Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events

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1Marion Bessin Liver Research Center, 2Cancer Research Center, and Departments of 3Medicine, 4Radiation Oncology, 5Pathology, and 6Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461; and 7Center for Gastrointestinal and Biliary Disease Studies and Program in Molecular Biology and Biotechnology, University of North Carolina School of Medicine at Chapel Hill, Chapel Hill, North Carolina 27514

Sigal, Samuel H., Pankaj Rajvanshi, Giridhar R. Gorla, Rana P. Sokhi, Romil Saxena, David R. Gebhard, J r., Lola M. Reid, and Sanjeev Gupta. Partial hepatectomy-induced polyploidy attenuates hepatocyte replication and activates cell aging events. Am. J. Physiol. 276 (Gastrointest. Liver Physiol.): G1260–G1272, 1999.—In understanding mechanisms of liver repopulation with transplanted hepatocytes, we studied the consequences of hepatic polyploidization in the two-thirds partial hepatectomy model of liver regeneration. Liver repopulation studies using genetically marked rodent hepatocytes showed that the number of previously transplanted hepatocytes did not increase in the liver with subsequent partial hepatectomy. In contrast, recipients undergoing partial hepatectomy before cells were transplanted showed proliferation in transplanted hepatocytes, with kinetics of DNA synthesis differing in transplanted and host hepatocytes. Also, partial hepatectomy caused multiple changes in the rat liver, including accumulation of polyploid hepatocytes along with prolonged depletion of diploid hepatocytes, as well as increased senescence-associated β-galactosidase and p21 expression. Remnant hepatocytes in the partially hepatectomized liver showed increased autofluorescence and cytoplasmic complexity on flow cytometry, which are associated with lipofuscin accumulation during cell aging, and underwent apoptosis more frequently. Moreover, hepatocytes from the partially hepatectomized liver showed attenuated proliferative capacity in cell culture. These findings were compatible with decreased proliferative potential of hepatocytes experiencing partial hepatectomy compared with hepatocytes from the unperturbed liver. Attenuation of proliferative capacity and other changes in hepatocytes experiencing partial hepatectomy offer novel perspectives concerning liver regeneration in the context of cell ploidy.

THE ABILITY TO REPOPULATE the liver with transplanted hepatocytes offers novel strategies to understand liver biology (18). Transplanted hepatocytes integrate in the liver parenchyma with physiologically regulated gene expression patterns (21, 22). Moreover, transplanted hepatocytes show the capacity to proliferate in the host liver, with proliferation regulated by the magnitude of host hepatocyte depletion in both acute and chronic settings (20, 31, 37, 41, 47). In contrast, transplanted hepatocytes do not proliferate much in the normal liver (17, 20). These findings indicate that genetically marked transplanted hepatocytes are effective reporters for analyzing reciprocal changes concerning proliferation and survival of host hepatocytes. Moreover, the ability to repopulate the diseased liver has generated interest in understanding how transplanted hepatocytes could be induced to proliferate in the intact normal liver. Among various perturbations, use of partial hepatectomy to induce proliferation in transplanted cells seemed appropriate (59). However, it has been unclear as to whether it is possible to amplify the transplanted hepatocyte mass by partial hepatectomy (17).

The two-thirds partial hepatectomy model, which induces compensatory hepatic hypertrophy, has been utilized extensively in studies of “liver regeneration” (6, 36). After partial hepatectomy, liver mass is restored rapidly, with most hepatocytes undergoing two to three rounds of DNA synthesis. In contrast, after hepatic ablation with toxins, liver regeneration may be associated with progenitor cell activations (8, 16), although the liver stem cell has not been isolated. Also, hepatocytes from adult animals could divide repeatedly after transplantation (20, 31, 37, 41, 47), suggesting extensive replication potential in hepatocytes; however, it is unknown whether all transplanted hepatocytes participated equally in this process or whether specific cell subsets proliferate preferentially.

In short-term studies, it has been established that partial hepatectomy leads to hepatic polyploidy, which refers to increased nuclear DNA content (1, 5, 14, 39, 49). Polyploidization is a feature of virtually all organs, including blood, muscle, cornea, thyroid, pancreas, endometrium, placenta, urinary bladder, and neural tissues (5, 25, 30, 34, 48). Furthermore, polyploidy is associated with hypertrophic responses in tissues, e.g., vascular muscle cells in hypertension, acinar cells in the lactating breast, endometrial cells in the gravid uterus, peripheral lymphocytes in human immunodeficiency virus infection, and with cell aging, as observed in cultured fibroblasts undergoing senescence (4, 29, 43). Similarly, the ploidy of hepatocytes increases in older animals (48). This is associated with greater proportions of cells exhibiting flow cytometric characteristics, such as increased autofluorescence, which reflects accumulation of the lipid peroxidation product lipofuscin, and wide-angle light scatter, which correlates with cytoplasmic complexity observed during cell differentiation (24, 50). Moreover, the normal adult liver itself contains hepatocytes with different degrees of ploidy, and cells with greater ploidy exhibit attenuated mitogenic activity (46). One reason could be that polyploid cells are at a proliferative disadvantage, as

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MATERIALS AND METHODS

Animals. Male F344 rats of 12–16 wk of age and 250–300 g weight (Harlan Sprague Dawley, Indianapolis, IN) were maintained under 14:10-h light-dark cycles. Animals were fed with standard pelleted Rodent Chow 5001 (PMI Feed, Richmond, VA) and allowed food ad libitum. C57BL/6j mouse recipients of hepatitis B virus surface antigen (HBsAg)-expressing transgenic hepatocytes (18–22, 45). We found that partial hepatectomy caused unexpected alterations in the host liver, including attenuation of proliferative potential, activation of senescence-associated β-galactosidase (SABG) (10), p21 expression (11), and apoptosis in hepatocyte subpopulations. This resulted in proliferation of transplanted hepatocytes in animals only when cells were transplanted subsequent to partial hepatectomy.

