Urinary excretion pattern of exosomal aquaporin-2 in rats that received gentamicin

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Abdeen A, Sonoda H, El-Shawarby R, Takahashi S, Ikeda M. Urinary excretion pattern of exosomal aquaporin-2 in rats that received gentamicin. *Am J Physiol Renal Physiol* 307: F1227–F1237, 2014. First published October 22, 2014; doi:10.1152/ajprenal.00140.2014.—Urinary exosomes are nano-sized vesicles secreted into urine from all types of renal epithelial cells and are known to contain possible biomarker proteins for renal diseases. Gentamicin has been reported to decrease the level of renal aquaporin (AQP)2, which is known to be mainly expressed in renal collecting ducts and excreted into the urine via exosomes. In the present study, we investigated whether urinary exosomal AQP2 could serve as a potential biomarker for gentamicin-induced nephrotoxicity, especially collecting duct cell dysfunction. Gentamicin was given to rats intraperitoneally once every day starting on *day 0*. Gentamicin significantly increased the plasma creatinine concentration from *day 5* and beyond. Also, gentamicin induced polyuria and a defective urine concentration mechanism on *day 7*, suggesting gentamicin-induced collecting duct cell dysfunction. Immunoblot analysis showed that gentamicin significantly increased urinary exosomal AQP2 excretion on *day 1* but decreased it on *day 7* compared with the control group. Similarly, increased excretion of exosomal tumor susceptibility gene 101 protein, frequently used as an exosome marker protein, was observed on *day 1*. However, gentamicin did not significantly affect the urinary excretion of exosomal tumor susceptibility gene 101 on *day 7*. Gentamicin slightly decreased renal AQP2 expression on *day 2* and markedly decreased it on *day 8*. These data strongly suggest that the use of urinary exosomal AQP2 as a biomarker may allow detection of gentamicin-induced collecting duct cell dysfunction. Furthermore, urinary exosomal AQP2 might also be useful for the early detection of gentamicin-induced renal injury in addition to collecting duct injury.

gentamicin; nephrotoxicity; exosomes; aquaporin-2; tumor susceptibility gene 101 protein

GENTAMICIN, an aminoglycoside antibiotic, is used for the treatment of many types of bacterial infection, especially those involving gram-negative bacteria. Gentamicin therapy is associated with two forms of toxicity, nephrotoxicity and ototoxicity, as side effects (18, 25). Gentamicin-induced nephrotoxicity has been reported to appear during 10–25% of therapeutic courses, and the typical clinical manifestation is nonoliguric renal insufficiency. After glomerularfiltration, gentamicin is thought to bind to anionic sites on the brush border of proximal tubule cells and is then endocytosed (31). Subsequently, gentamicin is trafficked by the cellular endosomal system and accumulates mostly in the lysosomes, Golgi, and endoplasmic reticulum. Gentamicin binds to lysosomal membrane phspholipids and alters their turnover and metabolism, resulting in lysosomal phospholipidosis (18). Once in the cytosol, presumably mainly mediated by lysosomal rupture, gentamicin is distributed to various intracellular organelles, resulting in their dysfunction. For example, gentamicin acts on mitochondria and then increases the production of ROS, lowers ATP synthesis, and activates the intrinsic apoptotic pathway, leading to cell death (18, 31). As proximal tubules are known to be the main site for the renal reabsorption of Na^+, amino acids, and glucose, gentamicin-induced nephrotoxicity is characterized by increased urinary excretion of Na^+, aminoaciduria, and glucosuria (18).

Besides this impairment of proximal tubule function, gentamicin has been reported to affect collecting duct function, as evidenced by gentamicin-induced polyuria (18). As renal water excretion is known to be regulated by the level of expression of aquaporin (AQP)2, a water channel protein distributed to renal collecting duct cells, gentamicin has been assumed to affect renal AQP2 function and/or expression. In fact, it has been reported that gentamicin reduces the renal expression level of AQP2 (17, 32). These data are also supported by the fact that gentamicin is detectable in the distal tubule and collecting duct cells in addition to proximal tubule cells (9).

Since traditional blood biomarkers of kidney injury, such as creatinine and blood urea nitrogen, do not allow early detection of gentamicin-induced nephrotoxicity, many groups have sought a novel biomarker that would make this possible (5, 6, 13, 24, 29, 36). As a result, several urinary biomarkers have been discovered, including cystatin C, neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, β_2-microglobulin, osteopontin, and others. Some of these are currently at an advanced stage of validation for diagnosis and prognostication of acute kidney injury, including gentamicin-induced nephrotoxicity (13) (http://c-path.org/programs/pstc/pstc-tools/#tab-content). However, most of these biomarkers originate from blood, proximal tubules, or dedifferentiated tubule cells, and therefore it is considered that they cannot be used to directly detect impairment of renal collecting duct function in response to treatment with gentamicin (3).

Exosomes are small nano-sized vesicles secreted from various cell types such as dendritic cells, B lymphocytes, endothelial cells, and epithelial cells (15, 27, 39). Urinary exosomes, known to be released into urine from all nephron segments, have been reported to include many types of renal functional proteins that are specifically expressed in each renal segment and are involved in renal reabsoption and secretion of water and solutes, representing a reservoir of biomarkers that may allow detection of dysfunction and/or cellular injury in each segment (4, 27). Some renal functional proteins in exosomes have been reported to be potential biomarkers for several renal-related diseases. These proteins include AQP1,

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which is expressed in the proximal tubule and descending thin limb cells (33) and is a marker of acute kidney injury, Na^+-K^+-Cl^- cotransporter 2, which is expressed in cells of the thick ascending limb and a marker of Bartter syndrome type I (10), Na^+-Cl^- cotransporter, which is expressed in cells of the distal convoluted tubule and is a marker of primary aldosteronism (38), pseudohypoaldosteronism type II, or Gitelman's syndrome (14), and AQP2, which is diagnostic of the defect in urinary concentrating function in patients with American cutaneous leishmaniasis (22).

In the present study, we examined whether urinary excretion of exosomal AQP2 was altered in rats that received gentamicin, leading to the discovery of a possible biomarker for detection of gentamicin-induced collecting duct cell injury. We also measured urinary excretion of tumor susceptibility gene 101 (TSG101) protein, which is commonly used as a marker protein of exosomes (16, 34, 35). We found that the excretion of urinary exosomal AQP2 was reduced in the late phase of gentamicin-induced renal injury, accompanied by a defect in the urinary concentration mechanism and decreased expression of renal AQP2, suggesting that urinary exosomal AQP2 might be useful for the detection of collecting duct cell dysfunction. In addition, unexpectedly, increased excretion of urinary exosomal AQP2 was observed in the early phase of gentamicin-induced renal injury, and this increase appeared to be related to

the number of exosomes excreted into the urine, indicating that urinary exosomal AQP2 might also be useful for the early detection of gentamicin-induced renal injury.

MATERIALS AND METHODS

Animals and drug administration. All animal experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" at the University of Miyazaki and conducted in compliance with the Law Concerning the Protection and Control of Animals (Japan Law No. 105, October 1, revised on June 22, 2005), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006), and the Guidelines for Animal Experimentation (the Japanese Association for Laboratory Animal Science, May 22, 1987).

