JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. 2008; **28**: 122–131
Published online 14 May 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/jat.1256

Renal gangliosides are involved in lead intoxication

Rossana Pérez Aguilar, Susana Genta and Sara Sánchez*

Departamento de Biología del Desarrollo, Instituto Superior de Investigaciones Biológicas (INSIBIO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) y Universidad Nacional del Tucumán, 4000 San Miguel de Tucumán, Argentina

Received 27 October 2006; Revised 20 February 2007; Accepted 21 February 2007

ABSTRACT: The biological effects of lead are well defined; however, neither the risk exposure level nor the subcellular mechanism of its action is completely clear. The present work was undertaken to investigate the effects of low level and long term lead exposure on the composition and expression of rat renal gangliosides. In order to identify ganglioside expression, frozen sections of kidneys were stained with monoclonal antibodies GMB16 (GM1 specific), GM28 (GM2 specific), AMR-10 (GM4 specific) and CDW 60 (9-O-Ac-GD3 specific). Strong reactivity was observed for GMB28, AMR-10 and CDW 60, while GMB16 developed only weak labelling in treated kidney compared with the control. The alterations in the expression of renal gangliosides observed by immunohistochemistry were accompanied by quantitative and qualitative changes in the thin layer chromatography of total gangliosides isolated from kidney tissues. Lead treatment produced a significant increase in 9-O-Ac GD3, a ganglioside involved in apoptotic processes. In agreement with this result, a significant decrease in the number of apoptotic glomerular cells was observed with the TUNEL assay. These findings lead us to suggest that alterations in renal gangliosides produced by low level lead exposure are associated with the apoptotic processes that take place in the kidney.

These findings provide evidence that low level and long term lead exposure produces renal ganglioside alterations with urinary microalbumin excretion. The results suggest that lead levels within the limits of biological tolerance already cause molecular renal damage without clinical signs of toxicity. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: lead; intoxication; kidney; rats; gangliosides; apoptosis

Introduction

Lead is one of the most common toxic metals present in the atmosphere and exposure to it is still a major medical problem in both environmental and occupational settings. In conditions of low level and long term lead exposure, potentially adverse health effects have been observed in both humans and experimental animals (WHO, 1987; Goyer, 1971; Bressler and Goldstein, 1991).

The central nervous system, liver and kidneys have been proved to be the main targets of lead toxicity, which causes important pathological changes (Beck, 1992; Goyer, 1992) and functional abnormalities such as cognitive and behavioural outcome, gastrointestinal toxicity and chronic renal failure.

Kidneys are quite vulnerable to toxic injury because they are exposed directly to the blood plasma via their open fenestrae (Kleinman *et al*., 1986). However, lead nephropathy is rarely considered in the differential diagnosis of chronic renal disease.

A previous work demonstrated that chronic lead exposure produces changes in the expression of extracellular proteins such as laminin-1 and fibronectin, in rat kidney, together with ultrastructural modifications in the glomerular basement membrane (Sanchez *et al*., 2001).

These early molecular alterations might induce flaws in the assembly and molecular architecture of the glomerular basement membrane, thus affecting the filtration process.

Among the constituents of the plasma membrane, gangliosides play a significant role as modulators of cellular responses in glomerular functions (Hakomori and Igarashi, 1993; Zeller and Marchese, 1992). These molecules are composed of a hydrophilic sialic acid containing oligosaccharide chain and a ceramide hydrophobic tail. They are inserted into the outer leaflet of the plasma membrane through their ceramide moiety (van Echten and Sandhoff, 1993). Gangliosides can act as receptors for a variety of molecules and have been shown to take part in cell–cell interactions, cell adhesion, recognition and signal transduction (Hakamori and Igarashi, 1995).

Individual tissues and cell types show different ganglioside patterns that change in response to variations in cellular morphology and function (Koizumi *et al*., 1988; Iwamori *et al*., 1984). Gangliosides are present in kidney tissue (Shayman and Radin, 1991) and have been characterized in various animal species (Karlsson *et al*.,

* Correspondence to: Sara Sánchez, Departamento de Biología del Desarrollo, INSIBIO, (CONICET-UNT), Chacabuco 461, 4000 San Miguel de Tucumán. Argentina.
E-mail: ssanchez@fbqf.unt.edu.ar
Contract/grant sponsor: CONICET; contract/grant number: CIUNT.

1973; Gasa and Makita, 1980; Tomono *et al*., 1984). The adult rat kidney contains major ganglioside species of GM3 and GD3 and minor components including GM4, GM2 and GD1a (Saito and Sugiyma, 2000a; Tadano-Aritomi *et al*., 1998; Saito and Sugiyama, 2001). The O-acetyl-derivative of GD3 has been shown to be concentrated in the glomerular region of adult rat kidney. This restricted localization suggests its possible role in specific kidney functions (Tadano and Ishizuka, 1981).

Although the composition and topological distribution changes of renal gangliosides are well documented (Spiegel *et al*., 1988), there are few published reports concerning changes under pathological conditions (Zhang and Kiechle, 2004).

Altered kidney ganglioside patterns have been reported in diabetes. This alteration in ganglioside expression was believed to be implicated in the development of glomerular hypertrophy caused by a hyperglycaemic condition (Kwak *et al*., 2003; Masson *et al*., 2005). Renal cell carcinomas display increased levels of GD1a, GM1 and GM2 compared with normal kidney cells (Saito *et al*., 2000). Higher ganglioside levels are correlated with the potential degree of metastasis (Saito *et al*., 1991; Ritter and Livingston, 1991). Nevertheless, information concerning the role of renal gangliosides during lead intoxication is lacking.

Assuming the importance of lead from an occupational as well as an environmental viewpoint, this study was undertaken to examine possible alterations in the composition and expression of renal gangliosides and to determine whether such alterations could represent one of the earliest changes associated with lead nephropathy.

Materials and Methods

Experimental Animals

An animal model of chronic lead toxicity *in vivo* was applied. The study was performed of adult male Wistar rats weighing ca. 210 g at the start of the treatment. They were divided into an experimental and a control group, each consisting of 10 animals. The animals were housed in individual cages. All of them were fed a standard diet. Animals in the experimental group were given 0.06% lead acetate in their drinking water for 4 months, whereas the controls received ordinary tap water. Each animal drank 25–30 ml of water per day. One day before being killed, the rats were individually placed in metabolic cages during the day and 24 h urine samples were collected. These samples were allowed to stand at 4 °C in the dark until analysis.

At the end of the experimental period the animals were weighed and killed with an overdose of ether and their kidneys were perfused *in situ* with 150 ml of cold PBS (100 mM, pH 7.4). The perfusion was carried out in antegrade direction (portal to cava vein). Kidneys were removed, weighed and observed macroscopically and then analysed. Experiments were performed three times with similar results.

