Sub-nephrotoxic doses of gentamicin predispose animals to developing acute kidney injury and to excrete ganglioside M2 activator protein

Yaremi Quiros[1,2,4], Laura Ferreira[3,4], Sandra M. Sancho-Martínez[1,2], José M. González-Buitrago[3], José M. López-Novoa[1,2,5] and Francisco J. López-Hernández[1,2,3,5]

[1]Unidad de Fisiopatología Renal y Cardiovascular, Departamento de Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain; [2]Instituto Reina Sofía de Investigación Nefrológica, Fundación Renal Íñigo Álvarez de Toledo, Madrid, Spain and [3]Unidad de Investigación, Hospital Universitario de Salamanca, Salamanca, Spain

We studied whether nephrotoxic drug administration sensitizes to acute renal failure (ARF) by administering a sub-nephrotoxic dose of gentamicin. This pre-treatment sensitized animals with no sign of renal injury to develop ARF when exposed to a second potential nephrotoxic drug, also given at sub-nephrotoxic doses that would be otherwise harmless to non-sensitized animals. We identified urinary ganglioside M2 activator protein (GM2AP) as a biomarker of an enhanced sensitivity to suffer ARF following sub-nephrotoxic treatment with gentamicin. Sub-nephrotoxic gentamicin did not alter renal *GM2AP* gene expression or protein levels, determined by reverse transcriptase-PCR, western blot, and immunostaining, nor was its serum level modified. The origin of increased GM2AP in the urine is thought to be a defective tubular handling of this protein as a consequence of gentamicin action. Hence, markers of acquired sensitivity may improve the prevention of ARF by enhancing our capacity to monitor for this condition, in a preemptive manner.

Kidney International (2010) **78,** 1006–1015; doi:10.1038/ki.2010.267; published online 18 August 2010

KEYWORDS: gentamicin; GM2AP; nephrotoxicity; sensitization to acute kidney injury; urinary markers

Correspondence: *Francisco J. López-Hernández, Unidad de Investigación, Hospital Universitario de Salamanca, Paseo de San Vicente, 58-182, 37007 Salamanca, Spain. E-mail: flopezher@usal.es*

[4]These authors share first authorship.
[5]These authors share senior authorship.

Received 4 January 2010; revised 9 June 2010; accepted 24 June 2010; published online 18 August 2010

Acute renal failure (ARF) is an extremely serious condition in which the renal excretory function abruptly falls within a few hours or days after an insult to the kidneys.[1,2] ARF still leads to death in 50% of the cases, a number that grows to 80% if multiorgan damage occurs.[3–6] The most common causes of ARF are renal ischemia, obstruction of the urinary ways, and drug nephrotoxicity.[7] A key determinant for a successful clinical handling of ARF is an early diagnosis, which significantly improves therapeutic intervention and outcome.[8,9] Traditionally, ARF has been diagnosed through measurable symptoms of renal dysfunction, such as the increase in serum creatinine and blood urea nitrogen (BUN) concentrations, or changes in the fractional excretion of sodium.[9] However, owing to compensatory adaptation, renal dysfunction only appears after an extensive loss of functional nephrons occurs.[10] Consequently, a new generation of biomarkers (mostly urine biomarkers) is under development, associated with early pathophysiological events underlying the incipient acute kidney injury (AKI), before it turns into an overt ARF. They, most significantly, include kidney injury molecule 1 (KIM-1), neutrophil gelatinase-associated lipocalin, and others.[9]

Gentamicin is an aminoglycoside antibiotic widely used against Gram-negative infections. The most important side effect of this drug is its nephrotoxicity,[11,12] which occurs in ~10–25% of therapeutic courses, despite correct dosage and hydration status monitoring.[13–15] Gentamicin nephrotoxicity may range from a mere transient renal dysfunction to a severe ARF. Gentamicin produces tubular lesions, affecting mostly the proximal segment, with no gross modification of glomerular structures. Tubular lesions vary from a mild sloughing of the brush border to a generalized tubular necrosis.[11,16,17] Besides, gentamicin has been shown to exert functional glomerular[12,18–22] and vascular effects[23–25] that, depending on the dose, contribute to a larger or lesser extent of renal dysfunction.[26]

However, it is not yet well characterized to what extent a subtoxic treatment with gentamicin sensitizes individuals to ARF, such as the ARF induced by subsequent sub-nephrotoxic

exposure to another potentially nephrotoxic agent. A typical clinical situation exists, where a patient treated with gentamicin showing no signs of renal disease is thereupon given another potentially nephrotoxic agent, such as another drug, or a diagnostic contrast medium, also within a theoretically subtoxic regime. These scenarios pose relevant clinical situations of special importance for its hidden nature, which should be addressed from the diagnostic and therapeutic perspectives. In this study, we demonstrate that a sub-nephrotoxic regime of gentamicin primes the rats to develop an ARF induced by a subsequent or concomitant exposure to sub-nephrotoxic doses of a second nephrotoxicant. We also show that the urinary level of ganglioside M2 activator protein (GM2AP) may be used to identify this condition. Urinary levels of GM2AP could also serve for the early diagnosis of gentamicin-induced ARF. Detection of the increased risk enables a preemptive handling of drug toxicity by anticipating situations that can result in injury, before the slightest alterations that are usually observed arise.

RESULTS

Characterization of a sub-nephrotoxic regime of gentamicin

A sub-nephrotoxic regime of gentamicin was identified to test whether it would induce sensitisation to ARF in the absence of a direct deleterious effect on the kidneys. After pilot studies, a regime of six daily consecutive doses of 50 mg/kg/day gentamicin (G-50 group) was observed to exert no obvious renal injury symptoms. It was further characterized to ensure the absence of nephrotoxicity. Survival during the whole treatment was identical to that of control rats (100%), whereas in rats treated with a nephrotoxic regime of gentamicin (150 mg/kg/day; G-150 group), which developed a clear ARF, survival decreased to 50%. Similarly, body weight increased by a 3–4% in control and G-50 animals, whereas it was reduced by 4–5% in G-150 rats. As shown in Figure 1, we were unable to find a single marker of renal damage or dysfunction in G-50 rats, when compared with controls. On the contrary, G-150 rats underwent a typical and overt ARF characterized by an increase in plasma creatinine concentration (Cr_{pl}) and BUN, proteinuria, increased fractional excretion of sodium, and the presence of urinary (i.e., increased *N*-acetyl-glucosaminidase (NAG) excretion) and renal tissue (KIM-1, plasminogen activator inhibitor 1, and vimentin) markers of tubular lesion (Figure 1a–f). A gross morphological examination of renal slices showed that renal parenchyma in G-50 was indistinguishable from that of control rats, whereas a clear tubular necrosis and obstruction was evident in G-150 rats (Figure 1g). These results indicate that the G-50 regime exerts no apparent deleterious action on the kidneys, as evaluated by the finest diagnostic methods available.

