

## Role of Bcl-xL in paracetamol-induced tubular epithelial cell death

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### Role of Bcl-xL in paracetamol-induced tubular epithelial cell death.

**Background.** Paracetamol overdose causes acute renal failure and chronic exposure to paracetamol has been linked to chronic renal failure. Recently, apoptosis induction has been identified as a possible mechanism of paracetamol nephrotoxicity.

**Methods.** Murine proximal tubular epithelial MCT cells were cultured in the presence of paracetamol. The effects of Bcl-xL overexpression, Bax antisense oligodeoxynucleotides, and different caspase inhibitors on cell death were studied.

**Results.** While paracetamol did not change the mRNA expression of the antiapoptotic gene *bcl-xL*, it decreased Bcl-xL protein levels. The decrease in Bcl-xL was prevented by lactacystin, but not by caspase inhibitors. Addition to the culture media of the survival factors present in fetal calf serum (FCS) increased Bcl-xL expression and decreased paracetamol-induced apoptosis. Overexpression of a human *bcl-xL* transgene decreased apoptosis induced by paracetamol by 60% at 24 hours and increased long-term cell survival. The constitutive expression of the viral caspase inhibitor CrmA decreased the rate of apoptosis by 60% at 24 hours and the broad-specific caspase inhibitor zVAD-fmk prevented paracetamol-induced features of apoptosis. However, caspase inhibitors did not prevent eventual cell death. Bax did not translocate to mitochondria and Bax antisense oligodeoxynucleotides were not protective.

**Conclusion.** Our results suggest that induction of apoptosis may underlie the nephrotoxic potential of paracetamol and identify Bcl-xL as a player in toxic tubular cell injury.

Paracetamol is an over-the-counter analgesic with nephrotoxic potential [1–6]. Paracetamol overdose is a cause of acute liver and renal failure in humans and experimental animals [2, 3, 7]. Acute nephrotoxicity by paracetamol is characterized by morphologic and functional (phosphaturia, low-molecular-weight proteinuria)

evidence of proximal tubular injury in humans and experimental animals [2, 3, 7]. Acute renal failure in the course of therapeutic paracetamol administration has also been described in alcoholics [3]. In addition, a chronic nephrotoxic effect of paracetamol is suggested by case-control studies [4–6]. These findings have led to the recommendation that paracetamol be used in limited amounts and for limited time periods, pending definitive confirmation of its nephrotoxic potential [8]. Research into the biologic basis of paracetamol nephrotoxicity has been encouraged by a National Kidney Foundation Ad Hoc Committee [8].

Tubular cell loss is a characteristic feature of both acute renal failure and chronic renal disease (reviewed in [9]). Tubular cell loss is observed when cell death predominates over mitosis. Apoptosis is an active form of cell death that offers the opportunity for therapeutic intervention [9, 10]. Paracetamol promotes hepatocyte and renal tubular cell apoptosis [11, 12]. In renal proximal tubular epithelial cells, paracetamol activates caspases-9 and -3. In addition there is evidence for the involvement of endoplasmic reticulum injury, including GADD153 translocation to the nucleus and proteolysis of caspase-12 [12]. Several endogenous proteins protect mammalian cells from apoptosis. One of the most important is Bcl-xL, a member of the Bcl-2 family [13]. Bcl-xL protects cells from a wide variety of apoptotic stimuli [13], and has recently been shown to be regulated in the course of nephrotoxic acute renal failure [14]. However, Bcl-xL does not offer protection, in a cell-specific fashion, against certain stimuli, such as death receptor-mediated apoptosis [15, 16]. Moreover, caspase-generated Bcl-xL fragments are proapoptotic [17]. The role of Bcl-xL in protection from nephrotoxic drugs had not been previously addressed.

Tubular cell injury is the main feature observed during paracetamol-induced renal failure both in humans and animal models [2, 3]. Because expression of the protective protein Bcl-xL has been shown to be regulated in tubular cells during experimental toxic acute renal failure [14], we investigated the role of Bcl-xL in paracetamol-induced

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apoptosis of cultured mouse renal proximal tubular epithelial cells.

## METHODS

### Cell lines and cell culture

MCT cells are a cultured line of proximal tubular epithelial cells harvested originally from the renal cortex of SJL mice [18]. The cells were maintained in culture in RPMI 1640 (Gibco, Grand Island, NY, USA), 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, at 37°C in 5% CO<sub>2</sub>, as previously described [18]. Human kidney 2 (HK-2) cells, an immortalized human proximal tubular epithelial cell line (ATCC, Rockville, MD, USA) were grown on the same media as the MCT cells plus 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL sodium selenite, and 5 ng/mL hydrocortisone. For experiments on the effect of paracetamol, cells were plated and then grown for 24 hours in RPMI-10% FCS. Then the media was replaced with fresh serum-free RPMI (RPMI-0%), unless otherwise stated. RPMI-0% contained the same supplements as the serum-supplemented RPMI with the exception of the FCS. A concentration of 300 µg/mL paracetamol was chosen for studies on the intracellular pathways of cell death because it is in the range found in clinical paracetamol intoxication and readily induces apoptosis in murine proximal tubular cells [12].

MCT cell lines constitutively expressing Bcl-xL, CrmA, or control vectors were established. Bcl-xL overexpressing MCT cells express two- to threefold more Bcl-xL than control vector-transfected cells and the rate of proliferation is slightly slower than control [14]. MCT cell lines stably overexpressing CrmA, cloned into pcDNA3.1, were a gift from T.M. Danoff (University of Pennsylvania) and E.G. Neilson (Vanderbilt University).

