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Establishment of the Anti-Klotho Monoclonal Antibodies and Detection of Klotho Protein in Kidneys

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A novel gene, *klotho* (*kl*), which is involved in the development of a syndrome resembling human aging in mice, was recently identified. The *kl* gene encodes a single-pass membrane protein whose extracellular domain carries homology to β-glucosidases. There also exists a splice variant of *kl* mRNA which encodes a putative secreted protein in both human and mouse. In this study, to characterize the physiological roles of Klotho protein, we established three monoclonal antibodies (mAbs) against the recombinant human Klotho protein. The mAbs are named KM2076 (rat IgG_2a), KM2119 (rat IgG_2b), and KM2365 (mouse IgG_1). In Western blots, KM2076 and KM2119 specifically recognized a 130 kDa Klotho protein in the mouse and human kidney membrane fractions. To detect the human Klotho protein, the sandwich-type ELISA system with KM2076 and KM2365 was established. Using the ELISA system, we detected the human Klotho protein as low as 20 ng/ml in the supernatant of Chinese hamster ovary cells (CHO cells), introduced the human *klotho* gene. KM2076 and KM2119 specifically gave a positive staining by immunohistochemical staining in paraffin or frozen sections of the kidneys from wild-type mice but not in those from *kl* mice. Strong staining was observed especially in cortical renal tubules of the mouse kidney, where expression of *klotho* transcripts overlaps. KM2076 also showed a similar reaction pattern in the paraffin sections of rat and human kidneys. The mAbs established in this paper will serve as useful analytical, pathological, and diagnostic tools to disclose the role of Klotho protein in the suppression of a syndrome resembling human aging.

Abbreviations used: mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; RT-PCR, polymerase chain reaction with reverse transcription; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

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A novel mouse model for human aging was recently established and the gene responsible for it was identified (1). A decrease in the expression of a novel gene *klotho* (*kl*) in mice, leads to a syndrome resembling human aging. These mice, *klotho* mice, exhibited multiple disorders, such as arteriosclerosis, osteoporosis, skin atrophy, pulmonary emphysema, short life-span and infertility. The *kl* gene encodes a novel single pass-membrane protein (1,014 a.a.) consists of an N-terminal signal sequence, a putative extracellular domain with two internal repeats (hKL1 and hKL2), a single membrane-spanning region, and a short intracellular domain. These internal repeats share the sequence similarity to the β-glucosidase enzymes (2, 3). The human homologue of the *kl* gene encoding a membrane protein (1,012 a.a.) was also isolated (4). From the cDNA analysis, it was found that there exists a splice variant of *kl* mRNA which encodes a putative secreted protein (human: 549 a.a., mouse: 550 a.a.) lacking the second internal repeat of the extracellular domain (hKL2), the transmembrane domain and the intracellular domain (4, 5). Expression of the mouse *kl* gene was detected only in kidney and brain by Northern blot analysis. But the lower expression was observed in the pituitary gland, placenta, skeletal muscle, urinary bladder, aorta, pancreas, testis, ovary, colon and thyroid gland by RT-PCR (1). On the other

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hand, the human *kl* gene transcript was expressed predominantly in the kidney, placenta, prostate and small intestine (4).

Recent studies showed that the pathophysiological significance of the Klotho protein in some rat models for human diseases such as (a) spontaneously hypertensive rat, (b) deoxycorticosterone acetate-salt hypertensive rat, (c) 5/6 nephrectomized rat, (d) non-insulin-dependent diabetes mellitus rat models (6). The expression levels of *kl* mRNA in the kidney in these rat models were significantly lower than controls, indicating that the expression of the *kl* gene in the kidney is regulated under sustained circulatory stress such as a long-term hypertension, diabetes mellitus, and chronic failure.

In this study, we immunized rats and mice with the human recombinant Klotho protein and established mAbs to detect Klotho protein in the western blots, ELISA system and immunohistochemical staining. These mAbs will serve as useful analytical, pathological and diagnostic tools to disclose the role of Klotho protein in the suppression of a syndrome resembling human aging.

