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Microarray analysis of thioacetamide-treated type 1 diabetic rats

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Abstract

It is well known that diabetes imparts high sensitivity to numerous hepatotoxicants. Previously, we have shown that a normally non-lethal dose of thioacetamide (TA, 300 mg/kg) causes 90% mortality in type 1 diabetic (DB) rats due to inhibited tissue repair allowing progression of liver injury. On the other hand, DB rats exposed to 30 mg TA/kg exhibit delayed tissue repair and delayed recovery from injury. The objective of this study was to investigate the mechanism of impaired tissue repair and progression of liver injury in TA-treated DB rats by using cDNA microarray. Gene expression pattern was examined at 0, 6, and 12 h after TA challenge, and selected mechanistic leads from microarray experiments were confirmed by real-time RT-PCR and further investigated at protein level over the time course of 0 to 36 h after TA treatment. Diabetic condition itself increased gene expression of proteases and decreased gene expression of protease inhibitors. Administration of 300 mg TA/kg to DB rats further elevated gene expression of proteases and suppressed gene expression of protease inhibitors, explaining progression of liver injury in DB rats after TA treatment. Inhibited expression of genes involved in cell division cycle (cyclin D1, IGFBP-1, ras, E2F) was observed after exposure of DB rats to 300 mg TA/kg, explaining inhibited tissue repair in these rats. On the other hand, DB rats receiving 30 mg TA/kg exhibit delayed expression of genes involved in cell division cycle, explaining delayed tissue repair in these rats.

In conclusion, impaired cyclin D1 signaling along with increased proteases and decreased protease inhibitors may explain impaired tissue repair that leads to progression of liver injury initiated by TA in DB rats.

Keywords: Cathepsin; Cyclin D1; Diabetes; Microarray; Thioacetamide; Tissue repair

Introduction

Type 1 as well as type 2 diabetes are known to potentiate hepatotoxicity of numerous structurally and mechanistically diverse hepatotoxicants such as thioacetamide (TA), $CHCl_3$, and CCl_4 (Hanasono et al., 1975; El-Hawari and Plaa, 1983; Sawant et al., 2004, in press). Previously, we have shown that 300 mg/kg dose of TA is a non-lethal dose in normal rats. However, administration of the same dose of TA to type 1 diabetic (DB) rats resulted in CYP2E1-mediated higher initial liver injury and 90% mortality (Wang et al., 2000a, 2000b). It was found that diallyl sulfide (DAS), a specific inhibitor of CYP2E1, equalized CYP2E1-mediated initiation of TA-induced liver injury in DB rats to the same level as found in non-diabetic (NDB) rats (Fig. 1). Further investigation revealed that even though DAS decreased initial liver injury, it did not rescue the TA-treated DB rats from 90% mortality because tissue repair remained suppressed and led to progression of liver injury and acute liver failure in these rats (Fig. 1) (Wang et al., 2001).

It could be argued that higher initial liver injury in TA-treated DB rats resulted in lower number of healthy hepatocytes for cell division, thereby inhibiting compensatory tissue repair. To investigate this possibility, an initial-equihepatotoxicity model was developed in which 30 mg TA/kg in DB rats was found to be non-lethal and initiated liver injury equal to the NDB rats receiving 300 mg TA/kg (Wang et al., 2000a). In spite of equal initial bioactivation-mediated liver injury, DB rats receiving 30 mg TA/kg exhibited progression of liver injury due to delayed tissue repair, resulting in delayed recovery from liver injury (Wang et al., 2000a). These findings indicated that impairment of tissue repair leading to progression of liver injury plays a determinant role in the final unfavorable outcome of hepatotoxicity. Therefore, the objective of the present study was to investigate the mechanism of impaired tissue repair and progression of liver injury in TA-treated type 1 DB rats.

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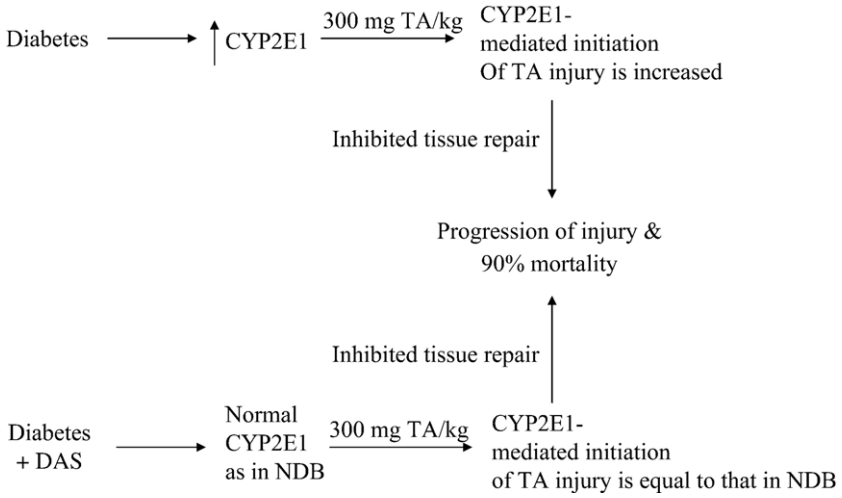

