Expression of cholesterol 7α-hydroxylase and Δ4-3-ketosteroid 5β-reductase genes in rat pancreatic hepatocyte-like cells

Yoshihisa Ando, Hideyuki Ide, Shuji Kosai, Ryo Kamimura, Yorio Maeda, Shushi Higashi, and Toshiaki Setoguchi

Department of Surgery I, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan

Abstract

Hepatocyte-like cells have been observed in the pancreas of the rat. We examined the bile acid biosynthetic function of these cells to determine whether they were real hepatocytes. This study investigated the existence of two liver-specific enzymes involved in bile acid biosynthesis (cholesterol 7α-hydroxylase and Δ4-3-ketosteroid 5β-reductase) in the hepatocyte-like cells. We could demonstrate cholesterol 7α-hydroxylase activity and its circadian rhythm in the hepatocyte-like cells. Northern blot analysis demonstrated the expression of messenger RNA for the 7α-hydroxylase and Δ4-3-ketosteroid 5β-reductase in the pancreatic hepatocyte-like cells. To measure the amount of the messenger RNA, we used the competitive polymerase chain reaction method for the 7α-hydroxylase. This quantitation revealed the existence of a circadian rhythm of cholesterol 7α-hydroxylase messenger RNA in the hepatocyte-like cells. These results indicated that bile acid biosynthesis was performed in the pancreatic hepatocyte-like cells as noted in the liver parenchymal cells. Induction of pancreatic hepatocyte-like cells has been investigated by many authors. It is thought that these hepatocyte-like cells develop via transdifferentiation (1–4). They can be induced by giving a copper-depleted diet to the rat (5, 6). These cells express the genes for albumin and some liver-specific enzymes (carbamoylphosphate synthase I and urate oxidase) (7, 8). However, the question of whether these cells function in bile acid biosynthesis, which is another important role of hepatocytes, has remained unanswered.

Cholesterol 7α-hydroxylase (C7αH) and Δ4-3-ketosteroid 5β-reductase (5βR) are liver-specific enzymes involved in bile acid biosynthesis (9, 10). C7αH is an initial and rate-limiting enzyme (11) and shows a circadian rhythm of messenger RNA (mRNA) expression (12–14).

We assayed C7αH activity in the microsomes and used Northern blot analysis for detecting C7αH and 5βR mRNAs expression in the pancreatic hepatocyte-like cells. We also used a competitive polymerase chain reaction (PCR) method (15) to quantitate the initial amount of mRNA and confirm whether the hepatocyte-like cells exhibit a circadian rhythm of C7αH mRNA expression.

MATERIALS AND METHODS

Induction of pancreatic hepatocyte-like cells

Hepatocyte-like cells were induced in the rat pancreas as reported previously (6). Male Fischer 344 rats (Charles River Breeding Laboratories, Yokohama, Japan) weighing 80–90 g were housed in an appropriate room with artificial light on a 12-h light/dark cycle. They were fed ad libitum with a normal diet which contained 6.5–8.0 mg of Cu/g (Clea, Tokyo, Japan) for control study, or a copper-deficient diet. The copper-deficient diet was prepared by adding triethylenetetramine tetrahydrochloride (Nacharai, Kyoto, Japan) at a final concentration of 0.6% (w/w) to a powdered copper test diet (16) which contained 0.3 to 0.4 mg of Cu/g (United States Biochemical Corp., Cleveland, OH). The animals were returned to a normal diet at the ninth week, and were then fed for 8 weeks before being killed. By 8 weeks on the copper-deficient diet, more than 90% of the acinar cells were replaced by adipose cells. Among these cells, the hepatocyte-like cells have been observed on histologic examination after copper repletion (6, 17).

Assay for cholesterol 7α-hydroxylase activity

C7αH activity was assayed according to the method of Ogishima and Okuda (18) using internal microsomal cholesterol as

Abbreviations: C7αH, cholesterol 7α-hydroxylase; cDNA, complementary DNA; 5βR, Δ4-3-ketosteroid 5β-reductase; mRNA, messenger RNA; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR.

1 To whom correspondence should be addressed.
the substrate (19). Microsomes were incubated at 37°C for 20 min with 0.1 m potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 20 mm cysteamine-HCl, 5 mm MgCl₂, 5 mm sodium isocitrate, 0.075 units of isocitrate dehydrogenase, and 0.5 mm NADPH in a final volume of 0.5 ml. After the termination of reactions, 7α-hydroxysterol was converted to 7α-hydroxy-4-cholesten-3-one having an intense absorption at 240 nm by adding cholesterol oxidase and subjected to straight-phase high performance liquid chromatography (HPLC) on a silica gel column (Wakosil 5SIL, 4.6 x 250 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The HPLC system used consisted of a pump (LC-6A, Shimadzu, Kyoto, Japan) equipped with a UV detector SPD-10A, Shimadzu) and an integration system (CLASS-LC10, Shimadzu). Protein concentration was determined by the method of Lowry et al. (20) using bovine serum albumin as the standard.

