Liver from Bone Marrow in Humans

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It has been shown in animal models that hepatocytes and cholangiocytes can derive from bone marrow cells. We have investigated whether such a process occurs in humans. Archival autopsy and biopsy liver specimens were obtained from 2 female recipients of therapeutic bone marrow transplantations with male donors and from 4 male recipients of orthotopic liver transplantations from female donors. Immunohistochemical staining with monoclonal antibody CAM5.2, specific for cytokeratins 8, 18, and 19, gave typical strong staining of hepatocytes, cholangiocytes, and ductular reactions in all tissues, to the exclusion of all nonepithelial cells. Slides were systematically photographed and then restained by fluorescence *in situ* hybridization (FISH) for X and Y chromosomes. Using morphologic criteria, field-by-field comparison of the fluorescent images with the prior photomicrographs, and persistence of the diaminiobenzidene (DAB) stain through the FISH protease digestion, Y-positive hepatocytes and cholangiocytes could be identified in male control liver tissue and in all study specimens. Cell counts were adjusted based on the number of Y-positive cells in the male control liver to correct for partial sampling of nuclei in the 3-micron thin tissue sections. Adjusted Y-positive hepatocyte and cholangiocyte engraftment ranged from 4% to 43% and from 4% to 38%, respectively, in study specimens, with the peak values being found in a case of fibrosing cholestatic recurrent hepatitis C in one of the liver transplant recipients. We therefore show that in humans, hepatocytes and cholangiocytes can be derived from extrahepatic circulating stem cells, probably of bone marrow origin, and such ''transdifferentiation'' can replenish large numbers of hepatic parenchymal cells. (Hepatology 2000;32:11-16.)

The existence of a bipotent hepatic progenitor or "stem" cell, capable of regenerating both hepatocytes and cholangiocytes in response to injury, has long been investigated in animal models and there now appears to be consensus that such a cell population does exist.[1-8] These cells are often referred to as "oval cells," and their intrahepatic location is likely to be the canal of Hering.[9] Recent data from our laboratory and that of Petersen et al. showing that hepatocytes[10,11] and cholangiocytes[11] can derive from cells of bone marrow origin in mice and in rats indicates that there is also an extrahepatic source of these bipotent cells.

The existence of hepatic stem cells in humans is less well established, although several studies support the concept. Ductular reactions in a variety of acute and chronic liver diseases have cells with similar phenotypes to hepatic stem cells in animal models.[12-22] Small epithelial cells derived from diseased human livers express the "hematopoietic" markers c-kit and CD34 and have stem-cell–like properties in culture.[23,24] Also, three-dimensional analysis of the architecture of the canals of Hering in normal and acutely injured human livers indicates that some or all of the cytokeratin 19 (CK19)-positive small cholangiocytes lining these canals constitute a functional stem cell population.[25]

Following on the reports of bone marrow cells functioning as hepatic stem cells in rodents,[10,11] we have investigated whether this process also contributes to hepatic regeneration in humans. Biopsy and autopsy liver specimens from human recipients of therapeutic bone marrow or liver transplants, in which there was gender discordance between donor and recipient, were analyzed for marrow-derived hepatocytes and cholangiocytes. Liver specimens from male recipients of female livers and female recipients of male bone marrow were analyzed using fluorescence *in situ* hybridization (FISH) for the Y chromosome to indicate cells that were engrafted from a circulating, bone marrow–derived population of cells.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded archival liver tissues were obtained from 2 female patients who received therapeutic bone marrow transplants from a male donor and 4 male recipients of orthotopic liver transplants from female donors. Positive control for Y chromosome FISH was a normal liver biopsy from a 43-year-old male patient before beginning methotrexate treatment for psoriasis. Negative control tissue for Y chromosome FISH consisted of normal liver tissue obtained at autopsy from a 26-year-old woman who expired suddenly from pulmonary embolism of unknown cause.