Sub-nephrotoxic gentamicin sensitizes to ARF

Under these sub-nephrotoxic circumstances, we tested whether the G-50 regime sensitizes rats to ARF, e.g., by reducing the nephrotoxicity threshold of another potential nephrotoxicant. We first used uranyl nitrate (UN), which we titrated for dose-nephrotoxic effect in pilot studies. A single dose of 0.5 mg/kg UN was found to lack nephrotoxic effects, which was confirmed in further experiments (Figure 2). However, when this dose of UN was administered to rats previously treated with G-50, a clear ARF ensued, which was not observed in control rats or in that treated with UN or G-50 alone. This ARF was characterized (Figure 2a–d) by an increase in Cr_{pl}, BUN and NAG excretion, proteinuria, and a decrease in creatinine clearance. This sensitization appears along with the first sub-nephrotoxic dose of gentamicin and lasts at least 1 week after gentamicin withdrawal. This is evidenced by the increase in serum creatinine in rats that were given the single dose of UN at the onset of the gentamicin regime, as well as in rats in which UN is administered 1 week after cessation of the gentamicin treatment (data not shown). Interestingly, the sensitization produced by gentamicin is also effective on other potentially nephrotoxic drugs, such as the antineoplastic cisplatin or the iodinated contrast medium iohexol. Figure 2e–f shows how rats, previously exposed to G-50 for 6 days, suffered a renal damage when subsequently exposed to iohexol (in 24 h) and cisplatin (in 2 days), both used at sub-nephrotoxic doses (titrated in previous pilot studies). This is evidenced by increased serum creatinine and BUN, and elevated NAG excretion.

GM2AP is identified in the urine as a marker of sensitized animals

Next, we performed a differential proteomic analysis comparing the urine of control rats and G-50 rats at the end of the treatment, before the administration of the second nephrotoxicant. The objective of this study was to identify whether proteins increased or decreased in the urine of G-50 (sensitized to AKI) compared with control (non-sensitized to AKI) rats, which might prospectively serve as biomarkers of gentamicin-induced sensitization to AKI. As shown in Figure 3a, both urinary proteomes were almost identical. However, a protein was clearly increased in the urinary proteome of G-50, which was unambiguously identified using liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as GM2AP. A polyclonal antibody raised in rabbits against an epitope found in rat and human GM2AP further confirmed the increased levels of GM2AP in the urine of G-50 using western blot analysis (Figure 3b). Furthermore, the urine of eight patients treated with gentamicin for at least 2 days was analyzed using western blot for the level of GM2AP, and compared with that of eight sex and age matching untreated individuals. A total of seven of eight gentamicin-treated patients, whereas only two of eight untreated controls, showed increased levels of GM2AP in the urine, also determined using western blot analysis (Figure 3c).

Early diagnosis capacity of GM2AP in gentamicin-induced AKI

We also decided to study the early diagnostic capacity of urinary GM2AP on an animal model of gentamicin-induced

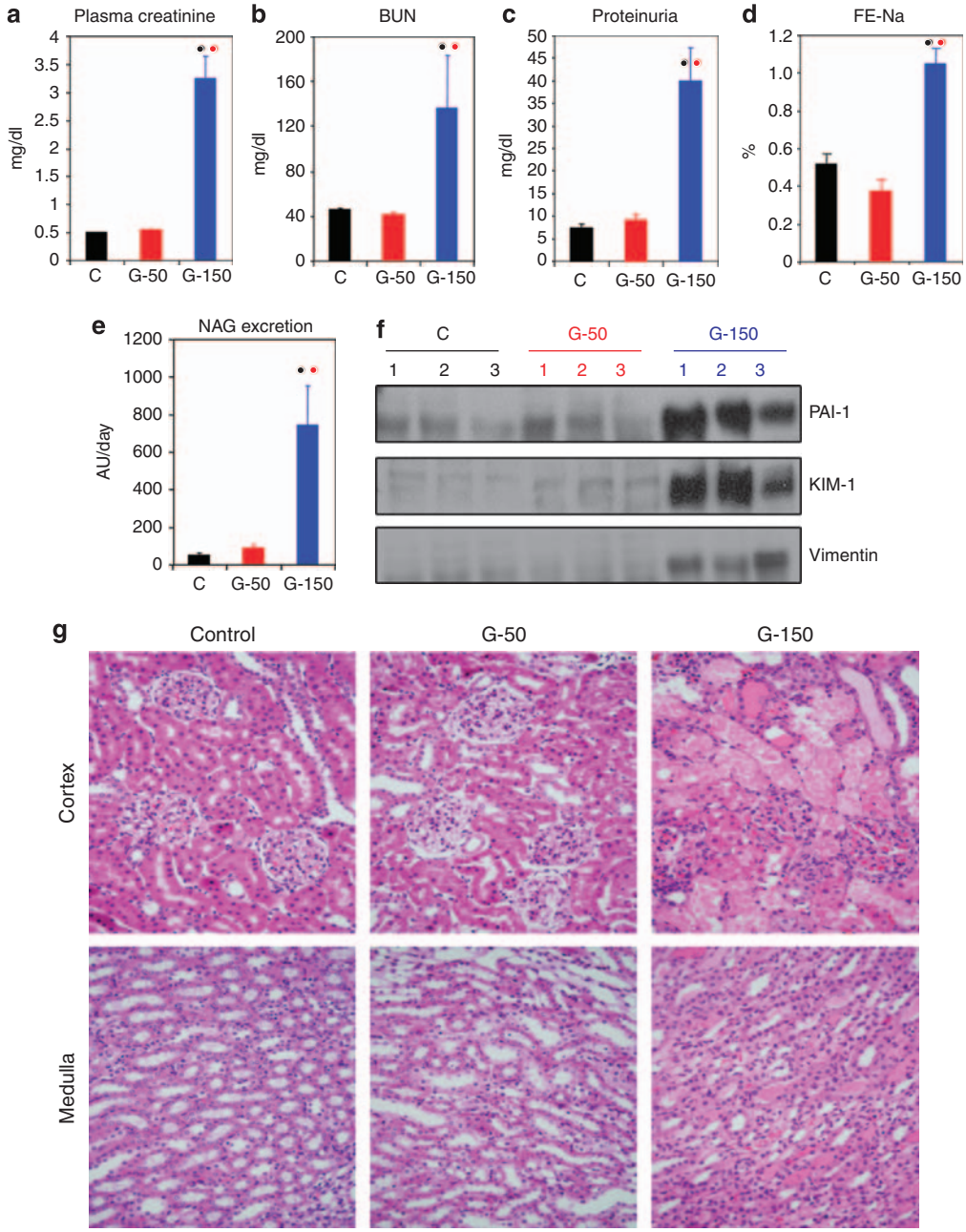

Figure 1 | Absence of renal effects after sub-nephrotoxic gentamicin. (**a**) Plasma creatinine concentration, (**b**) blood urea nitrogen concentration (BUN), (**c**) proteinuria, (**d**) fractional excretion of sodium (FE-Na), and (**e**) *N*-acetyl-glucosaminidase (NAG) excretion after 6 days of treatment with saline (C), 50 mg/kg/day gentamicin (G-50), or 150 mg/kg/day gentamicin (G-150); $n > 12$. (**f**) Representative images of western blot analysis of plasminogen activator inhibitor 1 (PAI-1), kidney injury molecule 1 (KIM-1), and vimentin levels in renal tissue homogenates from three randomly selected animals from C, G-50, and G-150 groups. (**g**) Representative images (400 ×) of the cortex and medulla of hematoxilin–eosin-stained renal sections from C, G-50, and G-150 rats ($n = 6$). ●, $P < 0.05$ with respect to C; •, $P < 0.05$ with respect to G-50.