Blood Samples

Blood was collected by cardiac puncture in heparinized tubes for lead analysis. Lead was estimated in the whole blood using an atomic absorption spectrophotometer Perkin Elmer A Analyst 600 with a graphite furnace.

Blood for haematological studies was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. The blood haemoglobin level (g dl^{-1}) was measured by optical density at 540 nm using Drabkin's (cyanmethaemoglobin) reagent. Other red blood cell indices were measured according to standard procedures using a Cell-DYN 3700 (Abbott) Haematology Analyser.

Blood for clinical chemistry studies was collected into tubes without an anticoagulant, allowed to clot and centrifuged to obtain serum. An Alcyon Analyser ISE (Abbott) and Axsym™ System (Abbott) were employed to measure creatinine and blood urea nitrogen.

Urinary Protein

Urine samples collected for 24 h were kept at 4 °C. Then they were centrifuged and used for microalbuminuria determination. The DCA 2000 microalbumin/creatinine reagent kit was used for the quantitative determination of albumin in urine (DCA 2000 Analyser, Bayer).

Urinary Delta-aminolevulinic Acid

Urinary delta-aminolevulinic acid (d-ALA) was estimated in urine samples according to Tomakunik and Ogata (1973). Delta-amino-levulinic acid reacted with acetylacetone and formed a pyrrole substance which reacted with dimethylaminobenzoese aldehyde. The coloured complex was measured spectrophotometrically at 553 nm using a Gilford Stasar III spectrophotometer. The results were expressed as mg g^{-1} creatinine.

Preparation and Chromatographic Separation of Gangliosides

Total glycosphingolipids were purified from the perfused kidneys of lead treated rats and normal control animals as described by Daniotti *et al*. 1991. Kidney samples were washed in cold PBS solution and the surrounding connective tissues were removed and immediately homogenized with an UltraTurrax homogenizer. Total lipids were

DOI: 10.1002/jat

extracted twice with 4 volumes of chloroform : methanol (C : M) (1 : 2; v/v) and the residue was re-extracted with C : M (2 : 1; v/v). The combined extracts were dried, resuspended in C : M : water (60 : 30 : 4.5; v/v) and desalted with a Sephadex G-25 column equilibrated with the same solvent mixture. The eluates were partitioned according to Folch *et al*. (1957) and the upper phase was completely dried, resuspended in C : M (4 : 1; v/v) and passed through a silicic acid column (Sigma Chemical Co., St Louis, MO, USA) equilibrated with chloroform. The column was washed with C : M (2 : 3; v/v). The eluate was dried and samples containing 20 nmol ganglioside sialic acid were resuspended in 15 µl C : M (2 : 3; v/v) and spotted on silica gel high performance thin layer chromatography (HPTLC) plates (HPTLC Silicagel 60 F 254, Merck, Darmstadt, Germany). Chromatograms were developed with C : M : $CaCl_2$ 0.25% (50 : 40 : 8.5 w/v) solvent systems. Gangliosides were visualized with resorcinol–HCl reagent. Neuroaminic acid bound to lipid was determined by the resorcinol method (Svennerholm, 1963). Densitometric analyses were performed as previously described Daniotti *et al*. (1991) Gangliosides were abbreviated according to Svennerholm (1963).

Tissue Processing for Immunohistochemistry

For indirect immunohistochemical staining, kidney tissue fixed overnight in 4% formaldehyde solution was cryoprotected with 30% sucrose solution and then quick-frozen and sectioned (6–8 µm) on a Bright 3020 cryostat. Sections were mounted on Histogrip-coated glass slides (Zimed Laboratories, Inc., San Francisco, CA) and allowed to dry for 30 min at 37 °C. Slides were stored at –20 °C before use.

Antibodies

The monoclonal antibodies used in this study, Mab GMB16, Mab GMB 28, Mab AMR-10 were kindly provided by Dr Tadashi Tai, Tokyo (Kotani *et al*., 1992); and CDW60 by Dr Bernhard Kniep, Germany (Kniep *et al*., 1992). These Mabs reacted strongly with the gangliosides GM1, GM2, GM4 and 9-O-Ac GD3, showing highly restricted binding specificities. Anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM)-FITC conjugate (antibodies developed in goats) were obtained from Sigma.

Indirect Immunofluorescence Microscopy

Serial kidney cryosections were hydrated with 5 min rinses with PBS. Then they were incubated with 3% bovine serum albumin (BSA)-PBS for 1 h at room temperature to prevent non-specific background staining. After blocking, they were treated for 2 h at room temperature with a 1 : 100 dilution of monoclonal antibody GMB16, GMB28, AMR-10 or CDW60. After rinsing with PBS, the sections were incubated with a 1 : 500 dilution of anti-mouse polyvalent immunoglobulin-FITC conjugate for 2 h at room temperature in the dark. The reaction was stopped by rinsing with distilled water. Then slides were mounted in Mowiol (Hoechst Verkauf Lackrohstoffe, Frankfurt, Germany) and observed with a Nikon Fluophot microscope with an appropriate FICT fluorescence. Control experiments without the primary antibodies were carried out as well.

In Situ Detection of Apoptotic Cells

Kidney cell apoptosis was measured in paraffin sections using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) method (Promega, Madison, WI, USA).

Statistical Analysis

The results are expressed as mean ± SE. Statistical differences were determined using Student's unpaired *t*-test. Differences were considered significant at $P < 0.05$.

Results

Blood Lead Concentration

After preliminary assays with different doses of lead acetate (0.01%, 0.06% and 0.1%), 0.06% was chosen on the basis that it caused blood lead levels to reach values within biological tolerance values.

Statistical analyses of data from blood lead concentration revealed that the animals that received 0.06% lead acetate in the drinking water for 4 months presented higher blood lead concentration levels than those of the controls (35.90 ± 11.80 vs 2.12 ± 0.71 µ dl^{-1}, $P < 0.001$).

Survival, General Condition and Diet Intake

Continuous lead exposure for 4 months did not cause mortality in rats. During the experimental period the animals appeared normal in their cage.

Individual observation outside the cage showed a fairly good general condition in lead treated animals, with no adverse symptoms that could be associated with abnormal autonomic central activity and behaviour.

Body weight gain for the treated group was less than that of the control group throughout the study. At the end of the experimental period, the body weight of treated

DOI: 10.1002/jat

rats was significantly different with respect to the control rats (323.0 ± 10.9 g vs 408.0 ± 15.2 g, $P < 0.05$).

However, no significant change in the kidney/body weight ratio was observed in lead treated rats compared with the controls. Macroscopic kidney examination revealed no alterations attributable to lead administration.

In the present study, lead administration caused no effect on daily standard rodent diet consumption compared with the control group (18.2 ± 0.3 vs 18.7 ± 0.2 g).