The identification of cell-specific proteins while performing FISH is difficult because of the extensive protease digestion required for FISH, which obliterates antigenic sites needed for antibody binding. Submitting all 3-micron specimens to a 2-step analytic process solved this dilemma. First, immunoperoxidase staining using CAM5.2 monoclonal antibody, specific for shared epitopes of CK8, 18, and 19, was performed according to standard techniques (see below). CAM5.2 strongly labels hepatocytes and cholangiocytes while leaving other intrahepatic cell populations (stromal cells, Kupffer

Abbreviations: CK-19, cytokeratin 19; FISH, fluorescence *in situ* hybridization; PBS, phosphate-buffered saline; DAB, diaminiobenzidine; SSC, sodium saline citrate; FITC, fluorescein isothiocyanate; GVHD, graft-versus-host disease.

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Received May 16, 2000; accepted May 18, 2000.

Supported in part by a Primary Biliary Cirrhosis Seed Grant from the American Liver Foundation and an award from the Mary Lea Johnson Research Institute (N.D.T.) and by a grant from the National Institutes of Health (D.S.K.).

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0270-9139/00/3201-0003$3.00/0
doi:10.1053/jhep.2000.9124

cells, stellate cells, lymphocytes, and cells of other hematopoietic lineages) negative.[20,26] Counterstaining with hematoxylin was followed by coverslipping. The sections were then systematically and thoroughly color photographed at magnification ×20, with images alternately centered on terminal hepatic venules and portal tracts. These color images were printed as 5 × 7-inch hard copy images. The second step of analysis involved removal of the coverslips from the slides and FISH staining for Y chromosome (see below).

Immunoperoxidase Staining for CK8, 18, and 19. After preparation of tissue sections with xylenes followed by hydration with graded alcohols (100%, 90%, and 70% EtOH in H_2O, respectively), sections were washed with water and phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by application of 0.6% hydrogen peroxide for 8 minutes at room temperature, and the slides were washed in PBS again. Tissues were then digested with pepsin (Biogenex, San Ramon, CA) for 10 minutes at room temperature and washed in PBS. Blocking serum (bovine serum albumin) was applied to the slides, and then they were incubated for 30 minutes at 37°C with primary mouse monoclonal CAM5.2 antibody (Biogenex) at a dilution of 1:10. Incubation with secondary biotinylated goat-anti-mouse antibody (Vector Laboratories, Burlingame, CA) was performed after washing again for 30 minutes at 37°C followed by application of avidin-biotin complex for another half hour at room temperature. Diaminiobenzidene (DAB) was used as the colorizing agent, and slides were counterstained with Mayer's hematoxylin.

FISH for Y Chromosome. Slides were deparaffinized in xylene (2 times) for 10 minutes, dehydrated, and air-dried. Slides were then incubated in 1 mol/L sodium thiocyanate in distilled water at 80°C, washed with water, fixed with EtOH, and air dried. Tissue was digested with pepsin 4 mg/mL (Sigma, St. Louis, MO) in 0.9% NaCl, pH 1.5 for 10 minutes at room temperature, washed with water, and rinsed in 2× sodium saline citrate (SSC) for 2 minutes at room temperature. Slides were then denatured with 70% formamide in 2× SSC at 75°C for 3 to 5 minutes, and quenched with 70% EtOH for 3 minutes, then dehydrated and air dried. Probe for X and Y chromosomes (Vysis Inc, Downers Grove, IL) was denatured for 5 minutes at 75°C, added to the denatured tissue, coverslipped, and incubated in a humid chamber overnight at 37°C. Slides were then washed according to the protocol provided by Vysis Inc. Briefly, slides were washed in 0.4× SSC/0.3% Nonidet P-40, agitated for 2 minutes at 73°C, transferred to 2× SSC/0.1% Nonidet P-40 at room temperature for 1 minute, and then drained, counterstained with 4,6-diaminidino-2-phenylidole (DAPI), and mounted with Vectorshield (Vector Laboratories).

Tissue Analysis. Counting of Y-positive nuclei was accomplished by systematically examining the FISH-stained tissue, field by field, under ×60 magnification, using an Olympus Provis (Tokyo, Japan) microscope equipped with a cooled CCD camera (Quantix Corp., Cambridge, MA) and specialized software (PSI Inc, League City, TX). The rhodamine signal was excited at 568 nm and emission collected above 585 nm. Images were pseudocolored using image processing software (Adobe Photoshop, San Jose, CA). Autofluorescence and fluorescein isothiocyanate (FITC) were excited at 488 nm, and emission was collected above 515 nm.