ARF. A time course experiment revealed that, in this model, GM2AP appears in the urine from the first day of treatment with overtly nephrotoxic doses of gentamicin, largely preceding not only classical markers such as serum creatinine, BUN, NAG excretion, or proteinuria, but also the new, earlier, and more sensitive urinary markers of AKI, KIM-1, and plasminogen activator inhibitor 1 (Figure 4a). Furthermore, the level of GM2AP in the urine progressively increased with time, which makes it potentially suitable for monitoring AKI evolution induced by gentamicin in a much more specific manner than other novel markers. GM2AP also appears in the urine of overtly nephrotoxic rats as a consequence of cisplatin or UN administration at toxic doses (Figure 4b). In the case of cisplatin, GM2AP appears in the urine in parallel or after KIM-1 (Figure 4c). These results indicate that urinary GM2AP, in the absence of damage

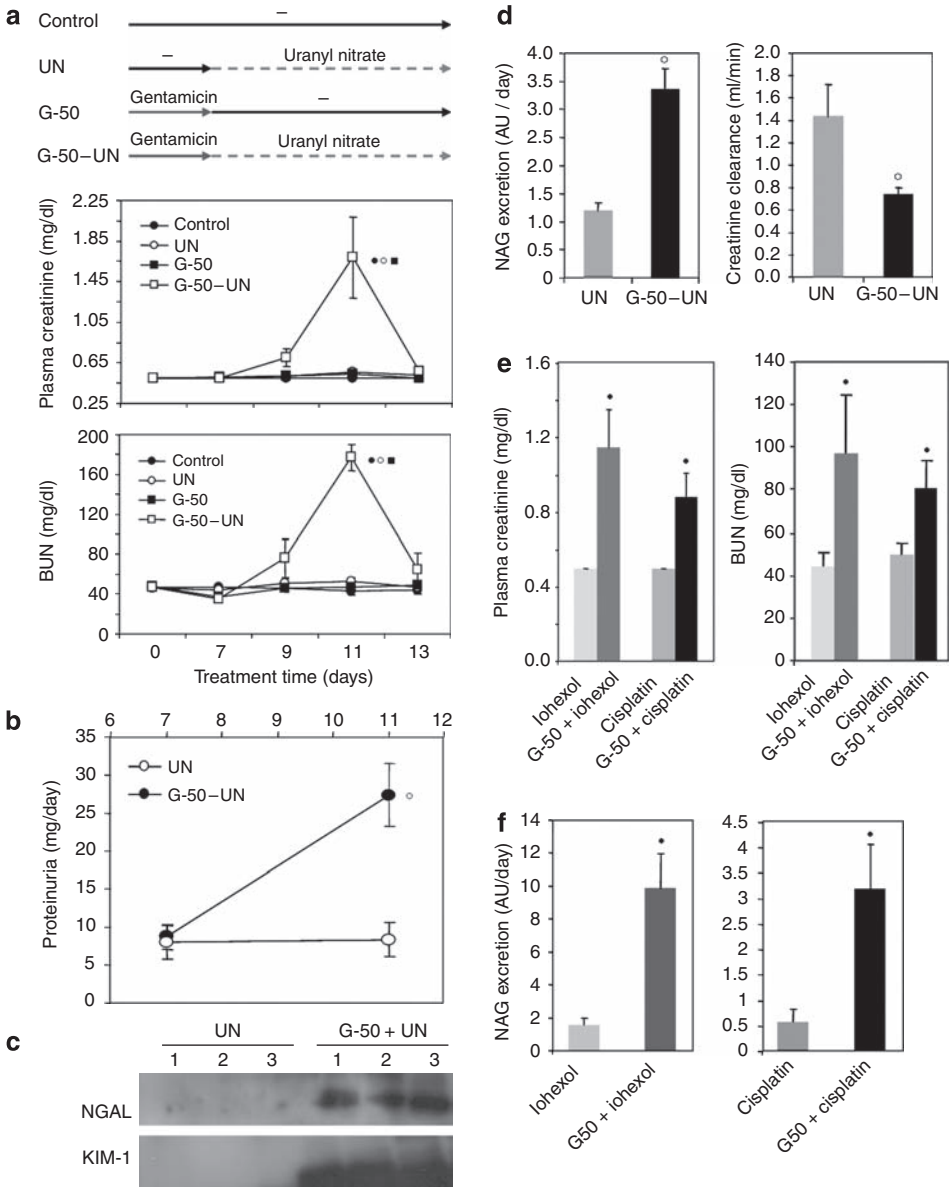

Figure 2 | Sub-nephrotoxic gentamicin sensitizes to acute renal failure. (**a**) Time-course evolution of plasma creatinine and BUN concentration, (**b**) proteinuria, (**c**) urinary neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1) levels, (**d**) *N*-acetyl-glucosaminidase (NAG) excretion, and creatinine clearance of rats treated as indicated in the upper, left panel of **a**; $n = 6$. ●, $P < 0.05$ with respect to C; ○, $P < 0.05$ with respect to uranyl nitrate (UN); and ■, $P < 0.05$ with respect to G. (**e**) Plasma creatinine and BUN concentration and (**f**) NAG excretion of rats treated as indicated in the left panel, in which uranyl nitrate was substituted by iohexol or cisplatin; $n = 4$; ●, $P < 0.05$ with respect to iohexol or cisplatin.

markers, likely reflects the increased risk of ARF induced by gentamicin, because further exposure to the drug produces an overt ARF.

Urinary GM2AP results from an altered renal handling of the filtered blood-borne protein

To unravel the origin of the urinary GM2AP, we studied the effect of gentamicin on the presence and production of GM2AP in renal tissue, the urine, and the blood. The immunohistochemical analysis of GM2AP distribution in renal tissue shows (Figure 5a and b) that this protein is mainly located in the renal cortex, with great selectivity, within the proximal tubules. The latter is evidenced by a perfect co-staining of GM2AP with the proximal tubule-restricted protein megalin, and the absence of co-staining with the distal tubule-borne protein calbindin. Sub-nephrotoxic gentamicin (G-50) did not modify the histological distribution or apparent expression level of GM2AP (Figure 5a and b). Western blot analysis of total GM2AP protein level in renal tissue homogenates (Figure 5c), as well as *GM2AP* gene expression (by reverse transcriptase-PCR; Figure 5d), confirmed the lack of effect of gentamicin on the renal expression of the protein. GM2AP was detected in the serum and its serum levels were not altered by G-50.