Biochemical Parameters

No significant changes in haematological parameters were noticed at the end of the experimental period in treated rats compared with the control group (Table 1). However, chronic lead exposure caused a significant increase in urinary d-ALA acid compared with the control group (1.55 ± 0.82 vs 0.48 ± 0.04 mg g^{-1}, $P < 0.05$).

Lead administration had no effect on chemical parameters such as serum creatinine and blood urea nitrogen. However, the addition of lead acetate to the drinking water caused microalbuminuria, characterized by a urinary albumin excretion of 26.5 ± 5.4 mg l^{-1} against 11.4 ± 1.5 mg l^{-1} ($P < 0.001$) for the control rats.

In agreement with Weir (2004), it is considered that the detection of microalbumin in the urine is an early signal of the onset of progressive renal disease.

Immunolocalization of Gangliosides in Kidney

The expression of different gangliosides in rat kidneys was studied using an immunofluorescence technique.

Figure 1 shows the immunostaining of GM1 ganglioside in sections of kidney using mouse MAb GMB16 antibody. The labelling was found mainly in the renal tubule. The relative intensity of the staining was lower in the kidney of lead treated rats (Fig. 1A) than in the controls (Fig. 1B). The distribution of the GM2 ganglioside was detected using mouse MAb GMB28 antibody. Strong reactivity was observed in the renal tubules and glomeruli of treated rats (Fig. 2A), whereas normal kidney tissue showed very weak staining (Fig. 2B).

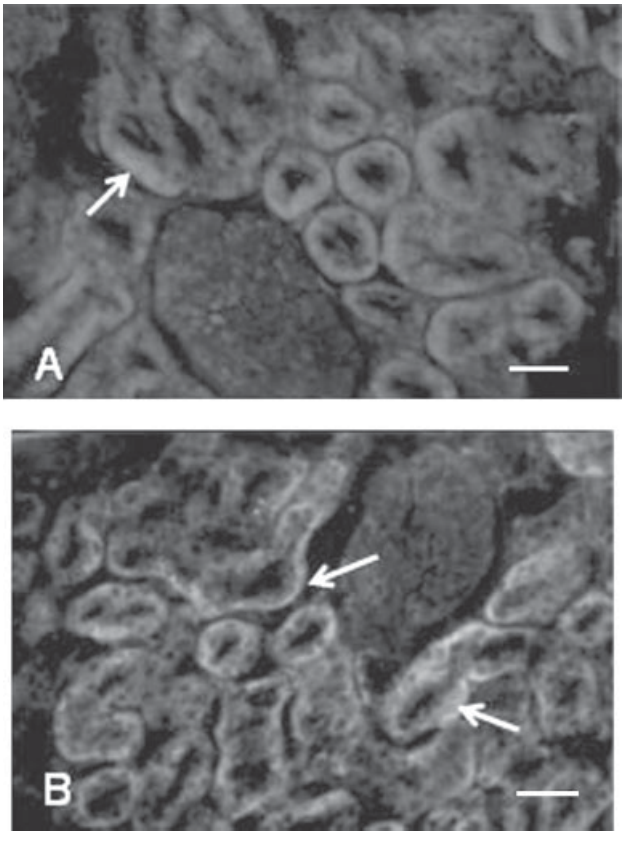

Figure 1. Immunofluorescence analysis of ganglioside GM1 expression in kidney tissue. Frozen sections of lead treated kidney (A) and control kidney (B) were immunostained using monoclonal antibody GMB16 and FITC labelled goat antimouse immunoglobulin antibody. Bar: 50 μm

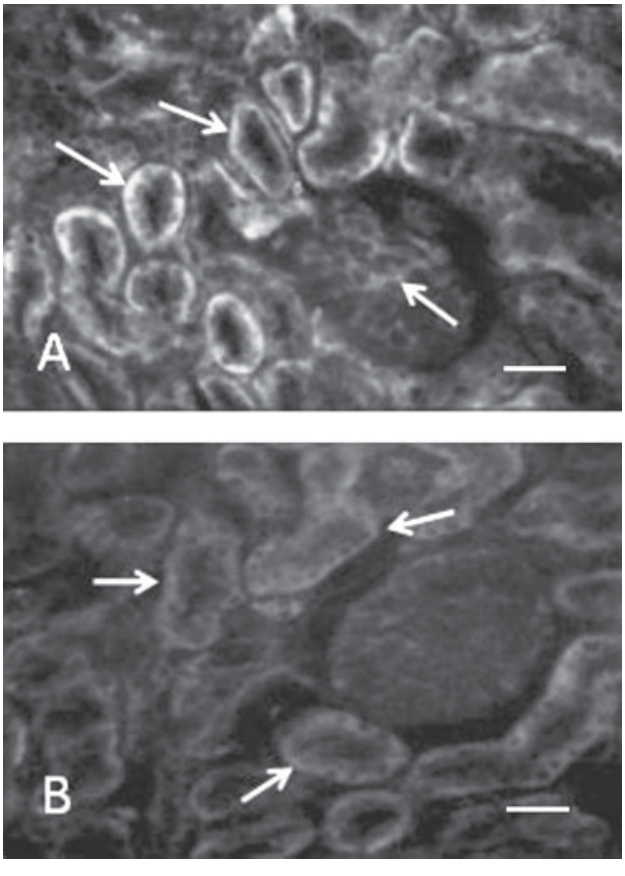

Figure 2. Immunofluorescence analysis of ganglioside GM2 expression in kidney tissue. Frozen sections of lead treated kidney (A) and control kidney (B) were immunostained using monoclonal antibody GMB28 and FITC labelled goat antimouse immunoglobulin antibody. Bar: 50 μm

DOI: 10.1002/jat

Table 1. Characteristics and biochemical parameters of study animals

	Lead treated rats	Control rats
Body weight (g)	323.0 ± 10.9[a]	408.0 ± 15.2
Kidney weight (g)	1.63 ± 0.13 NS	1.90 ± 0.10
Kidney/body weight	$4.7\ 10^{-3} \pm 5.010^{-4}$ NS	$4.0\ 10^{-3} \pm 6.0\ 10^{-4}$
Blood lead (μg dl^{-1})	35.90 ± 11.80[b]	2.12 ± 0.71
Urinary d-ALA (mg g^{-1} creatinine)	1.55 ± 0.82[a]	0.48 ± 0.04
Creatinine (mg l^{-1})	7.00 ± 0.65 NS	6.87 ± 1.51
Blood urea nitrogen (g l^{-1})	0.47 ± 0.14 NS	0.46 ± 0.10
Urine volume (ml 24 h)	10.8 ± 2.9 NS	11.5 ± 2.4
Urinary microalbumin (mg 24 h)	26.5 ± 5.4[b]	11.4 ± 1.5
Haematocrit (%)	78.0 ± 2.3 NS	78.7 ± 1.5
Haemoglobin (g dl^{-1})	15.2 ± 0.3 NS	15.2 ± 0.6
Apoptotic cells (%) – TUNEL	3.5 ± 0.3[b]	7.0 ± 0.2

Values are the mean ± SEM from 10 rats. Statistical analyses were performed using Student's *t*-test. NS, not significant; [a] $P < 0.05$; [b] $P < 0.001$.