Fig. 1. Inhibited tissue repair, not bioactivation-mediated initiation of liver injury, plays a determinant role in potentiation of thioacetamide (TA)-induced liver injury in type 1 diabetic (DB) rats. TA is bioactivated by CYP2E1 enzyme. Type 1 diabetes increases CYP2E1 protein levels by ~5- to 8-fold, thereby increasing the bioactivation of TA and initial liver injury induced by 300 mg TA/kg. This is accompanied by inhibited tissue repair that allows progression of liver injury and 90% mortality in the DB rats receiving 300 mg TA/kg. To investigate the role of bioactivation-mediated liver injury of TA in the final outcome, diallyl sulfide (DAS) was used to inhibit CYP2E1 in the DB rats to the same level as in non-diabetic (NDB) rats. Inhibition of CYP2E1 by DAS decreased initial liver injury as observed in NDB rats. In spite of decreased bioactivation-mediated liver injury of TA, liver injury progresses in DAS + TA-treated DB rats due to inhibited hepatic tissue repair, indicating that inhibited tissue repair, not bioactivation-mediated liver injury, plays a determinant role in the final outcome of liver injury (survival/death) in TA-treated DB rats (Wang et al., 2000b, 2001).

Hepatocytes respond to a toxicant challenge by altering various signaling pathways that result in transcriptional changes. Simultaneously altering numerous signaling pathways poses a challenge to researchers in identifying important signaling molecules which can be targeted to develop novel therapeutic avenues. The application of microarray technology to screen a wide array of genes involved in multiple signaling pathways represents a significant advance in identifying the key molecules to understand the mechanisms after toxicant challenge. Therefore, a comprehensive cDNA microarray approach was used as a hypothesis-generating tool to understand the mechanism of impaired tissue repair and progression of liver injury in TA-treated DB rats. Changes in gene expression pattern were examined at 0, 6, and 12 h after TA challenge in NDB rats receiving 300 mg TA/kg and in the DB rats receiving 30 and 300 mg TA/kg. The genes were classified into clusters, and leads from microarray experiments were further investigated over the extensive time course of 0 to 36 h by real-time RT-PCR and immunoblotting.

Materials and methods

Animals and maintenance. Male Sprague–Dawley rats (250 to 300 g) were obtained from our central animal facility. The rats received commercial rodent chow (Teklad rodent diet # 7002, Harlan Teklad, Madison, WI) and water ad libitum and were housed under controlled temperature (21 ± 1 °C), humidity (50 ± 10%), and a 12-h photoperiod. Animal care and use were in accordance with the NIH Guide for the care and use of laboratory animals.

Induction of diabetes and thioacetamide treatment. On day 0, rats received either a single dose of streptozotocin (STZ, 60 mg/kg, i.p.) or citrate buffer (1 ml/kg, i.p.). On day 9, plasma glucose level was measured, and the rats were considered DB if plasma glucose level was ≥200 mg/dl (mean 390 ± 112 mg/dl). On day 10, DB rats were divided into two groups. First group was treated with TA (300 mg/kg, i.p.), while the second group of DB rats was treated with 30 mg TA/kg, i.p. The control NDB group treated with citrate buffer received 300 mg TA/kg. At 0, 6, 12, 24, and 36 h after TA administration ($n = 4$ per group per time point), liver samples were collected and stored at −80 °C, until use. For both the DB groups receiving 300 as well as 30 mg TA/kg, one common 0-h control group, i.e., DB rats at 0 h, was used.

Isolation of RNA. Total RNA from frozen liver tissue was isolated using the RNA easy system (QIAGEN, Valencia, CA) as per the manufacturer's protocol. Isolated RNA was considered pure if the ratio of absorbance readings at 260 and 280 nm fell in the range of 1.7 to 2.1. $n = 3$ per group per time point was used for microarray experiments by pooling the samples in equal amount of RNA.

Expressed sequence tag (EST) libraries. Poly-A mRNA was isolated from total RNA via the Oligotex kit (QIAGEN, Valencia, CA). Construction of EST libraries was typically performed by using the cDNA Timesaver kit (Amersham Biosciences Inc., Sunnyvale, CA). In brief, 5 μg of poly-A was used to generate first strand cDNA with an anchored poly-T oligonucleotide [dT(18)-N] that also contained a NotI-specific restriction site. First strand cDNA was then purified via a phenol/chloroform extraction. After second strand synthesis, cDNA was again purified using phenol/chloroform extraction. Ligation of adaptors containing an EcoRI site was then performed, and the cDNA was digested with both NotI and EcoRI. The cDNA underwent size selection to give fragments with a minimum size of 400 bp. The resultant cDNA was repurified as described above and then directionally cloned into the NotI and EcoRI sites of pGEM11zf (Promega, Madison, WI).

Full-length libraries. Five micrograms of total RNA was dephosphorylated for 1 h using calf intestinal phosphatase (Stratagene, La Jolla, CA). The reaction was then cleaned up by a phenol/chloroform extraction followed by ethanol precipitation. The mRNA cap was then removed from the RNA using tobacco acid pyrophosphatase (Epicenter Technologies, Madison, WI). After phenol/chloroform extraction and ethanol precipitation, a custom RNA oligonucleotide sequence was then ligated to the mRNA using RNA ligase. A reverse transcription reaction was performed using Powerscript reverse transcriptase (BD Biosciences, San Jose, CA). The cDNA was amplified using the polymerase chain reaction (PCR), and products were checked on an agarose gel for amplification success and quality. The PCR products were purified using a PCR purification kit (QIA-GEN) and digested with AscI and PacI enzymes corresponding to sites incorporated during PCR. The PCI-neo vector (Promega)