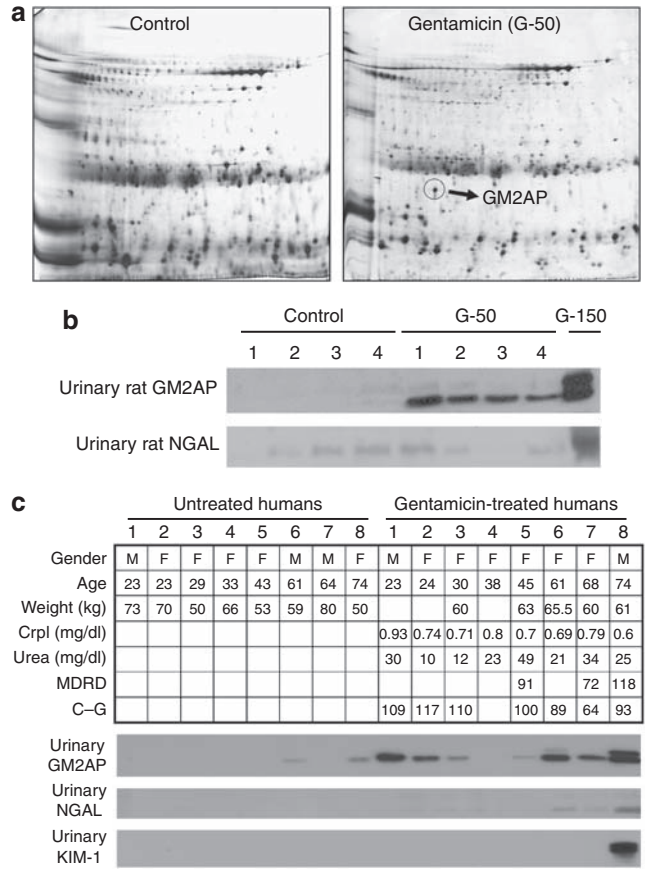

Figure 3 | Increased urinary level of ganglioside M2 activator protein (GM2AP) in gentamicin-sensitized rats and gentamicin-treated humans. (**a**) Representative bidimensional electrophoresis (2D) gels of urine from rats treated with saline (control) or 50 mg/kg/day gentamicin (G-50) during 6 days ($n = 4$). (**b**) Representative images of western blot analysis of GM2AP and neutrophil gelatinase-associated lipocalin (NGAL) in the urine of four randomly selected rats from control and G-50 groups, and an acute kidney injury-positive control (urine from a rat treated with 150 mg/kg/day gentamicin, otherwise as in G-50). (**c**) Representative images of western blot analysis of GM2AP, NGAL, and kidney injury molecule 1 (KIM-1) in the urine of eight patients treated with gentamicin and eight sex- and age-matched untreated individuals. Their gender, age, weight, plasma creatinine concentration (Cr_{pl}), blood urea concentration, and glomerular filtration rate estimated by the Modification of Diet in Renal Disease (MDRD) study equation or the Cockroft–Gault (C–G) equation are provided (when known).

However, G-150 treatment significantly increased serum GM2AP (Figure 5e).

In acute experiments with anesthetized rats, a bolus administration of a high dose of gentamicin induced a rapid increase in the urinary excretion of GM2AP (Figure 5g). The increased excretion declined after 3 h, probably correlating with the bioavailability of gentamicin. Interestingly, when, under similar circumstances, kidneys were perfused *in situ* with Krebs solution (instead of blood) by means of an extracorporeal circuit, gentamicin did not produce an increase in urinary GM2AP (Figure 5h). All together, these results indicate that the increase in urinary GM2AP produced by gentamicin is an acute effect which is tightly dependent on the presence of gentamicin and, most importantly, that the urinary GM2AP comes from the blood and not from renal tissues. These results can be explained by an altered renal handling of GM2AP (e.g., reduced reuptake) as the mechanism responsible for its increase in the urine. In fact, GM2AP appears in the urine shortly after treatment with maleate (Figure 5f), indicating that it is transported by the megalin complex (see discussion). This is further supported by the colocalization of GM2AP and megalin in proximal tubule cells within subcellular structures, probably being endocytosis vesicles, as revealed by confocal microscopy (Figure 5b; lower panel).

DISCUSSION

Our experiments show that gentamicin-induced sensitization to ARF, a condition hitherto largely underestimated, is distinctly differentiated from early and mild renal injury. It has a potentially high clinical relevance because it poses an unnoticed risk of ARF. The recognition of acquired sensitization to ARF as an existing and relevant pathological state makes obvious the necessity to identify tools to create a level of diagnosis for its detection and appropriate clinical handling. Our results also show that the increased urinary level of GM2AP is associated with the sensitization to AKI induced by sub-nephrotoxic gentamicin. They further show that an increased level of GM2AP also appears in the urine of rats undergoing an overt ARF and, in the case of gentamicin-induced AKI, urinary GM2AP appears earlier than other sensitive markers such as KIM-1 or plasminogen activator inhibitor 1. However, in the case of other nephrotoxicants, such as cisplatin, urinary GM2AP is elevated in parallel or even after the appearance early damage marker KIM-1. This fact has special importance because GM2AP might be exploited also for an etiological and selective diagnosis of AKI within the very early stages of the disease. The sub-nephrotoxic and the early nephrotoxic situations related to gentamicin treatments show the common characteristic of lacking the markers of tissue damage, while showing increased urinary levels of GM2AP. As such, monitoring the progressive increment of the urinary level of GM2AP from the onset of a gentamicin regime will provide means of detecting the increasing risk of an AKI burst as a consequence of further gentamicin administration or treatment with another potential nephrotoxicant.

In the case of the sensitization to AKI, GM2AP urinary excretion would serve as a diagnostic tool to discern which patients have acquired an increased risk as a consequence of a gentamicin regime, when contemplating the need of subjecting them to additional potentially nephrotoxic circumstances like the administration of other drugs. A typical and relevant case, from the clinical and socioeconomic point of views, is posed by the fact that 0.6–2.3% of not-at-risk patients undergoing a contrast radiography, with no previous history of renal disease, develop some degree of AKI.[27] We propose that a part of this patient group might be silently coursing

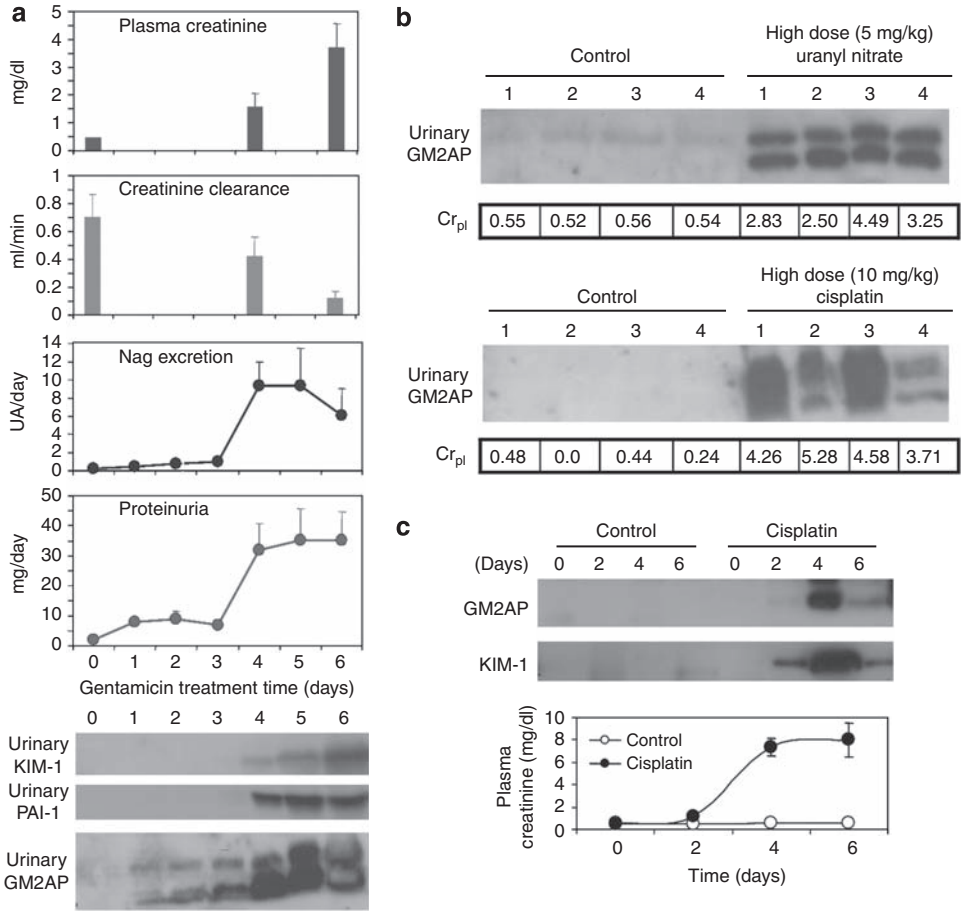

Figure 4 | Ganglioside M2 activator protein (GM2AP) in the urine of nephrotoxic rats. (**a**) Time-course evolution of plasma creatinine concentration (Cr_{pl}), creatinine clearance, urinary excretion of *N*-acetyl-glucosaminidase (NAG), proteinuria ($n = 6$), and representative ($n = 3$) images of western blot of urinary kidney injury molecule 1 (KIM-1), plasminogen activator inhibitor 1 (PAI-1), and GM2AP excretion of rats treated during 6 days with 150 mg/kg/day gentamicin. (**b**) Cr_{pl} and representative images of western blot analysis of urinary GM2AP of rats treated with saline (control) or a nephrotoxic dose of uranyl nitrate (5 mg/kg) or cisplatin (10 mg/kg). (**c**) Time-course evolution of Cr_{pl} ($n = 4$) and representative images of western blot analysis of urinary GM2AP and KIM-1 of rats treated with saline (control) or cisplatin (10 mg/kg).

