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Morphological and gene expression analysis in mouse primary cultured hepatocytes exposed to streptozotocin

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Abstract

Streptozotocin (SZ) is known to exert toxic effects not only on pancreatic islet beta cells but also on other organs including the liver. For analyzing direct effects of SZ on hepatocytes, we performed morphological analysis and DNA microarray analysis on mouse primary cultured hepatocytes. Hepatocytes were taken from non-treated Crj:CD-1(ICR) mice. The primary cultured hepatocytes were treated with SZ at concentrations of 0, 1, 3, 10, 30 and 100 mM. After the treatment for about 6 or 24 h, cell survival assay using tetrazolium salt (WST-1), light microscopic/electron microscopic analysis and gene expression analysis were performed. For the gene expression analysis, target (labeled cRNA) prepared from total RNA of the hepatocytes was hybridized to the GeneChip Murine Genome U74A V.2 (Affymetrix). The signal intensity calculation and scaling were performed using Microarray Suite Software Ver 5.0. IC50 of the cell survival assay was around 62 mM at 6 h exposure and 7 mM at 24 h exposure. Marked chromatin margination was observed in nuclei of the hepatocytes treated with SZ at concentrations of 3 or 10 mM. Gene expression analysis revealed similar expression changes to those of in vivo, i.e. up-regulation in cell proliferation/apoptosis related genes, and down-regulation of lipid metabolism related genes. These results potently supported the hypothesis that many of the hepatic alteration including histopathological and gene expression changes are induced by direct effect of SZ rather than by the secondary effect of the hyperglycemia or hypoinsulinemia.

Keywords: Streptozotocin; In vitro; Hepatocyte; Apoptosis; Gene expression; DNA microarray; Mouse

Introduction

Streptozotocin (SZ) has been attracting a great attention as a useful tool for the induction of diabetes mellitus and its complications in laboratory rodents (Sibay et al., 1971; Steffes and Mauer, 1984; Kume et al., 1992) because of its toxic action on islet β cells. However, SZ is known to exert toxic effects not only on pancreatic islet β cells but also on other organs including liver.

We have previously reported the details of SZ-induced hepatic lesions in the acute (6–48 h after the treatment) and the subacute (4–12 weeks after the treatment) phase (Kume et al., 1994a, b; Doi et al., 1997; Kume et al.,

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2004). Those studies characterized the pathological changes such as the appearance of oncocytic hepatocytes, cytomegalic hepatocytes and bile duct hyperplasia in the subacute phase. In the acute phase, SZ induced several hepatic changes including lipid peroxidation, mitochondrial swelling, peroxisome proliferation and inhibition of hepatocyte proliferation before the elevation of the serum glucose levels (Kume et al., 2004). We also analyzed molecular genetic changes in the liver before and after the induction of hyperglycemia using the Affymetrix GeneChip. Many of the up-regulated genes were categorized into cell cycle/apoptosis-related genes, immune/allergy-related genes and stress response/xenobiotic metabolism-related genes. On the other hand, genes related to glucose, lipid and protein metabolisms were down-regulated (Kume et al., in press). These morphological and genetic changes occurred before the induction of hyperglycemia. Therefore, it is suggested that those changes were attributable to the direct effects of SZ on hepatocytes rather than the secondary effects of diabetes or hyperglycemia.

Several reports have focused on the toxic mechanisms of SZ in islet cells in vitro (Ledoux and Wilson, 1984; Flament and Remacle, 1987; Eizirik et al., 1993; Turk et al., 1993; Bellmann et al., 1995), however, no researchers reported detailed changes in SZ-treated hepatocytes in vitro. Morphological examinations and gene expression analysis were performed on the SZ-treated mouse primary hepatocytes to clarify direct effects of SZ on hepatocytes.

Materials and methods

The study was approved by the Ethical Committee at Tanabe Seiyaku Co. Ltd., and all efforts were made to minimize animal suffering.

Animals

Two 8-week-old male Crj:CD-1(ICR) mice (Charles River Japan Inc., Kanagawa, Japan) were used.

Primary cultured hepatocytes

Hepatocytes were isolated from the mice with use of collagenase perfusion under pentobarbital anesthesia. The isolated hepatocytes were seeded at a density of 1.0×10^6 cells per 35 mm dish in 2 mL medium (William's E medium containing 5% FCS, 0.1 μM dexamethasone, 6.25 μg/mL insulin, 6.25 ng/mL transferrin, 6.25 ng/mL selenium, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL Matrigel). At 24 h after seeding, SZ was applied on the hepatocytes within the same medium for 6 or 24 h at 37 °C. Applied concentrations were selected as 0, 1, 3, 10, 30 and 100 mM. In all 4, 2, and 2 dishes of each concentration were provided for an analysis of cell survival rate, electron microscopic examination, and GeneChip analysis, respectively. Phase contrast micrographs were taken from the dishes for electron microscopic examination.

Cell survival rate (WST-1 assay)

After the 6 or 24 h-incubation with SZ, WST-1 (Wako Pure Chemical Industries Ltd., Osaka, Japan) was added for each dish at a final concentration of 15%, and the dish was incubated for another 3 h. Following the incubation, absorbance was read at a wavelength of 450 nm using a spectrophotometer (Bio-Rad Laboratories Inc., CA, USA). Percentage of survival cell was calculated using the following formula: (absorbance of treated dish/absorbance of control dish) × 100. For the calculation of the 50% inhibition concentration (IC50) value, concentration-response data were fit by non-regression analysis to sigmoid curves by using the GraphPad Prism program (GraphPad Software Inc., CA, USA).

Morphological examination

After the phase contrast micrographs were taken, hepatocytes were fixed with 2.5% glutaraldehyde and 2.0% formaldehyde, postfixed with 1% osmium tetroxide, and embedded in epoxy resin. Semithin sections were stained with toluidine blue and observed under a light microscope. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and observed under a JEOL-1210 electron microscope (JEOL Co. LTD., Tokyo, Japan).

RNA extraction

Total RNA was isolated as the manual of QIAGEN RNEASY kit (QIAGEN, CA, USA). For lysis of cells and tissues before RNA isolation, Buffer RLT with β-mercaptoethanol was added and incubated for 10 min at 37 °C. Total RNA was extracted by using QIAshredder spin column and RNeasy mini spin column. Absorbance rate of the sample at 260 nm/280 nm was determined.

Affymetrix GeneChip analysis

Total RNA was labeled as described in the GeneChip Expression Analysis Technical Manual (Affymetrix, CA, USA). mRNA was reverse-transcribed into cDNA using SuperScript Choice system (Invitrogen, Tokyo, Japan) and T7-(dT)24 primer (Amersham Biosciences, NJ, USA). The cDNA was converted to labeled cRNA using Bioarray HighYield RNA Transcript Labeling Kit