The Animal Care and Use Committee at Albert Einstein College of Medicine approved animal protocols.

To measure hepatocellular DNA synthesis, rats were injected intraperitoneally with 0.5 μCi/g body wt [3H]thymidine (70 Ci/mmol, ICN Radiochemicals, Irvine, CA) 1 h before killing the animals. The tissues were fixed in formaldehyde and embedded in paraffin. Sections were deparaffinized in xylene and subjected to autoradiography using NTB 2 emulsion (Eastman Kodak, Rochester, NY) with a 3-wk exposure at 4°C. Alternatively, animals were given 50 mg/kg bromodeoxyuridine (BrdU) (Boehringer Mannheim, Indianapolis, IN) for 2 h before obtaining tissues. BrdU incorporation was localized in cryostat sections with immunostaining using a commercial available antibody system (Amersham, North Chicago, IL). After tissues were blocked with 2% rabbit serum, antibody binding was detected with a supersensitive multilink antibody system using the peroxidase reporter (BioGenex Laboratories, San Ramon, CA), followed by color development with a Vectastain kit (Vector Laboratories, Burlingame, CA).

To localize transplanted cells, cryostat tissue sections were analyzed with histochemical staining for DPP IV and ATPase activities as described previously (45). To demonstrate DNA synthesis in transplanted hepatocytes, sections were stained first for DPP IV activity and then for BrdU incorporation, as described previously (20). The number of cells per portal area was determined in individual tissue sections for quantitative morphometry. A minimum of 50 portal areas was analyzed in each tissue. For additional analysis, changes in cluster sizes were determined by analyzing a minimum of 250 consecutive areas with transplanted hepatocytes in each tissue. For this purpose, transplanted cells in consecutive areas were scored for the number of cells per cluster as described.

To demonstrate whether preexisting polyploid nuclei rapidly underwent “cold mitoses” before the onset of DNA synthesis, 2-h pulses of colchicine (0.5 μg/kg body wt) (Sigma Chemical, St. Louis, MO) were administered intraperitoneally to three rats immediately after two-thirds partial hepatectomy. The animals were killed, and tissues were either frozen in methyl butane cooled to −70°C for cryostat sections or fixed in Formalin for routine histology.

Cell isolation. Cells were isolated by in situ perfusion of the liver via the portal vein with collagenase as previously described (45, 50). The liver cells were dispersed, filtered through an 85-μm tissue collector, and centrifuged for 5 min under 400 g at 4°C. To correlate morphometric and flow cytometric findings, tissue samples were obtained sequentially from representative animals at the time of partial hepatectomy, as well as immediately before cell isolation. For the latter, a small biopsy was obtained from the right posterior lobe immediately before liver perfusion.

Mitogenic responsiveness of cultured cells. Hepatocytes were isolated by collagenase perfusion from rats after 5 days and 1 mo of partial hepatectomy or sham-laparotomy (45). Cells were passed through an 80-μm Dacron mesh and purified by centrifugation twice at 50 g for 1 min each. After resuspension in RPMI 1640 culture medium (GIBCO, Grand Island, NY), cells were incubated 2 h at 37°C with or without 5 × 10^−8 M triiodothyronine (T3).

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The significance of hepatic polyploidy induced by partial hepatectomy has been unclear. We hypothesized that use of transplanted hepatocyte reporters will facilitate analysis of cellular changes after partial hepatectomy. Although in vivo studies with autoradiographic grain counts to analyze incorporation of radio-labeled nucleotides have been useful for analyzing DNA synthesis in tissues, such methods impose limitations in documenting differences in mitogenic activity of individual cells, as has been reviewed extensively (1).

In studies here, we specifically wished to analyze the effect of partial hepatectomy-induced polyploidy upon proliferation of transplanted cells. The expectation was that transplanted cells will proliferate after creation of a favorable microenvironment by partial hepatectomy. To test our hypothesis, we utilized our well-characterized transplantation systems in dipeptidyl peptidase IV (DPP IV)-deficient Fischer 344 (F344) rats and congenic mouse recipients of hepatitis B virus surface antigen (HBsAg)-expressing transgenic hepatocytes (18–22, 45). We found that partial hepatectomy caused unexpected alterations in the host liver, including attenuation of proliferative potential, activation of senescence-associated β-galactosidase (SABG) (10), p21 expression (11), and apoptosis in hepatocyte subpopulations. This resulted in proliferation of transplanted hepatocytes in animals only when cells were transplanted subsequent to partial hepatectomy.