The following caspase inhibitory peptides were used: the caspase-3 inhibitor, Z-Asp(OMe)-Glu(OMe)-Val-DL-Asp(OMe)-fluoromethylketone (DEVD-fmk), and the pancaspase inhibitor, Z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk), from Bachem (Bubendorf, Switzerland), the caspase-8 inhibitor, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (IETD-fmk), the caspase-9 inhibitor, Z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (LEHD-fmk), and the irreversible caspase-2 inhibitor, benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethyl ketone (z-VDVAD-fmk), from Calbiochem (San Diego, CA, USA). The caspase inhibitory peptides were dissolved in dimethyl sulfoxide (DMSO) and used at concentrations previously shown to protect from apoptosis in MCT cells or other cell systems [19]. Final concentration of DMSO was 0.05% and it did not influence MCT cell apoptosis [19]. Peptides or the proteasome inhibitor lactacystin (10 µmol/L) (Sigma

Chemical Co., St. Louis, MO, USA) were added to the cell culture 3 hours prior to paracetamol.

### Assessment of apoptosis

For quantification of cell death, 10,000 cells were seeded in 24-well plates (Costar, Schiphol-Rijk, The Netherlands) in RPMI-10%, and after 24 hours the cells were rested in RPMI-0% FCS for 24 hours. Thereafter, the cells were grown with the indicated stimuli in the latter medium. At defined time points, the cells were harvested from the wells. The nonadherent cells were pooled with the adherent cells, which were detached from the plate by gentle trypsinization. Apoptosis was quantified by flow cytometry analysis of DNA content in permeabilized, propidium iodide-stained cells, as previously described [20]. The percentage of cells with decreased DNA staining ( $A_0$ ), comprising apoptotic cells with fragmented nuclei, was counted. For expression of specific protection from paracetamol-induced apoptosis, apoptosis induced under control conditions (serum-free media with vehicle) was subtracted from apoptosis in the presence of paracetamol. Apoptosis in the presence of paracetamol alone was considered to be 100% and apoptosis in the presence of caspase inhibitor or Bcl-xL overexpression was expressed as a percentage of this [14].

To assess for the pyknotic nuclear changes seen in apoptosis, cells were plated onto Labtek<sup>TM</sup> slides (Nunc Inc, Naperville, IL, USA) in RPMI-10%. After 24 hours the media was changed to RPMI-0% and then grown for an additional 48 hours in the presence of paracetamol or vehicle. The cells were fixed in 10% buffered formalin and stained with hematoxylin-eosin.

### Colony-forming assay

The capacity of cells to survive and divide was assessed by a colony-forming assay. MCT cells were seeded in 12-well-plates and stimulated for 48 hours with 300 µg/mL paracetamol in the presence or absence of 200 µmol/L zVAD-fmk and others. They were then detached with trypsin-ethylenediaminetetraacetic acid (EDTA), washed in phosphate-buffered saline (PBS), seeded in Petri dishes and cultured in 10% FCS-RPMI for 7 days. They were then fixed in 5% formaldehyde/PBS and stained with 0.5% crystal violet/20% methanol for 2 minutes before being washed with water and photographed [19].

### Northern hybridization

For Northern blotting, 30 µg of total RNA were separated in 1% agarose gels containing 2.3% formaldehyde [21]. RNA was transferred to nylon membranes (GeneScreen Plus) (NEN, Perkin Elmer, Zaventem, Belgium) and prehybridized and hybridized at 65°C in 6× standard

saline citrate (SSC), 5× Denhardt's, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), 100 µg/mL salmon sperm DNA, and 100 µg/mL polyadenylic acid. The probes were labeled by random priming and added to the hybridization solution to a final activity of  $1.5 \times 10^6$  cpm/mL. After hybridization, blots were washed twice in 2× SSC for 15 minutes at room temperature, once in 2× SSC and 2% SDS for 45 minutes at 65°C, and once in 0.1× SSC and 0.1% SDS for 15 minutes at 65°C. The blots were then exposed to film at -70°C with the use of an intensifying screen. Blots were stripped and subsequently rehybridized with the probe for the housekeeping genes *G3PDH* or *28S*, to account for small loading or transfer variations. Autoradiograms were quantified using a GS-800 Calibrated Densitometer and Quantity One 4.4.0 software (Bio-Rad, Hercules, CA, USA), and the results expressed as arbitrary densitometry units related to the expression of *28S*. The probes for *bax* and *bcl-xL* have been described [14].

### Flow cytometry analysis of Bcl-x expression

For cytofluorography, cells were cultured in the presence of control medium or paracetamol. After washing the culture with PBS, adherent cells were detached with 2.2 mmol/L EDTA, 0.2% bovine serum albumin (BSA) in PBS, and  $5 \times 10^5$  cells. Single cell suspensions were fixed in 2% glutaraldehyde/PBS, washed in permeabilization buffer (0.01% saponin, FCS), incubated with 2 µg/mL polyclonal anti-Bcl-x<sub>S/L</sub> antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in permeabilization buffer for 30 minutes at 4°C, followed by incubation with 1:100 fluorescein isothiocyanate (FITC)-goat-antirabbit IgG for 30 minutes at 4°C. Control samples were stained with an anti-Bclx<sub>S/L</sub> antibody which had been previously incubated with a control Bclx peptide or with nonimmune rabbit IgG. Cells were then analyzed on a cytofluorograph. Dead cells and debris were excluded from analysis by selective gating based on anterior and right-angle scatter. At least 10,000 events were collected for each sample, and data were displayed on a logarithmic scale of increasing green fluorescence intensity.

### Western blot

Tissue or cell samples were homogenized in lysis buffer [50 mmol/L Tris HCl, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L ethyleneglycol tetraacetate (EGTA), 0.2% Triton X-100, 0.3% NP-40, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 µg/mL pepstatin A] then separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. After electrophoresis, samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp.,

Bedford, MA, USA), blocked with 5% skimmed milk in PBS/0.5% vol/vol Tween 20 for 1 hour, washed with PBS/Tween, and incubated with rabbit polyclonal anti-Bclx<sub>S/L</sub> (1:500) (Santa Cruz Biotechnology) or rabbit polyclonal anti-Bax antibodies (1:2000) (Pharmingen, San Diego, CA, USA), as described [14]. Antibodies were diluted in 5% milk PBS/Tween. Blots were washed with PBS/Tween and subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000) (Amersham, Aylesbury, UK). After washing with PBS/Tween the blots were developed with the enhanced chemiluminescence method (ECL) following the manufacturer's instructions (Amersham). Blots were then probed with antitubulin antibody and levels of expression were corrected for minor differences in loading.