MATERIALS AND METHODS

Specimens. Paraffin-embedded blocks of human tissues were kindly provided by Dr. T. Kamei of Yamaguchi Prefectural Central Hospital (Yamaguchi, Japan). Freshly isolated tissues from mice and rats were immediately fixed in 10% buffered formalin.

Expression of human recombinant Klotho protein. A SacII cDNA fragment which encodes a part of hKL1 (amino acids 55–261) and a cDNA fragment (KpnI-ApaI) which encodes a part of hKL2 (amino acids 801–954) were subcloned into a vector derived from pGHA2 (7). The recombinant protein was expressed in *Escherichia coli* in inclusion bodies and was purified from SDS-polyacrylamide gel.

Hybridoma production and purification of mAbs. Rat monoclonal antibody (mAb), KM2076, was established by immunizing rats with a part of hKL1 (amino acids 55–261), and that of KM2119 was established by immunizing rats with a part of hKL2 (amino acids 801–954). KM2365 was established by immunizing mice with a synthesized amino peptide (DDAKYMYYLKKFIMETLKAIKLDGV), which is a part of the human Klotho protein (8). The spleen cells were fused with mouse myeloma P3U1 cells, and the culture supernatants of the hybridomas were screened by ELISA for their ability to recognize the human recombinant Klotho protein. Hybridoma cells were cultured in RPMI growth medium, harvested by a brief centrifugation, and suspended in PBS. Four-week-old female BALB/c nude mice (CLEA Co., Tokyo, Japan) were given i.p. injections of the hybridoma cells. The mice were sacrificed 7 days later, and ascites fluid was collected. The mAbs were purified using protein G-Sepharose 4B gel beads (Zymed, San Francisco, CA).

Western blot analysis. Kidneys obtained either from human or from mice of 5-week-old wild-type or *klotho* mice were homogenized in the homogenize buffer (20 mM Tris-HCl at pH 7.5, 0.25 M Sucrose, 1 mM EDTA, 10 mM EGTA, 10 mM 2-mercaptoethanol) by 6 passes in a Tefronglass homogenizer. Each homogenate was centrifuged at 100,000 × g at 4°C for 10 min. The supernatant was centrifuged again at 100,000 × g at 4°C for 1 h. After the pellet was resuspended in homogenize buffer, membrane proteins were extracted with 1% Triton X-100. Samples from mice kidneys (20 μg) or from human kidneys (100 μg) were boiled for 5 min, with 1:4 dilution of the sample buffer containing 200 mM Tris-HCl (pH 6.8), 4% SDS, 100 mM DTT, 40% glycerol, and 0.04% bromophenol blue. Proteins in the samples were separated on SDS-PAGE gel (7.5–15% gradient) and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk in PBS and were incubated with the culture supernatant at 37°C for 1 h. They were washed and were incubated with peroxidase-conjugated anti-rat IgG antibody (Amersham) at 37°C for 1 h. For visualizing bands, the ECL Western blotting detection system (Amersham) was used.

Biotinylation of antibodies. Biotinylation of KM2365 was performed with Antibody Biotinylation Kit (American Qualex, San Clemente, CA). Briefly, a purified KM2365 mAb (5 mg) was dissolved in 0.6 ml of carbonate buffer concentrate. Six hundred microliters of biotin solution (1 mg/ml) was added into the antibody solution and gently shook for 1 h at room temperature. Unreacted biotin was removed by passing through G25 column. The biotinylated KM2365 was stabilized by adding BSA to a final concentration of 1% (w/v) and stored at 4°C until use.

