Editorial

Stem Cell INVESTIGATION

Noncoding RNAs as therapeutics for acetaminophen-induced liver injury

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Received: 30 August 2016; Accepted: 09 September 2016; Published: 11 October 2016.

doi: 10.21037/sci.2016.09.10

View this article at: http://dx.doi.org/10.21037/sci.2016.09.10

Acetaminophen-induced injury and acute liver failure as a clinically relevant model

Acetaminophen- (APAP) induced acute liver failure (ALF) is a major cause of morbidity and mortality in the US (1). Currently, the major therapeutic is N-acetylcysteine (NAC), which is highly effective when given within 8 h of drug overdose. However, late-presenting patients commonly have poor outcomes, and NAC is not effective in these patients. As such, additional therapeutics are urgently needed to treat late-presenting patients. Critical to the generation of new drugs is the continued development of our understanding of APAP-induced ALF in human patients. While a number of recent papers from our group and others have made considerable advances in understanding mechanisms of toxicity in patients and human hepatocytes (2-5), there is still a lack of actionable therapeutic targets. Liver transplantation is the gold standard procedure for treating late-stage ALF but this operation is expensive and comes with additional costs of lifelong anti-rejection medication. In addition, due to limited donor organs, this therapeutic option is not always available (1). There are also ethical issues to consider as a majority of APAP-induced liver injury cases are due to attempted suicide. It is thus imperative that new modalities of treatment, including novel pharmacological agents, be explored for patients who suffer from drug-induced liver injury and acute liver failure.

The mechanisms behind APAP-induced liver injury have been extensively examined over the last 40 years (6,7). APAP is typically non-toxic when taken at therapeutic dosages as it is largely metabolized through phase II metabolism by glucuronosyltransferases (glucuronidation) and sulfotransferases (sulfation), and then excreted via the urine (7). Minor levels of APAP are metabolized by cytochrome P450 2E1 (Cyp2E1), which results in the formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) (7). However, when sulfation is saturated (8), additional quantities of APAP are metabolized by Cyp2E1 and NAPQI accumulation initiates liver toxicity. Excess NAPQI causes significant depletion of glutathione, protein adduct formation especially in mitochondria (9,10) and a mitochondrial oxidative and nitrosative stress (11), which is then amplified by translocation of c-Jun N-terminal kinase (JNK) to the mitochondria (12,13). This triggers the opening of the mitochondrial permeability transition pore resulting in collapse of the membrane potential, cessation of ATP formation, and matrix swelling (14). The latter effect results in the outer membrane rupture with release of mitochondrial membrane proteins including apoptosis inducing factor and endonuclease G, which translocate to the nucleus and trigger DNA fragmentation (6). The extensive mitochondrial dysfunction and karyolysis are responsible for the necrotic cell death (6). A number of therapeutics have been proposed based on these mechanisms and the pathways that control them; however, new mechanisms that complement our current understanding are currently being investigated.

Micro-RNAs (miRs) in acetaminophen-induced liver injury

One potential novel avenue that has been explored to a limited degree thus far is the use of miRs. While early reports indicated that miRs were sensitive markers of toxicity, and might have predictive and diagnostic value in the treatment of APAP (2,15), there have been a few studies that attempted to understand how miR alterations affect

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APAP-induced liver injury. While it has been established that miRs control a number of cellular functions, the specific functions controlled by different miRs are largely still under investigation (16). Antagonism of miRs has therapeutic potential as they are targetable in a highly specific fashion, and their degradation can have profound effects on transcription of specific genes without affecting the genome. A few specific miRs have been indicated to potentially play a role in APAP toxicity. MiR-375 functions as a repressor of glucuronidation in the liver, and thus may predispose some patients to APAP-induced liver injury (17). Similarly, single-nucleotide polymorphisms in CYP2E1 are regulated in human liver by miR-570, which also may predispose some patients to excessive phase I metabolism, and thus APAP-induced liver injury (18). Given this information, novel studies aimed at understanding how miRs regulate the pathways and genes that control APAP-induced liver injury is imperative.

Drug-induced liver injury—new models, new methods

In a recent issue of *Stem Cells and Translational Medicine*, Szkolnicka and colleagues have attempted to advance both our understanding of APAP-induced liver injury in human cells, and simultaneously generate and evaluate a new therapeutic (19). They accomplished this through a number of novel experiments designed to generate a more robust system for assessing APAP toxicity. The authors were able to effectively differentiate human embryonic stem cells (hESCs) into hepatocyte-like cells, as demonstrated by the expression of HNF-4α and albumin, and, furthermore, showed that these cells express a number of functions imperative to APAP-induced liver injury including expression of cytochrome P450 enzymes necessary for APAP metabolic activation. While the cells had some differences in gene expression relative to cryopreserved primary human hepatocytes, they were noted as still expressing similar levels of critical genes and more importantly, the IC_{50} value for APAP toxicity reported in their cell line was similar to previously reported values in primary human hepatocytes (4). This was further confirmed by global miR analysis, wherein the hESCs were compared to primary human hepatocytes. Approximately 60% of all miRs were expressed at the same level in the hESC-derived hepatocytes, including miR-122, a liver specific miR that is expressed at very high levels (20). As such, the authors have largely generated a cell line similar to human hepatocytes that also expresses key features. New cell lines such as this are important for both the development of new therapeutics for APAP overdose and for understanding drug-induced liver injury in human patients. Currently, primary human hepatocytes are difficult to attain, expensive, and of very limited supply. While some newer cell lines, such as HepaRG cells recapitulate more of the facets of primary human hepatocytes (4,21-23), they still only express a relatively small degree of hepatocyte functional genes such as drug metabolizing enzymes and xenobiotic transporters (24). Human ESCs are highly proliferative, have an indefinite life span, and could thus be used to generate on a routine basis new and useful human hepatocyte-like cells that fairly accurately resemble primary human hepatocytes.