Male Sprague-Dawley rats aged 9 wk were purchased from Kyudo (Saga, Japan). All animals had free access to water and a normal pellet diet. Animals were randomly divided into two groups: a control group, which received daily vehicle (saline) via intraperitoneal injection; and a gentamicin group, which received daily gentamicin sulfate (160 mg·kg body wt^{-1}·day^{-1}, Sigma, St. Louis, MO) via intraperitoneal injection. At the start of administration (assigned as *day 0*), rats were 10 wk old and weighed ~400 g. Blood was collected on each day until the end of the experiment except on *day 6*. Urine was collected on *days 1–5* and *7*. During the urine collection, all animals were kept in metabolic cages and given free access to water. The urine
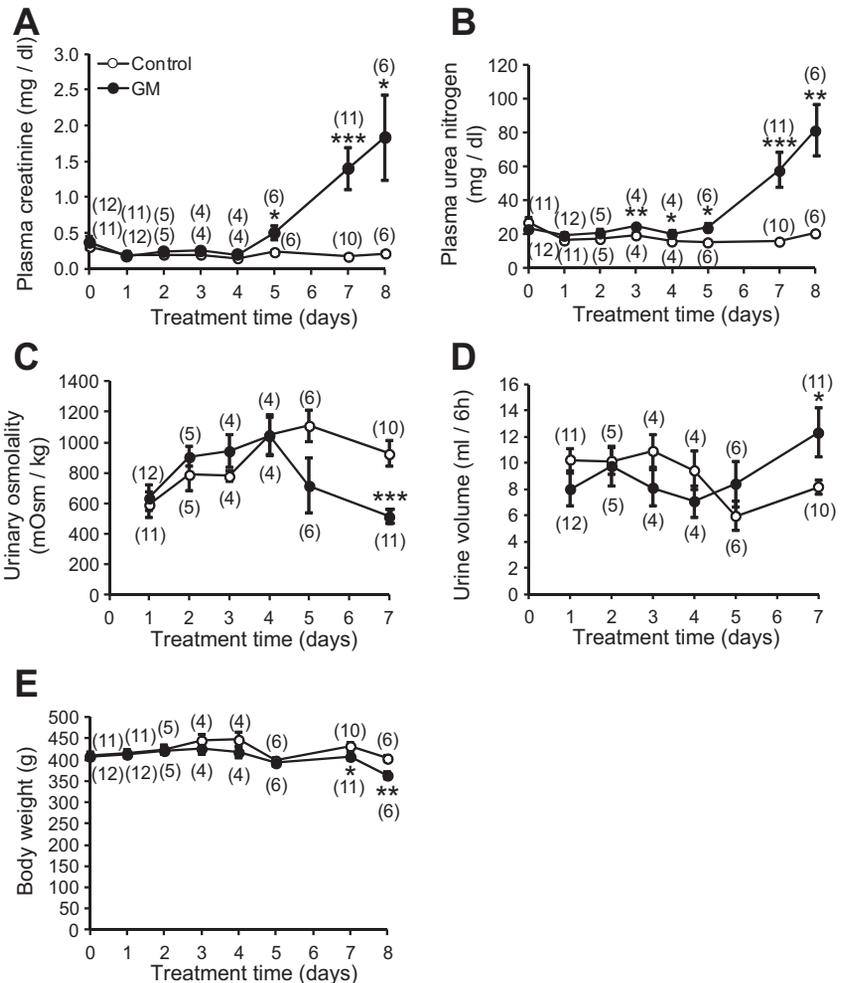

Fig. 1. Time course of changes in blood and urinary parameters and body weights of rats after treatment with genitamicin (GM). Changes in plasma creatinine concentration (*A*), urea nitrogen concentration (*B*), urinary osmolality (*C*), urine volume (*D*), and body weight (*E*) are shown. Data are expressed as means ± SE. Numbers in parentheses indicate numbers of animals tested. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the control group.

collected on *days 1, 2,* and *7* was used for the isolation of exosomes. Kidney samples were collected on *days 2* and *8*.

Analyses of blood and urine parameters. Creatinine and urea nitrogen concentrations were analyzed using an autoanalyzer (Fuji Film Medical, Tokyo, Japan). Urinary osmolality was measured using an automatic osmometer (Osmostation om-6060, Arkray, Kyoto, Japan).

Isolation of urinary exosomes. The procedure for the isolation of urinary exosomes was performed as previously described with slight modifications (12). Briefly, urine was collected from rats for 6 h with a collection tube containing a protease inhibitor mixture (60 μl of 130 mM EDTA, 70 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, and a complete protease inhibitor cocktail tablet) at room temperature. Immediately after collection, the urine was centrifuged at 1,000 *g* for 15 min, and the supernatant was centrifuged at 17,000 *g* for 15 min to remove the urinary debris. The resulting supernatant was retained, and the pellet was resuspended in 25 μl of isolation solution (250 mM sucrose and 10 mM triethanolamine) followed by the addition of 25 μl isolation solution containing DTT (200 mg/ml, Wako, Osaka, Japan) and then incubated at 37°C for 10 min. During the incubation with DTT, the sample was vortexed for 20 s every 2 min. Subsequently, the sample was mixed with isolation solution and centrifuged again at 17,000 *g* for 15 min. The first and second supernatants were combined and ultracentrifuged at 200,000 *g* for 1 h (Optima TL Ultracentrifuge; Beckman Instruments, Brea, CA). The resulting pellet was solubilized in a 10-fold diluted protease inhibitor mixture, and the suspension was then mixed with 4× sample buffer (8% SDS, 50% glycerol, 250 mM Tris·HCl, 0.05% bromophenol blue, and 200 mM DTT) followed by an incubation for 30 min at 37°C. These samples were stored at −80°C.

In our previous study (33) involving immunoblot analysis of exosomal protein samples, when we compared the time of urine collection with the urinary concentration of creatinine, there was no significant difference between these two approaches for normaliza-
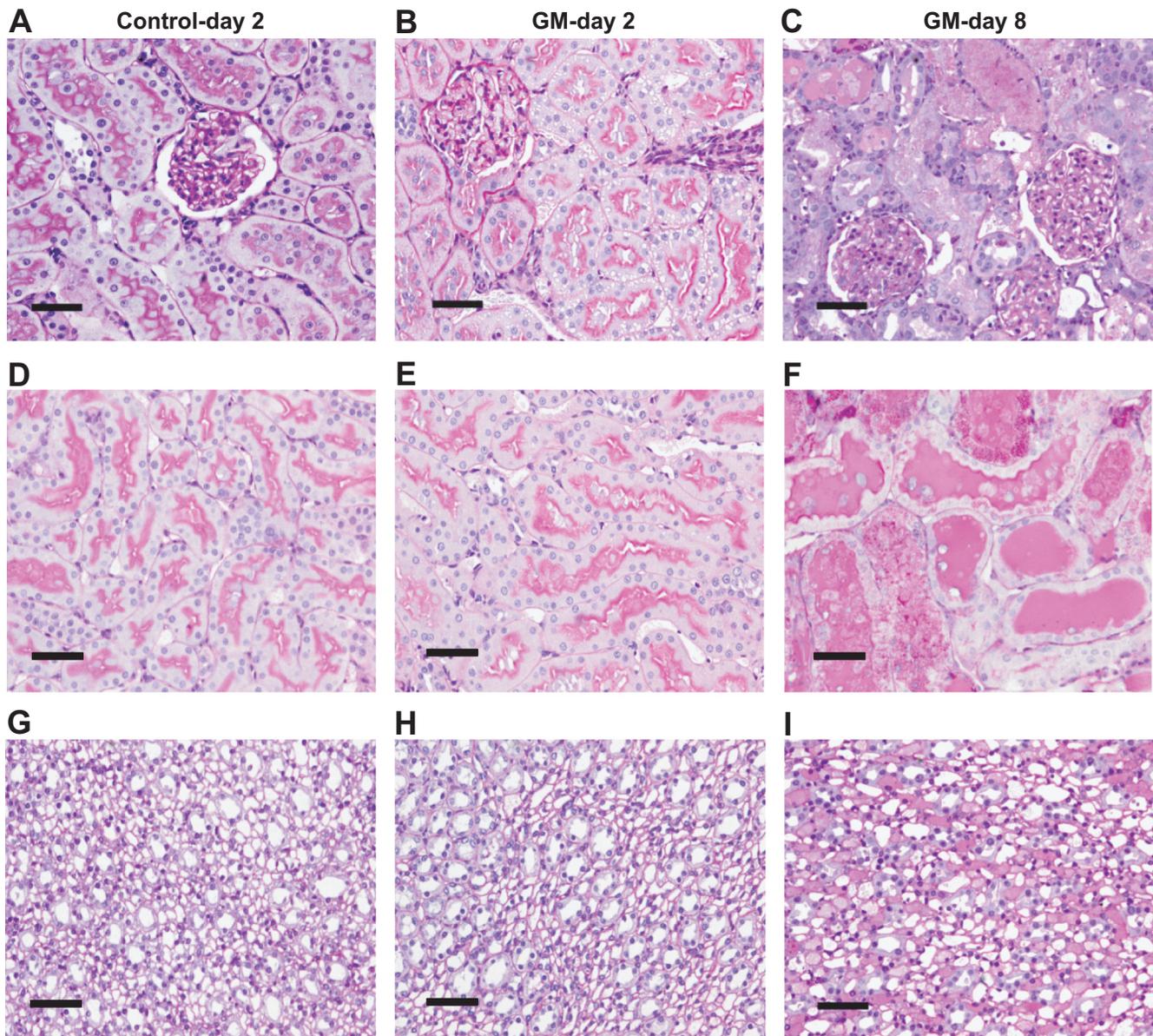

