Hepatic Microenvironment Affects Oval Cell Localization in Albumin-Urokinase-Type Plasminogen Activator Transgenic Mice

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Mice carrying an albumin-urokinase type plasminogen activator transgene (AL-uPA) develop liver disease secondary to uPA expression in hepatocytes. Transgene-expressing parenchyma is replaced gradually by clones of cells that have deleted transgene DNA and therefore are not subject to uPA-mediated damage. Diseased liver displays several abnormalities, including hepatocyte vacuolation and changes in nonparenchymal tissue. The latter includes increases in laminin protein within parenchyma and the appearance of cytokeratin 19-positive bile ductule-like cells (oval cells) both in portal regions and extending into the hepatic parenchyma. In this study, we subjected AL-uPA mice to two-thirds partial hepatectomy to identify the response of these livers to additional growth stimulation. We observed several changes in hepatic morphology. First, the oval cells increased in number and often formed ductules in the parenchyma. Second, this cellular change was accompanied by a further increase in laminin associated with single or clusters of oval cells. Third, desmin-positive Ito cells increased in number and maintained close association with oval cells. Fourth, these changes were localized precisely to uPA-expressing areas of liver. Regenerating clones of uPA-deficient cells appeared to be unaffected both by stromal and cellular alterations. Thus, additional growth stimulation of diseased uPA-expressing liver induces an oval cell-like response, as observed in other models of severe hepatic injury, but the localization of this response seems to be highly regulated by the hepatic microenvironment. (*Am J Pathol* 2003, 162:195–202)

Although the adult liver normally is a mitotically quiescent organ, hepatocytes transiently enter the cell cycle and proliferate to restore lost liver mass after parenchymal damage.^{1,2} The best experimental example of this type of regeneration follows two-thirds partial hepatectomy.³ In contrast, when there is a liver mitotic stimulus present but proliferation of hepatocytes is inhibited, liver regeneration

may occur by another process proposed to involve undifferentiated stem cells. To study this type of regeneration, several protocols have been developed in the rat and mouse in which hepatocyte mitoinhibition is combined with loss or destruction of liver parenchyma.^{4–8} In the rat, these treatments include oral gavage by 2-acetyl aminofluorene combined with two-thirds partial hepatectomy at the treatment midpoint;^{9–20} a single intraperitoneal injection of D-galactosamine;^{21,22} and chronic feeding of a choline-devoid, ethionine-supplemented diet.^{23–28} In the mouse, the protocol involves injection of the DNA-alkylating agent Dipin followed 2 hours later by two-thirds partial hepatectomy.^{29–32} Subsequent to each of these treatments, a population of small, cytokeratin 19-positive, ovoid cells with pale staining parenchyma are observed radiating out from terminal biliary ductules. These cells (termed oval cells) proliferate and migrate from portal regions into the parenchyma and do not disappear until liver regeneration is complete. Several lines of evidence from rodent studies suggest that oval cells are intermediate cells in a facultative stem cell lineage that can give rise to hepatocytes in cases of severe hepatic injury.^{10–12,14–17,19–22,27,28,30,31} It is worth noting, however, that the evidence supporting this hypothesis is not conclusive.^{9,13,24,32,33}

Transgenic mice in which urokinase-type plasminogen activator (uPA) expression is targeted to hepatocytes develop hepatocellular disease.^{34,35} Young albumin (AL)-uPA transgenic mouse liver appears pale compared to nontransgenic littermate liver, and hepatocytes contain rough endoplasmic reticulum vacuolations. Beginning at ~2 weeks of age, red foci of hepatocytes become visible in the transgenic liver; these foci gradually expand until the pale areas are replaced by confluent red nodules.³⁵ In contrast to pale liver, red liver lacks detectable transgene expression because of a stochastic event involving physical loss of integrated transgene DNA within individual hepatocytes.^{35,36} Transgene-deficient hepatocytes, liberated from the toxic effects of uPA expression, pref-