The study also examined whether any changes in the expression of GM4 gangliosides occurred in the kidney of the studied animals using the specific antibody AMR-10. Chronic lead administration led to a significant increase in the expression of the GM4 ganglioside, mainly in the glomeruli, and tubules exhibited a diffuse and discontinuous staining (Fig. 3A), in contrast with the weak and homogeneous staining label of the control animals (Fig. 3B).

CDW60 MAb is a monoclonal antibody that specifically recognizes 9-O-acetyl-GD3 ganglioside. Figure 4 shows the immunostaining of this ganglioside in kidney

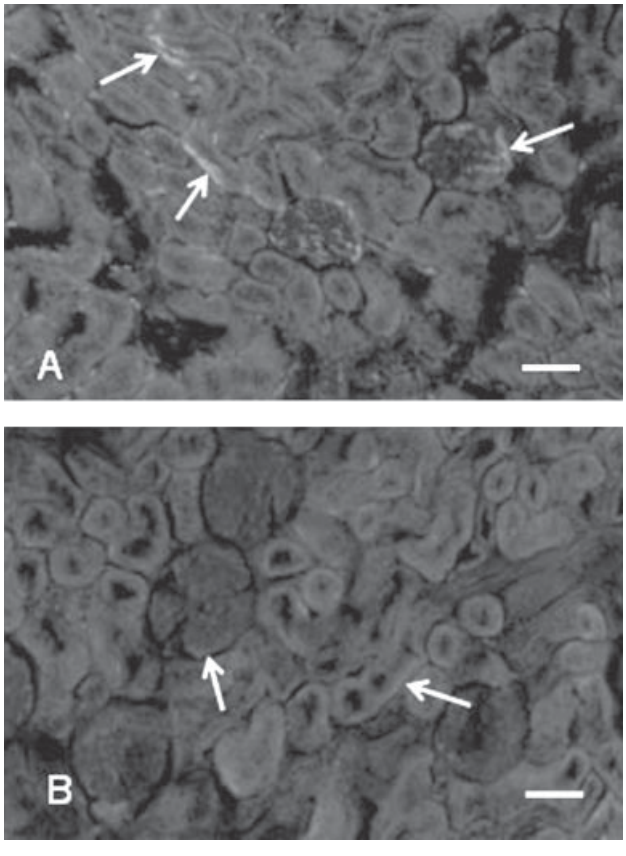

Figure 3. Immunofluorescence analysis of ganglioside GM4 expression in kidney tissue. Frozen sections of lead treated kidney (A) and control kidney (B) were immunostained using monoclonal antibody AMR10 and FITC labelled goat antimouse immunoglobulin antibody. Bar: 50 μm

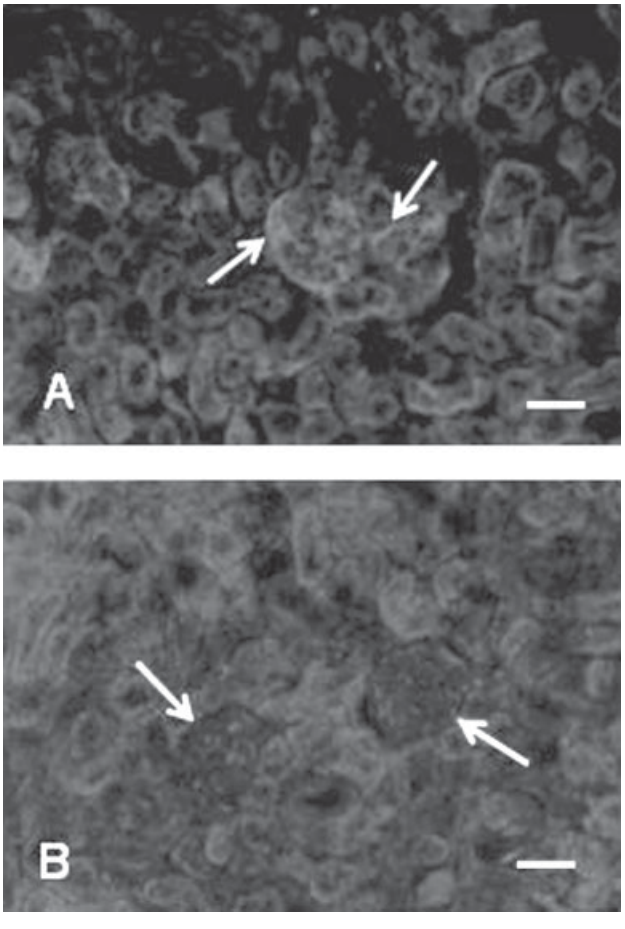

Figure 4. Immunofluorescence analysis of ganglioside 9-O-Ac-GD3 expression in kidney tissue. Frozen sections of lead treated kidney (A) and control kidney (B) were immunostained using monoclonal antibody CDW60 and FITC labelled goat antimouse immunoglobulin antibody. Bar: 50 μm

DOI: 10.1002/jat

Table 2. Gangliosides composition of lead treated and control rat kidneys

Gangliosides	Lead treated rats (%)	Control rats (%)
GM4	15.0 ± 0.5[a]	9.7 ± 2.1
GM3	15.6 ± 0.1 NS	15.8 ± 0.6
GM2	14.7 ± 0.5[a]	13.0 ± 0.5
GM1	9.3 ± 1.7[b]	17.6 ± 2.1
GD1a	15.0 ± 0.4[b]	12.9 ± 0.3
GD3	14.30 ± 0.03[b]	16.50 ± 0.08
9-O-Ac GD3	16.1 ± 0.4[b]	13.9 ± 0.1

The values represent the relative percentage of each ganglioside, determined by densitometric scanning. Statistical analyses were performed using Student's *t*-test. Data are an average of three separate experiments (mean ± SEM). NS, not significant; [a] $P < 0.05$; [b] $P < 0.005$.

sections from lead-treated and control rats. In all kidneys analysed, labelling was found only in the glomerular zone. Strong reactivity was seen in the glomeruli of treated rats (Fig. 4A), whereas normal kidneys showed very weak staining (Fig. 4B). This finding would indicate that chronic lead intoxication leads to a significant increase in the glomerular 9-O-acetylation of GD3.

