Histol Histopathol (1996) 11: 257-266

Histology and Histopathology

Invited Review

Expression of renin in coagulating glands

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Summary. The presence of an extrarenal or local renin-angiotensin system has been noted in several tissues, although their functions have not yet been clarified. Renin from the coagulating gland (CG), is the most recently discovered local renin, and is a significant subject for investigation with histological and molecular biological techniques. Dot-like reactions for renin are detected immunohistochemically in the epithelial cells of CGs of the strains C57BL/6 mice. Excretory products of some terminal lumina are also found to be positive for renin. Colloidal gold particles, indicating the presence of renin, are detected in the lysosomal granules, in which they are especially located on the crystalline structure. They are also observed in the production of series of exocrine granules. At the apical region, both renin-positive exocrine and lysosomal granules are secreted by exocytosis. In the development, immunoreactivity for renin is first detected at 6 weeks after birth. After that time, the number of renin-containing cells gradually increase throughout the development. In adults, several patterns of renin immunoreactivity are demonstrated in almost all epithelial cells of CGs. At 4 weeks after castration, renin-containing cells in terminal ducts are decreased and remain at very low levels. After testosterone injection, the numerical value of renin-containing cells is high at 1 week. Finally, renin mRNA is detected in the CGs by Northern blot analysis and hybridohistochemistry. These findings suggest that renin is synthesized depending on testosterone, and released by exocrine secretion.

Key words: Coagulating gland, Immunoelectron microscopy, Renin, Immunohistochemistry, *In situ* hybridization

Introduction

It is well known that the renal renin-angiotensin system (RAS) is involved in the control of blood pressure (Davis and Freeman, 1976). Classically, renin, a trigger enzyme in the RAS, is secreted from juxtaglomerular cells located at the site of the glomerular vascular pole in the kidney and changes angiotensinogen in circulating blood into angiotensin I. Angiotensin I is metabolized into angiotensin II by a converting enzyme localized in the lung, which is the most effective hormone regulating contraction of vascular smooth muscle.

Recently, renin has been reported to be synthesized and secreted in other organs or tissues apart from the kidney (Deschepper and Ganong, 1988). This extrarenal or local renin has been demonstrated, for example, by immunohistochemical or molecular biological techniques, in the pituitary and pineal glands (Haulica et al., 1975; Inagami et al., 1980; McKenzie et al., 1985), submandibular glands (Gresik et al., 1980; Misono et al., 1983), adrenal glands (Naruse et al., 1984; Mizuno et al., 1988; Kon et al., 1991), testes (Parmentier et al., 1983; Sealey et al., 1988), ovary (Do et al., 1988; Palumbo et al., 1989), uterus (Ferris et al., 1967; Hackenthal et al., 1980), oviduct (Eskildsen, 1972) and placenta (Poisner et al., 1981; Pinet et al., 1988). The functions and roles of these local renin are not well known except in the adrenal gland, in which all components of the RAS have been detected in rat tissues (Mendelsohn, 1982; Naruse and Inagami, 1982; Campbell and Habener, 1986). Briefly, renin secreted from the adrenocortical cells in the zona glomerulosa stimulates aldosterone and prostaglandin production via angiotensin II, although the tissue or cell type synthesizing it is still unknown (Doi et al., 1983; Urata et al., 1988).

Renin from the coagulating gland (CG), one of the male accessory sex glands, is the most recently described as a local renin, whose functional role remains uncertain (Fabian et al., 1989). However, it has been emphasized that renin expression in CG is significant, because large amounts of both mRNA and protein are detected in this organ (Kon et al., 1992a; Fabian et al., 1993).

The secretory granules of juxtaglomerular cells in the kidney have been classified as lysosomal granules, which cleave angiotensinogen into angiotensin I (Taugner et al., 1985b; Morris, 1988), and have been found to contain many different lysosomal enzymes, including acid phosphatase (Fisher, 1966; Kon et al.,

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1986), ß-glucuronidase (Gomba and Soltész, 1969), N-acetyl-ß-glucosaminidase (Soltész et al., 1979), and cathepsin B (Taugner et al., 1985a; Matsuda et al., 1989). To discover whether extrarenal renin is also contained in lysosomal granules, and where it is released are important for clarifying the function of CG renin. Production of renin is regulated by testosterone in the salivary gland (Oliver and Gross, 1967; Wagner et al., 1990). Additionally, it is known that testosterone increases blood pressure in spontaneously hypertensive castrated male rats (Cambotti et al., 1984). Therefore, apart from whether the effects is direct or not, it is possible that CG renin is also regulated by testosterone.

In the present review, using immunohistochemical, immunoelectron microscopical and molecular biological techniques, we show that renin is synthesized and released in CGs of inbred mice. The function of this enzyme in relation to the local RAS is discussed.

