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Gentamicin-Induced Apoptosis in Renal Cell Lines and Embryonic Rat Fibroblasts

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Gentamicin, an aminoglycoside antibiotic, induces apoptosis in the proximal tubule epithelium of rats treated at low, therapeutically relevant doses (El Mouedden *et al.*, *Antimicrob. Agents Chemother.* 44, 665–675, 2000). Renal cell lines (LLC-PK$_1$ and MDCK-cells) have been used to further characterize and quantitate this process (electron microscopy; terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling of fragmented DNA [TUNEL]; and DNA size analysis [oligonucleosomal laddering]). Cells were exposed for up to 4 days to gentamicin concentrations of up to 3 mM. Apoptosis developed, almost linearly, with time and drug concentration, and was (i) preventable within the time-frame of the experiments by overexpression of the anti-apoptotic protein Bcl-2, and by co-incubation with cycloheximide (MDKC but not LLC-PK$_1$ cells); (ii) associated with an increased activity of caspases (MDCK cells; *bcl-2* transfectants showed no increase of caspase activities and Z-VAD.fmk afforded full protection). Gentamicin-induced apoptosis also developed to a similar extent in embryonic fibroblasts cultured under the same conditions. In the 3 cell types, apoptosis (measured after 4 days) was directly correlated with cell gentamicin content (apoptotic index [~10 to 18% of TUNEL (+) cells for a content of 20 μg of gentamicin/mg protein; kidney cortex of rats showing apoptosis in proximal tubule epithelium typically contains ~10 μg of gentamicin/mg protein). Thus, gentamicin has an intrinsic capability of inducing apoptosis in eucaryotic cells. Development of apoptosis in proximal tubules of kidney cortex *in vivo* after gentamicin systemic administration is therefore probably related to its capacity to concentrate in this epithelium after systemic administration.

Key Words: **gentamicin; aminoglycosides; apoptosis; renal cell lines; fibroblasts.**

Aminoglycoside antibiotics are often essential for the treatment of severe infections due to Gram-negative bacteria, but they cause nephrotoxic reactions (Gilbert, 2000; Tulkens 1989). This adverse effect has been attributed to the development of an array of alterations in proximal tubule epithelium, followed by its destruction, thereby causing kidney dysfunction (Mingeot-Leclercq and Tulkens, 1999). Whereas, animals treated at-large with supra-therapeutic doses of gentamicin show extensive necroses of proximal tubules (Parker *et al*., 1982), those receiving low, more clinically relevant doses show a marked proliferation and de-differentiation of renal proximal tubules without evidence of necrosis (Laurent *et al*., 1983; Toubeau *et al.*, 1986). Using quantitative approaches, we now have demonstrated that apoptosis, i.e., the process of single, programmed cell death occurring in the absence inflammatory reaction (Saikumar *et al*., 1999; Wyllie, 1997), occurs on a wide scale in rats treated with such low doses of gentamicin (El Mouedden *et al*., 2000). In the same study, we also observed that other aminoglycosides with lower nephrotoxic potential, such as netilmicin, amikacin, or isepamicin, cause considerably less apoptosis when compared to gentamicin at equitherapeutic doses. This suggests to us that apoptosis may in fact be a key mechanism in the development of aminoglycoside toxicity at low therapeutic doses. Apoptosis is known to be activated by a cascade of both extrinsic and intrinsic factors and to be placed under tight genetic regulation. It has now been recognized as an important determinant of cell degeneration in many toxic events (Umanskii, 1996; Wyllie, 1997), including several instances of nephrotoxicity caused by drugs (Lau, 1999; Shihab *et al*., 1999) and environmental toxins (Ishido *et al.*, 1998). Apoptosis has unambiguously been recognized as an important factor in aminoglycoside-induced ototoxicity (Lang and Liu, 1997), another major adverse event associated with aminoglycoside therapy (Gilbert, 2000).

In order to gain a better insight into gentamicin-induced apoptosis and to begin unraveling its molecular mechanisms, we have now examined the ability of gentamicin to cause apoptosis in models of cultured cells. The usefulness of the LLC-PK$_1$ [porcine kidney] cells (derived from proximal tubules) to study the cellular fate and toxicity of gentamicin and other aminoglycosides has been highlighted in numerous studies. (Ford *et al.*, 1994; Hori *et al.*, 1984; Kohlhepp *et al.*, 1994; Oshima *et al.*, 1989; Sandoval *et al.*, 1998; Schwertz *et al.*, 1986; Steinmassl *et al.*, 1995). LLC-PK$_1$ cells have also been successfully used to study apoptosis and necrosis induced by cyclosporin A (Healy *et al*., 1998). Aminoglycosides have also

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been shown to cause specific alterations in other renal cell lines such as the MDCK (canine kidney) cells (Stiemer, 1989), and non-renal cells such as embryonic fibroblasts (Aubert-Tulkens *et al*., 1979). In the present study, LLC-PK_1 cells have therefore been used in comparison with MDCK cells and fibroblasts to explore the occurrence and specificity of the apoptotic response to gentamicin exposure. We also examined the relation between apoptosis and the capacity of gentamicin to accumulate in cells, on the one hand, and to cause a lysosomal phospholipidosis on the other hand. The latter alteration is one of the earliest and most conspicuous to be detected in proximal tubular cells of animals and man treated with low, clinically relevant doses of aminoglycosides (De Broe *et al*., 1984; Tulkens, 1986).

MATERIALS AND METHODS

Cell cultures and collection. Primary cultures of embryonic rat fibroblasts were initiated, maintained, and used exactly as described earlier (Aubert-Tulkens *et al*., 1979; Tulkens *et al*., 1974). LLC–PK_1 (Lilly Laboratories, Culture-Pig Kidney type1) cells ([ATCC CRL-1392], which originate and display some of the attributes of proximal tubular cells [Sepulveda and Pearson, 1982]), and MDCK (Madin-Darby Canine Kidney) cells ([ATCC CCL-34]), which most likely originate from distal tubules [Herzlinger *et al*., 1982]), were obtained from Professor H. De Smedt (Katholieke Universiteit Leuven, Louvain, Belgium) and subcultured every 4–5 days using 0.02% EDTA and 0.05% trypsin in an isotonic solution. All cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum in a humidified 5% CO_2-95% air atmosphere at 37°C. For experimental uses, cultures were initiated at a density of 5×10^4 cells per cm^2 and maintained for 2 days before addition of the test substances (gentamicin with or without cycloheximide or N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD.fmk; a nonspecific caspase inhibitor [Garcia–Calvo *et al*., 1998)]. At the end of the experiment, cells were collected by trypsinization in Ca^{++}/Mg^{++}-free Hank's balanced salt solution and pelleted by centrifugation at 200 × *g* for 10 min, followed by subsequent washing and repelletting in phosphate-buffered saline (PBS), unless stated otherwise.