RNA preparation

Each rat was killed under ether inhalation anesthesia. Resected tissues were frozen immediately in liquid nitrogen. Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method (21). Poly(A)+ RNA of the rat liver and pancreas were prepared by passing the total RNA fraction through an oligo(dt) cellulose column and eluting the residual RNA with the elution buffer (FastTrack 2.0 Kit, Invitrogen Corp., Carlsbad, CA) (22). In the pancreas of rats with the copper depletion-repletion regimen, almost all acinar cells were replaced with adipose cells but pancreatic hepatocyte-like cells grew in number. We therefore needed the pancreas from per depletion-repletion regimen, almost all acinar cells were replaced with adipose cells but pancreatic hepatocyte-like cells grew in number. We therefore needed the pancreas from one composite primer contained the upstream primer (forward primer) for target sequence linked to a 20-mer that annealed to opposite strand of C7cDNA. The other composite primer contained the downstream primer (reverse primer) for target sequence linked to a 20-mer that annealed to opposite strand of C7cDNA. The membrane was prehybridized for 3 h at 37°C, and hybridized overnight at 37°C with the radioactive probe. The Acl and Sma fragments of the C7c DNA (672 bp) and the Kpn and BglII fragments of the 5′R cDNA (714 bp) were prepared as probes. The sequences of the probes were located between the primers for PCR. The membrane was washed and exposed to an X-ray film for 5 min, and to an imaging plate of the bio-imaging analyzer system (23) (BAS 1000, Fuji Photo Film Co., Tokyo, Japan) for 1 min.

Preparation of the standard DNA for competitive PCR

The standard DNA was amplified by PCR with competitive primers. One composite primer contained the upstream primer (forward primer) for target sequence linked to a 20-mer that annealed to one strand of Acl-Smal fragment (672 bp) of C7cDNA. The other composite primer contained the downstream primer (reverse primer) for target sequence linked to a 20-mer that annealed to opposite strand of Acl-Smal fragment. The sequences of competitive primers were 5′-AATCGAAGGCAATGCTGGTGGCTCTCCAC3′ (nucleotides 514–537 of rat C7c DNA) and 5′-CCAGCCCAGTATCGCTGTTT CAGT-3′ (nucleotides 1535–1558 of rat C7cDNA), respectively. The sequences of forward primer and reverse primer for 5′R were 5′-AATGATGGAACGCATTCCGATGCA-3′ (nucleotides 77–101 of rat 5′R cDNA) and 5′-GGTATTGAGGATGTAC CTCCGACA-3′ (nucleotides 978–1001 of rat 5′R cDNA), respectively. The lengths of the amplicons of C7cDNA and 5′R cDNA were 1045 bp and 925 bp, respectively.

Reverse transcriptase polymerase chain reaction

RNA was reverse transcribed into first-strand cDNA. A 20 μl solution mixture containing: 10 μl of total RNA, 100 pmol of random hexamer, 50 mm Tris-HCl (pH 8.3), 75 mm KCl, 3 mm MgCl₂, 10 mm dithiothreitol, 0.5 mm dNTPs, 28 units of RNasin (Promega, Madison, WI), and 200 units of reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) was incubated at 37°C for 10 min, 42°C for 60 min, and heated at 95°C for 10 min. DNA amplification was carried out in a 50 μl solution mixture containing 10 mm Tris-HCl (pH 8.3), 50 mm KCl, 1.5 mm MgCl₂, 0.2 mm dNTPs, 0.4 μl each of forward and reverse primers, 2.0 units of Taq DNA polymerase (Promega, Madison, WI), and 1 μl of the first-strand cDNA solution. The mixture was overlaid with 50 μl of mineral oil and amplified in 25 sequential cycles at 94°C for 45 sec, 61°C for 45 sec, and then 72°C for 2 min with a DNA Thermal Cycler 480 (Perkin-Elmer, Foster, CA). All PCR components in the reaction were tested for positive contamination in a 50-cycle reaction lacking cDNA.

