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A novel function of connexin 32: marked enhancement of liver function in a hepatoma cell line

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Abstract

Connexin 32 (Cx32) is the main gap junction protein in hepatocytes and plays an important role in the regulation of signal transfer and growth control in the liver by constructing gap junction channels and gap junctional intercellular communication (GJIC). In this study, the human Cx32 gene was transfected into a hepatoma cell line (HepG2) that showed aberrant expression of Cx32 and was deficient in GJIC. Cx32-transfected HepG2 not only expressed a higher level of Cx32 mRNA, but also showed increased GJIC compared with HepG2 and vector-transfected HepG2. Furthermore, the liver functions of ammonia removal and albumin secretion of HepG2 were markedly enhanced with Cx32 gene transfection. It may be expected to improve the cellular functions of the hepatoma cell line by Cx32 gene transfection and serve to develop an efficacious bioartificial liver.

Keywords: Connexin; GJIC; Liver functions; Hepatoma cell line; HepG2

Gap junctions are transmembrane channels linking neighboring cells and providING the only pathway to transfer small hydrophilic cytoplasmic metabolites less than 1000 Da, growth modulators, and second messengers between the adjacent cells, in a process known as gap junctional intercellular communication (GJIC) [1]. GJIC was suggested to play a crucial role in maintaining tissue homeostasis and controlling growth, differentiation, embryogenesis, and several functions of different tissues [2–4]. Gap junctions are composed of two hemichannels and each hemichannel consists of six connexin (Cx) protein units. At present, there are greater than 16 different Cxs in vertebrate species and expression of some Cxs is organ specific [5]. In the liver, GJIC involves at least three different connexins, Cx32, Cx26, and Cx43, depending on the cell type and cell position in the lobule [6]. In vivo, Cx32 and Cx26 are expressed in parenchymal hepatocytes and the distribution of these Cx proteins is different within the liver lobules: Cx26 preferentially localizes in the periportal zone of the lobules, whereas Cx32 appears in most hepatocytes throughout the lobules and is the major component of liver gap junctions. Furthermore, many biological activities of the liver are spatially organized within the circulatory unit and several hepatic functions differ in periportal vs. pericentral hepatocytes, including carbohydrate, lipid, and nitrogen metabolism in addition to expression of gap junctions. Recently, several studies suggested that Cx32 expression had an inhibitory effect on hepatocarcinogenesis and transfection with Cx32 cDNA inhibits the growth of hepatoma cells [7–9]. However, it was not clear whether the recovery of GJIC by transfection of Cx32 gene would enhance the liver-specific functions of hepatoma cells, which would be very important in the research of liver disease therapy.

In the last two decades, with the development of cell biology and tissue engineering, a cell-based biohybrid artificial liver (BAL) was reported to be a promising approach to support patients with acute liver failure [10]. Primary human hepatocytes would be ideal for the cellular component of BAL, but it was limited by the

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worldwide lack of donor organs and the growth limitations of primary hepatocytes in vitro. To overcome the shortage of donor and avoid xenozoonosis risk, a hepatoma cell line (HepG2) derived from human-origin cells has good growth characteristics and less severe antigenicity, and was previously used for developing the BAL [11]. Although HepG2 maintains good liver-specific functions among hepatoma cell lines, the activities of the liver-specific functions of HepG2 were far lower compared with those of primary hepatocytes [12]. Cx32 is the major gap junction protein expressed in hepatocytes, but HepG2 is an aberrant expression of Cx32 and is deficient in GJIC. Therefore, we transfected the Cx32 gene into HepG2 and investigated the exchanges of GJIC and liver-specific functions of HepG2 in this study.

The results showed that Cx32 gene transfected in HepG2 improved the trafficking of Cx32 protein to the cytoplasmic membrane, clearly increased the GJIC, and enhanced the activities of ammonia removal and albumin secretion in the Cx32 gene transfected HepG2. This was the first finding that Cx32 could markedly enhance the liver-specific functions in a hepatoma cell line (HepG2).

Materials and methods

Cell culture. The human hepatoma cell line HepG2 from the Riken cell bank (Tokyo, Japan) was cultured at 37 °C under 5% CO_2/95% humidified air using minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1 mM non-essential amino acids (NEAA) (Gibco), 10% fetal bovine serum (FBS) (Intergen, NY), and 100 U/ml penicillin–streptomycin (Gibco).