Electron microscopy. Cell pellets were rinsed rapidly with PBS and fixed for 12 h at 4°C with 2% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Fixation was followed by 3 5-min rinses with 0.1 M sodium cacodylate buffer pH 7.4. Cells were post-fixed with a solution containing 1% OsO_4 and 2% $K_4Fe(CN)_6$ solution (both wt/vol), stained with 1% uranyl acetate, and pelleted in 2% agar. Pellets were dehydrated in graded ethanol solutions and embedded in Spurr resin. Ultrathin (60 nm nominal) sections were cut on a Reichert Ultracut microtome, collected on rhodanium 400-mesh grids, post-stained with uranyl acetate followed by lead citrate, and rinsed in water. Examination was carried out in a Philips CM-12 electron microscope at 80 kV.

Detection of DNA fragmentation by agarose gel electrophoresis. Fragmented DNA was isolated from cells as previously described (Wyllie, 1980). In brief, pelleted cells were lysed overnight at 50°C in 0.5% Triton X-100—1 mM, Tris-HCl—25 mM, and EDTA adjusted to pH 8. The lysates were centrifuged at 10,000 × *g* for 30 min and the supernatants containing fragmented DNA incubated with proteinase K (20 μg/ml) at 50°C overnight. DNA was extracted with phenol-chloroform-isoamyl alcohol (50:49:1), followed by precipitation with 0.1 vol of 3 M sodium acetate and 2.5 vol of 100% ethanol. The concentration of nucleic acid in each sample was determined by UV absorbance at 260 nm. The same amount of nucleic acid from each sample was treated with RNase (20 μg/ml) at 37°C for 3 h. Two μl of buffer (0.1 M Tris base—0.1 M, boric acid—0.02 M, and EDTA, pH 8.3 [TBE]) containing 0.02% bromophenol blue and 40% glycerol were added to 10 μg of DNA samples and loaded on 1.8% agarose gels. Samples were then resolved by electrophoresis (150 V for 2 h) using TBE as the running buffer. After electrophoresis, DNA was stained with ethidium bromide (0.5 μg/ml in distilled water) and visualized by fluorescence in UV light.

In situ *detection of DNA fragmentation and quantitative analysis of apoptosis.* A modification of the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) technique of Gavrieli *et al*. (1992) was used to detect DNA strand breaks *in situ*. Pelleted cells were rapidly rinsed with PBS containing 1% bovine serum albumin (BSA) and fixed in 4% paraformaldehyde for 30 min. After centrifugation and rinsing with PBS, cells were transferred to 96-well plates and treated with 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase activity. Apoptotic cells were detected by TdT-mediated extension of 3′OH ends of fragmented DNA, using fluorescein-labeled dUTP as a precursor, according to the instructions from the supplier. DNA-bound fluorescein was detected by reaction with anti-fluorescein antibody conjugated to peroxidase. Peroxidase activity in immunocomplexes was visualized by reaction of diaminobenzidine in the presence of H_2O_2. Cells were resuspended in PBS, spread on lysine-coated slides and allowed to dry out. Cells were then counterstained with methyl green, and the preparations mounted using permanent medium. Negative controls were performed by substituting distilled water for TdT enzyme in the preparation of the working solution and exhibited no immunostaining. Enumeration of apoptotic nuclei was made on slides picked at random from each experiment, using a Zeiss light microscope with a 40× objective and a 10× eyepiece. For calibration purposes, a square grid was mounted in one eyepiece, determining a square field of 0.04 mm^2. We counted all nuclei exhibiting a frank brown labeling. These nuclei most often displayed typical alterations such as pyknosis, crescent-like condensation of chromatin, or segregation into apoptotic bodies. Clusters of apoptotic bodies were given a single count. The incidence of apoptotic nuclei was given as the percentage relative to total nuclei (apoptotic index).

Cell transfection and selection. An RSV vector containing the human *bcl-2* gene and a hygromycin resistance gene (3′ phosphotransferase) was introduced into LLC-PK1 and MDCK-cells by means of LipofectAmine™ (cells transfected with the same vector, carrying the resistance gene to hygromycin, but no *bcl-2* gene were used as controls). Briefly, 60-mm dishes were seeded with 10^6 cells in 4 ml of complete culture medium. After 24 h, cells were exposed to a LipofectAmine™ reagent/DNA mixture (2.5 μg of DNA per dish) and incubated for 4 h, after which the medium was replaced with fresh medium for 2 days. Cells were then incubated and grown for 4 weeks in selection media (containing hygromycin sulfate at concentrations spanning from 100 to 1500 μg/ml) to obtain stable transfectants. Cloning was performed from cell populations surviving in 400 μg/ml hygromycin, and resistant populations were expanded in this selection medium. Prior to experiments, hygromycin-resistant cells were cultured in the absence of hygromycin for 1 or 2 passages. In control experiments, no cell survival was observed when non-transfected LLC-PK1 and MDCK-cells were cultured in medium containing 400 μg/ml hygromycin.

Western blotting. Pelleted cells were rinsed with PBS and cell lysis was obtained by a 1-h incubation on ice in 30 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml pepstatin, and 2 mg/ml leupeptin). After sonication and centrifugation, 100-μg protein aliquots of lysates were separated on 12% SDS–PAGE (80 V at 4°C, overnight) using 2.5 mM Tris–HCl, 19.2 mM glycine, and 1% SDS, pH 8.3 as a running buffer. Proteins were transferred onto a nitrocellulose membrane and blocked for 1 h with 3% BSA in 10 mM Tris–HCl, 150 mM NaCl, and 0.05% TWEEN®-20, pH 8 (TBST). The membranes were then incubated for 1 h with monoclonal mouse anti-Bcl-2 antiserum and for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG. Color was developed by incubation with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) according to the manufacturer's instructions (Promega Corp., Leiden, The Netherlands).

Determination of the activity of cysteine aspartate–specific proteases (caspases) activity. Pelleted cells were rinsed with PBS and suspended in lysis buffer (100 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.5, containing 10% sucrose, 0.1% 3-[(cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), 1 mM EDTA, 1 mM PMSF, 10 μg/ml pepstatin A and 10 μg/ml leupeptin) and left on ice for 30 min. Following sonication, lysates were centrifuged at 10,000 $\times$ *g* for 10 min (4°C) and supernatants were stored at –20°C. Activity was then determined in the supernatants by fluorimetric assay using the substrate DEVD–AMC, which releases the fluorochrome 7-amino-4-methyl coumarin (AMC). This substrate was originally described as being specifically cleaved by caspase 3; however, recent data and manufacturer's information shows that it can be also cleaved by caspases 6, 7, and 8). The appropriate amount of protein cell extracts (50 μg) was incubated in 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin at 30°C for 30 min with 20 mM acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) in a total volume of 0.5 ml. At the end of the incubation, the released fluorochrome was measured in a Perkin-Elmer LS-30 fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, UK) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Enzyme activity was expressed as arbitrary units relative to protein concentration.