Fig. 2. Renal histology after treatment with GM. Kidney sections from rats after 2 days of treatment with vehicle (control; *A, D,* and *G*) or GM (*B, E,* and *H*) or 8 days of treatment with GM (*C, F,* and *I*) were stained with periodic acid-Schiff reagent. Representative images of the cortex (*A–C*), outer medulla (*D–F*), and inner medulla (*G–I*) are shown. Bars = 50 μm.

tion. In addition, we had preliminarily observed that the amount of albumin in exosomes was increased in patients with proteinuria relative to healthy patients, suggesting that the total amount of protein in exosomes in rats with proteinuria was altered relative to animals without proteinuria. Furthermore, TSG101 has been used as a marker protein of exosomes, but its use as an internal control for quantification has not been fully validated. Therefore, for immunoblot analysis, each sample of urinary exosomal protein was loaded in each lane with the same amount of urine creatinine.

To determine an appropriate loading amount for the detection of target proteins, we preliminarily performed immunoblot analysis with urinary exosomal samples. This showed that the appropriate loading amount for AQP1 and AQP2 was 10–20 μg creatinine/lane and that for TSG101 and apoptosis-linked gene 2-interacting protein X (Alix) was >40 μg creatinine/lane.

Renal protein samples. The kidney was divided into cortex and medulla portions, and each part was mixed with zirconium beads (BioMedical Science, Tokyo, Japan) followed by homogenization for 5 min at 4°C in ice-cold homogenization buffer (300 mM sucrose, 1.3 mM EDTA, 25 mM imidazole, and a complete protease inhibitor cocktail tablet) using a shaker-type homogenizer (BioMedical Science). The homogenate was centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was then centrifuged at 200,000 g for 1 h at 4°C. The resulting 1,000-g supernatant (a whole cell lysate fraction) for analyzing TSG101 and 200,000-g pellet (a membrane-rich fraction) for analyzing both AQP2 and TSG101 were dissolved in homogenization buffer, mixed with 4× sample buffer, and incubated for 30 min at 37°C. The total protein concentration in each sample was determined using a Pierce BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) before the addition of 4× sample buffer. These samples were stored at −80°C. Each renal protein sample was loaded in each lane with the same amount of total protein for immunoblot analysis.

Immunoblot analysis. Urinary exosomal protein and renal protein samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. After being blocked with 5% skim milk in Tris-buffered saline-0.05% Tween (TBST), the membrane was incubated with 1.5% skim milk in TBST including a primary antibody, which was an antibody against AQP2 (catalog no. AQP-002, Alomone Labs, Jerusalem, Israel), TSG101 (catalog no. ab-125011, Abcam), AQP1 (catalog no. sc-20810, Santa Cruz Biotechnology, Santa Cruz, CA), Alix (catalog no. sc-49268, Santa Cruz Biotechnology), or β-actin (catalog no. sc-4778, Santa Cruz Biotechnology) at 30°C for 1 h. The membrane was then incubated with 1.5% skim milk in TBST including a peroxidase-conjugated anti-rabbit IgG antibody (catalog no. cs-7074, Cell Signaling Technology, Danvers, MA), anti-goat IgG antibody (catalog no. P0449, Dako Japan, Tokyo, Japan), or anti-mouse IgG antibody (catalog no. 1858413, Thermo Fisher Scientific, Rockford, IL) at 30°C for 45 min. Bands were visualized using a Super Signal chemiluminescence detection system (Thermo Fisher Scientific) and quantified using the WinRoof software package (version 5.7, Mitani, Tokyo, Japan) or ImageQuant TL software (GE Healthcare, Uppsala, Sweden). Corresponding control samples from animals treated with vehicle were always loaded in each gel for normalized quantification.

Histopathology and immunohistochemistry. Formalin-fixed, paraffin-embedded kidney sections (2 μm) were processed and stained with periodic acid-Schiff reagent (Muto Pure Chemicals, Tokyo, Japan). For immunostaining, sections were deparaffinized and rehydrated, and the antigen was retrieved by heating the slide in distilled water by autoclaving at 121°C for 5 min. After endogenous peroxidase had been inactivated using 3% H_2O_2 solution, the slide was incubated with anti-AQP2 antibody diluted 1:100 at 37°C for 1 h followed by an incubation with Envision System Labelled Polymer Reagent (Dako Japan) at 37°C for 45 min. The reaction product was visualized by treatment with 3,3′-diaminobenzidine tetrahydrochloride, and the slide was counterstained with hematoxylin.

Real-time PCR. Isolation of total RNA from each part of the kidney was performed using a RNeasy Protect Minikit (Qiagen, Tokyo, Japan) with DNase digestion. The total RNA concentration was determined spectrophotometrically (NanoDrop ND-1000 Spectrophotometer, Nano-Drop Technologies, Wilmington, DE). The isolated total RNA was reverse transcribed to cDNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Rat GAPDH, AQP2, and TSG101 were amplified using a Power SYBR Green RT-PCR Reagent Kit (Applied Biosystems, Carlsbad, CA) using the following primers: forward 5′-ACTCCCATTCCTCCACCTTT-3′ and reverse 5′-TTACTCCTTGGAGGCCATGT-3′ for GAPDH, forward 5′-TGTCTC-CTTCCTTCGAGCTG-3′ and reverse 5′-AGCTCTACAGTCACAGC-CTG-3′ for AQP2, and forward 5′-TGTGGCTCCTGGACACATAC-3′ and reverse 5′-ACTCTGACCGTGGGTGTTTC-3′ for TSG101. GAPDH was used for the normalization of AQP2 and TSG101 threshold cycles. Analysis of the expression of each gene was performed using a 7900HT system (Applied Biosystems).

