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Gentamicin-induced apoptosis in LLC-PK1 cells: Involvement of lysosomes and mitochondria

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Abstract

Gentamicin accumulates in lysosomes and induces apoptosis in kidney proximal tubules and renal cell lines. Using LLC-PK1 cells, we have examined the concentration- and time-dependency of the effects exerted by gentamicin (1–3 mM; 0–3 days) on (i) lysosomal stability; (ii) activation of mitochondrial pathway; (iii) occurrence of apoptosis (concentrations larger than 3 mM caused extensive necrosis as assessed by the measurement of lactate dehydrogenase release). Within 2 h, gentamicin induced a partial relocalization [from lysosomes to cytosol] of the weak organic base acridine orange. We thereafter observed (a) a loss of mitochondrial membrane potential (as from 10 h, based on spectrophotometric and confocal microscopy using JC1 probe) and (b) the release of cytochrome *c* from granules to cytosol, and the activation of caspase-9 (as from 12 h; evidenced by Western blot analysis). Increase in caspase-3 activity (assayed with Ac-DEVD-AFC in the presence of z-VAD-fmk]) and appearance of fragmented nuclei (DAPI staining) was then detected as from 16 to 24 h together with nuclear fragmentation. Gentamicin produces a fast (within 4 h) release of calcein from negatively-charged liposomes at pH 5.4, which was slowed down by raising the pH to 7.4, or when phosphatidylinositol was replaced by cardiolipin (to mimic the inner mitochondrial membrane). The present data provide temporal evidence that gentamicin causes apoptosis in LLC-PK1 with successive alteration of the permeability of lysosomes, triggering of the mitochondrial pathway, and activation of caspase-3.

Keywords: Apoptosis; Lysosomes; Aminoglycosides; Gentamicin; Mitochondria

Introduction

Since its original description at a cellular and functional level (Kosek et al., 1974), gentamicin-induced nephrotoxicity has been the subject of extensive studies that have unambiguously identified proximal tubular cells as the almost exclusive and specific site for drug accumulation and early signs of ultrastructural and histological alterations (see Gilbert, 2000; Mingeot-Leclercq and Tulkens, 1999 for reviews). Lysosomes are the first and most conspicuous site of accumulation of gentamicin in proximal tubules after in vivo administration (Giurgea-Marion et al., 1986; Silverblatt and Kuehn, 1979) as well as in cultured cells incubated with this antibiotic (Tulkens and Trouet, 1978). The relationship between this subcellular localization of gentamicin and the onset of subsequent cell alterations and toxicity is, however, still unknown. In this context, two major advances have been made over the last years. First, drug localization studies using morphological approaches showed that, besides lysosomes, gentamicin has also partially access to the Golgi complex from where it traffics to the endoplasmic reticulum, the cytosol, and the intermembrane space of mitochondria

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(Sandoval and Molitoris, 2004; Sandoval et al., 1998; Sundin et al., 2001). Second, apoptosis has been shown to occur early on in proximal tubules of animals treated with gentamicin under clinically-relevant conditions with respect to both dosages and treatment duration (El Mouedden et al., 2000a). This effect of gentamicin has been reproduced with both renal (LLC-PK1 and MDCK) and non-renal (fibroblasts) cell lines exposed to gentamicin, and was found to be directly related to the amount of drug accumulated by the cells (El Mouedden et al., 2000b).

Apoptosis is a highly regulated process that can be triggered by both exogenous and endogenous factors which can activate intracellular components such as caspases, AIF, cytochrome *c*, leading to the final condensation and fragmentation of the nucleus. Among the various subcellular organelles potentially involved in apoptosis, both lysosomes and mitochondria have been shown to send death signals through the activation of specific stress sensors (Ferri and Kroemer, 2001). For lysosomes, membrane rupture and release of acid hydrolases (as seen during oxidative stress or occurring through the action of oxidized lipids, lysosomal detergents, serum withdrawal, or Fas-ligation (Brunk et al., 1997; Guicciardi et al., 2000; Li et al., 2000; Neuzil, 2002; Yuan et al., 2002) appear critical. "Lysosomal pathway to apoptosis" is now an accepted terminology to describe this mechanism which seems to have been primarily identified in pathological situations (Guicciardi et al., 2004). For mitochondria, two main events leading to apoptosis are the disruption of the transmembrane potential (Lemasters et al., 1998) and the release of cytochrome *c* (Kluck et al., 1997; Zamzami et al., 1995), which contribute to formation of the so-called apoptosome leading to the successive activation of caspase-9 and of the excecutioner caspase-3.

In the present study, we have examined if gentamicin may alter lysosomal stability and trigger the mitochondrial pathway (loss of mitochondrial potential, release of cytochrome *c*, activation of caspase-9) in relation to the further activation of caspase-3 and the chromatin condensation. We have attempted to rationalize our observations by examining directly the effect exerted by gentamicin on model bilayers meant to represent the lysosomal and mitochondrial membranes when incubated at acid and neutral pH.

Methods

Materials. Dulbecco's Modification of Eagle's minimum essential Medium (DMEM) and Trypsin-EDTA were purchased from Life Technologies, Paisley, UK. Gentamicin sulfate (obtained as GEOMYCINE^{R}, the branded product registered for clinical usage in Belgium; mixture of C_1, C_{1a} and C_2 components) was kindly provided by Glaxo-SmithKline (Genval, Belgium) on behalf of Shering-Plough (Brussels, Belgium). Ac-DEVD-AFC, leupeptin, pepstatin, aprotinin, CHAPS, PMSF, DTT, oxidized glutathione, acridine orange, m-CCCP, sphingomyelin, cholesterol, calcein, Tris maleate, Tris base, NADH, pyruvate, bovine serum albumin (BSA) and cardiolipin were purchased from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylcholine and phosphatidylinositol (Grade 1) were purchased from Lipid Products (Nr Redhill, UK). In situ cell death detection kitR (TUNEL assay), DAPI and protein-A-agarose came from Boehringer-Mannheim (Mannheim, Germany; presently Roche Diagnostics, Basel, Switzerland). JC-1 came from Molecular Probes Inc. (Eugene, OR). z-VAD-fmk was purchased from Promega U.S. (Madison, WI). Laemmli buffer and Tween 20 were purchased from Bio-rad Laboratories (Hercules, CA), polyclonal rabbit IgG raised against a residues 287–306 of recombinant human anti-caspase-9 (cross-reacting with pig) came from Stressgen Biotechnologies Corp (Victoria, BC), polyclonal rabbit raised against residues 1–104 of horse cytochrome *c* from Santa Cruz Biotechnologies Inc (Santa Cruz, CA), peroxidase-labeled animal anti-rabbit IgG from BD Transduction Laboratories (San Diego, CA), the nitrocellulose membranes (Hybond C) from Amersham Biosciences, UK, Ltd. (Little Chalfont, Bucks., England) and the SuperSignalR West Pico Chemiluminescence Substrate from Pierce (Rockford, IL).