General morphology of CGs

The CGs in mice, showing a translucent colour, are located adjacent to the medial side of the seminal vesicles. Because it is a little difficult to separate CG from seminal vesicle, both tissues are recommended to be fixed together. With light microscopy, the CGs, differentiated from the seminal vesicles by paler eosin staining and slight dilatation of the lumen, are composed of single columnar or cuboidal epithelium. Many mucosal plicae are observed in the lumen. Because the structure of the CG in the mouse is ductal rather than acinar, the large dilated tubules located near the seminal vesicle and longitudinally along the seminal vesicle are identified as main duct, whereas the small tubules that are crossed or obliquely sectioned by longitudinal sections along the seminal vesicle and distributed to peripheral areas of the gland are identified as terminal ducts (Sugimura et al., 1986; Hayashi et al., 1991; Kon et al., 1995a).

In the electron microscopic observation of epithelial cells, a few microvilli are present at the apical boundaries, while basal regions are relatively flat where basal cells are sometimes localized, elongating their cytoplasmic processes between the epithelial cells. The epithelial cells in terminal ducts of CGs are found to be composed of large, oval nuclei containing one or two distinct nucleoli (Fig. 1). In the supranuclear region, the Golgi apparatus, divided into 3 or more areas, is well developed and immature exocrine secretory granules are also found. As a result, rough endoplasmic reticulum with large dilated cisternae is plentiful in the basal region (Chow and Pang, 1989; Kon et al., 1992a; Holterhus et al., 1993). Many exocrine secretory granules measuring about 600 nm in diameter and containing an electrondense core are demonstrated in the apical region of cells.

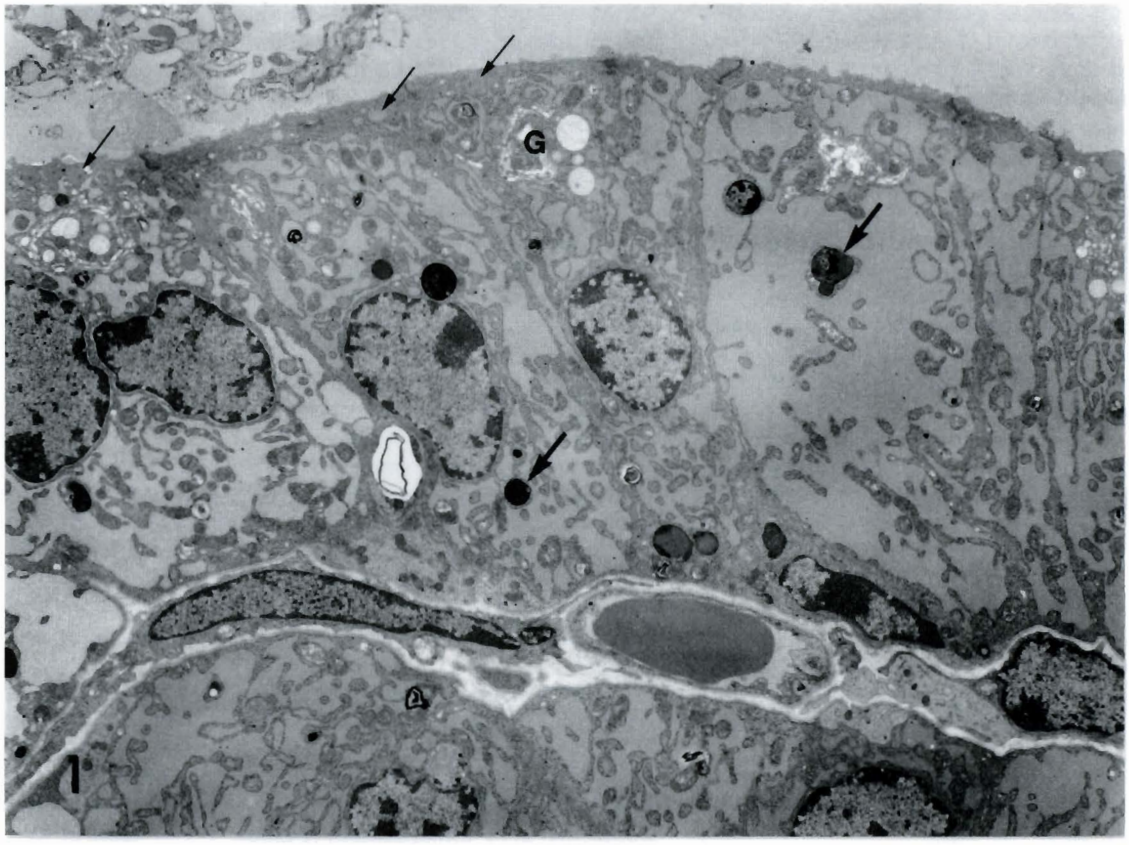

Fig. 1. Electron micrograph of C57BL/6 mouse coagulating gland. Golgi apparatus containing immature exocrine secretory granules is well-developed in the supranuclear region (G). Extensive rough endoplasmic reticulum is observed throughout the cytoplasm. Exocrine secretory granules are present in the apical region of cells (small arrows), and electrondense lysosomal granules are observed in the basolateral region (large arrows). Routine electron microscopical technique. x 3,700

