Hepatic Progenitor Populations in Embryonic, Neonatal, and Adult Liver[1] (43662)

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Abstract. **Oval cells, small cells with oval-shaped nuclei, are induced to proliferate in the livers of animals treated with carcinogens and are thought to be related to liver stem cells and/or committed liver progenitor cell populations. We have developed protocols for identifying and isolating antigenically related cell populations present in normal tissues using monoclonal antibodies to oval cell antigens and fluorescence-activated cell sorting. We have isolated oval cell-antigen-positive (OCAP) cells from embryonic, neonatal, and adult rat livers and have identified culture conditions permitting their growth in culture.[5–7] The requirements for growth of the OCAP cells included substrata of type IV collagen mixed with laminin, basal medium with complex lipids and low calcium, specific growth factors (most potently, insulin-like growth factor II and granulocyte-macrophage colony-stimulating factor), and co-cultures of embryonic, liver-specific stroma, strongly suggesting paracrine signaling between hepatic and hemopoietic precursor cells.[5–7] The growing OCAP cultures proved to be uniformly expressing oval cell markers but were nevertheless a mixture of hepatic and hemopoietic precursor cells.**

To separate the hepatic and hemopoietic subpopulations of OCAP cells, we surveyed known antibodies and found ones that uniquely identify either hepatic or hemopoietic cells. Several of these antibodies were used in panning procedures and fluorescence-activated cell sorting to eliminate contaminant cell populations, particularly hemopoietic and endothelial cells.[8] Using specific flow cytometric parameters, three cellular subpopulations could be isolated separately that were identified by immunochemistry and molecular hybridization assays as probable: (i) committed progenitors to hepatocytes; (ii) committed progenitors to bile ducts; or (iii) a mixed population of hemopoietic cells that contained a small percentage of hepatic blasts that are possibly pluripotent.[8,9] The hepatic precursor cells have been characterized using immunochemistry, flow cytometry, and molecular hybridization assays. The hepatic blasts are small (7–10 μm) cells with high nuclear to cytoplasmic ratios and with minimal complexity of the cytoplasm. Cultures of the committed progenitors were found to differentiate into cells with recognizable parenchymal cell fates.

We discuss our studies in the context of our model of the liver as stem cell and lineage system and suggest that a slow, unidirectional, terminal differentiation process, paralleling more rapid ones in the skin or gut, occurs at all times in the liver and is thought to vary primarily in kinetics during quiescent versus regenerative states. Aging is hypothesized to result in gradual reduction in the liver's regenerative potential due to loss of stem cells. The known zonal distribution in tissue-specific gene expression is interpreted to be a lineage-position-dependent process regulated by gradients of hormones or growth factors, soluble and insoluble matrix signals, and/or maturation-dependent changes in chromatin. [P.S.E.B.M. 1993, Vol 204]

Introduction and Background. The Architecture of the Liver Acinus and Its Relevance to Zonal Distribution in Gene Expression.

The structural and functional unit of the hepatic parenchyma is the acinus, which is organized like a

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wheel around two distinct vascular beds: six sets of portal triads, each with a portal venule, hepatic arteriole, and a bile duct, form the periphery and the central vein forms the hub (1–3). The parenchyma, effectively the "spokes" of the wheel, consist of plates of cells lined on both sides by the fenestrated sinusoidal endothelium. Blood flows from the portal venules and hepatic arterioles at the portal triads, through sinusoids which line plates of parenchyma, to the central vein. Based on this microcirculatory pattern, the acinus is divided into three zones: Zone 1, the periportal region; Zone 2, the central region; and Zone 3, the region surrounding the central vein or terminal hepatic venule. Hepatocytes display marked morphologic, biochemical, and functional heterogeneity based on their zonal location (4). Cells are smaller in Zone 1, intermediate in Zone 2, and larger in Zone 3, and there are variations in cellular morphology and in tissue-specific gene expression that show striking distribution patterns within the acinus. Ploidy and growth potential also vary with the zonal location: cells in Zone 1 are primarily diploid and show the maximum growth potential, tetraploid and with intermediate growth potential in Zone 2, and a mixture of tetraploid and octaploid and with the least growth potential in Zone 3 (1, 4).[9] The traditional explanation for the heterogeneity of cell size, growth potential and gene expression in hepatocytes is that it is due to the direction of blood flow across the liver acinus, resulting in a gradient within the sinusoidal microenvironment. However, as discussed in some detail in our reviews (1, 2), though the unidirectional perfusion model explains some aspects of hepatocyte heterogeneity, for example, glycogen and lipid metabolism, it has been discredited as an explanation for the position-dependent expression of most tissue-specific genes, especially those with discrete expression such as α-fetoprotein, connexins, α_2-microglobulin, and glutamine synthetase. The model we prefer is that hepatocyte heterogeneity is a manifestation of a lineage process (1–3), associated with cellular maturation in which stem cells or committed progenitors, located at or near the canals of Hering around each set of portal triads, produce daughter cells that undergo a unidirectional, terminal differentiation process ending at the central vein. The varying metabolic activities of the hepatocytes are, therefore, assumed to be age dependent.