Cells and treatments. All experiments were performed with LLC-PK1 (Lilly Laboratories Culture-Pig Kidney Type 1) cells as obtained from American Tissue Culture Collection (LGC Promochem, Teddington, UK) as ATCC CL-101. Cultures were grown in Dubelcco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum in an atmosphere of 95% air −5% CO_2. Cells were subcultured twice a week and used when reaching approximately 80% of confluence. All gentamicin solutions were adjusted to pH 7.4 prior being added to the culture medium.

Detection and enumeration of apoptotic cells. Cells were detached by trypsinization in Ca^{++}/Mg^{++}-free Hank's medium, pelleted by centrifugation at 1200 rpm for 10 min, washed three times with gentle resuspension and repelleting in ice-cold Phosphate Buffer Saline (PBS). Staining of DNA to reveal apoptotic bodies was made with 4′,6′-diamidine-2′-phenylindole (DAPI). Cells were resuspended in PBS, fixed in 4% paraformaldehyde for 30 min, spread on polylysine-coated slides, allowed to dry out for a few hours and incubated with the stain (1 μg/ml in methanol) for 30 min at 37 °C. Samples were then mounted in Mowiol/1, 4-diazabicyclo[2.2.2]octane (DABCO). Enumeration of apoptotic nuclei was made on slides picked up at random, using a Zeiss light microscope with 63 × oil-immersion objective, and counting about 500 cells. Clusters of apoptotic bodies were given as a single count. Data were expressed as the percentage of apoptotic nuclei relative to total number of nuclei counted.

Detection of necrotic cells. Necrosis was analyzed by following the release of lactate dehydrogenase (LDH) from cells into the medium after incubation with gentamicin as described earlier (Montenez et al., 1999). Results were expressed as the percentage of activity detected in the media over the sum of the activities in the media and in cells.

Measurement of caspase-3 activity. This was determined on the cell lysates (typically 50 μg protein per assay, as measured by a modified Bradford's method (Ramagli, 1999), using *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) (Thornberry et al., 1997) as previously described (El Mouedden et al., 2000b) except that the lysis buffer was made of 10 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], 2 mM EDTA [ethylene diamine tetraacetic acid], 0.1% CHAPS [3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulphonate], 5 mM DTT [1,4-Dithiotreitol], 1 mM PMSF [phenylmethylsulfonyl fluoride], 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM oxidized glutathione. Kinetic fluorometric measurement of the release of 7-amino-4-trifluoromethyl coumarin was made continuously over 120 min to check for assay linearity. Specificity for caspases was assessed by running parallel samples in the presence of the pan-inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk). In preliminary experiments, samples of lysates of control and gentamicin-treated cells were incubated with increasing concentrations of z-VAD-fmk. A plateau of inhibition was observed at about 100 μM, and this concentration was, therefore, used for all subsequent determinations. Because the extent of inhibition by z-VAD-fmk was low in control cells (about 30%), and did not exceed about 63% in gentamicin-treated cells (3 mM, 48 h), we checked to what extent the increase in activity, as detected with Ac-DEVD-AFC, was truly related to apoptosis. For this purpose, cells were exposed to campthothecin (10 mg/L) for 16 h, which induced marked apoptosis (about 50%) and a 30-fold increase in caspase activity (as measured with Ac-DEVD-AFC), which was inhibited to more 84% in the presence of z-VAD-fmk.

Assessment of lysosomal membrane integrity. The integrity of the lysosomal membrane and the maintenance of a lysosomal-cytosolic pH gradient was assessed using the acridine orange relocation technique (Zdolsek et al., 1990). In this approach, cells are incubated with a fluorogenic organic weak base which diffuses into cells and accumulates in lysosomes and related acidic vacuoles by proton-trapping (Olsson et al., 1989). This accumulation produces a change in the fluorescence emission of the probe (from green to red for acridine orange, due to concentration-dependent stacking of the molecules). Disruption of the lysosomal membrane and/or marked change in lysosome pH can, therefore, be assessed by measuring the change in emission ratio in comparison to controls and by visual inspection of the cells by confocal microscopy (Brunk and Svensson, 1999; Li et al., 2000; Olsson et al., 1989; Zdolsek et al., 1990). For our experiments and unless stated otherwise, cells were loaded with acridine orange (5 μg/ml) in complete growth medium for 15 min at 37 °C, rinsed with DMEM and then incubated with gentamicin in Hanks′ Balanced Salt Solution (HBSS). *O*-methylserine dodecylamide hydrochloride (MSDH), a lysosomotropic detergent, was used as a positive control (Li et al., 2000). At appropriate times, cells were rinsed off from gentamicin or MSDH in ice-cold PBS supplemented in 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$, and immediately observed using an Axiovert confocal microscope (Zeiss, Oberkochen, Germany) coupled to an MRC1024 confocal scanning equipment (Bio-Rad, Hemel, UK) operating at a λ_{exc} of 488 nm and at λ_{em} of 520 nm and 615 nm. For quantitative analysis, whole cells sheets were examined with a Fluorocount Microplate Fluorometer (Packard Instruments) with the excitation wavelength set of 485 nm and readings made at 530 nm and 620 nm. We checked that neither gentamicin nor MSDH interfered with the acridine orange fluorescence.

Disruption of mitochondria membranes potential. The mitochondrial potential ($\Delta\psi_m$) was measured using the fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1), (Reers et al., 1991) which exhibits a potential-dependent accumulation in mitochondria. As for acridine orange, the fluorescence of JC-1 is influenced by its concentration with a green light (530 nm) being emitted in dilute solutions, and a red light (595 nm) when molecules aggregate at larger concentrations. This method has been validated for a reliable analysis of $\Delta\Psi_m$ changes in mitochondria (Salvioli et al., 1997). For our experiments, control and gentamicin-treated cells were incubated with JC-1 (6.7 μg/ml [10 μM]; extemporaneously prepared from a 1 g/L stock solution in DMSO) for 30 min at 37 °C in the dark. Cells were then rinsed with PBS supplemented in 3.6 mM $CaCl_2$ and 3 mM $MgSO_4$, and examined by confocal microscopy (as described above for the acridine orange studies) for qualitative studies or in a Fluorocount Microplate Fluorometer (Packard Instruments), with excitation wavelength set at 485 nm and readings made at 530 nm and 610 nm for quantitative studies. Carbonyl cyanide m-chlorophenylhydrazone (m-CCCP), a protonophore and uncoupler of oxidative phosphorylation in mitochondria (Laval and Little, 1977) was used as positive control at 20 μM for up to 10 h (Lim et al., 2001). We checked that neither gentamicin, nor mCCCP interfered with JC-1 fluorescence.

Preparation of a granules fraction and a cell supernatant. A granule fraction, containing the bulk of mitochondria and a cell supernatant were prepared by differential centrifugation from homogenates of LLC-PK1 using the general procedures set up for other cultured cells in our

laboratory (Tulkens et al., 1974b). In brief, cells collected by gentle scraping in 0.25 M sucrose −20 mM Tricine pH 7.4–1 mM EDTA pH 7.4 (Hayashi et al., 2000) and homogenized in an all-glass Dounce tissue grinder. The resulting homogenate was subjected to 3 successive low speed centrifugations (600 × *g* for 10 min) to obtain a fraction containing nuclei and unbroken cells, followed by a high-speed centrifugation (145,000 g for 30 min) to yield a granules fraction and a final supernatant. The content of each fraction in marker enzymes of the mitochondria (cytochrome *c* oxidase [E.C. 1.9.3.1]) and of the soluble proteins (lactate dehydrogenase [E.C. 1.1.1.27]) was measured using previously described techniques (Renard et al., 1987; Tulkens et al., 1974a).

