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A simple method for the specific detection of Ren-1 renin

Akira Yabuki, Syusaku Suzuki, Mitsuharu Matsumoto, Kazuyuki Taniguchi, and Hayao Nishinakagawa

Department of Veterinary Anatomy, Faculty of Agriculture, and Institute of Laboratory Animal Sciences, Faculty of Medicine, Kagoshima University, Kagoshima, and Department of Veterinary Anatomy, Faculty of Agriculture, Iwate University, Morioka, Japan

A simple method for the specific detection of Ren-1 renin.

Background. Ren-1 and Ren-2 renin are expressed in the kidneys of all mice and in the submandibular gland of several mouse strains. The present study determined the usefulness of modified periodic acid silver-methenamine (PAM) staining for the specific detection of Ren-1 renin.

Methods. Conventional paraffin sections were prepared from kidneys of ICR, BALB/cA, C57BL/6Cr, C3H/HeN, DBA/2Cr, angiotensin II type 1a receptor gene knockout (AT1aKO) mice, Wistar rats and a human, and submandibular glands of C57BL/6Cr and DBA/2Cr mice. Sections were analyzed for the presence of renin using PAM and immunohistochemistry. PAM reactions were terminated at generally or weakly intense (weak PAM staining; W-PAM). In addition, kidneys of DBA/2Cr mice were fixed using various fixatives (formalin, PFA, PLP, Zamboni's, Bouin's, or Carnoy's) and treated using identical procedures.

Results. Although PAM-positive reactions were observed in juxtaglomerular (JG) cells, W-PAM reactions were particularly specific for these cells. These findings were observed in all mouse strains. Immunohistochemistry using mirror sections suggested that a W-PAM–positive reaction detected renin. This hypothesis was confirmed by the results from AT1aKO mice. Briefly, W-PAM detected an expansion of renin-positive areas in AT1aKO mice. Rat and human kidneys and mouse submandibular glands were negative for W-PAM. Levels of JG cell detection by W-PAM were similar in samples fixed in formalin, PFA, PLP, or Zamboni's.

Conclusions. The present findings show that W-PAM can identify Ren-1 renin, but not Ren-2, rat or human renin. The W-PAM method is useful for the specific detection of Ren-1 renin.

Renin is an important enzyme for controlling blood pressure and is highly localized in the juxtaglomerular (JG) cells of the kidney. Renin genes from several animal species have been cloned using molecular biological techniques [1–3]. A single renin locus has been identified in humans and rats. Some strains of mice possess a single renin locus (Ren-1), while others display duplicate renin loci (Ren-1 and Ren-2), with Ren-1 coding for kidney renin and Ren-2 coding for submandibular gland renin [4, 5]. Immunohistochemistry and in situ hybridization are powerful tools for assessing the distribution of expression for each type of renin [6–9]. However, immunohistochemistry requires a specific antibody, and in situ hybridization is not used routinely in many laboratories. In addition, although several classic staining methods have been employed to detect renin [10, 11], these methods lack specificity and restrict the fixative that can be utilized [10].

Periodic acid silver-methenamine (PAM) staining is a classic histochemical technique [12, 13], and the chemical mechanism of this stain is explained in detail by Rambourg [14, 15]. This stain is routinely used in histopathologic identification of glomerulonephritis because it can stain the glomerular basement membrane and mesangial cells efficiently and with high contrast. However, little is known about the specificity of PAM-staining for renin. The present report describes the usefulness of modified PAM stain for specific detection of Ren-1 renin.

METHODS

All experimentation proceeded in accordance with the Guidelines for Animal Experimentation of the Faculties of Medicine and Agriculture, Kagoshima University, Japan.

