50	Mass	N.A.	N	A	−	−	−	−	+ (f)	−
10	Sertoli cell tumour	3M	Scrotal swelling	3	Immature		+	+	+	−	−	+
11	Sertoli cell tumour	58	Mass	1×8×8	N	A	+	+	+	−	+	+
12	Fibroma	57	Mass	3	N.A.	N.A.	−	−	−	−	−	+
13	Fibroma	57	Mass	5	N.A.	N.A.	−	−	−	−	−	+
14	Granulosa cell tumour	42	Mass	6.5×5×4	N	A	−	−	−	−	+ (f)	−
15	Leydig cell hyperplasia	30	Azospermia	Biopsy	−	Sertoli only	+	+	+	−	−	−
16	Leydig cell hyperplasis	45	Crypto orchism	5	−	A	+	+	+	+ (f)	−	−
17	Sertoli cell hyperplasia	22	TF	(R)4 (L)3.5	−	none	−	−	−	−	−	+
18	Sertoli cell hyperplasia	22	TF	(R)3 (L)2	−	none	−	−	−	−	−	+

TF, Testicular feminization syndrome; NA, data not available; A, active spermatogenesis; N, normal; ↓, decreased; scc, P-450 (side chain cleavage); 3β, 3β-hydroxysteroid dehydrogenase; 17α, P-450 17α hydroxylase; aro, P-450 aromatase; CK, cytokeratin; Vim, Vimentin; (f), focal

Materials and methods

Cases studied in this series were 6 Leydig cell tumour, 5 Sertoli cell tumour, 2 fibroma, 1 granulosa cell tumour, 2 Leydig cell hyperplasia and 2 Sertoli cell hyperplasia associated with testicular feminization. They were all retrieved from surgical pathology files. Leydig cell hyperplasia and neoplasia were differentiated based on the fact that the hyperplastic cells fill and distend the pre-existing intertubular spaces without destroying or displacing the tubules (Mostofi and Price 1987). Histopathological classification of the neoplasms was based on that of Mostofi and Price (1987). Age, size and initial symptoms of these cases of testicular lesions obtained from medical records of the patients, when available, are summarized in Table 1. Due to the retrospective nature of the study, clinical endocrine data were usually not available. Adjacent non-neoplastic testis was available for histopathological examination in 15 cases. Histopathological findings of adjacent testis are summarized in Table 1.

Routinely processed formalin-fixed and paraffin-embedded tissues were processed to sections of 2.5 μm thick and mounted on clean glass slides. For immunohistochemistry, deparaffinized paraffin sections were put into methanol containing 0.3% hydrogen peroxide for 30 min in order to block endogenous peroxidase activity. They were washed in three changes of 0.01 M phosphate buffered saline (PBS), pH 7.2 for 5 min each and treated with normal goat or rabbit serum for 30 min at room temperature. Immunohistochemical staining procedure employed in this study was the biotin-streptavidin amplified method using the Histofine immunostaining system (Nichirei, Tokyo, Japan). After washing with 0.01 M PBS, sections were treated sequentially with primary antibodies, for 18 h at 4° C in a moist chamber, and with biotinylated goat anti-mouse or anti-rabbit immunoglobulin and peroxidase-conjugated streptavidin, each for 30 min at room temperature in a moist chamber. The characteristics of the primary antibodies employed in this study are summarized in Table 2. Sections were washed with cold PBS between incubations. A final wash was followed by immersion of the reacted sections for 5–10 min in a solution containing 0.05% TRIS-HCl pH 7.6, 0.66 mM 3,3′-diaminobenzidine and 2 mM hydrogen peroxide. Specific staining was identified by the presence of brown reaction products. The reacted sections were finally counterstained with 1% methyl green and mounted with a glycerol-gelatin water-soluble medium. Control