Liver Stem Cells and Their Transformed Counterparts, Oval Cells. Wilson and Leduc (5) first postulated the presence of liver stem cells in 1958. However, as in the hemopoietic field, the concept of a liver stem cell gained the most credibility from extensive studies of carcinogenesis (6–11). Rats exposed to a wide range of carcinogens display a remarkably uniform pattern of changes leading to the development of hepatocellular carcinoma: widespread hepatic necrosis and inflammation, followed by hepatocyte proliferation. Concomitantly, a population of small (7–15 μm) cells with scant cytoplasm and ovoid nuclei, termed oval cells (6–11), proliferate in the region of the portal triads. Subsequently, nodules of atypical hepatocytes appear, most of which are reorganized into the hepatic parenchyma. Several persist, however, and undergo malignant transformation. Though morphologically resembling bile duct cells, oval cells share many properties with fetal hepatocytes in that they contain α-fetoprotein and albumin and a number of fetal isoforms of enzymes (6, 8). Oval cells have been studied extensively (6–14) and have been established in culture (13, 14). Although they have limited differentiative ability, especially in culture, they have been shown to differentiate into cell types with some identifiable parenchymal markers if injected *in vivo* (11). Monoclonal antibodies (mAb) have been developed by Drs. Doug Hixson and Ron Faris (Rhode Island Hospital, Providence, RI) (9) that identify antigens on oval cells. Using these mAb, they (9) have identified cell populations in normal livers that are antigenically related to oval cells. These cell populations are referred to as oval cell-antigen-positive (OCAP) cells, and distinct populations are identified by including the number (or name) of the mAb; thus, OCAP-374.3 is that population identified by the mAb 374.3. Analogous to the antigenic characterization of the stages of hemopoiesis, Hixson *et al.* (9) proposed the existence of three subpopulations of OCAP cells based on their antigenic profile: stem cells able to differentiate either toward biliary or hepatocellular lineages and present in large numbers in rats up through embryonic Day 15; precursors committed toward a biliary lineage; and precursors committed to the hepatocellular lineage. The committed precursors are present increasingly after embryonic Day 15.

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[5] M. Agelli, E. Halay, and L. Reid. Isolation and characterization of oval cell antigen positive cells, OCAP cells (manuscript in preparation).

[6] M. Agelli, P. Sbarba, and L. Reid. Requirements for clonal growth of OCAP cells: Age- and tissue-specific forms of stromal feeder layers (manuscript in preparation).

[7] P. Holst, A. Ochs, S. Brill, E. Halay, R. Faris, D. Hixson, and L. Reid. Isolation and culture of liver OCAP cells, oval cell antigen-positive cells: Hepatic and hemopoietic precursors cells (submitted for publication).

[8] S. Sigal, S. Brill, L. Reid, R. Faris, D. Hixson, and P. Holst. Characterization and enrichment of fetal hepatoblasts by immunoadsorption ("panning") and fluorescence activated cell sorting (submitted for publication).

[9] S. Sigal, S. Gupta, D. Gebhardt, P. Holst, D. Neufeld, and L. Reid. Ploidy, cell cycle, granularity and autofluorescence during liver development: Evidence for a terminal differentiation process (submitted for publication).

Our Studies on Hepatic Progenitor Cells

In this symposium article, we summarize findings presented in detail in articles to be published elsewhere[5–9] on the isolation and characterization of liver progenitors from embryonic, neonatal, and adult livers. The evidence on liver progenitors has been used in the development of a model of the liver as a stem cell and lineage system described in detail in recent reviews (1–3). References to the literature constituting the background for our studies are given in these referred and review articles and will not be given here except as needed.

Isolation of OCAP Cells from Embryonic or Neonatal Livers. The details of the methodologies are given in articles to be published elsewhere[5–9] and are summarized here. We began our efforts to identify and isolate liver progenitor cells by enzymatically dispersing embryonic or neonatal livers and then sorting by fluorescence-activated cell sorting for cells uniformly expressing an oval cell antigen. We screened monoclonal antibodies to oval cell surface markers to identify ones that would yield sorted populations enriched for cells expressing albumin and α-fetoprotein.[5,6] Two qualifying by this criteria were mAb 374.3, identifying oval cell antigen 3 (OC3), and 270.38, identifying oval cell antigen 2 (OC2). These mAb were produced by Drs. Faris and Hixson of Rhode Island Hospital (9).