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Island, NY) and cell viability determination by trypan blue dye exclusion, hepatocytes were plated at 3 x 10^4 cells/cm^2 in dishes coated with rat tail collagen. The medium was supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT). Cells were stimulated with 20 ng/ml human hepatocyte growth factor (HGF, Genentech, San Francisco, CA). After 47 h of culture, [3H]thymidine (3 µCi, 70 Ci/mmol, ICN Radiochemicals) was added for 1 h. After washing twice with cold PBS, cells were lysed with sodium hydroxide, and DNA was precipitated as described (19). Aliquots of the redissolved DNA were subjected to either liquid scintillation counting for 3H activity or microquantitation of DNA by a fluorimetric assay. All experiments were done in at least triplicate.

Flow cytometry and cell sorting. Isolated cells were immunostained to identify contaminating nonhepatocyte cell types for 40 min at 4°C with mouse monoclonal antibody (MAb) OX-43 (15 µg/ml; MCA 276), MAb OX-44 (18 µg/ml; MCA 371), and MAb OX-1 (72 µg/ml; MCA 43) (all from Serotec, Indianapolis, IN) in 0.1% BSA (50). MAb OX-43 reacts with an antigen expressed by macrophages, endothelial cells, and red blood cells. MAb OX-44 reacts with cell membrane glycoprotein CD53 present on all rat myeloid and peripheral lymphoid cells and is related to the human CD37 leukocyte antigen, and MAb OX-1 was derived from rat thymocyte membrane glycoproteins and recognizes an antigen shared by all rat leukocytes. After being washed to remove excess antibody, cells were incubated for 40 min at 4°C with a heavy chain specific FITC-conjugated anti-mouse IgG (Southern Biotech, Birmingham, AL). Negative controls were stained with only the FITC-conjugated anti-mouse IgG.

A FACSTAR plus instrument equipped with a 100-µm nozzle (Becton Dickinson, San Jose, CA) was used for flow cytometry in the Cancer Research Center of the Albert Einstein College of Medicine. Fluorescence excitation at 488 nm was measured through a 530-nm FITC filter. Linear amplification was used for forward scatter, a measure of cell size, and four-decade logarithmic amplification for side scatter, a measure of cytoplasmic complexity. The instrument was calibrated by mechanical alignment of the optical bench at fixed amplifier and photomultiplier voltages so that measurements of fluorescent polystyrene beads (Fluoresbrite beads, 2.02 µm, Polysciences, Warrington, PA) fell in the same peak channels. For all analyses, propidium iodide (PI, 50 µg/ml stock solution) was added to cells, and only viable cells excluding the dye were analyzed and sorted. Cells were maintained at 4°C and sorted using Hanks’ balanced salt solution as sheath fluid. For each analysis, at least 10,000 events were collected, and data were analyzed with the Lysis II software.

To analyze cellular DNA content, highly granular and autofluorescent liver cells were sorted and centrifuged under 400 g for 5 min at 4°C. Nuclei were isolated from sorted cells with a detergent-trypsin method, stained with PI, and analyzed by flow cytometry as above (56). Pulse processing utilizing integrated areas vs. width of the DNA fluorescence pulses was used, and aggregates were excluded from the analysis. The cell cycle state was determined with the Verity: Modfit cell cycle analysis software (Verity Software, Topsham, ME). Nuclei from peripherally circulating rat lymphocytes were purified with Ficoll gradients (Pharmacia, Uppsala, Sweden) and included in each experiment as diploid DNA standards.

In situ demonstration of apoptosis. Cryostat tissue sections of 5-µm thickness were analyzed with a commercial assay as recommended by the manufacturer (In Situ Cell Death Detection, POD; Boehringer Mannheim). The assay is based on identification of DNA strand breaks that occur during apoptosis by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction utilizing terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay (13, 28).

In situ localization of SABG activity. Cryostat liver sections were fixed at room temperature for 10 min in 0.5% glutaraldehyde in PBS. The sections were washed and incubated at 37°C with the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl β-galactoside in either PBS or citric acid/sodium phosphate buffer, pH 6.0, according to Dimri et al. (10). Tissue sections were examined after incubation for up to 18 h and counterstaining with hematoxylin and eosin.

Immunostaining for p21 and p16. After cryostat tissue sections were fixed for 10 min in cold acetone, endogenous peroxidase activity was quenched with 1% hydrogen peroxide in PBS for 10 min. Tissues were then incubated with 1.5% rabbit serum in PBS at room temperature for 1 h. This was followed by incubation for 30 min at room temperature with commercially available anti-p21 and anti-p16 at 1 µg/ml (clones F-5 and F-12, respectively, Santa Cruz Biotechnology). After the tissues were washed with PBS, antibody binding was localized with a supersensitive multilink antibody system using the peroxidase reporter (BioGenex Laboratories).

Morphometric analysis. Six-micrometer-thick cryostat sections were prepared from liver tissues. To stain DNA with Feulgen (DNA Staining Kit, CAS, Elmhurst, NY), sections were placed in 5 N HCl for 60 min followed by the CAS staining solution for 60 min. Tissue sections were first washed with the rinsing solution provided and then with deionized water, placed in 1% HCl-alcohol for 5 min, dehydrated, and mounted in a permanent medium. Cellular DNA content was determined with the CAS 200 image analysis system and accompanying software after calibrating the system with a control slide of rat hepatocytes. The interactive CAS image analysis is based on the principle that the optical density of each nucleus is directly proportional to the DNA content (54). For morphometric analysis, 500 hepatocyte nuclei per sample were randomly chosen for DNA content analysis and DNA histograms generated.