Biochemical studies. Cell phospholipids were determined by assaying lipid phosphorus as described previously (Aubert-Tulkens *et al*., 1979). Proteins were measured according to Lowry's method or by Bradford's assay (using the Bio-Rad® dye reagent according to manufacturer's instructions).

Assay of gentamicin cellular uptake. Washed cell pellets were resuspended in 0.01% Triton X-100 and subjected to brief sonication to achieve complete dispersion. Gentamicin was then assayed by a disc-plate microbiological assay, which had been fully validated for the quantitative assay of aminoglycosides in lysates of cultured fibroblasts (Tulkens and Trouet, 1978). In the present study, the limit of detection was 0.8 μg/ml and the range of linearity of the zone diameter *vs.* the log of the antibiotic concentration extended from 0.8 to 10 μg/ml. Samples were prepared and appropriately diluted to systematically contain gentamicin concentrations within this range.

Sources of products. Dulbecco's modification of Eagle's minimum essential medium (DMEM), trypsin (1:250), LipofectAmine™ reagent, hygromycin sulfate, and foetal bovine serum were purchased from Life Technologies, Rockville, MD. Gentamicin sulfate (mixture of C_1, C_{1a}, and C_2/C_{2a} components in a weight ratio of 29.2:23.3:47.6) was kindly provided as powder for microbiological standard by Schering-Plough, Inc., Kenilworth, NJ. DEVD-AMC and N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone (Z-VAD-.fmk) were purchased from Calbiochem-Novabiochem, San Diego, CA. *In situ* cell death detection kit®, mouse anti-Bcl-2, and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin came from Boehringer-Mannhein, Mannheim, Germany (presently Roche Diagnostics, Basle, Switzerland). The plasmid containing the human *bcl-2* cDNA was a generous gift from Professor D.L. Vaux (Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia). Unless stated otherwise, all other products were purchased from Sigma Chemical Co., St-Louis, MO.

Statistical analyses. Two-group comparisons were performed by Student's *t*-test. When more than 2 groups were involved, a first statistical analysis was done by ANOVA, followed by Tukey (all groups) or Dunnett (test *vs.* control) *post hoc* tests.

RESULTS

Morphological and Biochemical Evidence of Gentamicin-induced Apoptosis

Examination of pellets collected from all 3 types of cells after 4 days of culture with 2 mM gentamicin revealed the presence of cells with ultrastructural alterations typical of apoptosis, i.e., shrunk cells displaying segregation of chromatin into discrete clumps abutting the nuclear membrane, whereas cytoplasmic organelles most often kept a normal appearance. We also noted the presence of numerous so-called apoptotic bodies consisting of membrane-bound entities containing intact organelles together with condensed chromatin. In addition to these alterations related to apoptosis, MDCK cells and fibroblasts, but not LLC-PK_1 cells, showed a typical lysosomal phospholipidosis (accumulation of electron-dense material in lysosomes, which, upon high magnification, displayed a multilayered appearance). These lysosomes were often grossly enlarged in fibroblasts, but kept on almost normal size in MDCK cells. In both MDCK cells and fibroblasts, nuclear changes characteristic of apoptosis could often be seen in cells also displaying clear signs of phospholipidosis. Typical pictures of these changes in all 3 cell types are shown in Figure 1. None of these morphological abnormalities were observed in control cells, except for a minimal level of apoptotic figures. The occurrence of a typical apoptotic process developing on a large scale in gentamicin-treated cells was further characterized by the demonstration of fragmented DNA. First, DNA from cells exposed to gentamicin was submitted to size analysis by gel electrophoresis. Results are presented in Figure 2 in comparison with control cells. In all 3 cell lines, incubation with gentamicin led to DNA breakdown and the appearance of "ladders" reflecting the production of oligonucleosomal fragments of discrete and decreasing lengths. Ladders were not seen in control cells at the same stage of culture. Next, we applied the immunocytochemical TUNEL procedure based on *in situ* 3′ OH-end tailing of double-strand breaks in DNA of apoptotic cells. Figure 3 clearly shows the occurrence of the characteristic features of apoptosis as detected by this technique in cell cultures, namely the staining of condensed, intact nuclei (pyknosis) as well as of clusters of apoptotic bodies.

Influence of Gentamicin Concentration and Duration of Incubation

Apoptosis was then quantitated, using the TUNEL technique in cultures of cells exposed to increasing gentamicin concentrations (up to 3 mM) for up to 4 days. Figure 4 shows that the incidence of apoptosis increased in all 3 cell lines in an almost direct relationship both with respect to the gentamicin extracellular concentration and to the duration of incubation, reaching values up to approx. 20–30% of all cells under our experimental conditions. In parallel, the gentamicin cell content was determined after 4 days of culture, demonstrating for all 3 cell lines a similar level of accumulation which was almost linearly related to the extracellular gentamicin concentration in the 0–3 mM range. The cellular drug contents were 28.9 $\pm$ 6.2, 26.7 $\pm$ 9.6, and 20.2 $\pm$ 6.7 μg/mg protein in fibroblasts, MDCK cells and LLC-PK_1 cells, respectively, after 4 days of culture (these levels correspond to intracellular to extracellular apparent con-