Cells uniformly expressing OC3 were isolated from embryonic, neonatal, or adult livers and were characterized by immunochemical, biochemical, and molecular hybridization assays. OCAP-374.3 cells were found to express markers appropriate for both hepatic and hemopoietic cells[5–9] as shown in Table I.

Culture Conditions for Survival and Growth of OCAP-374.3 Cells. Survival of the OCAP cells in culture required that the cells be plated onto collagen-coated transwells in a basal media supplemented with complex lipids and free fatty acids bound to bovine serum albumin.[5] Growth of the OCAP cells required, in addition, that the collagen be type IV (and ideally one mixed with laminin), that the basal medium have low calcium concentrations (<0.5 m*M*), and that the OCAP cells be co-cultured with stromal feeder layers derived from embryonic liver (E12-E18).[5,7] Stromal feeder layers from neonatal or adult livers were not active.[6,7] In the presence of the embryonic, liver-specific stromal feeder layers, a number of factors proved mitogenically active, including fibroblast growth factors, epidermal growth factors, tumor growth factor-α, insulin, insulin-like growth factor (IGF) I, interleukin 1, interleukin 6, and especially granulocyte-macrophage colony-stimulating factor (GM-CSF) and IGF-II. In addition, GM-CSF and IGF-II induced both diffuse proliferative growth and colony formation.

Table I. Characterization of Freshly Isolated OCAP-374.3 Cells

Oval cell markers	
374.3 (OC3)	100%
270.38 (OC2)	50–60%
Liver-specific markers	
α-Fetoprotein	5–10%
Albumin	20–25%
γ-Glutamyl transpeptidase	Neg (fetal, neonatal) 10–15% (adult)
Hemopoietic markers	
Common leukocyte antigen	1–5%
OX42 (MCA 275)	10–15%

Cultures of Growing OCAP Cells Indicate that They Are Mixed Hepatic and Hemopoietic Precursor Cells. After 2–3 weeks of culture, the OCAP cells retained their mixed phenotype of hemopoietic and hepatic markers. Even the colonies induced by GM-CSF and IGF-II consisted of mixed hepatic and hemopoietic cells. Oval cell markers identifiable with mAb 374.3 or 270.38 were evident on approximately 33% of the cells. Depending on the age of the host from which the OCAP cells derived, the fetal liver marker α-fetoprotein was expressed on 5–20% of the cells. Albumin was found on 20–30% of the cells. γ-Glutamyl transpeptidase was negative on the cultures derived from fetal or neonatal liver, but positive on up to half the cells in those derived from adult liver.[5,7] In parallel, the cultures also expressed classic hemopoietic markers, e.g., common leukocyte antigen (>50% of the cells), as well as OX42 (12%) and ED1 (40–50%), which recognize cells of the monocyte/macrophage lineage. Interestingly, the presence of hemopoietic cells was also evident in OCAP-374.3 cultures derived from adult liver.[5]

Identification of Antigens that Are Shared versus Unique to Hepatic versus Hemopoietic Cells. The early studies noted above indicated intriguing findings: the oval cell antigens OC2 and OC3 are expressed on both hepatic and hemopoietic cells. Preliminary studies were made to identify antibodies that would uniquely identify hepatic oval cells. However, all oval cell antigens tested proved to be shared between hemopoietic and hepatic cells. Since all mAb to oval cell markers resulted in mixed populations of hepatic and hemopoietic precursor cells, we changed our tack to do multiparametric fluorescence-activated cell sorting in which we sorted positively for an oval cell marker and negatively for one or more hemopoietic markers. This required efforts to identify mAb to markers unique to hemopoietic lineages. The studies resulted in an increasing appreciation for the considerable number of shared antigenic and biochemical markers summarized in Table II and found by us[7–9] or others (reviewed in Refs. 4, 9, 10).

