Cell transplantation causes loss of gap junctions and activates GGT expression permanently in host liver

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Gupta, Sanjeev, Pankaj Rajvanshi, Harmeet Malhi, Sanjeev Slehria, Rana P. Sokhi, Srinivasa R. G. Vasa, Mariana Dabeva, David A. Shafritz, and Andrew Kerr. Cell transplantation causes loss of gap junctions and activates GGT expression permanently in host liver. Am J Physiol Gastrointest Liver Physiol 279: G815–G826, 2000.—Cell transplantation into hepatic sinusoids, which is necessary for liver repopulation, could cause hepatic ischemia. To examine the effects of cell transplantation on host hepatocytes, we transplanted Fisher 344 rat hepatocytes into syngeneic dipeptidyl peptidase IV-deficient rats. Within 24 h of cell transplantation, areas of ischemic necrosis, along with transient disruption of gap junctions, appeared in the liver. Moreover, host hepatocytes expressed γ-glutamyl transpeptidase (GGT) extensively, which was observed even 2 years after cell transplantation. GGT expression was not associated with α-fetoprotein activation, which is present in progenitor cells. Increased GGT expression was apparent after transplantation of nonparenchymal cells and latex beads but not after injection of saline, fragmented hepatocytes, hepatocyte growth factor, or turpentine. Some host hepatocytes exhibited apoptosis, as well as DNA synthesis, between 24 and 48 h after cell transplantation. Changes in gap junctions, GGT expression, DNA synthesis, and apoptosis after cell transplantation were prevented by vasodilators. The findings indicated the onset of ischemic liver injury after cell transplantation. These hepatic perturbations must be considered when transplanted cells are utilized as reporters for biological studies.

hepatocyte; ischemia; injury; gene expression; vasodilatation

TRANSPPLANTED HEPATOCYTES integrate in the liver (20), repopulate the liver extensively in appropriate situations (24, 31, 37), and retain excellent function (22). In view of the therapeutic potential of hepatocyte transplantation (17), it is necessary to establish detailed mechanisms concerning liver repopulation with transplanted cells. Recent studies (21, 34) established that only a fraction of transplanted hepatocytes survive in the host liver. Although hepatocytes deposited in hepatic sinusoids integrated in the liver parenchyma, cells in portal spaces were cleared (21). In addition, it became apparent that there is temporary occlusion of portal vessels with cell emboli, which has the potential to cause hepatic ischemia. Subsequently, several days were required for transplanted cells to join host hepatocytes in the liver plate with the formation of conjoint plasma membrane structures. These findings indicate that the host liver undergoes perturbations after cell transplantation in hepatic sinusoids.

During our ongoing analysis of liver repopulation, we observed unexpected γ-glutamyl transpeptidase (GGT) expression in the host liver. In the normal adult liver, GGT is only expressed in biliary cells, whereas GGT is expressed in fetal hepatoblasts, precancerous or cancerous liver nodules, and malignant hepatocyte-derived cell lines, as well as “oval cells” arising in response to liver injury or carcinogenic treatments (2, 14, 23, 32). We were analyzing the fate of genetically marked cells in animals treated with the hepatotoxin D-galactosamine (GalN), which causes activation of progenitor liver cells expressing GGT (7). The hypothesis was that transplanted progenitor cells will differentiate into mature hepatocytes in a permissive microenvironment, and this was demonstrated with both pancreatic and hepatic epithelial cells after transplantation into the liver (8). Other studies examined the fate of transplanted hepatocytes in GalN-induced acute liver failure (19). Our expectation was that GGT expression might be activated in transplanted progenitor cells, but these cells matured into hepatocytes. However, despite maturation of progenitor cells into hepatocytes, we found that GGT expression was induced extensively in the host liver after cell transplantation. This prompted us to undertake further analysis of mechanisms underlying this observation. We used dipeptidyl peptidase IV-deficient (DPPIV–) Fischer 344 (F344) rats as hepatocyte recipients, which facilitates the localization of syngeneic normal hepatocytes in the liver, as previously described (35). Because one mechanism underlying GGT expression in host hepa-
lymphocytes could involve induction of hepatic ischemia, studies were conducted to analyze additional changes relevant to this process after cell transplantation.