Ganglioside TLC Analysis

Gangliosides extracted from kidneys rats were analysed by TLC chromatographic technique. As shown in Figure 5, the pattern of control (lane Ct) and treated kidney rats (lane Pb) revealed mainly the following ganglioside spots, defined according to their relative mobility. These TLC profiles showed quantitative differences when comparing treated and control animals. Table 2 shows the densitometric analysis of the TLC gangliosides profiles presented in Figure 5, demonstrating a significant decrease in the amount of GM1 and GD3 ganglioside, and an increase in GM4 and GD1a ganglioside in the kidney of lead treated rats compared with the controls. In contrast, only a slight increase in the GM2 ganglioside expression was detected in the treated rats. GM3 ganglioside was not affect by lead treatment, while the amount of 9-O-Ac-GD3 was strongly increased compared with the control rats.

Apoptosis Detection

In order to examine the possibility that chronic lead intoxication might be involved in apoptotic events, the TdT mediated dUTP Nick End Labelling (TUNEL) assay was used. The advantage of the TUNEL method is that it allows one to directly visualize cells exhibiting DNA fragmentation in tissue sections. Figure 6 shows apoptotic cells in treated (Fig. 6A, B) and normal kidneys (Fig. 6C, D).

Apoptotic cells were shown by TUNEL and were quantified by counting 1000 cells in at least ten microscopic fields.

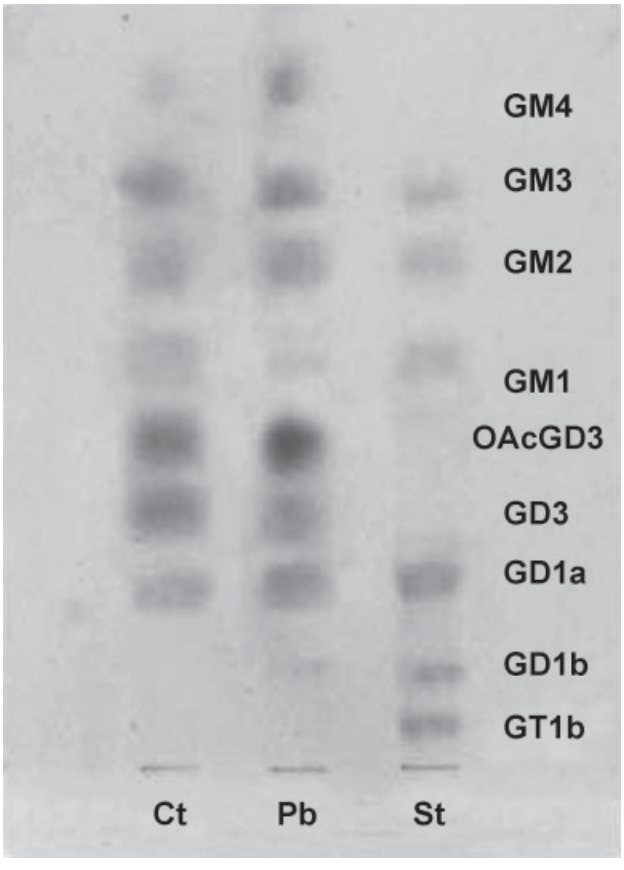

Figure 5. High performance thin layer chromatogram. HPTLC of total gangliosides obtained from lead treated kidney (Pb), normal kidney (Ct) and reference standard (St) was developed in solvent system of C : M : $CaCl_2$ 0.25% (50 : 40 : 8.5) and visualized with resorcinol : HCl reagent. The amount of gangliosides spotted in each lane is equivalent to 20 nmol ganglioside sialic acid. The profile presented is representative of three experiments

A statistically significant decrease in the number of apoptotic glomerular cells was observed in the kidney of treated rats compared with the control animals (3.5 ± 0.3 vs 7.0 ± 0.2%, $P < 0.001$).

