ARTICLES

Bilirubin Induces Apoptosis and Necrosis in Human NT2-N Neurons

ERIK HANKØ, THOR WILLY RUUD HANSEN, RUNAR ALMAAS, JULIE LINDSTAD, AND
TERJE ROOTWELT

*Department of Pediatric Research [E.H., R.A., J.L., T.R.], Department of Pediatrics [T.W.R.H., T.R.],
Rikshospitalet, 0027 Oslo, Norway, Department of Pediatrics [R.A.], Sykehuset Østfold, 1607 Fredrikstad,
Norway*

ABSTRACT

Studies on primary cultures of newborn rodent neurons have suggested that neuronal death induced by unconjugated bilirubin (UCB) is mainly apoptotic in nature. We exposed a human teratocarcinoma-derived cell line, NT2-N neurons, to different concentrations of UCB and albumin at a 1.5 molar ratio and used multiple, independent measures of cell damage to evaluate neuronal injury after 6, 24, and 48 h. Low doses of UCB (0.66, 2, and 5 μ M) induced a moderate loss of 3–4[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) cleavage accompanied by delayed morphologic changes consistent with apoptosis (2 and 5 μ M). Moderate concentrations of UCB (10 and 25 μ M) resulted in early (6 h) necrosis in a subset of neurons, while remaining neurons underwent progressive impairment of MTT cleavage and increasing lactate dehydrogenase (LDH) release accompanied by predominantly delayed apoptosis. High concentrations of UCB (100 μ M) induced severe impairment of MTT cleavage, extensive LDH release, and morphologic changes consistent with necrosis within 6 h. Used as a positive control for apoptosis, 2 μ M STS induced progressive impairment of MTT cleavage and morphologic changes consistent with apoptosis

over the entire observation period. DNA electrophoresis at 48 h was compatible with apoptosis both after treatment with STS and UCB concentrations ≤ 25 , but not at 100 μ M. Cleavage of poly (ADP-ribose) polymerase (PARP) was only seen in neurons treated with low UCB concentrations and STS. We conclude that UCB induces early necrosis at high and moderate concentrations and predominantly delayed apoptosis at low and moderate concentrations in cultured human NT2-N neurons. (*Pediatr Res* 57: 179–184, 2005)

Abbreviations

BSA, bovine serum albumin
EH, ethidium homodimer
HSA, human serum albumin
LDH, lactate dehydrogenase
MR, molar ratio
MTT, 3–4[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
UCB, unconjugated bilirubin

Bilirubin-induced encephalopathy in the newborn has been described for over a century. The term is mainly used to describe a picture of reversible lethargy and alterations in cerebral evoked potentials thought to be benign (1). The term *kernicterus* has been used to describe more severe clinical manifestations like seizures, opisthotonos, and hyperthermia in the acutely ill newborn, followed by neurologic sequelae in

survivors or death (1). The question of whether these manifestations share a common graded mechanism or reflect different entities is not settled (2).

Previously, neuronal death has been thought of as the result of either necrosis or apoptosis (3). However, it now seems that apoptosis and necrosis represent extreme ends of a spectrum of possible biochemical and morphologic deaths, so that a more dynamic boundary between apoptosis and necrosis has been suggested (3). Several studies have shown that it is often the magnitude of the initial insult, rather than the specific type of the stimulus, that determines whether the cell undergoes either type of cell death (3,4). Recently, several studies have reported that UCB primarily induces apoptosis both in primary cultures of fetal cortical and cerebellar rodent neurons (5,6), in cultures

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Correspondence: Erik Hankø, M.D., Rikshospitalet, Department of Pediatric Research, N-0027 Oslo, Norway; e-mail: erikh@klinmed.uio.no

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of fetal rodent astrocytes (7,8), and in murine hepatoma cells (9). Despite these studies, it remains unclear 1) whether the cells die by apoptosis, necrosis, or both; 2) whether the UCB concentration is decisive for the relative proportion of apoptosis and necrosis; and 3) how neuronal death evolves with time. Furthermore, all studies addressing the issue of UCB-induced cell death mechanisms so far have been undertaken in neuronal cultures from rodents or on non-neuronal cells. We have used a human teratocarcinoma-derived cell line, NT2-N cells, to study UCB-induced cell death. After treatment with retinoic acid and mitotic inhibitors, these cells develop into polarized, postmitotic, neuron-like cells that share many characteristics with CNS neurons (10). They express functional non-NMDA as well as NMDA-receptors (11), different types of calcium channels (12), and elaborate classical synaptic contacts (13). In this descriptive study, we determined the modes of cell death in NT2-N neurons and its temporal evolution after exposure to various concentrations of bilirubin. We demonstrate that bilirubin induces early necrosis-like cell death and delayed apoptosis-like cell death in a concentration-dependent manner in this cell line.