**MATERIALS AND METHODS**

**Cells.** Primary hepatocytes were isolated by in situ collagenase perfusion of the liver as described previously (35). Cell viability was documented by trypan blue dye exclusion and attachment to tissue culture plastic in RPMI 1640 medium containing penicillin, streptomycin, amphotericin B, and 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY). FBS was from Hyclone Laboratories (Logan, UT). Collagenase was from Boehringer Mannheim (Indianapolis, IN). A cell line derived from nonparenchymal cells of the young rat liver, designated G7FNRL cells, that contains a bacterial \( \beta \)-galactosidase has been described previously (30). These cells were cultured in RPMI 1640 medium with penicillin, streptomycin, amphotericin B, and 10% FBS.

For cell culture assays, primary rat hepatocytes were plated in tissue culture plastic dishes at density of \( 1 \times 10^5/\text{cm}^2 \). Cells were cultured for up to 48 h in RPMI 1640 medium containing 10% FBS and antibiotics. To demonstrate DNA synthesis, cells were exposed to 10 ng/ml recombinant human hepatocyte growth factor (hHGF) (Genentech, San Francisco, CA). DNA synthesis rates were measured in cultured cells after incubation with \([3H]\text{thymidine for 1 h, as described previously (30). To demonstrate responses to oxidant injury, cells were cultured for 48 h and then exposed for 1 h to 50 or 100 \( \mu \text{M tert-butyl hydroperoxide (t-BuOOH)} \) (Sigma Chemical, St. Louis, MO) (29). The cell viability was determined by measuring conversion of thiazolyl blue dye (MTT) as described previously (11).

**Animals.** Male F344 rats were obtained from the National Cancer Institute. The Special Animals Core of the Marion Bessin Liver Research Center provided DPP IV–F344 rats weighing 120–150 g. Both male and female rats were used for the studies. Approximately 90 rats were utilized for the studies. The animals were housed under 14:10-h light/dark cycles with standard pelleted rodent diet and water ad libitum. The Animal Care and Use Committee at Albert Einstein College of Medicine approved the experimental protocols, and animal use was in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. NIH 85–23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20505).

The animals were anesthetized with ether and used in groups of two to six rats. After hepatocyte transplantation, recipient animals were killed at various times, including at 1, 2, 4, 6, 8, 12, 16, 20, and 24 h, 2, 3, 4, and 7 days, 3 wk, and 3, 11, and 24 mo. Change in cell viability was determined by trypan blue dye exclusion. Data were also available from rats subjected to portal venography immediately after transplantation of 2 \( \times 10^7 \) hepatocytes into the spleen, as described previously (21).

**Histochemical assays.** Tissues were frozen in methylbutane cooled to –70°C, and 5-\( \mu \)-m-thick cryostat sections were prepared. DPP IV activity was visualized after fixing sections in cold chloroform and acetone (1:1 vol/vol) for 10 min followed by incubation with glycy1-L-proline-4-methoxy-2-naphthylamide substrate in the presence of 1 mg/ml fast blue BB salt for 30 min, as described previously (26). ATPase activity was used to colocalize bile canaliculi in the host and transplanted hepatocytes as reported previously (18). GGT activity was detected in tissue sections or cells fixed with ethanol/glacial acetic acid for 10 min at –20°C, according to Rutenberg et al. (38). The overall distribution of GGT activity in tissues was classified as being either grade 0 (absent), grade 1+ (scanty; an occasional cell per high power field positive), grade 2+ (moderate; 20–50 cells per high power field positive), or grade 3+ (extensive; >50 cells per high power field positive). Hepatic gap junctions were localized by immunostaining with the 7C6, \( \alpha \)-Cxs2 antibody, as described previously (20). In some studies, tissues were stained histochemically for DPP IV activity, followed by Cx32 immunostaining. Tissues were counterstained with hematoxylin or methyl green as appropriate.

**Cellular glutathione and catalase content.** All chemicals were from Sigma Chemical. Cells were cherished and stored at –80°C in 5% salicylic acid. For assays, cells were thawed to 4°C and disrupted by ultrasonication. Cell debris were eliminated by pelleting at 10,000 g for 10 min at 4°C. The supernatant was assayed for total glutathione content (1). To 100 \( \mu \)l supernatant, 800 \( \mu \)l of 0.3 mM niotinamide adenine dinucleotide phosphate, reduced form, 100 \( \mu \)l of 6 mM DTNB, and 0.5 U glutathione reductase were added. Changes in absorbance over 2 min at 412 nm were read spectrophotometrically. Glutathione standards were prepared with 100 \( \mu \)M stock solution in lineary range. Catalase activity was determined by a method described previously (27). Briefly, frozen cells were thawed on ice, ultrasonicated, and then centrifuged at 10,000 g for 10 min at 4°C. Catalase activity
was measured in the supernatant by adding 3 ml of H₂O₂-phosphate buffer and 10–40 μl sample in a silica cuvette, and time (t) for change in optical density from 0.450 to 0.400 was determined at 240 nm at room temperature. The catalase activity was calculated by the formula 17t/t = units/assay mixture. Protein content was assayed in aliquots using the Bradford assay. Each condition was in triplicate.