### Confocal analysis of Bax translocation

Cells were plated onto Labtek™ slides in RPMI-10%. After 24 hours, the medium was changed to RPMI-0% for 24 hours and then cells were incubated for 24 hours with the indicated stimuli. Then, cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton X-10 in PBS for 10 minutes each. After washing in PBS, cells were incubated overnight at 4°C with a rabbit polyclonal anti-Bax antibody (1:100) (Santa Cruz Biotechnology), followed by 1-hour incubation with appropriate FITC secondary antibody (1:200) (Sigma Chemical Co.). Cell nuclei were counterstained with propidium iodide.

### Bax antisense oligonucleotide

A phosphorothionate-modified antisense oligodeoxynucleotide (ODN) 5'-TGCTCCCCGGACCCGTCCAT-3' directed against the translation initiation region of mouse Bax mRNA and a control scrambled ODN 5'-TCGTCCGGCCACCGCTCACT-3', which has little complementarity with Bax mRNA but the same composition as the antisense ODN, were synthesized commercially (Metabion GmbH, Martinsried, Germany) [22]. Cells were treated with 300 µg/mL paracetamol in the presence or absence of Bax antisense ODN (20 µg/mL) or scramble ODN (20 µg/mL) [19, 21].

### Statistical analyses

Results are expressed as mean ± SEM. Significance at the 95% level was established using one-way analysis of variance (ANOVA) and Student *t* test. The presence of significant differences between groups was examined by a post hoc test (Bonferroni's method) by means of the SigmaStat statistical software (Jandel, San Rafael, CA, USA). Data represent three independent experiments.

## RESULTS

### Bcl-xL protein expression is down-regulated in paracetamol-treated tubular epithelial cells

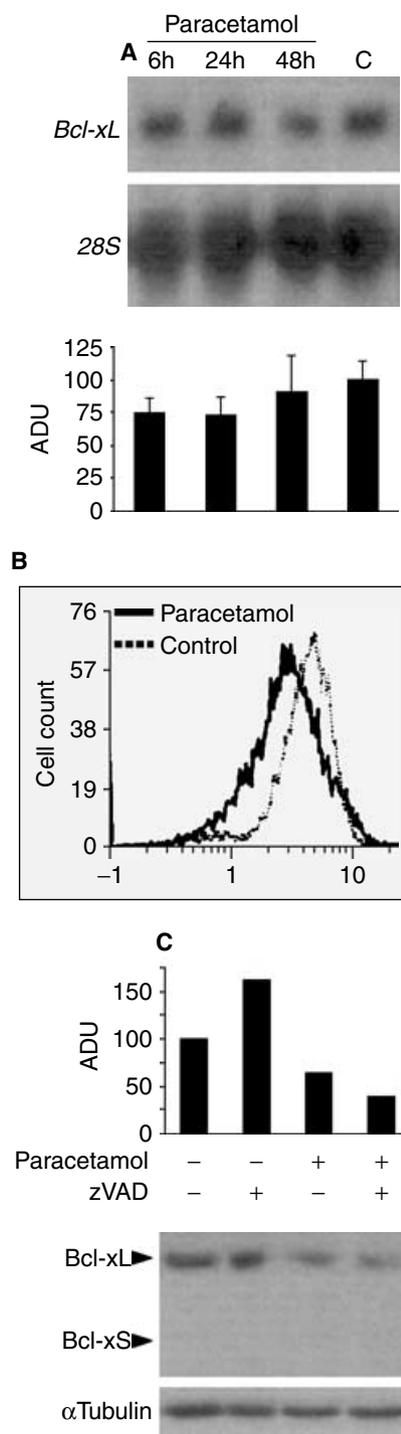
We investigated the participation of Bcl-xL in paracetamol-induced apoptosis. A paracetamol concentration of 300  $\mu\text{g}/\text{mL}$  was chosen for these experiments because it readily induces apoptosis in cultured tubular cells and it is in the range found in the serum of patients with clinically significant paracetamol intoxication [12, 23]. Incubation with 300  $\mu\text{g}/\text{mL}$  paracetamol did not significantly modify *bcl-xL* mRNA expression, as assessed by Northern blot, in MCT cells (Fig. 1A). However, paracetamol decreased the expression of Bcl-xL protein by 50% to 60% at 24 hours and 48 hours, as assessed by flow cytometry (Fig. 1B) and Western blot (Fig. 1C). No change in Bcl-xL protein expression was noted at earlier time points (8 hours, data not shown) when no increase in the rate of apoptosis was evident [12]. The proapoptotic splice isoform, Bcl-xS, was not detected in control or paracetamol-treated MCT cells (Fig. 1C).

To discard the hypothesis that paracetamol-induced changes in Bcl-xL protein and mRNA expression were cell line-specific we performed similar experiments on the human proximal tubular epithelial cell line HK-2 that is also sensitive to paracetamol-induced cell death (data not shown). There was not a major change in *bcl-xL* mRNA expression (Fig. 2A). We also found that paracetamol decreased Bcl-xL protein expression in HK-2, and the proapoptotic splice isoform, Bcl-xS, was not detected in control or paracetamol-treated HK-2 cells (Fig. 2B).