Immunoprecipitation and detection of cytochrome c the cell granules fraction and supernatant. Samples were incubated overnight at 4 °C in presence of anti-cytochrome *c* antibody (Santa Cruz Biotechnology) in a volume of 1 mL, thereafter mixed with 30 μL Protein-A-agarose for 2 h at 4 °C, and centrifuged at 16,000 × *g* for 5 min at room temperature. The pellets were washed at 4 °C with PBS, 0.5 M NaCl, and twice with PBS. The final pellets were resuspended in Laemmli buffer, mechanically disrupted by forcing them 5 times through a 20-gauge needle, and subjected to separation by electrophoresis in 18% polyacrylamide gels prepared in 25 mM Tris-192 mM glycine buffer pH 8.3 and using the same solution supplemented with 0.1% SDS as running buffer. Proteins were transferred to 6 × 8 cm nitrocellulose membranes (Hybond C). The membranes were blocked for 1 h in Tris-buffered saline containing 5% fat-free milk proteins and 0.1% Tween 20 (TBS-T), and incubated overnight at 4 °C with polyclonal anti-cytochrome *c* antibodies (diluted in TBS-T containing 5% of BSA). Membranes were then washed four times (5 min each) in TBS-T, incubated with peroxidase-labeled anti-animal IgG antibody in that medium supplemented with 5% fat-free milk proteins, then visualized with SuperSignal West Pico Chemiluminescence Substrate.

Immunodetection of caspase-9 detection. Cells were collected as described for caspase-3 activity measurement and resuspended in Laemmli buffer. Detection of caspase-9 was performed by Western blot using the same general technique as for cytochrome *c*, using a rabbit anti-caspase-9 polyclonal antibody that, according to the manufacturer, detects both the pro-caspase-9 and the 36 kDa protein corresponding to the processed large subunit of caspase-9. Densitometric analysis of the gels was made by scanning them with a commercial scanner and analyzing the images with a public software (Image J v. 1.31, National Institutes of Health, Bethesda, MD; http://www.rsb.info.nig.gov/ij).

Studies with liposomes. Small unilamellar vesicles were prepared with compositions mimicking (i) the lysosomal and the outer mitochondrial membranes (cholesterol/phosphatidylcholine/phosphatidylinositol/sphingomyelin molar ratio 5.5:4:3:4), and (ii) the inner mitochondrial membrane (with cardiolipin replacing phosphatidylinositol while all other lipids remained in the same molar proportions). Vesicles were prepared by sonication in 40 mM Tris maleate buffer (345 mosm/kg) adjusted to pH 5.4 to mimick the lysosomal environment, and at pH 7.4 (338 mosm/kg) to mimick the mitochondrial environment, and in the presence of purified calcein (Van Bambeke et al., 1993; 23.11 mM for liposomes prepared at pH 5.4 and 19.55 mM for liposomes prepared at pH 7.4). The liposomes concentration was then adjusted to 50 μM (using the average molecular weight of the constituent lipids), exposed to gentamicin, mixed thoroughly for 20 s, and allowed to stand at 37 °C up to 16 h. Leakage of entrapped calcein was monitored by the increase in fluorescence of the samples, resulting from the dilution and release of self-quenching of this tracer (Weinstein et al., 1977). All fluorescence determinations were performed on an LS 30 fluorescence spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, UK) using λ_{exc} and λ_{em} of 472 nm and 516 nm, respectively. The percentage of calcein released under the influence of drug was defined as $[(F_t - F_{contr}) / (F_{tot} - F_{contr})] \times 100$, where F_t is the fluorescence signal measured at time t in the presence of drug, F_{contr} is the fluorescence signal measured at the same time in the control liposomes, and F_{tot} is the total fluorescence signal obtained after complete disruption of liposomes by sonication (checked by quasi-elastic light spectroscopy).

Statistical analyses. The significance of the differences between paired values was tested by Student's *t* test, and that of two sets of data (kinetic studies) ANOVA analysis (Excel 97, Microsoft, Inc, Bellevue, WA). The significance of the differences between several sets of data was tested by ANCOVA analysis using XLSTATC version 6.0 (Addinsoft SARL, Paris, France). Correlations were calculated using GraphPad Prism version 2.1 for windows (GraphPad Software, San Diego, CA).

Results

Concentration-dependence of apoptosis and necrosis

In a first series of experiments, we examined the influence of increasing concentrations of gentamicin (1 to 12 mM) maintained for 24 h on apoptosis (DAPI staining) and cell necrosis (assessed by the release of LDH). Fig. 1 shows that the percentage of cells with evidence of apoptosis increased from about 2 to about 8% in the 0–3 mM range, with a more limited progression at larger concentrations (about 11% at 12 mM). In contrast, LDH release was not significantly different from controls up to a

gentamicin concentration of 3 mM, but markedly increased at larger concentrations.

Concentration- and time-dependence of apoptosis and activation of caspase-3

Having delineated the conditions in which necrosis became predominant (i.e., at a gentamicin concentration at 4 mM or above), we explored in more details the time- and concentration-dependence of the onset of gentamicin-induced apoptosis in the 0–3 mM range. We also measured the activity of caspase-3 (selected as one of the executioner caspases) in the same cells, Fig. 2 shows in combined fashion that both phenomena are time- and concentration-dependent. Considering the apoptosis process first (upper panel), a significant increase in the proportion of DAPI positive cells was already seen after exposing cells for 1 day at 1 mM of gentamicin, and this proportion increased steadily during the next 48 h. Larger concentrations of gentamicin (2 and 3 mM) accelerated the process and made it more intense with, however, a clear plateau after 48 h. Moving now to caspase-3 (lower panel), we see that the activity of caspase-3 showed a first, detectable but not statistically significant increase at 8 h before returning close to control values at 16 h. Thereafter, however, a marked activation was seen that proceeded, as for apoptosis, at a rate and an extent that were dependent on both gentamicin concentration and time of exposure. A maximal activity was obtained at 48 h for cells incubated with 1 and 2 mM gentamicin, and slightly earlier [30 h] for cells incubated with 3 mM gentamicin. Most conspicuously, a sharp decline was seen thereafter but the value reached at 72 h was still about twice that of the controls (this difference being statistically significant).