Quantitation of GGT activity in tissues. A commercial assay kit was used (Diagnosics Procedure 545, Sigma Chemical). Preweighed liver tissues were homogenized in PBS, pH 7.4, and incubated with l-glutamyl nitroanilide substrate for 37°C for 20 min. The reactions were conducted according to the manufacturer’s suggested protocol and stopped by the addition of glacial acetic acid. Sodium nitrite, ammonium sulfamate, and naphthylethlyenediamine were added, and resultant absorbance was measured at 540 nm for each condition against blanks containing equal amounts of unretracted liver tissue. The reaction in blank tubes was stopped by adding glacial acetic acid to the substrate before the addition of liver homogenate and other reagents. A standard curve was plotted using GTP calibration solution (Sigma Chemical, no. 545-10).

DNA synthesis assays. Animals were given 0.5 mCi/kg body wt [³H]thymidine (specific activity, 70 Ci/mmole; ICN, Irvine, CA) and 50 mg/kg ip bromodeoxyuridine (BrdU; Boehringer Mannheim, Indianapolis, IN) 1 and 2 h before death, respectively. To demonstrate mitotic spindles, animals were given colchicine (Sigma Chemical) in a dose of 0.5 mg/kg body wt 2 h before death. To detect [³H]thymidine incorporation, tissue sections were autoradiographed for 4 wk with NBT-2 emulsion (Eastman Kodak, Rochester, NY), as described previously (18). To detect BrdU incorporation, cryostat sections were fixed in cold ethanol or ethanol-acetic acid (99:1 vol/vol) and blocked with 2% rabbit serum followed by incubation for 1 h with anti-BrdU (Amersham Life Sciences). Tissue sections were autoradiographed at 4°C with NTB-2 emulsion (Eastman Kodak, Rochester, NY), as described previously (18). To detect BrdU incorporation, cryostat sections were fixed in cold ethanol or ethanol-acetic acid (99:1 vol/vol) and blocked with 2% rabbit serum followed by incubation for 1 h with anti-BrdU (Amersham Life Sciences, North Chicago, IL). Antibody binding was detected by a supersensitive multilink antibody system, using the peroxidase reporter (BioGenex Laboratories, San Ramon, CA), followed by color development with Vectastain (Vector Laboratories, Burlingame, CA). To localize BrdU incorporation in GGT-positive cells, tissues were first stained for GGT activity and then subjected to BrdU immunostaining. DNA synthesis in transplanted cells was analyzed by DPPIV staining followed by BrdU immunostaining.

In situ hybridization for α-fetoprotein mRNA. The recombinant plasmid pBAP700 containing α-fetoprotein (AFP) cDNA sequences derived from fetal rat mRNAs was originally provided by Dr. N. Fausto (7). After linearization with restriction enzymes, ³⁵S-labeled anti-sense and sense riboprobes were obtained with appropriate RNA polymerases. In situ hybridization was performed on 5-μm-thick paraformaldehyde-fixed cryostat sections as described (7). Hybridized sections were autoradiographed at 4°C with NBT-2 emulsion (Eastman Kodak). Tissue sections from frozen fetal rat liver were included as positive controls.

In situ demonstration of apoptosis. Cryostat tissue sections of 5-μm thickness were analyzed using a commercial kit with peroxidase detection (Boehringer Mannheim). The enzymatic reaction utilizes terminal deoxynucleotidyl transferasemediated dUTP nick-end labeling (TUNEL) assay (12). Tissues were fixed in cold ethanol for 10 min, followed by processing according to the manufacturer’s suggested protocol. The assay identifies DNA strand breaks that occur during apoptosis by labeling free 3’-OH termini with modified nucleotides. Tissues from at least two animals were analyzed for each time point reported.

Statistical methods. Data are presented as means ± SE or SD. The significance of differences was analyzed with the Student’s t-test, Mann-Whitney rank-sum test, or χ² test using SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). P < 0.05 was considered significant.