Table 2. List of primary antibodies

Antibody	Animal	Antigen	Dilution	Reference
P-450scc (side-chain cleavage)	Polyclonal (rabbit)	Bovine adrenal	1:500	Sasano et al. (1989a)
3β-HSD (hydroxysteroid dehydrogenase)	Polyclonal (rabbit)	Human placenta	1:350	Sasano et al. (1990a) Lorence et al. (1990)
$P\text{-}450_{17\alpha}$ (17α hydroxylase)	Polyclonal (rabbit)	Pig testis	1:300	Sasano et al. (1989b, c) Mason et al. (1986)
$P\text{-}450_{arom}$ (aromatase)	Monoclonal (mouse)	Human placenta	1:400	Sasano et al. (1989d) Mendelson et al. (1985)
Cytokeratin (KL-1)	Monoclonal (mouse)	Epidermal keratinocytes	1:50	Immunotech S.A. (Luminy, France)
Vimentin (clone V9)	Monoclonal (mouse)	Purified vimentin	1:1	Boehringer Mannheim Biochemica

sections were incubated with normal rabbit immunoglobulin for polyclonal antibodies and 0.01 M PBS for monoclonal antibodies instead of primary antibodies. Immunoreactivity observed was not present in these sections.

Results

Clinical, histopathological and immunohistochemical findings are summarized in Table 1. Patients' ages ranged from 3 months to 77 years. The initial clinical manifestation was a testicular mass in all the cases of tumour examined. Sertoli cell hyperplasia was observed in the intra-abdominal testis of patients who clinically manifested testicular feminization. Serum androgens and urinary 17-ketosteroids were examined in 5 cases (cases 1, 2, 5, 8 and 13) and all were within normal limits except for case 1 which showed slightly elevated 17-ketosteroid in 24 h urine with moderately increased levels of serum androgens. The size of the tumours ranged from 2 to 10 cm in greatest dimension. No foci of haemorrhage or confluent necrosis were observed in any of the cases examined. In Sertoli cell tumours in this series, the arrangement of the Sertoli cells varied, from well-differentiated tubular and/or cord-like structures to spindle-shaped cells. In cases 10 and 11, tumour cells with clear and/or vacuolated cytoplasm without Reinke's crystals were present between the tubules and bundles of spindle-shaped cells (Fig. 1).

Leydig cells were positive for all steroidogenic enzymes including P-450 aromatase ($P\text{-}450_{arom}$) in all Leydig cell tumours (Fig. 2). Tumour cells with clear and/or vacuolated cytoplasms in Sertoli cell tumours as shown in Fig. 1 were strongly positive for P-450 side chain cleavage ($P\text{-}450_{scc}$), 3β hydroxy steroid dehydrogenase (3β-HSD), and P-450 17α hydroxylase ($P\text{-}450_{17\alpha}$) (Fig. 3). These cells in Sertoli cell tumour were negative for $P\text{-}450_{arom}$. Other types of tumour cells in Sertoli cell tumour were negative for the enzyme, except for case 7, which showed $P\text{-}450_{scc}$ in the tumour cells forming tubular structures. Fibromas and granulosa cell tumour examined in this study were negative for steroidogenic enzymes.

Cytokeratin positivity was observed in 4 cases of Sertoli cell tumour. Immunoreactivity was present in the tubular epithelial-like tumour cells (Fig. 4), but also in the round or elongated tumour cells, which did not show the structures mentioned above. Cells positive for cytokeratin sometimes correspond to poorly differentiated tubular structures which were not otherwise conspicuous. Cytokeratin was negative in all the cases of Leydig cell tumour. Cytokeratin immunoreactivity was observed focally in granulosa cell tumour. Cytokeratin was negative in fibroma. Vimentin was positive in 5 cases of Leydig cell tumour and 2 cases of Leydig cell hyperplasia. Prominent vimentin immunoreactivity was observed in Sertoli cell hyperplasia and in seminiferous tubules of non-neoplastic testis and Leydig cell hyperplasia. Vimentin was positive in almost all the spindle-shaped tumour cells but focally in the tubular and/or epithelial like tumour cells in Sertoli cell tumours. Tumour cells with clear cytoplasm without Reinke's crystals seen in Sertoli cell tumours were positive for vimentin but negative for cytokeratin.