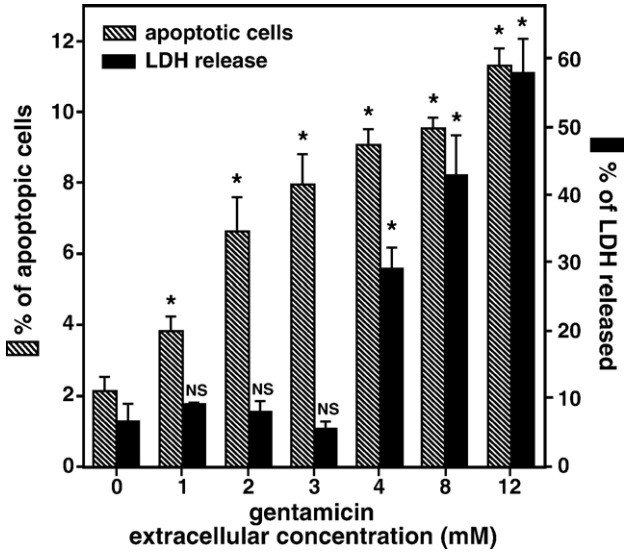

Fig. 1. Comparative increase in apoptosis index (DAPI staining) and in LDH release in LLC-PK1 cells exposed to increasing concentration of gentamicin (1 to 12 mM) for 24 h. Data are means ± SD of 3 independent determinations. NS and asterisks indicate the non-significant and significant changes, respectively, in treated cells compared to controls ($P < 0.05$; Student's paired tests).

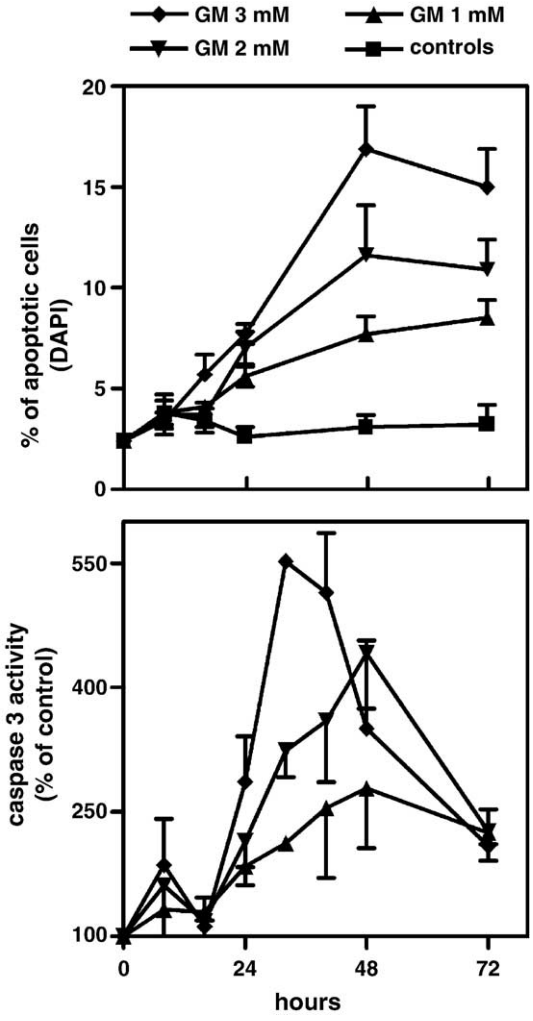

Fig. 2. Time-sequence of the development of apoptosis and activation of caspase 3 in LLC-PK1 cells exposed to increasing concentrations of gentamicin (GM; triangles, 1 mM; upside down triangles, 2 mM: diamonds, 3 mM). Values are given as arithmetic means ± standard deviation ($n = 3$; data points without bar have standard deviations smaller than the corresponding symbol size). Upper panel: detection of apoptosis by the DAPI staining (closed squares, untreated cells). Statistical analysis: the difference seen at 16 h between control cells and cells exposed to 3 mM gentamicin is significant ($P < 0.05$); all differences between control and treated cells are significant at 24 h or later ($P < 0.01$). Lower panel: change in the activity of caspase 3 in percent of the values observed in control cells (all values refer to z-VAD-fmk-inhibitable activities; mean activity in controls: 131 ± 19 μUnits/mg protein). Statistical analysis: (a) the changes seen between treated and control cells at 8 h are not significant; (b) from 24 h onwards, each set of values (corresponding to one treatment) is statistically significant from all others (corresponding to other treatments) and from controls by ANCOVA ($P < 0.05$).

Early lysosomal destabilization

Lysosomal destabilization was analyzed by the acridine orange relocation method using both spectrophotometric

analysis of whole cells and confocal microscopy. MSDH, a known lysosomotropic detergent was used as positive control (Li et al., 2000). As shown in Fig. 3, both gentamicin and MSDH produced an early (2 h) and steady (over the next 4 h) increase in the 530/620 nm ratio of the emitted light of acridine orange in whole cells. This increase was clearly concentration-dependent for gentamicin in the range investigated. Fig. 4 illustrates typical appearances and subcellular localizations of the tracer. In control cells (panels A and B), the tracer was almost exclusively found as red granules localized in the cytoplasm with a perinuclear distribution consistent with that of lysosomes, together with some faint green staining of the nucleoli. In cells treated with gentamicin for 3 h or more, red granules were still seen but many of them were surrounded by a yellow to green halo that seemed to smear into the cytoplasm. This feature, illustrated in panels C and D, was seen in significant proportion of cells incubated 3 h with 3 mM gentamicin. Other cells showed a more diffuse green staining in the cytoplasm together with a mixture of red and yellow granules (panel E; 4 h incubation with 2 mM gentamicin). These cells bore a striking similarity with those seen in large number upon treatment with MSDH (shown in panel F).

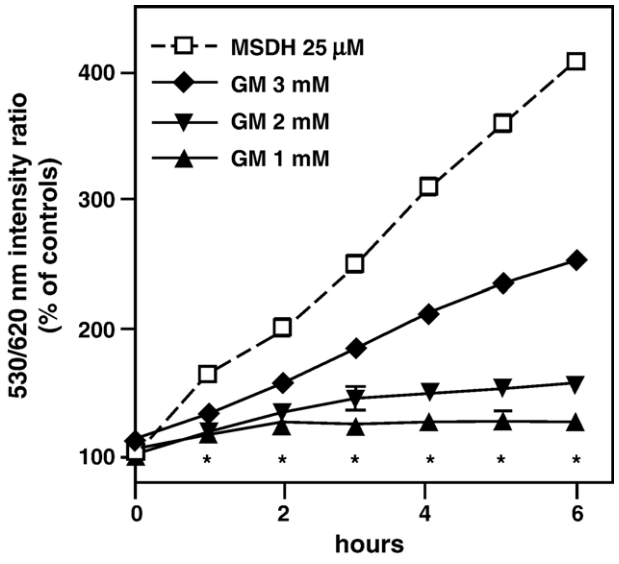

Fig. 3. Temporal variation of the 530 nm/620 nm fluorescence ratio of acridine orange in cells treated with gentamicin (GM; triangles, 1 mM; upside down triangles, 2 mM; diamonds, 3 mM) or MSDH (open squares; 25 µM) in percent of the values observed in controls. Cells were incubated with acridine orange (5 µg/ml) for 15 min and then returned to control medium (baseline) or to medium containing gentamicin or MSDH. Results are given as percentage of the values of controls (arithmetic means ± standard deviation; $n = 3$; when no SD bar is visible the standard deviation was smaller than the size of the symbol). A first analysis of all data points by ANOVA showed significant differences between the 4 groups ($P = 0.002$). Analysis by ANCOVA showed that each set of values (corresponding to one treatment) was significantly different from all others sets (corresponding to other treatments) or from controls ($P < 0.0004$). For cells treated with 3 mM GM, data points are significantly different from the corresponding controls as from 1 h ($P < 0.05$ by the Student's t test and for the other conditions from 2 h, denoted by asterisks at the bottom of the graph).