with an increased risk to AKI owing to a previous treatment or exposure to an environmental agent that has induced no clinical symptoms of renal lesions, such as a treatment with gentamicin. The urinary level of GM2AP could be used as a marker to detect this risk. Very interestingly, urinary GM2AP is found to be increased (to a variable degree) in most patients treated with gentamicin for at least 3 days, and whose estimated glomerular filtration rate and urinary levels of sensitive renal damage markers (e.g., neutrophil gelatinase-associated lipocalin, KIM-1) remain normal during analysis. Identification of markers of risk should be extended over other nephrotoxic drugs and insults, including renal ischemia and obstruction of the urinary ways.

GM2AP is an 18–24 kDa substrate cofactor for the lysosomal β-hexosaminidase A implicated in GM2 ganglioside metabolism.[28] It has also been proposed as an intra and intercellular glycosphingolipid transporter.[29–31] GM2AP is mainly driven to the lysosomes through typical trans-Golgi mannose-6-phosphate receptor-dependent[32] and -independent[33] pathways. About one third of the synthesized protein is secreted.[32] Because, even at low doses, gentamicin accumulates in the lysosomes, endosomes, and Golgi;[34–37] modulates lipid metabolism, signalling, and function;[14,38–41] and interferes with endosomal traffic,[42,43] it was tempting to think that the mechanism of gentamicin-induced increase in urinary GM2AP has to do with alterations in the cellular handling of this protein resulting in its higher secretion, production, or both. Our experiments indicate that, as a result of gentamicin treatment, the renal expression of GM2AP is not modified, and that the origin of the increased urinary level of GM2AP is not the renal tissue, but a blood origin. Owing to its low molecular weight, GM2AP is expected to cross the glomerular filtration barrier freely.[44–46] Our data point at an alteration in its tubular handling as the cause of its urinary appearance.

In the case of the sub-nephrotoxic regime of gentamicin (G-50), the increased urinary level of GM2AP is a highly specific event, being one of the very few proteins showing higher excretion than in control animals. Contrarily, in the urine of animals treated with nephrotoxic regimes of

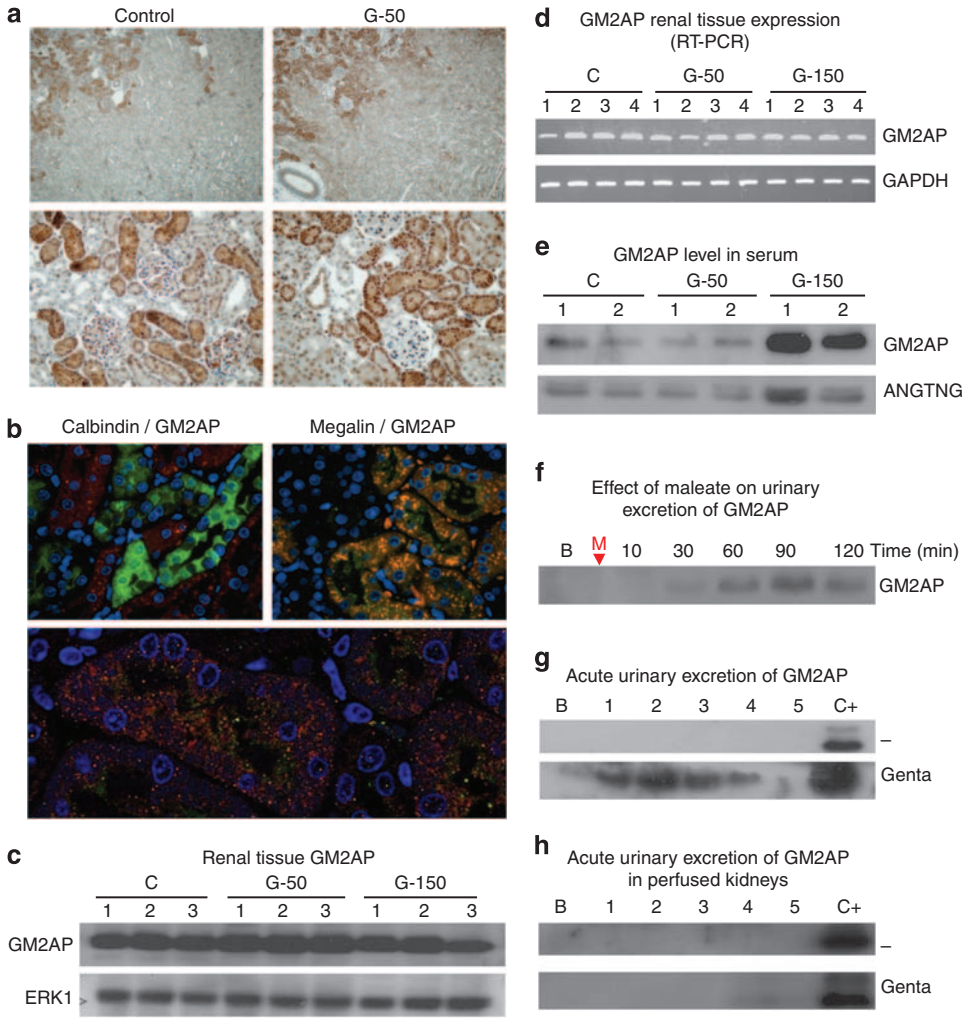

Figure 5 | An altered tubular handling of ganglioside M2 activator protein (GM2AP) causes its increase in the urine. (**a**) Representative images ($n = 3$) of renal tissue sections from rats treated with saline (C) or 50 mg/kg/day gentamicin (G-50) during 6 days, stained with anti-GM2AP. Upper panels (100 ×) show the cortex and medulla. Lower panels (400 ×) show an amplified area of the renal cortex. (**b**) In the upper panels, representative ($n = 3$) immunofluorescence co-staining images of GM2AP and calbindin (left), and GM2AP and megalin (right). In the lower panels, detailed confocal microscopy image of megalin and GM2AP co-staining. Red represents GM2AP, green represents calbindin or megalin, and orange shows co-staining. (**c**) Western blot analysis of GM2AP and extracellular signal-regulated kinase 1 (Erk-1) level in three randomly selected renal tissue homogenates from rats treated with saline (C), 50 mg/kg/day gentamicin (G-50), or 150 mg/kg/day gentamicin (G-150) during 6 days. (**d**) Reverse transcriptase-PCR (RT-PCR) amplification of the renal mRNA of GM2AP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from four randomly selected C, G-50, and G-150 rats. (**e**) Representative images of western blot analysis of GM2AP and angiotensinogen (ANGTNG) level in two randomly selected serum samples from C, G-50, and G-150 rats. (**f**) Representative images ($n = 5$) of western blot analysis of the time-course level of GM2AP in the urine of rats treated intravenously with a bolus of sodium maleate (400 mg/kg). (**g**) Representative images ($n = 4$) of western blot analysis of the time-course level of GM2AP in the urine of rats treated intravenously with a bolus of saline (C +) or 100 mg/kg gentamicin (Genta). (**h**) Representative images ($n = 4$) of western blot analysis of the time-course level of GM2AP in the urine of rats with their left kidney perfused with isosmotic solution (C +) or isosmotic solution containing 1.5 mg/ml gentamicin during 5 h. B, basal.