Serological assays. Blood was sampled at regular intervals by cutting the tail of animals, and sera were stored at −20°C for analysis. Serum HBsAg was measured with a commercially available RIA (AUSRIA II, Abbott Laboratories, North Chicago, IL) as described previously (17, 20). Baseline serum HBsAg levels were used to normalize HBsAg levels in individual animals.

Statistical analysis. The data were analyzed with the SigmaStat software (Jandel Scientific, San Rafael, CA) and are expressed as means ± SE. The significance of differences was analyzed by the Student’s t-test for normally distributed data and by the Mann-Whitney correlation tests for nonparametrically distributed data. A P value <0.05 was considered statistically significant.

RESULTS

Pattern of liver regeneration after partial hepatectomy. Autoradiographic analysis of [3H]thymidine incorporation showed a remarkable increase in labeling index within 24 h after partial hepatectomy, similar to previous experience (Fig. 1). Whereas only one or two cells incorporated [3H]thymidine in control animals, the fraction of [3H]thymidine-labeled cells 24 h after partial hepatectomy reached 30 ± 5%. [3H]thymidine
that serum HBsAg levels did not increase in this situation and indeed became lower than baseline levels (Fig. 2). This rather unexpected decline in serum HBsAg levels persisted throughout the duration of the experiment and became even more pronounced at late time points. The implications were that there was no increase in the transplanted hepatocyte mass after partial hepatectomy, similar to studies in DPPIV-deficient rats.

In another series of experiments, F344 rat hepatocytes were transplanted via spleen into either DPPIV-deficient control rats \( (n = 3; 2 \times 10^7\) cells each) or DPPIV-deficient rats immediately after two-thirds partial hepatectomy \( (n = 3; 0.66 \times 10^7\) cells each; cell numbers normalized for remnant liver mass). To establish the kinetics of cell proliferation after partial hepatectomy, additional animals were studied at 1, 2, 3, 5, and 7 days after partial hepatectomy and cell transplantation, along with a pulse of BrdU before killing the animals. There was extensive BrdU incorporation by host hepatocytes at 24 and 48 h after partial hepatectomy and cell transplantation, similar to thymidine incorporation shown in Fig. 1. However, there was no BrdU incorporation in transplanted cells at these times. BrdU incorporation was delayed in transplanted cells and was observed between days 3 and 5 after cell transplantation, when DNA synthesis was essentially over in host hepatocytes. BrdU incorporation in hepatocytes was associated with increased number of transplanted hepatocytes, albeit subsequent to the first week of cell transplantation. At 14 days, the number of transplanted cells increased from 4 \( \pm 5\) cells in control nonpartially hepatectomized rats to 9 \( \pm 6\) cells/portal area, along with larger transplanted cell clusters in partially hepatectomized rats compared with control animals \( (P < 0.05, t\)-test; Fig. 3, A and B). We interpreted these findings to indicate disproportionately

Fig. 2. Effect of partial hepatectomy on preexisting transplanted hepatocyte mass. Transgenic G26 HBV hepatocytes were transplanted into congenic C57BL/6J mice. Serum HBsAg was measured during a 4-wk baseline period followed by partial hepatectomy in six mice. The animals were then followed for another 6 wk with additional blood sampling. The data showed that serum HBsAg levels did not increase in this situation and indeed became lower than baseline levels (Fig. 2). This rather unexpected decline in serum HBsAg levels persisted throughout the duration of the experiment and became even more pronounced at late time points. The implications were that there was no increase in the transplanted hepatocyte mass after partial hepatectomy, similar to studies in DPPIV-deficient rats.
increased rates of proliferation in transplanted cells, which were derived from the normal liver without partial hepatectomy compared with host hepatocytes that had experienced partial hepatectomy.

To further examine this issue and to exclude potential confounding factors arising from complex initial changes occurring immediately after partial hepatectomy, we performed another experiment in DPPIV-deficient rats by transplanting $5 \times 10^7$ hepatocytes 4 days after partial hepatectomy, since hepatic DNA synthesis is virtually completed by this time. The differences in cluster sizes of transplanted hepatocytes were determined 1 and 12 wk after partial hepatectomy. Transplanted cells again formed larger clusters in partially hepatectomized animals at 12 wk compared with at 1 wk ($P < 0.05$, $\chi^2$ test; Fig. 3, C and D), in agreement again with discordant rates of cell proliferation compared with host hepatocytes. Such a difference in transplanted cell numbers was not observed in control animals with no partial hepatectomy.