Animals and preparation of tissue sections

All animals were housed in an open system room with a one-way airflow system (temperature, 22 ± 1°C; humidity, 55 ± 10%; light period, 07:00 to 19:00; ventilation, 12 cycles/h) at the Institute of Laboratory Animal Sciences, Faculty of Medicine, Kagoshima University, Japan. Animals received an autoclaved commercial diet (CE-2; Japan CLEA, Tokyo, Japan) and tap water ad libitum. All

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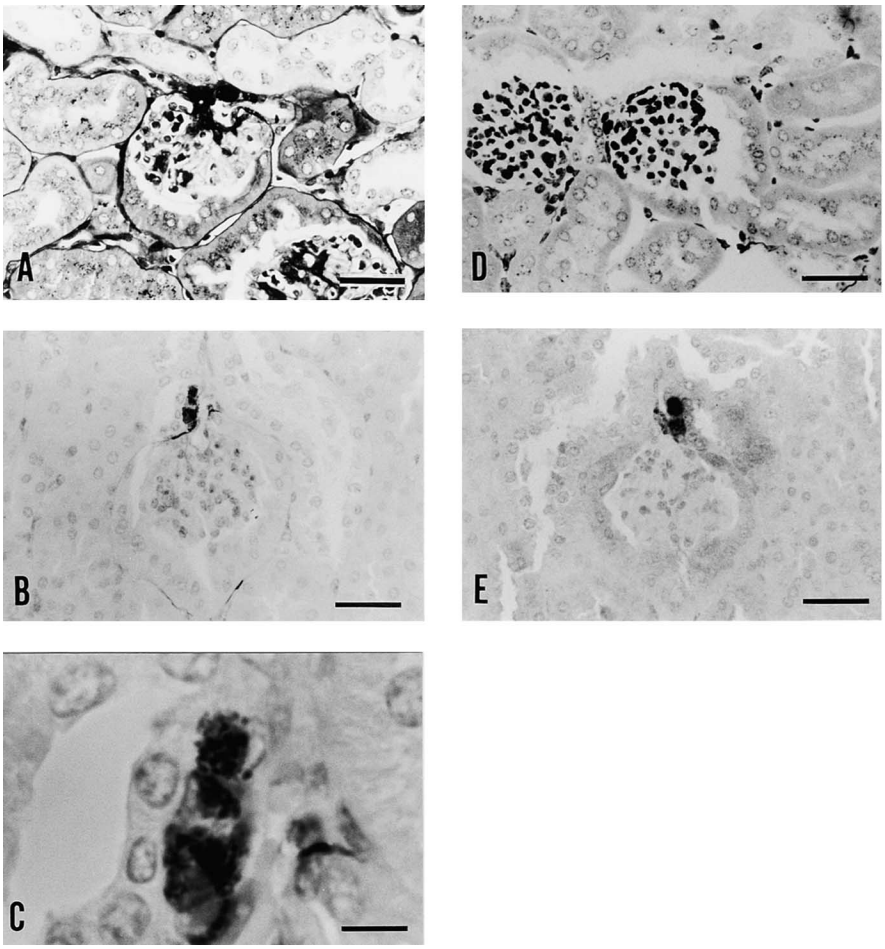

Fig. 1. Light micrographs of formalin-fixed kidney sections in male C57BL/6Cr mice. (*A*) G-PAM stain. (*B*) W-PAM stain. (*C*) Higher magnification view of B. (*D*) Silver-methenamine stain without periodic acid oxidation. (*E*) Immunohistochemical detection of renin (mirror to panel B). Bar = 50 μm (A, B, D and E) or 10 μm (C).

animals were sacrificed by exsanguination under anesthesia (mixture of ketamine and medetomidine).

To represent common strains of mouse, three- to four-month-old male and female Jcl:ICR, BALB/cA Jcl, C57BL/6Cr Slc, C3H/HeN Jcl, and DBA/2Cr Slc mice ($N = 3$) were utilized. Following sacrifice, their kidneys were quickly removed, sliced, and then immersed in 10% neutral formalin in 0.1 mol/L phosphate buffer (PB). To evaluate the effects of the fixatives, three female DBA/2Cr Slc mice (three months old) were used, and the kidney sections were fixed in formalin, 4% paraformaldehyde in PB (PFA), periodate-lysine-paraformaldehyde (PLP), Zamboni's, Bouin's or Carnoy's solutions. Following this procedure, five-month-old male angiotensin II type 1a receptor gene knockout (AT1aKO) mice and age-matched male C57BL/6Cr Slc wild-type mice ($N = 5$) were used, and the kidney sections were fixed in Zamboni's solution. AT1aKO mice were derived from Tanabe Seiyaku Co., Ltd. (Osaka, Japan), at three-months-old. The AT1aKO mouse has been shown to represent a model of high expression of kidney renin [16–18]. Moreover, to evaluate PAM-specificity to Ren-2, rat and human renin, submandibular glands of three-month-old male C57BL/6Cr Slc and DBA/2Cr Slc mice ($N = 3$) and kidneys of three-month-old male Wistar Slc rats ($N = 3$) were sampled and fixed in Zamboni's solution, and human adult normal kidney tissue slides (26-year-old male donor; paraformaldehyde fixation of tissue; 5 μm paraffin sections) were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

