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Steroid Sulfatase Appearance in Kidneys with or without Renal Cell Carcinoma: Examined by Immunohistochemistry, Enzyme-histochemistry, and *in situ* Hybridization

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Purpose: Steroid hormones are known to be potential endogenous carcinogens. Estrogen receptors have been identified in the kidney, raising unresolved question about involvement of steroid sulfatase (STS), an enzyme that converts soluble estrogen (E1S) to active estrogen (E2), in induction of renal cell carcinoma (RCC). We studied the renal distribution of STS activity, proteins and mRNA in surgical and autopsy specimens with or without renal cell carcinoma. Materials and methods: Immunohistochemical staining for STS and *in situ* hybridization for the corresponding mRNA were performed. The STS enzyme activity was assessed by ultrastructural histochemistry after incubation in 4-methylumbelliferyl sulfate. Results: Immunoreactivity for STS protein was seen in nontumor tissue adjacent RCC and showed some weak staining in tumor tissue. The ultrastructural reaction product which indicates enzyme activity was not present in tumors but was seen in adjacent tissue mainly in the endoplasmic reticulum of proximal tubule cells. *In situ* hybridization demonstrated STS mRNA primarily in proximal tubules of renal tissue adjacent to tumors but not in tumor tissue. Conclusion: STS is synthesized in the proximal tubules of the human kidney, and augmented STS activity may be related to induction of RCC.

Introduction

Steroid sulfatase (STS) is a microsomal enzyme catalyzing hydrolysis of 3β-hydroxysteroid sulfates, including dehydroepiandrosterone sulfate, cholesteryl sulfate, and sulfated forms of estrogen[1]. The STS gene is located on the short arm of the X-chromosome at a telomeric site, and escapes X-inactivation[2]. One of the important physiologic functions of STS is conversion of the transport forms to their active forms in target organs[3]. Recently, steroid hormones have been shown to influence cell proliferation, and they may be involved in cancer induction in several organ systems[4)~7)]. Soluble estrogen (E1S) is a common transport form in the circulation in adult women, and also circulates in small concentrations in men. Where present STS, E1S converts to active estrogen E2.

In the kidney, the proximal tubules have been suggested as a possible site of synthesis or action for steroid hormones[8]. In humans, many investigators have confirmed the presence of estrogen receptors in renal carcinoma tissue and suggested that estrogen is related to the origin and

development of this cancer[9)10)].

However, the presence and role of STS in the human kidney have not been fully investigated and little is known as to whether STS is related to induction of renal cell carcinoma (RCC). In the present study, we examined neoplastic and non-neoplastic renal tissue from patients with RCC by immunohistochemistry, enzyme histochemistry, and *in situ* hybridization.

Materials and Methods

Tissue

Heminephrectomy specimens including RCC and adjacent nonneoplastic areas were examined (n = 10; patient age, 51 to 72 years). For comparison, two heminephrectomy specimens from patients with renal tuberculosis and eight autopsy specimens with no evidence of kidney disease were examined.

Immunohistochemistry

Tissues used for immunohistochemistry, were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 20 min, and were embedded in paraffin, and sectioned at a 4 μm thickness. STS peptide was prepared according to the human STS sequence determined by Kawano et al[11)]. The 24-residue sequence has been confirmed by cDNA sequencing in a region corresponding to on the human X chromosome STS exon 10[11)], and is【H_2N-EASRPNIILVM-ADD-LGIGDPCYCG-COOH】. The peptide was suspended in saline, emulsified with Freund's complete adjuvant, and injected into rabbits. Serum was collected from immunized rabbits, and the IgG fraction was purified and stored at − 20 ℃. Sections were deparaffinized, incubated for 40 min in 0.3% H_2O_2 with methanol, incubated in normal human serum for 30 min, and then exposed to anti-STS antibody (1: 200) for 30 min at room temperature. Following incubation with biotinylated goat anti-rabbit IgG (1:200 ; Vector Laboratory, Tokyo, Japan) for 30 min, the sections were exposed to a streptavidin-biotinylated horseradish peroxidase complex (1:100 ; Vector Labs.) for 1 hr. The immunoreaction product was visualized with a solution of 20 mg of diaminobenzidine and 65 mg of NaN_3 dissolved in 100 ml of 0.05 M-Tris buffer (pH 7.6) containing 10 μl of 30% H_2O_2. Controls were prepared by replacing either the anti-STS antibody or the second antibody with PBS. Imunohistochemical staining with anti-proliferating cell nuclear antigen (PCNA) antibody (1:200 ; MBL, Nagoya, Japan) was performed in the same manner.