Southern blot analysis

After electrophoresis on 1.5% agarose gel containing 0.5 μg/ml of ethidium bromide, the DNAs were denatured in a solution containing 0.5 m NaOH and 1.5 m NaCl for 30 min. The agarose gel was then neutralized in a solution containing 1.5 m NaCl and 1.0 m Tris-HCl (pH 7.4). The DNAs were transferred to nylon membranes by blotting and fixed by exposure to ultraviolet irradiation. The membrane was prehybridized for 3 h at 37°C, and hybridized overnight at 37°C with the radioactive probe. The Acl and Sma fragments of the C7cDNA (672 bp) and the Kpn and BglII fragments of the 5′R cDNA (714 bp) were prepared as probes. The sequences of the probes were located between the primers for PCR. The membrane was washed and exposed to an X-ray film for 5 min, and to an imaging plate of the bio-imaging analyzer system (23) (BAS 1000, Fuji Photo Film Co., Tokyo, Japan) for 1 min.

Oligonucleotides used for amplification

The synthesized gene-specific primers for C7cDNA and 5′R cDNAs were purchased from Bio-Synthesis, Inc. (Lewisville, TX). The sequences of forward primer and reverse primer for C7cDNA were 5′-AATGAAAGGCAATGCTGGTGGCTCTCCAC3′ (nucleotides 514–537 of rat C7cDNA) and 5′-CCAGCCCAGTATCGCTGTTT CAGT-3′ (nucleotides 1535–1558 of rat C7cDNA), respectively. The sequences of forward primer and reverse primer for 5′R were 5′-AATGATGGAACGCATTCCGATGCA-3′ (nucleotides 77–101 of rat 5′R cDNA) and 5′-GGTATTGAGGATGTAC CTCCGACA-3′ (nucleotides 978–1001 of rat 5′R cDNA), respectively. The lengths of the amplicons of C7cDNA and 5′R cDNA were 1045 bp and 925 bp, respectively.

Competitive kinetic analysis of the amplification of the target cDNA and the standard DNA

The same amounts of C7cDNA and standard DNA were co-amplified with various cycles to determine the adequate cycle number for competitive PCR. The amounts of PCR product of the target cDNA and the standard DNA increased logarithmically parallel with each other up to 30 cycles and reached a plateau around 30 to 50 cycles. Therefore, 25 cycles was chosen for PCR.
Competitive PCR

Competitive PCR method was performed to determine the initial amount of mRNA. Briefly, total RNA was prepared from the liver or pancreas of the rat. Then, the first strand cDNA of C7αH was synthesized. A 1 μl aliquot of each serial dilution (1 x 10^-2.5, 1 x 10^-2.0, 1 x 10^-1.5, 1 x 10^-1.0, 1 x 10^-0.5, and 1 x 10^0 attomoles/μl) of the standard DNA was added to the PCR reaction mixture with 1 μl of the first strand cDNA solution. PCR was performed as noted above. After Southern hybridization of both the target amplicon (1045 bp) and the standard amplicon (692 bp), the intensity of the radioactivity of each band was analyzed using the bio-imaging analyzer system. A sample image obtained from this instrument is shown in Fig. 1A. The logarithm of the ratio (log [At/As]) of the intensity of radioactivity of the target band (At) to the standard band (As) was plotted as a function of the initial amount of standard DNA (Nos).

Statistical analysis

Statistical comparisons were made using the Student's t test for unpaired data. Differences were judged to be significant at the P < 0.01 or P < 0.05 level.

RESULTS

C7αH activity expressed in the pancreatic hepatocyte-like cells

The existence of the C7αH activity in the pancreatic hepatocyte-like cells was demonstrated using HPLC (Fig. 2). Four rats were killed at 10 am, and four at 10 pm. The specific activity of C7αH in the normal liver and pancreatic hepatocyte-like cells was 3.2-fold and 3.7-fold higher at 10 pm than at 10 am, respectively (Fig. 3). There were significant differences (P < 0.01) between the values. In the normal pancreas, the activity could not be detected.

Expression of C7αH and 5βR mRNA in the liver and the pancreas

Each rat was killed at 10 pm. Northern blot analysis demonstrated that C7αH and 5βR mRNAs were expressed in the pancreatic hepatocyte-like cells of the rats treated with copper depletion regimen but not in the normal pancreas of the rat fed a normal diet (Fig. 4). At least three species of mRNA of C7αH were detected in the normal liver and hepatocyte-like cells, including a prominent 3.6-kb mRNA and two less abundant mRNAs of 2.4 and 1.7 kb as previously reported (25). In the rats fed the copper-deficient diet, C7αH and 5βR mRNAs first appeared in the pancreas at the eighth week of treatment. In the rats fed a normal diet after copper depletion, both C7αH and 5βR mRNA levels increased dramatically in the pancreas (Fig. 5).
Quantitation of the amounts of expressed C7αH mRNA by competitive PCR

Three rats were killed at 10 am, and three at 10 pm. In the normal diet group, C7αH mRNA expressed in the liver was 2.4-fold higher at 10 pm than at 10 am (P < 0.01). Likewise, in the copper depletion-repletion diet group, pancreatic C7αH mRNA expression at 10 pm was 1.9-fold (P < 0.05) higher than at 10 am (Fig. 6). Negative control using total RNA isolated from a normal pancreas showed that there was no visualized target band on an agarose gel electrophoresis. Furthermore, Southern blot analysis also showed no visualized target band.