After fixation, sampled tissues were thoroughly washed in PB, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Serial or mirror sections cut at 3 μm were mounted on silane-coated glass, and analyzed for renin by PAM staining or immunohistochemistry. Wilson's crystal violet stain was applied to several kidney sections of AT1aKO and C57BL/6Cr wild-type mice [11] as a classical method for detecting JG cells.

PAM staining

Periodic acid silver-methenamine staining was performed according to the method described by Jones [12]. In brief, tissues were (*1*) deparaffinized and hydrated; (*2*) immersed in 0.5% periodic acid for 15 minutes; (*3*) washed in distilled water; (*4*) placed in Gomori's silver-methenamine solution at 58°C; (*5*) washed in distilled water; (*6*) toned in 0.2% gold chloride for two minutes;

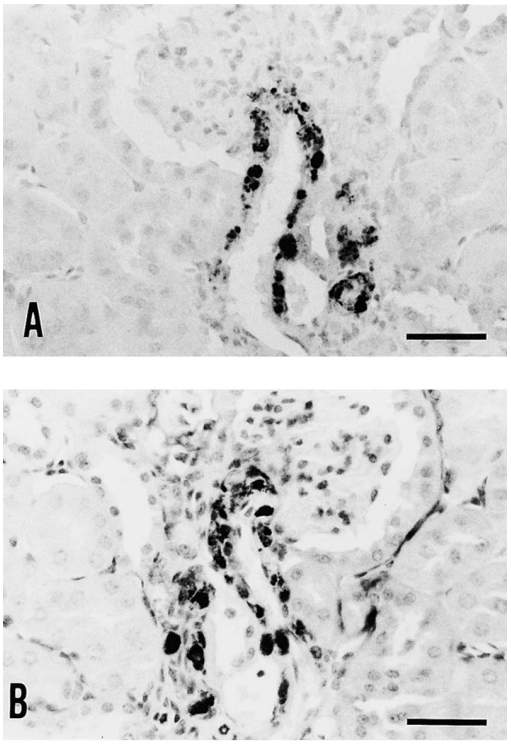

Fig. 2. Light micrographs of kidney sections in male AT1aKO mice. (*A*) Renin immunohistochemistry. (*B*) W-PAM stain (mirror to panel A). Bar = 50 μm.

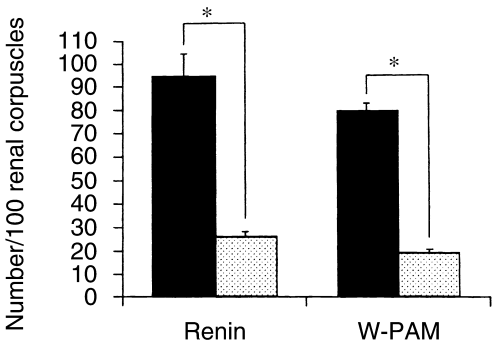

Fig. 3. Index of number of renin- and W-PAM–positive arterioles in kidneys of AT1aKO (■) and C57BL/6Cr (□) wild-type mice. Each column represents mean ± standard error. *Statistically significant difference, $P < 0.01$.

(*7*) washed in distilled water; (*8*) placed in 3% sodium thiosulfate for two minutes; (*9*) washed in distilled water; and (*10*) counterstained with hematoxylin.