Table II. Representative Markers Expressed by Hemopoietic and/or Hepatic Cells (with a focus on precursor cell populations)

Shared	
All known oval cell antigens	
Transferrins	
γ-Glutamyl transpeptidase	
Glucuronyl transferases (some isoforms)	
Glutathione-S-transferases, ligandins (some isoforms)	
cEBP	
Receptors for hormones/growth factors such as interleukin (IL)-1, IL-6, interferon, hepatocyte growth factor, insulin	
Multidrug resistance genes: MDR1 and MDR2	
Connexin 43	
Unique	
Hepatic	**Hemopoietic**
α-Fetoprotein	OX43 (MCA 276)
Albumin	OX44 (MCA 371, CD37)
Stem cell factor	c-*Kit*, receptor for Stem Cell Factor
Hepatic heparan sulfate proteoglycans (Syndecan/Perlecan)	Hemopoietic heparan sulfate proteoglycan (Serglycin)
IGF-I and -II	OX42 (MCA 275, CD11B)
Tumor growth factor-α and its receptor	GM-CSF, CSF
α1 and α5 Integrins	α4 Integrin
Connexins 26 and 32	Red blood cell antigen

Separation of Hepatic from Hemopoietic OCAP Cells. Using antigens identified as unique to hemopoietic versus hepatic cells, we developed new methods for isolating hepatic progenitor cells that included panning technologies followed by multiparametric fluorescence-activated cell sorting. The cell suspensions were panned to eliminate hemopoietic constituents using commercially available antibodies to rat red blood cells, to rat myeloid cells (OX43, OX44), and to endothelial cells (OX44).[8] From 85% (E15) to 95% (E18) of the cells in the original cell suspensions were eliminated in two to three 10-min panning procedures, enabling us to go onto the fluorescence-activated cell sorter with smaller numbers of cells (therefore, more efficient and rapid sorting), with cell populations significantly enriched for the cells of interest, and with cells of good viability. Thus, we used panning to reduce the cell numbers and fluorescence-activated cell sorting to handle the efficiency and thoroughness of selecting the cell populations of interest. By eliminating most of the major irrelevant cell populations, such as the hemopoietic cells, we enriched dramatically for parenchymal cells, resulting in a cell population that contained more than 75% α-fetoprotein and albumin-positive cells. A summary of the characterization of the phenotype of such a subpopulation is given in Table III and is summarized from data presented elsewhere.[8,9]

Subpopulations of OX43/44$^-$ Cells. The cell population negative for OX43 and OX44 (hemopoietic and endothelial cell markers) could be further subdivided by expression of oval cell markers and by extent of granularity, the latter a measure of cellular complexity as detected by the fluorescence-activated cell sorter side scatter function.[8,9] Table IV shows the essential parameters distinguishing the several subpopulations in the OX43- and OX44-negative population at E15.

Cultures of Sorted Hepatic Precursor Cell Populations. In ongoing studies, we are identifying culture conditions for the hepatic progenitor cells.[10] Freshly isolated, sorted hepatic progenitor cell populations are 7–10 μm in diameter, have high nuclear to cytoplasmic ratios seen as a thin rim of cytoplasm surrounding the nucleus, have few cytoplasmic organelles (e.g., mitochondria, ribosomes), resulting in low granularity as measured by the fluorescence-activated cell sorter side scatter function, intensely express fetal markers such as α-fetoprotein, weakly express albumin, and do not express adult-specific markers such as glycogen. After 1–2 weeks in culture on matrix substrata and in media supplemented with hormones and growth factors, the cells are considerably larger in size, have a significant increase in cytoplasmic volume and cytoplasmic organelles, form bile canaliculi throughout the cultures, have a decrease in expression of fetal markers such as α-fetoprotein, and have a significant increase in expression of adult-specific markers such as albumin. Therefore, specific matrix and hormonal conditions have been found that cause the cells to differentiate into more mature parenchymal cells.

Discussion

Isolation and Characterization of Hepatic Progenitor Populations. We have developed methods for the isolation of early hepatic progenitor cells that are either committed progenitors for hepatocytes or bile ducts or are early hepatic blasts that may be pluripotent.[5–9] The committed progenitors were found capable of differentiating in culture under appropriate matrix and hormonal conditions to more mature cells with recognizable parenchymal cell fates.

The conditions for growth of the early hepatic blasts in culture are still being defined but are likely to involve signals derived from hemopoietic precursors and from embryonic, liver-specific stromal feeders. Studies[5,7] in which growth requirements were identified for oval cell antigen-positive cells, mixed hepatic and hemopoietic precursor cell populations, indicated that

[10] S. Brill, I. Zvibel, L. M. Reid, and P. A. Holst. Isolation of fetal liver parenchymal cells by multiparametric flow cytometry (manuscript in preparation).