DISCUSSION

Recent studies have shown that marked involution of pancreatic acinar tissue in the rat during copper depletion is secondary to apoptosis (26, 27). The ductular and...
periductular (oval) cells, which contain albumin transcripts and proliferate during acinar cell loss after copper depletion treatment, are considered to be precursors of pancreatic hepatocyte-like cells (28, 29). The mechanisms for transdifferentiation of ductular and oval cells into hepatocyte-like cells are not clear. The pancreatic hepatocyte-like cells show morphologic features characteristic of the liver parenchymal cells and can produce the liver-specific proteins, carboxymethylphosphate synthase I and urate oxidase, as well as albumin (7, 8). Bile canaliculi have also been observed by electron microscopy (1, 3). Whether pancreatic hepatocyte-like cells act functionally like normal hepatocytes with respect to bile production, an essential function of the liver, is another interesting question. It was thus worthwhile to study whether cholesterol 7α-hydroxylase (C7αH), which is specific to the hepatocytes and responsible for the production of bile acids from cholesterol, is produced in the pancreatic hepatocyte-like cells.

Bile acid production is dependent upon several factors: the level of bile acids returning to the liver via the enterohepatic circulation, hormonal factors, and modulation of activity by the formation of covalent bonds to the enzyme (30, 31). Whether the circadian rhythm of C7αH activity is regulated pre-translationally or post-translationally is still controversial (12, 13). Recently, a correlation between the enzyme activity and mRNA level was demonstrated which points to pre-translational enzyme regulation (14). The existence of the C7αH mRNA in the pancreatic hepatocyte-like cells seems, therefore, to be essential for the production of this enzyme in these cells.

In our study, Northern blot analysis demonstrated the expression of mRNAs of enzymes for bile acid biosynthesis (C7αH and 5βR) in the pancreatic hepatocyte-like cells. Furthermore, the results from RT-PCR demonstrated that mRNAs of both enzymes appeared at the eighth week of copper depletion treatment and these expressions were more prominent after copper repletion.

It is considered that circadian rhythm of C7αH mRNA is transcriptionally regulated by albumin D-element-binding protein (DBP), which is a liver-enriched transcription activator protein (32). It is thought that a stringent circadian rhythm of DBP protein level is regulated by the function of the hypothalamic-pituitary-gonadal axis (33). It is not clarified whether the negative feedback regulation of bile acids biosynthesis has an influence on the circadian regulation of C7αH. Vlahcevic et al. (34) reported that not only enzyme activity but also gene expression of C7αH was increased by administration of cholestyramine and inhibited by administration of bile acids in rat liver. These facts indicate that negative feedback regulation of C7αH is also transcriptionally performed by bile acids. In the animals of the present study, the pancreatic hepatocyte-like cells are less flooded with bile acids absorbed in the intestine than liver parenchymal cells. Whether absorbed bile acids which affect negative feedback in production of bile acids in the liver parenchymal cells play the same role in the pancreatic hepatocyte-like cells is an interesting problem, though the cells are small in amount compared with the liver parenchymal cells and therefore play a smaller role for the total sterol metabolism.

In the normal rat liver, the specific activity and gene expression of C7αH were significantly 3.2-fold and 2.4-fold higher at 10 pm than at 10 am, respectively. This circadian rhythm is consistent with previous reports (12, 14). We analyzed the activity and initial amount of mRNA of C7αH in the pancreatic hepatocyte-like cells of the rats. As for the quantitation of the mRNA, recent studies have demonstrated the accuracy and precision of the competitive PCR methods to quantify mRNA from a small number of cells (24, 35). Also, in the pancreatic hepatocyte-like cells, the specific activity and expression of C7αH mRNA were significantly 3.7-fold and 1.9-fold higher at 10 pm than at 10 am, respectively. It was certain that circadian rhythms of cholesterol 7α-hydroxylase activity and mRNA expression existed in the pancreatic hepatocyte-like cells as observed in normal liver cells.

These results obtained from our study indicate a possibility that bile acid biosynthesis is performed in the pancreatic hepatocyte-like cells of the rat on copper depletion-repletion regimen. 


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