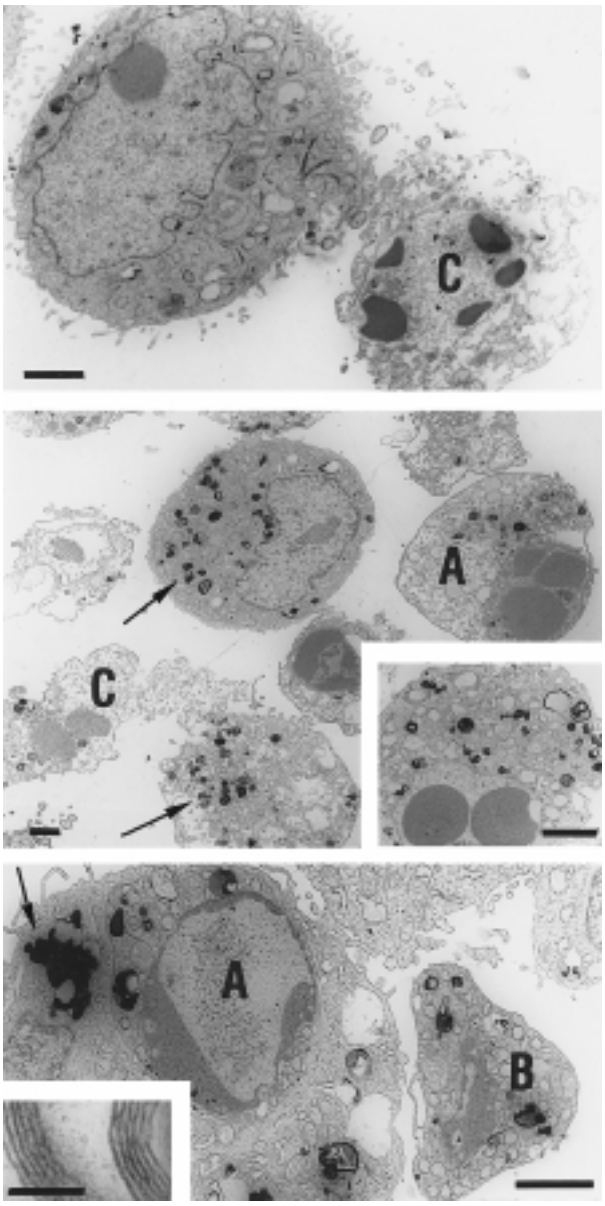

FIG. 1. Electron micrographs showing typical apoptotic alterations observed in LLC-PK_1 cells (top panel), MDCK cells (middle panel) and embryonic rat fibroblasts (lower panel) after 4 days of culture in the presence of 2 mM gentamicin. Apoptotic cells are clearly visible in all 3cell types (A) Nucleus containing chromatin in clump(s) often abutting the nuclear membrane; (B) apoptotic body containing condensed nuclear material, intact mitochondria and other organelles surrounded by a membrane; (C) cells undergoing rupture with clear evidence of condensation of chromatin and fragmentation of the nucleus. A lysosomal phospholipidosis was clearly detected in MDCK cells and fibroblasts (arrows pointing to electron-dense material in lysosomes; this material displays a lamellar appearance upon high magnification (inset of bottom panel). The inset in the middle panel shows that lysosomal phospholipidosis and apoptosis may develop in the same cell (MDCK cells). Lysosomes are grossly enlarged in fibroblasts but display a normal size in MDCK cells. In contrast to MDCK cells and fibroblasts, no ultrastructural evidence of phospholipidosis was seen in LLC-PK_1, whether in apoptotic cells or in cells with normal nuclei (upper panel). Bars are 2 μm (main photograph and inset of middle panel) and 0.1 μm (inset of lower panel).

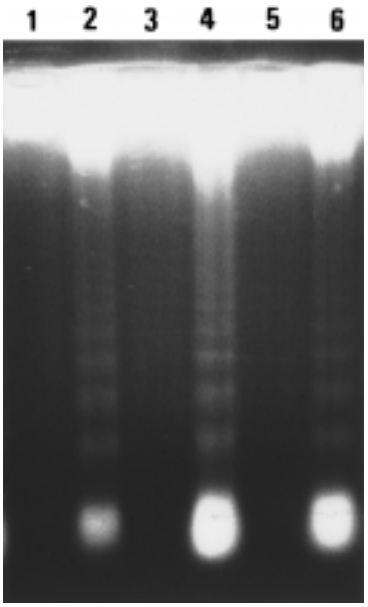

FIG. 2. Agarose gel electrophoresis of DNA extracted from embryonic rat fibroblasts (lanes 1 and 2), MDCK (lanes 3 and 4) or LLC-PK_1 cells (lanes 5 and 6). In each case, 10 μg of cell DNA was submitted to electrophoresis in 1.8% agarose gel. Cells corresponding to lanes 2, 4, and 6 were cultivated in the presence of 2 mM gentamicin for 4 days. Lanes 1, 3, and 5 correspond to control cells at the same stage of culture.

centration ratios of approx. 6.4, 5.7, and 4.3, and to clearance rates of 315, 285, and 215 $\mu l \times (\text{mg protein})^{-1} \times h^{-1}$ for each of these cells, respectively (see Tulkens and Trouet [1978] for details on methodology). Figure 5 shows that there was a highly significant correlation between apoptosis, quantitated by TUNEL, and drug accumulation in all 3 types of cells, with LLC-PK_1 cells demonstrating, however, a higher (~1.5-fold) intrinsic sensitivity.

Correlation with Gentamicin-induced Phospholipidosis

As shown earlier in both fibroblasts (Aubert-Tulkens *et al.*, 1979) and LLC-PK_1 cells (Schwertz *et al.*, 1986), gentamicin causes a marked cellular phospholipidosis when present in the culture medium for more than a few days. To examine whether a link could be established between this metabolic alteration

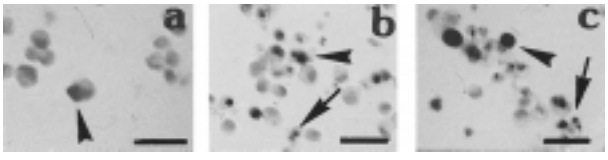

FIG. 3. *In situ* labeling of apoptotic death in embryonic rat fibroblasts (a), MDCK cells (b), and LLC-PK_1 cells (c) exposed to 2 mM gentamicin for 4 days using the terminal deoxynucleotidyl transferase (TdT)/fluorescein-dUTP nick-end labeling (TUNEL) technique. Arrowheads point to TUNEL-positive nuclei demonstrating cell pyknosis (condensation of nuclear material); arrows point to cells with fragmented, TUNEL (+) nuclei. Bars are 20 μm.