Plasmid construction and transfection. Using genomic DNA extracted from HepG2 as template, the human connexin genes were amplified by polymerase chain reaction (PCR) using primers Cx32F (5′-ATGAACTGGACAGGTTTGTAGACCTTGCTC-3′) and Cx32R (5′-TCAGCAGGCCGAGCAGCGG-3′). These amplified gene fragments were isolated and inserted into the pTARGET mammalian expression. HepG2 cells were transfected with the Cx32/pTARGET plasmid or empty vector as a control using FuGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instructions with minor modification. After continuously culturing for two days, transfectants were selected by adding 1.3 mg/ml geneticin (Life Technologies, Frederick, MD) in the culture medium for one week. Individual transfected clones were prepared by limiting dilution cloning in 96-well plates and then culturing as for HepG2.

RT-PCR. Total RNA was isolated from cells cultured on the seventh day with TRIzol reagent according to manufacturer's instructions. The cDNA was prepared from 1 μg of total RNA by reverse transcription using a commercially available First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). After proper optimization of PCR conditions, subsequent PCR was performed with 1 μl cDNA in 20 μl reaction mixture (10× PCR buffer 2 μl, dNTP 1.6 μl, each primer 2 μl, *Taq* DNA polymerase 0.2 μl, and distilled water). The conditions for RT-PCR were equilibration at 37 °C for 15 min, followed by an initial denaturation at 95 °C for 1 min, 25 cycles of 95 °C for 1 min, 60 °C for 1 min, 70 °C for 2 min, and final extension of 70 °C for 5 min. Electrophoresis of PCR products was done on 1.5% agarose gel for the visualization of connexin after staining with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA). Images were captured using an image scanner and analyzed using NIH Image software. The primers used in this study were as follows:

hCx32	forward 5′-ATGAACTGGACAGGTTTGTACACCTTGCTC-3′
	reverse 5′-TCAGCAGGCCGAGCAGCGG-3′
hCx26	forward 5′-ATGGATTGGGGCACGC-3′
	reverse 5′-TTAAACTGGCTTTTTTGACTTCCC-3′

Immunocytochemical stainings. Immunocytochemical staining of Cx32 protein was performed using the VECTASTAIN ABC kit (Vector Laboratories, Inc. Burlingame, USA) following the manufacturer's instruction with some modification. Briefly, cells grown on the glass coverslips were fixed in cold pure acetone for 5 min. The acetone-fixed specimens were blocked in diluted normal blocking serum in Dulbecco's phosphate-buffered saline (PBS) at room temperature for 30 min and incubated with polyclonal rabbit anti-connexin 32 (Zymed Laboratories, San Francisco, CA) overnight at 4 °C. Protein–antibody complexes were visualized by the biotin/streptavidin/peroxidase method with diaminobenzidine tetrahydrochloride (DAB) (Vextor Laboratories, Burlingame, USA) as the chromogen. All slides were viewed with a Nikon microscope (Nikon, Japan).

Scrape-loading/dye transfer assay to measure GJIC. The scrape-loading/dye transfer (SLDT) technique was adapted after the method of El-Fouly et al. [13]. Briefly, when the cells grew into confluent monolayer cells in 35-cm dishes, cell dishes were loaded with 0.05% Lucifer Yellow (Molecular Probes, Eugene, OR, USA) in PBS (+) solution and scraped immediately with a sharp blade after rinsing with PBS (+). After incubating for 5 min at 37 °C, cells were washed with PBS (+) and monitored using a fluorescence microscope. The dye spreading distance was measured from the cell layer at the scrape to the edge of the dye front that was visually detectable.

Liver-specific function assay. The functions of the hepG2 and Cx32 transfected cells were evaluated by measuring ammonia removal and albumin secretion. For the ammonia removal activities of these cells, the cells were cultured in MEM with 5 mM ammonium chloride. After the exchange of the medium containing ammonium, the concentration of ammonia in the medium was measured at 0 and 24 h, respectively, using the indophenol method (an ammonia assay kit, Wako Pure Chemicals, Japan). The albumin secreted into the culture medium was detected by enzyme-linked immunosorbent assay kit (Exocell, Philadelphia, PA).

Statistical analysis. Student's t test was used to compare the samples. Statistical significance was represented by $p < 0.05$. Values were means ± SD. Three cultures were run for each case and all experiments were repeated at least twice.