Decrease of mitochondrial transmembrane potential

The change of mitochondrial membrane potential ($\Delta\psi_m$) was monitored by measuring the shift in the fluorescence of the emitted light of JC-1, using, as for the acridine orange relocation studies, both spectrophotometric and confocal microscopy approaches. m-CCCP, a protonophore known to collapse the transmembrane mitochondrial potential (King et al., 1999; Laval and Little, 1977), was used as internal control. Fig. 5 shows the changes of the 610/530 nm ratio of the emitted light in whole cells as the percentage of that observed in control cells. As anticipated, the protonophore m-CCCP produced a fast and marked decrease of this ratio that remained low for several hours. In contrast, no significant effect of gentamicin was seen up to 8 h, after which a significant decrease (20–30%) was seen which persisted up to 24 h. Fig. 6 shows that JC-1 was distributed on a peripheral fashion in control cells appearing as elongated structures with an exclusively red color. In contrast, cells treated 12 h with 2 mM gentamicin showed a mixture of red and green structures. High magnification revealed that both structures were present in the same cells. In m-CCCP treated cells, most of the fluorescence was of green color, with a large part distributed in a diffused fashion in the cytosol, but also appearing associated with small, rounded shaped structures. Red structures were scarce.

Release of cytochrome c from mitochondria and activation of caspase-9

The release of cytochrome c in the cytosol resulting from activation of mitochondrial pathway was examined by Western blot analysis of granules and supernatant fractions prepared from control and cells exposed to gentamicin (2 mM) for 12 h. The granule fractions contained 90.4 ± 8.3 and 83.9 ± 4.7% of the activity of cytochrome oxidase, and 1.5 ± 1.3 and 2.0 ± 1.9% of the activity of the lactate dehydrogenase of the original homogenates obtained from control cells and cells incubated 12 h with gentamicin, respectively, whereas the supernatants contained 1.3 ± 1.7 and 2.5 ± 0.4% of the activity of cytochrome oxidase, and 96.6 ± 0.85 and 94.6 ± 1.87% of that of the lactate dehydrogenase ($n = 3$). Fig. 7 shows a typical image of a Western blot obtained with the granules fraction and the supernatant from control cells and cells treated with 2 mM gentamicin for 12 h. Cytochrome c was clearly detected in the supernatant in larger amounts than in control cells, whereas it was decreased in the granules fraction. In parallel, whole cell samples were examined by Western blot analysis for occurrence of processed, activated caspase-9. A distinct reaction product was seen in cells incubated for 12 and 16 h with 2 mM gentamicin at an apparent Mr of 36

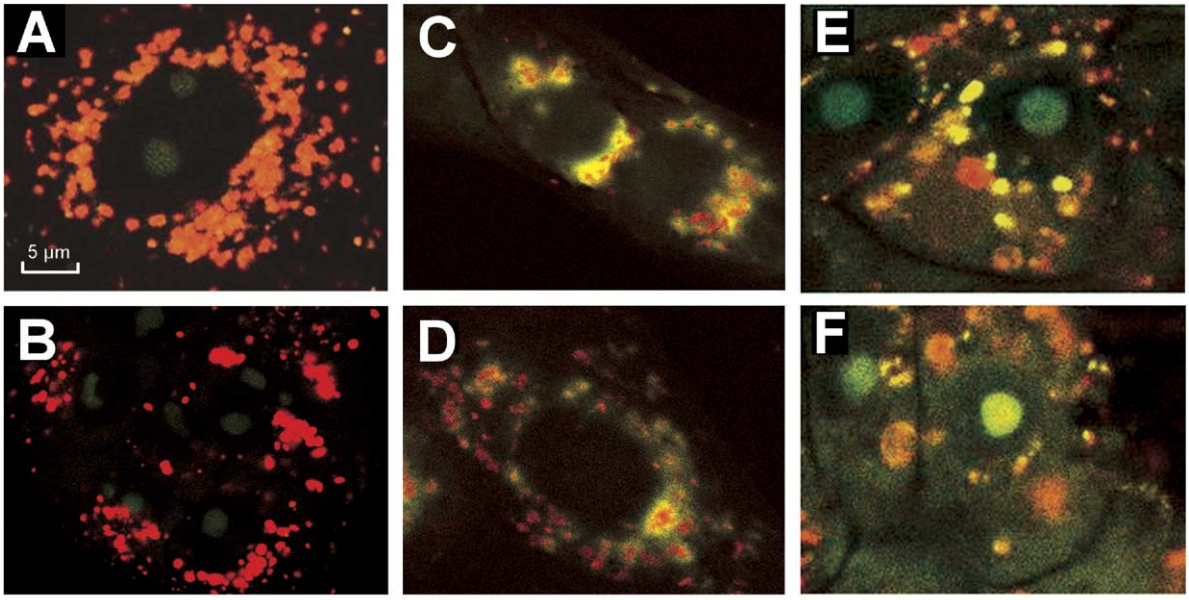

Fig. 4. Appearance of acridine orange-loaded LLC-PK1 cells in confocal microscopy. Cells were exposed to acridine orange (5 µg/ml) for 15 min and then returned to control medium for 3 h (A, B), or exposed to gentamicin (C and D, 3 mM, 3 h; E, 2 mM, 4 h) or MSDH (F, 25 µM, 3 h).

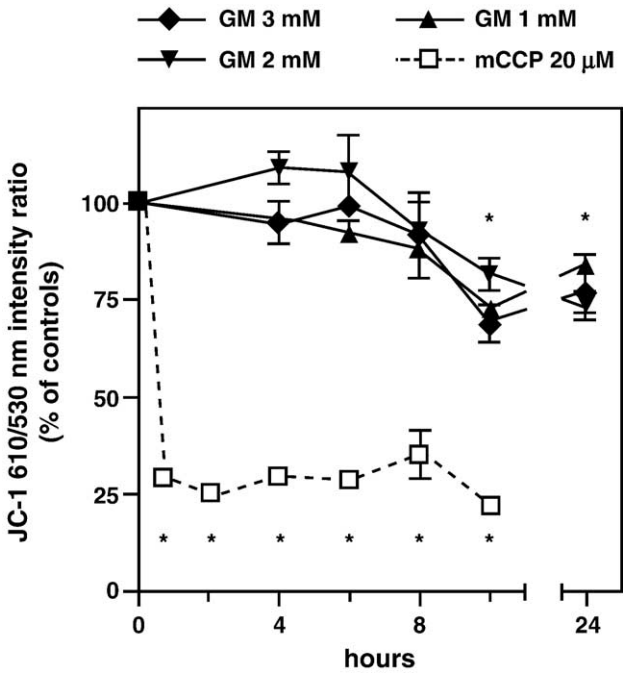

Fig. 5. Temporal variation of the 610 nm/530 nm fluorescence ratio of JC-1 in cells treated with gentamicin (GM 1 mM, triangles; GM 2 mM, upside down triangles; GM 3 mM, diamonds) or m-CCCP (open squares, 20 µM) in percent of the value observed in controls. Cells were incubated with gentamicin or m-CCCP for the times indicated in the abscissa and then exposed to JC-1 (6.7 µg/ml) for 30 min at 37 °C. Results are given as percentage of the values of control (arithmetic means ± standard deviation; $n = 3$; data points without bar have standard deviations smaller than the corresponding symbol size). All values from cells treated with m-CCCP are significantly different from control values. For gentamicin-treated cells, differences from control values are significant at 10 and 24 h ($P < 0.05$) and indicated by an asterisk on the graph to denote this point.

