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Comparative study of renin expression in the coagulating glands of C57BL/6 and Balb/c mice

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ABSTRACT

The comparative localisation of renin in the genital organs, especially in the coagulating glands of male mice of the strains C57BL/6 and Balb/c, was investigated using immunocytochemical, immunoelectron microscopical and Northern blot techniques. Dot-like reactions for renin, of varying diameters, were detected immunocytochemically in the epithelial cells of coagulating glands of C57BL/6 mice, but not in those of Balb/c mice. In both strains, many electron dense granules differing in content and morphology were observed in the epithelial cells of the coagulating glands. Crystalline materials were sometimes contained in these granules. Colloidal gold particles indicating the presence of renin were detected in the electron dense granules of C57BL/6 mice, in which they showed a heterogeneous distribution and were especially located on the crystalline structure. No positive reaction was detected in these crystalline structures in Balb/c mice. Renin mRNA was detected in the coagulating glands of both C57BL/6 and Balb/c mice by Northern blot analysis. However, the expression in C57BL/6 coagulating glands was stronger than that in Balb/c. These findings suggest that renin is synthesised and released in the coagulating glands.

INTRODUCTION

It is well known that the renal renin-angiotension system is involved in control of blood pressure (Davis & Freeman, 1976). Recently renin, a proteolytic enzyme expressed mainly in the kidney, has been reported to be synthesised and released in other organs or tissues outside of the kidney (Deschepper & Ganong, 1988). This extrarenal 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), ovaries (Do et al. 1988; Palumbo et al. 1989), uterus (Ferris et al. 1967; Hackenthal et al. 1980), oviduct (Eskildsen, 1972) and placenta (Polsner et al. 1981; Pinet et al. 1988). The function of extrarenal renin has been partially clarified in these papers. For instance, the angiotensin series synthesised with the cleavage of angiotensinogen by local renin is reported to stimulate aldosterone synthesis in the adrenal gland (Nakamaru et al. 1985).

In our laboratory, variations in renin expression between animal strains have been investigated by immunohistochemical and Northern blot analyses using mouse tissues, especially in reproductive tissues (unpublished results). From these investigations, it has been noted preliminarily that renin is detectable in the coagulating gland, one of the male reproductive organs. Furthermore, differences in renin expression in the submandibular glands of different animal strains have been demonstrated by molecular biological analysis (Masuda et al. 1982; Field & Gross, 1985). From these results, it has been established that production of murine renin is controlled by 2 types of genes, namely Ren-1 and Ren-2. Ren-1 gene is known to be related mainly to the expression of renin in...
kidney tissue 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 which except for ICR (closed colony) are all inbred strains.

It has been further reported that physiologically renin is one of the proteolytic, i.e. lysosomal enzymes cleaving angiotensinogen into angiotensin I, which is the strongest autacoid causing smooth muscle contraction (Davis & Freeman, 1976). For instance, the secretory granules of juxtaglomerular cells in the kidney, being the main source of renin, have been classified as lysosomal granules (Taugner et al. 1985; Morris, 1988), and found to contain many different lysosomal enzymes, including acid phosphatase (Fisher, 1966; Kon et al. 1986a), beta-glucuronidase (Gomba & Soltész, 1969), and N-acetyl-beta-glucosaminidase (Soltész et al. 1979). These findings suggest that extrarenal renin is also contained in lysosomal granules.

In the present study, using immunohistochemical, immunoelectron microscopical and Northern blot techniques, it was investigated whether renin was synthesised and released in male reproductive organs, especially coagulating glands of 2 types of inbred mice. The function of this enzyme in relation to the local renin-angiotensin system is discussed.