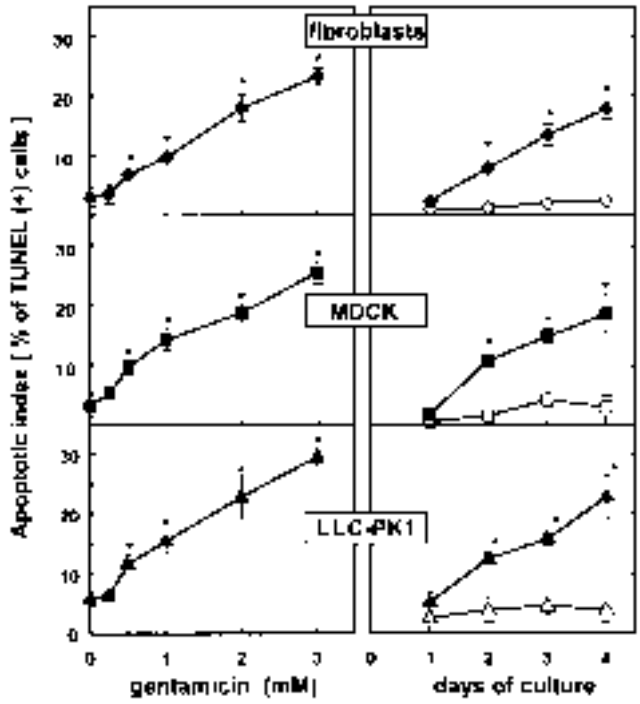

FIG. 4. Determination of apoptotic index in embryonic rat fibroblasts (upper panels), MDCK cells (middle panels), and LLC-PK_1 cells (lower panels) incubated for 4 days in the presence of increasing concentrations of gentamicin (left panels) or incubated for up to 4 days (right panels) with a fixed gentamicin concentration (2 mM; closed symbols) or without drug (open symbols). Apoptosis was quantified by counting TUNEL-positive cells (or clusters of apoptotic bodies) and expressing the values relative to total cell counts. Values are means ± SD of 3 experiments. Asterisk signifies $p < 0.05$ as compared to control.

and apoptosis, we assayed phospholipids in all cell pellets obtained from the experiments, in which the time- and dose-dependency of apoptosis had been examined. Figure 6 shows that phospholipidosis and apoptosis were highly correlated in both fibroblasts and MDCK cells under all conditions investigated. In contrast, and as suggested from the images obtained in the electron microscope, LLC-PK_1 cells showed only a modest increase of their phospholipid content (maximum ~9% of phospholipid increase), making the correlation between apoptosis and phospholipidosis difficult to assess in these cells under our experimental conditions.

Roles of Protein Synthesis, Bcl-2 Protein and Caspases

Apoptosis is, in most cases, described as an active process requiring protein synthesis and the activation of specific proteolytic enzymes (cysteine-aspartate-specific proteases [caspases]) involved in the cleavage of an array of critical cellular substrates, which then result in the initiation of apoptosis on the one hand and several of the biochemical and morphological changes associated with apoptotic death on the other (Stennicke and Salvesen, 1998). Apoptosis is also controlled by agonist and antagonist gene products, among which Bcl-2 proteins play a key role. In particular, the *bcl-2* protooncogene encodes a protein acting as an antagonist of apoptosis (Kroemer, 1997). In the next series of experiments, we therefore characterized gentamicin-induced apoptosis with respect to these factors, using a quantitative approach based on TUNEL technique.

Effect of Protein Synthesis Inhibition

To test for the role of protein synthesis in gentamicin-induced apoptosis, cells were incubated for 3 days in the presence of cycloheximide (1 μM), gentamicin (2 mM), or their combination. As shown in Table 1, cycloheximide alone caused no significant increase of apoptosis in rat fibroblasts or MDCK cells. Cycloheximide also completely prevented the apoptosis induced by gentamicin in these 2 types of cells, bringing the apoptotic index to a level very close to that seen with cycloheximide alone (fibroblasts) or even somewhat lower (MDCK cells). In sharp contrast, LLC-PK_1 cells displayed a marked sensitivity to cycloheximide, which, by itself, caused a larger increase in the apoptotic index than that observed with gentamicin alone. The combination of cycloheximide and gentamicin was more than additive in these cells.

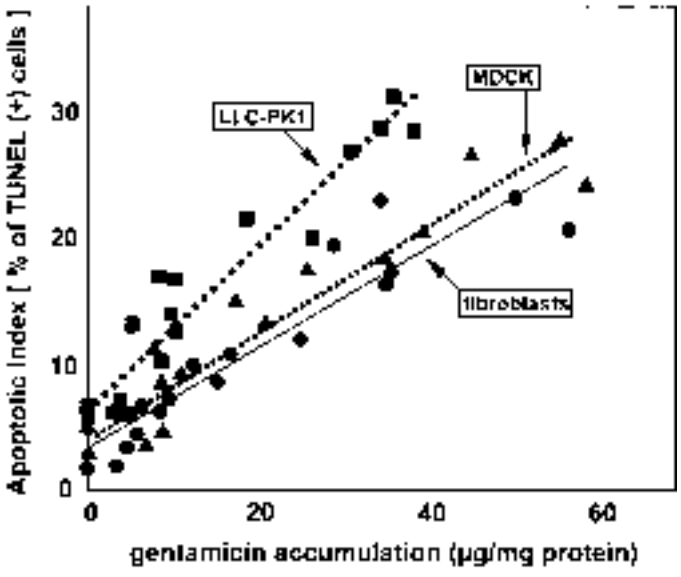

FIG. 5. Correlation between apoptotic index and gentamicin accumulation in rat fibroblasts (circles), MDCK cells (triangles), or LLC-PK_1 cells (squares) upon exposure to gentamicin (0–3 mM) for 4 days. Apoptosis was quantitated by TUNEL as in Figure 4, and gentamicin was assayed by a microbiological method. $R = 0.943$ ($p < 0.001$), 0.954 ($p < 0.001$), and 0.945 ($p < 0.001$) for fibroblasts, MDCK cells, and LLC-PK1 cells, respectively. LLC-PK_1 cells significantly differ from both fibroblasts and MDCK cells, which show no difference from each other.

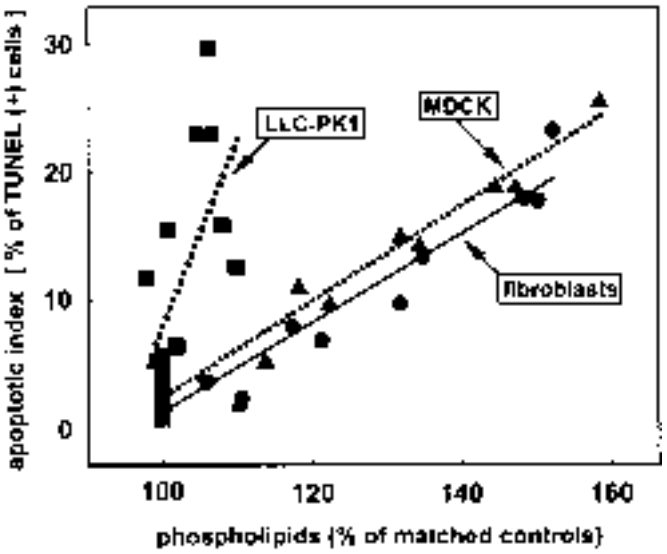

FIG. 6. Correlation between apoptotic index and cell phospholipid content in rat fibroblasts (circles), MDCK cells (triangles), and LLC-PK_1 cells (squares) upon exposure to gentamicin (0–3 mM; 1 to 4 days of culture). Apoptosis data and the experimental conditions are from those in Figure 4. $R = 0.921$ ($p < 0.001$), 0.942 ($p < 0.001$), and 0.725 ($p = 0.01$) for fibroblasts, MDCK cells, and LLC-PK_1 cells respectively. LLC-PK_1 cells significantly differ from both fibroblasts and MDCK cells ($p < 0.001$), which show no significant difference from each other.