MATERIALS AND METHODS

Materials. Dulbecco's medium modified by Eagle (DMEM), fetal bovine serum (FBS), and PBS were purchased from Invitrogen (Carlsbad, CA). Penicillin and streptomycin were also purchased from Invitrogen, while polylysine and Matrigel were purchased from BD Biosciences (San Jose, CA). Bilirubin, BSA, MTT, retinoic acid, uridine, 5-fluoro-2'-deoxyuridine, and cytarabine were from Sigma Chemical Co. (St. Louis, MO). BSA was dissolved in deionized water. MTT was dissolved in PBS with glucose. The fluorescent dyes Hoechst 33342 and EH (both from Molecular Probes, Eugene, OR) were diluted in DMSO to 5 mg/mL and 2 mM stocks, respectively.

NTera2/c1.D1 (NT2) cells were cultured as previously described (14). In short, cells were plated at a density of 2.3×10^6 per T 75 flask and fed twice weekly with DMEM (4500 mg/L glucose) with 10% FBS, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 10 μ M retinoic acid for 5 wk. The cells were then split 1:4 and grown for 2 d more in identical medium without retinoic acid in T 162 flasks. Neuronal cells growing atop a monolayer of non-neuronal cells were dislodged with trypsin and plated in 12-well plates pretreated with polylysine and Matrigel at a density of 3×10^6 cells per well. The cells were then fed weekly for 3 wk more with DMEM, containing 5% FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin, and mitotic inhibitors (10 μ M uridine, 10 μ M 5-fluoro-2'-deoxyuridine, and 1 μ M cytosine- β -arabinofuranoside) to reduce the amount of non-neuronal cells. After 3 wk, the cells were fed with identical medium without inhibitors for 1 wk.

UCB treatment of NT2-N neurons. Before each experiment, UCB was dissolved in 0.1 M NaOH and deionized water and added to a BSA solution to obtain a UCB/BSA molar ratio (MR) of 1.5 or 3. All handling of bilirubin was performed in dim light. Except for experiments performed to compare MTT cleavage at 24 h (Fig. 1), all experiments were performed with a 1.5 MR. Serum free DMEM with high glucose, containing penicillin 100 IU/mL and streptomycin 100 μ g/mL was added to each well before the addition of UCB or control solution. Cells exposed to concentrations of NaOH and BSA equivalent to the concentrations for cells treated with 100 μ M UCB served as controls. pH in the incubation medium dropped slightly during incubation from an average of 7.59 ($\pm$ 0.057) before incubation to 7.56 ($\pm$ 0.065) at 6 h and to 7.52 ($\pm$ 0.054) at 24 h. As UCB solubility dropped with decreasing pH, pH was not adjusted. In UCB concentrations $\geq$ 5 μ M, sparse aggregates could be detected in an otherwise homogeneous, UCB-stained incubation medium. UCB/BSA MR 3 displayed more pronounced aggregation. Considerable aggregation was displayed by 0.66 μ M UCB without addition of BSA. The cultures were incubated for 6, 24, or 48 h at 37°C in a mixture of 95% room air and 5% CO₂.

Figure 1. MTT cleavage in NT2-N neurons exposed to different UCB concentrations (0.66–250 μ M) for 24 h. Two different molar ratios of UCB/BSA (1.5 and 3) were compared. The concentration of 0.66 μ M UCB did not contain albumin. All UCB concentrations induced significant reduction in MTT cleavage compared with controls (*t* test, *p* < 0.05). No add (no additives) represents cultures exposed to serum-free medium only. Controls represent the addition of NaOH and BSA in doses equivalent to neurons treated with 100 μ M UCB. Data are given as means $\pm$ SD.

MTT reduction. After incubation, the medium was aspirated and the cultures were washed twice with PBS. Next, 0.5 mL of the MTT solution (0.5 mg/mL) was added, and the plates were returned to the incubator (37°C in 5% CO₂). After 60 min, the MTT solution was aspirated and replaced with 0.5 mL of DMSO to dissolve the formazan crystals produced by MTT cleavage. Absorbance was read at 570 nm and background at 660 nm was subtracted in a Titertek multiscan Plus MK1-ELISA reader, and analyzed by the program Genesis (LabSystems and Life Sciences International Ltd., Haverhill, Sussex, UK). Results are given in percentage of MTT cleavage in controls.