gentamicin, cisplatin, and UN, the increased GM2AP urinary levels might be the result of a less specific and defective renal handling of proteins leading to an overt and unselective proteinuria. Gentamicin is transported into the proximal tubule epithelial cells by the apical protein–endocytic transport complex megalin/cubilin.[47] The complex megalin/cubilin has been shown to mediate the reabsorption of a wide variety of proteins.[48] Gentamicin competes with filtered proteins for this transporter and augments their urinary excretion,[21,49–52] even in an acute manner.[51]

As such, it is reasonable to hypothesize that a plausible origin of the increased urinary excretion of GM2AP in sub-nephrotoxic circumstances is a submaximal competition of gentamicin for the GM2AP tubular transporter (likely the megalin/cubilin complex). Indeed, our results suggest that GM2AP is reabsorbed in the proximal tubule by the megalin complex. First, megalin and GM2AP colocalize in spotty subcellular structures in proximal tubule cells, which are likely the endosomal vesicles. Second, acute treatment with maleate produces a rapid appearance of GM2AP in the urine.

Maleate treatment has been shown to induce the shedding of megalin into the urine and to impair megalin-mediated reabsorption.[53] In fact, both maleate[54] and gentamicin[55,56] have been used experimentally to inhibit megalin-dependent endocytosis. It is further hypothesized that under mild competition circumstances, only a few proteins showing the least affinity for the system would be displaced, GM2AP being one of the first. This is supported by the uneven effect of gentamicin on the increased excretion of different proteins, the excretion of ones being increased by orders of magnitude that of others.[51] Increasing competition (i.e., by higher gentamicin concentrations) would bring about the displacement of more proteins that, together with a dysfunctional necrotic tubular epithelium, would produce a full range proteinuria like that seen during overtly nephrotoxic circumstances with gentamicin and other drugs.

In conclusion, our study opens new possibilities for the improvement of AKI diagnosis by providing a potential marker of the enhanced risk of AKI induced by gentamicin, a condition that has hitherto been mostly uncharacterized, quite underestimated, and impossible to diagnose, but which might have a high clinical and socioeconomic repercussion. The capacity to detect and prospectively quantify AKI risk through the determination of biochemical parameters may help to overturn drug-induced sensitization to AKI. However, the most important result of this work is the realization, through this proof-of-concept, of new possibilities for a more rational and personalized utilization of drugs in the clinical practice, by anticipating the increased susceptibility and risk of damage, injury, or alteration posed by drugs even in the absence of detectable signs of toxicity.

METHODS

Reagents

Unless otherwise indicated, products were purchased from Sigma. Gentamicin sulphate was kindly provided by Schering-Plough. For the preparation of an anti-GM2AP serum, female New Zealand White rabbits were injected with a synthetic immunogen corresponding to the rat and human GM2AP partial peptide sequence SWDNCDEGKDPAVI. After three immunizations, the serum was purified through a HiTrap TM Protein G HP column (GE Healthcare Bio-Sciences AB, Madrid, Spain) and kept at −20 °C for further use.

Animal models and experimental protocols

In-house bred, female Wistar rats weighing 190–230 g were divided into the following experimental groups: (i) control: rats treated intraperitoneally during 7–13 days with saline (0.9% NaCl), once daily, (ii) G-50: rats treated with 50 mg/kg/day gentamicin during 6 days, (iii) G-50-NU: rats treated with 50 mg/kg/day gentamicin during 6 days and on the seventh day, treated with a single intraperitoneal dose of 0.5 mg/kg UN or cisplatin (10 mg/kg) or iohexol (2.1 g I/kg, GE Healthcare), (iv) NU: rats treated intraperitoneally with saline (0.9% NaCl) once daily during 6 days and on the seventh day, treated with a single intraperitoneal dose of 0.5 mg/kg UN or cisplatin (10 mg/kg) or iohexol (2.1 gI/kg), and (v) G-150: rats treated with 150 mg/kg/day gentamicin during 6 days. Rats were individually allocated in metabolic cages under controlled conditions. Urine and plasma were obtained and stored at −80 °C. Only for western blot studies, some plasma was depleted of albumin through Murine Albumin Depletion Columns (Qiagen, Madrid, Spain) at the moment of use. At the end of treatments, kidneys were perfused with heparinized saline and immediately dissected. Half of the kidney was fixed in paraformaldehyde for histological studies. The remaining renal mass was frozen at −80 °C for ulterior western blot and gene expression studies.

Characterization of renal function

Plasma and urine creatinine, (Cr_p and Cr_u, respectively) and BUN concentrations were determined using an automated analyzer (Reflotron, Roche Diagnostics, Barcelona, Spain). This method has a lower detection limit of 0.5 mg/dl for creatinine. Creatinine clearance (Cl_{Cr}) = $Cr_u \times$ 24-hour urine output $\times Cr_p^{-1}$. Urine protein concentration was measured by the Bradford method,[57] and urine NAG activity by a commercial enzymatic test (Roche Diagnostics). Sodium (Na) concentration was measured by indirect potentiometry in a Modular Analytics System (Roche Diagnostics). Fractional excretion of sodium (FE-Na) was calculated as (100 × urinary Na × Cr_p)/(plasma Na × Cr_u).

Histological studies

Paraformaldehyde-fixed tissue samples were immersed in paraffin, cut into 5 μm-thick slices and stained with (i) hematoxylin and eosin, (ii) anti-GM2AP, (iii) anti-megalin, (Santa Cruz, Santa Cruz, CA, USA) and (iv) anti-calbindin (Santa Cruz); followed by horseradish peroxidase-, fluorescein isothiocyanate- or cyanine 3-conjugated secondary antibodies.

Western blotting

Protein extracts were obtained from renal tissue homogenates in lysis buffer (25 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mmol/l NaCl, 1% Igepal CA-630, 10 mmol/l $MgCl_2$, 1 mmol/l EDTA, 2% v/v glycerol, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 5 mmol/l phenylmethylsulfonyl fluoride, 5 μM NaF, 1 mmol/l Na_3VO_4, pH = 7.5). A total of 50 μg of tissue lysates, 21 μl of albumin-deprived plasma, or a volume of urine from each animal corresponding to the same excretion fraction were subject to polyacrylamide gel electrophoresis, transferred to an Inmobilon-P membrane (Millipore, Madrid, Spain) and hybridized with antibodies against KIM-1 (R&D Systems, Minneapolis, MN, USA and Santa Cruz), neutrophil gelatinase-associated lipocalin (MBL, Woburn, MA, USA), bone morphogenetic protein 7 (Santa Cruz Biotechnology), plasminogen activator inhibitor 1 (BD Biosciences, Madrid, Spain), vimentin (Dako, Glostrup, Denmark), GM2AP.