Sandwich ELISA for the detection of Klotho protein. Klotho protein level in the supernatant was determined with the sandwich ELISA as follows. Fifty microliters of KM2076 (10 μg/ml) in PBS was added into each well of the 96-well ELISA plate and incubated overnight at 4°C, and the wells were washed with PBS for 3 times and flooded with 1% BSA-PBS for 1 h at room temperature. The secreted isoform of human Klotho protein was constitutively expressed using CHO cells (unpublished data) and the supernatant (40 μl) was diluted and applied into each well and incubated overnight at 4°C. The supernatants were then aspirated and the wells were washed with 0.05% Tween 20 in PBS for 5 times. Fifty microliters of biotinylated KM2365 (10 μg/ml) in 1% BSA-PBS were added and incubated for 2 h at room temperature. After the wells were thoroughly washed with 0.05% Tween 20-PBS for 5 times, 50 μl of avidin-biotin-peroxidase complex (Vectastain DAB kit: Vector Laboratories Inc., Burlingame, CA) were added and incubated for 1 h at room temperature. After washed with 0.05% Tween 20-PBS for 5 times, 100 μl of ABTS solution with 0.1% H_2O_2 were applied for 10 min at room temperature, and the absorbance at 415 nm and 490 nm was measured.

In situ hybridization. ^{35}S-Labeled riboprobes were prepared from the full-length cDNA for mouse *klotho* (1). Antisense and sense [^{35}S]cRNAs were synthesized from linearlized plasmids in the presence of [^{35}S]UTP (1289 Ci/mmol; New England Nuclear Corp; Boston, MA). *In situ* hybridization was performed as described previously (9). In brief, sections were postfixed with 4% PFA-PBS for 15 min. After washing with PBS, sections were digested with 1 μg/ml proteinase K (37°C for 15 min) in PBS and again treated with 4% PFA-PBS (10 min). Sections were then sequentially washed with PBS, incubated with 0.2 N HCl (10 min), again washed with PBS, acetylated with 0.25% acetic anhydride in the presence of triethanolamine (0.1 M, 10 min), dehydrated with increasing concentrations of ethanol, and air dried. Hybridization with ^{35}S-labeled complementary RNAs (cRNAs; ca. 5×10^7 cpm/ml) were performed in a humidified chamber in a solution containing 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 50 mM dithiothreitol, 0.25% sodium dodecyl sulfate, and 200 μg/ml transfer RNA (18 h; 55°C).

After hybridization, sections were washed briefly with 5× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) at 50°C, 50% formamide–2× SSC (50°C for 30 min), and 10 mM Tris-HCl (pH 7.6)-500 mM-1 mM EDTA (TNE; 37°C; 10 min). Sections were then treated with 10 μg/ml ribonuclease-A in TNE (37°C; 30 min). After being washed with TNE, sections were incubated once with 2× SSC (50°C; 20 min) and twice with 0.2× SSC (50°C; 20 min), dehydrated with increasing concentrations of ethanol, and air-dried. Slides were then placed on X-ray films (Fujifilm, Tokyo, Japan) and film autoradiographs were obtained after overnight exposure. Slides were dipped into NTB-2 (Eastman Kodak, New Haven, CT) and stored at 4°C for specific times estimated from the intensity of the expression

on the X-ray film. After development, sections were counterstained with hematoxylin and eosin and mounted.

Immunoperoxidase staining (paraffin sections). Paraffin sections were dewaxed in xylene, hydrated through graded alcohol solutions and washed with PBS. Endogenous peroxidase was blocked by immersion in 0.3% (wt/vol) hydrogen peroxide in absolute methanol for 30 min at room temperature. The following steps were carried out using a Vectastain ABC kit. Briefly, after washed with PBS for 5 times, nonspecific binding was blocked with VECTASTAIN RABBIT Normal Serum for 20 min at room temperature. Then KM2076 (10 μg/ml) were applied on sections and incubated for 1 h at room temperature. Then sections were washed with PBS (5 times) and were incubated with anti RAT IgG. DAB substrate was diluted to the half and applied. Sections were incubated 2 to 4 min according to the time predetermined and the reaction was terminated by immersing sections into deionized water for 5 min. Sections were counterstained either with hematoxylin or with methyl-green, and dehydrated with increase concentration of ethanol solution and then with xylene and mounted.