Table III. Phenotype of Original Cell Suspension versus Cells after Sorting and/or Panning

Markers	Original	Panned	Sorted OX43/44$^-$ cells
Liver specific			
α-Fetoprotein	2%	15%	75%
18.11	26%	—	—
270.38	36%	—	—
Albumin	2%	15%	75%
γ-Glutamyl transpeptidase	Positive	—	Granular = 33% (agranular = <1%)
Glycogen	E15-Negative; E18-Rare faint cells; E20-Rare positive cells	E15-Negative; E18-Faint in parenchymal cells; E20-Strongly positive in parenchymal cells	Not tested
Hemopoietic markers			
OX42	9%	E14 = 6%; E15 = 3%	<3%
OX43/44	88–95% (depending on embryonic age)	50–80% (depending on embryonic age)	<1%
Serglycin	Strongly positive	Strongly positive	Neg

Note: Assays: immunofluorescence, molecular hybridization studies, histochemical, and biochemical assays.

Table IV. Subpopulations of OX43/OX44$^-$ Cells at E15

Agranular cells (<50 AU[a])
- Mostly hemopoietic cells (cells not recognized by OX43 or OX44) and a small percentage (3–5%) of hepatic blasts that intensely stain for α-fetoprotein, weakly stain for albumin, and are negative for γ-glutamyl transpeptidase

Granular cells (>50 AU[a])
- OC3$^-$: committed progenitors to hepatocytes (positive for albumin and for α-fetoprotein; negative for γ-glutamyl transpeptidase)
- OC3$^+$: committed progenitors to bile duct cells (negative for albumin; positive for α-fetoprotein; strongly positive for γ-glutamyl transpeptidase)

[a] Granularity measured by the side scatter function on the fluorescence-activated cell sorter and subdivided into 256 arbitrary units (AU). Increasing granularity is a measure of cellular complexity due to cytoplasmic organelles.

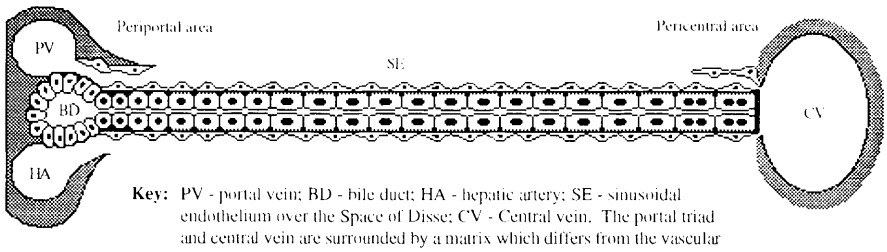
there are strict requirements for substrata of type IV collagen and laminin, serum-free basal medium with low calcium (<0.5 mM) and supplemented with complex lipids (mixtures of free fatty acids bound to bovine serum albumin as well as high density lipoprotein), and embryonic liver stromal feeder layers. In this context, a number of factors facilitated colony growth and/or a diffuse proliferative growth of the OCAP cells, including fibroblast growth factors, epidermal growth factors, tumor growth factor-α, platelet-derived growth factor, several interleukins, and especially GM-CSF and IGF-II. GM-CSF, hepatocyte growth factor, and IGF-II proved the most potent, inducing 20–25 colonies/10^5 cells as well as diffuse proliferative growth.

Although extensive studies are still required to characterize these hepatic progenitor populations and to complete the efforts to identify precise growth conditions in culture for the hepatic blast cells, we believe that our evidence increasingly supports the interpretation of the liver as a stem cell and lineage system with parallels to lineages in the skin, gut, and hemopoietic tissues and varying from them primarily in the kinetics of the lineage in the quiescent and regenerative states.

Lineage Model of the Liver. Based on extensive evidence summarized in recent reviews (1–3), we have hypothesized that the liver, even in the adult, contains progenitor cells located at or near the canals of Hering at each of the portal triads. These cells give rise to daughter cells that undergo a unidirectional, terminal differentiation process beginning at the portal triads and ending at the central vein (1, 2). Contrary to current opinion (8), in which liver stem cells are considered to be a silent cell population except after carcinogenic treatments, we agree with models proposed by Sell, Pierce, and van Potter (6, 7, 15) in which the liver is assumed to parallel lineage systems in the skin, gut, and bone marrow. In the lineage model, the liver progenitor cell populations are thought to contribute to the turnover of the liver cells in all physiological states, including quiescence and regeneration, and varying only in the kinetics. The plates of parenchymal cells are, therefore, thought to be lineages of maturing liver cells with age-dependent size, ploidy, growth, and differentiative potential. The heterogeneity of gene expression in the liver is interpreted as a lineage phenomena of "early," "intermediate," and "late" genes.

Figure 1 shows a schematic diagram of this lineage model.