Evaluations and statistical analysis. All evaluations were performed in a nonblinded manner, but we performed all experiments and analyzed the data objectively and impartially. All quantitative data are presented as means ± SE. Statistical comparison between control and gentamicin groups was performed by t-test, and differences at $P < 0.05$ were considered to be statistically significant.
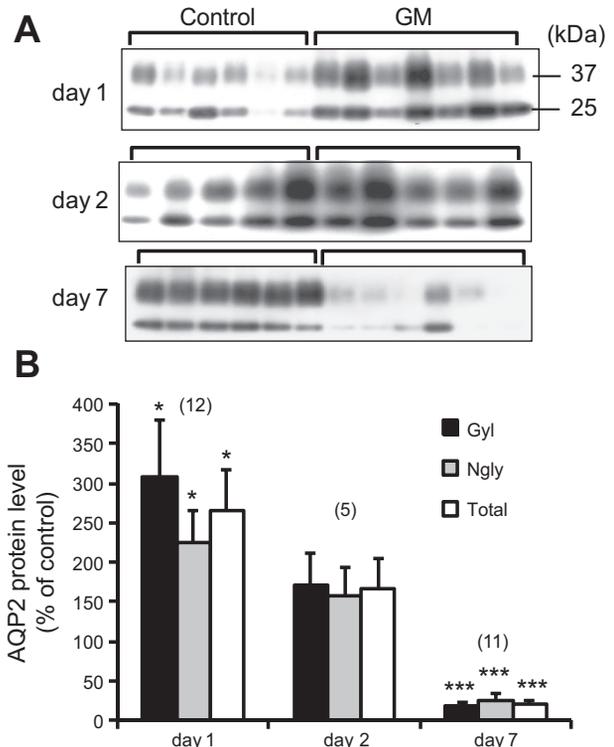

Fig. 3. Time course of change in urinary excretion of exosomal aquaporin (AQP)2 after GM treatment. *A*: representative immunoblots illustrating the time course of changes in urinary exosomal AQP2 levels. Six-hour urine samples were collected after 1, 2, and 7 days of treatment with vehicle (control) or GM. Each sample was loaded with the same amount of creatinine (20 μg/lane). Two bands are evident, with the upper band being the glycosylated form and the lower band being the nonglycosylated form. *B*: quantitative data obtained from immunoblot analysis of urinary exosomal AQP2. Each value is expressed as a percentage of the mean urinary exosomal AQP2 level in the control group. Gly, Ngly, and total indicate the data for glycosylated, nonglycosylated, and glycosylated + nonglycosylated bands, respectively. Data are expressed as means ± SE. Numbers in parentheses indicate the numbers of animals tested. *$P < 0.05$ and ***$P < 0.001$ compared with the control group.

RESULTS

Blood and urine parameters and body weight. During the experimental period, biochemical parameters of blood and urine were measured. As shown in Fig. 1*A*, gentamicin significantly increased the plasma creatinine concentration on *day 5* or later. On *day 3* or later, gentamicin significantly increased the urea nitrogen concentration (Fig. 1*B*). The gentamicin group also showed a significant decrease in urinary osmolality and an increase in urine volume on *day 7* compared with the control group (Fig. 1, *C* and *D*). A significant loss of body weight was also observed on *days 7* and *8* (Fig. 1*E*).

Kidney histopathology. Figure 2 shows the histology during the experimental period. On *day 2*, slight and restricted vacuolization was observed only in the cortex (Fig. 2*B*) in response to treatment with gentamicin. On *day 8*, marked kidney tubule injury was evident in the gentamicin group (Fig. 2, *C*, *F*, and *I*), including tubule dilatation, loss of the brush border, tubule necrosis, and cast formation in all kidney regions. Also, in the interstitial region adjacent to the injured tubules, variable degrees of lymphocytic infiltration were observed (data not shown). In contrast to these pathological findings, there was no gross alteration in the glomeruli.

Blood and urine parameters and histological analyses indicated that our model had no obvious kidney injury until 3 days of treatment with gentamicin (*day 2*) and that the renal injury was progressive thereafter, including polyuria. These alterations in the response to gentamicin with a similar dose resemble those previously reported (8, 29).

Urinary exosomal and renal AQP2. Next, we examined whether gentamicin affected the urinary excretion of exosomal AQP2 protein. In this examination, each sample of urinary exosomal protein was loaded in each lane with the same amount of urinary creatinine. When we measured the total amount of urinary creatinine excreted, mean ± SE values were 5.5 ± 0.1 mg/6 h ($n = 11$) on *day 1*, 5.6 ± 0.9 mg/6 h ($n = 5$) on *day 2*, and 6.0 ± 0.3 mg/6 h ($n = 10$) on *day 7* for the control group and 4.9 ± 0.5 mg/6 h ($n = 12$) on *day 1*, 5.1 ± 0.6 mg/6 h ($n = 5$) on *day 2*, and 4.9 ± 0.6 mg/6 h ($n = 11$) on *day 7* for the gentamicin group, and these values did not differ significantly between the control and gentamicin groups at each corresponding time point. As shown in Fig. 3, immunoblot analysis revealed that the excretion of urinary exosomal AQP2 protein was significantly increased on *day 1* compared with the control group. On *day 2*, the level of urinary exosomal AQP2 in the gentamicin group was increased by 150% relative to the control group, but the difference did not reach statistical significance. In contrast to the results obtained on *days 1* and *2*, the urinary excretion level of exosomal AQP2 on *day 7* was dramatically decreased in the gentamicin group. When we examined the glycosylated and nonglycosylated forms of AQP2 separately (Fig. 3*B*), gentamicin evenly affected both forms.

Next, we examined the level of renal AQP2 protein. Since our previous study (33) had clearly detected a change in the level of renal protein 1 day after the change in the level of urinary exosomal protein, in this experiment, we examined the levels of renal protein on *days 2* and *8*. Figure 4 shows representative immunoblots and a quantified data summary. Levels of AQP2 protein in the cortex and medulla were slightly reduced on *day 2*, and those on *day 8* were markedly reduced (Fig. 4). When we separately measured the glycosylated and nonglycosylated forms of AQP2 after treatment with gentamicin (Fig. 4, *B* and *D*), the glycosylation form in the cortex and both forms in the medulla were significantly reduced on *day 8*. However, since the cortical nonglycosylated form also tended
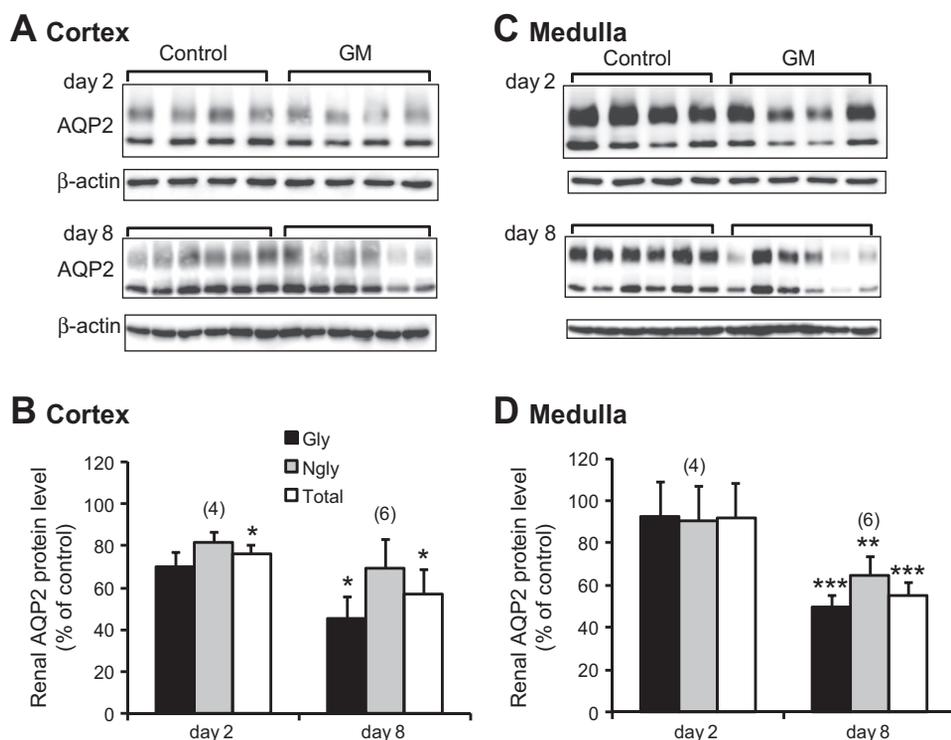