Results

Functional GJIC in HepG2 enhanced by Cx32 gene transfection

HepG2 cells were transfected with Cx32/pTARGET plasmid DNA using FuGENE6 transfection reagent and the transfectants were obtained by selection with geneticin. Expressions of Cx mRNAs were first detected using RT-PCR (Fig. 1). As shown in Fig. 1A, Cx32 mRNA was detected and showed different levels among the HepG2, Cx32 gene- and empty vector-transfected cells, while the Cx26 mRNAs were almost not detected in all cells. The image analysis showed the level of Cx32

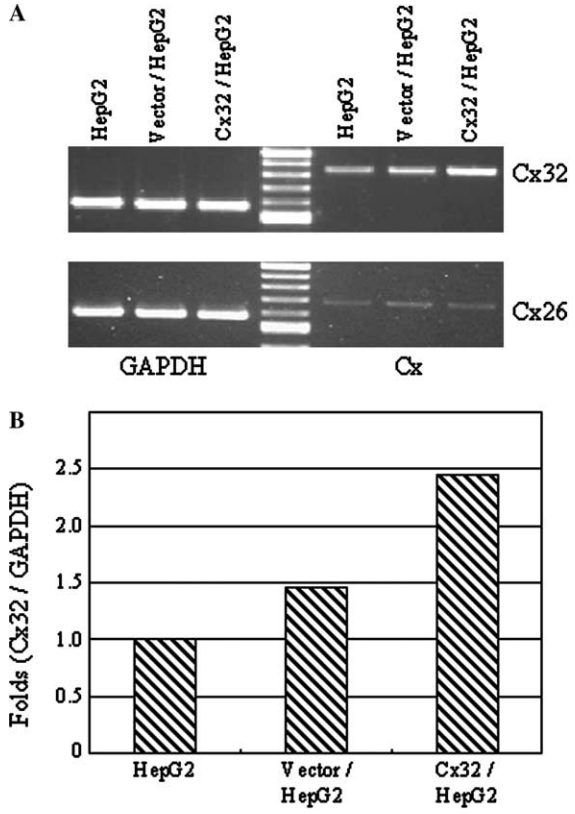

Fig. 1. Establishment of Cx32 and vector transfected HepG2. (A) RT-PCR analysis of Cx32 and Cx26 gene expression in HepG2, vector-transfected cells (Vector/HepG2), and Cx32 gene transfected cells (Cx32/HepG2). (B) Image assay of Cx32 gene expression in RT-PCR. Relative densities were standardized to that of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

mRNA expressed in the Cx32 gene transfected cells was enhanced 2.5- and 1.7-fold in comparison with the HepG2 and empty vector transfected cells, respectively (Fig. 1B). The abilities of functional GJIC in the cells were investigated by the scrape-loading dye transfer technique. The distances of lucifer yellow spreading reflected the functional GJIC in the cells, and the longer distance of dye spreading indicates the higher functional GJIC in the cells. As shown in Fig. 2, the distance of lucifer yellow spreading in Cx32 gene transfected cells was clearly greater than those in HepG2 and empty vector transfected cells. Thus, the distance of dye spreading in Cx32 gene transfected cells was 2.8- and 1.8-fold longer than those in HepG2 and empty vector transfected cells, respectively (Fig. 3). It could be concluded that the Cx32 gene transfection not only increased the expression of Cx32 mRNA, but also significantly enhanced the functional GJIC in HepG2.

Localization of Cx32 protein before and after Cx32 gene transfection

To confirm the contribution of Cx32 protein for the formation of functional GJIC after the Cx32 gene transfection, the localizations of Cx32 protein in the cells were further observed by immunocytochemical staining. The photographs of A, B, and C in Fig. 4 show the localization of Cx32 protein in HepG2, empty vector transfected cells, and Cx32 gene transfected cells, respectively. These results demonstrated that the Cx32 protein was expressed in all the cells, but the localizations of Cx32 protein were clearly different among them. Thus, the Cx32 protein was localized in the cell borders and formed many small gap junction plaques in the Cx32 gene transfected cells, however, the Cx32 protein was limited in the cytoplasm and hardly detected the gap junction plaques in the HepG2 and empty vector transfected cells. The results in the present study suggest that the trafficking of Cx32 protein to the cell membrane in HepG2 was enhanced by Cx32 gene transfection and then increased the functional GJIC in Cx32 gene transfected cells.