Immunoperoxidase staining (frozen sections). Mice were either anesthetized with ether or killed by rapid cervical dislocation. Kidneys were removed, cut into slices in 4% paraformaldehyde (PFA)/PBS and further fixed in the same solution overnight at 4°C. They were rinsed sequentially with PBS containing 5 and 10% sucrose for 4 h and then with the same buffer containing 15% sucrose overnight at 4°C. Tissues were placed in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen with liquid nitrogen. Frozen sections (6 μm thickness) were prepared and mounted on silane-coated glass slides. To block the endogenous peroxidase, sections were first washed 3 times in cold 10% sucrose/PBS solution (5 min each) and then incubated in 0.3% H_2O_2 in methanol for 15 min at room temperature and rinsed off with PBS. Nonspecific binding was blocked by incubating sections with 10% nonimmune goat serum (Zymed Laboratories, Inc., CA) for 10 min at room temperature. KM2076 (2 μg/ml in 1% BSA/PBS) was applied on sections and they were incubated overnight at 4°C. After rinsed with cold 10% sucrose/PBS three times, sections were incubated with peroxidase-conjugated anti body (goat anti-rat IgG(H + L) HRP, Chemicon International Inc.) for 4 h at room temperature. After washed with cold 10% sucrose/PBS three times, DAB solution was diluted to the half and was applied for 1 min at room temperature as described in the Vectastain DAB kit protocol. Sections were washed and stained with hematoxylin.

Fluorescence staining (frozen sections). Sections were prepared as described in immunoperoxidase staining (frozen sections) except for using avertin (0.8 ml/head) as anesthetic. After washing out O.C.T. compound with PBS, nonspecific binding was blocked with 10% goat serum for 15 min. KM2119 (ascites, 100× dilution with 3% FCS-PBS) incubation were done for 1 h followed by washing with PBS. Sections were incubated with Alexa Fluor 488 goat anti-rat IgG(H + L) conjugate (200× dilution with 3% FCS-PBS, Molecular Probes, Inc., OR) for 30 min (dark) and washed with PBS. Samples were observed with a Leica TCS SP confocal laser scanning microscope (absorbance 488 nm and emission 520 nm).

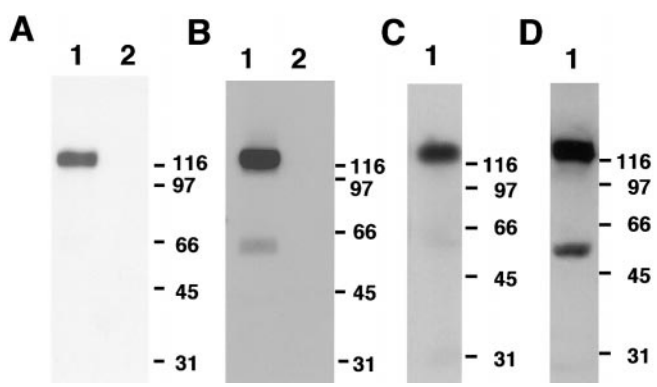


FIG. 1. Immunoblot analysis of the Klotho protein in the kidneys. Immunoblot analysis of the Klotho protein in the kidneys of wild (lane 1) and *kl* mice (lane 2) with (A) KM2076 and (B) KM2119. Immunoblot analysis of the Klotho protein in the human kidneys (lane 1) with (C) KM2076 and (D) KM2119.