RESULTS

Host hepatocytes expressed GGT immediately after cell transplantation. When injected into the spleen, hepatocytes migrated immediately to the liver and were found in hepatic sinusoids within 1 h, as documented previously (21, 35). We found that in contrast with the normal adult liver, cell transplantation caused extensive GGT expression in host hepatocytes. This became apparent as early as 2 h after cell transplantation (Fig. 1). Hepatocytes expressing GGT were observed primarily in perportal areas, although GGT-positive cells were also present in midzonal positions of the liver lobule. There was a characteristic distribution of GGT expression, with hepatocytes becoming positive in areas of the liver distal to transplanted cells. On the other hand, transplanted cells themselves remained GGT negative. Surprisingly, extensive GGT expression in host hepatocytes was found in all tissues from hepatocyte recipients, including at early (2–24 h and 2–4 days) and intermediate times (7 days and 3 wk), as well as late times (3, 11, and 24 mo). Indeed, tissues analyzed at 11 mo after cell transplantation still showed significant GGT expression in hepatocytes, similar to that observed at earlier times (Fig. 1D). Moreover, the overall pattern of GGT expression was essentially unchanged with time. GGT-positive cells remained distributed in perportal areas of the liver with no GGT activity in perivenous areas. In addition, the proportion of cells containing GGT activity was similar at early and late times, with GGT expression ranging from grade 2+ to 3+ on histological analysis. Together, these findings indicated that once GGT expression was induced in the liver, hepatocytes expressed this activity permanently.

Biochemical quantitation showed that in comparison with the normal liver, GGT content of the liver after cell transplantation increased markedly (Table 1). Additional experiments were conducted to determine whether GGT expression was induced by transplantation of fewer hepatocytes. For this purpose, groups of three animals each received 1 × 10⁶, 1 × 10⁷, or 2 × 10⁷ cells via the spleen. Compared with the normal liver, all treatments, including injection of 1 × 10⁶ hepatocytes, caused marked increases in GGT activity (range, 5.2- to 5.7-fold, P < 0.05).

To document whether this mode of GGT activation was a general biological feature, tissues from mice and rabbits previously subjected to cell transplantation were also analyzed. GGT expression was present in host hepatocytes in a pattern similar to that in F344 rats, male C57BL/6J mice, and male New Zealand White rabbits (Fig. 2A). The latter were transplanted with HepG2 human hepatocellular carcinoma cells, as previously reported (18). Moreover, GGT expression
was induced in the liver when nonparenchymal epithelial cells, FNRL, were transplanted, indicating that hepatocytes were not necessary for this effect (Fig. 2B).

The overall magnitude of GGT expression was comparable with that after treatment of rats with phenobarbital for 4 days, similar to results published previously (17). In this instance, hepatocytes in zone 1 constituting ~10–20% of the liver lobe expressed GGT. A variety of additional studies were performed to determine further methods of GGT activation (Table 2). Injection of saline or of ultrasonically fragmented hepatocytes did not cause GGT activation. In response to either hHGF infusion or turpentine injection, only an occasional hepatocyte became GGT positive, without any zonal preference. However, extensive GGT expression was induced by intrasplenic injection of inert latex beads, which were distributed in portal areas.

**Table 1. Activation of hepatic GGT expression**

<table>
<thead>
<tr>
<th>Condition</th>
<th>GGT Activity, units/mg liver</th>
<th>Relative GGT Expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>0.6 ± 0.1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Time after cell transplantation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h after cell transplantation</td>
<td>9.6 ± 2.1</td>
<td>16 ± 3.5</td>
<td>0.007</td>
</tr>
<tr>
<td>2 wk after cell transplantation</td>
<td>21.6 ± 6.1</td>
<td>36 ± 10</td>
<td>0.009</td>
</tr>
<tr>
<td>24 mo after cell transplantation (mean)</td>
<td>17.2</td>
<td>15.6</td>
<td>28</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3–5 animals/group, except 24 mo after cell transplantation, where data are from only 2 rats. GGT, γ-glutamyl transpeptidase. P values obtained from t-tests.

Fig. 1. γ-Glutamyl transpeptidase (GGT) expression in the host Fischer 344 (F344) rat liver. A: GGT expression in the normal liver showing activity restricted to bile duct cells (arrow). B: liver from an animal 2 h after cell transplantation, with extensive GGT expression (red staining of cell membranes) in host hepatocytes primarily in periportal areas and also in midzonal locations. Note absence of GGT expression in a group of transplanted hepatocytes (arrow), which were identified by their location in portal areas or inside sinusoidal spaces. Inset: a higher magnification view of this area; arrow, GGT-negative transplanted hepatocytes in sinusoids. C: GGT expression 2 wk after cell transplantation with activity in bile duct cells (arrow), as well as in periportal hepatocytes. D: liver from a rat 11 mo after cell transplantation showing persistence of GGT expression in hepatocytes in periportal areas.
areas, similar to transplanted hepatocytes (Fig. 2C). GGT expression was observed in areas distal to where latex beads were lodged. In view of the inert nature of latex beads, these findings were suggestive of a mechanical effect underlying GGT expression, such as attenuation of sinusoidal blood flow by cell emboli.