The Driving Forces of the Lineages. In all well-characterized lineage systems (e.g., hemopoiesis, skin, gut), the determinants of a cell's position within the lineage and its phenotype at that position have been



Key: PV - portal vein; BD - bile duct; HA - hepatic artery; SE - sinusoidal endothelium over the Space of Disse; CV - Central vein. The portal triad and central vein are surrounded by a matrix which differs from the vascular basement membrane; see table below.

Matrix

Portal spaces: Col I, III, V, VI, VII; fibronectin; undulin; elastin. *Bile duct and vascular basement membrane*: Col IV; laminin; basement membrane HSPG; liver-specific HSPG; perlecan; entactin suspected. *HSPG: Heparan Sulfate Proteoglycan*	*Space of Disse*: Col I, III (sparse mid-acinar, more prominent in portal and central areas), VI, Col IV is present without other basement membrane components; fibronectin; undulin; tenascin; liver-specific HSPG; *additionally found on hepatocyte microvilli*: Col I, III, V, VI; fibronectin; liver-specific HSPG; syndecan; perlecan.	*Central spaces (matrix is continuous with the Space of Disse)*: Col I, III, V, VI; fibronectin; undulin. *Vascular basement membrane*: Col IV, laminin, liver-specific HSPG; perlecan.

Oval Cell Antigens:

OC.2 (270,38), OC.3 (374,3), HBD.1 (270,26).	H.4 (368.7), HBD.1 (270,26).	H.4 (368.7), H.5 (374.8), HBD.1 (270,26).	H.4 (368.7), HBD.1 (270,26).

Genes Expressed:

Bile Duct: GST-P, laminin; GGT, DPP-IV, desmoplakin.	*Periportal*: glutaminase, αfetoprotein, connexin 26.	*Expression uniform or in a gradient*: albumin, transferrin, α1 antitrypsin, tyrosine aminotransferase, connexin 32, cell CAM 105, DPP-IV, desmoplakin, L-CAM	*Pericentral*: α2 microglobulin (MUP), glutamine synthetase, 3-oxoacylCoA thiolase (induced), many P450's, pyruvate kinase.

Distibution of Metabolic Functions (Periportal versus Pericentral):

gluconeogenesis (glucose output), fatty acid oxidation, urea synthesis, ketogenesis, cholesterol synthesis, sulfations, bile acid secretion, monooxygenation	glycolysis (glucose uptake), fatty acid synthesis, glutamine synthesis (ammonia detoxication), biotransformation, glucuronidation

Distribution of Cellular Characteristics (Periportal versus Pericentral):

increased mitochondria; diploid; small; mononuclear; high growth stimulation by growth factors	increased smooth ER; polyploid; larger; binuclear; low growth-stimulation by growth factors

Figure 1. The hepatic acinus.

shown to include a combination of (i) autonomous intracellular mechanisms that are division number or time dependent, such as chromatin rearrangements or methylation events; (ii) gradients of soluble (autocrine, paracrine, or endocrine) factors; and (iii) gradients of insoluble extracellular matrix signals (1, 2).

Matrix Chemistry Gradients Are Defining Features of Epithelial Lineages and an Important Driving Force for Cellular Maturation. In well-characterized lineages, the matrix chemistry produced by progenitors is distinct from that by the maturing or mature cells. In general, the progenitors are associated with type IV collagen and forms of laminin and of heparan sulfate proteoglycans; more mature cells are associated with fibrillar collagens (gut) or with the loss of collagens (skin, hemopoietic cells). All maturing epithelia are associated with fibronectins and highly sulfated heparan sulfate proteoglycans as well as chondroitin sulfate and dermatan sulfate proteoglycans. Recent studies by Martinez-Hernandez and Amenta (16) and by others (18) have revealed that the extracellular matrix of the liver has many parallels with that in well-known lineage systems: the matrix associated with the presumptive liver progenitors, the ductular cells of the canals of Hering, is a classic fetal form of basement membrane consisting of type IV collagen, laminin, and forms of heparan sulfate proteoglycans. In the space of Disse, along the liver plates, there is a decline in the amount of matrix, a reduction in the amount of laminin and type IV collagen, and a conversion to fibrillar collagens, fibronectins, and more highly sulfated forms of proteoglycans such as a heparin proteoglycan and dermatan sulfate proteoglycan. The absence of the classic basement membrane structure in the space of Disse has been the reason for the liver being referred to as an "epithelioid" tissue, the assumption being that all epithelia are associated with basal laminae. However, an alternative way of interpreting these findings is to consider the liver as a proper epithelium with parallels to the skin: in both tissues, only the progenitors are associated with a basal lamina, and in both the more mature cells are associated with a decline in the amount of matrix and a conversion to fibrillar collagens and/or fibrillar collagen-associated matrix components (fibronectins, highly sulfated proteoglycans). In the epidermis and intestine, stem cells and their lineage of daughter