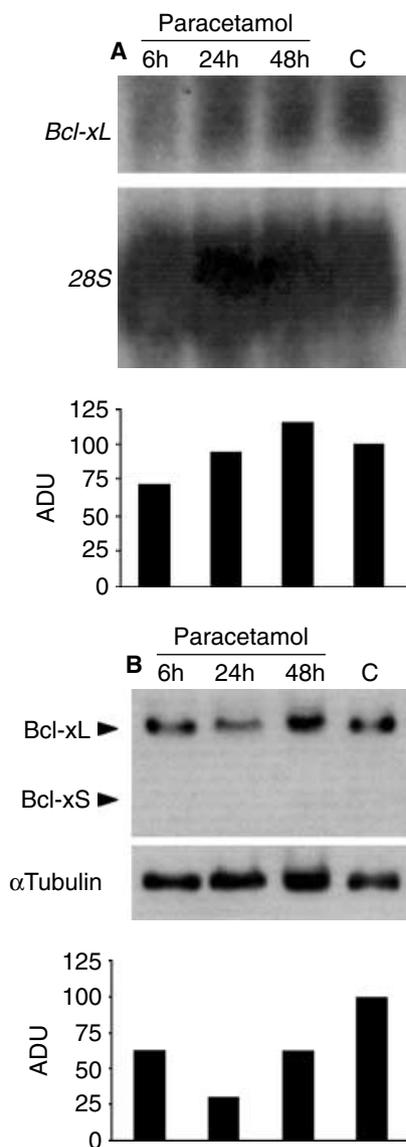
It has been previously published that caspases are capable of cleaving Bcl-xL into fragments with proapoptotic activity [17]. However, caspase inhibition did not prevent the fall in Bcl-xL expression in MCT cells treated with paracetamol (Fig. 1C). By contrast, the highly specific proteasome inhibitor lactacystin partially prevented the fall in Bcl-xL expression (Fig. 3A). In addition, lactacystin decreased the percentage of hypodiploid cells induced by paracetamol by approximately 50% at 24 hours (Fig. 3B). Serum, a stimulus that increased Bcl-xL protein expression [14], also protects from paracetamol-induced apoptosis (specific apoptosis induced by paracetamol in the presence of 10% FCS was  $38\% \pm 12\%$  of the value obtained under serum free conditions) ( $P < 0.05$ ).

### Overexpression of Bcl-xL protects tubular epithelial cells against paracetamol-induced apoptosis

To further delineate the role of Bcl-xL in paracetamol-induced apoptosis, Bcl-xL was stably overexpressed in transfected MCT cells (Fig. 4A and B). Cells stably overexpressing Bcl-xL were protected from features of apoptosis induced by paracetamol (Fig. 4C), including when cells were incubated in the presence of high doses of



**Fig. 1. Modulation of Bcl-xL expression in MCT paracetamol-treated cells.** (A) *Bcl-xL* mRNA expression of paracetamol-treated MCT cells (300  $\mu\text{g}/\text{mL}$  for 6 to 48 hours). Data were normalized for 28S expression. Diagram represents mean  $\pm$  SEM of two experiments with samples done in duplicate.  $P > 0.5$  versus all groups. (B) Paracetamol (300  $\mu\text{g}/\text{mL}$ , 48 hours) decreases Bcl-xL protein levels in MCT cells as assessed by flow cytometry. Mean cell fluorescence decreased by 50% in paracetamol-treated cells. Control is nonimmune rabbit IgG. (C) Paracetamol (300  $\mu\text{g}/\text{mL}$ , 24 hours) decreases Bcl-xL protein level in MCT cells as assessed by Western blot. The decrement in Bcl-xL expression is not prevented by inhibition of caspases with 200  $\mu\text{mol}/\text{L}$  zVAD-fmk. Bcl-xS is not detected in MCT cells. Data are representative of three experiments. ADU is arbitrary densitometry units.

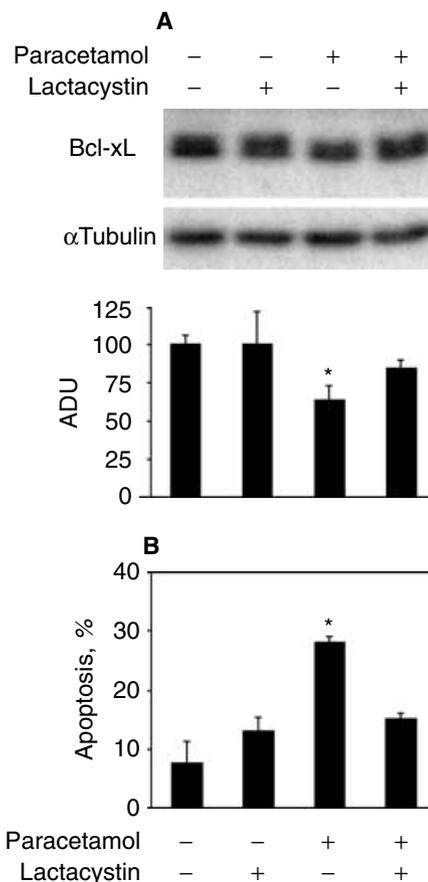


**Fig. 2. Modulation of Bcl-xL expression in human kidney-2 (HK-2) paracetamol-treated cells.** (A) *Bcl-xL* mRNA expression of paracetamol-treated HK-2 cells (300  $\mu$ g/mL for 6 to 48 hours). Data were normalized for 28S expression. (B) Paracetamol (300  $\mu$ g/mL) decreases Bcl-xL protein expression in HK-2 cells as assessed by Western blot. Data were normalized for tubulin expression. ADU is arbitrary densitometry units.

paracetamol (1000  $\mu$ g/mL) or during long incubation periods (48 hours).