LDH release. LDH release to the supernatant was assayed with a kit (Roche Molecular Biochemicals, Mannheim, Germany). The sum of the LDH activity in the supernatant and the lysed cells in two wells were used as a measure of total LDH activity for all wells in the actual plate. The measurements were performed at 492 nm in a Titertek Multiscan Plus MK1-ELISA reader and analyzed by the program Genesis. In separate experiments, $\leq$ 25 μ M UCB was shown not to interfere with the LDH assay when compared with incubation medium alone. The concentration of 100 μ M UCB distorted the values by <3% of the total LDH activity of a typical plate and was not corrected for. In this model, LDH release has been shown to be highly correlated with the energy-failure indicator hypoxanthine (15). LDH release is expressed as a percentage of total LDH release.

Assessment of nuclear morphology with fluorescent dyes. The combination of the two fluorescent dyes, Hoechst 33342 and EH, was used to assess nuclear morphology and membrane integrity, respectively, as previously described (16). Hoechst 33342 stains the nuclei of all living and dead cells, while nuclei of cells with damaged cell membranes, as seen in necrosis, are stained by EH. All dying and dead cells eventually lose nuclear and plasma membrane integrity, so-called "secondary necrosis" (17). After incubation, the neurons were washed twice with PBS and incubated with the two dyes for 15 min. An

UV excitation filter (340–380 nm) and a green excitation filter (515–560 nm) were used for each motive. The cells were visualized by an inverted fluorescent microscope (model DM IRB, Leica Microscopy, Herburg, Switzerland) and photographed with a digital camera (Kodak, DC 120 ZOOM Digital Camera, Eastman Kodak, Rochester, NY) for later analysis. Neurons with intact membranes (exclusively stained with Hoechst) displaying uncondensed nuclear chromatin were considered undamaged. Shrunken nuclei with condensed chromatin and intact membranes were labeled as condensed. Nuclei with distinct fragmentation (at least two distinct lobuli) were considered fragmented. Condensed nuclei with uptake of EH were labeled as condensed with disrupted membrane. In general, cells of all categories remained adherent to the dish, and detached nuclei accounted for <2% of the total cell count. All cells were counted by the same investigator. A subset of neurons was counted by an independent investigator to an interobserver agreement of >95%. Prevalence of the different morphologic categories is given in percentage of the total number of cells counted for each experimental condition (at least 800 cells).

DNA fragmentation analysis. After 48 h of treatment, the cells were treated with trypsin, harvested, centrifuged, washed with cold PBS, centrifuged again, and the supernatant fluid was aspirated before storage at -70°C . H_2O_2 (500 μM) induced total necrotic cell death within 24 h (microscopic evaluation), and was therefore used as a positive control for necrosis. For analysis, cells were incubated in a hypotonic buffer with a mild detergent [1% Triton X-100, 50 mM Tris-HCl (pH 7.9), 10 mM EDTA, 50 $\mu\text{g}/\text{mL}$ RNase A] for 10 min. Fragmented DNA was separated from intact nuclear DNA by centrifugation for 10 min at 16,000 g. The fragmented DNA in the supernatant fluid was purified in a Promega wizard PCR Preps DNA Purification System (Promega, Madison, WI) and visualized by agarose gel electrophoresis and ethidium bromide staining.

PARP cleavage. Neurons combined from three wells (a total of 9×10^6 cells) were washed once with PBS, suspended in 150 μL of sample buffer (62.5 mM Tris/HCl, pH 6.8; 6 M urea; 10% glycerol; 2% SDS; 0.00125% bromophenol blue; 5% β -mercaptoethanol), sonicated for 15 s, and incubated at 65°C for 15 min. Proteins were separated by SDS-PAGE on gels containing 8% polyacrylamide and blotted (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad, Hercules, CA) onto a nitrocellulose membrane (Hybond-C Extra; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The membrane was incubated with monoclonal anti-PARP antibodies (BIOMOL, Plymouth, PA), visualized with goat anti-mouse IgG-AP (Santa Cruz Biotechnology, Santa Cruz, CA) and read with a Kodak scanner (Kodak Digital Science Image Station 440 CF; Eastman Kodak).

