Radiocontrast-induced DNA fragmentation of renal tubular cells in vitro: role of hypertonicity

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Abstract

Background. Radiocontrast-induced nephropathy is a clinically important complication of invasive cardiological procedures. It has been associated with DNA fragmentation of renal tubular cells, which is a hallmark feature of programmed cell death (apoptosis). We investigated the mechanism of this DNA fragmentation in an in vitro model of radiocontrast cytotoxicity on renal epithelial cells.

Methods. Madin Darby canine kidney (MDCK) cell monolayers were incubated (for 2–8 h) with isosidone doses (37–111 mg iodine/ml) of the highly hyperosmolal, ionic radiocontrast agent diatrizoate or of the less hyperosmolal, non-ionic substance iopamidol. Mannitol, urea, and NaCl control media of corresponding hyperosmolality were used to evaluate the contribution of hypertonicity, hyperosmolality and/or ionic strength to radiocontrast toxicity. DNA fragmentation was assessed using fluorescence-activated cell sorting (FACS), agarose gel electrophoresis and terminal deoxynucleotidyl transferase-mediated deoxyuridine nick end labelling (TUNEL), cell morphology was analysed in Giemsa-stained cytospins.

Results. Diatrizoate induced concentration- and time-dependent DNA fragmentation of MDCK cells which was associated with morphological signs of apoptosis. Cycloheximide (1 μg/ml) did not prevent diatrizoate-induced DNA fragmentation, indicating that it is not dependent on protein synthesis. Diatrizoate-mediated cell death was associated with cell detachment from the tissue culture matrix. However, the DNA fragmentation is not a consequence of cell detachment since the prevention of cell attachment on agarose-coated dishes induced significantly less DNA fragmentation than diatrizoate. Iopamidol caused no detectable DNA breakdown. In contrast, hypertonic mannitol and sodium chloride, but not hyperosmolal urea, induced DNA fragmentation in MDCK cells, albeit less than diatrizoate.

Conclusions. The DNA fragmentation of MDCK cells induced by diatrizoate is related to its hypertonicity in this in vitro model of radiocontrast cytotoxicity. Nuclear disintegration with subsequent cell death may contribute to the pathophysiology of radiocontrast-induced nephropathy, particularly in the hypertonic/hypoxic environment of the renal medulla. The present results underscore the importance of avoiding hyperosmolal urine states in patients at high risk of radiocontrast-induced nephropathy.

Key words: apoptosis; cytotoxicity; hypertonicity; MDCK; radiocontrast; renal failure

Introduction

Radiocontrast-induced nephropathy is a complication of intravascularly applied radiocontrast agents, particularly in patients with compromised renal function [1,2]. Patients undergoing cardiac catheterization often have pre-existing renal insufficiency due to diabetes mellitus, hypertension and/or generalized atherosclerosis. The pathogenesis of radiocontrast-induced nephropathy is not clear. In vivo radiocontrast agents may induce renal hypoperfusion resulting in hypoxic cell damage in the renal medulla, where the cells operate at the brink of hypoxia even under physiological conditions [3–5]. In a rat model of radiocontrast-induced nephropathy the cellular injury in the renal medulla consisted of extensive DNA fragmentation, which has been attributed to medullary hypoxia [6]. Nucleosomal DNA breakdown is a hallmark feature of programmed cell death [7,8].

In addition to haemodynamic mechanisms, direct cytotoxic effects of radiocontrast agents may contribute to radiocontrast-induced nephropathy [9–12]. We investigated the mechanism of radiocontrast-induced cell death in an in vitro model of radiocontrast cytotoxicity on the permanent renal epithelial Madin Darby...
canine kidney (MDCK) cell line. We assessed nucleosomal DNA fragmentation as an indicator of programmed cell death by a variety of independent methods: morphological features of apoptosis (e.g. cell shrinkage, chromatin condensation, nuclear pyknosis and fragmentation) were analysed with Giemsa staining, while direct evidence of DNA fragmentation was obtained using fluorescence-activated cell sorting (FACS) [13], agarose gel electrophoresis [7], and terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end labeling (TUNEL) [14].

The results of the present study show that the in vitro toxicity of radiocontrast agents is associated with DNA fragmentation even in the absence of hypoxia. Furthermore we provide evidence that the DNA fragmentation in this model is related to the hypertonicity, but not the iodine content of the radiocontrast agents.