Y chromosomes were identified as turquoise dots in nuclei, X chromosomes were identified as red dots. Sections through the nuclei could have one, the other, or both, and multiple copies of each were sometimes identified within a single cell, which is indicative of polyploidy. Hepatocyte cytoplasm autofluoresced green (bilirubin metabolites), red (protoporhphyrins), and yellow (bilirubin and lipofuscin). Nuclear zones were usually devoid of this autofluorescence. Background fluorescence of precipitated DAB, still adherent to the slide after FISH protease digestion, could be identified in all tissues examined as it is protease resistant.[27,28]

Fields were matched to corresponding photomicrographs of the CAM5.2 immunostained slides, relying on location of the tissue on the slide, identity of nuclear positioning, DAB stain, and typical hepatocyte autofluorescence, thereby identifying specific CAM5.2-labeled hepatocytes and cholangiocytes. Hepatocytes and cholangiocytes could be distinguished from each other by morphology, location (parenchymal vs. intraportal), and intensity/density of DAB staining (cholangiocytes greater than hepatocytes). Ductular reactions with cells of intermediate morphology, however, were identified in many livers with mild to severe hepatic disease.

Cell counts of Y-positive hepatocytes and cholangiocytes were obtained using only those cells reliably classified on the basis of the features noted above. Periportal regions were examined separately from pericentral areas, as recognized by comparison with the initial photomicrographs. Since nuclear sampling was incomplete in 3-micron thin sections, "correction factors" were obtained from counting of cells in the normal male tissue, expressed as the fraction of all hepatocyte and cholangiocyte nuclei, respectively, which were positive in the male positive control specimen.

RESULTS

Liver tissues were obtained from needle biopsy or from autopsy specimens. Two of the samples examined were from female recipients of male bone marrow transplants and 4 were from male recipients of livers from female donors. Clinical information regarding the recipients, the reason for sampling of liver tissue, and diagnoses pertaining to these specimens is summarized in Table 1. Using both cytogenetic and FISH analysis, bone marrow engraftment in the bone marrow transplant recipients was 100%.

As expected, the CAM5.2 monoclonal antibody labeled hepatocytes and cholangiocytes only, leaving Kupffer cells, endothelial cells, stromal cells, and blood cells negative (Figs. 1-3). Histologically, 3 liver specimens (Table 1; specimens 3, 4, and 6) from liver allografts showed features of extrahepatic large bile duct obstruction, with a periportal ductular reaction, portal edema, and focal bile duct dilatation, confirmed clinically. One liver allograft (specimen 5) showed features of

TABLE 1. Clinical Information Regarding Transplant Recipients

Specimen No.	Type of Specimen	Type of Transplant	Patient's Age at Transplant	Reason for Transplant	Time From Transplant to Liver Sampling	Liver Diagnoses
1	Autopsy	BMT	24	CML	13 mos	Hx of mild GVHD; cholestasis, no GVHD at death
2	Biopsy	BMT	23	CML	4.5 mos	Mild, nonspecific parenchymal inflammation
3	Biopsy	OLT	34	PSC	2 yrs	Features of biliary obstruction, possible recurrent primary sclerosing cholangitis, mild
4	Biopsy	OLT	56	A-1-AT def.	1.5 mos	Features of biliary obstruction, mild
5	Biopsy	OLT	52	Hepatitis C	4 mos	Fibrosing cholestatic recurrent hepatitis C
6	Biopsy	OLT	41	Hepatitis C	1 mo	Features of biliary obstruction, mild

Abbreviations: BMT, bone marrow transplantation; CML, chronic myelogenous leukemia; OLT, orthotopic liver transplantation; PSC, primary sclerosing cholangitis; A-1-AT, alpha-1 antitrypsin deficiency.