STS experiments. STS is a general protein kinase inhibitor that has been widely used to induce apoptosis in many different cell lines. NT2-N neurons were exposed to 2 μM STS to serve as a positive control for apoptosis. After 6, 24, or 48 h, cells were analyzed with MTT cleavage, LDH release, and nuclear morphology. DNA electrophoresis and PARP cleavage were performed after 48 h.

Statistics. Data are given as mean $\pm$ SD. Cells from at least eight wells from at least three separate experiments were examined for all experimental conditions except for UCB concentrations and time points where additional experiments were considered without significance (UCB 2 μM at 6 h, $n = 6$, and UCB 100 μM at 48 h, $n = 3$). All comparisons between two groups were performed with t tests. Welch correction was applied if the groups had different standard deviations. A two-tailed $p < 0.05$ was considered statistically significant. MTT cleavage in neurons treated with UCB/BSA MR of 1.5 and 3 was compared with ANOVA. Bonferroni corrections were not applied.

RESULTS

MTT cleavage. MTT cleavage was used as a measurement of neuronal viability. Increasing UCB concentrations caused a dose-dependent decrease in neuronal viability as measured with MTT reduction at 24 h (Fig. 1). There were no differences between controls and neurons treated without additives. UCB/BSA MR 3 induced significantly more reduction in MTT cleavage at 24 h than neurons treated with UCB/BSA MR 1.5 ($p < 0.0001$).

As shown in Figure 2, all UCB concentrations (UCB/BSA MR 1.5) induced a decline in MTT cleavage compared with controls already at 6 h. Unlike all higher UCB concentrations, 2 μM UCB did not induce further impairment thereafter. During incubation with 2 μM STS, MTT cleavage was comparable to values achieved with low UCB concentrations at 6 h, but decreased thereafter to low levels comparable to those seen with UCB concentrations ≥ 25 μM UCB.

LDH release. Loss of cell membrane integrity associated with necrosis was assessed with LDH release to the supernatant (Fig. 3). A minor increase in LDH release was induced at 24 h with 2 and 5 μM UCB. Treatment with 10 and 25 μM UCB induced significant LDH release within 6 h, which continued to increase thereafter. In neurons treated with 100 μM UCB, LDH release reached high levels within 6 h ($63 \pm 5\%$), indicating a rapid cell demise in the majority of neurons. During STS treatment, LDH release was moderate and reached maximum values at 24 h ($31 \pm 5\%$).

Nuclear morphology. Figure 4 shows a selection of pictures of neurons exposed to different UCB concentrations (UCB/BSA MR 1.5) or 2 μM STS. Figure 5 shows controls (BSA and NaOH only) displaying a minor loss in undamaged nuclei not different to cells exposed to serum deprivation alone (data not shown). Whereas all UCB concentrations ≥ 5 μM induced a moderate but significant loss of undamaged nuclei at 6 h, only 100 μM UCB caused morphologic changes in a majority of cells at this time. In all other UCB concentrations and STS-treated neurons, the most prominent loss in morphologically undamaged neurons was found between 6 and 24 h. Early morphologic changes (6 h) were mainly seen as condensed nuclei with disrupted membrane after exposure to UCB concentrations ≥ 10 μM UCB. This morphologic appearance dominated in cells treated with 25 and 100 μM UCB at later time points, but only reached $12 \pm 6\%$ in STS-treated neurons at 48 h. Beyond 6 h, the proportion of fragmented nuclei increased with time throughout the experiment for all UCB concentrations ≤ 10 μM ($p < 0.05$). UCB induced a maximum proportion of fragmented nuclei in neurons exposed to 10 μM at 48 h ($37 \pm 11\%$), whereas STS induced $61 \pm 6\%$ fragmentation at this time point. A subset of fragmented nuclei (ap-

Figure 2. MTT cleavage in percentage of controls after treatment with different UCB concentrations (UCB/BSA molar ratio 1.5) or 2 μM STS for 6, 24 and 48 h. Data are given as means $\pm$ SD. * ($p < 0.05$) denotes the earliest time point when significant difference from controls was reached for each concentration. + ($p < 0.05$) denotes significant difference from the previous time point in cells exposed to the same treatment.

Figure 3. LDH release in percentage of total after treatment with different UCB concentrations (UCB/BSA molar ratio 1.5) or 2 μ M STS for 6, 24, and 48 h. Data are given as mean $\pm$ SD. * ($p < 0.05$) denotes the earliest time point when significant difference from controls was reached for each concentration. † ($p < 0.05$) denotes significant difference from the previous time point in cells exposed to the same treatment.