Subjects and methods

Cell culture

MDCK II cells were obtained from Dr K. Simons (EMBL, Heidelberg, Germany). The cells were cultured under normoxic conditions at 37 °C in a humified atmosphere containing 5% CO₂. The culture medium was MEM (Minimum Essential Medium, Life Technologies, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all reagents from Eurobio, Les Ulis, France). Confluent MDCK cell monolayers grown in T25 tissue culture flasks (Greiner, Frickenhausen, Germany; Nunc, Roskilde, Denmark) were washed once with HBSS (Hank's buffered salt solution, Eurobio, Les Ulis, France) and incubated under normoxic conditions for 2–8 h with the experimental solutions. All cells (detached and adherent) were harvested by media collection, res. trypsinization followed by centrifugation for 8 min at 150 g. The resulting pellets were washed twice and evenly resuspended in 5 ml HBSS. Cells from 1 ml of the suspension (about 1.8–2.0 × 10⁶ cells) were spun onto glass microscope slides and processed for morphological evaluation using a standard Giemsa staining protocol. The cyto-}

Experimental solutions

MDCK cell monolayers were incubated under normoxic conditions for 2–8 h with isodine concentrations (37–111 mg iodine/ml i.e. 10–30% of the highly hyperosmolar, ionic radiocontrast agent diatrizoate (Schering, Berlin, Germany) or of the less hyperosmolar, non-ionic compound iopamidol (Byk Gulden, Konstanz, Germany). Since the induction of DNA fragmentation has been related to changes in cell volume in other experimental systems [15], we tested mannitol control solutions (non-ionic) of corresponding hyperosmolality. To evaluate a possible effect of ionic strength, MDCK cells were also incubated with hyperosmolar NaCl solutions. Both mannitol and NaCl solutions cause cell shrinkage by their hypertonicity. As an additional control experiment the toxicity of equally hyperosmolar urea solutions was analysed. Because of the cell membrane permeability of this solute, urea solutions are hyperosmolar, but not hypertonic and therefore do not induce significant changes in cell volume. To exclude an induction of DNA fragmentation due to dilution of growth factors in the experimental media, the growth medium of the cells was diluted with 0.9% NaCl thereby maintaining iso-osmolality, res. isotonicity. MDCK cells were coincubated with cycloheximide (CHX, 1 or 10 μg/ml, Fluka, Buchs, Switzerland) and the hypertonic solutions in order to assess the influence of protein synthesis on the DNA breakdown. Osmalalities of all experimental solutions were measured at room temperature using a vapour pressure osmometer (Wescor, Logan UT, USA). Table 1 shows the osmolalities of the culture media containing the radiocontrast agents in the studied concentrations.

Table 1. Osmolality and iodine concentration of the culture media containing radiocontrast agents in the studied concentrations. Osmolality was measured at room temperature with a vapour pressure osmometer (mOsm/kg)

<table>
<thead>
<tr>
<th>Radiocontrast concentration</th>
<th>Iodine content</th>
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<tbody>
<tr>
<td></td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>(37 mg/ml)</td>
</tr>
<tr>
<td>Diatrizoate</td>
<td>450</td>
</tr>
<tr>
<td>Iopamidol</td>
<td>365</td>
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</table>
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'hypodiploid' nuclei was determined as described by Nicoletti et al. [13].

**DNA electrophoresis**

The internucleosomal DNA fragmentation was directly demonstrated using agarose gel electrophoresis according to the method described by Szondy [16]. Suspended cells were centrifuged (8 min, 150 g) and the pellets were resuspended in 250 μl of ice-cold Tris-EDTA buffer (TE, 15 mM Tris, 1 mM EDTA, pH 8.0). The suspensions were transferred into microfuge tubes and incubated with 250 μl ice-cold lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) for 30 min at 4°C. After centrifugation for 15 min at 13 000 r.p.m. at 4°C, the supernatants containing the fragmented, cytoplasmic DNA were transferred into new Eppendorf tubes. The cytoplasmic DNA was precipitated overnight at −20°C by addition of 75 μl 2 M NaCl and 1 ml 96% ethanol per tube. The precipitates were centrifuged (15 min, 13 000 r.p.m., 4°C) and dried at room temperature. They were then redissolved in 500 μl TE and incubated with 10 μg RNase A (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C. After proteinase K treatment (200 μg, 1 h, 37°C; Boehringer Mannheim, Mannheim, Germany) the DNA was re-precipitated overnight, centrifuged and dried as described above. The pellet was dissolved in 16.7 μl TE and 3.3 μl load buffer (30% glycerol, 0.25% bromphenol blue in TE, pH 8.0). The DNA samples were loaded onto a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and subjected to agarose gel electrophoresis in TBE (90 mM Tris, 90 mM boric acid, 3 mM EDTA) at 100 V. The DNA ladders were visualized by UV illumination.