Effect of Bcl-2 Protein Overexpression

MDCK and LLC-PK_1 cells were transfected with a recombinant plasmid carrying the human *bcl-2* gene, to obtain stable lines over-expressing the Bcl-2 protein. Analysis by SDS–PAGE and Western blotting confirmed the enhanced expression of Bcl-2 protein in both lines selected after transfection (Fig. 7). We then examined the ability of Bcl-2 to protect from gentamicin-induced apoptosis or to delay its onset by analyzing DNA of *bcl-2*-transformed LLC-PK_1 and MDCK cells treated with gentamicin. As illustrated in Figure 8, MDCK or LLC-PK_1 cells over-expressing Bcl-2 did not exhibit visible DNA laddering after 4 days of gentamicin exposure, whereas DNA breakdown associated with apoptosis was evident in cells transfected with a control plasmid (containing only the hygromycin-resistance gene) and exposed to gentamicin for the same period of time. Quantification of apoptosis was then made using the TUNEL technique and the results are shown in Table 2. Cells over-expressing Bcl-2 protein and exposed to gentamicin for 4 days showed an apoptotic index close to that seen for untransfected cells not exposed to gentamicin. Interestingly enough, MDCK cells over-expressing Bcl-2 protein developed a more extensive accumulation of phospholipidosis (approximately 20% higher; $p < 0.005$) than untransfected cells when exposed to 2 mM gentamicin [data not shown]. Cells transfected with the control plasmid, and which did not over-express Bcl-2 protein, developed apoptosis and phospholipidosis to a level similar to untransfected cells. This control rules out the possibility that the 3′ phosphotransferase encoded in the hygromycin-resistance gene present in the plasmid, and which inactivates hygromycin, could also have inactivated gentamicin (although gentamicin lacks a 3′ OH function and should therefore not be a substrate for 3′ phosphotransferase, it could nevertheless have been recognized by the enzyme and be inactivated by binding, as demonstrated for other aminoglycosides lacking a 3′ OH function [tobramycin, e.g., see Mingeot-Leclercq *et al*., 1999]).

Role of Caspases in Gentamicin-induced Apoptosis

In the present study, the activity of caspases was globally assayed in MDCK cells by measuring the cleavage of the fluorogenic substrate DEVD-AMC. As shown in Table 3, exposure of these cells to gentamicin resulted in an almost 2-fold increase in activity. This increase was not seen in cells over-expressing Bcl-2 protein (cells transfected with the control vector [lacking the *bcl-2* gene] showed an increase in caspase activities similar to that of non-transfected cells). The role of caspases in gentamicin-induced cell apoptosis was further explored by examining the effect of the irreversible inhibitor Z-VAD.fmk. Cells were exposed to gentamicin for 2 days and then maintained in the presence of Z-VAD.fmk and

TABLE 1
Influence of Cycloheximide on Gentamicin-induced Apoptosis

	Apoptotic index (% of TUNEL (+) nuclei)			
	Controls	Cycloheximide	Gentamicin	Cycloheximide + gentamicin
Fibroblasts	2.0 ± 0.3	4.6 ± 1.3	13.4 ± 1.8[a]	4.8 ± 1.4[b]
MDCK cells	2.8 ± 1.4	3.7 ± 1.2	14.9 ± 1.3[a]	1.3 ± 0.3[b]
LLC-PK_1 cells	4.6 ± 1.5	28.7 ± 1.3[a,c]	15.8 ± 1.1[a]	52.8 ± 3.9[a,c,d]

Note. Cells were exposed to cycloheximide (1 μM), gentamicin (2 mM), or their combination for 3 days.
[a]Significantly larger than control ($p < 0.01$).
[b]Significantly lower than gentamicin alone ($p < 0.001$).
[c]Significantly larger than gentamicin alone ($p < 0.01$).
[d]Significantly larger than cycloheximide alone ($p < 0.001$).

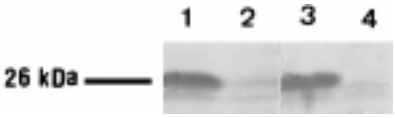

FIG. 7. Western blot analysis of the expression of Bcl-2 protein in MDCK cells (lanes 1 and 2) and LLC-PK_1 cells (lanes 3 and 4) transfected with a control plasmid (only carrying a hygromycin resistance gene; lanes 1 and 3) or with a plasmid containing also the *bcl-2* gene (lanes 2 and 4). Whole protein extracts obtained from stable cell lines were separated by SDS–PAGE, transferred to nitrocellulose membranes, and revealed by incubation with a mouse anti Bcl-2 monoclonal antibody and exposure to alkaline phosphatase-conjugated anti-mouse IgG. The position of Bcl-2 is indicated by the bar.

gentamicin for 2 additional days. As shown in Table 4, the presence of Z-VAD.fmk during the 2 last days of culture almost completely prevented the development of apoptosis during this period. In parallel with being protected against apoptosis, MDCK cells exposed to Z-VAD.fmk and gentamicin developed an accumulation of a phospholipids that was significantly more important (~20% larger, $p < 0.05$) than that seen in the absence of Z-VAD.fmk [data not shown].

DISCUSSION

The present study unambiguously shows that gentamicin causes apoptosis in 2 renal-cell lines from different species and histological origins, as well as in rat embryonic fibroblasts after a few days of culture. Apoptosis is thought to develop in 2 distinct phases, occurring in succession; namely a first, reversible phase of commitment, which is under the control of death antagonists and agonists like the members of the Bcl-2 protein family, and is associated with the activation of a certain number of cysteine-aspartate-specific proteases (caspases), mainly caspases 2, 8, 9, and 10), and a second, irreversible phase of execution, which involves the activation of other caspases, mainly caspases 3, 6, and 7 (Kroemer, 1997; Stennicke and Salvesen, 1998). This process results in the activation of a series of apoptosis effectors, including specific endonucleases (caspase-activated endonucleases [CAD]; Enari *et al.*, 1998). These will perform the internucleosomal DNA cleavages that will cause the appearance of the specific nuclear morphological alterations typifying apoptotic cell death.