The following PAM-staining intensities were determined under light microscopy while samples were in the silver-methenamine solution. (*1*) Generally intense PAM (G-PAM) reactions were terminated when the basement membrane of glomeruli or tubules stained positively. (*2*) Weak PAM (W-PAM) reactions were terminated when the basement membrane of the outer layer of several glomerular capsules began to stain positively. The primary difference between G-PAM and W-PAM was the timing of reaction termination in the silver-methenamine solution. In several sections, periodic acid oxidation was omitted prior to the silver-methenamine reaction. Whenever submandibular gland sections were stained with PAM, kidney sections were stained at the same time as a control for staining intensities.

Immunohistochemical procedure

Immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA). For detection of renin, rabbit antiserum raised against recombinant renin (supplied by Dr. Kazuo Murakami, University of Tsukuba, Japan), was used as the primary antibody. The samples were incubated with the primary antibody (diluted 1:10,000) overnight at 4°C and then with the secondary antibody, biotinylated goat anti-rabbit Ig G (H+L; Vector Laboratories), diluted 1:200, for 30 min at room temperature. Immunoreactivity was detected by a 0.025% 3,3′-diaminobenzidine–0.003% H_2O_2 solution, and counter staining was performed with hematoxylin.

Semiquantitative procedure

In the kidneys of AT1aKO and C57BL/6Cr wild-type mice, numbers of renin- or W-PAM-positive arterioles were semiquantitatively analyzed according to the procedure described in previous reports [18, 19]. Briefly, the total number of renin-positive or W-PAM-positive arterioles and the total number of renal corpuscles were counted in each section (3 sections selected every 30 μm for each animal). Values were expressed per 100 renal corpuscles, and statistically analyzed using the Student *t* test.

All light micrographs in this study were taken using a special filter for enzyme-labeled immunohistochemistry (IF436; Olympus, Inc., Tokyo, Japan) to reduce nuclear color.

RESULTS

In the kidneys of common strains of mouse, G-PAM–positive reactions were observed in the glomerular basement membrane, tubular basement membrane, mesangial cells, connective tissue, erythrocytes, tubular cytoplasmic granules, and JG cells (Fig. 1A). W-PAM–positive reactions were highly localized in the JG cells (Fig. 1B), and granular components were observed under higher magnification (Fig. 1C). Although connective tissue, erythrocytes, and tubular granules reacted positively in the absence of periodic acid oxidation, JG cells were negative (Fig. 1D). Immunohistochemical analysis of W-PAM mirror sections identified renin-positive areas that matched