Many electrondense lysosomal granules, distinct from the exocrine granules and varying in morphological content are observed, especially in the lateral and basal regions of epithelial cells. Crystalline structures are demonstrated in these granules. They are composed of electron-lucent and electrondense regions measuring about 8 nm long and 4.5 nm in width, respectively (Kon et al., 1992a). It is generally known that the crystalline

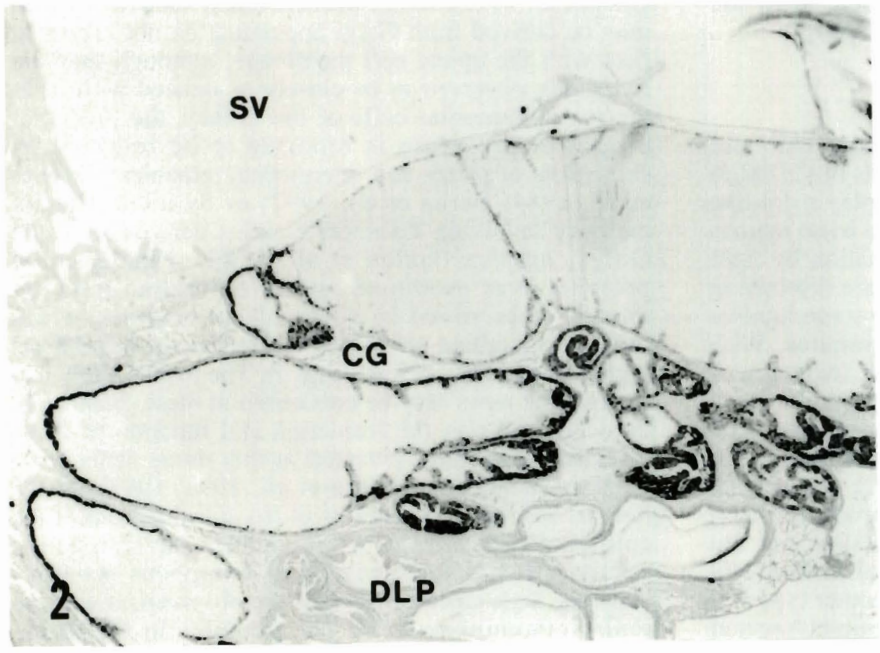

Fig. 2. Low magnification of renin immunohistochemistry in a mature coagulating gland (CG), seminal vesicle (SV) and dorsolateral prostate (DLP). Immunoreactivity is restricted in CG epithelial cells (Kon et al., 1995a). x 38