#### Certain inhibitors of caspases prevent features of apoptosis induced by paracetamol

zVAD-fmk, a broad-spectrum inhibitor of caspases, prevented features of apoptosis induced by paracetamol such as internucleosomal DNA degradation or the presence of hypodiploid cells in permeabilized, propidium-stained cells [12]. However, the nuclear morphology of zVAD-fmk-, paracetamol-exposed cells was abnormal

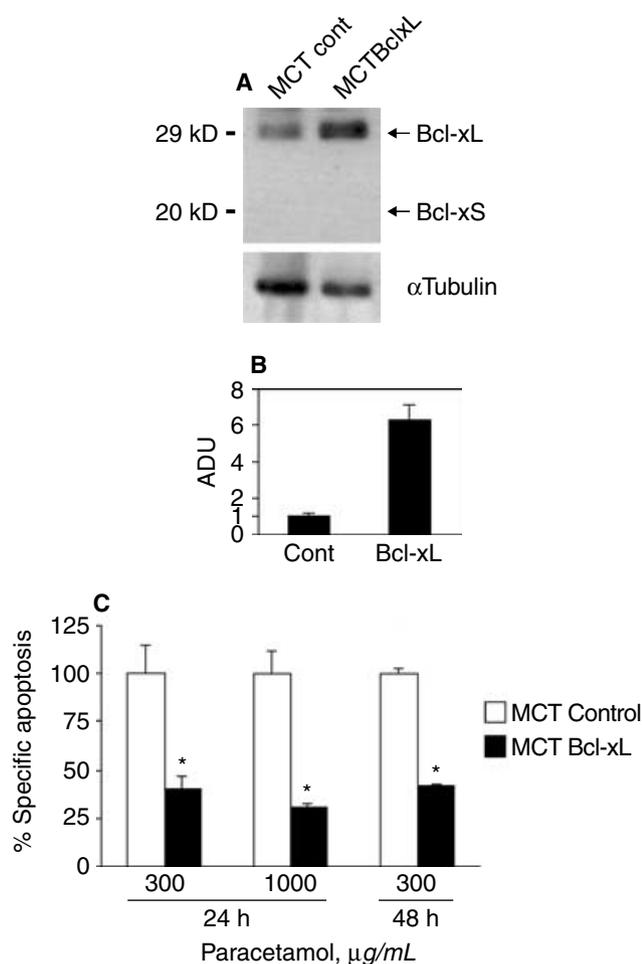


**Fig. 3. Lactacystin partially prevents the decrease in Bcl-xL expression and prevents features of apoptosis.** MCT cells were treated with 300  $\mu$ g/mL paracetamol and/or lactacystin 10  $\mu$ mol/L for 24 hours. Bcl-xL expression and apoptosis were studied. (A) Representative Western blot. Diagram represents mean  $\pm$  SEM of three Western blots. Data were normalized for tubulin expression. ADU is arbitrary densitometry units. (B) Apoptosis was studied by flow cytometry of permeabilized, propidium iodide-stained cells at 24 hours (mean  $\pm$  SEM). \* $P < 0.05$  versus other groups.

(Fig. 5A). Overexpression of the viral protein CrmA only delayed paracetamol-induced apoptosis (Fig. 5B). By contrast, specific inhibitors of caspases-2, -3 and -9 (zVDVAD-fmk, zDEVVD-fmk, and zLEHD-fmk, respectively) did not prevent apoptosis (data not shown).

#### Bcl-xL, but not inhibitors of caspases, increase long-term cell survival after exposure to paracetamol

Some lethal stimuli can induce both caspase-dependent apoptosis, and, if caspases are inhibited, eventual caspase-independent cell death. Caspase inhibition may not improve long-term cell survival when apoptosis is induced by these stimuli [24, 25]. We tested the ability of Bcl-xL overexpression and zVAD-fmk to increase long-term MCT cell survival. Bcl-xL overexpressing cells were able to adhere to culture substrata and proliferate after treatment with paracetamol, while zVAD-fmk-treated cells



**Fig. 4. Bcl-xL overexpression protects from paracetamol-induced apoptosis.** (A) MCT-transfected cells overexpress Bcl-xL but not Bcl-xS. Western blot. (B) Data were normalized for tubulin expression. ADU is arbitrary densitometry units. (C) Bcl-xL-overexpressing cells were protected from apoptosis induced by paracetamol when compared with control cells. Apoptosis was studied by flow cytometry of permeabilized, propidium iodide-stained cells. Data are expressed as percentage of specific protection, where paracetamol-specific cell death was considered to be 100% in control MCT cells. Mean  $\pm$  SEM. \* $P < 0.05$  versus control. Data represent three independent experiments.

were not (Fig. 6). The same negative results were observed when specific inhibitors for caspase-8 (zIETD-fmk), -2, -9, and -3 were used (data not shown).

#### Bax is not involved in paracetamol-induced cell death

In contrast to Bcl-xL, Bax is a proapoptotic member of the Bcl-2 family. In normal conditions Bax resides in the cytoplasm of the cells. Some apoptotic stimuli induce its translocation to the mitochondrial outer membrane where it antagonizes the protecting effect of Bcl-xL and promotes apoptosis. No changes in *bax* mRNA expression were noted after the addition of paracetamol (Fig. 7A). Also, Bax protein expression did not significantly change from 8 hours to 48 hours incubation with