Early GGT expression is associated with evidence of ischemic events, including disruption of hepatic gap junctions. Portal venography immediately after cell transplantation showed attenuation of major portal vein radicles (Fig. 3). These findings were similar to those reported elsewhere (21) with restoration of nor-

Table 2. Effect of various treatments on hepatic GGT expression

<table>
<thead>
<tr>
<th>Condition Tested</th>
<th>Relative GGT Expression</th>
</tr>
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<tbody>
<tr>
<td>Intrasplenic injection of normal saline</td>
<td>–</td>
</tr>
<tr>
<td>Intraportal infusion of hHGF</td>
<td>+/–</td>
</tr>
<tr>
<td>Intraperitoneal injection of turpentine</td>
<td>+/–</td>
</tr>
<tr>
<td>Intrasplenic injection of fragmented hepatocytes</td>
<td>–</td>
</tr>
<tr>
<td>Intrasplenic injection of intact nonparenchymal liver epithelial cells</td>
<td>+</td>
</tr>
<tr>
<td>Intrasplenic injection of inert latex beads</td>
<td>+/–</td>
</tr>
<tr>
<td>Intrasplenic injection of latex beads or cells with nitroglycerine</td>
<td>–</td>
</tr>
</tbody>
</table>

hHGF, human hepatocyte growth factor.

Fig. 2. Relationship of GGT expression to cell-type independent and mechanical events. Data shown are from animals at 2 h after various manipulations. A: GGT expression in the liver after intrasplenic transplantation of $1 \times 10^7$ G7-HepG2 cells in a rabbit. Note GGT-expressing cells in periportal areas, as well as in midzonal locations. B: GGT expression in the liver after intrasplenic transplantation of $2 \times 10^7$ nonparenchymal liver (FNRL) cells. C: latex beads in portal areas of the liver (arrow), similar to the localization of transplanted cells, associated with GGT expression in periportal areas.

Fig. 3. Hepatic ischemia after cell transplantation. Portal venography immediately after transplantation of $2 \times 10^7$ hepatocytes in a rat showing extensive attenuations of portal vein radicles (arrowheads). The contrast material was injected into the superior mesenteric vein (smv). Visualization of the splenic vein (spl v) as well as tributaries of the superior mesenteric vein indicates onset of portal hypertension. The portal vein (pv) is more distended than normal.
Fig. 4. Ischemia-related changes in the host liver. A: liver from a rat 24 h after cell transplantation showing abnormal areas with ischemic necrosis (encircled by arrowheads at top right and bottom left). The arrow points to transplanted cells in a portal area. B: liver from a rat 48 h after cell transplantation displaying normal histology. A and B were stained with hematoxylin and eosin. C: extensive loss of Cx32 immunostaining in hepatocytes 2 h after cell transplantation in the area indicated by *. This area contained transplanted cells, which were localized simultaneously by dipeptidyl peptidase IV (DPP IV) staining. Cx32 activity is preserved in other parts of the liver lobule (brown color). Inset: a higher power view with transplanted cells in hepatic sinusoids containing red-colored DPP IV activity (straight arrow). The curved arrow points to occasionally preserved gap junctions, whereas most of the gap junctions were lost. Note that transplanted hepatocytes are negative for Cx32 activity because cell dissociation disrupts Cx32 expression. D: a corresponding section from C showing GGT activation in the area where gap junction activity was lost. E: liver from an animal treated with nitroglycerine before cell transplantation with preservation of Cx32 staining. The animal was killed 2 h after cell transplantation. F: liver from an animal treated with nitroglycerine before cell transplantation showing absence of GGT expression in hepatocytes at 2 h. GGT staining is present in bile ducts (short arrow indicating red color). Transplanted hepatocytes situated in portal areas were negative for GGT expression (long arrow). Similar findings were obtained after treatment of animals with phentolamine.
mal portal vasculature within 1 day after cell transplantation.