A Cortex

C Medulla

B Cortex

D Medulla

Fig. 4. Renal expression of AQP2 after GM treatment. Typical immunoblots for renal AQP2 and β-actin in the renal cortex (*A*) and medulla (*C*) are shown. Kidney samples were collected after 2 and 8 days of treatment with vehicle (control) or GM. Each sample was loaded with the same amount of protein (2 μg/lane). Quantitative data were obtained from immunoblot analyses of renal AQP2 in the cortex (*B*) and medulla (*D*). After normalization to the corresponding level of β-actin protein, each normalized value was expressed as a percentage of the mean value of the renal AQP2 protein level in the control group. Data are expressed as means ± SE. Numbers in parentheses indicate numbers of animals tested. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the control group.

to be reduced on *day 8*, gentamicin appeared to similarly affect both forms.

The renal expression of AQP2 protein was also examined by immunohistochemistry (Fig. 5). On *day 2*, there were no significant differences in renal AQP2 expression levels in the cortex and outer medulla between control and gentamicin groups. In the inner medulla, slightly reduced expression was observed in the gentamicin group (Fig. 5*H*). On *day 8*, gentamicin had remarkably decreased the level of AQP2 expression in the outer and inner medulla (Fig. 5, *F* and *I*). These immunohistochemistry data supported the immunoblot data described above. Interestingly, there was increased apical ex-
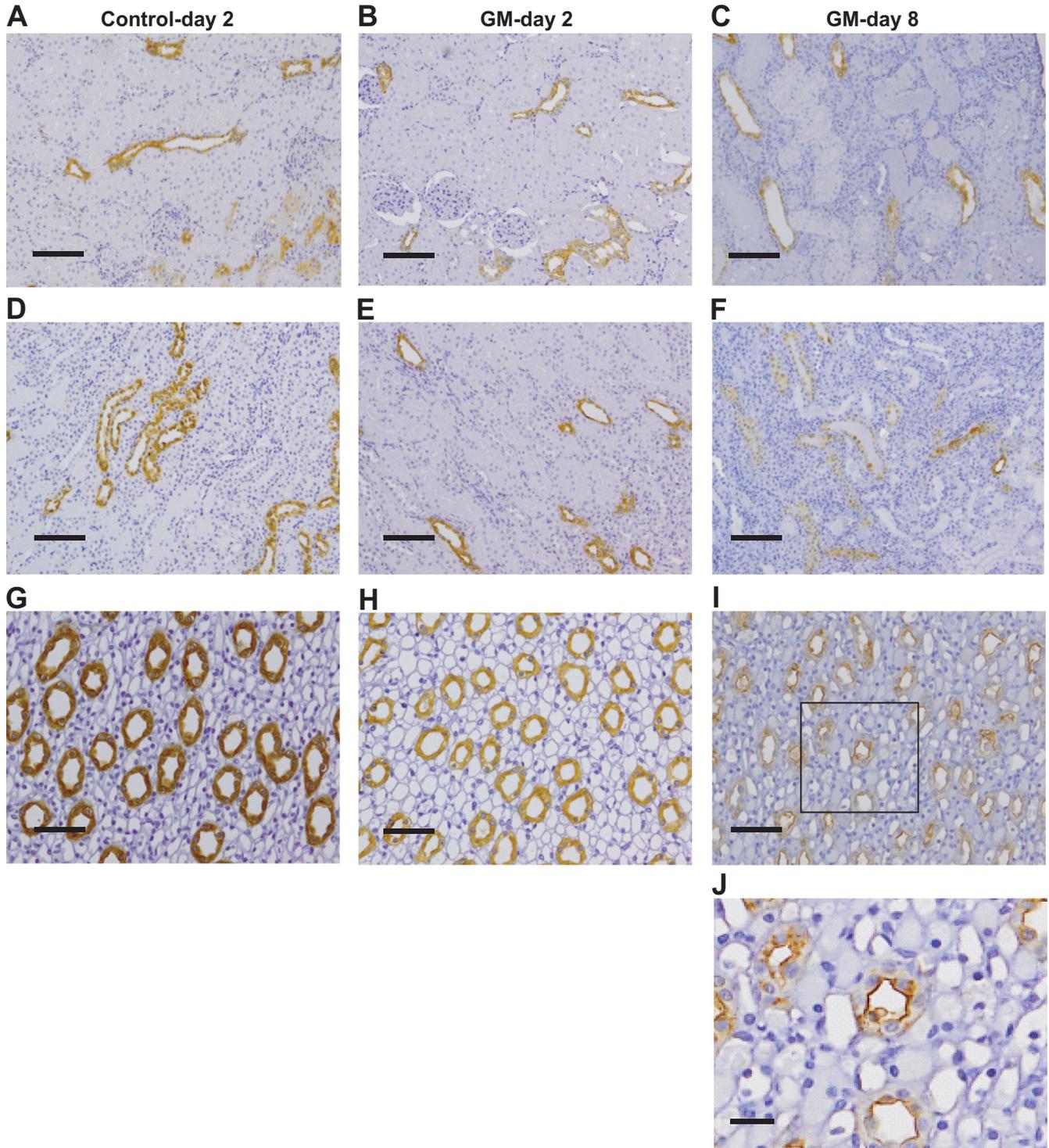

Fig. 5. Immunohistochemistry of renal AQP2 after GM treatment. Kidney sections from rats after 2 days of treatment with vehicle (control; *A*, *D*, and *G*) or GM (*B*, *E*, and *H*) or 8 days of treatment with GM (*C*, *F*, *I*, and *J*) were stained with anti-AQP2 antibody. Representative images of the cortex (*A–C*), outer medulla (*D–F*), and inner medulla (*G–J*) are shown. The box in *I* indicates the region of high magnification in *J*. Brown staining indicates the presence of AQP2. Bars = 100 μm in the cortex and outer medulla, 50 μm in the inner medulla, and 20 μm in the high-magnification image.

pression of AQP2 in the inner medulla in the gentamicin group on *day 8* (Fig. 5*J*).

Urinary exosomal and renal TSG101. TSG101 has been commonly used as a marker protein of exosomes in urine (16, 34, 35). Therefore, we examined the abundance of urinary exosomal TSG101. Figure 6 shows representative immunoblots and quantified data. Interestingly, urinary exosomal TSG101 excretion was significantly increased on *days 1* and *2* but not on *day 8* compared with control group.

Next, we examined the renal protein level of TSG101 by immunoblot analysis on *days 2* and *8* (Fig. 7). As shown in Fig. 7, *A–D*, the renal TSG101 level in the whole cell lysate fraction in the gentamicin group was significantly decreased in the cortex and tended to be decreased in the medulla on *day 2*. On *day 8*, levels of both cortical and medullary TSG101 were significantly decreased.