**Statistical analysis**

The data were analysed using single factor ANOVA and two-sample F-test to determine variance. Student’s t test was used where applicable.

**Results**

**Diatrizoate-induced DNA fragmentation**

Diatrizoate-induced concentration- and time-dependent DNA fragmentation in MDCK cells. Figure 1 shows the concentration-dependence of this DNA breakdown during a 4-h incubation period using FACS analysis of isolated nuclei stained with propidium iodide. The percentage of nuclei with reduced DNA content ('hypodiploid') was compared with the DNA content of the normal cell population. Even at a concentration of 10% (37 mg iodine/ml) diatrizoate induced a significant (P < 0.01) DNA breakdown compared to control medium. Increasing concentrations of diatrizoate (20 and 30%) induced further concentration-dependent DNA fragmentation (P < 0.01).

The time-dependence of diatrizoate-induced DNA fragmentation is shown in Figure 2 using the same FACS protocol. MDCK cell monolayers were incubated with 20% diatrizoate (74 mg iodine/ml) for 2–8 h. Compared to control monolayers incubated in parallel with regular growth medium the percentage of 'hypodiploid' nuclei was significantly increased starting 2 h after exposure to diatrizoate and continued to rise progressively up to the maximum studied period of 8 h, at which time >80% of nuclei were 'hypodiploid' (Figure 2).

Apoptotic cells in Giemsa-stained cytospins from MDCK cell monolayers were identified by their morphological features (Figure 3). Confluent MDCK cell monolayers were incubated with 20% diatrizoate or regular growth medium. Characteristic morphological signs of apoptosis (cell shrinkage, nuclear pyknosis, chromatin condensation, phagocytosis by neighbouring cells) were detected frequently in diatrizoate-treated cells (Figure 3C), but were exquisitely rare in control cells (Figure 3A).

In addition to the cytomorphological evidence of apoptotic cell death we examined the cytospins for DNA fragmentation using the TUNEL method. Figure 3D demonstrates a representative example of a cytospin from a diatrizoate-treated monolayer in which several TUNEL-positive nuclei/nuclear fragments stand out, whereas cells from the control monolayer showed only very little evidence of nuclear disintegration (Figure 3B).

The internucleosomal DNA fragmentation was directly demonstrated by agarose gel electrophoresis of cytoplasmic DNA isolated from diatrizoate-treated
and control MDCK cell monolayers. Figure 4 shows a representative experiment: DNA isolated from a diatrizoate-treated monolayer (and from a monolayer treated with hypertonic mannitol, see below) exhibits the characteristic approximately 200 bp internucleosomal DNA cleavage pattern (DNA 'laddering'), which is not seen in two independent, untreated control monolayers.

**Iodine content and dilution of growth factors**

To evaluate the role of iodine concentration, isiodine doses of the less hyperosmolar, non-ionic radiocontrast agent iopamidol were also tested. Since growth factor deprivation may induce DNA fragmentation [17,18], we studied the effects of media dilution using isotonic NaCl. MDCK cell monolayers were incubated for 6 h with the experimental solutions and DNA fragmentation was analysed by FACS.

In contrast to diatrizoate, neither isiodine doses of iopamidol nor media diluted to a similar degree with NaCl induced detectable DNA fragmentation (Figure 5).

**Hypertonicity and/or ionic strength**

Since diatrizoate and iopamidol are different with respect to osmolality and ionic strength, we tested the effects of control solutions of similar hypertonicity, hyperosmolality or ionic strength on cellular DNA breakdown. Therefore we used mannitol as a non-ionic, membrane-impermeable substance (non-ionic hypertonicity), urea as a non-ionic, membrane-permeable compound (normotonic hyperosmolality) and sodium chloride, as a functionally membrane-impermeable control solute (ionic hypertonicity). MDCK cells were incubated for 2–8 h with experimental media and the DNA fragmentation was detected using FACS. The results are shown in Figure 6: like diatrizoate, hypertonic mannitol and sodium chloride solutions also induced significant DNA fragmentation in MDCK cells \(P < 0.01\) at 6 h. However, at all time points diatrizoate induced more DNA fragmentation than NaCl, which in turn caused more extensive DNA breakdown than mannitol. These differences became significant \(P < 0.01\) after 6 h of incubation. Urea, which is hyperosmolar, but not hypertonic, caused only a minor increase in the rate of DNA fragmentation, which was not significant at the \(P < 0.01\) level after 8 h of incubation (Figure 6).