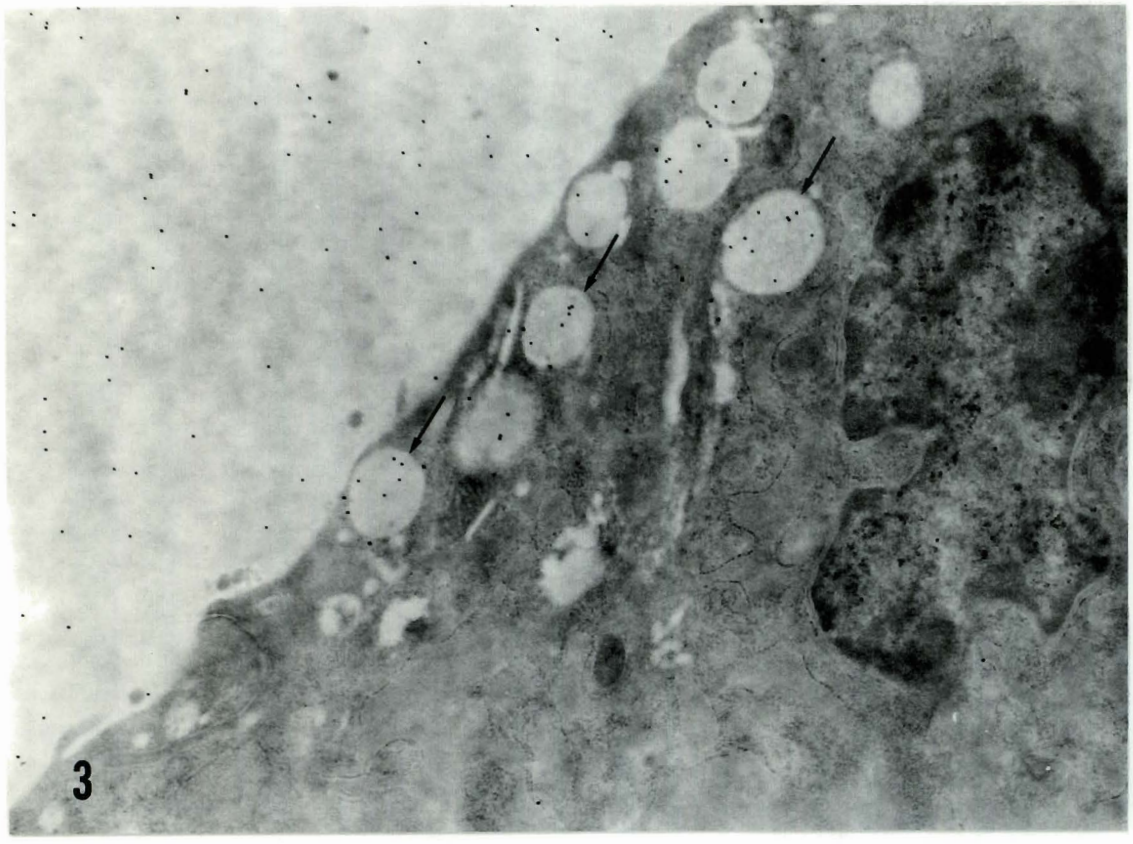

Fig. 3. Immunoelectron micrograph of the apical region in CG. Gold particles labelling renin are distributed in the exocrine granules (arrows). Lowicryl K4M (Chemische Werke Lowi, Germany) resin was used for embedding. x 20,000

structures found in the cells/tissues are due to the condensation and packing of pure protein materials without modification by other materials (Fawcett, 1981). In the juxtaglomerular cells of the kidney, crystalline structures observed in the small granules, the so-called juvenile granules, subsequently disappear with the maturation of secretory granules (Barajas, 1966).

Exocrine function in CG renin

Immunohistochemically, renin-positive cells are observed in the epithelial lining of the CGs of C57BL/6 mice (Fig. 2). The immunoreactivities display a dot-like shape of varying diameter in the lateral and basal regions of epithelial cells. However, with examination by light microscopy, immunoreactivities for renin are detected in the apical portion of epithelial cells and in some luminal products as well as in the basolateral granules. With immunoelectron microscopical observation, two types of exocrine secretory pathway for renin are proposed (Kon et al., 1995b). The first type is a general exocrine pathway, in which renin protein is targeted with other materials into apical exocrine granules (Fig. 3). Briefly, it is supposed that Golgi vacuoles are produced from Golgi lamellae, and that they fuse together and are transported to and released from the apical surfaces via the cell membrane of epithelial cells. The other type is a lysosome-dependent pathway, in which renin is targeted to lysosomal granules (Fig. 4). Generally, lysosomal granules, also produced by the Golgi complex, fuse with smaller excreting granules, and materials are exocytosed from the apical cell membrane like those in other lysosome-containing cells (Lejeune and Vercammen-Grandjean, 1979). Renin-positive small vacuoles (which may be derived from Golgi apparatus) do not appear to fuse with the apical cell membrane, although they are frequently observed to be closely associated with it. In the juxtaglomerular cells of the kidney, the precursor prepro or pro-renin is reported to be released by exocytosis of proto- and intermediate granules (Taugner et al., 1984; Berka et al., 1992) or by a constitutive pathway involving transport vesicles derived from the Golgi complex (Galen et al., 1983). Finally renin proteins over produced by CG epithelial cells are thought to be stored in electrondense granules, which include crystalline structures located in the basolateral region (Kon et al., 1992a) (Fig. 5). The results show that the enzyme renin may be condensed in these granules in the CGs, and that the formation and function of these crystalline structures detected in this tissue differ from those in the kidney (Berka et al., 1992, 1993). In the luminal spaces or the pockets of the apical regions of the epithelial cells, which are thought to have an active secretory function, considerable amorphous materials showing high electron density are observed to express renin immunoreactivity. The granules in the juxta-