kDa (consistent with the known molecular weight of the large subunit of active caspase-9; densitometric analysis: 3.97 ± 0.01 [$n = 3$] increase over controls). This reaction product was not seen in cells incubated with gentamicin for 8 h only.

Changes in liposomes permeability

The permeabilizing potential of gentamicin towards lipid bilayers was examined on liposomes by measuring the release of entrapped calcein and the ensuing increase of its fluorescence due to its dequenching. Fig. 8 shows that gentamicin (2 mM) induced a fast (4 h) and marked increase in the fluorescence signal in liposome preparations when their composition mimicked that of the lysosomal membrane and the pH was adjusted to 5.4. Additional experiments were performed to explore the concentration-dependence of this release in the 0.001–3 mM range. Data could be fitted to a sigmoidal function ($R^2 = 0.9505$) with an EC_{50} of 22.5 µM (95% confidence interval, 6.7–75 µM). As shown in Fig. 8, the release of calcein proceeded at a slower pace when the pH was raised to 7.4. It was markedly delayed when examined at pH 7.4 with liposomes of a composition mimicking the inner membrane of mitochondria (i.e., in which phosphatidylinositol had been replaced by cardiolipin). In all cases, however, a similar extent of release (±60%) was obtained at 16 h.

Discussion

The data presented in this paper show that the onset of apoptosis induced by gentamicin on renal cell line is

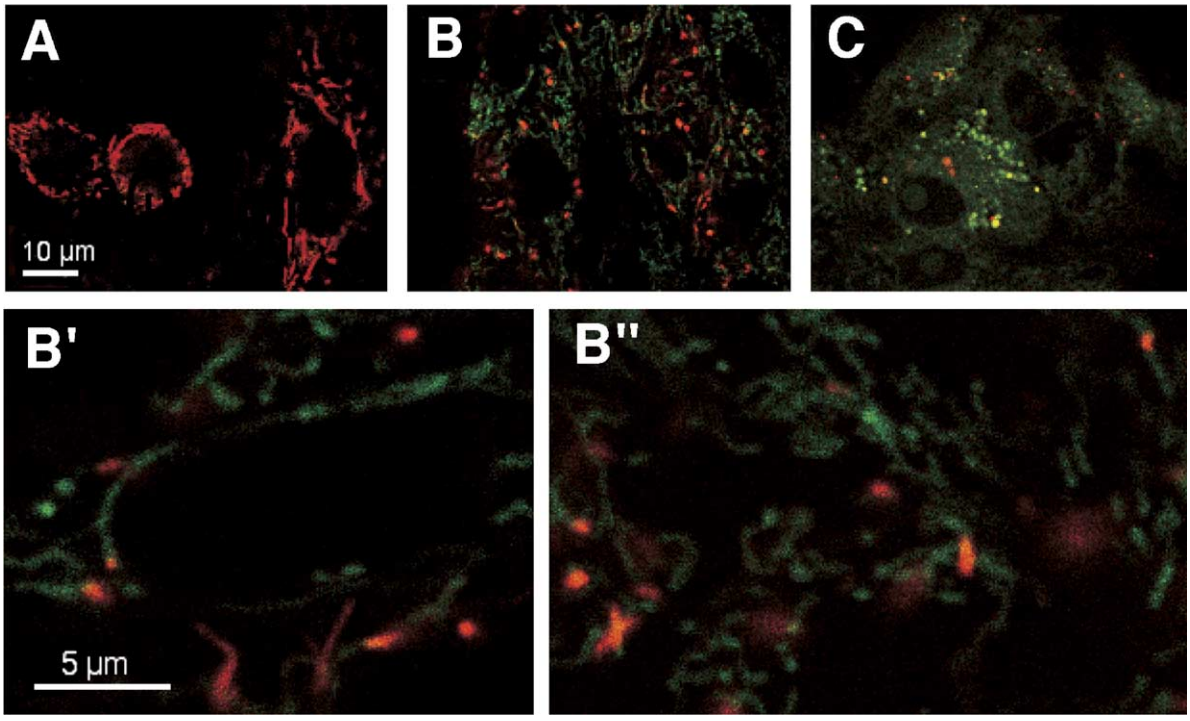

Fig. 6. Appearance of JC-1-loaded LLC-PK1 cells in confocal microscopy. A: control cells; B, B′, B″: cells incubated with gentamicin (2 mM, 12 h); C: cells incubated with m-CCCP (20 µM, 2 h). Cells were thereafter exposed to JC-1 (6.7 µg/ml) for 30 min at 37 °C.

preceded by lysosomal destabilization followed by definite signs of mitochondrial alterations known to be related to the activation of the mitochondrial pathway of apoptosis. This clearly appears from the examination of the data presented here, namely the successive (i) shift in acridine orange emission spectrum and its subcellular redistribution from lysosomes to cytosol), (ii) change in emission spectrum of the mitochondrial probe JC-1 associated with a change in subcellular localization of cytochrome *c*, and evidence of activation of capase-9, and (iii) fragmentation of nuclei and increase in activity of caspase-3. Apoptosis has been shown to develop at concentrations (0–3 mM) at which necrosis was still absent, demonstrating that it is a specific event most likely related to the triggering of the apoptotic machinery by gentamicin. While the present study does not provide a definitive model of pathogenic progression, it nevertheless provides a temporal sequence of alterations that can be used for further analysis of causal relationships.

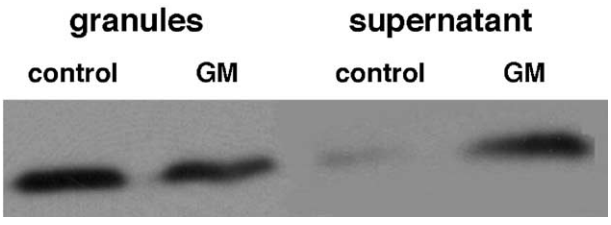

Fig. 7. Release of cytochrome *c* from mitochondria in cells exposed to gentamicin as demonstrated by Western blot of a granule fraction and a supernate prepared from control cells and cells incubated with 2 mM gentamicin for 12 h. Equivalent amounts of protein from each sample (158 µg) were mixed with 5 µL of polyclonal anti-cytochrome *c* IgG (1:200 dilution of a 200 µg protein/ml).