Initially, we tried to demonstrate localization of TSG101 in the kidney using an immunohistochemistry technique. However, the commercially available antibodies did not work on our formalin-fixed paraffin-embedded sections. Therefore, using immunoblot analysis, we examined the renal protein level of TSG101 in the membrane-rich fraction. As shown in Fig. 7, *E–H*, the degree of reduction in the level of renal TSG101 in the membrane-rich fraction in the gentamicin group was less marked than that in the whole cell lysate fraction.

Renal AQP2 and TSG101 mRNAs. The data from protein analyses showed that gentamicin altered renal expression levels of AQP2 and TSG101 proteins. Therefore, we examined the expression levels of renal AQP2 and TSG101 mRNAs using a real-time PCR technique. As shown in Fig. 8, gentamicin significantly decreased the level of AQP2 mRNA and tended to decrease that of TSG101 mRNA in both the renal cortex and medulla on *day 2*. On *day 8*, levels of cortical and medullary AQP2 and medullary TSG101 mRNAs were not affected by gentamicin, but the level of TSG101 mRNA in the cortex was significantly increased.

Urinary exosomal AQP1 and Alix. As mentioned above, the urinary excretion of both exosomal AQP2 and TSG101 on *day 1* was increased, suggesting that the total number of exosomes was increased on *day 1*. Therefore, we examined other urinary exosomal proteins, including AQP1 and Alix, on *day 1* (2, 27). As shown in Fig. 9, excretion of both exosomal proteins was significantly increased in the gentamicin group, suggesting that the number of exosomes in the urine was increased on *day 1*.

DISCUSSION

Exosomes are known to be released into the extracellular space by fusion of the outer membrane of multivesicular bodies (MVBs), late endosomes, with the cell surface (15, 27, 39). The endosomal sorting complex required for transport (ES-CRT) machinery executes the biogenesis of MVBs, and the ESCRT pathway has been viewed as a means by which transmembrane proteins are degraded in vacuoles/lysosomes (11, 30). The ESCRT machinery comprises four protein complexes, ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, and the process involved in biogenesis of MVBs, assisted by the ESCRT machinery, is currently proposed to be as follows (1, 11, 30). ESCRT-0 captures ubiquitinated cargo proteins at the endosome membrane, and this binding then initiates invagination of the endosome membrane. Thereafter, the cargo proteins are sequentially affected by ESCRT-I and ESCRT-II, and the membrane neck of the forming vesicle is then stabilized. The membrane neck is further narrowed by the action of ESCRT-III and then scissored out of the endosome membrane by recruitment of the vacuolar protein sorting 4 complex to ESCRT-III, resulting in the formation of intraluminal vesicles. This endosome, which contains small intraluminal vesicles, is known as the MVB. Fusion of MVBs with lysosomes results in the degradation of the cargo proteins. When MVBs are trafficked to the plasma membrane and fuse with the plasma membrane, the intraluminal vesicles, termed exosomes, are released into the extracellular space. TSG101 is a component of ESCRT-I and responsible for binding ubiquitinated cargo proteins (1, 11, 30). Alix, which is not a member of ESCRT proteins but is associated with them, has been reported to have the potential to link ESCRT-I and ESCRT-III (2). As TSG101 and Alix play important roles in the biogenesis of MVBs, many studies have used these proteins as markers of exosomes (16, 34, 35, 40).

AQP2 is the vasopressin-regulated water channel in kidney collecting duct cells (21). AQP1 is expressed in the proximal tubule, descending thin limb, and capillary endothelium, and it is thought to play an important role in isosmolar water reabsorption in those segments and the maintenance of renal medullary hypertonicity (20). Although the renal distributions of TSG101 and Alix have not been studied extensively, an immunoblot study (27) has clearly indicated that TSG101 is richly distributed in the renal cortex, whereas Alix is expressed
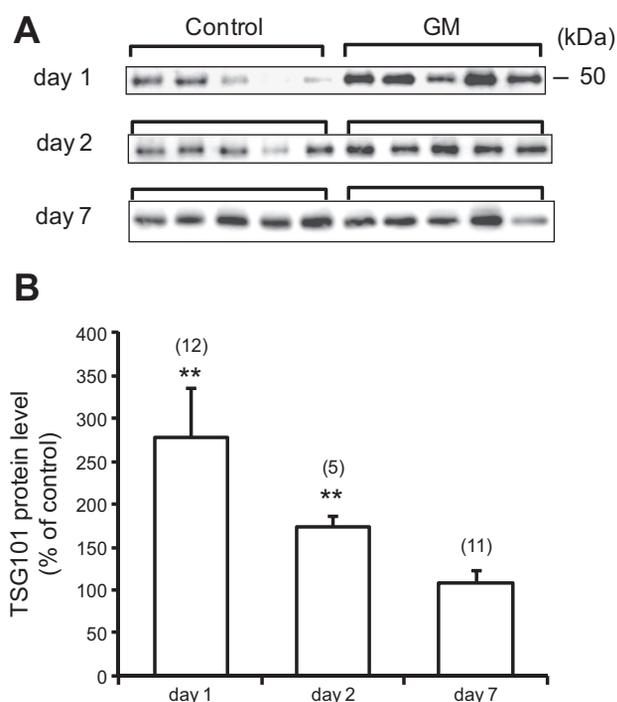