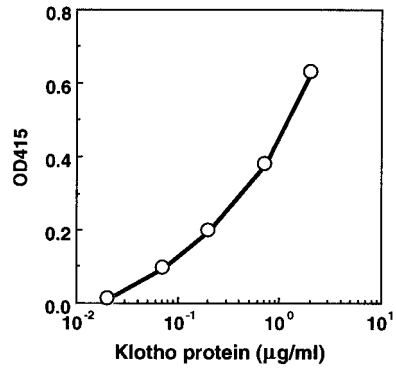
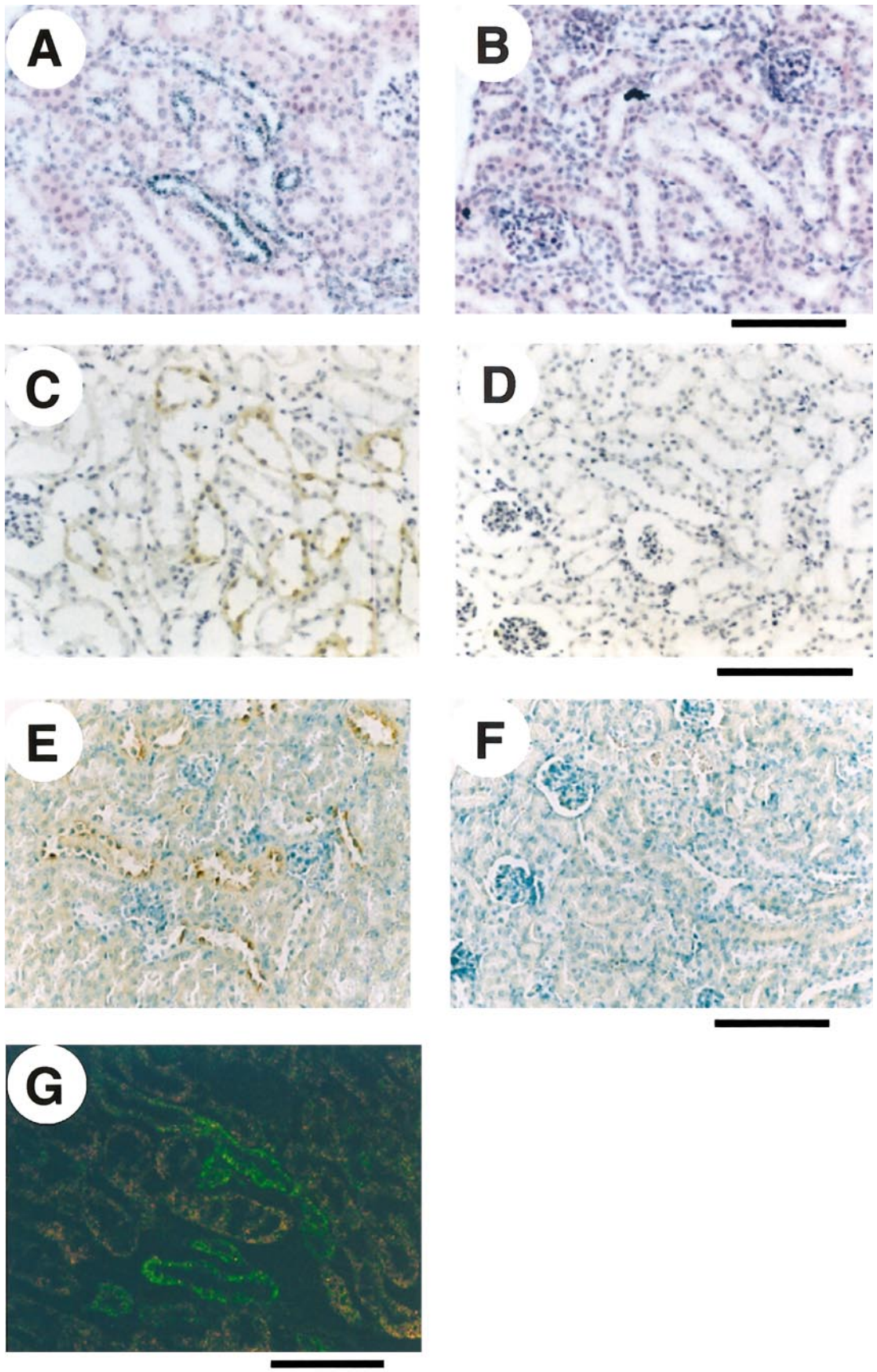


FIG. 2. Standard curve of sandwich ELISA for the detection of the Klotho protein.

RESULTS AND DISCUSSION

Establishment of anti-human Klotho mAbs. Here, we presented the establishment of a mAbs against Klotho protein. We immunized rats and mice with the recombinant human Klotho protein or a synthesized amino peptide and obtained three mAbs (KM2076, KM2119 and KM2365) as described in Materials and Methods (8). Matsumura *et al.* (4) reported that there are two transcripts which apparently arise from alternative splicing of *kl* mRNA. One transcript encodes a single-pass membrane protein with internal repeats (hKL1, hKL2). The other transcript encodes only hKL1 domain corresponding to a secreted-form of Klotho protein. As the immunized antigen for KM2119 was a part of hKL2 domain, this antibody does not recognize the secreted form. On the other hand, the immunized antigen for KM2076 and KM2365 was a part of hKL1, these mAbs could react with both the membrane and the secreted form of Klotho protein.