Discussion

Testicular sex cord-stromal tumours are very rare but a large number of pathologists and urologists have been interested in these neoplasms because of their endocrine features or ability to produce various sex steroids (Ga-

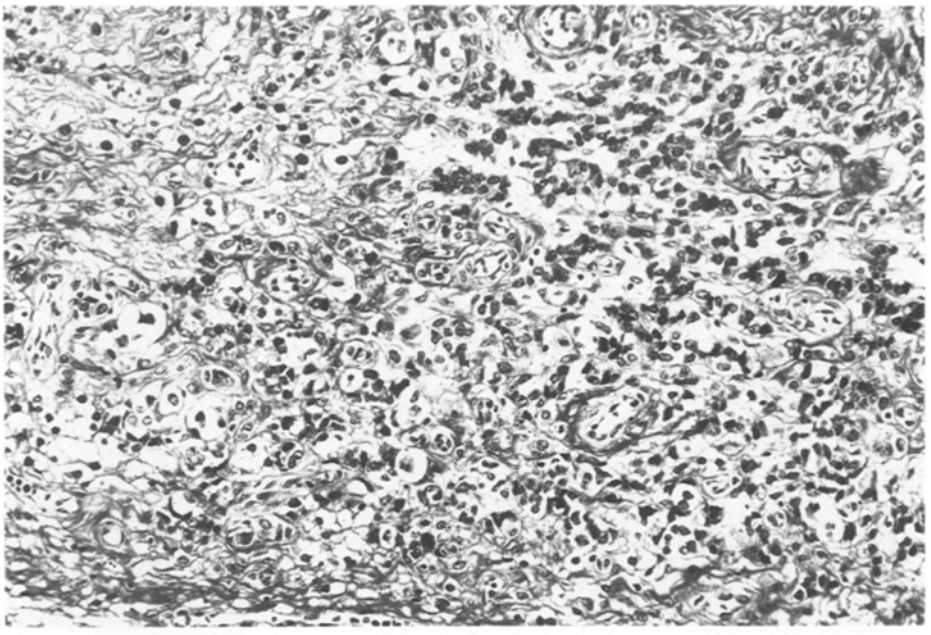


Fig. 1. Sertoli cell tumour (case 11). Tumour cells with clear cytoplasm and vesicular nuclei were observed. Haematoxylin and eosin, ×300

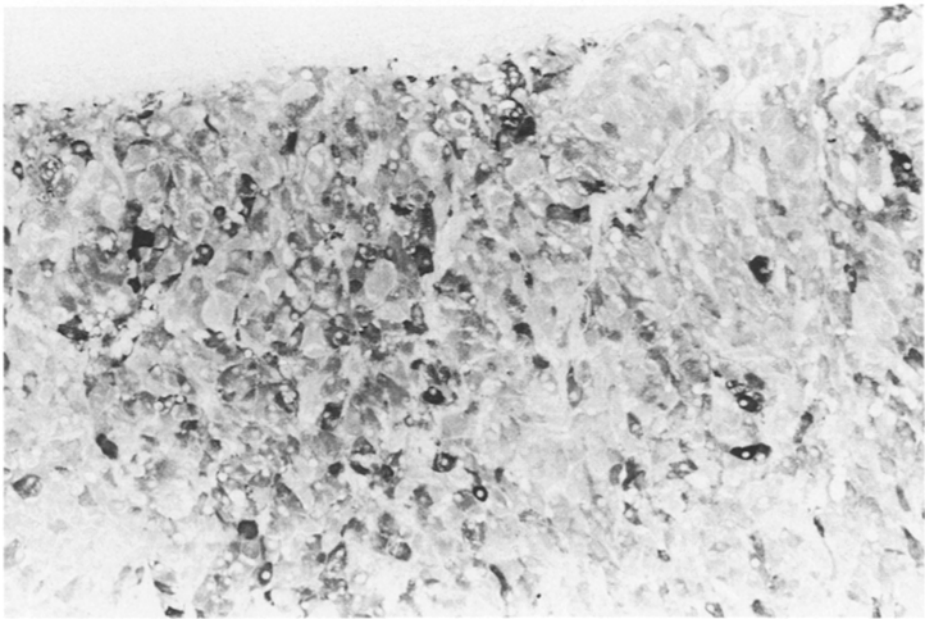


Fig. 2. Immunohistochemistry of 3β-hydroxysteroid dehydrogenase in Leydig cell tumour (case 4). ×200

brilove et al. 1975; Caldamone et al. 1979; Kaplan et al. 1986; Hatakeyama 1990) and their multiple differentiation capacity. Testicular sex cord-stromal lesions have been reported to be associated with various clinical endocrine abnormalities and biochemical studies also confirmed steroidogenic capability of Leydig cell tumour of the testis (Mostofi and Price 1987). Therefore, it is important to correlate diverse histological patterns in the testicular sex cord-stromal lesions with sex steroid biosynthesis. So doing it is possible to study which cells are responsible for producing what steroids, and thus to gain a better understanding of neoplastic steroidogenesis.