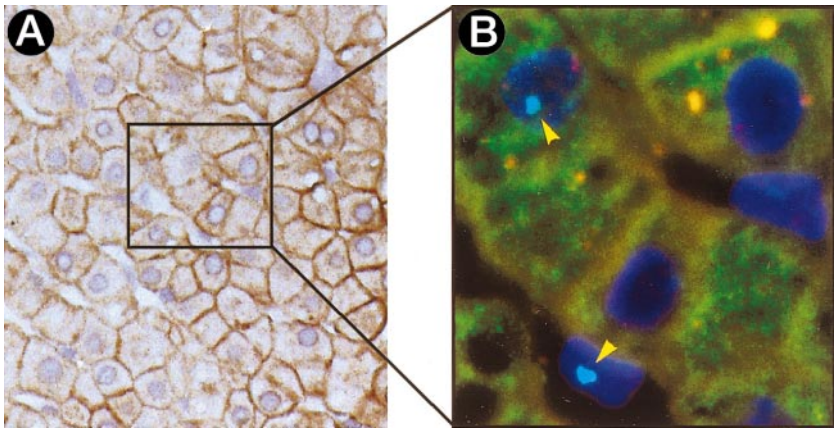

FIG. 1. (A) Immunohistochemical staining of normal male liver parenchyma with monoclonal antibody CAM5.2, specific for CK8, 18, and 19. Hepatocytes are positive with dim cytoplasmic and dark membranous staining. Sinusoidal cells, such as Kupffer cells, lymphocytes and other hematopoietic cells, endothelial cells, and stellate cells, are negative. (DAB, Mayer's hematoxylin counterstain; original magnification ×20). (B) FISH for centromeres of X (*red*) and Y (*turquoise*) chromosomes, with DAPI (*blue*) nuclear counterstain, of the same section of tissue in (A). Morphology, persistence of brown DAB stain indicating cytokeratin positivity, and cytoplasmic autofluorescence identify the hepatocytes. The hepatocyte in the upper left (*yellow arrow*) has X and Y chromosomes. A similarly labeled nucleus in the lower left (*yellow arrow*) is a sinusoidal cell of undetermined type. The absence of chromosomal staining in other nuclei is a result of partial sampling of nuclei in the 3-micron thin tissue section. (FITC, rhodamine, DAPI; original magnification ×100).

the fibrosing cholestatic variant of hepatitis C, characterized by marked portal expansion by fibrosis, an extensive ductular reaction, and cholestasis of the liver parenchyma with increased sinusoidal cellularity. No features of acute or chronic rejection were present in any of the allograft specimens. The livers of the 2 bone marrow transplant recipients showed mild, nonspecific changes: cholestasis in specimen 1 and a mild parenchymal and portal mononuclear infiltrate in specimen 2. Neither of these specimens showed features of graft-versus-host disease (GVHD), veno-occlusive disease, or recurrent neoplasia.

Marked protease digestion was required for full demonstration of Y chromosome centromeres. This yielded a blurring of nuclear contours and, where cell densities were particularly thick (inflammatory infiltrates, some ductular reactions), individual cell counts could not be reliably obtained. However, the digestion did not completely eliminate cytoplasmic features. Green (FITC filter) and red (Cy3.5 filter) autofluorescence caused by bilirubin and porphyrin metabolites allowed for visualization of hepatocyte contours. Moreover, the DAB, deposited where CK8, 18, and 19 had been present originally, also yielded a signal in the Cy3.5 and FITC filters, reinforcing the appearance of the contours of hepatocytes, cholangiocytes, and ductular reactions (Figs. 1-3). This effect of residual DAB on fluorescence has been reported previously.[27,28]

Results of the cell counts for Y-chromosome–positive hepatocytes, cholangiocytes, and ductular reactions are summarized in Table 2. Y-positive hepatocytes and cholangiocytes were consistently identified in the male control tissue, whereas none were identified in the female control tissue. Only some of the hepatocyte and cholangiocyte nuclei in the male liver tissue were positive due to partial sampling of nuclei in the 3-micron thin tissue sections. To adjust cell