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erentially proliferate and eventually clonally repopulate the liver parenchyma. Similar to other rodent models in which there is both liver damage and inhibition of hepatocyte proliferation, the hepatotoxic effects of uPA expression also produce an oval cell response. A population of small, basophilic, generally ovoid cells was observed extending outward from portal areas into the parenchyma in regions of pale, diseased liver.³⁵ Interestingly, oval cells appeared to be absent from red, transgene-deficient parenchyma. After two-thirds partial hepatectomy of AL-uPA liver at a stage when both pale and red tissues were present, ³H-thymidine labeling indicated that both types of hepatocytes proliferate; however, red liver hepatocytes did so at a higher rate.³⁵ These results suggested that the ability of transgene-expressing, diseased hepatocytes to proliferate may be impaired, and that, as in other models of severe liver damage combined with hepatocyte mitoinhibition, the oval cell compartment therefore was activated.

The observation that oval cells were excluded from regenerative hepatocyte foci in AL-uPA transgenic mice suggested that the local hepatic microenvironment could influence the oval cell response. The objective of the studies described here was to identify local tissue changes that accompanied the oval cell response in AL-uPA liver, and to determine whether these changes also were excluded from regenerative tissue.

Materials and Methods

Transgenic Mice

The 1944-6 line of AL-uPA transgenic mice used in these studies was described previously,^{34,35} and has been assigned the following genetic designation: Tg(Alb-1 Plau)145Bri. Mice heterozygous for the transgene were used in this study. The transgene was generated by joining the murine AL enhancer/promoter to the mouse urokinase-type plasminogen activator (uPA) genomic coding sequence, with poly-A addition signal provided by the human growth hormone gene. Mice were maintained as hybrids between C57Bl/6 and FVB (attempts to produce inbred congenic transgenic mice on either background failed because of the development of increased transgene-mediated lethality). AL-uPA mice were identified by polymerase chain reaction, using a forward probe specific for uPA, 5'-GCGATTCTGGAG-GACCGCTTATC-3', and a reverse probe specific for the human growth hormone gene, 5'-TTAGGACAAGGCTGTGGGGCACTG-3'. Genomic DNA extracted from tail tissue was amplified in a 25- μ l reaction mixture using the following conditions: 92°C for 2 minutes; 35 cycles of: 45 seconds at 92°C, 1 minute at 60°C and 1 minute at 72°C; and 72°C for 5 minutes. Transgene DNA displayed an amplified product band of 300 bp on an agarose gel. Mice were housed in Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities, and all husbandry and experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and

approved by the Institutional Animal Care and Use Committee.

Two-Thirds Partial Hepatectomy

AL-uPA transgenic mice and nontransgenic littermates between 25 and 43 days of age were anesthetized, and two-thirds partial hepatectomy was performed by surgical removal of the left and median liver lobes.³ Only mice with <50% red, regenerated parenchyma at the time of surgery (as estimated visually) were included in the study.

Tissue Procedures

Mice were euthanized by CO₂ between 1 and 15 days after surgery and liver was collected for histochemical and immunohistochemical analysis. To label cells undergoing DNA synthesis, mice received an intraperitoneal injection of 200 mg/kg body weight 5-bromo-2'-deoxyuridine (BrdU) 1 to 2 hours before euthanasia. Separate pieces of each liver lobe were fixed in 10% neutral buffered formalin at room temperature overnight or in Carnoy's fixative at room temperature for 1 to 2 hours. Fixed tissue was transferred to 70% ethanol; embedded in paraffin; sectioned at 5 μ m; mounted on a slide; stained with hematoxylin and eosin (H&E), Gomori's reticulum, or immunohistochemically; and then examined microscopically.