A key, initiating role of lysosomes in gentamicin-induced apoptosis in proximal tubular cells as well as in cultured cells was suspected (El Mouedden et al., 2000a, 2000b) based on the fact that this antibiotic most conspicuously accumulates in lysosomes both in vivo (Giurgea-Marion et al., 1986; Silverblatt and Kuehn, 1979) and in vitro (Sundin et al., 2001; Tulkens and Trouet, 1978). We now present direct evidence that lysosomes may suffer from early and significant destabilization of their membrane, as evidenced by our data with acridine orange. Acridine orange is a fluorescent dye that emits in green in diluted solutions but for which molecular stacking induces its emission to be shifted to red at high concentrations (Millot et al., 1997). As a weak organic base, acridine orange concentrates in the lysosomes of cultured cells (Olsson et al., 1989) explaining both the typical distribution and emission wavelength seen in control cells. A shift towards a green color, and a cytoplasmic staining are therefore highly indicative of either (i) a dissipation of the lysosome-cytosol pH gradient (suppressing the motive force that maintains the dye in lysosomes), or (ii) a loss of the integrity of the lysosomal membrane which will also result in the loss of transmembrane pH gradient. We have no simple way to distinguish between these two non-mutually-exclusive hypotheses from the present data. We may, nevertheless, probably dismiss the possibility of a simple and selective loss of lysosomal/cytosol pH gradient. Gentamicin, indeed, has been shown not to induce significant change of lysosomal pH in fibroblasts incubated for up

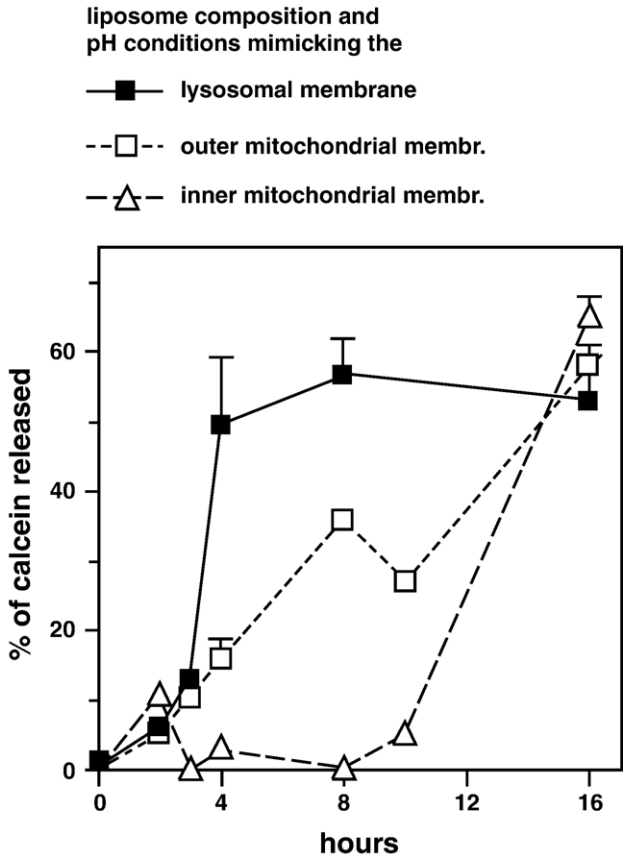

Fig. 8. Time-dependence of calcein release from liposomes (50 μM in average lipid) upon incubation at 37 °C with gentamicin (2 mM). The membrane compositions and pH were adjusted to mimic the lysosomal, outer mitochondrial and inner mitochondrial membranes, and their corresponding environments (closed squares, cholesterol/phosphatidylcholine/sphingomyelin/phosphatidylinositol [5.5:4:3:4 molar ratio], pH 5.4); open squares, same composition but pH 7.4; triangles, phosphatidylinositol was replaced by cardiolipin, pH 7.4). Results are given as the amount of calcein released in percentage of the maximal releasable amount (determined by complete disruption of the liposomes). Data are arithmetic means ± standard deviation ($n = 3$; when no SD bar is visible the standard deviation was smaller than the size of the symbol). A first analysis of all data points by ANOVA showed no significant differences between groups ($P = 0.41$). Analysis by ANCOVA, however, showed that each set of values (corresponding to one condition of liposome composition and/or of pH) was significantly different ($P < 0.001$) from all others or from controls (no gentamicin added) ($P < 0.001$). For each data point corresponding to liposomes of a composition mimicking that of the lysosomal or outer mitochondrial membrane, all values are significantly different from the corresponding controls as from 2 h of contact with gentamicin. For liposomes of a composition mimicking that of the inner mitochondrial membrane, significant differences are only observed at 2, 4, 10, and 16 h.

to 24 h in the presence of 2.2 mM of gentamicin, based on studies using the endocytizable pH probe FITC dextran (Oshima et al., 1986). In contrast, our results with MSDH, which induces a leakage of acridine orange out of lysosomes as in gentamicin-treated cells, support the concept of membrane disruption. MSDH, indeed, is a detergent that accumulates in lysosomes and produces bilayer disruption when exposed to acidic pH (Firestone et al., 1979; Li et al., 2000). Because lysosomes form a heterogenous population with respect to endocytosis and potential damage to membranes (Nilsson et al., 1997), and since gentamicin was present all along our experiments, the overall process may appear continuous when examined by spectrophotometry. The relocalization of acridine orange has already been extensively used to evidence the loss of lysosomal membrane integrity by L-Leucyl-L-Leucine methyl ester (Uchimoto et al., 1999). Besides MSDH, the role of lysosomal disruption as an initial event in triggering apoptosis has also been clearly observed with a variety of membrane-damaging compounds or molecules that accumulate in lysosomes or bind to lysosomal membrane, such as digitonin (Ishisaka et al., 1998), α-tocopheryl-succinate (Neuzil et al., 1999), 6-hydroxydopamine (Takai et al., 1998), synthetic retinoid (Zang et al., 2001), bile salts (Roberts et al., 1997), sphingosine (Kagedal et al., 2001). We also know that apoptosis can be selectively triggered by direct damage to lysosomes resulting from photooxidation (Brunk et al., 1997), or oxidative stress induced by naphthazarin (Roberg and Ollinger, 1998), or hydrogen peroxide (Brunk et al., 1995; Nilsson et al., 1997; Ogawa et al., 2004).