Liver-specific functions in HepG2 improved by Cx32 gene transfection

For determining the effect of Cx32 gene transfection on the liver-specific functions in HepG2, the albumin secretion ability and ammonia removal activity were continuously monitored in the HepG2, empty vector transfected cells, and Cx32 gene transfected cells, respectively (Fig. 5). Albumin secretion, which was used as a marker for protein synthesis in the liver, showed greater amounts of albumin detected in Cx32 gene transfected cells than HepG2 and empty vector transfected cells (Fig. 5A). Furthermore, ammonia removal activity, which represents the detoxification potentiality of the liver, was significantly higher in the Cx32 gene transfected cells than HepG2 and empty vector transfected cells during the 14 days of culture with 5 mM ammonium chloride (Fig. 5B). It was suggested that the small molecular ammonium was effectively eliminated through the gap junctional channels formed by Cx32 in HepG2. The enhancement of liver-specific functions of HepG2 was suggested to relate to the increasing functional GJIC by Cx32 gene transfection.

Discussion

HepG2 cells, a human hepatoma cell line, are deficient in GJIC due to the aberrant expression of Cx32 and low expression of Cx26. In in vivo and in vitro models, low or no functional GJIC was observed in various kinds of hepatocarcinoma and hepatoma, which was suggested to be involved in the malignant phenotype of cancer and tumor cells [14]. In vivo, normal rodent hepatocytes express Cx32 and Cx26, but only Cx32 expression is constant across the liver lobule [6]. Thus, liver gap junction channels composed of Cx32 are

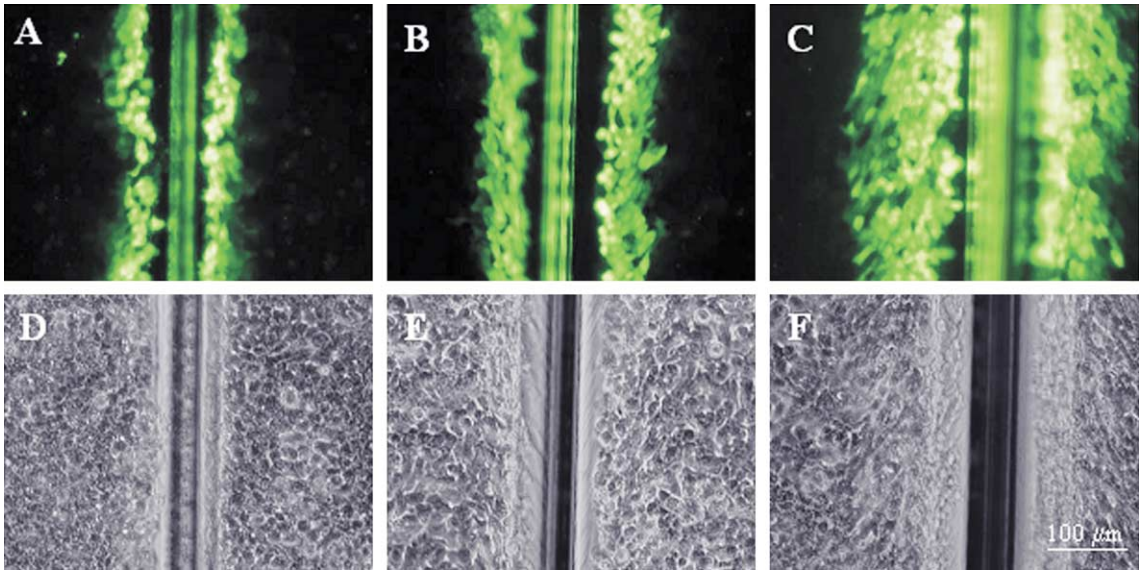

Fig. 2. Fluorescent (A–C) and phase-contrast (D-F) photographs of HepG2 (A and D), vector-transfected HepG2 (Vector/HepG2) (B and E), and Cx32-transfected HepG2 (Cx32/HepG2) (C and F) in the assay of scrape loading and dye transfer (SLDT), same scale in (A–F).

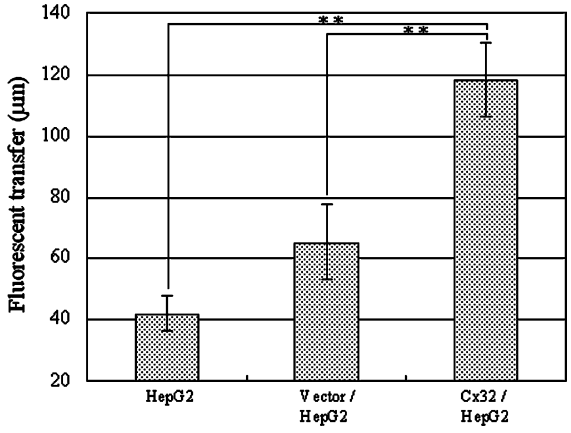

Fig. 3. Functional GJIC in HepG2, vector-transfected HepG2 (Vector/HepG2), and Cx32-transfected HepG2 (Cx32/HepG2) measured by the SLDT method at the seventh day of culture. Values are expressed as means from 20 determinations (**$P < 0.01$).

suggested to be important to maintain the normal phenotype of hepatocytes. Therefore, to reduce malignant phenotype and improve liver-specific functions in HepG2, we transfected human Cx32 gene into HepG2 to enhance functional GJIC.