Fig. 6. Change in urinary excretion of tumor susceptibility gene 101 (TSG101) protein after GM treatment. *A*: representative immunoblots illustrating the time course of changes in urinary exosomal TSG101 levels. Six-hour urine samples were collected after 1, 2, and 7 days of treatment with vehicle (control) or GM. Each sample was loaded with the same amount of creatinine (40 µg/lane for *day 1* and 120 µg/lane for *days 2* and *7*). *B*: quantitative data obtained from immunoblot analysis of urinary exosomal TSG101. Each value is expressed as a percentage of the mean urinary exosomal TSG101 level in the control group. Data are expressed as means ± SE. Numbers in parentheses indicate numbers of animals tested. **$P < 0.05$ compared with the control group.
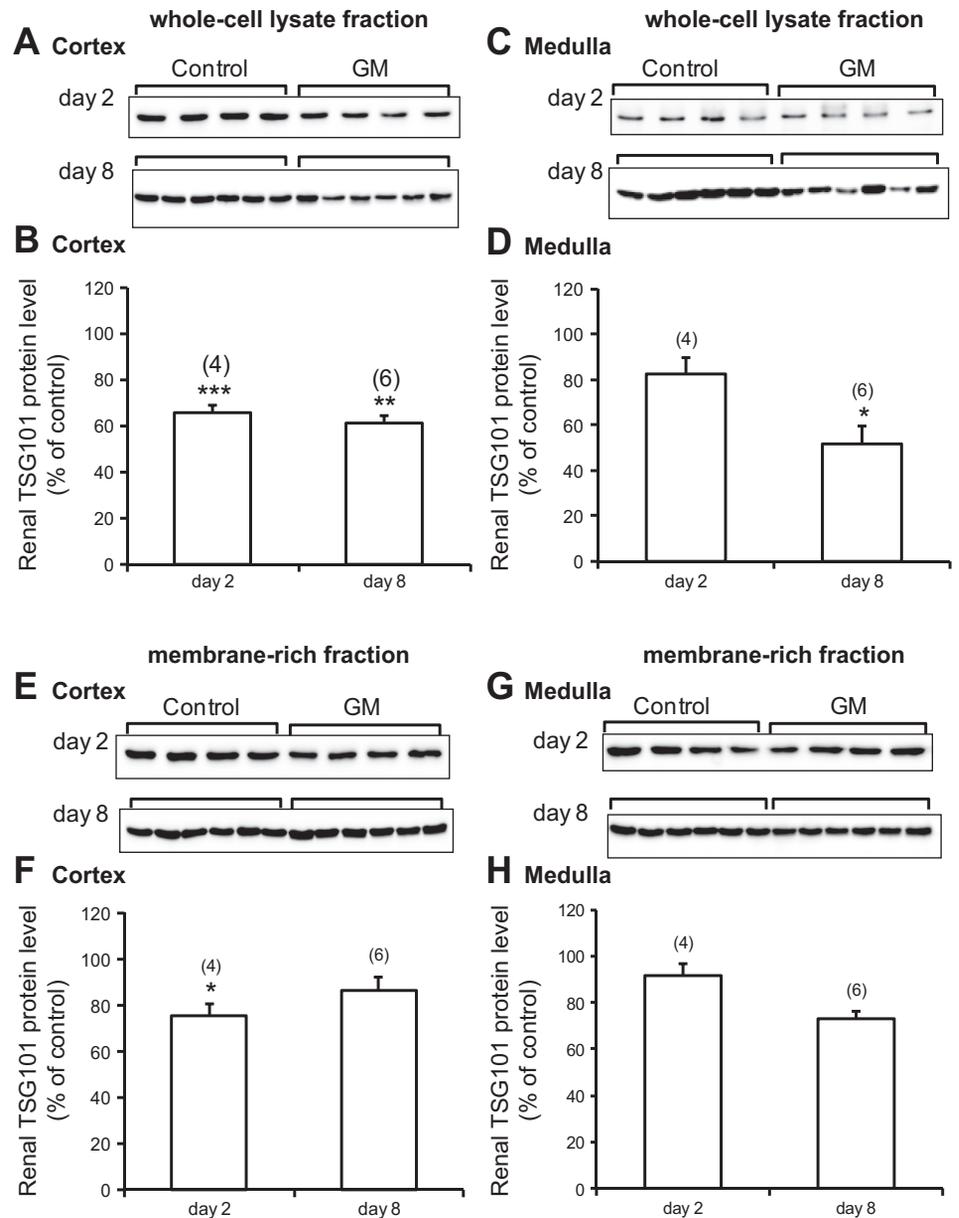
Fig. 7. Renal expression of TSG101 after GM treatment. Typical immunoblots for renal TSG101 in the renal cortex (*A* and *E*) and medulla (*C* and *G*) are shown. Whole cell lysate fraction (*A* and *C*) and membrane-rich fraction (*E* and *G*) samples were obtained from the kidney after 2 or 8 days of treatment with vehicle (control) or GM. Each sample was loaded with the same amount of protein (40 μg/lane). Quantitative data were obtained from immunoblot analyses of renal TSG101 in the whole cell lysate fraction (*B* and *D*) and membrane-rich fraction (*F* and *H*). Data for the cortex (*B* and *F*) and medulla (*D* and *H*) are shown. After normalization to the corresponding level of β-actin protein, each normalized value was expressed as a percentage of the mean value of the renal TSG101 protein level in the control group. Data are expressed as means ± SE. Numbers in parentheses indicate numbers of animals tested. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with the control group.

abundantly in the inner medulla. Therefore, the exosomal proteins examined in this study, including AQP1, AQP2, TSG101, and Alix, are thought to be derived from most types of renal epithelial cells. Our present data indicated that increased excretion of exosomal AQP2 and TSG101 was observed after 1-day treatment with gentamicin. Similarly, on *day 1*, excretion of urinary exosomal AQP1 and Alix was increased. As urinary excretion of all exosomal proteins examined here was increased, it was strongly suggested that treatment with gentamicin increased a greater number of exosomes released from all renal epithelial cells.

Recently, urinary excretion of exosomal AQP2 has been reported to be increased, in part, in a vasopressin-dependent manner (12, 35), and therefore the enhanced excretion of urinary exosomal AQP2 on *day 1* was thought to be mediated by the action of vasopressin. When vasopressin acts on the specific vasopressin type 2 (V2) receptor in renal collecting ducts, AQP2 rapidly accumulates in the apical plasma membrane, and subsequently the vasopressin-V2 receptor pathway increases the expression of renal AQP2 protein via enhanced transcription of AQP2 mRNA, both of which contribute to increased water reabsorption (20, 21). In the present study, we did not observe any increase in the apical expression of AQP2 (Fig. 5, *B*, *E*, and *H*) on *day 2*. Furthermore, the amount of renal AQP2 mRNA and protein was not increased and, in fact, was slightly decreased on *day 2*. Therefore, it seems unlikely that the vasopressin V2 receptor pathway was involved in the enhanced excretion of exosomal AQP2 on *day 1*.

Parolini et al. (26) examined the effect of pH on exosome release from melanoma cells. Using different pH conditions, they observed an increase of exosome release into the medium at pH 6, 3 days or later after the change in pH, but no increment was detected 2 days after the medium had been changed to an acidic pH. When we checked urinary pH during gentamicin dosing, we observed that the treatment slightly acidified the urine from *days 1* to 7 [control group: 7.09 ± 0.08 (n = 6) on
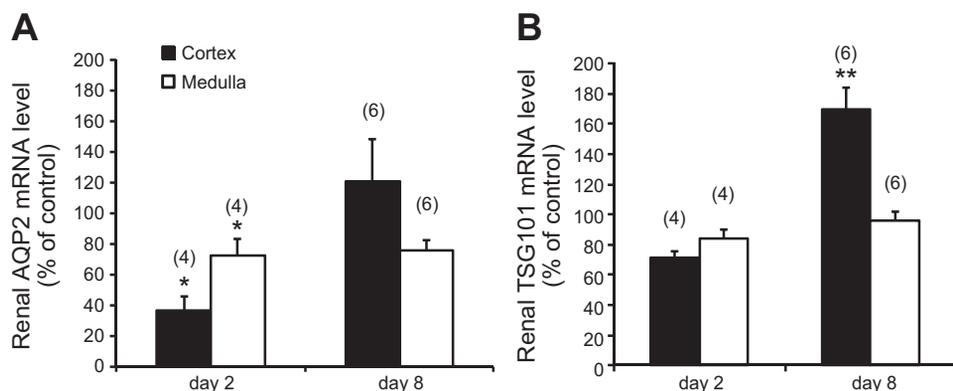
A

B

Fig. 8. Time course of changes in renal expression of AQP2 and TSG101 mRNAs after GM treatment. *A* and *B*: renal AQP2 (*A*) and TSG101 (*B*) mRNA levels in the cortex and medulla on *days 2* and *8* were determined using a real-time PCR assay. Each value is expressed as a percentage of the mean value of renal AQP2 and TSG101 mRNA levels in the control group. Data are expressed as means ± SE. Numbers in parentheses indicate numbers of animals tested. *$P < 0.05$ and **$P < 0.01$ compared with the control group.

day 1, 7.50 ± 0.07 ($n = 7$) on *day 2*, and 7.31 ± 0.14 ($n = 6$) on *day 7*; and gentamicin group: 6.54 ± 0.19 ($n = 6$) on *day 1*, 6.81 ± 0.08 ($n = 7$) on *day 2*, and 6.88 ± 0.08 ($n = 6$) on *day 7*]. On the other hand, we have previously observed the increased excretion of urinary exosomal AQP2, accompanied by urinary alkalinization, within 2 h after the treatment of rats with sodium bicarbonate (12). Furthermore, in a preliminary experiment, urinary acidification with ammonium chloride, an agent for acidifying urine, did not alter, or even reduced, the excretion of urinary exosomal AQP2 (data not shown). Therefore, it was unlikely that the urinary acidification with genitamicin was involved in the increased excretion of exosomal proteins.