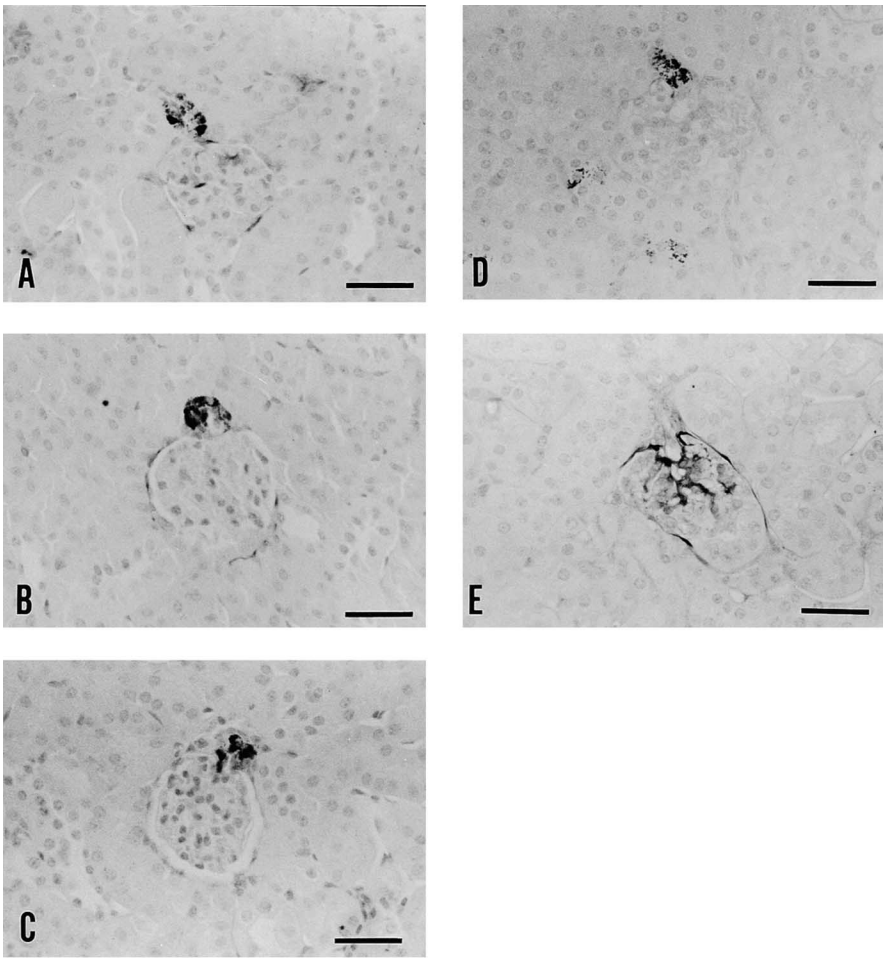

Fig. 4. Light micrographs of W-PAM stained kidney sections from female DBA/2Cr mice, fixed using (*A*) PFA, (*B*) PLP, (*C*) Zamboni's, (*D*) Bouin's, or (*E*) Carnoy's solutions. Bar = 50 μm.

the W-PAM positive JG cells (Fig. 1 B, E). The results were observed in all mice used in the present study.

In the kidneys of AT1aKO mice, the renin-positive area was substantially larger than that in C57BL/6Cr wild-type mice. Renin-positive reactions were observed not only close to the vascular poles, but also in arterioles distant from the vascular poles (Fig. 2A). The index of numbers of renin-positive arterioles in AT1aKO (94.8 ± 9.3) was about 3.7-fold greater than that in C57BL/6Cr (25.9 ± 1.9; Fig. 3). For W-PAM staining, positive arterioles in AT1aKO were much wider than those in C57BL/6Cr, and the localization of these matched that of renin-positive arterioles (Fig. 2B). The index for numbers of W-PAM–positive arterioles in AT1aKO (79.8 ± 3.2) was about 4.2-fold greater than that in C57BL/6Cr (18.9 ± 1.2; Fig. 3). In sections stained with Wilson's crystal violet, staining results were unstable and assessment of positive granules proved difficult.

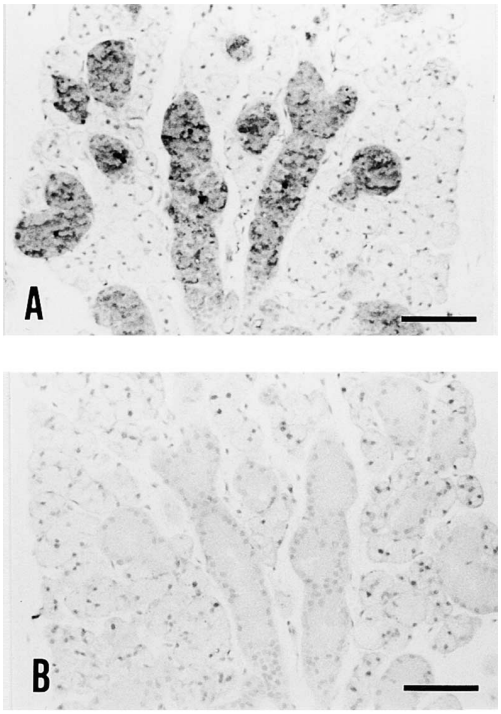

Fig. 5. Light micrographs of submandibular gland sections in male DBA/2Cr mice. (*A*) Renin immunohistochemistry. (*B*) W-PAM stain in DBA/2Cr (mirror to panel A). Bar = 100 μm.