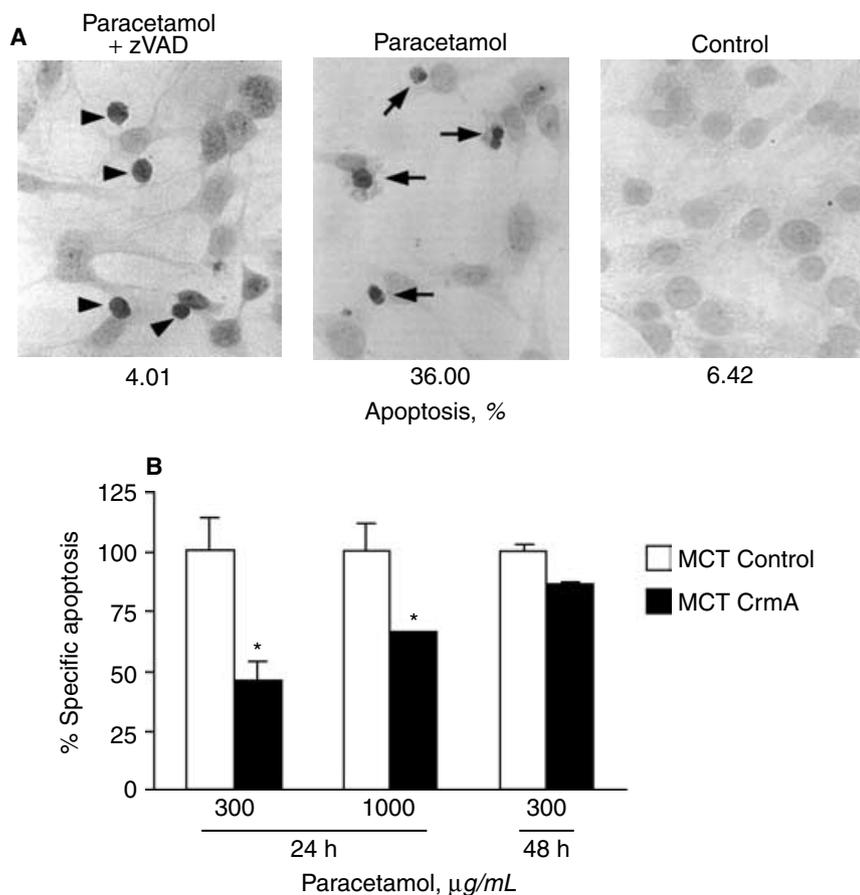
paracetamol, as assessed by Western blot (Fig. 7B). Furthermore, we did not detect any translocation of Bax to the mitochondria of MCT cells treated with paracetamol (Fig. 7C). Our group has previously shown that this apoptotic pathway is active in MCT cells [19]. In addition, suppression of Bax protein expression by means of Bax antisense ODNs was not protective (a method previously used in our laboratory to successfully inhibit MCT cell death induced by other nephrotoxins [19]) (Fig. 7D).

#### DISCUSSION

The understanding of the apoptotic pathways activated in the course of tubular cell death induced by different stimuli may provide the basis for the therapeutic targeting of apoptosis in the course of acute or chronic renal injury. Nephrotoxins such as paracetamol engage several molecular pathways for apoptosis, including loss of protective intracellular molecules and activation of caspases. The present study shows that the protective protein Bcl-xL plays a role in paracetamol-induced apoptosis in murine kidney tubular epithelial cells. They further pinpoint Bcl-xL as a potential therapeutic target, as opposed to caspases or Bax, for certain forms of tubular injury, such as paracetamol injury.

Three main molecular pathways for apoptosis have been described. They are initiated by deprivation of survival factors, by activation of death receptors or by the presence of other causes of cellular stress, such as nephrotoxins (reviewed in [9]). The role of Bcl-xL in the regulation of apoptosis initiated by deprivation of survival factors or by lethal cytokines, such as tumor necrosis factor (TNF), has been previously explored in renal tubular epithelium [14]. In both pathways, there is a decrease in the expression of *bcl-xL* mRNA and protein [14]. Moreover, loss of Bcl-xL protein expression was noted in some tubular epithelial cells during experimental toxic acute renal failure induced by a folic acid overdose [14]. We now report that a nephrotoxic drug decreases Bcl-xL in apoptotic tubular epithelium. These findings suggest that a decrease in Bcl-xL levels is a common event in tubular cell death induced by different mechanisms and point to Bcl-xL as a therapeutic target in nephrotoxicity and other forms of tubular injury. Indeed, overexpression of Bcl-xL protected from paracetamol-induced apoptosis and, as previously reported also protects against TNF-induced apoptosis [14]. Naturally occurring compensatory overexpression of Bcl-xL was observed in some tubular cells in an in vivo model of acute renal failure characterized by spontaneous recovery of tubular integrity [14].

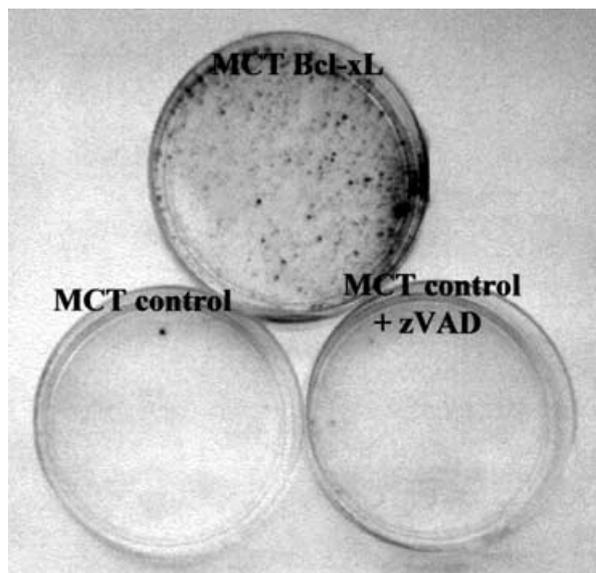
Paracetamol decreased Bcl-xL protein without significantly changing its mRNA levels. Caspases are able to degrade Bcl-xL in the course of apoptosis, generating proapoptotic fragments that may contribute to cell death [17]. However, inhibition of caspases did not prevent the