Enzyme-histochemistry

The 10 μm thick and 1 mm^2 wide tissues were incubated with 2% glutaraldehyde in 0.1 M cold cacodylate buffer (pH 7.2) containing 5% sucrose for 30 min. Specimens then were rinsed with 0.1 M cold cacodylate buffer (pH 7.2) containing 5% sucrose before incubated for 1 hr at 37 ℃ in medium containing 1 mM 4-methylumbelliferyl sulfate (MUS) as substrate[11)], 1% barium chloride as the capturing metal source, 0.1 M imidazole-HCl buffer (pH 7.5), and 5% sucrose. After washing twice in 0.1 M cacodylate buffer (pH 7.2), specimens were postfixed with 1% OsO_4 at 4 ℃ for 30 min, embedded in epoxy resin, and examined by electron microscopy. Controls were prepared using a medium without MUS.

Probes for hybridization

A human STS probe was cloned from human placenta[12)]. The clone has been mapped using a somatic cell hybrid panel and by *in situ* hybridization to Xp22.3.A 2.7-kb cDNA including a fragment of exon 10 originally cloned into the vector pUC18 (total size 5.4 kbp) then was digested with the restriction enzyme EcoR1. The 2.7-kb fragment of STS probe was coupled with digoxigenin by random-primed labeling. To determine the optimal dilution of anti-digoxigenin antibody for probe visualization, the digoxigenin-conjugated probe was transferred to a nitrocellulose filter

and incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody. Ten nanograms of STS cDNA could be detected with a 1:500 dilution of concentrated antibody. A 1:250 dilution was employed for *in situ* hybridization.

***In situ* hybridization**

For hybridization, the anti-digoxigenin antibody (Bio Cell Research Laboratories, Cardiff, UK) was conjugated to 5 nm gold particles instead of alkaline phosphatase. Tissue samples were fixed with 4% PFA in 0.1 M PBS and 30% sucrose for 12 hr. Cryostat sections of frozen samples (10 μm thick) were mounted onto slides coated with gelatin. The sections were washed three times in 0.1 M PBS, treated with 0.2 N HCl for 20 min, and then they were treated with 1 mg/ml of proteinase K for 15 min and incubated in 4% PFA for 5 min. Sections then were treated twice with 2 mg/ml of glycine for 15 min and 40% deionized formamide for 5 min. After sections were incubated in tris-ethylenediaminetetraacetic acid (TE) buffer, prehybridization was performed at 37 ℃ for 1 hr in a humid chamber with hybridization buffer containing deionized formamide (final concentration, 40%), 10 mM Tris/HCl (pH 7.4), 0.5 M NaCl, 1 mM EDTA (pH 7.4), 1×Denhardt's solution, sonicated salmon sperm DNA (5 mg/ml), yeast tRNA (10 mg/ml), and 50% dextran sulfate.