Urinary differential proteomic studies

Urine proteins were precipitated and isoelectrically focused (500–8000 V) through 18-cm long immobilized pH gradient (IPG) strips, pH 4–7 (GE Healthcare). Then, proteins in IPG strips were separated by polyacrylamide gel electrophoresis, fixed, and silver stained. The spots of interest were in-gel digested with porcine trypsin (Promega, Madrid, Spain). Peptides were extracted and injected in a liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometer QSTAR XL (Applied Biosystems, Carlsbad, CA, USA) with an 1100 micro high-performance liquid chromatography (Agilent, Madrid, Spain). MS/MS spectra were obtained. Mass spectrometry was confirmed

by matrix-assisted laser desorption/ionization time-of-flight. Protein identification was performed with the MASCOT software (http://www.matrixscience.com) against non-redundant protein sequence databases (Swiss Prot and National Center for Biotechnology Information).

Gene expression analysis

reverse transcriptase-PCR amplification of GM2AP and glyceraldehyde 3-phosphate dehydrogenase was performed with the following primers: for rat GM2AP, 5′-ATCAAAAGCCTCACGCTCCA-3′ and 5′-TATT TGCCCTTGAGAGAGGC-3′; for rat glyceraldehyde 3-phosphate dehydrogenase, 5′-GTGGTCATGAGCCCTTCCA-3′ and 5′-AACTC CCTCAAGATTGTCAGCAA-3′. PCR conditions were: $1 \times (95\,^{\circ}C \times 4\,min)$; $30 \times (95\,^{\circ}C \times 1\,min + T_m \times 1\,min)$; $1 \times (72\,^{\circ}C \times 10\,min)$; where T_m was 55 °C for GM2AP and 55.9 °C for glyceraldehyde 3-phosphate dehydrogenase.

Excretion of GM2AP

In a set of experiments, rats were anesthetized and after a single injection of gentamicin (100 mg/kg), sodium maleate (400 mg/kg) or saline through the jugular vein, urine fractions were collected from the bladder during the last 15 min of each subsequent hour, during 5 h. In another set of experiments, rats were anesthetized and an extracorporeal circuit for kidney perfusion was set up, as described[58] with some modifications. Briefly, the renal artery, vein, and ureter of the right kidney were ligated. The renal artery and vein of the left kidney and the urinary bladder were canulated. Oxygenated and warm (37 °C) Krebs-dextran (40 g/l of dextran (molecular weight 64K–76K) in Krebs solution (118.3 mmol/l NaCl, 4.7 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 0.026 mmol/l EDTA, 11.1 glucose, pH = 7.4)) or Krebs-dextran containing 1.5 mg/ml gentamicin was perfused through the renal artery at 3 ml/min, and was discarded through the renal vein. Urine fractions were also collected as before. All urine samples were kept at −80 °C until assayed by western blot for the presence of GM2AP.

Human samples

The urine and blood from eight unselected patients treated for at least 3 days with gentamicin, and the urine from eight sex- and age-matched untreated donors was obtained from volunteers from the Hospital Universitario de Salamanca (Spain). Glomerular filtration rate was estimated by the Modification of Diet in Renal Disease (MDRD) study or the Cockroft–Gault (C–G) equations.

Statistical analysis

Data are expressed as the average ± s.e.m. of the indicated number of experiments. Statistical analysis was performed by the Scheffe's test or the Kruskal–Wallis test, as appropriate. $P < 0.05$ was considered statistically significant.

DISCLOSURE

JML-N and FJL-H are minority shareholders of Bio-inRen, S.L., a biotech company holding license of the patent on the use of GM2AP as a marker for the diagnosis of kidney injury.

ACKNOWLEDGMENTS

We thank Schering-Plough (Madrid, Spain) for the kind provision of gentamicin sulphate; Immunostep for helping in the anti-GM2AP antibody production; and Dr P. Miramontes (Hospital Universitario de Salamanca, Spain) for human urine samples.

This study was supported by grants from the Instituto de Salud Carlos III (Retic 016/2006, RedinRen to JML-N, and FIS grant PI081900 to FJL-H).

REFERENCES

1. Kellum JA, Levin N, Bouman C *et al.* Developing a consensus classification system for acute renal failure. *Curr Opin Crit Care* 2002; **8**: 509–514.
2. Bellomo R, Kellum JA, Ronco C. Defining and classifying acute renal failure: from advocacy to consensus and validation of the RIFLE criteria. *Intensive Care Med* 2007; **33**: 409–413.
3. Neild GH. Multi-organ renal failure in the elderly. *Int Urol Nephrol* 2001; **32**: 559–565.
4. Block CA, Schoolwerth AC. The epidemiology and outcome of acute renal failure and the impact on chronic kidney disease. *Semin Dial* 2006; **19**: 450–454.
5. Kellum JA, Hoste EA. Acute kidney injury: epidemiology and assessment. *Scand J Clin Lab Invest Suppl* 2008; **241**: 6–11.
6. Waikar SS, Liu KD, Chertow GM. Diagnosis, epidemiology and outcomes of acute kidney injury. *Clin J Am Soc Nephrol* 2008; **3**: 844–861.
7. Binswanger U. Acute renal failure: changing causes? *Kidney Blood Press Res* 1997; **20**: 163.
8. Devarajan P. Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of kidney disease. *Scand J Clin Lab Invest Suppl* 2008; **241**: 89–94.
9. Vaidya VS, Ferguson MA, Bonventre JV. Biomarkers of acute kidney injury. *Annu Rev Pharmacol Toxicol* 2008; **48**: 463–493.
10. Mueller PW, Price RG, Finn WF. New approaches for detecting thresholds of human nephrotoxicity using cadmium as an example. *Environ Health Perspect* 1998; **106**: 227–230.
11. Mingeot-Leclerq MP, Tulkens P. Aminoglycosides: nephrotoxicity. *Antimicrob Agents Chemother* 1999; **43**: 1003–1012.
12. Martínez-Salgado C, López-Hernández FJ, López-Novoa JM. Glomerular nephrotoxicity of aminoglycosides. *Toxicol Appl Pharmacol* 2007; **223**: 86–98.
13. Kacew S, Bergeron MG. Pathogenic factors in aminoglycoside-induced nephrotoxicity. *Toxicol Lett* 1990; **51**: 241–259.
14. Laurent G, Kishore BK, Tulkens PM. Aminoglycoside-induced renal phospholipidosis and nephrotoxicity. *Biochem Pharmacol* 1990; **40**: 2383–2392.
15. Leehey DJ, Braun BI, Tholl DA *et al.* Can pharmacokinetic dosing decrease nephrotoxicity associated with aminoglycoside therapy. *J Am Soc Nephrol* 1993; **4**: 81–90.
16. Nakakuki M, Yamasaki F, Shinkawa T *et al.* Protective effect of human ulinastatin against gentamicin-induced acute renal failure in rats. *Can J Physiol Pharmacol* 1996; **74**: 104–111.
17. Xie Y, Nishi S, Iguchi S *et al.* Expression of osteopontin in gentamicin-induced acute tubular necrosis and its recovery process. *Kidney Int* 2001; **59**: 959–974.
18. Baylis C. The mechanism of the decline in glomerular filtration rate in gentamicin-induced acute renal failure in the rat. *J Antimicrob Chemother* 1980; **6**: 381–388.
19. Avasthi PS, Evan AP, Huser JW *et al.* Effect of gentamicin on glomerular ultrastructure. *J Lab Clin Med* 1981; **98**: 444–454.
20. Schor N, Ichikawa I, Rennke HG *et al.* Pathophysiology of altered glomerular function in aminoglycoside-treated rats. *Kidney Int* 1981; **19**: 288–296.
21. Coimbra TM, Lachat JJ. Analysis of urinary albumin excretion in gentamicin-treated rats. *Nephron* 1988; **49**: 154–159.
22. De-Barros-e-Silva ML, Varanda WA, Lachat JJ *et al.* Glomerular permeability to macromolecules in gentamicin-treated rats. *Braz J Med Biol Res* 1992; **25**: 409–417.
23. Klotman PE, Yarger WE. Reduction of renal blood flow and proximal bicarbonate reabsorption in rats by gentamicin. *Kidney Int* 1983; **24**: 638–643.
24. Goto T, Fujigaki Y, Sun DF *et al.* Plasma protein extravasation and vascular endothelial growth factor expression with endothelial nitric oxide synthase induction in gentamicin-induced acute renal failure in rats. *Virchows Arch* 2004; **444**: 362–374.
25. Secilmis MA, Karatas Y, Yorulmaz O *et al.* Protective effect of L-arginine intake on the impaired renal vascular responses in the gentamicin-treated rats. *Nephron Physiol* 2005; **100**: 13–20.
26. Hishida A, Nakajima T, Yamada M *et al.* Roles of hemodynamic and tubular factors in gentamicin-mediated nephropathy. *Ren Fail* 1994; **16**: 109–116.
27. Mehran R, Nikolsky E. Contrast-induced nephropathy: definition, epidemiology, and patients at risk. *Kidney Int Suppl* 2006; **100**: S11–S15.