Figure 4. The combination of the two fluorescent dyes Hoechst 33342 (5 ng/mL), and EH (EH) (2 nM) was used to assess nuclear morphology and membrane integrity respectively after treatment with UCB (UCB/BSA molar ratio 1.5) or 2 μ M STS after 6, 24 or 48 h. Undamaged nuclei (white arrow), condensed nuclei with preserved cellular membranes (blue arrow), and fragmented nuclei (orange arrow) displayed uptake of EH as a sign of secondary necrosis. Condensed nuclei with disrupted membranes, displaying uptake of EH (yellow arrow), were considered necrotic.

proximately 25% irrespective of treatment) displayed uptake of EH as in “secondary necrosis.” Neurons exposed to STS and all UCB concentrations $\leq 25 \mu$ M showed a gradual increase in the proportion of neurons displaying nuclear condensation until 24 h, which was stable thereafter.

DNA electrophoresis. DNA fragmentation is considered a hallmark in apoptosis, however, the extent varies greatly (18). STS induced high molecular weight (HMW) DNA fragments,

Figure 5. Nuclear morphology in percentage of total cells counted for different UCB concentrations (UCB/BSA molar ratio 1.5) or 2 μ M STS after 6, 24, or 48 h. Data are given as mean $\pm$ SD. * ($p < 0.05$) denotes the earliest time point when significant difference from controls was reached for each concentration. † ($p < 0.05$) denotes significant difference from the previous time point in cells exposed to the same treatment.

and distinct DNA laddering after 48 h (Fig. 6). Five micromolar UCB (UCB/BSA MR 1.5) induced HMW DNA fragments and a faint but distinct DNA ladder not seen after treatment with higher UCB concentrations. There was a dose-dependent loss in the fraction of HMW fragments in cells treated with 10 and 25 μ M UCB. Neither treatment with 100 μ M UCB nor necrosis control (500 μ M H_2O_2) induced DNA fragmentation.

Figure 6. DNA electrophoresis after incubation with different UCB concentrations (UCB/BSA molar ratio 1.5) or 2 μ M STS for 48 h. Lane 1, DNA markers; lane 2, cells incubated in medium without additives; lane 3, cells from control cultures (incubated with BSA/NaOH only); lane 4, cells treated with 500 μ M H_2O_2 (necrosis control); lane 5, cells treated with 2 μ M STS (apoptosis control); lanes 6–9, cells treated with UCB 5, 10, 25, and 100 μ M. High molecular weight (HMW) fragments were induced by STS and UCB ≤ 25 μ M. Only STS and 5 μ M UCB induced bands of small DNA fragments (DNA ladder).

PARP Cleavage. Proteolytic cleavage of PARP from a 116-kDa polypeptide to an 85-kDa fragment is an important event in at least one apoptotic pathway (19). Western blots demonstrated a strong presence of the 85-kDa fragment after STS treatment at 48 h (Fig. 7). Faint but distinct PARP cleavage was also visible after treatment with 5 μ M UCB, but not for higher concentrations (UCB/BSA MR 1.5), nor in controls.

DISCUSSION

The principal findings of the present study were that low and moderate UCB concentrations induced early necrosis in a

subset of neurons followed by late apoptosis-like cell death in human NT2-N neurons. Higher concentrations of UCB induced rapid metabolic demise and necrotic cell death. These results are in accordance with numerous *in vitro* studies showing that exposure to a variety of different toxic stimuli such as glutamate, methylmercury, or acidosis induce early necrotic cell death or late apoptotic cell death, depending on the intensity of the insult (20–22).

Little is known about which brain bilirubin concentrations are required under what conditions to cause damage *in vivo*. However, autopsy material and electrophysiological studies indicate that brain bilirubin concentrations may exceed 25 μ M (23,24). A main concern in the study of UCB toxicity *in vitro* is to keep the bilirubin molecule in solution, as the aqueous solubility of UCB has been estimated as low as 70 nM (25). The most common approach to this problem has been to add albumin. In the present study, experiments with UCB/BSA MR of 3 induced a slightly greater reduction of cell viability as measured with MTT reduction than MR 1.5 at 24 h. This difference was smaller than expected, possibly due to a more pronounced aggregation of unbound bilirubin for the higher MR, confirmed by light microscopy. All further experiments were performed with a UCB/BSA MR of 1.5. Consequently, the total bilirubin level overestimates the levels of unbound bilirubin during incubation.