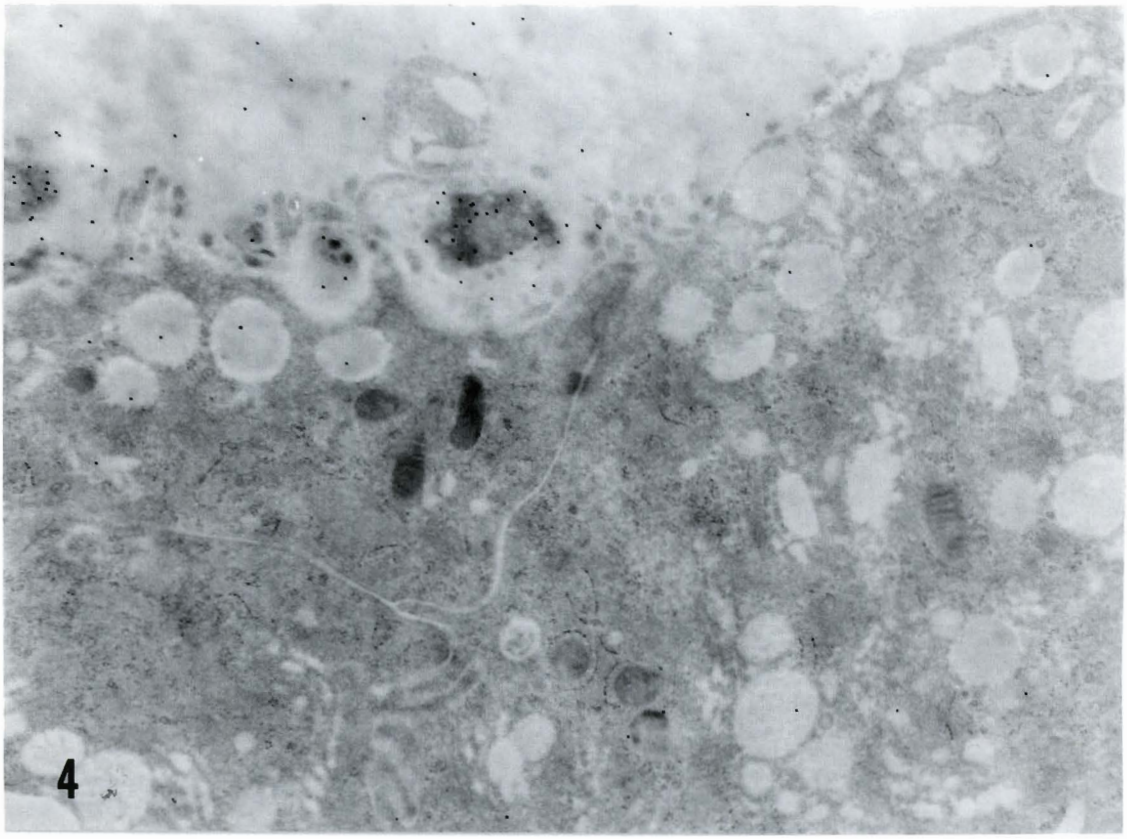

Fig. 4. Exocytosis of a lysosomal granule using Lowicryl K4M embedding medium. x 20,000

glomerular cells are suggested to be released into blood or lymphatic circulation by specialized exocytosis. For example, the mature secretory granules are released from channel-like invaginations via the cell membrane (Peter, 1976), by a vacuolated exocytosis pathway (Berka et al., 1992; Kon et al., 1992b), or by diacrine secretion (Galen et al., 1983; Kon et al., 1992b). In any case, because secretion is first toward the intercellular spaces, the secretory style of renin in the kidney is classified as an endocrine or paracrine function.

Localization patterns of extrarenal renin expression are divided into 3 types according to the functions proposed: 1) neuroendocrine organs (Naruse et al., 1985; Deschepper and Ganong, 1988); 2) cardiovascular tissues (Barrett et al., 1981; Swales et al., 1983; Taylor et al., 1988); and 3) exocrine organs (Tanaka et al., 1980; Hirose et al., 1983; Penschow and Coghlan, 1993). The first is represented by the brain, especially the pituitary gland (Inagami et al., 1980; McKenzie et al., 1985; Ganong, 1993). In the pituitary gland, it is known that renin and angiotensin II are located in gonadotrophs, especially in the same secretory granules with luteinizing hormone, although the production site of angiotensinogen is not clear (Deschepper and Ganong, 1988). Because the administration of angiotensin II to rat pituitary tissue in vitro increases the release of prolactin and adrenocorticotropic hormones, angiotensin II activated by renin has been thought to regulate their secretion, in addition to affecting blood pressure, and to stimulate aldosterone secretion and water intake (Steele et al., 1981). Secondly, very low-levels of renin-like activities have been detected in mouse and rat hearts and arterial walls (Swales et al., 1983; Dzau and Re, 1987). It has been proposed that renin causes myocardial and smooth muscle cell hypertrophy or hyperplasia via angiotensin II formation (Naftilan et al., 1991; Holtz, 1993). Finally, submandibular gland renin is the only well-known model of exocrine gland renins. Although the real physiological function of submandibular gland renin is not entirely understood, it may be that salivary gland renin can be released into the circulation (Menzie et al., 1974; Penschow and Coghlan, 1993). The granular convoluted tubule cells of the mouse submandibular glands also produce many other physiological autacoids, including epidermal growth factor, nerve growth factor, kallikrein, proteinase A and tonin (Gresik et al., 1980; Ledoux et al., 1982; Rall et al., 1985; Wilson et al., 1986; Penschow and Coghlan, 1993). Because it has been reported that renal/pancreatic kallikrein of the submandibular gland is secreted from both the basolateral and apical surfaces (Penschow and Coghlan, 1993), the possibility of bipolar secretory pathways for CG renin cannot be ignored. Recently, evidence that the renal proximal tubules may produce renin has been reported in a molecular biological study (Moe et al., 1993). Angiotensinogen is also localized in the proximal