The results of the RT-PCR and SLDT assay in the present study showed that the levels of Cx32 mRNA expressed in Cx32 gene transfected cells were increased greater than twofold compared with HepG2 (Fig. 1) and the functional GIJC was also markedly enhanced by Cx32 gene transfection in HepG2 (Figs. 2 and 3). Analyses of chemically induced rat liver tumors suggest that Cx32 gene is rarely mutated in these tumors but the expression of the Cx32 protein is often reduced or the Cx32 protein is abnormally localized in these cells [15]. In the present study, although the Cx32 protein expression by Western blotting assay showed almost no change even after Cx32 gene transfection (data not shown), a clear difference in the localization of Cx32 protein was observed between before and after Cx32

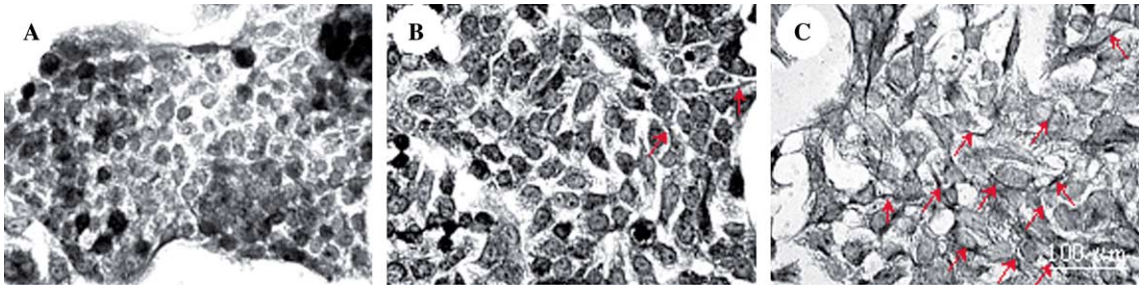

Fig. 4. Localization of Cx32 protein in HepG2 (A), vector-transfected HepG2 (B), and Cx32-transfected HepG2 (C). More gap junction plaques were detected in Cx32 gene transfected cells (arrow) than HepG2 and empty vector transfected cells. Immunocytochemical staining of Cx32 protein was performed with a Vectastain ABC Kit and polyclonal rabbit anti-connexin 32, same scale in (A–C).

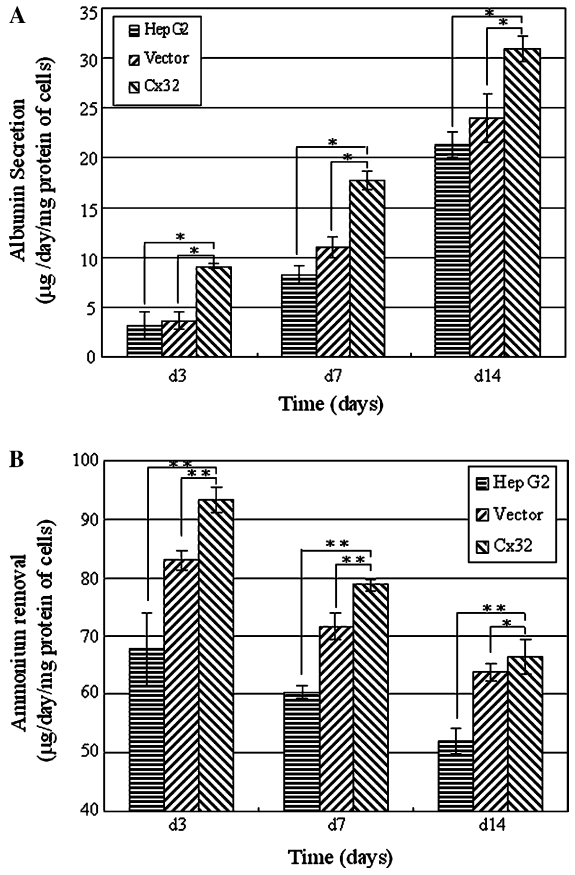

Fig. 5. Liver functions of albumin secretion (A) and ammonia removal (B) of HepG2, vector-transfected HepG2, and Cx32-transfected HepG2 examined on the 3rd, 7th, and 14th day of culture (*$P < 0.05$, **$P < 0.01$).