Partial hepatectomy induced cellular changes. In studies conducted in parallel with cell transplantation analysis, we first examined whether hepatocytes with flow cytometric characteristics of fetal cells (lower cytoplasmic granularity and autofluorescence) appeared in the liver after partial hepatectomy (50). Liver cells were isolated from control animals ($n = 3$) or animals 24 h ($n = 3$), 5 days ($n = 4$), or 30 days ($n = 3$)
after two-thirds partial hepatectomy and subjected to flow cytometry (Fig. 4). Parenchymal cells were grouped into discrete populations: RA1 cells with a more differentiated phenotype and RA2 cells, which represented a less mature phenotype, as described previously (50). At 24 h after partial hepatectomy, greater accumulation of RA1 cells was apparent. Although this was most obvious at 24 h after partial hepatectomy, RA1 cells also accumulated at 5 or 30 days after partial hepatectomy. Data from two experiments showed that cytoplasmic granularity increased from 4.2 or 4.3 arbitrary geometric mean units (AU) in control rats ($n = 2$) to 25 AU in rats $24$ h after partial hepatectomy. Similarly, cellular autofluorescence increased from 80 and 96 AU in control rats to 198 and 190 AU in rats at 24 h and to 180 and 170 AU in rats at 5 days after partial hepatectomy ($< 0.05$, $\chi^2$ test). In cells isolated from rat livers after 30 days of partial hepatectomy, a granular pattern of cytoplasmic granularity and autofluorescence continued to be evident. Alterations in cytoplasmic granularity, autofluorescence, and polyploidy in hepatocytes after partial hepatectomy resembled maturational changes in the normal adult liver and were quite distinct from fetal hepatoblasts (50).

To determine whether flow cytometric evidence of maturation in hepatocytes was compatible with other evidences associated with cell aging, tissues were stained histochemically for SABG activity at reaction pH 6.0 (10). In the normal rat liver, only occasional cells were positive for SABG activity. However, in the liver from rats 5 days after two-thirds partial hepatectomy, up to 8–12 cells/high-power field showed diffuse cytoplasmic staining for SABG activity ($< 0.001$, Mann-Whitney rank correlation). In contrast, no $\beta$-galactosidase activity was visualized when tissues were stained at a pH of 7.4. Increased SABG in hepatocytes after partial hepatectomy was in agreement with the induction of cell-aging events and substantiated flow cytometry observations.

Ploidy of liver cells advanced after partial hepatectomy. To determine changes in ploidy, we isolated nuclei after sorting highly granular and autofluorescent RA1 parenchymal liver cells. In control rats, isolated hepatocyte nuclei contained predominantly tetraploid and less frequently diploid DNA (Fig. 5 and Table 1). Nuclei with higher ploidies, such as octaploid DNA content, were rare. At 24 h after partial hepatectomy, active DNA synthesis was observed in both diploid and tetraploid nuclear fractions. To document the possibility...
that polyploid hepatocytes immediately underwent mitosis without prior DNA synthesis (9), we administered colchicine to rats immediately after either two-thirds partial hepatectomy (n = 4) or sham laparotomy (n = 2). Neither control nor partially hepatectomized animals showed increased mitosis at 2- or 5-h time points.

The cessation of DNA synthetic activity in hepatocytes by 5 days was in agreement with the well-established kinetics of DNA synthesis after partial hepatectomy (Table 1) (6, 26, 36). However, the relative proportion of diploid nuclei in hepatocytes declined within 24 h after partial hepatectomy and remained so subsequently at 5 days, as well as 30 days after partial hepatectomy (n = 3). At 5 days after partial hepatectomy, hepatocyte nuclei shifted to higher ploidy classes, with significantly fewer diploid and greater tetraploid or octaploid nuclei in comparison with normal rats. The shift of hepatocytes after partial hepatectomy to greater ploidy, along with decline in diploid DNA content, suggested that diploid or tetraploid cells advanced to tetraploid and octaploid ones, respectively. This interpretation was supported by the evidence for DNA synthesis in both diploid and tetraploid nuclear fractions, as well as accumulations of octaploid nuclei at 24 h after partial hepatectomy. However, the octaploid nuclear fraction was smaller in animals at 5 days after partial hepatectomy compared with at 24 h after partial hepatectomy. This finding indicated to us that a number of cells with advanced ploidy were lost after 24 h of partial hepatectomy. Such depletion could have occurred by either death of polyploid hepatocytes or generation of daughter cells with lower ploidy. The latter should have resulted in amplification, or at least maintenance to original levels, of diploid and/or tetraploid peaks, which was, however, not observed.

To document that nuclear DNA content in our samples correlated with nuclear size, we compared the diameter of hepatocyte nuclei obtained by forward scatter on flow cytometry with the nuclear DNA content. A positive correlation was present between nuclear size and ploidy level. The nuclear size shifted to higher classes after 24 h and 5 days of partial hepatectomy, which was again in agreement with data obtained by nuclear DNA analysis.

Partial hepatectomy increased hepatocyte losses. The change in the size of hepatocyte nuclei was reflected in tissue sections obtained from individual animals before and after partial hepatectomy (Fig. 6). Although hepatocyte nuclei in normal rats were of a uniform size in a given part of the liver lobule, hepatocytes exhibited significant nuclear pleomorphism, corresponding to increased ploidy in animals after either 5 or 30 days of partial hepatectomy. Furthermore, there was morphological evidence for apoptosis in the liver with visualization of scattered apoptotic bodies after partial hepatectomy. In situ assays to demonstrate DNA fragmentation in nuclei verified ongoing apoptosis in remnant hepatocytes (Fig. 7), a significant process implying losses of some hepatocytes, presumably those with most advanced ploidy or maturation.

