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COMMENTARY

Bilirubin Induced Apoptosis *In Vitro*: Insights for Kernicterus

Commentary on the article by Hankø et al. on page 179

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Despite a wealth of studies on hyperbilirubinemia and brain injury, many fundamental questions regarding the mechanisms of bilirubin-induced cell damage and cell death remain unanswered, their details incompletely understood, and their potential clinical relevance not fully characterized (1–5). Does bilirubin neurotoxicity reflect interference with single or multiple pathways (2,3)? Is there a unifying mechanism to explain bilirubin intoxication (1,2)? Why are only selected cell types (and not all neurons) readily "poisoned" by bilirubin (5)? What is the sequence and relative importance of toxic events in bilirubin induced cell injury and cell death (5)?

Neuronal cell damage in kernicterus is classically described as eosinophilic necrosis, but this literature largely predates recognition of programmed cell death or apoptosis and on closer inspection describes a spectrum of histologic and cytologic alterations across time of which necrosis may be but a final common pathway or end point (6). Of particular interest in kernicteric human neonates are early, subacute neuropathologic changes akin to apoptosis including nuclei with increased nuclear density and a lack of inflammation (6), the latter a hallmark of apoptosis. Thus, apoptosis, an active energy (ATP) requiring, highly organized, controlled process of cell self-destruction may represent an important means of bilirubin-induced cell death particularly during the early phases of injury. In contrast, later neuropathologic changes of kernicterus are characterized by a predominance of neuronal cell loss, prominent reactive astrocytosis, and necrosis (6).

Hanko and colleagues in a compelling paper in this issue of *Pediatric Research* determined the modes of cell death in human NT2-N neurons and their temporal evolution following exposure to various concentrations of unconjugated bilirubin (UCB) *in vitro* (7). As the investigators hypothesized and others as cited by the authors suggest, apoptosis and necrosis may represent ends of a cell death spectrum where the magnitude of the insult determines whether a cell undergoes apoptosis or necrosis (7–9). Indeed, Hanko and coworkers demonstrated that increasing UCB concentration from <1 to 250 μM at an UCB to BSA ratio of 1.5 caused a dose dependent increase in NT2-N neuronal cell death *in vitro*. Moreover they observed that: *1*) low concentration [5 μM] UCB exposures were associated with apoptosis as evidenced by fragmented nuclei, DNA laddering, and cleavage of the DNA repair enzyme and death substrate poly (ADP) ribose polymerase (PARP); whereas *2*) moderate [10 μM] to high [$\geq$ 25 μM], UCB concentrations were associated with morphologic nuclear variants typical of necrosis and an absence of DNA laddering and PARP cleavage. Consistent with these findings are the observations of Silva and colleagues who noted that although short duration (4 h) UCB exposures resulted in similar degrees of apoptosis and necrosis in rat astrocytes *in vitro*, necrosis predominated over apoptosis at higher UCB concentrations and longer exposure times (10). These reports are compatible with the notion that the magnitude and duration of insult, in this case UCB concentration, is critical to the death pathway activated and that stimuli that lead to apoptosis are typically milder than those that cause necrosis.

Others also suggest that apoptosis plays a role in bilirubin-induced cytotoxicity *in vitro* (10–15) in murine neurons, astrocytes, and capillary endothelial cells, and that programmed cell death in this context appears to be caspase dependent (although caspase independent alternative cell death mechanisms cannot be entirely excluded at this time). Such evidence includes activation of caspase 3 (15), a key distal effector of apoptosis; PARP cleavage (11,15); decreased mitochondrial transmembrane potential (15); increased mitochondrial cytochrome *c* release (15); DNA fragmentation (11); terminal deoxynucleotididyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) (10); Hoescht nuclear staining (15), and transmission electron microscopic ultrastructural nuclear changes (11). This growing body of literature suggests that enhancing our understanding of bilirubin-induced apoptosis is an important goal to more fully characterize the pathogenesis of bilirubin neurotoxicity. Such insights could hold potential

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therapeutic benefit given that the cascade of events in programmed cell death can be interrupted.