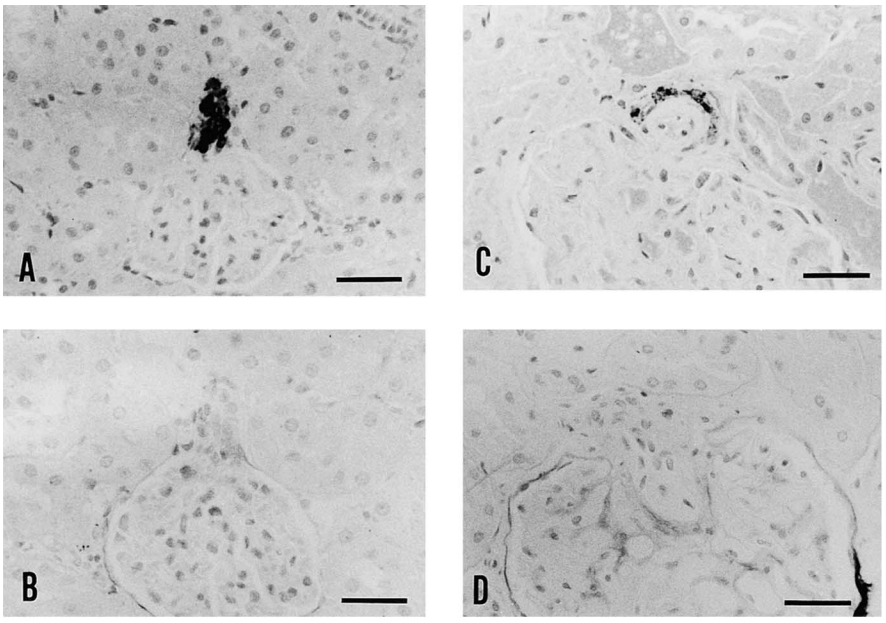

Fig. 6. Light micrographs of kidney sections in male Wistar rat and human. (*A*) Renin immunohistochemistry in Wistar rat. (*B*) W-PAM stain in Wistar rat (mirror to panel A). (*C*) Renin immunohistochemistry in human. (*D*) W-PAM stain in human. Bar = 50 μm.

Variations were observed between samples prepared using different fixatives. Although positive reactions in the basement membrane of the outer layer of glomerular capsules were observed for all fixatives, time-to-staining differed for each fixative as follows: formalin ≠ PFA ≠ PLP < Zamboni's << Bouin's << Carnoy's. Efficient detection of JG cells by W-PAM was observed for samples fixed in PFA, PLP, or Zamboni's (Fig. 4A-C), as well as formalin (Fig. 1B). Although positive reactivity was observed in JG cells for samples fixed in Bouin's solution, specificity for JG cells was lower than in samples fixed in formalin, PFA, PLP, or Zamboni's solution. In the Bouin's samples, many granules were detected in the tubules as well as JG cells (Fig. 4D). JG cells were PAM-negative in sections fixed in Carnoy's, regardless of the staining criterion (Fig. 4E). Fixatives also affected immunohistochemistry results. The intensity of the positive reactions was as follows: Bouin's > Zamboni's > formalin ≠ PFA ≠ PLP > > Carnoy's. Background staining was particularly high in Carnoy's sections, which hampered identification of renin-positive reactions.

Submandibular glands were examined in male DBA/2Cr and C57BL/6Cr mice. In immunohistochemistry, detection of renin was different based on strain, and positive reactions were detected in the granular portions of excretory ducts from DBA/2Cr (Fig. 5A). No immunoreactivity was observed in the submandibular glands of C57BL/6Cr. On the other hand, staining results of W-PAM were identical between DBA/2Cr and C57BL/6Cr, and no positive reactions were observed in the submandibular glands (Fig. 5B). In the kidneys of Wistar rats and human, renin-positive JG cells were detected by immunohistochemistry, and no positive reactions were observed in JG cells under W-PAM (Fig. 6).