### Table 1. DNA content in sorted RAT hepatocyte nuclei after two-thirds partial hepatectomy

<table>
<thead>
<tr>
<th></th>
<th>Controls, %total</th>
<th>Partial Hepatectomy, %total</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>5 days</td>
</tr>
<tr>
<td>Total diploid</td>
<td>0.3 ± 0.2</td>
<td>19.1 ± 2.1*</td>
</tr>
<tr>
<td>Diploid G0-late G1</td>
<td>29.9 ± 0.2</td>
<td>15.6 ± 2.4*</td>
</tr>
<tr>
<td>Diploid S</td>
<td>0.3 ± 0.1</td>
<td>3.5 ± 0.9*</td>
</tr>
<tr>
<td>Total tetraploid</td>
<td>0.6 ± 0.5</td>
<td>80.9 ± 2.1*</td>
</tr>
<tr>
<td>Greater ploidies</td>
<td>68.3 ± 0.9</td>
<td>48.3 ± 3.4*</td>
</tr>
<tr>
<td>Tetraploid G0-late G1</td>
<td>68.3 ± 0.9</td>
<td>48.3 ± 3.4*</td>
</tr>
<tr>
<td>Tetraploid S</td>
<td>0.4 ± 0.1</td>
<td>8.3 ± 2.6*</td>
</tr>
<tr>
<td>Tetraploid G2/M</td>
<td>1.1 ± 0.7</td>
<td>24.3 ± 2.1*</td>
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<tr>
<td>Plus greater</td>
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<tr>
<td>ploidies</td>
<td>0.0 0.1± 0.2</td>
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Values are means ± SE from 2 or 3 Fischer 344 rats each. *P < 0.05 vs. controls (χ² test).

**Fig. 5.** Analysis of ploidy in hepatocyte cell nuclei by flow cytometry. A: hepatocytes from control animals showed major peaks of diploid and tetraploid DNA content. B: within 24 h of partial hepatectomy, proportion of diploid nuclei declined and that of nuclei containing octaploid or greater amount of DNA increased. C: this change persisted at both 5 (shown here) and 30 days after partial hepatectomy, although accumulation of cells with octaploid or greater DNA content was relatively less prominent compared with B. Analysis was performed on RAT cell fractions indicated in Fig. 4. Rapid decrease in fraction of cells showing advanced polyploidy indicates early losses of these cells (between 24 h and 5 days after partial hepatectomy).
Further evidence for partial hepatectomy-induced attenuation of proliferative capacity in hepatocytes. To determine the proliferative capacity of hepatocytes isolated from normal rats (n = 2) or rats subjected to two-thirds partial hepatectomy (n = 4), we analyzed growth factor-induced DNA synthesis. In parenchymal cells from control rats, exposure to HGF increased DNA synthesis by 5- to 20-fold, whereas in hepatocytes isolated from rats after either 5 or 30 days of partial hepatectomy, the replicative capacity was significantly attenuated and DNA synthesis increased by only 2- to 4-fold (P < 0.05; Fig. 8). Analysis of liver biopsies from these animals with Feulgen staining before and after two-thirds partial hepatectomy also verified that parenchymal cell ploidy had shifted to higher classes after partial hepatectomy (P < 0.05, χ² test).

Partial hepatectomy induced p21 but not p16 expression in hepatocytes. We documented whether increased hepatocyte ploidy was associated with changes in cell cycle regulated genes, such as p16 and p21, overexpression of which is known to alter cell cycle progression (11, 44). In the normal rat liver, p21 immunostaining was exhibited by only rare hepatocytes (Fig. 9). At 30 h after two-thirds partial hepatectomy, which is associated with significant hepatic DNA synthesis, only occasional hepatocytes showed p21 activity. In contrast, at 5 days after partial hepatectomy, there was considerable p21 expression in hepatocytes, although p21 expression did not show any zonal preference in the liver lobule. The findings indicated that p21 could be involved in regulating cell cycling after partial hepatectomy. In contrast, immunostaining of tissues did not show p16 expression in either normal liver or liver subjected to partial hepatectomy.

DISCUSSION

These findings show remarkable differences in the proliferative activity of host and transplanted hepatocytes in the context of partial hepatectomy. The number of transplanted hepatocytes was not increased when partial hepatectomy was performed subsequent to cell transplantation and engraftment of transplanted cells, which requires several days (22), whereas transplanted cell numbers increased when partial hepatectomy preceded cell transplantation. Studies in rats and mice provided similar results, with a decrease in serum HBsAg levels in hepatocyte recipients after subsequent partial hepatectomy. Serum HBsAg levels in the transgenic transplantation system used correlate with the mass of transplanted hepatocytes (17, 20). We interpret the data to indicate that in the former condition transplanted hepatocytes were exposed to the same processes that influenced host hepatocytes with an inability to divide. In contrast, in the latter situation, transplanted hepatocytes were spared from the changes affecting host hepatocytes, and thus cells could proliferate significantly. We found that partial hepatectomy induced multiple changes, including polyploidy, SABG and p21 expression, as well as apoptosis in hepatocytes. Moreover, we found that mitogenic responses were attenuated in hepatocytes isolated from the partially hepatectomized liver. It is noteworthy that in previous studies concerning proliferation of transplanted hepatocytes, infusion of HGF was ineffective, despite significant DNA synthesis in the liver, whereas ablation of host hepatocytes, such as with carbon tetrachloride (CCl₄) was effective (20).