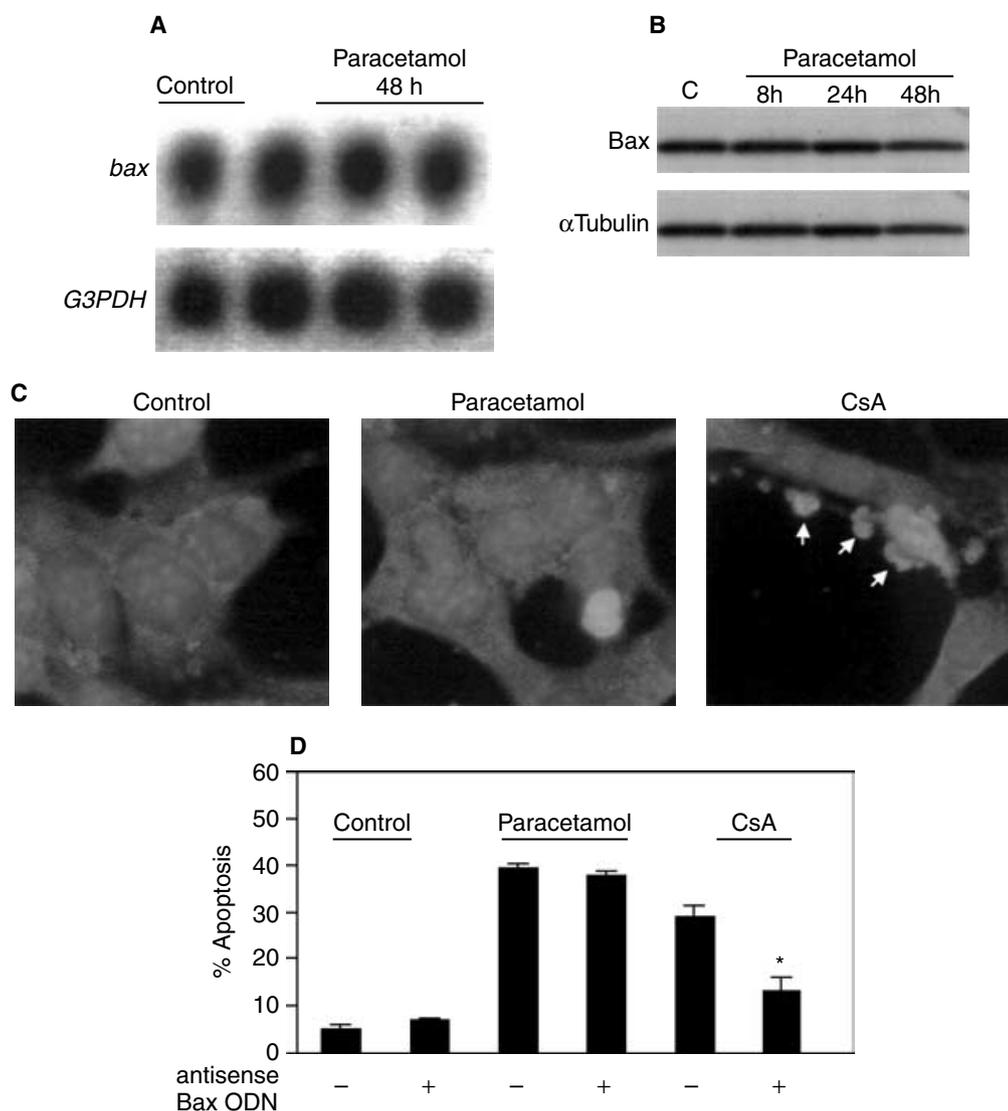
**Fig. 5. Effect of peptide and viral inhibitors of caspases on paracetamol-induced apoptosis.** (A) Although zVAD-fmk prevents nuclear morphologic features of apoptosis, it does not preserve normal nuclear morphology. Arrowheads show that the nuclear morphology of zVAD-fmk + paracetamol-treated cells is different to that characteristic of apoptotic paracetamol-treated cells (arrows show condensed, shrunken, picnotic nuclei). However, it also differs to that of control cells (hematoxylin and eosin, original magnification  $\times 400$ ). Apoptotic cell death at the indicated conditions is also shown (flow cytometry of permeabilized, propidium iodide-stained cells). (B) Effect of CrmA on apoptosis induced by paracetamol. Mean  $\pm$  SEM. \* $P < 0.05$  versus control. Apoptosis was quantified by flow cytometry analysis of DNA content in permeabilized, propidium iodide-stained cells. Data represent three independent experiments.

decrease in Bcl-xL in MCT cells exposed to paracetamol. This raises the possibility of the involvement of other proteases in Bcl-xL degradation in this system [26–28]. The role of proteasome activation in apoptosis is cell- and stimulus-specific [29–31]. In different settings, in nonrenal cells, the proteasome contributes to the degradation of specific Bcl-2 family members, and its net effect on apoptosis induction may depend on whether it regulates proapoptotic (such as tBid and Bik) or antiapoptotic Bcl-2-like proteins (such as Bcl-2) [29–34]. In this regard, our data suggest that the overall effect of proteasome inhibition in tubular cells may result in protection from apoptosis. This information may be relevant to the recently published observation that the proteasome inhibitor lactacystin protects renal tubular cells *in vivo* from acute ischemic damage [35].

Bcl-xL is thought to function either by directly inhibiting Apaf-1-mediated activation of caspase-9 or by attenuating release of mitochondrial cytochrome c through interaction with Bax [16, 36, 37]. Caspases are cysteine proteases that participate in the initiation and execution phases of the apoptotic program [38]. During apoptosis caspase zymogens are activated sequentially by proteolysis. Caspase-9 becomes activated in a protein complex formed by Apaf-1 and cytochrome c, following



**Fig. 6. Bcl-xL, but not inhibitors of caspases, preserve long-term survival of paracetamol-treated tubular cells.** Bcl-xL-MCT and control MCT cells were exposed to 300  $\mu\text{g/mL}$  paracetamol for 48 hours, in the presence or absence of zVAD-fmk, then trypsinized, washed, seeded in Petri dishes, and allowed to grow for 7 days, then they were stained with crystal violet. Bcl-xL overexpression, but not caspase inhibition, increased the number of long-term surviving cell colonies. Data are representative of three independent experiments.



