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A Protective Function of the Calcium Binding Protein S100A4 in Rat Hepatic Injury Due to Carbon Tetrachloride

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Abstruct: F The role of the S100A4 in rat hepatocites was clarified by measuring detailed changes in serum ALT values and intracellular Ca^{2+} after CCl_4 administration. In this study, we further showed that the calcium binding protein S100A4 might be a protein signal involved in the response to protect cells against CCl_4 intoxication. S100A4 mRNA expression levels abruptly increased to a high level in normal liver at 1 day after CCl_4 administration, and then gradually reduced to the control level by day 3. This change is the opposite to that of intracellular Ca^{2+}.

Key words: Basic research, S100A4, hepatic injury, intracellular Ca^{2+}

Introduction

In general, cells normally maintain cytoplasmic Ca^{2+} at very low levels, such as 10^{-7} M, but the concentration briefly raises several folds in response to physiological stimuli. The role of Ca^{2+} ions in cells has been well established to regulate a wide variety of cellular processes, such as hormonal responses and cell death. Numerous studies have documented that intracellular Ca^{2+} plays a potential role in chemically induced liver injury, including that caused by carbon tetrachloride (CCl_4). Schanne et al. (1979) concluded that intracellular accumulation of calcium is the final common pathway by which toxic cell death occurs. However, we showed that the intracellular Ca^{2+} acts the roles of cytoprotection in hepatocytes. That is, the serum ALT activity increased up to a peak value within 1 day after i.p. administration of CCl_4 at 0.2 ml/kg, and then successively decreased to the control level after 3 days. The intracellular Ca^{2+} concentration then responded to the CCl_4 intoxication with a biphasic increase, that is, a first Ca^{2+} peak at 1 hour that quickly increases to a steeply peak, and a second Ca^{2+} peak that gradually increases up to a peak at 3 days, and recovered the control level by 4 days. We showed that the second Ca^{2+} peak acts protectively against the hepatic injury.

In this study, we investigated the characterization of the calcium binding protein, which well known to associate with intracellular Ca^{2+}.

Materials and Methods

Chemicals

CCl_4, BSA, SDS and fura 2-AM were purchased from Wako Co. (Osaka, Japan). The PCR kit, agarose gel, molecular weight markers and ethidium bromide were purchased from Takara Bio Inc. (Shiga, Japan). All other reagents were of the highest grade available from commercial sources.

Animal treatment

Male Donryu ratswere obtained from SLC Co. Ltd. (Shizuoka, Japan).

Isolation of hepatocytes

Hepatocytes were prepared from fed rats by recirculating collagenase perfusion of the liver essentially as described by Seglen (1972). Briefly, livers were perfused with Ca^{2+}-free Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mM glucose, 1 mM $CaCl_2$ and 0.2 mg/ml collagenase. The liver was then removed and gently agitated in Krebs-Ringer bicarbonate, pH 7.4, containing 5 mM glucose and 1 mM $CaCl_2$. Cells were washed, and resuspended in Krebs-Ringer bicarbonate, pH 7.4, containing 5 mM glucose and 1 mM $CaCl_2$. The hepatocytes were prepared to $3x10^6$ cells/ml.

RNA Isolation and RT-PCR

Total RNA was extracted from rat livers at each time point after CCl_4 administration by homogenization in guanidium isothiocyanate, and then purified using acid phenol-chloroform. The samples were digested , and followed by the RT-PCR cycles. Amplified PCR products were electrophoresed , and photographed. The relative amount of mRNA transcripts was determined using an image scanner. Densitometric analysis of each captured image was performed, and normalized for the ß-actin content.

Vector construction for S100A4-BFP reporer

The S100A4 gene was inserted to the plasmid pQBI 50-fc1 constructed reporter BFP (blue fluorescence protein) gene as following. The S100A4 gene was amplified from RNA excluding the stop codon using PCR by the two primers containing with restriction enzymes EcoR V at 5' site and Hind III at 3' site. The PCR product was cleaned, cut with EcoR V and Hind III, and treated with calf intestinal alkaline phosphatase. The vector pQBI 50-fc1 also was digested with with EcoR V and Hind III. Both DNA fragments were gel purified and ligated together with T4 DNA Ligase (Invitrogen). The ligation priducts were transformed into E.coli JM109 competent cells and plated onto Luria-Bertani (LB)-ampicillin. Transformed colonies were picked, and further incubated in 1 ml SOC for 1 hour. An aliquot of 5 ml of fresh LB-carbenicillin (50 µg/ml) was inoculated with 100 µl of the SOC mixture. Cells were growth at 30°C with shaking for 24 hours. The vector was prepared from harvested cellsIt was confirmed using digestion gel pattern due to exclusive restriction enzyme Kpn I that the S100A4 DNA is inserted to the vector pQBI 50-fc1.

Fluorescence microscopy

A culture transformed with pGRFP harboring ligated sea DNA was harvested for cells that were fixed on a coverslip with 10% formaldehyde. Cells were photographed using a fluorescence microscope with attached Nikon digital camera. Illumination was by arc lamp light filtered to the blue range (450-490 nm).

Results

We studied roles of S100A4 on the intracellular Ca^{2+} increase after the CCl4 hepatic injury.

RT PCR used RNA prepared from rats treated CCl_4.

In this study, we examined whether the calcium binding proteins calmodulin, calpain, calcineurin and S100A4 took part in the induction of this system, by using semi-quantitative RT-PCR.

