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Epigenetic changes in the rat livers induced by pyrazinamide treatment ☆

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Abstract

Drug-induced liver injury, including drug-induced hepatotoxicity during the treatment of tuberculosis infection, is a major health problem with increasingly significant challenges to modern hepatology. Therefore, the assessment and monitoring of the hepatotoxicity of antituberculosis drugs for prevention of liver injury are great concerns during disease treatment. The recently emerged data showing the ability of toxicants, including pharmaceutical agents, to alter cellular epigenetic status, open a unique opportunity for early detection of drug hepatotoxicity. Here we report that treatment of male Wistar rats with antituberculosis drug pyrazinamide at doses of 250, 500 or 1000 mg/kg/day body weight for 45 days leads to an early and sustained decrease in cytosine DNA methylation, progressive hypomethylation of long interspersed nucleotide elements (LINE-1), and aberrant promoter hypermethylation of placental form glutathione-*S*-transferase (*GSTP*) and $p16^{INK4A}$ genes in livers of pyrazinamide-treated rats, while serum levels of bilirubin and activity of aminotransferases changed modestly. The early occurrence of these epigenetic alterations and their association with progression of liver injury specific pathological changes indicate that alterations in DNA methylation may be useful predictive markers for the assessment of drug hepatotoxicity.

Keywords: DNA methylation; Pyrazinamide; Liver injury; Rat

Introduction

Drug-induced severe hepatotoxicity is a major health problem with increasingly significant challenges to modern hepatology (Holt and Ju, 2006; Björnsson, 2006). It can arise at various stages during the development of a drug or, in many cases, even after its approval. Drug-induced liver injury has been a long-standing concern in the treatment of tuberculosis infection, especially during treatment of latent tuberculosis (Durand et al., 1996; Lenaerts et al., 2005; McElroy et al., 2005; Younossian et al., 2005; Ijaz et al., 2006; Saukkonen et al., 2006). The hepatotoxic effects, including fatal liver injury, of most commonly used antituberculosis drugs, such as isoniazid, rifampicin, and pyrazinamide, resulted in the revision of the US guidelines for treatment of latent tuberculosis (McCarthy, 2001). Therefore, the assessment and monitoring of the hepatotoxicity of antituberculosis drugs for prevention of liver injury are great concerns during disease treatment. Additionally, isoniazid and rifampicin are considered as non-genotoxic rodent hepatocarcinogens (Nie et al., 2006), though they have not been recognized as carcinogens for humans (Skakun and Tabachuk, 1991).

Current clinical diagnosis of drug-induced liver injury depends on non-specific elevation in liver test results, in particular, increases in serum aminotransferases activity and bilirubin levels (Björnsson, 2006). This proven approach is successfully employed in the assessment of hepatotoxicity of newly developed drugs; however, the accuracy of this method is limited for monitoring liver injury induced by pharmacological agents during treatment of chronic diseases and for determining long-term prognosis. Furthermore, this methodology does not allow the elucidating and understanding of the underlying pathophysiological and biochemical mechanisms of drug-induced liver dysfunction and the prediction of the outcome of drug-induced liver injury. This illustrates clearly the crucial need for uncovering underlying mechanisms of drug-induced liver injury for

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improvement of its diagnostic accuracy. More importantly, further studies are needed to determine the long-term prognosis of drug-induced hepatotoxicity (Björnsson, 2006).

The recently emerged data showing the ability of toxicants, including pharmaceutical agents, to alter cellular epigenetic status open a unique opportunity for early detection of drug hepatotoxicity. Importantly, it has been suggested that drug-induced epigenetic changes, especially alterations in global DNA methylation status, as well as changes in methylation status of particular genes, may be more sensitive indicators of liver toxicity than classic parameters used for toxicity assessment, and they may be useful predictive biomarkers for toxicities (Moggs et al., 2004; Watson et al., 2004). Furthermore, the early appearance of epigenetic changes may give an advantage for timely detection of toxic potential compared to the currently used indicators.

Based on these considerations, the present study was undertaken to determine (a) whether or not the drug-induced hepatotoxicity is associated with alteration in DNA methylation status, (b) whether the assessment of alteration in DNA methylation may be used as a biomarker for detection and monitoring of drug-induced liver injury.

We demonstrate that exposure of male Wistar rats to antituberculosis drug pyrazinamide for 45 days leads to an early and sustained decrease in cytosine DNA methylation, progressive hypomethylation of long interspersed nucleotide elements (LINE-1), and aberrant promoter hypermethylation of placental form glutathione-*S*-transferase (*GSTP*) and $p16^{INK4A}$ genes in livers of pyrazinamide-treated rats. Importantly, these changes were associated with the progression of pathomorphological changes indicative for liver injury.