28. Meier EM, Schwarzmann G, Furst W *et al.* The human GM2 activator protein. A substrate specific cofactor of beta-hexosaminidase A. *J Biol Chem* 1991; **266**: 1879–1887.
29. Mahuran DJ. The GM2 activator protein, its roles as a cofactor in GM2 hydrolysis and as a general glycolipid transport protein. *Biochim Biophys Acta* 1998; **1393**: 1–18.
30. Mundel TM, Heid HW, Mahuran DJ *et al.* Ganglioside GM2-activator protein and vesicular transport in collecting duct intercalated cells. *J Am Soc Nephrol* 1999; **10**: 435–443.
31. Yanai H, Yoshida H, Tomoro Y *et al.* The possible contribution of a general glycosphingolipid transporter, GM2 activator protein, to atherosclerosis. *J Atheroscler Thromb* 2006; **13**: 281–285.
32. Rigat B, Wang W, Leung A *et al.* Two mechanisms for the recapture of extracellular GM2 activator protein: evidence for a major secretory form of the protein. *Biochemistry* 1997; **36**: 8325–8331.
33. Glombitza GJ, Becker E, Wilhelm Kaiser H *et al.* Biosynthesis, processing, and intracellular transport of GM2 activator protein in human epidermal keratinocytes. *J Biol Chem* 1997; **272**: 5199–5207.
34. Silverblatt FJ, Kuehn C. Autoradiography of gentamicin uptake by the rat proximal tubule cell. *Kidney Int* 1979; **15**: 335–345.
35. Beauchamp D, Gourde P, Bergeron MG. Subcellular distribution of gentamicin in proximal tubule by immunogold labeling. *Antimicrob Agents Chemother* 1991; **35**: 2173–2179.
36. Sandoval R, Leiser J, Molitoris BA. Aminoglycoside antibiotics traffic to the Golgi complex in LLC-PK1 cells. *J Am Soc Nephrol* 1998; **9**: 167–174.
37. Myrdal SE, Johnson KC, Steyger PS. Cytoplasmic and intranuclear binding of gentamicin does not require endocytosis. *Hear Res* 2005; **204**: 156–169.
38. Oshima M, Hashiguchi M, Shindo N *et al.* Biochemical mechanisms of aminoglycoside cell toxicity. I. The uptake of gentamicin by subcultured skin fibroblasts and the alteration of lysosomal enzyme activities. *J Biochem* 1986; **100**: 1575–1582.
39. Schacht J. Molecular mechanisms of drug-induced hearing loss. *Hear Res* 1986; **22**: 297–304.
40. Kacew S. Cationic amphiphilic drug-induced renal cortical lysosomal phospholipidosis: an *in vivo* comparative study with gentamicin and chlorphentermine. *Toxicol Appl Pharmacol* 1987; **91**: 469–476.
41. Ramsammy LS, Josepovitz C, Kaloyanides GJ. Gentamicin inhibits agonist stimulation of the phosphatidylinositol cascade in primary cultures of rabbit proximal tubular cells and rat renal cortex. *J Pharmacol Exp Ther* 1988; **247**: 989–996.
42. Giurgea-Marion L, Toubeau G, Laurent G *et al.* Impairment of lysosome-pynocytic vesicle fusion in rat kidney proximal tubules after treatment with gentamicin at low doses. *Toxicol Appl Pharmacol* 1986; **86**: 271–285.
43. Van Bambeke F, Tulkens PM, Brasseur R *et al.* Aminoglycoside antibiotics induce aggregation but not fusion of negatively-charged liposomes. *Eur J Pharmacol* 1995; **289**: 231–233.
44. Deen WM, Lazzara MJ, Myers BD. Structural determinants of glomerular permeability. *Am J Physiol Renal Physiol* 2001; **281**: F579–F596.
45. Deen WM, Lazzara MJ. Glomerular filtration of albumin: how small is the sieving coefficient? *Kidney Int Suppl* 2004; **S63**: S64.
46. Haraldsson B, Sorensson J. Why do we not all have proteinuria? An update of our current understanding of the glomerular barrier. *News Physiol Sci* 2004; **19**: 7–10.
47. Schmitz C, Hilpert J, Jacobsen C *et al.* Megalin deficiency offers protection from renal aminoglyoside accumulation. *J Biol Chem* 2002; **277**: 618–622.
48. Christensen EI, Birn H. Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. *Am J Physiol Renal Physiol* 2001; **280**: F562–F573.
49. Valette JP, Nicot G, Charmes JP *et al.* Low molecular weight proteins as urinary markers of aminoglycoside nephrotoxicity in man. *Proc Eur Dial Transplant Assoc* 1979; **16**: 597–602.
50. Nicot G, Merle L, Valette JP *et al.* Gentamicin and sisomicin – induced renal tubular damage. *Eur J Clin Pharmacol* 1982; **23**: 161–166.
51. Bernard A, Viau C, Ouled A *et al.* Effects of gentamicin on the renal uptake of endogenous and exogenous protein in conscious rats. *Toxicol Appl Pharmacol* 1986; **84**: 431–438.
52. Negri AL. Proximal tubule endocytic apparatus as the specific renal uptake mechanism for vitamin D-binding protein/25-(OH)D3 complex. *Nephrology (Carlton)* 2006; **11**: 510–515.
53. Bergeron M, Mayers P, Brown D. Specific effect of maleate on an apical membrane glycoprotein (gp330) in proximal tubule of rat kidneys. *Am J Physiol Renal Fluid Electrolyte Physiol* 1996; **271**: F908–F916.
54. Nagai J, Tanaka H, Nakanishi N *et al.* Role of megalin in renal handling of aminoglycosides. *Am J Physiol Renal Physiol* 2001; **281**: F337–F344.
55. Cui S, Verroust PJ, Moestrup SK *et al.* Megalin/gp330 mediates uptake of albumin in renal proximal tubule. *Am J Physiol* 1996; **271**: F900–F907.
56. Nagai J, Katsube T, Murakami T *et al.* Effect of gentamicin on pharmacokinetics of lysozyme in rats: interaction between megalin substrates in the kidney. *J Pharm Pharmacol* 2002; **54**: 1491–1496.
57. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
58. López-Novoa JM, Santos JC, Villamediana LM *et al.* Renal catabolism of 125I-glicentin. *Am J Physiol* 1986; **250**: E545–E550.