**Fig. 7. Bax is not involved in MCT paracetamol-induced apoptosis.** (A) No change in the expression of *bax* mRNA was noted after 48-hour culture in the presence of 300  $\mu$ g/mL paracetamol. Northern blot. (B) Bax protein expression did not significantly change from 8 hours to 48 hours following the addition of 300  $\mu$ g/mL paracetamol, as assessed by Western blot. (C) Confocal microscopy revealed that Bax (green fluorescence) did not translocate to mitochondria in response to paracetamol. Orange fluorescence is nuclei stained with propidium iodide. Note aggregated Bax in cells exposed to cyclosporine A (CsA), 10  $\mu$ g/mL for 24 hours (arrows) (magnification 360 $\times$ ). (D) Suppression of Bax protein expression by means of Bax antisense oligodeoxynucleotides (ODNs) did not prevent paracetamol-induced apoptosis at 24 hours. Apoptosis was quantified by flow cytometry analysis of DNA content in permeabilized, propidium iodide-stained cells. \* $P < 0.05$  versus CsA alone. CsA was used as a positive control because it induces MCT cell death with Bax translocation and Bax antisense ODNs efficiently protect MCT from cell death caused by this nephrotoxin [19]. Data represent three independent experiments.

release of the latter from mitochondria [38]. Caspase-12 may also activate caspase-9 in the absence of cytochrome c release [39]. Caspase-9, in turn, activates downstream effector caspases such as caspase-3 [38]. We have previously reported that caspase-3, -9, and the specific endoplasmic reticulum stress-activated caspase-12 are activated during MCT paracetamol-induced apoptosis [12]. ZVAD-fmk, a broad-spectrum peptide inhibitor of caspases, prevented paracetamol-induced nuclear features of apoptosis [12]. This property is not shared by specific inhibitors of caspases-3 and -9, suggesting that

activation of these specific caspases is not central for the development of apoptosis induced by paracetamol. These findings contrast to the necessary role of caspases-9 and -3 in cyclosporine A (CsA)-induced apoptosis in the same cells [19]. In extrarenal cells zVAD-fmk blocks death receptor-mediated apoptosis and eventual cell death by preventing the first step of this pathway, caspase-8 activation [38]. By contrast, in some forms of apoptosis zVAD-fmk retards cytolysis, but is incapable of preventing disruption of the plasma membrane during protracted cell culture, even in conditions in which it completely blocks

nuclear apoptosis (chromatin condensation and DNA fragmentation), leading to a loss of clonogenicity [24, 26]. In this sense, in tubular epithelial cells treated with paracetamol, zVAD-fmk did not promote long-term survival. In addition CrmA, a potent and selective inhibitor of group I caspases (caspase-1, -4, and -5) and most group III caspases (caspase-8, -9, and -10) [40] afforded only partial, transitory protection from paracetamol-induced apoptosis. These results are in contrast with the long-term protection and maintenance of proliferative potential afforded by caspase-3 and -9 specific inhibitors to MCT cells treated with the nephrotoxin CsA [19]. The failure of specific caspase-9 inhibitors to prevent apoptosis and the failure of pancaspase inhibition to promote long-term cell survival suggests that caspase inhibition is not the basic mechanism of the protective effect of Bcl-xL. The observed protective effect of Bcl-xL on clonogenicity and the lack of effect of caspase inhibition despite the disappearance of features of apoptosis is reminiscent of the effects of Bcl2 in other models of cell death [24].

Bcl-xL may also prevent cytochrome c release from mitochondria [16]. For this protective function, interaction with the lethal factor Bax and the relative levels of expression of Bcl-xL and Bax are important [37]. Bax resides in the cytoplasm of healthy cells. Certain apoptotic stimuli, such as CsA in tubular epithelium, induce the translocation of Bax to the mitochondria, where it promotes the release of cytochrome c to the cytoplasm, contributing to the formation of the apoptosome, which leads to the activation of caspase-9 [19]. However, Bax expression was not increased in tubular cells treated with paracetamol and no translocation of Bax to mitochondria was observed. Furthermore, suppression of Bax protein expression by means of Bax ODNs, an approach that successfully rescued MCT cells from CsA-induced cell death [19], did not affect paracetamol toxicity. These results, together with the lack of cytochrome c release from mitochondria during paracetamol-induced apoptosis of tubular epithelium [12], suggest that there is not a role for Bax in paracetamol toxicity in murine tubular cells and that the protective effect of Bcl-xL is not related to its ability to interact with Bax or prevent mitochondrial events.

Paracetamol induces features of endoplasmic reticulum (ER) stress in tubular epithelium [12]. ER stress has recently emerged as an apoptosis inducer (reviewed in [41]). Depending on the apoptotic stimulus, multiple signalling pathways could emerge from the ER to promote cell death via caspase-dependent and -independent means [41]. There are possible roles for Bcl2-related proteins, such as Bax in these processes [41]. However, the lack of long-term protection by caspase inhibition or anti-Bax strategies in this model indicates that additional, Bcl-xL-sensitive pathways are important. In this regard, recent papers have described the interaction of Bcl-xL

with a series of apoptotic regulatory proteins located at the ER. These proteins include, but may not be limited to, BAP31, RTN-XS, and NSP-C, and ER-specific BH3-only family members such as Spike [42–44]. The precise consequences of these interactions have not been fully characterized yet.

While most research in the pathogenesis of paracetamol and analgesic-induced renal injury has focused on adverse effects to the renal medulla, both clinical evidence of proximal tubular injury [2, 3, 7] and recent cell culture data [12], including the present report, indicate that other parts of the nephron may be the target of the drug. The present manuscript contributes to further understanding nephrotoxin-specific pathways in cell death of tubular epithelium. Bcl-xL protects murine tubular epithelial cells from paracetamol-induced apoptosis. In this regard, Bcl-xL appears to protect cultured tubular cells from lethal stimuli ranging from death receptor engagement to nephrotoxin exposure. This points to Bcl-xL as a therapeutic target in nephrotoxicity and other forms of tubular injury. However, the protective effect of Bcl-xL on paracetamol toxicity appears to differ from canonical Bcl-xL actions on caspase-dependent and Bax-dependent pathways and may be related to ER-stress specific pathways.

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