Increased proliferation of transplanted cells followed induction of apoptosis and other changes in the host liver after partial hepatectomy, which are associated with cell aging (polyploidy, increased cellular autofluorescence and cytoplasmic complexity, SABG and p21 expression). Although the transplanted hepatocyte number increased modestly, indicating limited rounds of cell division in the study period (possibly 2–3), the implications of this change will probably be broad. At 5 days after partial hepatectomy, shift of hepatocytes to greater ploidy, along with decline in diploid DNA content, suggested that nuclei with diploid or tetraploid DNA converted to tetraploid and octaploid ones, respectively. This interpretation was supported by observa-
tions of DNA synthesis in both diploid and tetraploid nuclear fractions, as well as by the accumulation of octaploid or more DNA-containing nuclei at 5 days after partial hepatectomy. In the normally aging liver, cells accumulate lipofuscin, which contributes to cytoplasmic autofluorescence (48). Lysosomal accumulation of lipofuscin is a well-recognized feature of postmitotic cells undergoing aging-associated oxidative damage (24). Although morphometric analysis of nuclear size yielded indirect evidence of increasing hepatic ploidy with aging and after partial hepatectomy (1, 5), the biological significance of this finding was unclear previously.

Recently, it was proposed that mature hepatocytes may dedifferentiate and acquire characteristics of the fetal phenotype through cold mitoses to diploid DNA states (9). However, we were unable to identify such a process after partial hepatectomy. In contrast, although tetraploid DNA peaks were restored after partial hepatectomy, diploid DNA peaks were not restored to control levels, indicating an advance in the ploidy of some, but not all, diploid hepatocytes. Intriguingly, in studies described by Brodsky and Uryvaeva (5), when CCl4 was administered to animals 2 days after partial hepatectomy, no hepatic ablation was observed, whereas this was not so either 1 day after or subsequent to 2 days after partial hepatectomy. This absence of CCl4-induced hepatotoxicity at 2 days after partial hepatectomy would be in agreement with the early loss of polyploid cells capable of metabolizing this toxin, similar to our findings. Changes in cytochrome P-450 expression related to liver regeneration could be invoked as an alternative mechanism to account for decreased toxicity from CCl4; however, CCl4 was effective at 1 day after partial hepatectomy, which represents the peak of DNA synthesis phase. The possibility of preferential or early loss of hepatocytes with the most advanced polyploidy is supported by direct evidence, with recent time-lapse observations showing apoptosis in cultured cells undergoing polyploidy (12).

Fig. 7. Analysis of apoptosis in liver with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. A: liver removed at time of partial hepatectomy showed apoptosis in only an occasional cell (arrowhead). B: liver at 5 days after partial hepatectomy showed far more apoptotic nuclei (arrowheads). P = Portal area. C: DNA fragmentation in "TUNEL-positive" hepatocyte nuclei in partially hepatectomized liver. Arrows show cells undergoing apoptosis. D: negative control liver in which terminal deoxynucleotidyl transferase was omitted from reaction with no staining. In 35 consecutive high-power fields (HPF) (magnification, ×200), overall rate of apoptosis was increased by 2.6-fold at 5 days after partial hepatectomy (apoptosis in tissue removed at partial hepatectomy, 1.2 ± 1.1 vs. 3.1 ± 1.9 cells/HPF; in tissue from same animal after partial hepatectomy; P < 0.001, paired t-test). Magnification: A, B, and D, ×200; C, ×1,000.
Although the genetic mechanisms regulating polyplody have not been defined, p21 overexpression in hepatocytes after partial hepatectomy may offer a regulatory paradigm for this process. We did not find evidence for activation of p16-related mechanisms (44). Originally identified as a senescence factor, p21 regulates cell cycle progression through complex mechanisms (11), including by association with cyclin-dependent kinases (CDK) and inhibition of DNA polymerase delta-dependent DNA replication. In transgenic mice with p21 overexpression, p21 complexes with cyclin D1 and CDK4, leading to inhibition of hepatocyte entry into S phase during postnatal growth and partial hepatectomy (58). Interestingly, the size of the liver lobule and hepatic mass are decreased in p21 transgenic mice. Hepatocytes became hyperploid, and there is marked proliferation of oval cells, which could be in agreement with the depletion of polyploid hepatocytes, especially because proliferating oval cells and rare nodular foci of regenerating hepatocytes may not express p21. Undoubtedly, the situation after two-thirds partial hepatectomy is complex, with release of myriad signals, such as abrupt changes in hepatic ploidy after partial hepatectomy were verified by Feulgen analysis of biopsies from individual donors.

Fig. 8. DNA synthesis rates were significantly attenuated in rat hepatocytes isolated after either 5 days or 1 mo of two-thirds partial hepatectomy (P < 0.05, t-test). Data are from cells isolated 1 mo after partial hepatectomy and exposed to human hepatocyte growth factor (HGF) for 48 h. Cells were not fractionated by flow cytometry or other methods and represent a mixed population of hepatocytes. Changes in hepatic ploidy after partial hepatectomy were verified by Feulgen analysis of biopsies from individual donors.