MATERIALS AND METHODS

Light microscopy

Coagulating glands from 5 male C57BL/6 and 5 Balb/c mice aged from 6 to 12 months were used for light microscopy investigation. Under ether anaesthesia, the animals were killed by cervical dislocation, the coagulating glands removed with the seminal vesicles, and fixed in Bouin’s solution overnight. Paraaffin sections (4 μm) were prepared routinely, deparaffinised and reacted with immunohistochemical techniques using rabbit antimouse submandibular gland renin antiserum (Kon et al. 1986b). Briefly, the rehydrated sections were immersed in methanol solution containing 0.1 % H₂O₂ for 30 min and then incubated with the following sera or solutions: (1) normal nonimmunised goat serum diluted 1:100 for 1 h at room temperature; (2) antirenin antiserum diluted 1:2000 overnight at 4 °C; (3) goat anti-rabbit IgG antiserum diluted 1:100 for 1 h at room temperature; (4) rabbit peroxidase-antiperoxidase complex diluted 1:100 for 1 h at room temperature; and finally (5) 0.02 % 3,3’-diaminobenzidine tetrahydrochloride containing 0.0035 % H₂O₂ in 0.05 M tris-HCl buffer (pH 7.6) for less than 5 min. After immunohistochemistry, the sections were stained lightly with haematoxylin, dehydrated and mounted under cover slips. Control sections were incubated with pre-absorbed antiserum (50 μg per 100 μl of diluted antiserum) (Fukushi et al. 1982) or nonimmunised rabbit serum substituted for the primary antiserum.

Electron microscopy

For electron microscopy, 3 C57BL/6 and 3 Balb/c mice were used. The coagulating glands were removed immediately after death, cut into 1 mm³ cubes, immersion fixed in 2.5 % glutaraldehyde buffered with 0.1 M cacodylate sodium and HCl (pH 7.3) for 3–5 h at 4 °C, and postfixed with 1 % osmium tetroxide for 1 h at 4 °C. The samples were dehydrated with graded alcohol solutions and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a Hitachi HU-12A electron microscope. Part of the coagulating gland tissue was fixed with 2.5 % glutaraldehyde only, embedded in epoxy resin and examined immunoelectron microscopically with postembedding methods using antimouse submandibular gland renin antiserum (Kon et al. 1991). Briefly, ultrathin sections mounted on nickel grids were floated on phosphate buffered saline (PBS), antirenin serum diluted 1:2000 with PBS overnight for 4 °C, and subsequently protein A-coated gold solution (15 nm in diameter) (EY Laboratories Inc. USA) diluted 1:10 with PBS. Control sections were incubated with PBS. After light double staining with uranyl acetate and lead citrate, the ultrathin sections were observed with the same electron microscope.

Northern blotting

The extraction of total RNA was carried out according to Birnboim’s (1988) method. Briefly, the liver, kidney and coagulating gland of C57BL/6 and the kidney and coagulating gland of Balb/c mice were removed immediately after death, homogenised with Polytron and incubated with 500 μl of 0.1 % Protease K solution. After the centrifugation of samples at 12 krpm for 10 min, total RNA was extracted with phenol/chloroform, secondarily with chloroform, incubated at 0 °C for 8 h with 7.5 μl of 2 M acetic acid and 1 ml of LiCl/ethanol and dissolved in 30 μl of distilled water. Each aliquot (6 μl) of total RNA was electrophoresed on a 1 % neutral agarose gel and transferred to a Gene Screen Plus membrane. Hybridisation was carried out at 60 °C for 24 h in 1 M NaCl, 1 % SDS,
Fig. 1. Immunohistochemistry for renin in C57BL/6 (a) and Balb/c mouse (b) coagulating glands. In the coagulating gland of C57BL/6 mice, renin immunoreactivities are observed in the epithelial lines (a), but are not present in Balb/c mice (b). ×34. CG, coagulating gland; SV, seminal vesicle.

