Isolated Small Rat Hepatocytes Express both Annexin III and Terminal Differentiated Hepatocyte Markers, Tyrosine Aminotransferase and Tryptophan Oxygenase, at the mRNA Level

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We recently showed that annexin III is expressed in isolated small rat hepatocytes but, not in parenchymal hepatocytes. In the present study, we used reverse transcription polymerase chain analysis to examine the annexin III mRNA level in isolated small rat hepatocytes and parenchymal hepatocytes. Annexin III mRNA was detected in isolated small hepatocytes, but not in isolated parenchymal hepatocytes, confirming the presence of annexin III expression in isolated small rat hepatocytes at the mRNA level and indicating that the absence of annexin III expression in isolated parenchymal hepatocytes is due to the absence of annexin III mRNA. Furthermore, we examined the mRNA level of tyrosine aminotransferase and tryptophan oxygenase, two terminally differentiated hepatocyte markers. mRNA for these markers was detected in both parenchymal hepatocytes and small hepatocytes.

Key words: small rat hepatocyte; annexin III; differentiated hepatocyte phenotype

Small hepatocytes represent a minor population of cells that have been identified among cultured rat hepatocytes. These cells have a high replication potential in some defined media and differentiate into cells expressing either α1-antitrypsin, a differentiated hepatocyte marker protein, or biliary cell markers, such as BD1 and cytokeratins 7 and 19. Small hepatocytes have also been found in normal and diseased human liver tissue, and express differentiated hepatocyte phenotypes. Therefore, human small hepatocytes are expected to be useful for cell therapy and as carrier cells for gene therapy in diseased liver.

Recently, we attempted to identify the proteins that are specifically expressed in small hepatocytes, but not in parenchymal hepatocytes. We discovered that annexin III is expressed in isolated small rat hepatocytes, but not in parenchymal hepatocytes.

In the present study, we used reverse transcription polymerase chain reaction (RT-PCR) analysis to examine whether the difference in annexin III expression in these cells was due to a difference in mRNA levels. We also measured the mRNA levels of tyrosine aminotransferase (TAT) and tryptophan oxygenase (TO), two terminally differentiated hepatocyte markers, in isolated small rat hepatocytes to examine the hepatic differentiation state of the cells.

MATERIALS AND METHODS

Isolation of Small and Parenchymal Rat Hepatocytes

Fractions of small hepatocytes and parenchymal hepatocytes were prepared from adult male Wistar strain rats (Japan SLC Co. Ltd., Shizuoka, Japan) weighing 180–200 g by in situ perfusion of the liver with collagenase, followed by differential centrifugation and Percoll gradient centrifugation using a method reported by Tateno et al. All animal care and procedure protocols were approved by the institutional animal care committee.

RT-PCR Analysis

Total RNA was extracted from cells washed with phosphate-buffered saline using a QuickPrep Total RNA Extraction kit (Amersham Biosciences, U.S.A.) according to the manufacturer’s protocol. Approximately 3 μg of RNA per sample was reverse-transcribed using a THERMOScript™ RT-PCR System (Invitrogen Life Technologies, U.S.A.) and oligo(dT)20 in a final volume of 40 μl, according to the manufacturer’s protocol. Subsequently, 1 μl of cDNA corresponding to 12.5, 25 and 50 ng of template RNA was amplified using the THERMOScript™ RT-PCR System in a final volume of 20 μl per reaction, according to the manufacturer’s protocol, for 20 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and polymerization for 1 min at 72 °C using each set of 5’ and 3’ primers (Table 1). The PCR products were separated on a 2% agarose gel, stained with SYBR Green 1, and visualized with a Fluoroomager 595 (Amersham Biosciences). The annexin III, TAT, and TO PCR product specificities were confirmed by DNA sequencing using an ABI Prism 377 Sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

Figure 1 shows the PCR products prepared from RNA derived from isolated small and parenchymal rat hepatocytes. The bands of the PCR products corresponded to those predicted by use of the respective primers. The PCR product of annexin III was detected in small hepatocytes, but not in parenchymal hepatocytes and its intensity increased depending on the amount of cDNA used. On the other hand, almost the same quantity of PCR products for albumin, a universal marker of hepatocytes, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, were detected depending on the amount of cDNA used in both small hepatocytes and parenchymal hepatocytes.
These results indicate that annexin III mRNA is expressed in isolated small hepatocytes, but not in parenchymal hepatocytes. The absence of annexin III mRNA in the parenchymal hepatocytes was consistent with our previous findings and the finding that annexin III mRNA was not detected in rat liver when examined using a Northern blot analysis. The probable reason for the failure to detect annexin III mRNA in the latter study, in spite of the fact that small hepatocytes are a cell component of the liver, was the use of the whole liver in the analysis sample, reducing the detection sensitivity because the population of small hepatocytes in the liver is extremely small (less than 3% of the population of parenchymal hepatocytes in our preparation). On the other hand, a few reports have examined the expression patterns of other annexins in rat liver, although no reports have examined the expression of any annexins in small hepatocytes, except for our previous findings. The expression of annexin I and II was detected during the perinatal period, peaking at postnatal day 1 and gestational day 20, respectively, and reaching almost undetectable levels thereafter. The expression of annexin V and VI was detectable at low levels during the fetal period and steadily detected at high levels thereafter.