DOI: 10.1002/jat

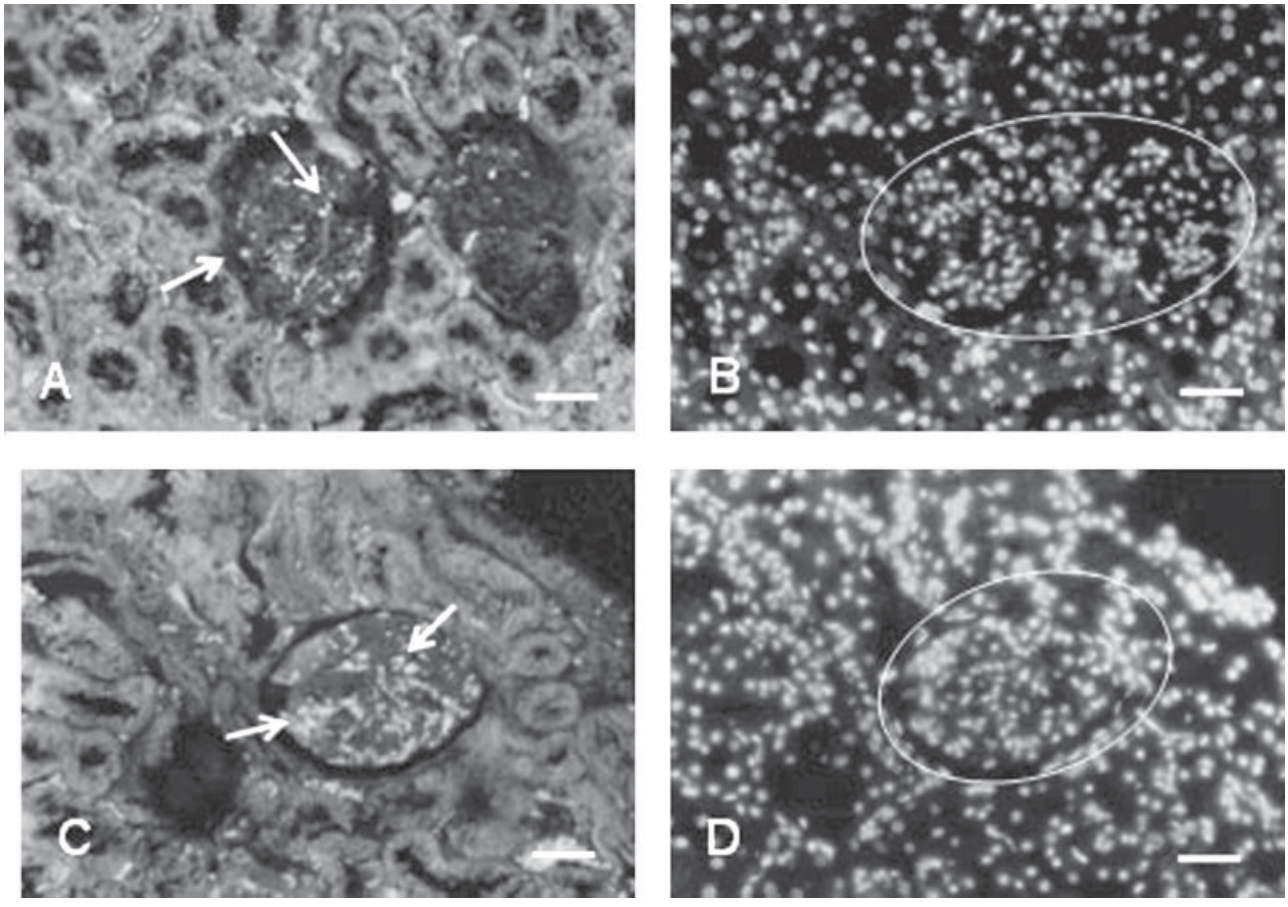

Figure 6. TUNEL analysis of renal apoptotic processes. (A–C) Apoptotic nuclei staining by fluorescein-12 dUTP at the 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT). (B–D) Cells stained by 4′-6-diamidino-2-phenylindole (DAPI). DAPI stains both apoptotic and non-apoptotic cells. (A, B) Lead treated rats; (C, D) Control rats. Bar: 25 μm

Discussion

The toxic effects of lead have been studied for many years, but the cellular targets of action are not completely established (Oberley *et al*., 1995; Stohs and Bagchi, 1995; Van Den Heuvel *et al*., 2001). Nephropathy due to chronic exposure to lead is manifested by dysfunction of renal tubules, which can lead to chronic renal failure (Nolan and Shaikh, 1992). A previous work demonstrated that chronic lead exposure caused changes in the extracellular protein expression and ultrastructural modifications such as a decrease in the thickness of the glomerular basal membrane in rat kidney (Sanchez *et al*., 2001).

In order to increase understanding of molecular alterations produced by lead, the studies focused on gangliosides. These molecules are believed to be integral for the dynamics of many cell membrane events, including cellular interactions, signalling and trafficking.

The present study demonstrated characteristic changes in ganglioside patterns on TLC in the rat kidney after chronic lead intoxication at low exposure levels. The ganglioside pattern was mainly characterized by a decrease in the GM1 gangliosides as well as by a mild increase in GM4 and GM2 gangliosides, but the strongest alteration for ganglioside expression was observed in the 9-O-acetylated modified form of the GD3 ganglioside, which is over expressed.

Anti-ganglioside antibodies have become a useful tool for localizing gangliosides in the cell (Schwarz and Futerman, 1997, 2000). MAbs GM 16, GMB 28 and AMR 10 showed highly restricted binding specificities, reacting only with gangliosides used as immunogens, GM1, GM2 and GM4, respectively. The monoclonal antibody raised against GM1 was capable of staining the kidneys at the tubular level, whereas the anti GM2 and anti GM4 antibodies were detected at the tubules and glomeruli. The intensity of the labelling varied according to the gangliosides analysed. MAb GMB 28 and AMR 10, which specifically recognize GM2 and GM4 gangliosides, respectively, showed strong reactivity, whereas Mab GM16 (GM1-specific) developed very weak labelling.

DOI: 10.1002/jat

The immunostaining is in agreement with the results obtained through TLC.

GD3 is a minor ganglioside in most normal tissues (Sugiyama and Saito, 1999; Saito and Sugiyama, 2002) that is highly expressed only during development and in pathological conditions such as cancer and neurodegenerative disorders (Prokazova and Bergelson, 1994; Saito and Sugiyama, 2000b; Mennel *et al*., 2000).

In mammalian cells, the intracellular accumulation of ganglioside GD3 contributes to mitochondrial damage, a crucial event during the apoptotic process (De Maria *et al*., 1997; Melchiorri *et al*., 2002). GD3 recruits mitochondria to the apoptotic programme and the relevant GD3 targets are under bcl-2 control (Rippo *et al*., 2000). The results revealed that chronic lead exposure did not affect the amount of GD3, as shown in the TLC. However, in our experimental conditions, a marked increase in 9-O-acetyl-GD3 expression was observed only in the glomerular zone. GD3 contains two sialic acid residues sequentially attached to the galactose residues. The most common post-synthetic modification of GD3 is O-acetylation at the C9 position of its terminal sialic acid (Klein and Roussel, 1998; Malisan *et al*., 2002). O-acetylation does not change the charges of the gangliosides but diminishes their polarity, and thus changes their TLC mobility. The results demonstrated that lead administration increased the intensity of a band corresponding to GD3 with O-acetyl groups, enhancing its TLC mobility. This modification could affect the proapoptotic activity of GD3, which would be an example of the gain/loss of the proapoptotic function regulated by acetylation in a lipid mediator (Malisan *et al*., 2002). This study examined the apoptotic activity of the renal tissue using the TUNEL assay. It was demonstrated that lead treatment caused a significant decrease in the number of apoptotic glomerular cells together with an increase in 9-O-acetyl GD3.

On the basis of these findings, it is believed that the increase in GD3-O-acetylation could represent a strategy to attenuate the normal renal apoptotic process and could therefore contribute to cell survival during lead exposure.

Renal ganglioside expression patterns have been studied and characterized in several animal species (Saito and Sugiyama, 2000a; Tadano and Ishizuka, 1981; Spiegel *et al*., 1988; Shayman and Radin, 1991). However, there is little information concerning the expression of these molecules and their participation in pathological processes (Tsuboi *et al*., 2003; Hakomori and Handa, 2002; Iwabuchi *et al*., 1998). As far as we know, there is only evidence that cisplatin, an antineoplasic and nephrotoxic agent, causes modification of gangliosides in the lung and breast (Basu *et al*., 2004; Yoshida *et al*., 2002; Kiura *et al*., 1998). This is the first work that reports studies of the expression of renal gangliosides in chronic experimental lead intoxication. The fact that a differential distribution of gangliosides was observed within the nephron led us to suggest that the increase or decrease in the amount of a given ganglioside might indicate the degree of kidney compromise during lead intoxication. An important fact to be borne in mind is that the changes in the expression of gangliosides occur at low levels of lead exposure and with very low blood lead concentration levels.

The fact that the urinary delta-ALA was increased in the treated rats indicates that chronic administration of low levels of lead in our experimental model produced a certain degree of inhibition in the enzymes in the heme synthesis pathway. Interestingly, anaemia is not apparent; although no enzymatic inhibitory assay was carried out, it is believed that a good candidate could be the cytosolic enzyme δ-aminolevulinic acid dehydratase, which is readily inhibited by lead (Tomokuni *et al*., 1993).

The slight increase in urinary albumin excretion found in our treated animals was probably due to an isolated defect in charge selectivity. Gangliosides are involved in the maintenance of the charge selective filtration barrier of glomeruli (Simons *et al*., 2001) due to their electronegative charge. Consequently, the changes in renal gangliosides in treated animals could explain the presence of microalbuminuria, which is considered an early sign of renal alteration (Weir, 2004).