The mechanisms underlying urinary exosome release remain poorly studied. One possible reason for this is that many researchers have focused on the discovery of new biomarkers among urinary exosomal proteins and RNAs (4, 28, 37). Another reason for the lack of progress in understanding the mechanisms is that current methods for the identification and quantification of urinary exosomes are time consuming and only semiquantitative (4). Recently, a new method based on the analysis of Brownian motion in solution, known as nanoparticle tracking analysis, for counting and measurement in size for urinary exosomes, has been developed (23). In future studies, the combination of this new method with fluorescent labeling for specific markers of exosomes will help to shed light on the mechanisms of urinary exosome release, including

a possible mechanism underlying the increase of exosomal proteins in the early phase of gentamicin-induced nephrotoxicity.

On *day 2*, cortical total AQP2 was significantly decreased, whereas medullary AQP2 was slightly (not significantly) decreased. At the same time point, both cortical and medullary AQP2 mRNAs were decreased and the degree of the decrease was larger in the cortex than in the medulla. Therefore, a larger reduction of the AQP2 mRNA level in the cortex might reflect the level of protein expression.

In the present study, a decrease in the excretion of urinary exosomal AQP2 was observed on *day 7*. Along with this change, a defect in the urinary concentration mechanism and polyuria were observed on *day 7*, and a reduced level of renal of AQP2 expression was evident on *day 8*. These findings indicate that gentamicin-induced polyuria is mediated by a reduction in the renal abundance of AQP2 and that exosomal AQP2 can be used as an indicator of impairment of renal collecting duct function. In support of this, we have previously observed that excretion of urinary exosomal AQP1 was decreased at 96 h after unilateral renal ischemia-reperfusion and that this was positively correlated with renal AQP1 abundance (33).

As shown in Fig. 2, the renal cellular architecture was largely destroyed on *day 8*, and, at the same time point, the results of RT-PCR showed that the level of renalAQP2 mRNA was not significantly altered, whereas immunohistochemistry

A
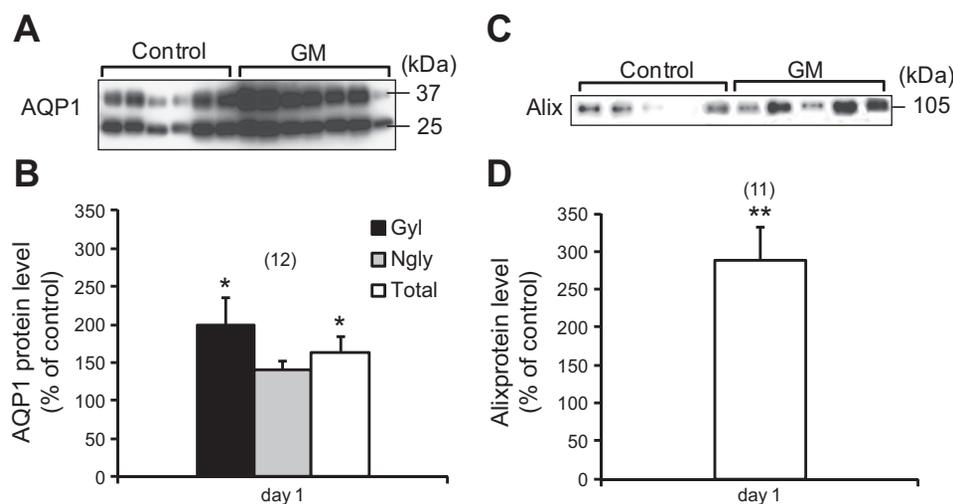

B

C

D

Fig. 9. Changes in the levels of exosomal AQP1 and apoptosis-linked gene 2-interacting protein X (Alix) after 1 day of GM treatment. Typical immunoblots for urinary exosomal AQP1 (*A*) and Alix (*C*) are shown. Six-hour urine samples were collected after 1 day of treatment with vehicle (control) or GM. Each sample was loaded with the same amount of creatinine (10 μg/lane for AQP1 and 120 μg/lane for Alix). Two bands are evident in *A*, with the upper band being the glycosylated form and the lower band being the nonglycosylated form. Quantitative data were obtained from immunoblot analyses of urinary exosomal AQP1 (*B*) and Alix (*D*). Each value is expressed as a percentage of the mean urinary exosomal AQP1 or Alix level in the control group. Data are expressed as means ± SE. Numbers in parentheses indicate numbers of animals tested. *$P < 0.05$ and **$P < 0.01$ compared with the control group.

clearly demonstrated enhancement of apical AQP2 expression in medullary collecting ducts. Therefore, it is considered that the structural damage, but not the decreased level of mRNA, might be related to the reduction in the total level of renal AQP2 expression and that this reduction initiates a compensatory mechanism to maintain the reabsorption of water, mediated by an increase in the apical level of AQP2 expression.

The level of renal TSG101 in the whole cell lysate fraction was significantly reduced, whereas that in the membrane-rich fraction was slightly reduced, on *day 8*. As a result, exosomal excretion of TSG101 might not be decreased on *day 7*. Since TSG101 is recognized to assist endosomal trafficking, the difference of TSG101 abundance between the whole cell lysate fraction and the membrane-rich fraction suggest that cytosolic TSG101 was recruited to membrane-associated organelles, such as endosomes, associated with ESCRT complexes in the late phase of gentamicin-induced nephrotoxicity. So far, it has been reported that functional ESCRT machinery, including TSG101, is required to maintain renal epithelial cell polarity (7). Therefore, it is considered that maintenance of the TSG101 level in the ESCRT machinery might be required to maintain epithelial organization against the toxic insult caused by gentamicin in the late phase. This notion is also supported by the fact that cortical TSG101 mRNA was increased on *day 8* in the gentamicin group.

Nephrotoxicity caused by exposure to drugs, including gentamicin, has been estimated to contribute to 19–25% of all cases of acute kidney injury (19). Therefore, considerable effort has been made to discover novel biomarkers for the early detection of such injury. In recent years, numerous molecules have been described and investigated as candidate biomarkers, and most of them are related to injury of primary sites in the kidney, such as the glomerulus or proximal tubule, which together represent the major sites of drug-induced toxicity. Therefore, these biomarkers cannot be used to directly detect injury to the collecting tubule. Exceptionally, urinary calbindin D28, a vitamin D-dependent Ca^{2+}-binding protein that appears to be selectively expressed in the distal tubule and proximal part of the collecting duct, is thought to be a marker of injury to sites where it is expressed (3). However, it has been reported that the level of urinary cabindin D28 is significantly increased after 3 days of gentamicin dosing or later, suggesting that this may be related to both proximal and distal tubule injury. Furthermore, it has been reported that urinary excretion of calbindin D28 depends on the serum concentration of Ca^{2+} (13). In the present study, we unexpectedly found that urinary excretion of exosomal AQP2 and TSG101 was increased on *day 1*, indicating that both exosomal proteins can be used to detect early renal impairment due to gentamicin. Moreover, on *day 7*, urinary excretion of exosomal AQP2 was remarkably decreased, accompanied by a defect in the urinary concentration mechanism and polyuria, partially reflecting the renal expression level of this protein and indicating that it can also be used to detect later gentamicin-induced nephrotoxicity. We anticipate that these findings will contribute to the future clinical application of urinary exosomal AQP2 and TSG101 as a panel of diagnostic markers for patients with gentamicin-induced nephrotoxicity.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.A., H.S., S.T., and M.I. performed experiments; A.A., H.S., and M.I. analyzed data; A.A., H.S., R.E.-S., and M.I. interpreted results of experiments; A.A. and M.I. prepared figures; A.A. and M.I. drafted manuscript; M.I. conception and design of research; M.I. edited and revised manuscript; M.I. approved final version of manuscript.

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