Fig. 2. High magnification of immunoreactive renin in the C57BL/6 coagulating gland. The immunoreactivities are demonstrated as a dot-like structure of varying diameter in the lateral and basal regions of epithelial cells. Sometimes they are detected on the epithelial nuclei.
Fig. 3. Low (a) and high (b) magnification electron micrographs of C57BL/6 mouse coagulating gland. a. Some microvilli are present at the apical boundaries of the epithelial cells, while their basal regions are flat. Golgi apparatus containing immature exocrine secretory granules is well-developed in the supranuclear region, as is extensive rough endoplasmic reticulum in the basal region. Many exocrine secretory granules are present in the apical region of cells, and electron dense granules are observed in the lateral and basal regions (arrows). Bar, 2 μm. b. Crystalline structures in an electron dense granule. They are composed of electron-lucent and electron-dense regions measuring about 8 nm and 4.5 nm in width, respectively, and have accumulated in a parallel array at several sites in the granule. Bar, 0.2 μm.
Fig. 4. Immunoelectron micrograph demonstrating renin in the coagulating gland of the C57BL/6 mouse. Positive reactions for renin are observed in the electron dense granules (arrows) located in the lateral and basal regions of the epithelial cell, but not in exocrine granules. N, nucleus; L, lumen. Bar, 2 µm.
10% dextran sulphate, 300 μg/ml of denatured salmon sperm DNA, and 5 ng/ml of 32P labelled mouse Ren-1 DNA probe (prepared to 535 base pairs with Eco R1/Hin dIII restriction enzymes). The membrane was washed twice with 2 × SSC (1 × SSC, 0.15 M-NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 10 min, twice with 2 × SSC containing 1% SDS at 60 °C for 30 min, and twice with 0.1 × SSC at room temperature for 30 min. The membrane was then subjected to autoradiography at −80 °C for 48 h with an intensifying screen.

RESULTS

The coagulating glands of mice are located adjacent to the medial side of the seminal vesicles. On light microscopy, the coagulating glands, differentiated from the seminal vesicles by paler eosin staining and slight dilatation of the lumen, were composed of single columnar or cuboidal epithelium: mucosal plicae were observed in the lumen. No differences in these morphological characteristics existed between C57BL/6 and Balb/c mice.

With immunohistochemical staining, immuno-reactivities for renin were observed in the epithelial lining of the coagulating glands of C57BL/6, but not Balb/c mice (Figs 1a, b). The immunoreactivities displayed a dot-like shape of varying diameter in the lateral and basal regions of epithelial cells (Fig. 2). Sometimes they were detected on the epithelial cell nuclei. No immunoreactivity was detected in the seminal vesicles of either C57BL/6 or Balb/c mice.

In the electron microscopic study of epithelial cells, a few microvilli were present at the apical boundaries, while basal regions were relatively flat at which site basal cells were sometimes localised (Fig. 3a). In the supranuclear region, the Golgi apparatus was well-developed and contained immature exocrine secretory granules which were being actively produced. As a result, rough endoplasmic reticulum with large dilated cisternae were plentiful in the basal region. Many exocrine secretory granules measuring about 600 nm in diameter and containing an electron dense core were demonstrated in the apical region of cells. No differences in the general ultrastructural morphology related to exocrine mechanisms were observed between the coagulating glands of C57BL/6 and Balb/c mice.
Many electron dense granules distinct from the exocrine granules and varying in morphological content were observed, especially in the lateral and basal regions of epithelial cells. Many of these, which could possibly be categorised as lysosomes, were round or oval in shape, or sometimes spindle-shaped or with an irregular contour. Crystalline structures were demonstrated in these granules (Fig. 3b). They were composed of electron-lucent and electron-dense regions measuring about 8 nm and 4.5 nm in width, respectively, and had accumulated in a parallel pattern in some parts of the granules. Sometimes they were divided into 2-4 groups within a granule. It was occasionally observed that the electron-dense granules and the secondary lysosomal granules were fused. These granules containing crystalline structures were observed more frequently in the coagulating gland of the C57BL/6 mouse than in that of Balb/c mice. Moreover, the crystalline structures of Balb/c mice were demonstrated only in the peripheral regions just below the granular membrane.

In the coagulating gland of the C57BL/6 mouse, no colloidal gold particles indicating the presence of renin were detected immunoelectron microscopically in the exocrine secretory granules in the apical regions of epithelial cells (Fig. 4). However, they were demonstrated in the electron-dense granules, where they showed a heterogeneous distribution and were located especially on the crystalline structures (Fig. 5a). In the Balb/c mouse, colloidal gold particles were detected neither in the exocrine granules, nor in the electron dense granules containing crystalline structures (Fig. 5b).

In the Northern blot analysis using Ren-1 cDNA, signals were detected not only in the kidney but also in the coagulating glands of both C57BL/6 and Balb/c mice. The expression in C57BL/6 coagulating glands was more intense than that in Balb/c (Fig. 6).