gene transfection in HepG2 by immunocytochemical stainings (Fig. 4). The majority of Cx32 protein was localized in the cell borders and formed small gap junction plaques in the Cx32 gene transfected cells, whereas it was limited to the cytoplasm and nucleus before Cx32 gene transfection. Furthermore, the morphologies of the cells showed that Cx32 gene transfected cells grew as a monolayer with the spreading cell shape, while the HepG2 grew as clusters with the spherical cell shape. Cx protein expression, gap junction assembly, and its function are controlled by the transcription, translation, and post-translational modification. In addition, recent studies have suggested that they are also associated with tight junction components and elements of the cytoskeleton [16–18]. Although the precise role of the cellular morphology in gap junctional channel formation between the cells is not clear at present, the results of the present study suggest that the trafficking, assembly of Cx32, and functional GJIC in the cellular membrane are enhanced by forced expression of Cx32 in HepG2.

Furthermore, Cx32 gene transfection into HepG2 markedly enhanced the liver-specific functions of ammonia removal and albumin secretion with accompanying increase in the functional GJIC (Fig. 5). Ammonia removal activity and albumin secretion ability are typical differentiated functions of the liver, but these functions in HepG2 were significantly lower than those of hepatocytes in vivo. The defect in albumin production was reported to be due to the reduction or absence of albumin gene transcription in some hepatoma cells, and the structure of the albumin gene was detected in all MH1C1, FAO, and 3924A rat hepatoma cells, but a different albumin expression was found to correlate well with methylation state of the albumin gene [19]. The results in the present study showed that the transcription of albumin gene in HepG2 may be enhanced with the increase in the functional GJIC by Cx32 gene transfection, and the albumin production was increased (Fig. 5A). In addition, the studies of ammonia removal activity in HepG2, Cx32 gene transfected cells, and vector transfected cells showed that urea was not detected in the culture media of all cells, and the ability of ammonia removal was higher in the absence than the presence of 4 mM glutamine in the media in all cells (data not shown). In the intact liver, the two major ammonia-detoxification systems, urea and glutamine synthesis, are anatomically present in periportal and pericentral hepatocytes, respectively [20]. In functional terms, this organization represents the sequence of a periportal low-affinity but high-capacity system (ureogenesis) and a pericentral high-affinity system for ammonia detoxification (glutamine synthesis). Therefore, the present results suggested that HepG2 eliminated ammonia via the high-affinity pathway of glutamine synthesis, and the capacity enhanced with the increase in the functional GJIC by forced expression of Cx32, which could be similar to the characters of Cx32 high-expressional pericentral hepatocytes. Furthermore, glutamine is an essential nutrient as a major source of energy and nitrogen for mammalian cells, which would be useful for the development of bioartificial liver. These results showed that the ammonium metabolic activity and albumin secretion in HepG2 were related to the functional gap junctional channel composed of Cx32 proteins. Other studies reported distinct biological roles of the highly homologous Cx proteins in correlations of Cx mRNA isoform expression with the degree of hepatic cellular differentiation (in RLC, FTO.2B, and WB-F344 cell lines), and suggested that Cx gene expression may be a marker of hepatic development: as hepatocytes differentiate, the proportions of Cx43 and then Cx26 mRNA decrease while that of Cx32 mRNA increases [21]. Moreover, the diffusion of second messengers through gap junction channels composed of Cx32 in liver is suggested to be a major determinant for the establishment of metabolic coupling between neighboring hepatocytes and for the proper distribution of signals involved in the promotion of liver-specific functions.

Taken altogether, the recovery of Cx32 expression could be proposed to have an effect on enhancing the liver-specific functions in hepatoma cells, in addition to improving the biological safety of hepatoma cells for the application as tissue engineered artificial liver by the inhibition of malignant growth of tumor cells of HepG2.

In conclusion, this study is the first to report a clear increase in the functional GJIC in HepG2 by transfection of Cx32 gene, and the subsequently enhanced liver-specific functions of ammonia detoxification and albumin synthesis in the Cx32 gene transfected HepG2. It may be expected to improve cellular functions of the hepatoma cell line by Cx32 gene transfection and serve to develop an excellent biohybrid-artificial liver.

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