Fig. 9. Hepatic p21 expression after two-thirds partial hepatectomy. A: F344 rat liver removed at time of partial hepatectomy showing only rare cells with p21 expression (arrows in A, B, and C). B: p21 expression at 30 h after partial hepatectomy in scattered groups of hepatocytes. C: at 5 days after partial hepatectomy, p21 expression became quite extensive and was apparent in a large number of hepatocytes. D: a negative control in which anti-p21 antibody was omitted showing no staining. Magnification, ×400.
perfusion and circulating levels of various hormones, growth factors, and cytokines (36). Changes in extracellular matrix components, cell signaling, ambient physical conditions, or other factors, may regulate cellular gene expression and differentiation in portions of the liver lobule (51). Hepatic polyploidy and apoptosis in transforming growth factor-α (TGF-α) transgenic mice are in agreement with the potential involvement of soluble signals in the regulation of hepatocyte turnover (57). Similarly, catecholamines induce polyploidy in cultured hepatocytes, which resembles responses in cultured fibroblasts and smooth muscle cells (7, 15, 32, 35, 43). After two-thirds partial hepatectomy, serum catecholamine, as well as TGF-α levels are known to increase (36). How these extracellular factors might affect p21 and other cell cycle regulatory molecules will require further analysis. Nevertheless, the possibility of impaired cytkinetic ability in polyploid cells is in agreement with the observations of repeated S phase transitions without intervening mitosis or cytokinesis in other somatic mammalian cells (2, 23). We believe that p21 overexpression after partial hepatectomy is in agreement with roles for p21 in G2/M arrest, polyplidization, and terminal differentiation, similar to other systems (11, 58).

The attenuated proliferative capacity in growth factor-stimulated hepatocytes after partial hepatectomy was in agreement with observations in cell types undergoing senescence (7). Previously, evidence has been provided for hepatocytes undergoing hypertrophy and polyploidization in culture and for decreased proliferative capacity in polyploid hepatocytes (15, 35). Age dependency of proliferative capacity is further reinforced by studies with fetal, neonatal, or “small” diploid adult hepatocytes (38). This reduced regenerative potential with cell aging might contribute to impaired recovery after partial hepatectomy and severe hepatitis in older subjects observed clinically, as well as experimentally (3, 6, 42). Similarly, orthotopic liver transplantation from older donors is associated with inferior outcomes (33). Nonetheless, our findings are not in conflict with previous studies showing the capacity of the liver to regenerate after repeated partial hepatectomies (27, 52, 53). We interpret the data to indicate that many, albeit not all, hepatocytes exhibit attenuated proliferative capacity and life span after partial hepatectomy. It is likely that epithelial renewal would involve replacement of polyploid cells with other cells. On the other hand, recurrent exposure to polyploidizing events should amplify this process. Indeed, repeated partial hepatectomies seem to amplify polyploid change in the liver (53). Also, although partial hepatectomy leads to DNA synthesis in most hepatocytes, cells of higher ploidy classes incorporate DNA less avidly or with slower kinetics (5), similar to our findings concerning HGF-induced DNA synthesis in cultured cells. Although adult mouse hepatocytes, as well as rat hepatocytes, have been shown to be capable of extensive proliferation when host hepatocytes are depleted significantly (20, 37, 41, 47), it is not possible to determine whether polyploid cells could have repopulated the liver in these settings. We believe that further analysis of this issue can be addressed directly by isolating polyploid cells, as shown recently (46), followed by cell transplantation in suitable hosts to document their proliferative potential and fate.

The findings have implications in respect with the analysis of liver regeneration using the two-thirds partial hepatectomy model. Our studies imply that hepatocytes in the liver remnant have suffered from signals directing advance along differentiation pathways. Therefore, in studies concerning analysis of liver regeneration, changes in DNA synthesis rates should be coupled with the analysis of changes in hepatocyte numbers. Use of genetically marked reporter hepatocytes, as shown here, will be an appropriate strategy for this analysis. Our findings will also help interpret how partial hepatectomy can facilitate induction of hepatic oncogenesis in combination with specific drugs or chemical toxins. In these situations, partial hepatectomy would serve synergistic roles by inducing hepatocyte polyploidy and depletion of polyploid hepatocytes to allow emergence of oncogenic cell clones. Finally, partial hepatectomy in the setting of mito-inhibitory block of hepatocytes, such as with pyrrolizidine alkaloids or radiation, may help by augmenting depletion of host hepatocytes due to further polyploid change (18, 31), thereby creating conditions for transplanted cell proliferation and extensive liver repopulation.

We greatly appreciate the assistance of Rosina Passela, Dinish Williams, Pat Holst, as well as Drs. Sanjeev Slehria, K. Schlesinger, and J. J. Steinberg.

S. H. Sigal and P. Rajvanshi made equal contributions to this work. The studies were supported in part by the Irma T. Hirsch Trust and National Institutes of Health (NIH) Grants R01-DK-46952 (to S. Gupta), P30-DK-14296 (to Marion Bessin Liver Center), and P30-CA-13330 (to Albert Einstein Cancer Center). L. M. Reid was supported in part by American Cancer Society Grant BE-92C, National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1-DK-44266, the Council for Tobacco Research Grant 1897, NIH Center for Advanced Study in the Biological and Behavioral Sciences and The Arnold and Mabel Beckman Foundation (UNC) School of Medicine Grant DK-34987, Glaxo-Wellcome Pharmaceuticals, and the UNC School of Medicine. S. H. Sigal was a recipient of American Liver Foundation Fellowship Award. Additional support to L. M. Reid was from Renaissance Cell Technologies, North Carolina Biotechnology Center Grant, NIH Grant RO1-DK-52851, and Johns Hopkins Foundation. The flow cytometry studies were initiated by S. H. Sigal in L. M. Reid’s laboratory at Albert Einstein College of Medicine.

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Received 31 December 1997; accepted in final form 29 January 1999.

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