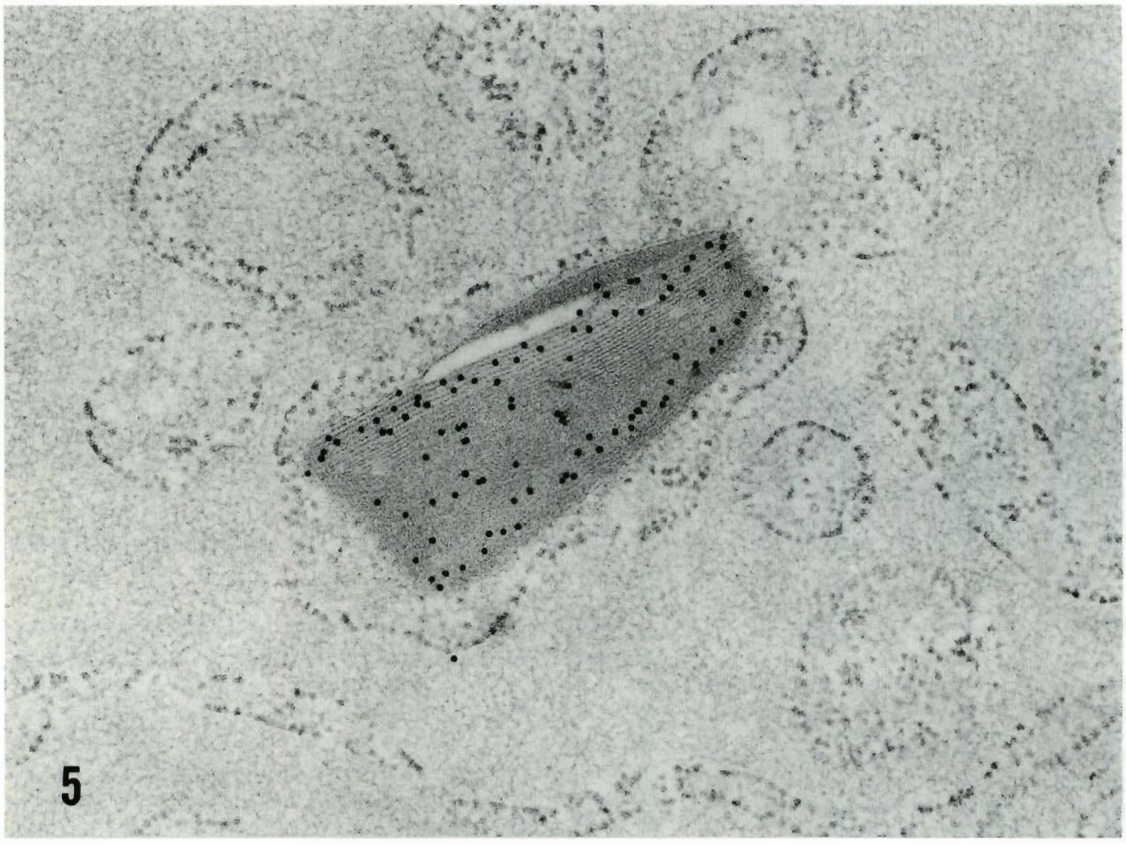

Fig. 5. High magnification immunoelectron micrograph in CG. Colloidal gold particles indicating presence of renin are observed in the electrondense lysosomal granule, where they are located particularly in the crystalline structures. Epoxy resin was used for embedding. x 52,000

tubules in the case of transgenic mice (Fukamizu et al., 1994). These results may suggest the existence of a third exocrine tissue for renin localization.

Relationship between renin expression and testosterone in CGs

Recently, it has been reported that steroid hormones, especially testosterone, influence renin synthesis in the submandibular gland, gonadotrophic cells of the anterior pituitary gland and other extrarenal tissues, although intrarenal renin is not influenced by testosterone (Oliver and Gross, 1967; Naruse et al., 1986; Wagner et al., 1990). Testosterone secreted from testicular interstitial cells is known to act on sex accessory glands, such as CGs, to directly activate exocrine secretion (Cunha et al., 1987). In this part, the relationship between renin expression and testosterone in CGs of C57BL/6 mice is discussed.

During development, immunoreactivity for renin is not detected in the CGs of animals younger than 5 weeks old. At 6 weeks after birth, a few renin-containing cells expressing a very weak reaction are demonstrated in some epithelial lines. At 7-8 weeks after birth, the terminal ducts of the CG showing lightly pinkish secretory products in the lumen have increased in size. The epithelial cells containing abundant cytoplasm and round-shaped nuclei are observed to be columnar in form, and sometimes to have mitotic figures. Almost all cells express a weak renin immunoreaction, whereas some cells contain small granular materials in their basolateral regions (Kon et al., 1995a). In adults, renin immunoreactivities are demonstrated throughout almost all epithelial cells in CGs (Fig. 6). There are several patterns of renin immunoreactivity in CGs. Excretory products in some terminal ducts are also found to be positive for renin. Sometimes immunoreactivity to the epithelium of the proximal urethra (possibly an opening site of the CG duct) is observed.