The authors went on to look at how antagonism of specific miRs could potentially ameliorate the effects of APAP by focusing on enhancing phase II metabolism. The authors hypothesized that the downregulation of phase II metabolizing genes such as sulfotransferases by antagonizing miRs could potentially enhance detoxification of APAP, and protect against APAP-induced liver injury. Antagonism of miR-324 resulted in increased expression of sulfotransferase 2A1, a reduction in the amount of glutathione depletion, and increased cellular survival in APAP-treated cells (19). The authors interpreted these data to mean there was enhanced phase II metabolism and consequently less phase I metabolism (NAPQI formation) with less GSH depletion, and less cell death. While this interpretation is largely valid, it should be noted that potential off-target effects of antagonism of miR-324 directly on phase I metabolism were not assessed, and thus, the protective mechanism could also be through reduced metabolism of APAP by cytochrome P450 enzymes. The authors attempted to validate their claims by applying patient plasma onto their hESC-derived hepatocytes that were either transfected with the miR-324 antagomir or a control transfection. Szkolnicka and colleagues showed increased ATP levels in the miR-324 antagomir-treated cells and an increase in caspase 3/7 activity after exposure to this plasma, and infer that there is a shift from necrosis to apoptosis (19). However, there are a number of issues with this experiment. First, the application of human plasma from an unknown time point in the patients' course likely contains any number of other toxic metabolites released from dying cells which are largely out of their normal context without a functional immune system. Second, the minimal increase in caspase 3/7 activity may not even be responsible for appreciable levels of cell death, especially

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given that these cells appear to have a substantial baseline activation of caspases when exposed to control serum. Given that apoptosis is only a minor factor of cell death after APAP overdose (3,5), these results do not reflect the situation in patients. Third, cells were only exposed to diluted (20%) patient plasma. Given that the plasma was obtained after the injury when the patients' hepatocytes did not experience toxicity from being exposed to 100% of this plasma, these results indicate a higher susceptibility of the cell line. Fourth, and most importantly, the authors noted that the plasma did not actually contain APAP. This means that whatever caused the toxicity, it was not induced by the reactive metabolite of APAP indicating that the protection in the miR-324 antagomir-transfected cells was not due to the sulfotransferase 2A1 overexpression. It would have been important to assess the actual mechanism of toxicity and protection in this experiment.

In summary, Szkolnicka and colleagues used hESC-derived hepatocytes to study mechanisms and potential therapeutic approaches for human APAP-induced liver injury in patients. The authors showed that miR-324 antagomir transfection increased sulfotransferase 2A1 and attenuated APAP-induced cell death. While these experiments documented that targeting phase II enzymes can limit APAP toxicity, this therapeutic approach has limited value in patients. The current clinical antidote NAC is highly effective during the metabolism phase of APAP overdose by scavenging NAPQI and even beyond by scavenging reactive oxygen (25). Novel therapeutic approaches including use of noncoding RNAs need to target later events in the mechanism of cell death or promote regeneration in order to limit injury and acute liver failure in APAP overdose patients.

Acknowledgements

Work in the authors' laboratory was supported in part by the National Institutes of Health grants DK070195 and DK102142, and by a grant from the National Institute of General Medical Sciences (P20 GM103549) of the National Institutes of Health. Additional support came from the "Training Program in Environmental Toxicology" T32 ES007079 from the National Institute of Environmental Health Sciences.

Footnote

Provenance: This is a Guest Editorial commissioned by Editor-in-Chief Zhizhuang Joe Zhao (Pathology Graduate Program, University of Oklahoma Health Sciences Center, Oklahoma City, USA).
Conflicts of Interest: The authors have no conflicts of interest to declare.

Comment on: Szkolnicka D, Lucendo-Villarin B, Moore JK, *et al.* Reducing Hepatocyte Injury and Necrosis in Response to Paracetamol Using Noncoding RNAs. Stem Cells Transl Med 2016;5:764-72.

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doi: 10.21037/sci.2016.09.10
Cite this article as: Woolbright BL, Jaeschke H. Noncoding RNAs as therapeutics for acetaminophen-induced liver injury. Stem Cell Investig 2016;3:54.

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