The data presented in this paper suggest that gentamicin acts at a level where Bcl-2 can still block or delay this cascade, which would rank the drug as an early initiator of apoptosis. This first conclusion is reinforced by our observation that gentamicin-induced apoptosis requires active protein synthesis in at least 2 of the 3 types of cells studied (fibroblasts and MDCK cells). Although the elements involved in the execution phase indeed preexist within the cell (Weil *et al.*, 1996) and only need to be triggered by appropriate initiation, effective gene transcription, and/or translation of the corresponding mRNA are necessary to produce the factors that allow the process to move from the early steps of the commitment phase

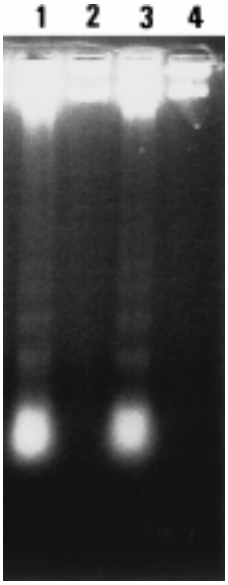

FIG. 8. Agarose gel electrophoresis of DNA extracted from transformed MDCK cells (lanes 1 and 2) and LLC-PK_1 cells (lanes 3 and 4). Stable cell sublines obtained from transfected cells were exposed to 2 mM gentamicin for 4 days and analysis was made as explained in the legend of Figure 2. Lanes 1 and 3, cells transfected with a control plasmid carrying only the hygromycin resistance gene. Lanes 2 and 4, cells transfected with a plasmid carrying both the hygromycin-resistance gene and the *bcl-2* gene. Over-expression of *bcl-2* was checked as shown in Figure 7.

(initiation) to the onset of the execution phase (Umanskii, 1996). Inhibition of protein synthesis has also been shown to suppress apoptosis induced in proximal tubular cells by nephrotoxins other than gentamicin, such as cisplatin (Takeda *et al.*, 1998), and in streptomycin-induced apoptosis in vestibular hair cells (Nakagawa *et al.*, 1998).

The proteins that must be produced in rat fibroblasts, MDCK cells, or proximal tubule cells for apoptosis to occur remain to be determined. However, the fact that cycloheximide acts as a protectant against gentamicin-induced apoptosis in rat fibro-

TABLE 2
Influence of Bcl-2 Overexpression on Gentamicin-induced Apoptosis

	Apoptotic index (% of TUNEL (+) cells)	
	Bcl-2 (+) transfectants[a]	Bcl-2 (–) transfectants[b]
MDCK cells	$3.3 \pm 1.0^{+,\S}$	21.0 ± 5.2
LLC-PK_1 cells	$5.4 \pm 2.9^{+,\S}$	22.9 ± 1.3

Note. Cells were exposed to gentamicin (2 mM) for 4 days.
[a]Cells transfected with a *bcl-2* gene-containing vector.
[b]Cells transfected with a control vector.
[+]Significantly lower than the Bcl-2 (-) group ($p < 0.001$).
[§]Not significantly different from untransfected cells not exposed to gentamicin (see Fig. 4).

TABLE 3
Activity of Caspases in Control MDCK Cells and in Transfected MDCK Cells Exposed to Gentamicin

Activity (fluorescence arbitrary units)[a]			
Control cells[b]	Untransfected cells[c]	Bcl-2 (+) transfectants[c,d]	Bcl-2 (–) transfectants[c,e]
266.9 ± 11.4	444.4 ± 11.6*	290.3 ± 5.2	410.6 ± 12.9*

[a]Using DEVD-AMC as substrate (this substrate is cleaved by caspases 3, 6, 7, and 8).
[b]Untransfected cells not exposed to gentamicin.
[c]Gentamicin-treated cells (cells incubated for 4 days with 2 mM gentamicin).
[d]Cells transfected with a *bcl-2* gene-containing vector.
[e]Cells transfected with a control vector.
*Significantly larger than control cells ($p < 0.001$) and than Bcl-2(+) transfected cells ($p < 0.001$).

blasts and MDCK cells virtually excludes the possibility that the cytotoxicity of gentamicin could be related to an inhibition of protein synthesis, as suggested from studies with the atypical aminoglycoside hygromycin, G-418 (Buchanan *et al*., 1987); G-418, however, also causes apoptosis by itself in a Bcl-2-preventable manner (Chen *et al*., 1995). We have no simple explanation to offer for the unanticipated finding that LLC-PK_1 cells undergo apoptosis in the presence of cycloheximide alone, especially since these cells have been claimed to show marked protein synthesis inhibition when exposed to gentamicin (Okuda *et al*., 1992). Possibly, LLC-PK_1 cells constitutively express the elements of apoptosis machinery and cycloheximide as well as gentamicin could act by abolishing the production of short-lived proteins acting as apoptosis antagonists.

Where and how gentamicin initiates apoptosis was not established by our experiments. Yet, the fact that apoptosis develops slowly over time and in strict correlation with cellular drug levels strongly suggests that it is the cell-associated drug which is acting as the initiator. Aminoglycosides have been previously shown to slowly enter both fibroblasts and LLC-PK_1 cells and to accumulate in their lysosomes (Ford *et al.*, 1994; Tulkens and Trouet, 1978). Because the clearance of gentamicin by all 3 cell types was essentially similar in our conditions, we suspect that the drug enters these cells by a common mechanism. For fibroblasts, this mechanism has been shown to be fluid-phase pinocytosis, i.e., a constitutive process of uptake of non-diffusible solutes occurring in all eucaryotic cells (Mukherjee *et al.*, 1997). Gentamicin uptake in fibroblasts is indeed non-saturable (viz. the present experiments and Tulkens and Trouet, 1978), and its rate of influx is very similar to that of an authenticated marker of fluid-phase endocytosis in the same cells (Cupers *et al.*, 1994). Cell fractionation and morphometric studies have shown that gentamicin pinocytosed by fibroblasts and accumulated in lysosomes reaches, therein, concentrations at least 30-fold larger than in the extracellular medium (Aubert–Tulkens *et al.*, 1979).

Less is known about the concentration of gentamicin in the lysosomes of LLC-PK_1 cells, where part of the drug may traffic to the Golgi vesicles (Sandoval *et al.*, 1998), but animal studies have revealed a huge accumulation of gentamicin in lysosomes of proximal tubular cells (Giurgea-Marion *et al.*, 1986), suggesting that this property is probably very general. Gentamicin stored in lysosomes, as well as in Golgi vesicles, is expected to become fully protonated and therefore strongly polycationic, due to the acidic pH (~5 to ~6) prevailing in these compartments (Anderson and Pathak, 1985; Ohkuma and Poole, 1978). The pK_a's of the ionizable groups in aminoglycosides span from ~5.5 to 9.5 (Claes *et al*., 1977). It is probably in this context that the role of intracellular gentamicin as an initiator of apoptosis must be examined.