Typical results of western blots were shown in Fig. 1. KM2076 specifically recognized a 130 kDa Klotho protein exists in the membrane fraction from the wild-type mouse kidney, but not that from the *kl* mice (Fig. 1A). KM2076 also recognized specifically a 130 kDa Klotho protein in the membrane fraction of human kidney (Fig. 1C). KM2119 also recognized the 130 kDa Klotho protein in the membrane fraction from the wild-type mouse kidney (Fig. 1B) and the human kidney (Fig. 1D), but not that from the *kl* mice (Fig. 1B). Interestingly, KM2119 additionally recognized a 60 kDa protein as well as a 130 kDa one in the wild-type mouse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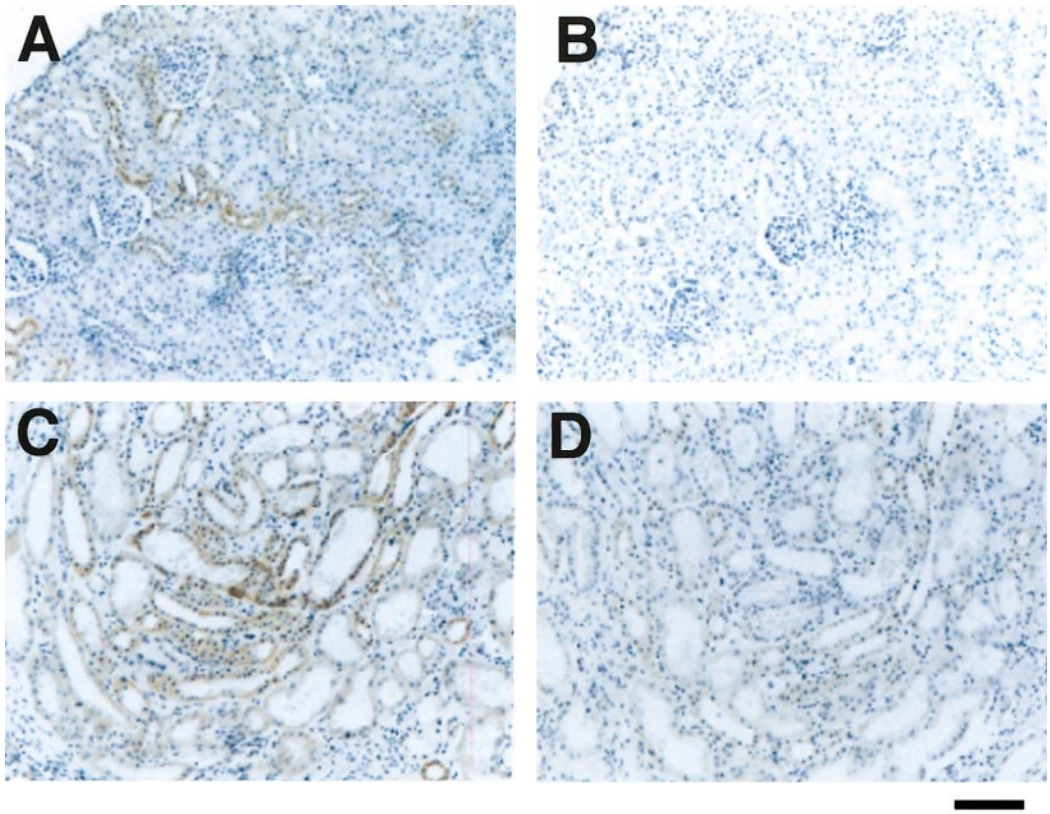

FIG. 4. Immunoperoxidase staining of kidneys with KM2076. Paraffin sections of (A and B) rat or (C and D) human kidneys. (A and C) KM2076 and the secondary mAb were sequentially applied as described under Materials and Methods. (B and D) The secondary antibody but not KM2076 was applied. Sections were counterstained with hematoxylin. Bar represents 100 μm.