Various attempts have been made to study steroidogenesis on tissue sections of testicular stromal lesions. Electron microscopic examination revealed well-developed smooth endoplasmic reticulum and mitochondria with tubular cristae in Leydig cells or in cells resembling luteinized stromal cells without Reinke's crystals (Dadoune et al. 1967). However, these observations are still non-specific and at best provide indirect evidence of steroid production or steroidogenic capacity, and cannot demonstrate what types of hormones are produced in what types of tumour cells. Attempts have been made to identify steroid hormone production by both histochemistry of the enzymes and immunohistochemistry of steroids. Histochemical studies in testicular sex cord-stromal lesions include intracytoplasmic demonstration of the lipid, which may weakly suggest, but by no means indicate, the presence of steroidogenesis and demonstration of certain enzymes, such as β-hydroxybutyric acid dehydrogenase, which is known to play a role in the formation of testosterone (Jones et al. 1967). Despite these attempts, histochemical studies of the steroidogen-

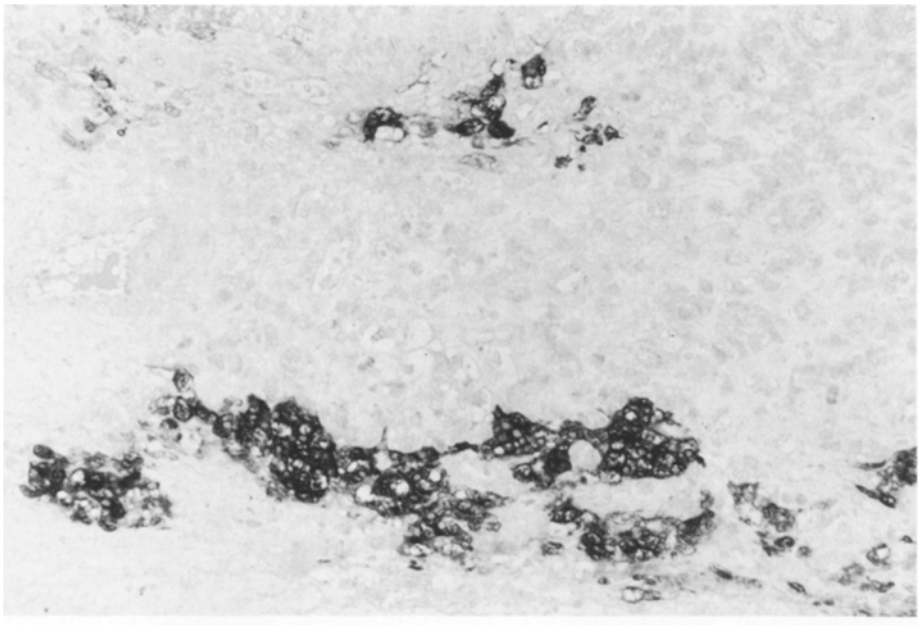


Fig. 3. Immunohistochemistry of P-$450_{17\alpha}$ (17α-hydroxylase) in Sertoli cell tumour (case 11). The same case as in Fig. 1. $\times 300$