One of the most early and conspicious alterations related to the accumulation of gentamicin in lysosomes was the development of a phospholipidosis which is directly related to the capacity of the intralysosomal polycationic drug to bind to phospholipid bilayers (see Laurent *et al*, 1990 for review, Mingeot-Leclercq *et al*., 1988 and Piret *et al.*, 1992 for a discussion of the molecular mechanisms). This phospholipidosis was clearly observed here for fibroblasts and MDCK cells but developed only to a minimal extent in LLC-PK_1 cells in our

TABLE 4
Influence of the N-Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl-ketone (Z-VAD.fmk, a non-specific inhibitor of caspases) on Gentamicin-induced Apoptosis in MDCK Cells

Apoptotic index (% of TUNEL [+] nuclei)		
Control cells[a]	No Z-VAD.fmk[b]	Plus Z-VAD.fmk[c]
2.9 ± 0.7	22.9 ± 1.9*	1.4 ± 0.6

[a]No treatment (cells incubated with Z-VAD.fmk alone for the last 2 days of culture did not show significant change in apoptotic index).
[b]Gentamicin-treated cells (cells were treated for 4 days with 2 mM gentamicin only).
[c]Gentamicin-treated cells (cells were treated for 2 days with 2 mM gentamicin and incubation was then carried on in the same medium, supplemented with 50 μM Z-VAD.fmk, for 2 additional days).
*Significantly larger than control ($p < 0.001$).

conditions, suggesting that it is probably not the key initiator of apoptosis. Lysosomal phospholipidosis may, however, develop also in LLC-PK_1 cells if incubation is prolonged (Schwertz *et al.*, 1986). This set of data therefore suggests that it is rather the drug accumulation *per se* which is most critical. It may be envisioned that the large concentrations of gentamicin in lysosomes (30 mM or more [see above]) will eventually cause a destabilization of their membrane resulting in their *in situ* disruption, which, by itself, is likely to trigger apoptosis. We showed indeed that millimolar concentrations of gentamicin permeabilizes liposomes at pH 5.5 (Van Bambeke *et al*., 1993). We know of at least one clear example where disruption of lysosomes *in situ* (by photooxidation) causes apoptosis (Brunk *et al*., 1997), probably by the release of the lysosomal proteases and endonucleases in the cytosol (Ohsawa *et al*., 1998). We also know that the relocation of the lysosomal proteases, cathepsins B and D in the cytosol, triggers apoptosis (Ohsawa *et al.*, 1998), possibly by direct activation of caspases (Vancompernolle *et al.*, 1998). In this context, it may even be speculated that phospholipidosis, which entails a partial binding of gentamicin to the intralysosomally accumulated phospholipids, may actually be protective, since it will reduce the amount of free drug available for binding to the lysosomal membrane. This could be the basis for the higher susceptibility of LLC-PK_1 cells compared to the other 2 cell types studied here.

At first glance, the hypothesis of gentamicin-induced release of lysosomal proteases and of a direct activation of caspases may seem to fail to account for the protective effect, which acts upstream of the caspases involved in the execution phase. Yet, we cannot exclude that lysosomal disruption activates caspase 8, which is involved in the initiation phase (the substrate used to assay for caspase activity is cleaved by this enzyme as well as by caspases 3, 6, and 7, and the inhibitor Z-VAD.fmk is a non-specific inhibitor of most caspases). We may also envisage that lysosomal destabilization triggers mitochondrial events such as the so-called "permeability transition" (Lemasters *et al.*, 1998) and/or the release of the apoptosis-inducing-factor (AIF) (Susin *et al.*, 1999), which will then cause apoptosis in a Bcl-2-preventable or -retardable manner. Other alternative explanations than a lysosomal origin in the triggering of gentamicin-induced apoptosis must, however, also be envisioned. Gentamicin, indeed, has been shown to modulate membrane enzyme activities, to cause changes in membrane fluidity and increases in cytosolic Ca^{++} concentration, and to directly alter the pericellular membrane and mitochondrial membranes (Holohan *et al.*, 1988; Jutila *et al.*, 1998; Okuda *et al.*, 1992; Van Bambeke *et al.* 1993). All these changes are known to trigger apoptosis (Lang and Liu, 1997; Umankskii, 1996).

The data presented here provides, however, no direct support for any of these potential mechanisms although they do not rule them out either. Conversely, the data plead against a potential role for gentamicin binding to megalin or other cell surface constituents. Binding to megalin and acidic phospholipids undoubtedly takes place in proximal tubular cells *in vivo* and is responsible for preferential uptake of aminoglycosides by these cells (Moestrup *et al.*, 1995). Yet, the rates of gentamicin influx observed here in LLC-PK_1 and in MDCK cells, which are slightly lower than those of fibroblasts, preclude a major role of membrane binding in gentamicin uptake in these cells, under our conditions. In addition, there is no evidence of the presence of megalin in fibroblasts, and we show here that these cells are only slightly less sensitive to gentamicin when compared to LLC-PK_1, as far as apoptosis is concerned.

The toxicological implications of our findings need to be critically assessed. We see that a ~20% apoptotic index is reached in LLC-PK_1 cells when these contain 20 μg of gentamicin per mg of protein. In comparison, animals treated for 10 days with a clinically relevant dose of gentamicin (10 mg/kg), and which demonstrate an extensive apoptotic process in their proximal tubule epithelium, contain ~10 μg of drug per g of cortex (El Mouedden *et al.*, 2000; see the discussion in this paper for estimates of the equivalents of human and animal doses of aminoglycosides). Because the drug that accumulated in kidney *in vivo* was found almost exclusively in proximal tubules (Kuhar *et al.*, 1979), where it localizes mainly in lysosomes (Giurgea-Marion *et al.*, 1986), these cortical levels will translate into local drug concentrations of ~15 to 50 mM (see Laurent *et al.*, 1983 for estimates of the fractional volume of proximal tubules and Wilmotte *et al.*, 1983 for measurements of the aggregated volume of lysosomes in the kidney cortex of rats treated for 10 days with gentamicin at 10 mg/kg). Thus, the lysosomal concentrations of gentamicin at the level of both the target cells (proximal tubules) and in the cultured cells are of the same order of magnitude. This clearly supports the validity of the cultured cells model to study the *in vivo* situation. The fact that gentamicin-induced apoptosis is observed in the 2 renal cell lines to a similar extent, i.e., disregarding their cytological origin, as well as in fibroblasts, coupled with the observation that apoptosis has been recognized as an important determinant in aminoglycoside-induced toxicity towards hair cells in the cochlea (Lang and Liu, 1997) also indicates that we are probably dealing with an intrinsic toxicological property of aminoglycosides of potentially broad significance.

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