BSA, as used in the present study, has a lower affinity for UCB than HSA (26). On the other hand, BSA-bound UCB have shown slower rates of dissociation and cellular interactions in cultured hepatoblastoma cells (26). Furthermore, UCB affinity for HSA is dependent on impurities of UCB and on buffer composition, and is inversely correlated to HSA concentrations (27). How this applies to UCB affinity for BSA is not settled. Hence, due to differences in binding affinities, and the fact that UCB often forms metastable solutions where the precipitation level is unpredictable, the choice of species of albumin may influence toxicity of UCB in otherwise identical conditions. In the present study, a stepwise increase in total bilirubin with a fixed UCB/BSA MR (1.5) induced a graded toxic effect of bilirubin, indicating a proportional increase also for the unbound fraction of UCB.

The execution of apoptosis is an energy-requiring process (3). In neurons treated with ≤ 10 μ M UCB, metabolic activity was maintained as measured by MTT cleavage throughout the observation period, concomitant with nuclear fragmentation and condensation compatible with apoptosis. Despite almost total cell death at 48 h ($>94\%$), as indicated by nuclear morphology, LDH release was moderate after treatment with STS. A possible explanation for this finding is that apoptosis results in a compaction of the cell membrane without the spillage of cell contents into the culture medium before secondary necrosis (17). All UCB concentrations induced a significant proportion of condensed nuclei with disrupted membrane and a significant increase in LDH release compared with controls within 24 h, consistent with a component of early necrotic cell death. Neurons treated with 25 μ M UCB, however, displayed a coexistence of a variety of different shapes not entirely typical of apoptosis or necrosis combined with a

Figure 7. Western blot analysis of PARP in NT2-N neurons after 48 h incubation with different UCB concentrations (UCB/BSA molar ratio 1.5) or 2 μ M STS. The band at 116 K is intact PARP and the band at 85 K represents the cleaved fragment. STS and UCB 5 μ M cleaved PARP (arrows). PARP cleavage was not seen in cells treated with UCB ≥ 10 μ M UCB, in necrosis controls (500 μ M H_2O_2), or in negative controls.

late LDH release, suggesting a component of secondary necrosis.

DNA fragmentation in apoptosis is a two-step process in which DNA is first cleaved into large fragments of 50–300 kb (HMW fragments) that subsequently are cleaved into smaller oligonucleosomes (the DNA ladder) (18). Only the first step is considered essential in apoptosis (18). NT2-N neurons have been reported never to produce DNA ladders, although morphologic changes consistent with apoptosis can be induced (19). In the present study, 5 μ M UCB, like STS, induced not only HMW fragments, but also DNA ladders; 10 and 25 μ M UCB induced HMW fragments only. This finding further substantiates that low and moderate UCB concentrations induce apoptosis-like cell death. PARP cleavage by caspase-3 is vital for the appropriate function of the apoptotic machinery in at least one pathway to apoptosis (19). PARP cleavage induced by 5 μ M UCB (and STS), as found in the present study, indicates a possible role for caspase-3 in bilirubin-mediated apoptosis in human NT2-N neurons.

The present study is the first to investigate the relation between UCB-induced apoptosis and necrosis in a human neuronal cell line. The importance of species differences was recently underscored when tumor necrosis factor-related apoptosis-inducing ligand was found to induce apoptosis in human CNS (28). Previously, this agent had been thought to be safe as it did not cause neuronal damage in laboratory animals. In general, our findings are in agreement with previous findings in nonhuman cells. Seubert *et al.* (9) investigated UCB-induced cell death (1–100 μ M UCB and a fixed concentration of 40 μ M BSA) in three different murine hepatoma cell lines. Assessed with nuclear morphology, they found a dose-dependent increase of both apoptosis and necrosis at 24 h, with an increase in the relative proportion of necrotic cells at higher concentrations. In a study on cortical neurons from newborn rats by Grojean *et al.* (5), 0.4 μ M UCB (without added albumin) induced predominately apoptotic cell death (16% apoptotic *versus* 8% necrotic cells at 96 h). On the other hand, relatively high UCB concentrations (17 and 86 μ M UCB/HSA MR 3) were recently reported to induce apoptosis in fetal rat neurons after only 4 h (8).

In conclusion, the present study demonstrated that UCB induces delayed apoptosis and early necrosis in human NT2-N neurons. In neurons exposed to low UCB concentrations (≤ 10 μ M), apoptosis was the major form of cell death. The shift in cell death mechanisms within a clinically relevant concentration range, as found in the present study, urges further *in vitro* and *in vivo* investigations in the hope that a better knowledge of UCB toxicity may yield novel therapeutic approaches.

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