In this experimental model, microalbuminuria was found to be an early marker of renal alteration. This fact agrees with the works of Assadi (2005) and Abid *et al*. (2001) who have reported microalbuminuria as a predictor of renal disease. However, no changes were found in blood urea nitrogen or serum creatinine levels, although these biochemical indicators are used in occupational lead poisoning studies (Staessen *et al*., 1992).

Traditionally, lead poisoning was recognized when classical symptoms were present and blood lead levels were high. However, based on our findings, lead nephropathy already occurs in the absence of symptoms or with minimal biochemical changes at the cellular and molecular levels.

Acknowledgements—This research was supported by CONICET and CIUNT grants to Sara Sánchez. We wish to thank Virginia Méndez for proofreading.

References

Abid O, Sun Q, Sugimoto K, Mercan D, Vincente JL. 2001. Predictive value of microalbuminuria in medical ICU patients: results of a pilot study. *Chest* **120**: 984–988.

Assadi FK. 2005. Value of urinary excretion of microalbumin in predicting glomerular lesions in children with isolated microscopic hematuria. *Pediatr. Nephrol.* **20**: 1131–1135.

Basu S, Ma R, Boyle PJ, Mikulla B, Bradley M, Smith B, Basu M, Banerjee S. 2004. Apoptosis of human carcinoma cells in the presence of potential anti-cancer drugs: III. Treatment of Colo-205 and SKBR3 cells with: cisplastin, tomoxifen, melphalan, betulinic acid, L-PDMPO, L-PPMP and GD3 ganglioside. *Glycoconj. J.* **20**: 536–577.

Beck BD. 1992. An update on exposure and effects of lead. *Fundam. Appl. Toxicol*. **18**: 1–16.

Bressler JP, Goldstein GW. 1991. Mechanisms of lead neurotoxicity. *Biochem. Pharmacol*. **41**: 479–484.

Daniotti JL, Landa CA, Rosner H, Maccioni H. 1991. GD3 prevalence in adult rat retina correlates with the maintenance of a high GD3/GM2 synthase activity ratio through development. *J. Neurochem*. **57**: 2054–2058.

De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, Rippo MR, Testi R. 1997. Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. *Science* **277**: 1652–1655.

Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation of total lipides from animal tissues. *J. Biol. Chem*. **226**: 497–509.

Gasa S, Makita A. 1980. Characterization of gangliosides from equine kidney and spleen. *J. Biochem. (Tokyo)* **88**: 1119–1128.

Goyer RA. 1971. Lead and the kidney. *Curr. Top. Pathol*. **55**: 147–176.

Goyer RA. 1992. Nephrotoxicity and carcinogenicity of lead. *Fundam. Appl. Toxicol*. **18**: 4–7.

Hakomori S, Handa K. 2002. Glycosphingolipid-dependent cross-talk between glycosynapses interfacing tumor cells with their host cells: essential basis to define tumor malignancy. *FEBS Lett*. **531**: 88–92.

Hakomori S, Igarashi Y. 1993. Gangliosides and glycosphingolipids as modulators of cell growth, adhesion and transmembrane signaling. *Adv. Lipid Res*. **25**: 147–162.

Hakomori S, Igarashi Y. 1995. Functional role of glycosphingolipids in cell recognition and signalling. *J. Biochem. (Tokyo)* **118**: 1091–1103.

Iwabuchi K, Yamamura S, Prinetti A, Handa K, Hakomori S. 1998. GM3-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate–carbohydrate interaction in mouse melanoma B16 cells. *J. Biol. Chem*. **273**: 9130–9138.

Iwamori M, Shimomura J, Tsuyuhara S, Nagai Y. 1984. Gangliosides of various rat tissues: distribution of ganglio-N-tetraose-containing gangliosides and tissues characteristic composition of gangliosides. *J. Biochem. (Tokyo)* **95**: 761–770.

Karlsson KA, Samuelsson BE, Steen GO. 1973. The sphingolipid composition of bovine kidney cortex, medulla and papilla. *Biochim. Biophys. Acta* **316**: 317–335.

Kiura K, Watarai S, Ueoka H, Tabata M, Gemba K, Aoe K, Yamane H, Yasuda T, Harada M. 1998. An alteration of ganglioside composition in cisplatin-resistant lung cancer cell line. *Anticancer Res*. **18**: 2957–2960.

Klein A, Roussel P. 1998. O-acetylation of sialic acids. *Biochimie* **80**: 49–57.

Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR. 1986. Basement membrane complexes with biological activity. *Biochemistry* **25**: 312–318.

Kniep B, Peter-Katalinic J, Flegel W, Northoff H, Rieber EP. 1992. CDW60 antibodies bind to acetylated forms of ganglioside GD3. *Biochem. Biophys. Res. Commun*. **187**: 1343–1349.

Koizumi N, Hara A, Vemura K, Taketomi T. 1988. Glycosphingolipids in sheep liver, kidney and various blood cells. *J. Exp. Med*. **59**: 21–31.

Kotani M, Ozawa H, Kawashima I, Ando S, Tai T. 1992. Generation of one set of monoclonal antibodies specific for a-pathway ganglio-series gangliosides. *Biochim. Biophys. Acta* **1117**: 97–103.

Kwak DH, Rho YI, Kwon OD, Ahan SH, Song JH, Choo YK, Kim SJ, Choi BK, Jng KY. 2003. Decrease of ganglioside GM3 in streptozotocin induced diabetic glomeruli of rats. *Life Sci*. **72**: 1997–2006.

Malisan F, Franchi L, Tomassini B, Ventura N, Condo I, Rippo MR, Rufini A, Liberati L, Nachtigall C, Kniep B, Testi R. 2002. Acetylation suppresses the proapoptotic activity of GD3 ganglioside. *J. Exp. Med*. **196**: 1535–1541.

Masson E, Wiernsperger N, Lagarde M, El Bawad S. 2005. Glucosamine induces cell-cycle and hypertrophy of mesangial cells: implication of gangliosides. *Biochem. J*. **388**: 537–544.

Melchiorri D, Martini F, Lococo E, Gradini R, Barletta E, De Maria R, Caricasole A, Nicoletti F, Lenti L. 2002. An early increase in the disialoganglioside GD3 contributes to the development of neuronal apoptosis in culture. *Cell Death Differ*. **9**: 609–615.

Mennel HD, Bosslet K, Geissel H, Bauer BL. 2000. Immunohistochemically visualized localisation of gangliosides Glac2 (GD3) and Gtri2 (GD2) in cells of human intracranial tumors. *Exp. Toxicol. Pathol*. **52**: 277–285.