Notably, ischemic areas were observed in the liver 24 h after cell transplantation (Fig. 4). In several lobules, in which portal areas were filled with transplanted cells, less intensely stained host hepatocytes were observed. Occasionally, areas with clearly obvious ischemic necrosis distal to portal areas containing transplanted cells were noted (Fig. 4A). These ischemic changes were no longer observed in the liver 48 h after cell transplantation (Fig. 4B). To determine whether additional changes occurred in the liver parenchyma during initial deposition of cells, we examined the integrity of gap junctions. There was extensive loss of immunostaining with our Cx32 antibody (Fig. 4, C and D). The disruption of gap junction activity was observed primarily in perportal hepatocytes, in a pattern similar to the distribution of GGT activity after hepatocyte transplantation. These gap junction-deficient areas were apparent within 2 h of cell transplantation and disappeared within 24 h after cell transplantation.

We reasoned that if GGT expression was induced by mechanical perturbations blocking blood flow through the liver microcirculation, use of vasodilators might be helpful in preventing or decreasing induction of GGT expression. To test this possibility, nitroglycerine was infused before, during, and after hepatocyte transplantation. Interestingly, this manipulation prevented loss of gap junction activity (Fig. 4E), as well as GGT activation in host hepatocytes (Fig. 4F). To demonstrate whether other vasodilators could also exert this effect, tissues were analyzed in which phentolamine was administered to the animals before cell transplantation. GGT expression was abrogated under these circumstances as well. Biochemical quantitation of hepatic GGT activity in animals treated with vasodilators before cell transplantation showed a marked decline in similar to the distribution of GGT activity after hepatocyte transplantation. These gap junction-deficient areas were apparent within 2 h of cell transplantation and disappeared within 24 h after cell transplantation.

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GGT content (2.8 ± 0.2 vs. 16 ± 3.5 units/mg in corresponding controls, P < 0.01).

**DNA synthesis in response to cell transplantation.** Analysis of hepatic DNA synthesis with either [³H]thymidine or BrdU incorporation showed similar kinetics in host hepatocytes. Rats injected with saline alone showed no evidence for significant DNA synthesis, similar to untreated control animals (0.1–0.2% labeled hepatocytes). In contrast, host hepatocytes showed unscheduled DNA synthesis, as indicated by BrdU labeling (Fig. 5). Scattered host hepatocytes with or without GGT expression and other adjacent GGT-positive hepatocytes also showed DNA synthesis. To determine the kinetics of this change, a series of animals were analyzed. Hepatic DNA synthesis first became apparent at 24 h (2 ± 2% labeling), became most pronounced at 48 h (5 ± 3% labeling, P < 0.001, χ² test), and was not detected subsequent to 72 h after cell transplantation. Interestingly, transplanted hepatocytes were not observed to be undergoing DNA synthesis at these early times, i.e., 24 or 48 h after cell transplantation (Fig. 5A). The findings were verified by analysis of BrdU labeling in tissue sections stained first for DPPIV activity. Moreover, although DNA synthe-

![Table 3. Response to oxidant stress in cultured hepatocytes](image-url)

<table>
<thead>
<tr>
<th>tert-Butyl Hydroperoxide Treatment</th>
<th>Control Hepatocytes</th>
<th>Hepatocytes from rats after cell transplantation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM</td>
<td>1.04 ± 0.05</td>
<td>1.99 ± 0.12</td>
<td>0.006</td>
</tr>
<tr>
<td>50 μM</td>
<td>0.20 ± 0.02</td>
<td>1.49 ± 0.42</td>
<td>0.03</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.43 ± 0.04</td>
<td>1.24 ± 0.21</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD. MTT, thiazolyl blue dye. P values obtained from t-tests.
sis was not analyzed in the long term, the number of transplanted cells did not change in recipients between 3 and 11 mo.

Absence of AFP expression in liver after cell transplantation. Expression of GGT and DNA synthesis in perportal cells led us to consider whether progenitor cells had become activated. Therefore, we analyzed AFP mRNA expression in the liver by in situ hybridization. Although the control fetal rat liver contained cells with abundant AFP mRNA expression, the liver of adult rats receiving transplanted hepatocytes never showed hybridization signals at 12, 24, 48, or 3 wk of cell transplantation (Fig. 6). The experiments were repeated twice with similar data. Moreover, we did not observe oval cells or expansions of biliary cells in the normal liver after cell transplantation. These findings are consistent with an absence of progenitor cell activation.