Paraffin sections of Carnoy's-fixed tissue were stained immunohistochemically with rat monoclonal antibody to cytokeratin 19 (TROMA 3; kindly provided by Dr. Rolf Kemler, Max Planck Institute für Immunobiologie, Freiburg, Germany), rat monoclonal antibody to BrdU (MAS-250P; Accurate Scientific, Westbury, NY), polyclonal rabbit antiserum to laminin (L-9393; Sigma Chemical Co., St. Louis, MO), or polyclonal rabbit antiserum to desmin (A0611; DAKO, Carpinteria, CA). Tissue sections were rehydrated in descending concentrations of ethanol and endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol. Tissue used for BrdU and desmin immunohistochemistry was microwaved to boiling for 10 minutes in 0.1 mol/L of Tris, pH 9.0, for antigen retrieval. The antibodies were diluted in phosphate-buffered saline plus 0.1% nonfat dried milk and applied at room temperature for 12 to 20 hours. Primary antibody dilutions were as follows: anti-cytokeratin 19 rat monoclonal TROMA 3, 1:100; anti-BrdU, 1:40; anti-laminin, 1:100; anti-desmin, 1:150. Tissue was incubated with either biotinylated anti-rat (HK393-9T; BioGenex, San Ramon, CA) or biotinylated anti-rabbit (HK336-9R, BioGenex) secondary antibody in a species-specific manner. The label was peroxidase-conjugated (HK330-5K, BioGenex) or alkaline phosphatase-conjugated streptavidin (HK331-5K, BioGenex). Color development was performed with diaminobenzidine peroxidase substrate (D-4293, Sigma Chemical Co.) or New Fuchsin (HK183-5K, BioGenex). Finally, sections were counterstained for 2 minutes in hematoxylin, dehydrated through graded alcohols, and mounted under glass cov-

erslips. On some sections double immunohistochemistry was performed with TROMA 3 and either anti-BrdU, anti-laminin, or anti-desmin. For these sections, New Fuchsin color detection was used with TROMA 3 to stain cytokeratin 19-positive cells red, and diaminobenzidine color detection was used with the second primary antibody to yield a brown stain.

BrdU-Labeling Indices

Tissue sections stained immunohistochemically with both anti-BrdU and anti-cytokeratin 19 TROMA 3 were examined under a microscope with an eyepiece reticle. For each hepatocyte or cytokeratin 19-positive oval cell contained within the reticle grid, the BrdU-labeling status was recorded. Hepatocytes present in transgene-expressing parenchyma or regenerating parenchyma were counted separately. For each liver examined, counts from at least 500 of each cell type were added together to determine the percentage of BrdU labeling in diseased hepatocytes, healthy hepatocytes, or oval cells.

Statistical Analysis

Data were analyzed using GraphPad Prism version 2.0 (GraphPad Software, San Diego, CA). One-tailed Mann-Whitney rank sum tests were performed, with significance recognized when P was <0.05 .

Results

Oval Cell Response in AL-uPA Transgenic Mice

To characterize the oval cell response and associated stromal changes in AL-uPA transgenic mice, liver was collected from 36 AL-uPA mice and 26 nontransgenic littermates at 0 (newborn), 2, 4, 6, 8, and 10 weeks of age. Liver parenchyma in nontransgenic mice sacrificed at all time points appeared normal (Figure 1A). Microscopic examination of liver sections stained with H&E showed that transgenic mice between 0 and 2 weeks of age displayed mild hepatocyte vacuolation that was not present in nontransgenic littermates (data not shown). In contrast, as reported previously,^{34,35} starting at 4 weeks of age there appeared to be two cellular populations present in the liver of transgenic mice (Figure 1, B and C). In areas of regenerating parenchyma, the cells appeared mildly pleiomorphic but otherwise resembled normal hepatocytes. In contrast, transgene-expressing pale areas contained small hepatocytes with prominent cytoplasmic vacuolation. At interfaces between healthy and diseased parenchyma, it appeared that healthy tissue was mildly compressing the diseased tissue, consistent with expansion of the regenerative foci (data not shown). By the time transgenic mice were 8 to 10 weeks of age, the livers were mostly repopulated by confluent foci of regenerating hepatocytes.