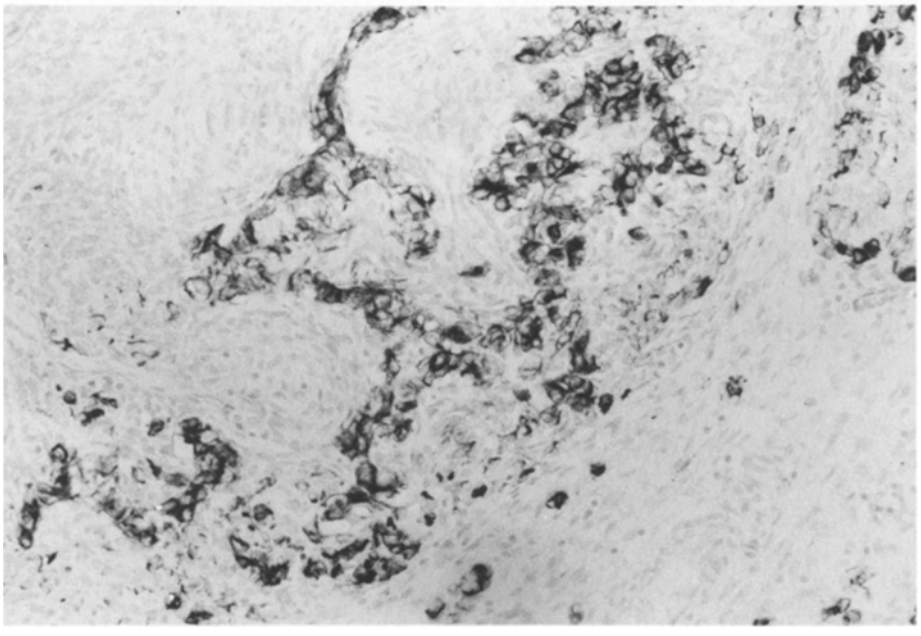


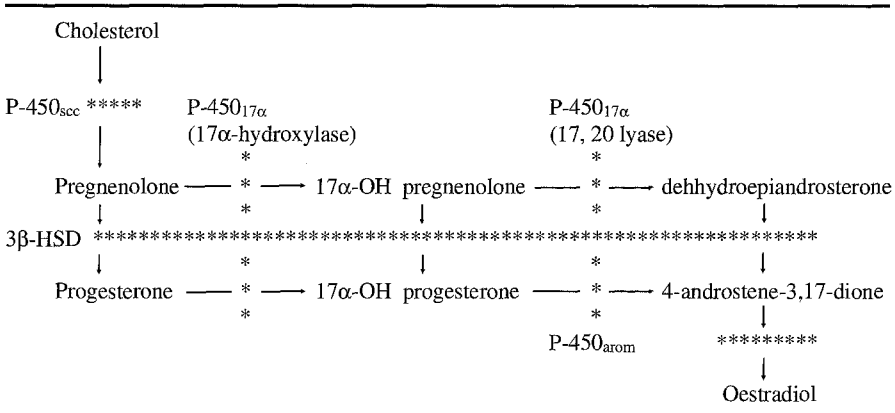
Fig. 4. Cytokeratin immunohistochemistry in Sertoli cell tumour (case 7). Immunoreactivity was present in the tumour cells forming epithelial like structure, $\times 200$

ic enzymes are well known to be associated with various methodological difficulties, and interpretation of results can be very difficult (Sasano 1975; Ishimura et al. 1988). With the development of antibodies against specific steroid hormones, immunohistochemical studies of sex steroids were reported in testicular Sertoli-Leydig cell tumour (Kurman 1978). However, immunohistochemical study of steroids involves numerous technical problems (Sasano et al. 1990a). In addition, it is generally not known whether antisteroid antibodies recognize the hormones in the cells in which the steroids are synthesized, stored or bound to receptor (Kurman et al. 1984).

Attempts to develop methods directed specifically toward staining steroids or toward histochemical activities of the enzymes, therefore, subsequently gave way to techniques directed toward illustrating the enzyme systems specifically involved in steroidogenesis. Sasano and Sasano (1990) demonstrated immunolocalization of all the steroidogenic enzymes involved in adrenocortical steroidogenesis in human adrenal and its disorders and normal ovary (Sasano et al. 1989d, 1990b) and ovarian sex cord-stromal tumour (Sasano et al. 1989e, 1990a). Immunolocalization of steroidogenic enzymes can demonstrate expression of the enzymes directly in formalin-fixed paraffin-embedded specimens and is currently considered as the most suitable morphological technique to study human steroidogenesis. However, when formalin-fixed and paraffin-embedded blocks are collected from different institutions, as in this study, the results must be interpreted with caution because of possible differences in the type and mode of fixation.