Further evidence for injury to host hepatocytes. Our observations concerning the presence of mitoses in colchicine-pretreated animals were interesting. Despite DNA synthesis in the liver after hepatocyte transplantation, cells in mitosis were not observed in metaphase. On the other hand, we found scattered GGT-positive and GGT-negative host hepatocytes with nuclear fragmentation (Fig. 7). Colocalization studies showed that DPP IV-positive transplanted cells neither incorporated BrdU nor exhibited nuclear fragmentation at these times. Notably, such nuclear fragmentation was not observed in animals treated with nitroglycerine before cell transplantation. To determine whether the change in host cells was compatible with the onset of apoptosis, in situ TUNEL assays were performed. These studies showed increased apoptosis rates in the liver 24 and 48 h after cell transplantation. In the normal control liver, only an occasional cell (1–2 cells/section) showed apoptosis. In contrast, 5 ± 3 apoptotic cells were observed per high power field (×100) in hepatocyte recipients (P < 0.01, t-test). Furthermore, hepatocyte apoptosis was not observed in animals pretreated with nitroglycerine.

Biological effects of GGT expression in hepatocytes. To determine the significance of GGT expression, hepatocytes were isolated from F344 rats that 3 wk previously received 5 × 10⁷ cells via the spleen. Trypan blue dye exclusion showed that cell viability was >85%, similar to cells from control animals not treated with cell transplantation. Analysis of GGT expression in cytospun preparations showed that 30–40% of hepatocytes from transplanted animals were GGT positive. In contrast, hepatocytes from control animals did not express GGT. The glutathione content was greater in hepatocytes from transplanted animals (39 ± 1 μM glutathione/μg protein) compared with hepatocytes from control animals (21 ± 1 μM glutathione/μg protein, P < 0.05), whereas catalase activity was not different (87 ± 8 vs. 98 ± 7 units/mg protein). Analysis of DNA synthesis in cultured hepatocytes showed that cells from transplanted animals responded to hHGF with a four- to ninefold increases in DNA synthesis. In contrast, hepatocytes from control animals showed 6- to 15-fold increases in DNA synthesis after stimulation with hHGF, which was not significantly different from transplanted animals.

On the other hand, hepatocytes from transplanted animals were more resistant to oxidant injury with t-BuOOH (Table 3). The data shown are from triplicate conditions and were obtained by exposing cells to t-BuOOH for 1 h after cells had been in culture for 48 h. Hepatocytes from animals treated with cell transplantation showed greater viability without any exposure to t-BuOOH and showed significantly greater resistance to 50 and 100 μM concentrations of t-BuOOH, which has been well characterized for its toxic effects on hepatocytes (29). Together, these findings indicate that activation of GGT expression in the liver was associated with greater resistance of cells to oxidative injury.

**DISCUSSION**

We consider it most likely that mechanisms related to hypoxia or ischemia-reperfusion were responsible for GGT activation, loss of gap junction activity, and other changes after cell transplantation. Embolization of cells in hepatic sinusoids would be consistent with hypoperfusion, as well as hypoxia in areas distal to transplanted cells. Subsequent restoration of the microcirculation with resumption of blood flow would trigger ischemia-reperfusion events.

Acute and chronic hypoxia is known to induce hepatic GGT expression (40). Hepatic ischemia leads to peripheral release of GGT with peak blood levels between 20 and 30 h after restoration of hepatic blood flow (25), although our studies showed that hepatocyte membrane-bound GGT is expressed much earlier (within 2 h). GGT was activated in the liver by transplantation of relatively few cells, such as 1,000,000–5,000,000 cells. We believe this to indicate that extensive occlusions of portal vein radicles are not required for inducing ischemic changes in the liver. The ability to reproduce induction of GGT by inert latex beads and its abrogation by vasodilation with nitroglycerine and its prolongation by hypoperfusion, as well as hypoxia in areas distal to transplanted cells, are also in agreement with ischemia-reperfusion-type mechanisms.

GGT is localized in cell membrane domains in many extrahepatic tissues, including, for instance, kidney, pancreas, spleen, heart, and brain, and is thought to play a role in amino acid transport across cell membranes (14). Although drugs, such as phenobarbital and phenytoin, as well as alcohol, are known to induce GGT expression, the precise pathophysiological significance of this change is unclear, especially because of complex alterations accompanying these perturbations (39, 45). GGT expression may be regulated by multiple factors, including animal strain, hormones, cell differentiation states, and tissue-specific mechanisms, as suggested by studies (4, 5, 9, 10, 42, 48) in intact animals and cultured cells. The peculiar susceptibility of female F344 rats to GGT expression has previously been reported (4), although in our experience, GGT
expression was observed after cell transplantation in both male and female rats, male mice, and male rabbits, exhibiting the broad nature of the finding.