Immunohistochemical staining of AL-uPA and nontransgenic livers collected at various ages was used to assess the oval cell response and accompanying stromal

changes. In nontransgenic mouse liver, cytokeratin 19-positive cells (biliary epithelial cells) were confined to portal areas (Figure 1A). In contrast, in AL-uPA transgenic mice, increased numbers of cytokeratin 19-positive cells were present exclusively in diseased, transgene-expressing parenchyma (Figure 1, B and C). These cells morphologically resembled the oval cells described in other rat and mouse models of severe liver disease. Normal liver parenchyma lacks distinct, continuous laminin-containing basement membranes in the liver sinusoids. Therefore, as expected, strong laminin staining was present only around vessels and bile ducts in the portal area of nontransgenic mice (Figure 1D). In contrast, AL-uPA transgenic mice displayed increased levels of laminin protein specifically in diseased parenchyma, often surrounding cytokeratin 19-positive cells but also surrounding hepatocytes (Figure 1, E and F). Finally, the frequency of desmin-positive Ito cells was increased markedly in diseased tissue of AL-uPA transgenic mice compared to nontransgenic mouse liver (Figure 1G) or healthy regenerating parenchyma (Figure 1, H and I). In general, all changes in stromal characteristics were localized precisely to diseased parenchyma of AL-uPA transgenic mice. The severity of these changes generally increased in the AL-uPA mice as they aged, until final liver remodeling restored relatively normal hepatic architecture once regeneration was complete. After this occurred, these hepatic abnormalities no longer were observed in AL-uPA transgenic mice (data not shown).

Two-Thirds Partial Hepatectomy Increases the Extent of the Oval Cell Response

If the AL-uPA mouse oval cell lineage has developed in response to uPA-mediated liver damage, then increasing the mitotic stimulus might be expected to amplify the oval cell reaction. To test this hypothesis, AL-uPA and control nontransgenic mice were subjected to two-thirds partial hepatectomy at a stage (between 25 and 43 days of age) when transgenic liver consisted of both regenerating and diseased parenchyma. Mice then were euthanized between 1 and 15 days after surgery and the morphological characteristics of liver collected at each time point were assessed (Table 1). In the first week after partial hepatectomy, the number of oval cells in diseased hepatic parenchyma did not increase significantly *versus* prehepatectomy levels (Figure 1J). In contrast, in this line of AL-uPA transgenic mice, the oval cell response peaked at 9 to 11 days after hepatectomy, when there was extensive oval cell presence throughout all of the diseased parenchyma (Figure 1K). Oval cells present at this stage often formed duct-like structures within the diseased parenchyma, a phenomenon that is observed infrequently in unhepatectomized AL-uPA liver. In striking contrast, no oval cells were observed in regenerating hepatocyte foci in transgenic mice (Figure 1K) or in nontransgenic mice after two-thirds hepatectomy (Figure 1L).

Next, immunohistochemistry was used to assess stromal characteristics in AL-uPA transgenic mice after partial hepatectomy. At the peak of the oval cell response,

Figure 1. Oval cell response and associated hepatic changes in AL-uPA mice. **A–I:** Each column displays neighboring liver sections that were stained immunohistochemically with antibodies against cytokeratin 19 (**A–C**), laminin, (**D–F**), or desmin (**G–I**). **A, D, and G:** Liver from a 27-day-old nontransgenic mouse. In normal parenchyma, cytokeratin 19-positive cells (biliary epithelial cells) (**A**) and prominent laminin staining (**D**) are restricted to portal regions. Cytoplasmic processes of desmin-positive Ito cells (**arrow**) occasionally are visible in the perisinusoidal spaces (**G**). **B, E, and H:** Liver from a 27-day-old AL-uPA mouse. **Arrowheads** mark the edge of a focus of regenerating, transgene-deficient hepatocytes. Staining for cytokeratin 19-positive cells (**B**) and laminin protein (**E**) is present in diseased hepatic parenchyma. Increased numbers of desmin-positive Ito cells (**H**) also are present in diseased parenchyma; the **arrow** marks a desmin-positive Ito cell in the regenerative focus. Regenerating parenchyma displays reduced or no staining with each antibody. **C, F, and I:** Liver from a 42-day-old AL-uPA mouse. **Arrowheads** mark the edge of a regenerative focus. Staining for cytokeratin 19-positive cells (**C**), laminin protein (**F**), and Ito cells (**I**) is further increased exclusively in diseased parenchyma of older AL-uPA transgenic mice. The **arrow** marks a desmin-positive Ito cell (**I**) in the regenerative focus. **J, K, and L:** Appearance of oval cells in AL-uPA mice after partial hepatectomy. Liver sections were stained immunohistochemically for cytokeratin 19. **Arrowheads** mark the edges of regenerative foci. **J:** Liver morphology in an AL-uPA transgenic mouse at 7 days after hepatectomy. Cytokeratin 19-positive cells are present in diseased hepatic parenchyma, occasionally forming ducts. **K:** Liver morphology in an AL-uPA transgenic mouse at 9 days after hepatectomy. Extensive numbers of cytokeratin 19-positive cells are present in diseased parenchyma, frequently forming ducts. **L:** Liver morphology in a nontransgenic mouse at 11 days after hepatectomy. Only biliary epithelial cells in the portal region stain for cytokeratin 19. Original magnifications, $\times 200$.