cells are positioned in a polarized organization that makes the cellular and matrix gradient apparent (reviewed in Refs. 1 and 2). The stem cells are located in the base of intestinal crypts or in special epidermal compartments of the skin. Adjacent to the stem cells are cells of the amplification compartment. These transitional cells move upward in the intestinal crypts or outward in the epidermis and undergo rapid proliferation, allowing for extensive cellular amplification. As the cells proliferate and mature, they are pushed away (skin) from the proper basement membrane and have mature cells, the squames, associated with less matrix (containing only proteoglycans and adhesion proteins) or upward (intestine) onto matrix whose composition changes in mature cells, the enterocytes. This process creates a "flow" of cells with varying degrees of maturity from the basal stem cells to the outer mature cells.

Implications of the Model of the Liver as a Stem Cell and Lineage System. In brief, the developmental profiles of many (but not all) tissue-specific genes parallel and complement the position-dependent pattern of gene expression seen in the liver plates within the acinus (reviewed in Refs. 1 and 4). For example, connexin 26 is expressed in fetal liver and is restricted to periportal localization in adult life, whereas connexin 32 is expressed primarily in adult liver and is expressed throughout the acinus (18). Similarly, many other liver-specific genes expressed early in development, early genes, are found throughout most of the parenchyma in early fetal life; with increasing age of the animal, expression of these genes is restricted to cells around the portal triad. Genes expressed at intermediate stages of development are present in adult liver in the middle of the acinus. Genes activated and expressed late in development, late genes, are expressed pericentrally in adult liver. There are important and intriguing variations on this basic pattern. For example, glutamine synthetase is expressed by all of the parenchymal cells early in development but is eventually restricted to a single cell layer around the central vein, a pattern shown to be strictly lineage position specific and not governed by direction of blood flow across the acinus (19). Recent studies (20) have suggested that an endothelial cell-derived matrix component(s) is a critical regulator of this tissue-specific gene. Therefore, the strictly pericentral localization is apparently due to the gradient of matrix molecules in the liver acinus.

In our reviews (1, 2), we discuss the numerous implications of the lineage concept for an understanding of liver biology or of clinical relevance. Here we present only three that are representative:

1. In vitro parenchymal culture. The ability of the parenchymal cells to divide and the number of cell divisions of which they are capable are predicted to be strictly lineage-position dependent. Therefore, periportal parenchymal cells should have greater division potential than pericentral ones (a prediction confirmed in studies by Gebhardt and associates [21]). This would explain the longstanding mystery of why primary cultures of liver, the most renown regenerative organ in the body, show such limited cell division in culture (22). Only the stem cells or their immediate descendants should be clonable.

2. Gene expression. Studies comparing liver-specific gene expression in embryonic versus adult tissues (reviewed in Refs. 1, 2, and 4) define several classes of genes: those diagnostic of the compartments (stem cell, amplification, differentiation); those expressed across compartmental boundaries (e.g., zonal); and those expressed early, middle, or late in the lineage but discretely in one or a few cells. Each class can be initially activated and expressed at very low, constitutive levels. Subsequently, the gene expression becomes transcriptionally regulatable by exogenous stimuli. Thus, stem cells and their transformed counterparts, hepatomas, are predicted both to express and to be able to regulate early genes such as α-fetoprotein and IGF-II, but not genes expressed later in the lineage. Minimally deviant tumor cells are hypothesized to be those that have progressed sufficiently through the lineage to have regulatable early genes and either constitutively or minimally transcriptionally regulatable expression of the liver's intermediate genes. By this model, no hepatoma should express late genes, since full progression through the lineage requires undisturbed regulation of differentiation, growth, and cell cycling, and this has indeed been observed (reviewed in Refs. 1 and 7). Posttranscriptional regulation should be evident at all stages of the lineage, although it too is likely to be influenced by lineage-dependent phenomena. Therefore, the lineage model easily accounts for the observation that most of the distinctions in gene expression between hepatomas and hepatocytes are distinctions in the regulation of fetal versus adult genes.