The role of apoptosis in bilirubin-induced cell injury is of increasing interest as well in light of recent findings suggesting that ATP-binding cassette (ABC) transport proteins may limit the degree of bilirubin-induced apoptosis *in vitro* (16,17). Gennuso and coworkers report that inhibition of murine astrocyte multidrug resistance associated protein-1 (MRP-1), an efflux pump of glutathione, xenobiotics, and UCB (18), during UCB exposure *in vitro*, is associated with increased levels of astroglial apoptosis (16). A preliminary report suggests an analogous cellular pro-apoptotic effect of MDR1 P-glycoprotein, an ABC transporter of xenobiotics and possibly UCB (19), inhibition *in vitro* (17). In this regard, both MRP-1 and MDR1 P-glycoprotein are expressed in the developing human CNS, MDR1 P-glycoprotein in brain capillary endothelial cells and perivascular astrocytes of the blood-brain barrier, MRP-1 and MDR1 P-glycoprotein in choroid plexus epithelium of the blood-CSF barrier, and both in cells of the CNS parenchyma not associated with blood vessels including astrocytes within white matter, large pyramidal cells of the brainstem and thalamus, and Purkinje cells of the cerebellum (20). These findings taken together suggest that ABC transport proteins may play a neuroprotective role against bilirubin-induced apoptosis. Whether these effects are mediated by directly limiting the cellular uptake of UCB, extruding UCB before it has a chance to exert its cytotoxic effect, or by a mechanism independent of its bilirubin transport function (21) is unclear but merits further study. Similarly, whether up-regulation of ABC transporter expression in cells of the CNS may further attenuate bilirubin-induced injury and cell death is worth exploring.

It will be important, however, to substantiate that bilirubin-induced apoptosis observed *in vitro* is a phenomenon that occurs *in vivo*. Programmed cell death depends on the confluence of a number of factors and may be different in cell lines exposed to bilirubin *in vitro* compared with cells *in vivo*. To date reported evidence for bilirubin induced apoptosis *in vivo* is limited to morphologic findings under light microscopy in the Gunn rat animal model of neonatal jaundice and kernicterus, more specifically, apoptotic nuclear profiles in cerebellar Purkinje cells in homozygous hyperbilirubinemic animals (22). These animals were treated with sulfadimethoxine (200 mg/kg ip) to enhance the blood concentration of free UCB, increase brain tissue bilirubin accumulation, and thereby neuronal cell injury (22). Although nuclear morphologic features are considered reliable criteria for defining apoptosis, complementary studies using biochemical or molecular assays of apoptosis have yet to be reported in the Gunn rat model of kernicterus or in human kernicteric postmortem tissue samples. Specific markers for apoptosis including *1*) caspase activation, *e.g.*, activated caspase-3 using either immunohistochemical or Western immunoblotting techniques, and *2*) detection of apoptotic (annexin-V positive; propidium iodide negative) and necrotic (propidium iodide positive) cells using flow cytometry would help to substantiate that bilirubin induced apoptosis is a relevant phenomenon *in vivo*. An important challenge in efforts to document bilirubin induced apoptosis *in vivo* will be the rapid clearance of apoptotic cells from tissue, *i.e.* only a few cells are likely to be observed undergoing apoptosis at a single time point. Therefore, assessment at multiple time points may be necessary to detect programmed cell death *in vivo*. Even with such an approach the rapid phagocytosis of apoptotic cells *in vivo* will make an accurate assessment of the contribution of apoptosis to overall cell death *in vivo* difficult to quantify (may in fact preclude it). Documentation of bilirubin induced apoptosis *in vivo*, however, is necessary to substantiate the potential clinical relevance of apoptosis to kernicterus even if the results of such studies will likely underestimate its contribution.