the cytokeratin 19-positive oval cells are found primarily in ductules confined specifically to diseased parenchyma (Figure 2, A and B). Reticulum staining, which identifies interstitial collagen, was strong in diseased parenchyma but was primarily excluded from the foci of

healthy cells that are repopulating the liver (data not shown). Increased staining for laminin protein (Figure 2C) and desmin-positive Ito cells (Figure 2D) also was restricted to diseased parenchyma. Furthermore, double immunohistochemistry to identify cytokeratin 19-positive

Table 1. Oval Cell Response in Diseased Parenchyma of AL-uPA Transgenic Mice*

Days after partial hepatectomy	Number of mice	Oval cell score [†] X ± SD	Duct formation score [‡] X ± SD
Unhepatectomized [§]	13	2.7 ± 1.2	1.8 ± 0.9
1–4	9	2.2 ± 0.9	1.6 ± 0.7
5–7	8	2.8 ± 1.1	2.4 ± 1.2
9–11	8	4.5 ± 1.4 [¶]	4.3 ± 1.5 [¶]
13–15	3	3.2 ± 1.5	2.3 ± 0.6

*The oval cell response was classified by microscopic examination of paraformaldehyde-fixed, paraffin-embedded sections stained with hematoxylin and eosin or anti-cytokeratin 19 antibody. For each liver, the prevalence of the oval cell response and the frequency of oval cell incorporation into ducts were scored separately on scales of 1 to 5. Each liver was assessed separately by two investigators and a mean value of the two scores was assigned to the liver. Control livers collected from 20 nontransgenic mice after partial hepatectomy showed no evidence of oval cells.

[†]1, Oval cells are absent or rare within diseased parenchyma; 5 = extensive oval cell presence throughout all zones of diseased liver; 2 to 4 represent intermediate responses. Representative oval cell response scores for Figure 1: panel J, 3; panel K, 5; panel L, 1.

[‡]1, Oval cells never are present in ducts; 5, nearly all oval cells are incorporated into ducts; 2 to 4 represent intermediate responses. Duct formation scores for Figure 1: panel J, 3; panel K, 5; panel L, 1.

[§]Data from unhepatectomized AL-uPA mice sacrificed at a median of 31 days of age.

[¶]Statistically significant difference by Mann-Whitney rank sum test when compared with scores from unhepatectomized AL-uPA mice: oval cell score, $P = 0.004$; duct formation score, $P = 0.002$.

oval cells and either laminin (Figure 2E) or desmin-positive Ito cells (Figure 2F) demonstrated that oval cells closely associate with laminin protein and Ito cells.