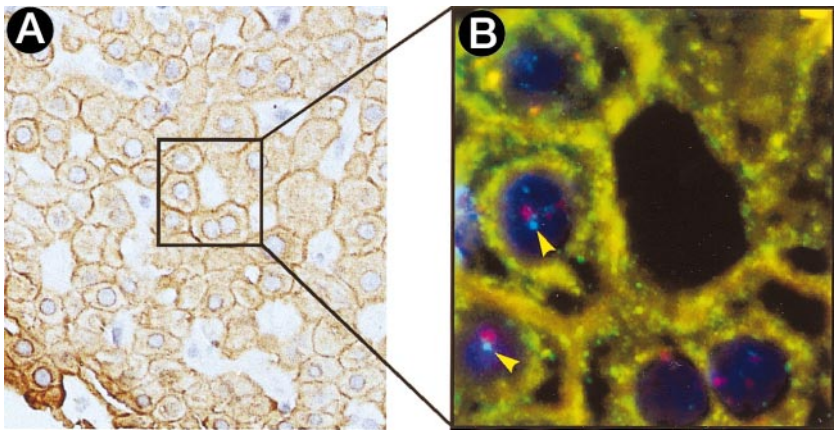

FIG. 2. (A) Immunohistochemical staining with CAM5.2 of a female liver allograft in a male recipient (specimen 5). This liver displayed features of the fibrosing cholestatic variant of recurrent hepatitis C, reflected here in mildly increased sinusoidal cellularity. (DAB, Mayer's hematoxylin counterstain; original magnification ×20). (B) FISH for X and Y chromosomes of the corresponding field in (A). Two hepatocytes (*yellow arrows*) contain Y chromosomes (*turquoise*) indicating origin from the recipient of the liver transplant. These cells were part of a small cluster of Y-positive hepatocytes in the periportal region. Because of partial nuclear sampling, the number of Y-positive cells in this area is probably underestimated. (FITC, rhodamine, DAPI counterstain; original magnification ×100).

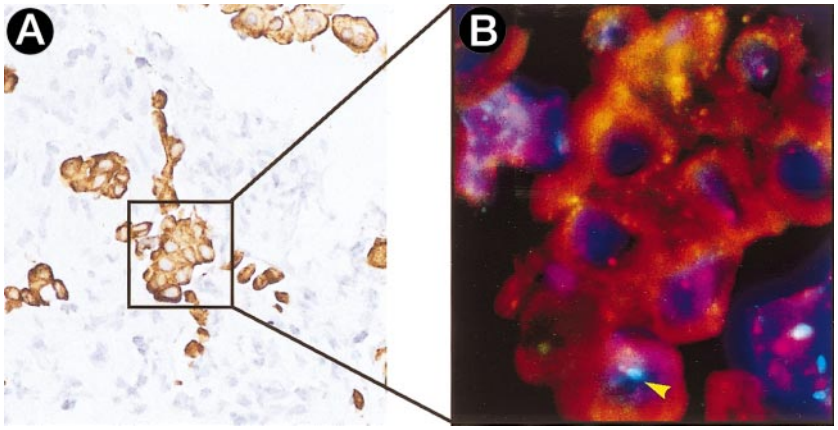

FIG. 3. (A) A region of the ductular reaction in liver specimen 5, with fibrosing cholestatic recurrent hepatitis C, immunostained with CAM5.2. The surrounding stroma of the portal tract is markedly expanded by fibrosis and infiltrated by a predominantly mononuclear cell infiltrate. The stromal and inflammatory cells do not stain for CAM5.2. Proliferating cholangiocytes of the ductular reaction show dense cytoplasmic staining for cytokeratins. (DAB, Mayer's hematoxylin counterstain; original magnification ×20. (B) FISH for X and Y chromosomes show X chromosomes (*red*) in many cholangiocyte nuclei, but the cholangiocyte at the bottom (*yellow arrow*) also contains a Y chromosome (*turquoise*) indicating origin from the male recipient rather than from the female donor. Y-positive nuclei in the lower right belong to inflammatory cells. The cluster of DAB-positive cells at the upper left are part of the ductular reaction, although individual nuclei cannot be distinguished due to crowding and overlapping of the cells. (FITC, rhodamine, DAPI counterstain; original magnification ×100).

counts from the study tissues, correction factors were derived from the cell counts in this positive control male tissue, thereby accounting for the sectioning artifact. In the male control, 19% and 29% of the hepatocytes (Fig. 1) and cholangiocytes, respectively, had a clear Y chromosome signal in the nucleus. Thus, cell counts of hepatocytes and cholangiocytes were adjusted, dividing by a factor of 0.19 and 0.29, respectively, yielding likely estimates of the absolute cell counts in the experimental tissue specimens (Table 2). No ductular reactions were seen in either male or female control tissues. Y-positive, CAM5.2-negative cells were identified in all specimens, presumably representing hematopoietic cells, stromal cells, and Kupffer cells (Fig. 1, lower arrow).