Whereas the potential role of lysosomes in initiating apoptosis is now widely accepted, there is no consensus as to whether lysosomal rupture can directly result in the activation of the executioner caspases or whether the lysosomal pathway needs to be relayed by the mitochondrial one (Guicciardi et al., 2004). In the present work, we demonstrate that lysosomes are destabilized after 2 h of incubation of the cells with gentamicin, and that the mitochondrial pathway leading to apoptosis is activated at 10 h. We do not know to what extent lysosomal changes are related to mitochondrial activation. But we cannot exclude either that lysosomal-mitochondrial cross-talk constitutes an amplifying loop in the apoptotic process. A loss of mitochondrial potential heralds indeed an alteration in the mitochondrial permeability properties and is known, since several years, to precede the onset of apoptosis (Lemasters et al., 1998). Recently, another group of investigators (Sandoval and Molitoris, 2004) using rhodamine B hexyl ester as probe also described a loss of mitochondrial potential in LLC-PK1 cells incubated with 1 mg/ml gentamicin (approximately 2.15 mM). A significant decrease was observed after 4 h and progressed over time to reach 20% of the control values at 8 h. In comparison, the modest effect exerted by gentamicin on the mitochondrial potential in our study may seem surprising at first glance. It could be related to the use of a different probe (and JC-1 used here may actually be one of the most faithful probes in this respect) (Salvioli et al., 1997). But, in a more general context, it could simply be related to the fact that gentamicin is a low apoptosis inducer the influence of which on mitochondria is expected to be weaker than that of stronger inducers. It is interesting to note that sphingosine, another weak apoptosis inducer, causes also only a modest decrease in mitochrondrial potential (Kagedal et al., 2001). Our morphological studies also showed that gentamicin differently affected

individual mitochondria within the same cell, suggesting that the globally modest decrease seen in the spectrophotometric analysis could actually cover major local changes.

The main point, however, is that the change in mitochondrial potential demonstrated here is accompanied by a partial redistribution of cytochrome *c* from the granules fraction to the cytosol and by the activation of caspase-9. Cytochrome *c* released from mitochondria is a well-known trigger of apoptosis because it can interact in the cytosol with the Apaf-1 protein and the inactive form of caspase-9 to form the apoptosome (Li et al., 1997). This leads to activation of caspase-9 (Kandasamy et al., 2003; Zou et al., 1997) and other caspases, including caspase-3. The mitochondrial pathway to apoptosis is known to be blocked by the overexpression of Bcl-2 (Wang et al., 2004). Our previous study demonstrated that gentamicin-induced apoptosis in LLC-PK1 cells is largely prevented in cells overexpressing Bcl-2 (El Mouedden et al., 2000b). Thus, one may probably exclude a direct effect on caspase-3 without mitochondrial relay in gentamicin-induced apoptosis.

We do know whether the triggering of the mitochondrial pathway is due to a direct effect of the antibiotic on mitochondria, or whether it results from some other mechanism. The first hypothesis implies that gentamicin has access to the mitochondria, which may simply result from its release from lysosomes as we suggest here. It is interesting to note, in this context, that gentamicin is capable of permeabilizing liposomes with a composition that mimics that of the outer mitochondrial membrane at pH 7.4. While this process is less rapid than at pH 5.4, it is nevertheless faster than what is observed for liposomes of a composition mimicking the inner leaflet of the mitochondrial membrane. The latter observation is consistent with data (Mather and Rottenberg, 2001) showing that aminoglycosides have a larger affinity for phosphatidylinositol (one of the acidic phospholipids present in lysosomal and outer mitochondrial membrane) than for cardiolipin (which is abundant in the inner mitochondrial membrane). It remains, however, to be seen whether gentamicin can also destabilizes the lysosomal membrane in cultured cells or in renal tissue, although the concentrations needed (EC_{50} of 22.5 μM at acid pH) are probably easily reached (see discussion in El Mouedden et al., 2000a, 2000b).

Possibly also, gentamicin could have a direct access to mitochondria without rupture of lysosomes thanks to its retrograde transport from the Golgi complex through the endoplasmic reticulum from where translocation of the drug to the cytosol was shown to occur (Sandoval and Molitoris, 2004). Retrograde transport of drugs, and participation of the Golgi structures and the endoplamic reticulum in their intracellular trafficking is indeed a rather common event which has been advocated in other cases of drug-induced toxicities such a those observed with the cationic amphiphiles (Ruben et al., 1991). Incidentally, one of the reasons for gentamicin to be a weak apoptosis inducer may actually be the fact that only a small part of the cell-associated drug eventually reaches mitochondria (probably less than 10%, as judged by the most recent morphological data obtained with LLC-PK1 (Sandoval and Molitoris, 2004).

Finally, other factors released from lysosomes than gentamicin it-self could also elicit a loss of mitochondrial potential. Lysosomal proteases, for instance, can activate Bid or other cytosolic pro-enzymes which would then induces the release of cytochrome *c* (Brunk et al., 2001; Guicciardi et al., 2000; Roberg et al., 1999; Stoka et al., 2001; Zhao et al., 2001), inducing thereby the formation of the apoptosome and the subsequent caspases activation. Formation and release of reactive oxygen species, which have been implicated in the development of apoptosis by gentamicin both in kidney glomeruli (Martinez-Salgado et al., 2004) and in the organ of Corti (Nagy et al., 2004), could also play a crucial role.

One may wonder why apoptosis seems to reach a plateau and does not proceed further after 1 to 2 days of culture with gentamicin. This plateau may actually be only apparent, since cells undergoing apoptosis are likely to quickly detach from the growing surface and will, therefore largely escape detection. The potential presence of cells truly refractory to gentamicin is, in any case, unlikely since for higher concentrations, apoptotic death shift to necrotic death as shown by LDH release measurement. A key-factor in determining the type of cell death (necrosis vs. apoptosis) mediated by the lysosomal pathway seems to be the magnitude of lysosomal permeabilization and, consequently, the amount of proteolytic enzymes released into the cytosol (Li et al., 2000). A complete breakdown of the organelle with release of high concentrations of lysosomal enzymes into the cytosol results in unregulated necrosis, whereas partial, selective permeabilization triggers apoptosis (Guicciardi et al., 2004).

In spite of these uncertainties, the present study opens potentially interesting perspectives for the unraveling of gentamicin-induced renal alterations. Although direct extrapolation from in vitro to in vivo data remains a risky exercise, LLC-PK1 cells have been widely used as a model for the study of various aspects of gentamicin-induced nephrotoxicity (Hori et al., 1984; Kohlhepp et al., 1994; Sandoval et al., 1998; Schwertz et al., 1986) and apoptosis (Shino et al., 2003; Yano et al., 2003). In the present study, large gentamicin concentrations (1–3 mM; approximately 0.5 to 1.4 g/L) have been used, which may raise questions about the significance of our results with respect to the situation prevailing in vivo (gentamicin typical serum concentrations are indeed in the 1–20 μM range). As explained earlier (El Mouedden et al., 2000b), these concentrations simply allow to obtain in LLC-PK1 cells cellular and intralysosomal drug concentrations of the same order of magnitude as those observed in proximal tubular cells of animals receiving clinically-relevant doses of gentamicin and in which apoptosis has been demonstrated (El Mouedden et al., 2000a; most interestingly, this study shows that increasing the dose administered to animals to supraclinical ones shifts the response to necrosis as observed

here). Since apoptosis is generally considered as a pathological event when developing in kidney after exposure to drugs (Lieberthal et al., 1996; Tanimoto et al., 1993; Thevenod, 2003), the present data may provide the so far missing link between gentamicin accumulation in lysosomes (Giurgea-Marion et al., 1986; Silverblatt and Kuehn, 1979) and the onset of the cascade of cellular events leading to functional and pathologic signs of gentamicin nephrotoxicity (Gilbert, 2000; Tulkens, 1986).

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