**Cell detachment and DNA fragmentation**

Diatrizoate-mediated cell death was associated with extensive cell detachment. Since it has been reported that the loss of cell-matrix interactions can induce DNA fragmentation in MDCK cells [19], we tested whether the loss of cell attachment by itself can explain the diatrizoate-induced DNA fragmentation. In order to prevent cell attachment, trypsinized cells from confluent MDCK cell monolayers were washed with MEM (containing 10% FCS as trypsin inhibitor) and seeded onto agarose-coated surfaces, which are known to prevent cell attachment [20]. Cells were then incubated with regular growth medium or 20% diatrizoate in MEM. Preventing cell attachment caused an insignificant increase in DNA fragmentation compared to that of attached cells under similar conditions \(P > 0.05\) for both regular medium and diatrizoate. Figure 7). Diatrizoate caused significantly more DNA breakdown in adherent cells than the prevention of cell attachment under control conditions \(P < 0.01\), Figure 7), suggesting additional mechanisms besides cell detachment.

**DNA fragmentation and protein synthesis**

Since programmed cell death depends on protein synthesis in some experimental models of apoptosis [21,22], we investigated whether inhibition of protein synthesis by cycloheximide (CHX) can prevent diatrizoate/hypertonicity-induced DNA fragmentation in MDCK cells. A 6-h coincubation of confluent MDCK cell monolayers with CHX and hypertonic solutions did not prevent DNA fragmentation (Figure 8). Under isotonic conditions a low concentration of cycloheximide \(1 \mu g/ml\) had no effect on DNA fragmentation. However, CHX significantly \(P < 0.01\) increased the DNA breakdown induced by diatrizoate, mannitol and hypertonic NaCl medium. A higher concentration of
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Fig. 3. Cell morphology and DNA fragmentation. Cytospins of control (A,B) and diatrizoate-treated (C,D, 20%, 6 h) MDCK cells. Left (A,C), giemsa staining. Arrows show characteristic morphological signs of apoptosis such as cell shrinkage, nuclear pyknosis, and chromatin condensation. Right (B,D) DNA nick end labelling (TUNEL counterstained with Harris’ haematoxylin). Arrows show TUNEL-positive nuclei/nuclear fragments.

CHX (10 μg/ml) caused DNA fragmentation even under control conditions, and significantly increased the hypertonicity-induced DNA breakdown (data not shown). Thus, CHX not only failed to prevent, but caused DNA fragmentation in this experimental model of radiocontrast/hypertonicity-induced DNA breakdown.

Discussion

Apoptosis plays an important role in the kidney. This is true not only during embryonic development [23,24] and regeneration [25], but also under pathological conditions like cystic renal diseases [26] and acute renal failure [27]. Apoptotic cells have been documented in experimental animals after renal ischaemia [28], and in a rat model of radiocontrast nephropathy [6]. In this latter model the radiocontrast-induced renal injury was associated with DNA fragmentation of renal tubular cells in the thick ascending limb of Henle’s loop. This was attributed to medullary hypoxia, which is an important pathogenic factor for radiocontrast-induced nephropathy [4].
Fig. 5. Effects of iodine concentration and dilution of growth factors on DNA fragmentation (FACS analysis). MDCK cell monolayers were incubated for 6 h with experimental solutions. Control medium, MEM; diatrizoate, 74 mg iodine/ml (20%); iopamidol, 74 mg iodine/ml (20%); 0.9% NaCl, MEM diluted 1:4 with isotonic NaCl solution. Mean ± SEM; n as indicated; *P < 0.01.

However, in addition to local (medullary) hypoxia and/or medullary hypoperfusion [29] direct cytotoxic effects may likewise contribute to the pathogenesis of radiocontrast-induced nephropathy [9,11,12]. The cytotoxicity of radiocontrast agents on isolated renal tubular segments in vitro is aggravated by hypoxia [10] and hypoxia has been shown to induce DNA fragmentation of MDCK cells in vitro [30]. Here we tested the hypothesis that radiocontrast agents may induce DNA fragmentation of MDCK cells even in the absence of hypoxia. Previously we have shown that MDCK cells are susceptible to the toxic effects of radiocontrast agents and that their cytotoxicity is associated with phenotypic alterations resulting in the redistribution of polarized MDCK cell surface marker proteins [12].