Finally, double immunohistochemistry was performed on liver sections using anti-cytokeratin 19 to identify oval cells and anti-BrdU to stain cells that had incorporated BrdU into replicating DNA (Figure 2G). The BrdU-labeling index was determined for oval cells, diseased hepatocytes, and healthy hepatocytes (Table 2). In unhepatectomized mice, the indices were not different. After hepatectomy, labeling indices increased significantly for each cell type compared to indices in unhepatectomized AL-uPA mice. This increase persisted for healthy but not diseased hepatocytes through day 11 after hepatectomy. Oval cell proliferation also increased after hepatectomy, including during days 9 to 11 after hepatectomy, corresponding to the period of ductule formation. (Lack of a significant increase in oval cell proliferation at days 5 to 7 after hepatectomy was associated with a highly variable response among that group of mice, and we do not believe that it indicates a biphasic mitotic response to partial hepatectomy.) Labeling indices were not significantly different from unhepatectomized values beyond 13 days after hepatectomy.

Discussion

The proliferation of transgene-deficient healthy hepatocytes to repopulate diseased AL-uPA liver indicates that there is a chronic hepatic growth stimulus present in these mice. Proliferation of normal hepatocytes transplanted into the liver of these and similar mice also supports this conclusion.^{37,38} In this study, we found that both uPA-deficient and uPA-expressing hepatocytes proliferate at a similar rate; however, diseased hepatocytes cannot divide at a rate that balances their death, because eventually they are replaced by healthy cells. After partial hepatectomy, proliferation of both types of hepatocytes increased, but this response was sustained only by the healthy hepatocytes. Proliferation of cytokeratin 19-positive oval cells also increased, indicating that the magnitude of the growth stimulus influences the extent of this

cellular response. However, the oval cell response occurs exclusively in diseased parenchyma of AL-uPA transgenic mice. This response resembles in several ways the oval cell response reported in other rodent hepatic-injury models. First, in both AL-uPA mice and 2-acetyl aminofluorene-treated rats, the peak oval cell response occurs 9 to 11 days after the growth stimulus is maximized by partial hepatectomy.^{10,11} Second, the arrangement of oval cells into parenchymal ductules also has been reported in several models of liver disease.^{11,15,22,26,30,39} Finally, in the 2-acetyl aminofluorene/partial hepatectomy model, oval cells and Ito cells proliferate and maintain close physical contact while spreading into parenchyma in a coordinated manner.^{39–41} In this report, we additionally demonstrate a co-localization between oval cells and laminin. Because reactivation of the AL-uPA transgene (and development of associated cellular toxicity) likely would accompany hepatocellular differentiation of oval cells, we could not address whether this lineage transition occurred in our model.

The morphological observations described above suggest a link between stroma in the hepatic microenvironment and the oval cell response. This interaction may occur via several mechanisms. Alison and colleagues⁸ have speculated that Ito (or stellate) cells⁴² may affect the oval cell response by 1) secreting metalloproteinases⁴³ specific for basement membrane proteins so that oval cells can invade damaged parenchyma; 2) secreting multiple growth factors [including hepatocyte growth factor^{44,45} and transforming growth factor- β ^{39,46}] to promote oval cell migration and differentiation; 3) creating a microenvironment featuring stromal components that resemble periportal regions, so that oval cells retain biliary-like properties early during regeneration; and 4) synthesizing and secreting laminin chains to stimulate endothelial invasion and sinusoid formation.⁴⁷ In addition, our observation that laminin fibers were associated with oval cells may be significant because adhesive interactions between cells and extracellular matrix molecules, including laminin, are proposed to be a critical

Figure 2. Neighboring liver sections from an AL-uPA transgenic mouse at 11 days after hepatectomy. **Arrowheads** mark the edge of a regenerative focus. Sections were stained with H&E (**A**) or immunohistochemically with anti-cytokeratin 19 (**B**), anti-laminin (**C**), or anti-desmin (**D**). Double immunohistochemistry shows co-localization of cytokeratin 19-positive cells (red stain) and laminin protein (brown stain) in diseased parenchyma (**E**). A similar association was seen between cytokeratin 19-positive cells (red) and desmin-positive Ito cells (brown) (**F**). **G:** Section stained immunohistochemically with anti-cytokeratin 19 (red stain) and anti-BrdU (brown stain) to identify oval cells and hepatocytes undergoing DNA synthesis. BrdU injected 1 to 2 hours before euthanasia was incorporated into DNA of cells undergoing DNA synthesis. Original magnifications, $\times 400$.

element in cell migration.⁴⁸ Of course, oval cells also may participate in modifying the local tissue environment.