In our present study of immunolocalization of steroidogenic enzymes in testicular sex cord-stromal lesions, Leydig cell tumour exhibited expression of all the en-

Table 3. Steroid synthetic pathway



zymes involved in the synthesis of androgen and oestrogen from cholesterol (Table 3). This result indicates that Leydig cell tumour can produce oestrogens and androgens from cholesterol by itself. The finding is also consistent with biochemical studies of various steroidogenic enzyme activities in Leydig cell tumours (Mostofi and Price 1987). This finding in Leydig cell tumour is very similar to that of steroid cell tumour not otherwise specified of the ovary (Sasano et al. 1989d, 1990b) and suggests that steroid cell tumour not otherwise specified, although Reinke's crystals are not observed, has steroidogenic characteristics similar to testicular Leydig cell tumour. Absence of immunoreactivity of steroidogenic enzymes in fibroma and granulosa cell tumour of the testis is consistent with the findings in ovarian counterparts of these neoplasms (Sasano et al. 1989d, 1990b). In Sertoli cell tumour, tumour cells resembling luteinized cells or cells with clear and vacuolated cytoplasm have been reported to be present, sometimes admixed with spindle-shaped stromal cells (Mostofi and Price 1987). These cells do not usually have crystalloid of Reinke and pigments which are frequently observed in Leydig cells. Immunolocalization of steroidogenic enzymes in these cells in Sertoli cell tumour is similar to that of Leydig cell tumour but P-450$_{arom}$, which converts adrogens to oestrogens, was negative in contrast to Leydig cell tumour. Therefore, these cells observed in Sertoli cell tumour are considered to have the capacity to produce sex steroids, at least up to androgens. Interestingly, immunohistochemical patterns of expression of steroidogenic enzymes in these cells, i.e. the presence of P-450$_{scc}$, 3β-HSD and P-450$_{17\alpha}$ and the absence of P-450$_{arom}$, are similar to thecomatous cells in typical thecoma and luteinized tumour cells in luteinized thecoma in the ovary (Sasano et al. 1989d, 1990b). Further investigations will be necessary to clarify the similarity of steroidogenesis between the two cell types of these gonadal neoplasms.

Gonadal sex cord-stromal tumours are known to have multiple differentiation potential and many differentiation markers have been applied for immunohistochemical study. Among these markers, antibodies to intermediate filament proteins have been widely and successfully applied in the characterization of the cellular nature of ovarian sex cord-stromal tumours (Miettinen et al. 1983, 1985; Aguirre et al. 1986; Benjamin et al. 1987; Saitoh et al. 1989; Lastarria et al. 1990). However, immunohistochemical study of intermediate filaments in testicular sex cord-stromal tumour has not been reported except for the report of Miettinen et al. (1986) who studied one case of spindle cell testicular stromal tumour. In our present study, Leydig cell tumours expressed only vimentin and Sertoli cell tumour expressed both cytokeratin and vimentin. In Sertoli cell tumour, as was expected, the degree of cytokeratin expression appeared to correlate with epithelial differentiation of the tumour. Cytokeratin was also positive in the tumour cells with subtle and minimum epithelial differentiation. These results are consistent with the report of immunohistochemistry of intermediate filaments on ovarian sex cord-stromal tumours described above and that on fetal testis (Benjamin et al. 1987). In our study, Sertoli cells associated with Sertoli cell hyperplasia as well as Sertoli cells in the seminiferous tubules of non-neoplastic testis examined demonstrated only vimentin positivity, which is consistent with the report of Franke et al. (1979). Therefore, the presence of cytokeratin and the occcasional absence of vimentin in the tumour cells forming epithelial or cord-like structures in testicular Sertoli cell tumour may be consistent with the multiple differentiation capacity of the neoplasm as observed in ovarian Sertoli cell tumour (Zaloudek and Norris 1984; Aguirre et al. 1986).

In conclusion, antibodies to steroidogenic enzymes successfully localized the sites of specific steroid hormone production and antibodies to vimentin and cytokeratin revealed differences in their expression in testicular sex cord-stromal lesions. These findings shed new insights into the biological characteristics of these rare but interesting testicular neoplasms.

Acknowledgements. The authors are grateful to Drs. Shigeru Arai (Yamagata University School of Medicine, Yamagata), Masayuki Fukase (Shounai Hospital, Tsuruoka), Teiichi Motoyama (Niigata University School of Medicine, Niigata), Etsuo Okazaki (Niigata City Hospital, Niigata), Shizaki Saiki (Seiroka Kokusai Hospital, Tokyo), Toshiaki Manabe (Kawasaki Medical College, Kurashiki) and Kazunori Miura (Kumamoto National Hospital, Kumamoto) for having provided the cases for our study.

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