Materials and methods

Animals, treatment, and tissue preparation. Male Wistar rats were obtained from the Institute of Pharmacology and Toxicology of the Academy of Medical Sciences of Ukraine breeding facility (Kyiv, Ukraine), housed in a temperature-controlled room (24°C), and given *ad libitum* access to water and laboratory diet. In the preliminary study, the rats (body weight 160–200 g) were allocated randomly and treated with pyrazinamide at doses of 250, 500, or 1000 mg/kg/day body weight or vehicle by oral gavage for 45 days. The low dose corresponded to human therapeutical dose taking into account species sensitivity according to U.S. Food and Drug Administration Guidance available at http://www.fda.gov/cder/guidance/index.htm, and the second and third doses exceeded human dose by 2- and 4-fold, respectively. Bedding, feed, water, and animal maintenance were identical for all rats. Five rats per group were sacrificed after 45 days of pyrazinamide initiation; prior to sacrifice, blood was taken from narcotized rats. The livers were excised, frozen immediately in liquid nitrogen, and stored at −80 °C for subsequent analysis. The liver slices from median lobe were fixed in 10% neutral buffered formalin for 48 h, processed, embedded in paraffin, sectioned at 4 μm, and mounted on glass slides. The sections were stained with hematoxylin and eosin for histopathological examination.

For the main study, a concentration of 250 mg/kg/day of pyrazinamide and treatment for 15, 30 or 45 days were selected. After exposure, 5 rats per control or treated groups were sacrificed at indicated time intervals, and blood and liver tissues samples were collected as described above. Additionally, 5 rats from control and treated groups were sacrificed 30 days after the rats received the last pyrazinamide treatment.

All animal experimental procedures were carried out in accordance with animal study protocol approved by the Institutional Animal Care and Use Committee.

Analyses of serum bilirubin content and aminotransferase activities. The total serum bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined by standard methods according to the manufacturer's instructions (PLIVA-Lachema, Brno, Czech Republic).

Determination of global DNA methylation status. The extent of global DNA methylation was evaluated with a radiolabeled [^{3}H]dCTP extension assay as described previously (Pogribny et al., 1999).

Methylation analysis of LINE-1 repetitive elements. Methylation status of LINE-1 was determined by the combined bisulfite restriction analysis (COBRA) as described previously (Eads and Laird, 2002), including bisulfite modification of genomic DNA, PCR amplification, and digestion of PCR products with restriction enzymes *Bst*UI or *Rsa*I (Tryndyak et al., 2007).

Determination of the $p16^{INK4A}$ methylation status by methylation-specific PCR (MSP). The methylation status of the promoter and first exon of the $p16^{INK4A}$ gene was determined by MSP analysis; the primer sequences and PCR conditions have been previously described (Swafford et al., 1997). The PCR products were separated on 3% high resolution agarose gels (Sigma, St. Louis, MO), stained with ethidium bromide, and photographed. Negative control PCR amplifications were performed by using both sets of modified primers with untreated DNA. The absence of PCR products confirmed that unmodified DNA could not be amplified with modified sets of primers.

Determination of the GSTP promoter methylation status by methylation-sensitive PCR. Genomic DNA was digested with *Hpa*II or *Bst*UI cytosine-methylation-sensitive restriction endonuclease followed by PCR amplification of a 168 bp fragment of the *GSTP* promoter region. In our previous study, we have shown that CpG sites located within this fragment are differentially methylated in normal and injured liver tissues (Steinmetz et al., 1998). By using primers that flank the *Hpa*II or *Bst*UI cleavage sites within the 5′ region of the GSTP gene, quantitative recovery of PCR product will vary directly with the extent of *Hpa*II- or *Bst*UI-induced DNA breaks at unmethylated CCGG or CGCG sites, respectively. One microgram of genomic DNA was treated with 20 units of *Hpa*II or *Bst*UI restriction endonuclease (New England Biolabs, Ipswich, MA) for 16 h according to the manufacturer's instructions. The primer sequences were: the sense primer 5′-TCATCGTCCACGCAGCTTTGA-3′ and the antisense primer 5′-CCTTCTCTGTTTTGTCCCCCAGAAC-3′. The semi-quantitative aspect of the procedure was verified by a linear increase in PCR product recovery with increasing cycle number and DNA template concentration. The PCR products were separated on 3% agarose gels (Sigma), stained with ethidium bromide, photographed, and the band intensity was analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results are presented as ratio of PCR product recovery after digestion DNA with the appropriate restriction endonuclease to undigested DNA.

Statistical analysis. Results are presented as mean ± S.D. Statistical analyses were conducted by two-way ANOVA, using treatment and weeks as fixed factors. P-values < 0.05 were considered significant.

Results

Effect of different doses of pyrazinamide on the serum bilirubin levels, activity of aminotransferases, and liver DNA methylation in pyrazinamide-treated rats

In the present study, rats were exposed to pyrazinamide in order to determine association between the drug-induced liver pathology and perturbation in genome-wide and region-specific DNA methylation. Increased levels of serum bilirubin and activities of ALT and AST are established clinical indicators of liver damage (Durand et al., 1996; Lenaerts et al., 2005). Treatment of rats with pyrazinamide in doses of 250, 500 or 1000 mg/kg/day body weight led to minor changes in serum ALT and AST activities (Fig. 1, panels B and C). After 45 days of