Hybridization then was performed at 37 ℃ overnight with digoxigenin-labeled STS cDNA in TE buffer at a concentration of approximately 200 pg/ml of hybridization buffer. After washing, sections were rinsed five times in 50% formamide/2× SSC for a total of 60 min in a shaker at 37 ℃. Sections then were blocked with 0.5% bovine serum albumin including 0.5% sheep IgG at room temperature for 1 hr and incubated for 3 hr at room temperature with 5 nm colloidal gold-labeled sheep anti-digoxigenin-antibody at a dilution of 1:250. The sections then were washed three times for 15 min in 0.075% Brij 35 (Sigma Chemical, St. Louis, MO) in PBS. After the antibody reaction was completed (30 min), the sections were processed for silver enhancement with a kit (British Bio Cell scopy. International, UK) with an incubation time, 15 min. The sections were examined by epipolarized light microscopy.

Results

Immunohistochemistry

Anti-STS antibody showed staining in adjacent nonneoplastic renal tissue in cases with RCC (Fig. 1A, B). STS staining was evident mainly in proximal tubules as opposed to distal tubules. Weak positive staining occurred in some area of the carcinomas (Fig. 1C). Immunoreactivity was not observed in glomeruli, vessels, or interstitial or pelvic tissues. Immunoreactivity was absent from all areas of the kidney in specimens from nontumor cases.

Proliferating cell nuclear antigen (PCNA) was demonstrated in cells of the proximal tubules in tumor cases (Fig. 2A, B)

Enzyme-histochemistry

In tissue specimens from nine patients with RCC, STS enzyme activity was demonstrated in nonneoplastic proximal tubules (Fig. 3A~D). Reaction product was evident mainly in membranes of the rough endoplasmic reticulum and nuclear envelope. No activity was observed in mesangial, endothelial, or epithelial cells of glomeruli. No reaction product appeared in Bowman's capsule or in the walls of vessels. No STS activity was demonstrated anywhere in renal specimens from cases without RCC.

***In situ* hybridization**

The STS probe hybridized mainly in cells of the proximal tubules in the adjacent noneoplastic tissue in cases with RCC (Fig. 4). No STS mRNA was demonstrated in the tumors. No STS mRNA was detected in mesangium, epithelial cells, or cells of Bowman's capsule. RNase-treated control