Nolan CV, Shaikh ZA. 1992. Lead nephrotoxicity and associated disorders: biochemical mechanisms. *Toxicology* **73**: 127–146.

Oberley T, Friedman AL, Moser R, Siegel F. 1995. Effect of lead administration of developing rat kidney. *Toxicol. Appl. Pharmacol*. **131**: 94–107.

Prokazova NV, Bergelson LD. 1994. Gangliosides and atherosclerosis. *Lipids* **29**: 1–5.

Rippo MR, Malisan F, Ravagnan L, Tomassini B, Condo I, Costantini P, Susin SA, Rufini A, Todaro M, Kroemer G, Testi R. 2000. GD3 ganglioside as an intracellular mediator of apoptosis. *Eur. Cytokine Netw*. **11**: 487–488.

Ritter G, Livingston PO. 1991. Ganglioside antigens expressed by human cancer cell. *Semin. Cancer Biol*. **2**: 401–409.

Saito S, Nojiri H, Satoh M, Ito A, Ohyama C, Orikasa S. 2000. Inverse relationship of expression between GM3 and globo-series gangliosides in human renal cell carcinoma. *Tohoku J. Exp. Med*. **190**: 271–278.

Saito S, Orikasa S, Ohyama C, Satoh M, Fukushi Y. 1991. Changes in glycolipids in human renal cell carcinoma and their clinical significance. *Int. J. Cancer* **49**: 329–334.

Saito M, Sugiyama K. 2000a. Tissue specific expression of c-series gangliosides in the extraneural system. *Biochim. Biophys. Acta* **1474**: 88–92.

Saito M, Sugiyama K. 2000b. Specific ganglioside changes in extraneural tissues of adult rats with hypothyroidism. *Biochim. Biophys. Acta* **1523**: 230–235.

Saito M, Sugiyama K. 2001. Gangliosides in rat kidney: composition, distribution and developmental changes. *Arch. Biochem. Biophys*. **386**: 11–16.

Saito M, Sugiyama K. 2002. Characterization of nuclear gangliosides in rat brain: concentration, composition, and developmental changes. *Arch. Biochem. Biophys*. **398**: 153–159.

Sánchez S, Pérez Aguilar R, Genta S, Aybar M, Villecco E, Sánchez Riera A. 2001. Renal extracellular matrix alterations in lead-treated rats. *J. Appl. Toxicol*. **21**: 417–423.

Schwarz A, Futerman AH. 1997. Determination of the localization of gangliosides using anti-ganglioside antibodies: comparison of fixation methods. *J. Histochem. Cytochem*. **45**: 611–618.

Schwarz A, Futerman AH. 2000. Immunolocalization of gangliosides by light microscopy using anti-ganglioside antibodies. *Methods Enzymol*. **312**: 179–187.

Shayman JA, Radin NS. 1991. Structure and function of renal glycosphingolipids. *Am. J. Physiol*. **260**: 286–302.

Simons M, Schwarz K, Kriz W, Miettinen A, Reiser J, Mundel P, Holthofer H. 2001. Involvement of lipid rafts in nephrin phosphorylation and organization of the glomerular slit diaphragm. *Am. J. Pathol*. **159**: 1069–1077.

Spiegel S, Matyas GR, Cheng L, Sacktor B. 1988. Asymmetric distribution of gangliosides in rat renal brush border and basolateral membranes. *Biochim. Biophys. Acta* **938**: 270–278.

Staessen JA, Lauwerys RR, Buchet JP, Bulpitt CJ, Rondia D, Vanrenterghem Y, Amery A. 1992. Impairment of renal function with increasing blood lead concentrations in the general population. The Cadmibel Study Group. *N. Engl. J. Med*. **327**: 151–156.

Stohs ST, Bagchi D. 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med*. **18**: 321–336.

Sugiyama K, Saito M. 1999. Growth- and development-dependent expression of gangliosides in rat hepatocytes and liver tissues. *Biol. Chem*. **380**: 413–418.

Svennerholm L. 1963. Chromatographic separation of human brain gangliosides. *J. Neurochem*. **10**: 613–623.

Tadano K, Ishizuka I. 1981. Isolation and partial characterization of a novel disulfoglycosphingolipid from rat kidney. *Biochem. Biophys. Res. Commun*. **103**: 1006–1013.

Tadano-Aritomi K, Kubo H, Ireland P, Hikita T, Ishizuka I. 1998. Isolation and characterization of a unique sulfated ganglioside, sulfated GM1a, from rat kidney. *Glycobiology* **8**: 341–350.

Tomakunik K, Ogata M. 1973. Simple method for determination of urinary delta amino levulinic acid measured by a new method. *Clin. Chim. Acta* **47**: 323–324.

Tomokuni K, Ichiba M, Fujishiro K. 1993. Interrelation between urinary delta-aminolevulinic acid (ALA), serum ALA, and blood lead in workers exposed to lead. *Ind. Health* **31**: 51–57.

DOI: 10.1002/jat

Tomono Y, Naito I, Watanabe K. 1984. Glycosphingolipid patterns of rat kidney. Dependence on age and sex. *Biochim. Biophys. Acta* **796**: 199–204.

Tsuboi N, Utsunomiya Y, Kawamura T, Kikuchi T, Hosoya T, Ohno T, Yamada H. 2003. Shedding of growth-suppressive gangliosides from glomerular mesangial cells undergoing apoptosis. *Kidney Int.* **63**: 936–946.

Van Den Heuvel RL, Leppens H, Schoeters GE. 2001. Use *in vitro* assay to assess hematotoxic effect of environmental components. *Cell Biol. Toxicol.* **17**: 107–116.

van Echten G, Sandhoff K. 1993. Ganglioside metabolism. Enzymology, topology and regulation. *J. Biol. Chem.* **68**: 5341–5344.

Weir MR. 2004. Microalbuminuria in type 2 diabetics: an important, overlooked cardiovascular risk factor. *J. Clin. Hypertens.* **6**: 134–141.

World Health Organization. 1987. *Air Quality Guidelines for Europe, European Series* No. 23. Vol 45–48. World Health Organization: Geneva; 242–261.

Yoshida S, Kawaguchi H, Sato S, Ueda R, Furukawa K. 2002. An anti-GD2 monoclonal antibody enhances apoptotic effects of anti-cancer drugs against small cell lung cancer cells via JNK (c-Jun terminal kinase) activation. *Jpn J. Cancer Res.* **93**: 816–824.

Zeller CB, Marchese RB. 1992. Gangliosides as modulators of cell function. *Am. J. Physiol.* **262**: 1341–1355.

Zhang X, Kiechle F. 2004. Glycosphingolipids in health and disease. *Ann. Clin. Lab. Sci.* **34**: 3–13.

DOI: 10.1002/jat