Y-positive hepatocytes (Fig. 2) and cholangiocytes (Fig. 3) were identified in varying numbers in all livers from recipients of bone marrow or liver transplants. Some of the cells of ductular reactions were also Y-positive (Fig. 3). These Y-positive hepatocytes were identified as isolated single cells that were scattered throughout the lobules, in all but specimen 5. In specimen 5, Y-positive hepatocytes were noted individually and also in close proximity to one another, giving the impression of clustering (Fig. 2). In this specimen there was periportal predominance to the Y-positive hepatocytes not seen in the other specimens (Table 2). Y-positive cholangiocytes were often identified singly, although some duct profiles contained more than one such cell.

TABLE 2. Hepatic Engraftment of Y-Chromosome–Positive Cells

	Hepatocytes		Cholangiocytes		
Specimen	Count	% (adjusted %)	Count	% (adjusted %)	Ductular Reaction
Male control	42/216	19 (100%)	21/73	29 (100%)	NA
Female control	0/245	0 (0%)	0/123	0 (0%)	NA
1	12/622	2 (10%)	6/124	5 (17%)	NA
2	6/596	1 (5%)	2/182	1 (4%)	NA
3	13/416	3 (16%)	4/78	5 (18%)	Focal cells
4	11/308	3.6 (19%)	2/126	2 (5%)	Focal cells
5 (total)		Avg: 8 (40%)	15/137	11 (38%)	Clustered cells
(periportal region)	62/509	12 (64%)			
(pericentral region)	12/396	3 (16%)			
6	21/521	4 (21%)	6/115	5 (18%)	Focal cells

Abbreviation: NA, not applicable.

DISCUSSION

Using serial analysis of liver samples with immunohistochemistry followed by FISH, we show that human bone marrow–derived cells can differentiate into mature hepatocytes and cholangiocytes. The tissues examined came from two clinical situations that are roughly analogous to the animal models that have previously shown this process.[10,11] Two specimens were from female recipients of therapeutic bone marrow transplants with male donor cells. Neither patient had a history of significant prior liver damage; one experienced a single episode of mild GVHD and histologic cholestasis at the time of death, the other had no history of prior liver disease and a mild, nonspecific inflammatory infiltrate at the time of liver biopsy. These histories are similar to the clinical course of the female mice on which we reported previously. The female mice underwent lethal irradiation followed by complete marrow engraftment with male whole bone marrow cells with no overt hepatic injury.[10] Thus, in both of these human subjects and in the experimental animals, engraftment by bone marrow cells as hepatocytes could be seen in the absence of severe injury, suggesting that such movement might occur as a low level, baseline, physiologic phenomenon. Unlike in the mice, marrow engraftment as cholangiocytes was noted in both of these patients. Interestingly, the patient with a longer period of engraftment also had a higher percentage of engrafted cells.

The male recipients of female liver allografts are similar to the third experiment reported by Petersen et al., in which L21-6 antigen–positive rats were transplanted with L21-6–negative livers, with oval cell proliferation induced by carbon tetrachloride toxicity.[11] Although we did not have gender mismatched liver allografts in which massive necrosis had

taken place, which would have been a more precisely analogous clinical situation, we were able to select 4 biopsy specimens in which there were ductular reactions in response to other types of injury. Three (specimens 3, 4, and 6) had mild changes of large bile duct obstruction, confirmed clinically, including periportal ductular reactions, portal tract edema, and bile duct dilatation. In these cases, as in the work by Petersen et al., recipient-derived hepatocytes and cholangiocytes were identified. Still more striking, specimen 5 was from a patient with the fibrosing cholestatic variant of recurrent hepatitis C, one of the most severe forms of recurrent disease.[29] This specimen was characterized by marked expansion of portal tracts, a prominent ductular reaction, and severe cholestasis indicative of hepatocyte damage secondary to the viral infection. As in the corresponding experimental work, clustering of Y-positive hepatocytes could be identified.