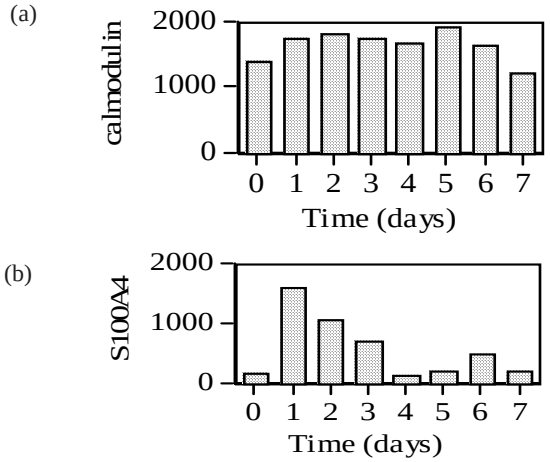

Fig. 1. Gene expression level in rat liver after CCl_4 adminstration.

(a) S100A4
Sense 5'-CTG GATGTAATAG TGTCCACCTT CCA-3'
Antisense 5'-TTGC TCAGCACTGT GCACATGTAT-3'
(b) Calmodulin
Sense 5'-CTG GATGTAATAG TGTCCACCTT CCA-3'
Antisense 5'-TTGC TCAGCACTGT GCACATGTAT GA-3'

As shown in Fig. 1a, S100A4 was notably induced 1 day after CCl_4 administration, S100A4 mRNA expression level abruptly increased to 9.0-fold the level in normal liver at 1 day, and then gradually reduced to the control level by day 3. In contrast, calmodulin and calpain were usually expressed at high levels, but did not respond to CCl_4 administration. Calcineurin could not be detected by gel electrophoresis of the RT-PCR products (not shown data). Thus, S100A4 may be involved with a protein responsible for the intracellular Ca^{2+} increase after CCl_4 intoxication

Cloning of S100A4 and its gene expression

The vector pQBI 50-fc1 (S100A4) was electrotransformed into hepatocytes and incubated in L-12 and F-15 mixture containing proline and 5% calf serum for 3-16 hours. The hepatocytes were loaded with fura2-Am , andobserved with fluorescence photomicroscope.

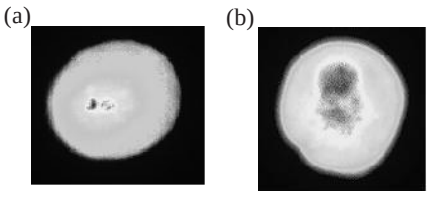

Fig. 2. Effect of S100A4 protein on the intracellular Ca^{2+}.

Then, it was confirmed that the S100A4 protein expressed initially at 3 hours, and increased by 16 hours. As shown in Fig. 2, the fluorescence of the intracellular Ca^{2+} increased significantly in the cells at 16 hours, but did not in the control cells. Thus, the result might indicate that expression levels of the protein S100A4 associated with intracellular Ca^{2+} increase.

Changes on expression of S100A4 in hepatocytes after CCl_4 treatment

The results showed that the expression of S100A4 in hepatocytes increases after the CCl4 treatment by day 3, and recovered day 4 (Fig. 3). That is, this is reversed with that of S100A4 mRNA.

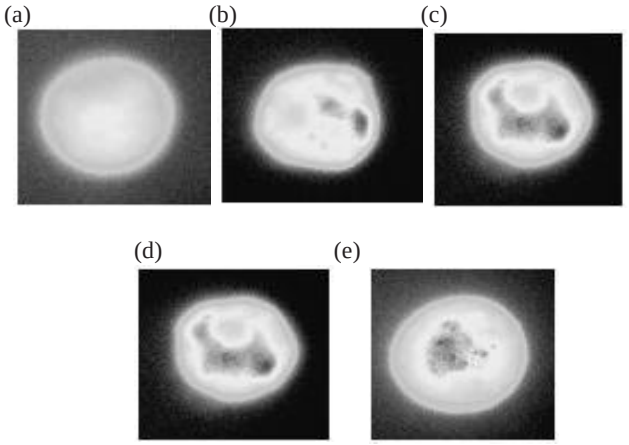

Fig. 3. Time course of S100A4-BFP fusion protein in hepatocytes prepared from rats treated with 0.05 mM CCl_4. The cells were loaded with electroporetion method, and cultured for sixteen hours. (a) Control, (b) day 1, (c) day 2, (d) day 3 and (e) day 4.

Discussion

Schanne et al. (1979) concluded that intracellular accumulation of calcium is the final common pathway by which chemically toxic cell death occurs. However, we found that the intracellular Ca^{2+} acts the roles of cytoprotection in hepatocytes. In this study, we showed that the calcium binding protein S100A4 might be a protein signal involved in the response to protect cells against CCl_4 intoxication. *S100A4* mRNA expression levels abruptly increased to a high level in normal liver at 1 day after CCl_4 administration, and then gradually reduced to the control level by day 3. This associated in the opposite way with that of intracellular Ca^{2+}. This might indicate that the S100A4 protein is suppressive to regulate the intracellular Ca^{2+} level. The S100A4 protein is present at high levels in cells executing proliferation and cancer metastasis, but is present at a relatively low level in normal differentiated cell types. However, no one reports study S100A4 associating with hepatic injury induced with CCl_4.

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