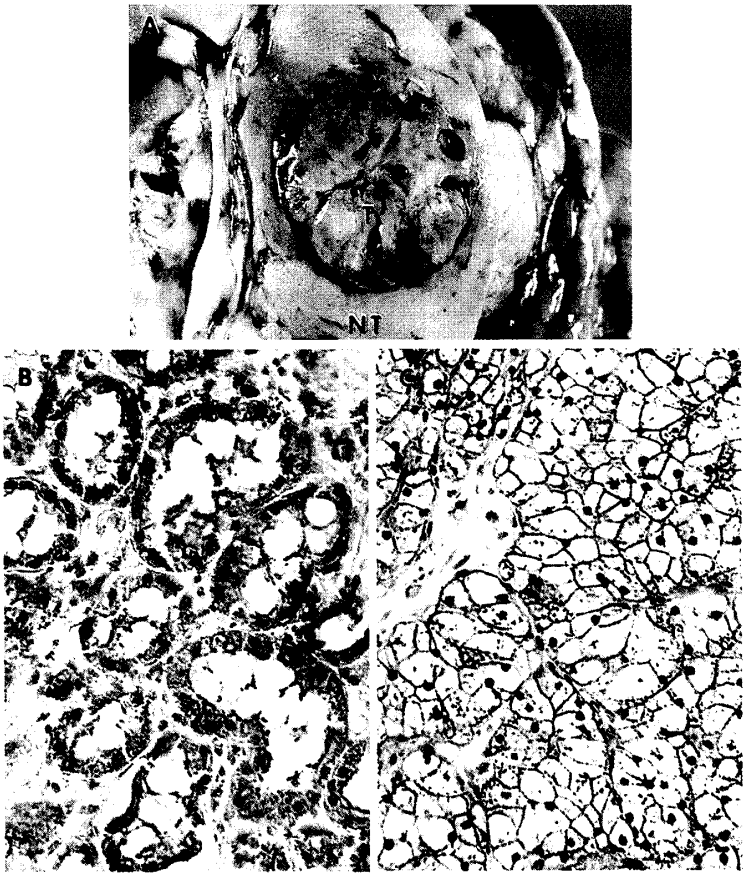

Fig. 1 Immunohistochemistry in case (Case 59 y, female) of renal cell carcinom (RCC)
A: specimen from a patient with RCC. T: tumor tissue, NT: adjacent nontumor tissue.
B: Immunohistochemistry for steroid sulfatase (STS) was positive in proximal tubule cells in nontumor tissue (original magnification, ×200). Nine of ten RCC cases showed a similar proximal tubular pattern of reactivity.
C: Anti-STS antibody weakly stained portion of the carcinoma. (original magnification, ×200).

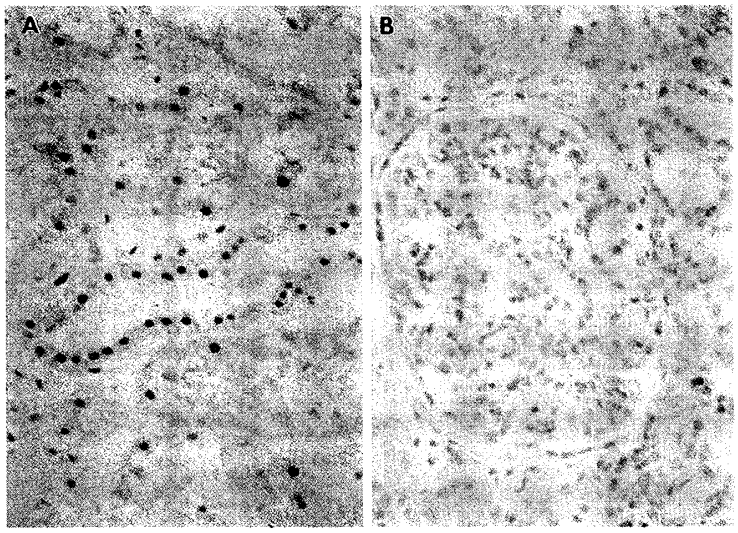

Fig. 2 Result of staining for proliferating cell nuclear antigen (PCNA)
A: illustrates positive nuclei in several proximal tubular cells (Case 69y, female). **B**: Virtually no signal was demonstrated within glomerular structures.
(original magnification in both panels, ×200).

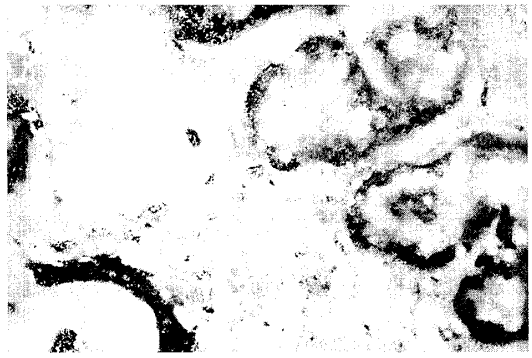

Fig. 4 *In situ* hybridization
The section was examined by epipolarized light microscopy. Steroid sulfatase (STS) mRNA was seen predominantly in proximal tubule cells. Gold color area indicate the signal for STS mRNA (original magnification ×200, Case 51y, male).