Different distributions of hepatocyte engraftment suggest at least 2 pathways for this process. First, as in our experimental animals, engrafted hepatocytes were most often scattered throughout the parenchyma, usually appearing as isolated cells. This pattern is most suggestive of a process whereby hepatic stem cells enter through the circulation and intercalate randomly into preexistent liver cords directly as hepatocytes, without an obvious oval-like cell intermediate. Such a process is known to occur in animal models of liver cell transplantation.[30] On the other hand, where a marked ductular reaction takes place in response to injury (the equivalent of oval cell proliferation in animals), the extrahepatically derived stem cells appear to enter as oval-like cell intermediates, perhaps through the canals of Hering.[17,25] With subsequent expansion and differentiation, clusters of extrahepatic hepatocytes are formed. Thus, hepatic regeneration may have multiple and perhaps overlapping pathways to accomplish cell replacement and organ repair.

Differences in the extent of engraftment seem to correlate with the degree of hepatic injury. When injury was subclinical or mild, lower levels of hepatocyte and cholangiocyte engraftment occurred. With severe injury, as was seen in specimen 5, significantly increased engraftment occurred; up to 64% of hepatocytes in the periportal regions and up to 38% of cholangiocytes were recipient derived (adjusted values). Even in the most mild conditions, however, there was significant engraftment, ranging from 5% to 21% for hepatocytes and 4% to 18% for cholangiocytes, after adjustment for nuclear sampling artifact.

The occurrence of this process in mouse and human recipients of bone marrow transplants strongly suggests that these hepatic stem cells are marrow derived. However, we cannot exclude the possibility that there is a circulating population of cells, obtained from intramarrow vascular spaces at the time of marrow harvesting, that is not of bone marrow origin. This latter possibility is unlikely because the $CD34^+lin^-$ marrow population, 200 of which were capable of the same degree of hepatocyte engraftment as 20,000 whole bone marrow cells,[10] is present at only very low frequency (less than 0.05% of cells) in the peripheral blood.[31] Therefore, bone marrow remains the likeliest source of these hepatic stem cells. The identity of the extrahepatic cells capable of developing into liver cells is not yet known, but hepatocyte engraftment from $CD34^+$ bone marrow cells suggests that hematopoietic stem cells may have this prehepatocyte potential.

Before obtaining the data presented herein, it was not clear whether the high degree of hepatocyte engraftment that occurred in murine bone marrow transplant recipients was caused by lodging of bone marrow–derived cells in the liver at the time of intravenous injection or because of endogenous seeding of cells from the engrafted bone marrow. The engraftment of both hepatocytes and cholangiocytes in the 4 male recipients of female livers shows that this process occurs endogenously, and venous injection probably does not contribute substantively to the engraftment.

In the last few years there has been increasing evidence that adult cells have far greater differentiative plasticity than previously thought, with bone marrow turning into skeletal muscle,[32] skeletal muscle back into bone marrow,[33] brain into blood[34] and back again,[35,36] bone marrow into liver,[10,11] and with the cloning of mammals from adult cells.[37] Although these occurrences in different mammals (including mice, rats, and sheep) have been suggestive, it is impossible to know of the human relevance of the findings without studying human tissues directly. Exploiting the therapeutic "experiments" of gender-mismatched bone marrow and liver transplantation, we conclude that one of these experimental results, that of bone marrow cells differentiating into liver cells, does in fact occur in humans. Perhaps others do as well.

The data confirm that bipotent hepatic stem cells exist in humans and that they can be of extrahepatic, most likely bone marrow, origin. This finding opens new avenues of research regarding the increasingly complex, multiple mechanisms of hepatic regeneration. In particular, the marked degree of hepatic engraftment from extrahepatic cells in cases of severe liver injury indicates that there may be therapeutic utility for bone marrow transplantation to correct defects in hepatocyte metabolic or synthetic function.

Acknowledgment: The authors are grateful to Esperanza Papadopoulos, Department of Hematology/Oncology, Memorial-Sloan Kettering Cancer Center, for helping us to access the liver tissue from bone marrow transplant recipients; to David Ward, Department of Genetics, Yale University School of Medicine, for use of the fluorescence microscope; and to Katie Henderson for assistance with figure preparation.

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