(Fig. 1B) and the human (Fig. 1D) kidneys. It might be possible that a part of extracellular domain of Klotho protein was cleaved and the rest of Klotho protein near KL2 domain was recognized as 60 kDa protein.

Sandwich ELISA for the detection of Klotho. The supernatant of CHO cells expressing human *klotho* cDNA was serially diluted, and the level of Klotho protein was determined with the sandwich ELISA. As shown in Fig. 2, the value of A415 corresponded well to the concentration of Klotho protein. Using the ELISA, we concluded that the Klotho protein as low as the concentration of 10 to 20 ng/ml could be detected.

Localization of the klotho transcript and Klotho protein. Strong signals of *klotho* transcripts were observed on the cortical renal tubules of the wild-type mouse kidney, but not those of the *kl* mouse (Fig. 3A, B). The interesting point is that not all the cells in the cortical renal tubules express the mRNA. These signals are specific for *kl* transcripts, because we did not detect any signals with the *kl* sense probes (data not shown). The pattern of localization of *kl* transcripts was similar to that reported earlier (1).

To select mAbs available for the immunohistochemical detection of Klotho protein, we first stained kidneys from wild-type and *kl* mice. Among mAbs tested, KM2076 specifically stained the cortical renal tubules of wild-type mice kidney in both frozen (Fig. 3C) and paraffin sections (Fig. 3E) by immunoperoxidase staining. We concluded that the staining was specific for Klotho protein, because we did not detect any staining

FIG. 3. Expression of *klotho* mRNA and Klotho protein in the mouse kidneys. (A and B) Expression of mRNA for *kl* in the kidney of 7-week-old mouse. *In situ* hybridization was performed on sections with ^{35}S-labeled cRNA probes that was specific for mRNA for *kl*. (A) Bright-field image with the kidney from the wild-type mouse. Expression of *kl* mRNA was detected as the dense localization of black grains on the renal tubules. (B) Bright-field image with the kidney from *kl* mice. (C and D) Immunoperoxidase staining of kidneys with KM2076. Frozen sections of kidneys isolated from (C) wild-type or (D) *kl* mice were stained with KM2076. Sections were counterstained with hematoxylin. (E and F) Immunoperoxidase staining of kidneys with KM2076. Paraffin sections of kidneys isolated from (E) wild-type or (F) *kl* mice were stained with KM2076. Sections were counterstained with methyl-green. (G) Fluorescence staining of the wild-type mouse kidney with KM2119. Green signals represent signals from Klotho staining. The bars under B, F, and G represent 100 μm for A, B, E, F, and G, and the bar under D represents 50 μm for C and D.

on the cortical renal tubules of *kl* mice kidney (Figs. 3D and 3F). KM2119 also specifically stained the cortical renal tubules of wild-type mice kidney on frozen sections by fluorescence staining (Fig. 3G). The localization of Klotho protein in the mouse kidney was similar to that of *kl* transcripts. Using KM2076, we further stained the paraffin sections of the rat (Figs. 4A and 4B) and the human (Figs. 4C and 4D) kidneys. KM2076 also gave specific staining on the cortical renal tubules of rat (Fig. 4A) and human (Fig. 4C) kidneys.

In conclusion, among the monoclonal antibodies established, KM2076 can be used to detect human Klotho protein for the Western blot analyses. Also KM2076 can be used to monitor the secreted form of human Klotho in ELISA system with the combination with KM2365. KM2119, which detects the KL2 part of Klotho, gave an additional 60 kDa band as well as 130 kDa band in the samples from human and mouse kidneys in Western blot. For immunohistochemical analysis, KM2076 gave a specific staining of Klotho in mouse, rat, and human kidney sections. KM2119 also gave a similar result in mouse kidney sections by using fluorescence staining. Besides the characterization above, we also have a preliminary data of the availability of KM2076 and KM2119 for the immunoprecipitation of Klotho protein.

These antibodies will be powerful tools for understanding the function of Klotho.

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