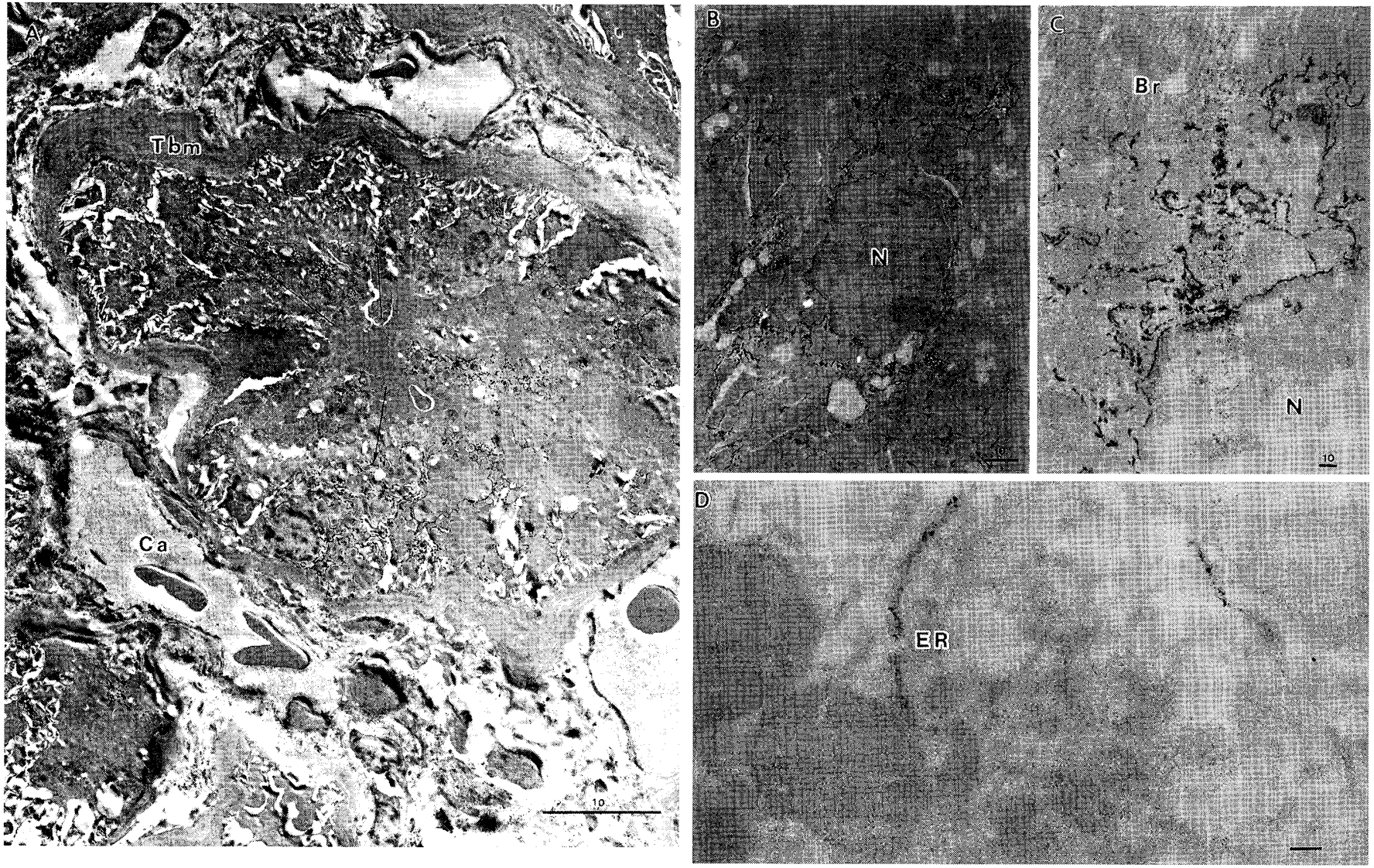

Fig. 3 Enzyme histochemistry demonstrated steroid sulfatase (STS) activity in cells of the proximal convoluted tubules In a normal appearing area (A, center) in a resected kidney from woman with renal cell carcinoma. These products were located both in relations to nuclear envelope (B) and analong the membranes of the endoplasmic reticulum (B, C, D). Bars equal 1 μm except when marked 10 (10 μm). Arrows indicate reaction products. Tbm: tubular basement membrane, Ca: peritubular capillary, N: nuclei of proximal tubules, Br: brush border, ER: rough endoplasmic reticulum.

and sense control sections revealed no mRNA signals. Renal tissue from cases without RCC did not demonstrate any evidence of STS mRNA.