**FIG. 6. Northern blot analysis for coagulating gland renin mRNA.** Renin mRNA is detected in the coagulating glands of both C57BL/6 and Balb/c mice, but the expression in C57BL/6 coagulating glands is stronger than that in Balb/c. A, Coagulating gland of C57BL/6; B, kidney of C57BL/6; C, liver of C57BL/6; D, coagulating gland of Balb/c; E, kidney of Balb/c.

**DISCUSSION**

The expression of the renin gene, particularly the Ren-1 gene, in the coagulating gland has been previously reported by Fabian et al. (1989). (In that report, the coagulating glands were described as the sex accessory glands.) In the present study, this result was confirmed by immunohistochemistry, immunoelectron microscopy and Northern blotting. The functions of extrarenal renin have not yet been completely clarified. It is known that the renin found in the Leydig cells of the rat testis and the angiotensin I-converting enzyme, part of the renin-angiotensin system 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 lowest levels during the post-oestrous phase (Hackenthal et al. 1980). In the oviduct of the rabbit, oestadiol administration resulted in a marked increase in renin release (Eskildsen, 1972). These findings suggest that the renin-angiotensin system in reproductive tissue is dependent on sexual maturation and plays some role in the regulation of the reproductive system.

Angiotensinogen is the only substrate for renin. The main functional autacoid in the renin-angiotensin system is angiotensin II, which is synthesised by the cleavage of angiotensinogen. The role of circulating angiotensin II is the control of blood pressure and the maintenance of water–mineral balance (Davis & Freeman, 1976). Another function of angiotensin II is the synthesis of prostaglandin in several organs (Needleman et al. 1975; Shereske & Aiken, 1980). Moreover, prostaglandin has been shown to stimulate steroidogenesis in the adrenal gland (Saruta & Kaplan, 1972), and renin release in association with beta-adrenergic receptors (Suzuki & Hashiba, 1986). It is assumed that angiotensin II produced outside the kidney plays a function in regulating local homeo-
stasis. In the present study, it is possible that coagulating gland renin also plays a role in production of the angiotension series, and functions in association with prostaglandin. The function of prostaglandin found in seminal fluid is in the contraction of smooth muscle in the uterus and vagina and in the decrease of blood pressure in the body, while angiotension II functions in the contraction of smooth muscle and the increase of blood pressure. These findings may suggest that renin/angiotension/prostaglandin work in local-feedback and local-enhancing mechanisms in the reproductive tissue.

The present study investigated the differences in renin expression and ultrastructural granular morphology between C57BL/6 and Balb/c mice. In C57BL/6 mice, renin protein/mRNA were detected by both immunohistochemistry and Northern blot analysis, while in Balb/c mice they were not demonstrated by immunohistochemistry. Moreover, it was observed that the condition and frequency of the crystalline structures in dense granules differed between C57BL/6 and Balb/c mice. These findings lead to the following 3 hypotheses. First, it may be said that Northern blot analysis is more sensitive for detecting mRNA than immunohistochemistry using antigen-antibody binding. Secondly, the antigenic determinants of local renin, especially in coagulating glands of Balb/c mice, differ from those of renal renin. Indeed, preliminary immunohistochemical investigations using the coagulating glands of other mouse strains (ICR, A/He) revealed variable expression patterns (Kon et al., unpublished observations). These findings may suggest that there are unknown DNA signals expressing/inhibiting extrarenal local renin, as well as Ren-1 and Ren-2, depending on the mouse strain. Finally, it is possible that renin mRNA is not converted to a protein form, although it is expressed in this tissue. Further studies are required in these areas.

From electron microscopy, it is known generally that the crystalline structures found in the cells/tissues are due to the condensation packing of purely protein materials without modification by other materials (Fawcett, 1981). In the juxtaglomerular cells of the kidney, crystalline structures observed in the small granules, so-called juvenile granules, subsequently disappear with the maturation of secretory granules (Barajas, 1966). In the present study of C57BL/6 mice, the crystalline structures were observed in many granules of variable size, not only in juvenile granules but also in mature-type granules. The results may show that the enzyme renin is condensed in these granules in the coagulating glands, and that the formation and function of these crystalline structures detected in this tissue differs from those in the kidney. In any case, it should be emphasised that renin expression in the coagulating gland is most significant in the investigation of the local renin-angiotensin system.

REFERENCES