REFERENCES

1. Cashore WJ 1990 The neurotoxicity of bilirubin. Clin Perinatol 17:437–447
2. Hansen TWR 2000 The pathophysiology of bilirubin toxicity. In: Maisels MJ, Watchko, JF (eds) Neonatal Jaundice. Harwood Academic Publishers, Amsterdam, pp 89–104
3. Karp WB 1979 Biochemical alterations in neonatal hyperbilirubinemia and bilirubin encephalopathy: a review. Pediatrics 64:361–368
4. Levine RL 1983 The toxicology of bilirubin. In: Levine RL, Maisels MJ (eds), Hyperbilirubinemia in the Newborn, Report of the Eighty-fifth Ross Conference on Pediatric Research, Columbus, OH, pp 39–44
5. Wennberg RP, Ahlfors CE, Rasmussen LF 1979 The pathochemistry of kernicterus. Early Hum Dev 3:353–372
6. Ahdab-Barmada M 2000 The neuropathology of kernicterus: definitions and debate. In: Maisels MJ, Watchko JF (eds) Neonatal Jaundice. Harwood Academic Publishers, Amsterdam, pp 75–88
7. Hanko E, Hansen TWR, Almaas R, Lindstad J, Rootwelt T 2004 Bilirubin induces apoptosis and necrosis in human NT2-N neurons. Pediatr Res 57:179–184
8. Leist M, Nicotera P 1998 Apoptosis, excitotoxicity, and neuropathology. Exp Cell Res 239:183–201
9. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA 1995 Apoptosis and necrosi- 2 distinct events induced, respectively, by mild and intense insults with N-Methyl-D-Aspartate or nitric-oxide superoxide in cortical cell-cultures. Proc Natl Acad Sci USA 92:7162–7166
10. Silva RFM, Rodrigues CMP, Brites D 2001 Bilirubin-induced apoptosis in cultured rat neural cells is aggravated by cheonodeoxycholic acid but prevented by urodeoxycholic acid. J Hepatol 34:402–408
11. Aikin E, Clower B, Tibbs R, Tang J, Zhang J 2002 Bilirubin produces apoptosis in cultured bovine brain endothelial cells. Brain Res 931:168–175
12. Grojean S, Koziel V, Vert P, Daval JL 2000 Bilirubin induces apoptosis via activation of NMDA receptors in developing rat brain neurons. Exp Neurol 166:334–341
13. Rodrigues CM, Sola A, Silva R, Brites D 2000 Bilirubin and amyloid-beta peptide induce cytochrome c release through mitochondrial membrane permeabilization. Mol Med 6:936–946
14. Rodrigues CMP, Sola S, Castro RE, Laires PA, Brites D, Moura JJG 2002 Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis. J Lipid Res 43:885–894
15. Rodrigues CMP, Sola S, Brites D 2002 Bilirubin induces apoptosis via the mitochondrial pathway in developing rat brain neurons. Hepatology 35:1186–1195
16. Gennuso F, Fernetti C, Tirolo C, Testa N, L'Espiscopo F, Caniglia S, Morale MC, Ostrow JD, Pascolo L, Tiribelli C, Marchetti B 2004 Proc Natl Acad Sci USA 101:2470–2475
17. Watchko JF, Ziegler J, Daood MJ, Balkundi D 2003 Inhibition of human MDR1 P-glycoprotein increases bilirubin-induced cell apoptosis *in vitro.* Pediatr Res 53:400A
18. Rigato I, Pascolo L, Fernetti C, Ostrow JD, Tiribelli C 2004 The human Multidrug Resistance-associated Protein MRP1 mediates ATP-dependent transport of unconjugated bilirubin. Biochem J 15:335–341
19. Watchko JF, Daood MJ, and Hansen TWR 1998 Brain bilirubin content is increased in P-glycoprotein deficient transgenic null mutant mice. Pediatr Res 44:763–766
20. Daood MJ, Ahdab-Barmada M, Watchko JF 2004 Comparison of multidrug resistance protein-1 (MRP-1) and P-glycoprotein (Pgp) expression in the developing human central nervous system: cellular and tissue localization. Pediatr Res 55:460A–461A
21. Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW 1998 The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. Proc Natl Acad Sci USA 95:7024–7028
22. McDonald JW, Shapiro SM, Silverstein FS, Johnston MV 1998 Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. Exp Neurol 150:21–29