Discussion

In the present study, renal STS activity was demonstrated in cases with RCC, specifically in nonneoplastic proximal tubules. Our results confirmed that STS is synthesized at this site in humans, and showed its' activity to be augmented in kidneys harboring RCC. Heterogeneity of human STS has been reported by Simard et al, who have identified rapidly and slowly mobile isozymes by electrophoresis in fibroblast extracts[13]. The rapidly mobile isoform of STS is believed to lack enzymatic activity, while the less mobile isoform shows high activity[13].

Our histochemical results using MUS indicated that the STS in proximal tubules of cancers cases were active form to a substantial extent. According to high E2 activity which is generated by this active form of STS, proliferation of proximal tubular cells may be started. The proximal tubule has been suggested as a possible site of renal synthesis or action for steroid hormones by Stumpf et al[8] who injected ^{3}H-labeled estradiol into rat, and found radioactivity to be concentrated in the proximal but not distal tubules. E1S, the transport form of estrogen, therefore may be converted to the active form E2 by STS in the proximal tubules of the human kidney. Generally, regulatory mechanisms keep STS activity at a low level in tissues, and local increases in STS activity result in activation of estrogen, which in the proximal tubule might induce RCC. RCC occurs more frequently in males than in females. Generally, estrogen concentrations are low in males, resulting in less systemic feedback suppression to counter any local consequences of increased renal STS activity[14].

Although the pathogenesis of RCC is unclear, this cancer is believed to originate from proximal tubule[15]. By estrogen administration, Li et al induced renal carcinoma in the hamster[4]. In humans, many investigators have confirmed the presence of estrogen receptors in renal carcinoma tissue and suggested that estrogen is related to the origin and development of this cancer[9,10]. Thus increase in STS activity might be related to the cause of RCC through augmented E2 level in the proximal tubule. Alternatively, RCC might produce some factor by paracrine pattern and induce STS activity in the proximal tuble, resulting further development of the cancer.

Positive immunoreactivity for PCNA, a cofactor of DNA polymerase-delta, was demonstrated in the proximal tubule, where STS was also disclosed for immunoreactivity. Thus it may be suppressed that the augmentation of STS activity may be related to the proliferation of the cells in the proximal tubule of the kidney.

However, the present paper is the first to demonstrate augumented STS activity in proximal tubules in cases with RCC. This increased activity may have pathogenic importance.

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Steroid sulfatase の腎細胞癌とその非腫瘍部組織における発現
—免疫組織化学・酵素組織化学・*in situ* hybridization による検討—

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［目的］腎近位尿細管は，ホルモンの標的器官として注目されている．性ステロイドホルモンが renal cell carcinoma (RCC) の発症原因になるか否かについての詳細はいまだ不明である．循環血液中に存在する性ステロイドホルモンは糸球体で濾過されないが，例えば estrogen sulfate (E1S) は糸球体で濾過され，近位尿細管で再吸収される．そこで，E1S のステロール環 3β 硫酸基を，水酸基に置き換える steroid sulfatase (STS) の腎近位尿細管における局在について RCC 腎，健常腎において検討した．［方法］STS-peptide を合成し，polyclonal 抗体を作製し，免疫組織化学法を行った．STS mRNA を digoxigenin 標識法で観察した．STS 活性は 4-methyl-umbelliferyl sulfate を基質とした組織化学的方法で検出した．［結果］免疫組織化学および mRNA は近位尿細管を中心に RCC 腎で陽性所見が見られた．STS 活性は核膜，小胞体に局在した．活性は管腔側に強く発現した．［結論］硫酸基をもつ E1S は近位尿細管で STS により活性化され，受容体と結合してその作用を発現すると考えられた．性ステロイドホルモンは細胞の増殖分化発生に影響するため，RCC 腎の近位尿細管におけるこのような STS の局在は興味深い．