DISCUSSION

The present study investigated the staining of mouse kidneys using different intensities of PAM stain, G-PAM and W-PAM. W-PAM clearly detected JG cells and these results were confirmed under immunohistochemical analysis of mirror sections. From these findings, we hypothesized that W-PAM could detect renin granules in the JG cells of mouse kidney, and W-PAM might represent a simple method for detecting renin in mouse kidney. However, to confirm this hypothesis, clarification was required as to whether W-PAM–positive granules in JG cells truly represented renin granules. Therefore, the kidneys of AT1aKO mice were investigated, as they have been demonstrated to show overexpression of renin and marked expansion of renin immunoreactive areas in the kidneys [16–18]. We examined kidneys of AT1aKO and age-matched C57BL/6Cr wild-type mice using mirror sections or semiquantitative methods. As a result, areas of arterioles containing W-PAM–positive granules were much wider in AT1aKO mice than in wild-type mice, and the index of numbers of W-PAM–positive arterioles demonstrated higher values for AT1aKO. These results from W-PAM staining matched those for immunohistochemistry for renin. These findings strongly suggest that W-PAM–positive reactions in JG cells of mouse kidney truly represent renin granules.

We also evaluated the suitability of specific fixatives to detect JG cells by W-PAM. Of the six fixatives, formalin, PFA, PLP, and Zamboni's provided the best results. On the other hand, Carnoy's was entirely unsuitable. Carnoy's solution is a mixture of acetic acid, chloroform, and methanol, and this fixative has been recommended previously for PAM staining [12]. However, Carnoy's

is unsuitable for fixation of low-weight molecular substances. The molecular weight of mouse kidney renin is relatively low, approximately 40 kD [20, 21], and the protein may have been exposed during tissue preparation. Bouin's solution also was found to be inappropriate for the detection of JG cells using W-PAM, despite its usefulness for immunohistochemistry. The specificity of W-PAM for JG cells was lower in sections fixed in Bouin's than in formalin, PFA, PLP, or Zamboni's, and many positive granules were detected in the tubules. In a previous study these PAM-positive granules were identified as lysosomes [22], and the present investigation showed that these granules were detected in absence of periodic acid oxidation. For W-PAM evaluation, samples fixed in Bouin's required a longer reaction time in silver-methenamine solution than formalin, PFA, PLP, or Zamboni's. The detection of tubular granules in tissues fixed in Bouin's therefore results from an oxidation-independent reaction that is faster than the PAM-positive reaction of the basement membrane of the outer layer of the glomerular capsule.

Furthermore, we evaluated whether W-PAM can detect Ren-2 renin in the submandibular gland. Expression of Ren-2 renin differs based on strain [4, 5], and the present study investigated the submandibular glands of male C57BL/6Cr (a major strain possessing only Ren-1) and DBA/2Cr (a major strain displaying both Ren-1 and Ren-2) mice. As a result, the submandibular glands of neither strain demonstrated W-PAM-positive reactions, while those of DBA/2Cr displayed positive immunoreactions for renin. In addition, we evaluated whether W-PAM could detect renin from rats and human, and renin of these species did not react with W-PAM. These findings confirm that W-PAM reaction is specific for Ren-1 renin, and is non-reactive for Ren-2, rat or human renin. Ren-1 renin is reportedly a glycosylated protein, while Ren-2 renin is not glycosylated [21]. Structural differences between Ren-1, Ren-2, rat and human renin genes also have been reported [23]. Although the regions of peptides or genes associated with the W-PAM reaction to Ren-1 renin are unknown, the simple W-PAM method presented in our present study should prove useful as a routine histochemical procedure for specific detection and classification of Ren-1 renin.

In conclusion, we emphasize the simplicity of the W-PAM technique. The chemical composition of solutions used in W-PAM did not differ from the original PAM staining protocol. Determination of the appropriate reaction termination point is relatively easy, as positive reactivity of the basement membrane proceeds from the outer layer of the glomerular capsule in all kidney sections, regardless of sex, strain of mouse, or fixative. In addition, types of tissue fixative for this method are relatively unrestricted, and the formalin, PFA, PLP, or Zamboni solutions are recommended.

Reprint requests to Akira Yabuki, D.V.M., Ph.D., Department of Veterinary Anatomy, Faculty of Agriculture, Kagoshima University, 21-24 Korimoto 1, Kagoshima-shi, Kagoshima 890-0065, Japan.
E-mail: yabu@vet.agri.kagoshima-u.ac.jp

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