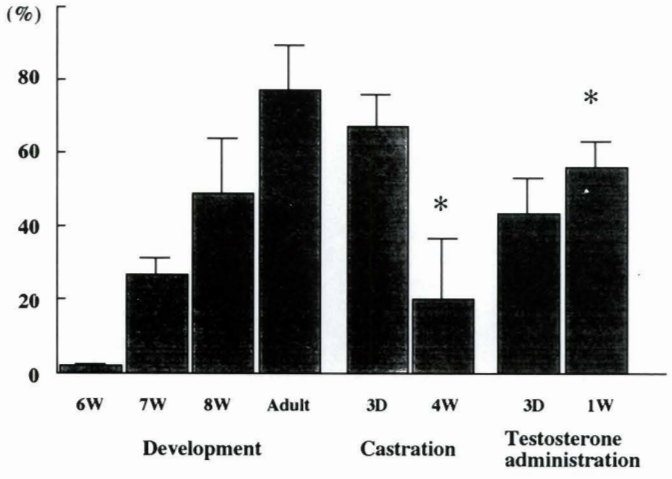

Fig. 6. Statistical changes in the ratio of renin-positive cells per 1000 epithelial cells in the terminal ducts of the CGs during development, following castration and following testosterone administration to castrated mice. Bars represent mean +/- standard error. A statistically significant difference ($p<0.05$) between the value at 4 wks after castration and that at 1 wk after testosterone administration is shown by asterisks. (The data from Kon et al., 1995a were modified).

Numerous papers have been published about the immunohistochemical expression of renin during development, especially its pre- and perinatal changes in kidneys (Kon et al., 1989, 1994; Graham et al., 1992; Dodge, 1993). According to these reports, expression of renin in the kidney has been shown to be responsive to homeostatic adaptations from pre- to postnatal life. As for the development of renin a few weeks after birth, the dynamics of testicular renin have only been reported using rats (Parmentier et al., 1983). These observations provide the morphological evidence that renin-containing cells in mice are physiologically developed after birth, especially during adolescent periods in the CG.

Morphological changes of sex accessory glands after castration have been reported by many investigators (MacKenzie et al., 1963; Sugimura et al., 1986; Holterhus et al., 1993). At 3-4 weeks after castration, both CG and seminal vesicle are small and atrophic, whose luminal spaces in terminal portions are narrowed. Immunohistochemically, renin-containing cells are not demonstrated in terminal ducts, whereas weak renin immunoreactivity is still observed in the main duct (Kon et al., 1995a). From these results, 3 possibilities could be suggested: 1) testosterone effect on expression of renin is heterogeneous in the CG; 2) there are two types of renin-expressing cells (testosterone-dependent and -independent renins) in CG; and 3) the terminal duct is the actual production site of renin, whereas the main duct is the storage or uptake site.

At 1 week after testosterone administration to castrated mice, large numbers of renin-positive cells are detected (Kon et al., 1995a). It appears that the expression of CG renin is mainly regulated by the testis, especially by testosterone. However, it should not be forgotten that other cellular or humoral factors are related to changes in expression of renin in the CG. In the testis, it is known that Sertoli cells constituting testicular tubules secrete androgen-binding protein (Attramadal et al., 1981; Pelliniemi et al., 1981), and that spermatocytes contain angiotensin AT_1 receptor (Paxton et al., 1993). The maintenance of renin expression in CGs might be multifactorial.

Northern blot analysis and hybridohistochemistry for CG renin

Immunohistochemistry was first conceived by Coons et al. (1942), has been modified by several investigators and institutes, and is established as a useful tool for the detection of biochemical materials. Generally, the cells expressing a certain mRNA have been reported to be protein secreting cells. The use of serial sections for immunohistochemistry and hybridohistochemistry (*in situ* hybridization) reveals, however,

more important information (Lloyd et al., 1992; Bachmann et al., 1993). For example, if the immuno/hybridohistochemistry result is -/+ in a certain cell, then the cell can produce the protein but not store it in its cytoplasm. Additionally, if immuno/hybridohistochemistry is +/-, circulating proteins from the blood or intercellular spaces can be taken up by the cell. These results suggest that double staining and employing serial sections for immunohistochemistry and hybridohistochemistry are useful and necessary tools for the detection of protein-expressing/synthesizing cells (Guitteny et al., 1988; Ronnekleiv et al., 1989). To obtain evidence of synthesizing cells, it is necessary to detect mRNA, coding the target proteins on the tissue sections. In recent years, we have been investigating how to improve the methodology for detection of tissue mRNA by using the renin gene in mice (Kon et al., 1993).