Because livers of young AL-uPA transgenic mice are chimeric, featuring both diseased parenchyma and foci of healthy, transgene-deficient hepatocytes, they provide a unique opportunity to evaluate the link between the

hepatic microenvironment and the oval cell response. In these mice, oval cells are tightly restricted to regions of liver disease, indicating that local parenchymal damage is sufficient for induction of these cells. There are several potential explanations for the exclusion of oval cells from healthy parenchyma. First, signals that induce or permit

Table 2. BrdU-Labeling Indices of Oval Cells and Hepatocytes in AL-uPA Transgenic Mice*

Days after partial hepatectomy	Number of mice	% BrdU labeled; X $\pm$ SD		
		Oval cells	Diseased hepatocytes	Healthy hepatocytes
Unhepatectomized [†]	7	5.7 $\pm$ 1.8	5.6 $\pm$ 4.0	4.8 $\pm$ 2.6
2–4	7	10.5 $\pm$ 5.1 [§]	12.7 $\pm$ 4.0 [§]	10.9 $\pm$ 2.8 [§]
5–7	8	8.7 $\pm$ 6.5	7.3 $\pm$ 3.6	10.4 $\pm$ 6.4 [§]
9–11	6	10.4 $\pm$ 4.7 [§]	5.8 $\pm$ 1.2	8.1 $\pm$ 2.2 [§]
13–15	4	4.3 $\pm$ 0.7 [‡]	4.3 $\pm$ 1.4	10.0 $\pm$ 6.3

*The BrdU-labeling index for each cell type was determined by microscopic examination of paraformaldehyde-fixed, paraffin-embedded sections stained immunohistochemically with anti-BrdU and anti-cytokeratin 19 antibodies (photomicrograph in Figure 2G).

[†]Euthanized at 41 to 44 days of age.

[‡]n = 3.

[§]Statistically significant increase by Mann-Whitney rank sum test when compared with labeling index of the same cell type in unhepatectomized AL-uPA mice ($P < 0.05$).

oval cell proliferation may be present exclusively in diseased parenchyma. Alternatively, the presence of multifocal liver disease may induce a systemic proliferative signal, but local factors within healthy, regenerative tissue may suppress oval cell proliferation. Finally, although Ito cells were observed in parenchyma of regenerating foci, these foci may not contain oval cells or their precursors. This absence is especially likely if regenerative nodules do not have proper bile duct architecture, because liver stem cells are thought to be associated with terminal biliary ductules.^{4,6–8} In addition, for each of these cases, migration of oval cells into regions of normal tissue also would have to be impaired to maintain the zonal restriction that we observed.

The presence of a boundary effect that excludes oval cells from healthy tissue implies that there is local control of oval cell proliferation and migration within hepatic parenchyma. The best candidates to maintain such a tight boundary would not be diffusible signaling molecules, capable of long-range activity, but rather highly local interactions such as cell-cell or cell-extracellular matrix contact. The precise co-localization of oval cells, Ito cells, and laminin protein in AL-uPA mice suggests that liver stroma may serve this role. A possible explanation for the differences in stromal composition between regenerative and diseased tissue is that Ito cells are activated only in response to local hepatocellular damage instead of systemic signals. In this scenario, parenchymal damage not only would initiate the oval cell response by producing the mitotic stimulus, but also would provide the local signal that regulates the distribution of the response.

In summary, AL-uPA transgenic mice provide an additional rodent model of hepatic disease in which oval cells can be studied. In these mice, oval cells were associated closely with Ito cells and increased laminin protein, providing evidence that stroma may participate in the oval cell response after severe hepatic injury. Most importantly, the availability of mice with chimeric livers allowed us to discover that oval cells were excluded from foci of healthy hepatocytes that lacked the stromal changes. This observation provides strong evidence that the local hepatic microenvironment influences the cellular response in liver disease.

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