Use of AFP expression as a marker for progenitor cell activation suggested that this did not account for GGT expression in our hepatocyte recipients. Also, there was no morphological evidence for progenitor cell activation in the liver, such as the appearance of oval cells. Recent studies (5, 9) demonstrated that GGT expression may be regulated by cell differentiation states at the transcriptional level, through promoter interactions with specific cellular factors. These studies indicate that GGT expression is increased when cells undergo differentiation rather than dedifferentiation, which is again compatible with the absence of progenitor cell activation in our animals. Among other regulatory mechanisms, it has been shown that glucocorticoids induce and thyroid hormones suppress GGT expression in the liver by transcriptional or other mechanisms, including alterations in cell differentiation states (9). We do not know whether cell transplantation altered the local availability, incorporation, or metabolism of these hormones in the liver.

GGT expression can also be induced by growth factor activation (10, 42). We did not study changes in the local release of specific growth factors or cytokines after cell transplantation, although studies by Yazigi et al. (47) have suggested that HGF is expressed in areas adjacent to transplanted hepatocytes. Nonetheless, we found that hHGF infusion alone did not induce GGT expression after cell transplantation. Similarly, our studies using turpentine to induce an acute phase response along with cytokine release (3, 15), including that of interleukin-6, which plays a role in liver regeneration (6), showed no activation of GGT. Our inability to induce GGT expression by injection of disrupted cells also suggested that soluble factors released from hepatocytes themselves were unlikely to be responsible for this change.

Previous analysis (46) of GGT-positive hepatocytes isolated from hepatic nodules arising in response to carcinogenic treatments demonstrated that these cells possessed greater proliferative capacity. However, hepatocytes influenced to express GGT in our studies did not proliferate in the liver. Indeed, our findings are most compatible with the persistence of GGT-positive hepatocytes without change in the overall mass of these cells, similar to the fate of transplanted hepatocytes in animals (16). Although we observed unscheduled DNA synthesis in host hepatocytes after cell transplantation, DNA synthesis was observed in GGT-negative hepatocytes, as well as GGT-positive hepatocytes. Moreover, we have found no evidence for oncogenesis in several F344 rats subjected to cell transplantation during up to two years of observation (n = 12; S. Gupta, unpublished observations).

The prolonged duration of GGT expression in the liver after cell transplantation was most intriguing. We found that increased hepatic GGT expression was still present at 2 years after a single session of cell transplantation. Although we do not know the basis for indefinite persistence of GGT expression in hepatocytes, previous studies (41) showed that hepatocytes expressing GGT were protected against glutathione depletion and oxidative stress. Thus one could speculate that if ischemia-reperfusion-related oxidative stress occurred in the liver after cell transplantation, GGT expression might represent a protective event, which could increase cell survival. Our findings were in agreement with this possibility. In hepatocytes isolated from animals subjected to cell transplantation, greater resistance to t-BuOOH injury is compatible with survival advantages, rather than increased proliferation of cells, as indicated by the absence of greater DNA synthesis in these hepatocytes compared with hepatocytes from the unperturbed normal rat liver.

We found it remarkable that the activity of Cx32, which is a major constituent of hepatic gap junctions, was rapidly, albeit transiently, altered after hepatocyte transplantation. Although hepatic Cx32 expression is regulated in the context of partial hepatectomy, drug-induced neoplasia, and other situations (33, 43, 44, 49), changes in Cx32 expression after ischemia-reperfusion are directly relevant to our observations. Recent studies (13), published subsequent to the performance of our experiments, showed that after ischemia hepatic Cx32 expression is attenuated within 1–4 h after reperfusion. Similarly, cell transplantation in the presence of vasodilators prevented the loss of hepatic Cx32 expression in the liver. Our findings concerning Cx32 expression are in agreement with the onset of ischemia-reperfusion injury in the liver after hepatocyte transplantation. Angiographic analysis indicated attenuation of portal radicles after cell transplantation that is transient (21).

Findings in this study will be helpful in understanding changes occurring during liver repopulation and have possible implications in basic hepatic biology and pathophysiology. For instance, it should be of interest to analyze how cells induced to express GGT after hepatocyte transplantation differ from GGT-positive cells arising in response to carcinogenic treatments. Fractionation of GGT-positive hepatocytes from the liver not subjected to carcinogenic treatments should help advance our knowledge in this area. Our findings should also be helpful in defining whether changes in the host liver after cell transplantation could be of significance in cell therapy, such as in acute liver failure. Could it be that in the setting of significant liver injury, e.g., after drug or other toxicity, cell transplantation could worsen the situation initially by inducing further ischemia-reperfusion injury? Finally, our findings stress the need for caution in conducting and interpreting cell transplantation studies utilizing GGT as a marker, especially where analysis of the fate of progenitor liver cells is concerned (28).

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