3. Implications of lineage mechanisms on disease processes. Based on these concepts, one may interpret the known phenotypic heterogeneity in tissues as resulting from lineage position, hormone, and matrix variations. By implication, disease processes should also be influenced by these phenomena, and there is much evidence to support this idea (1, 7, 23). Some viruses, for instance, have been found to exhibit activity, regulation, and cell lysis which is dependent upon the state of differentiation of the host cell. Drugs and radiation also show differential effects on the various stages of the lineage: stem cells and/or early precursors may have mechanisms that protect them from damage by viruses, by radiation, and drugs. For example, there is evidence that early precursors of normal hemopoietic cells express the multidrug resistance 1 gene (MDR1), and are therefore inherently drug resistant, while the more mature cells with remaining replicative ability do not

express multidrug resistance and are thus more readily damaged and killed by cytotoxic agents.

Complementing these concepts has been the renewed appreciation for the hypotheses of Pierce and van Potter (7, 15), which suggest that stem cells are the targets for oncogenic events. Within the context of the stem cell and lineage model, Pierce's hypothesis can be reinterpreted to suggest that oncogenes (e.g., carcinogens, viruses, radiation) kill mature cells at the end of the lineage and, as a result, induce chronic regenerative responses from the stem cells and early precursors (1). The result of this chronic proliferative state is an increased probability that a stem cell or one of its immediate descendants will undergo a transformation event. Therefore, some oncogenes do not necessarily target early cells specifically, but rather indirectly increase the opportunities for malignant transformation. Consequently, we assume that tumor cells are transformed stem cells or early precursor cells and will have a phenotype similar to normal cells at that stage of the lineage. Depending upon the lineage position and the mechanisms by which these early cells are transformed, they may be either unable to differentiate or able only partially to progress through their lineage. Thus, minimally deviant or well-differentiated tumors are those that can progress the most, whereas anaplastic tumors represent early stem cells that are blocked entirely from going through the lineage. Therefore, most of the so-called "tumor markers" are not genes activated or induced by a transformation event but rather normal genes representative of immature cell populations, and tumor progression is predicted to be a reverse lineage phenomenon.

An alternative view of the "streaming model" of the liver. One of the maturational models of the liver, that proposed by Arber *et al.* (24), incorporates a hypothesis that the liver cells are "streaming" from the portal triads to the central vein. The streaming model has provoked considerable debate and controversy, and in a recent study (25) investigators have claimed to have evidence refuting it. The debates have revolved around distinct sets of evidence that offer opposite interpretations: Arber *et al.* (24) found that labeling of cells begins at the portal triads and slowly progresses across the acinar plates, data used to support the streaming model. Ponder *et al.* (25) found that adult hepatocytes, marked with retroviral vectors and introduced into the liver, remained at positions throughout the acinus and were capable of proliferation after partial hepatectomy. They suggest that these data argue against the streaming model. An elegant series of studies from Brinster's laboratory (26)[11] may resolve this controversy. Sandgren *et al.* (26) prepared transgenic mice using a urokinase gene coupled to an albumin promoter. The transgenic mice are born with white livers in which all mature parenchymal cells are missing. Those that live do so because of a small percentage of cells that lose the transgene and go on to reconstitute the livers with mature cells. They have described the reconstitution process as a series of colonies that expand outward, having portal triads at the periphery and a central vein at the centers.[11] Thus, migration phenomena of stem cells is outward (not inward toward the central vein) until some acinar dimensions are reached dictated by unknown mechanisms. The resulting daughter cells then undergo maturation not requiring movement toward the central vein. Therefore, an alternative model could be a slow loss of cells at the central vein and compensatory migration of cells outward at the portal triads to maintain the acinar dimensions.

[11] J. A. Rhim, E. Sandgren, J. Degen, R. Palmiter, and R. Brinster. Replacement of diseased mouse liver by hepatic cell transplantation in albumin-urokinase transgenic mice (manuscript in preparation).

Summary

The mechanisms governing liver growth and differentiation are likely to parallel that in all other tissues, including those of rapidly renewing tissues such as gut and skin. The lessons learned from the numerous studies on these other tissues is that they are organized architecturally and mechanistically around stem cells and lineages of their descendants that undergo a unidirectional, terminal differentiation process involving apoptosis or programmed cell death. Cell size, ploidy, growth potential, and tissue-specific gene expression are position dependent along the lineage. Success with our efforts and those of others to isolate liver progenitors and to establish them in culture should offer considerable opportunities to analyze this lineage process.

This article is dedicated to Harriel Martinez, whose passionate concern for animals and whose energetic and gifted assistance immeasurably contributed to our studies in all projects, especially these stem cell studies. Her untimely death this year at the age of only 49 was a loss to us all. We will remember especially her devotion and the exuberance she demonstrated in everything she did.

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