In contrast, the PCR products of TAT and TO were detected not only in the parenchymal hepatocytes, but also in the small hepatocytes. The intensity increased depending on the amount of cDNA used. As for the TAT and TO activities, it is uncertain whether the results of the present study reflect these activities in the small hepatocytes, since these enzyme activities in small hepatocytes have not been previously examined. In contrast, these activities have been detected in adult rat liver. As the mRNA levels of TAT and TO in the parenchymal hepatocytes were almost the same as those in the small hepatocytes, it is likely that these activities are detectable in small hepatocytes at almost the same levels as those in parenchymal hepatocytes.

TAT expression is virtually absent in the fetus; the accumulation of mRNA is followed by an increase in enzyme activity after birth, when the transcription of the gene is activated. TO expression is also virtually absent in the fetus; the accumulation of mRNA is followed by an increase in enzyme activity 14 d after birth, when the transcription of the gene is activated. On the other hand, albumin mRNA expression has been detected in rat embryo on gestational day 9.5—10.5. Based on their characteristic expression patterns, TAT and TO are used as terminal differentiated hepatocyte markers from hepatic progenitor cells or bone marrow-derived stem cells to hepatic cells. In this respect, the present results showing the expression of TO and TAT mRNA in small rat hepatocytes were unexpected, because small hepatocytes are generally believed to be hepatic progenitor cells, based on their differential capacity as described in the introduction. This discrepancy between the results of the present study and the previous findings suggests that the above findings may have derived from the experimental designs that were used, since isolated small hepatocytes were used for the RT-PCR analysis, but the phenotypes were examined in cultured small hepatocytes. In this respect, it is noteworthy that all the small hepatocytes expressed α1-antitrypsin for at least for 4 h after plating, did not express α1-antitrypsin for the next 7 d of culture, and then expressed α1-antitrypsin after the 10 d of culture. Since α1-antitrypsin mRNA is detected as early as gestational day 13 in rat embryo, this marker is classified as an early differentiated hepatocyte marker. Therefore, this finding indicates that isolated small hepatocytes express an early differentiated hepatocyte marker, consistent with the present results. On the other hand, retorsine-exposed rats can re-

### Table 1. Sequence for PCR Primers in the 5´ to 3´ Orientation

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Accession No.</th>
<th>Nucleotide No.</th>
<th>Sequences</th>
<th>Predicted product size (bp)</th>
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<tr>
<td>Annexin III #2</td>
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<td></td>
<td></td>
<td>549–568</td>
<td>GTCGAGCCTGGTGGCCAAAT</td>
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<tr>
<td>TAT</td>
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<tr>
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Fig. 1. RT-PCR Analysis of Annexin III, TAT and TO mRNA in Isolated Small Hepatocytes and Isolated Parenchymal Hepatocytes

Total RNA was extracted from isolated small rat hepatocytes and parenchymal hepatocytes and analyzed by RT-PCR, as described in the Materials and Methods section. The panel shows the RT-PCR results of a representative electrophoresis gel. The experiment was performed in triplicate.
place their entire mass after undergoing a 2/3 surgical partial hepatectomy (PH) through the emergence and expansion of a population of small hepatocyte-like progenitor cells (SHPCs).20) SHPCs do not express mRNA for α1-antitrypsin 7 d after PH and exhibit mRNA expression patterns that are indistinguishable from those of fully differentiated hepatocytes 14 d after PH.11) Therefore, SHPCs do not express TAT mRNA comparable to that of fully differentiated hepatocytes 14 d after PH. Therefore, the present results suggest that small hepatocytes initially dedifferentiate from cells expressing highly differentiated hepatocyte phenotypes before beginning to differentiate during culture in retrorsine-exposed and PH-ized rats.

In conclusion, we demonstrated that annexin III mRNA was expressed in isolated small rat hepatocytes, but not in parenchymal hepatocytes, and that isolated small rat hepatocytes also express TAT and TO mRNA, two markers of highly differentiated hepatocyte phenotypes, indicating that the small hepatocytes have highly differentiated phenotypes. Since small hepatocytes can differentiate into parenchymal hepatocytes or biliary epithelial cells, as described in the introduction, such plasticity in phenotypes, including the dedifferentiation suggested by the results of the present study, may be regarded as one of the characteristic features of small hepatocytes that parenchymal hepatocytes do not share.

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REFERENCES