The present findings show that the ionic radiocontrast agent diatrizoate induces time- and concentration-dependent DNA fragmentation of MDCK cells in vitro under normoxic conditions (Figures 1–4). This was to a large extent related to its hypertonicity, since equally hyperosmolar but less hypertonic urea solutions failed to induce DNA fragmentation (Figure 6). The iodine content of the radiocontrast agent was irrelevant for the induction of DNA breakdown, as equal concentrations of the non-ionic, less hypertonic substance iopamidol did not result in DNA cleavage (Figure 5). However, the DNA fragmentation depended not solely on colligative properties of the media; ionic strength may mediate part of the toxic effects of diatrizoate since NaCl solutions were more toxic than equally hypertonic mannitol media (Figure 6). A significant role of growth factor deprivation due to media dilution was excluded by incubating the cells with isotonically diluted media without apparent toxicity or phenotypic alterations (Figure 5).

Incubation with high concentrations of diatrizoate induced cell detachment. The loss of cell-matrix interactions can induce a specific form of apoptosis in MDCK cells for which the term ‘anoikis’ has been coined [19]. However, anoikis was observed in MDCK cells after 8 h [19], whereas the hypertonic solutions in the present study induced DNA fragmentation in as little as 2–4 h (Figures 2, 6). Even though cell detachment by itself was ineffective in causing DNA breakdown in the time frame of our experiments, there was a trend towards an increased toxicity of the hypertonic media in non-adherent cells (Figure 7). This is consistent with several reports that programmed cell death may be connected with cell-matrix interactions [31].

Cycloheximide failed to protect MDCK cells from diatrizoate/hypertonicity-induced DNA fragmentation (Figure 8). In contrast, CHX prevented glucocorticoid-induced apoptosis in thymocytes [16,21] and stress-associated programmed cell death in L cells [22]. On the other hand, cycloheximide also failed to inhibit the
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Fig. 7. Effect of inhibition of cell adhesion on DNA fragmentation. Cells were plated on agarose (non-adherent, closed bars) or on tissue culture plastic (adherent, open bars) and incubated for 6 h with control medium or 20% diatrizoate. Under control conditions and during incubation with 20% diatrizoate the DNA fragmentation rates of adherent and suspended MDCK cells were not significantly different (NS, \(P>0.05\)). Diatrizoate treatment of adherent cells caused significantly more DNA breakdown than the prevention of cell adhesion alone (\(P<0.01\)). Mean \(\pm\) SEM; n as indicated.

Fig. 8. Effect of cycloheximide (CHX, 1 \(\mu\)g/ml) on hypertonicity-induced DNA fragmentation. Confluent MDCK cell monolayers were incubated for 6 h with experimental solutions either in the presence or absence of 1 \(\mu\)g/ml cycloheximide (open bars, without CHX; closed bars, with CHX). Control medium, MEM; 0.9% NaCl, MEM diluted with 20% isotonic NaCl solution; diatrizoate, 20%; mannitol, hypertonic NaCl, same hyperosmolality as 20% diatrizoate. n as indicated; *\(P<0.01\).

Conclusions

Diatrizoate induces time- and concentration-dependent DNA fragmentation of MDCK cells \textit{in vitro} under normoxic conditions which is mainly due to its hypertonicity/ionic strength. The diatrizoate-induced DNA fragmentation in renal epithelial cells \textit{in vitro} suggests that programmed cell death plays a role in radiocontrast-induced acute renal failure, particularly in the hypertonic/hypoxic environment of the renal medulla. The present results provide further rationale for avoiding hyperosmolar urine states in patients undergoing invasive cardiological procedures, especially in patients with pre-existing renal failure.

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References

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anoikis of MDCK cells [19] and was toxic in other experimental cell systems [32–34], including the present one. Even though low concentrations of CHX were relatively innocuous on MDCK cell monolayers under control conditions, the combined incubation with CHX and hypertonic solutions not only failed to protect the cells from DNA fragmentation but increased the rate of DNA breakdown significantly (Figure 8).

In agreement with \textit{in vivo} observations that have demonstrated apoptotic tubular cells in acute renal failure [6,35] our results suggest that DNA fragmentation of renal tubular cells may contribute to radiocontrast-induced nephropathy. The present study demonstrates for the first time that this can occur in the absence of hypoxia and/or hypoperfusion. Furthermore, it is independent of the iodine content, but correlates with the hypertonicity/ionic strength of the radiocontrast agents. Hence, these data provide an explanation at the cellular level for the clinical experience that non-ionic, less hypertonic radiocontrast agents may be less nephrotoxic than ionic, hyperosmolar compounds.
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ionic and non-ionic contrast media in 1196 patients: a random-

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