In the Northern blot analysis using Ren-1 cDNA, signals are detected not only in the kidney but also in the coagulating gland of C57BL/6 mice (Kon et al., 1992a). In the kidneys, the immunoreactivity for renin is restricted to the juxtaglomerular cells. Using the serial sections, positive signals from hybridohistochemistry with the antisense probe are also detected in the same juxtaglomerular cells. No cells are detected by the staining using the sense probe. In the CGs, immunoreactive deposits of renin are localized in the basolateral granules of the epithelial cell lines. By the survey of serial sections using hybridohistochemistry, positive signals are observed in the same epithelial cells, and the distinctive reactions are demonstrated to be in basolateral regions rather than apical regions (Fig. 7).

Proposed function of CG renin

Differences in renin expression in the submandibular glands of different animal strains have been demonstrated by molecular biological analysis (Masuda et al., 1982; Field and Gross, 1985). From these results, it has been established that production of murine renin is controlled by 2 types of genes, Ren-1 and Ren-2. Ren-1 gene is known to be related mainly to the expression of renin in kidney tissues, and all mouse strains possess this gene, while Ren-2 gene has been expressed only in association with renin production in the submandibular glands of SWR, AKR, DBA and ICR mice. The expression of the renin in the CG is reported to be coded by Ren-1 gene (Fabian et al., 1989).

It is known that the renin found in the Leydig cells of the rat testis and the angiotensin I-converting enzyme, part of the RAS released from the male reproductive tract, are dependent on and regulated by pituitary hormones (Parmentier et al., 1983). In the uterus of mice, renin concentrations vary with the ovarian cycle, peaking during oestrus and reaching the lowest levels during the postoestrus phase (Hackenthal et al., 1980). In the oviduct of the rabbit, oestradiol administration resulted in a marked increase in renin release (Eskildsen, 1972). The practical hormone in RAS is angiotensin II, which is synthesized by the cleavage of angiotensinogen. The function of angiotensin II is the production of prostaglandin in several organs as well as the control of blood pressure and the water-mineral balance (Needleman et al., 1975; Davis and Freeman, 1976). Arachidonic acid, a precursor of prostaglandins produces a consistent increase in renin release in the kidney in several animals (Larsson et al., 1974; Weber et al., 1975; Bolger et al., 1976). Moreover, prostaglandin has been shown to stimulate steroidogenesis in the adrenal gland (Saruta and Kaplan, 1972), and renin release in association with ß-adrenergic receptors (Suzuki and Hashiba, 1986). Additionally, an acute reduction in renal perfusion pressure increases the release of renal prostaglandins (McGiff et al., 1970; Jackson et al., 1982), which directly stimulate renin release from

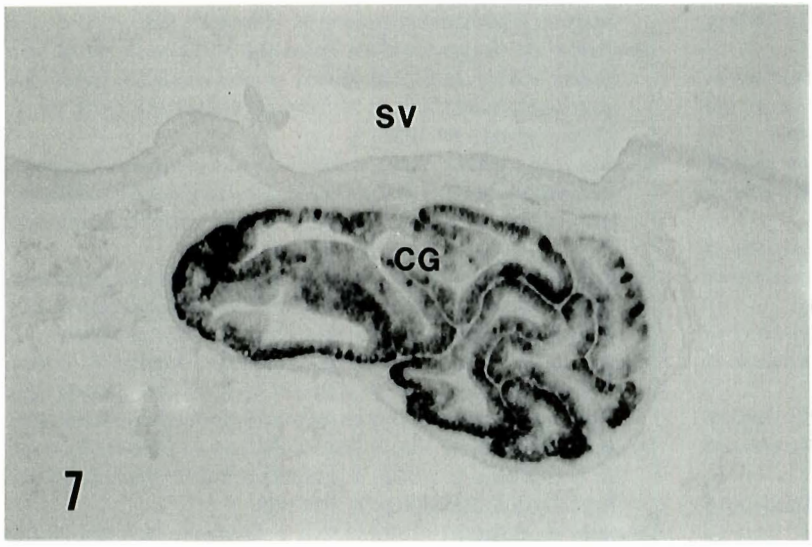

Fig. 7. Hybridohistochemistry for Ren-1 mRNA in CG of C57BL/6 mouse. The positive signals are mainly detected in the infranuclear region of the epithelial cells (CG). No positive signals are observed in the epithelial cells of the seminal vesicle (SV). x 120

juxtaglomerular cells. The function of prostaglandin found in seminal fluid is for the contraction of smooth muscle in the uterus and vagina and for the decrease of blood pressure in the body, while angiotensin II functions in the contraction of smooth muscle and the increase of blood pressure. It is assumed that angiotensin II produced outside the kidney has a function in regulating local homeostasis. In the present review, it is possible that CG renin also plays a role in production of the angiotensin series, and functions in association with prostaglandin.

These findings suggest that the renin-angiotensin-prostaglandin system may be dependent on male sexual maturation and may affect certain aspects of female reproduction as well, working in local-feedback and local-enhancing mechanisms. Further studies are required to clarify the function of CG renin.

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