Gut

*Leading article*

# What keeps hepatocytes on the straight and narrow? Maintaining differentiated function in the liver

The liver in general and hepatocytes in particular maintain an awesome array of function—synthesis, catabolism, intermediary metabolism, and detoxification. Recent work has enlarged our knowledge of the factors that permit this range of function to be expressed, and in particular the complex inter-relations of cellular and non-cellular elements in the liver that contribute to this. The functions expressed by the liver obviously vary over time. For example, growth and regeneration are associated with a functional repertoire that differs from that of mature adult liver, as in most tissues proliferation is associated with reduced expression of normal differentiated function and expression, generally short-lived, of cell cycle and other replication associated genes.[1] Disease alters function, although there is some support for the "intact hepatocyte" hypothesis suggesting that in chronic disease the surviving hepatocytes continue to express a relatively normal functional array.[2] In acute disease, however, there are many changes in function such as the upregulation of acute phase protein production as well as the initiation of replication to regenerate liver mass.

Understanding the conditions necessary for a normal hepatocyte to express its full functional repertoire is important as science strives to recreate normality in a diseased liver. That understanding is also fundamental to attempts to establish fully functional cultures of liver cells, whether for experimental studies or with the aim of creating a bio-artificial liver. This article highlights three main areas relevant to maintaining expression of normal differentiated hepatic function: the multiplicity of cell types present, the relation between cells and non-cellular elements, particularly extracellular matrix (ECM), and intracellular events that modulate specific gene expression.

## The subpopulations of the liver

Hepatocytes comprise the main metabolically active cells of the liver, the majority of hepatic mass, and about 80% of the cell number. The hepatocyte cords are separated from the portal blood by the sinusoidal lining cells, comprised predominantly of endothelial cells and of Kupffer cells of macrophage lineage. Despite the predominant role of hepatocytes in protein synthesis in the liver, some of the protein synthetic function resides in non-parenchymal cells—for example, binding proteins for insulin-like growth factors are sinusoidally expressed.[3] The hepatic stellate cells lie in the perisinusoidal space and play a major role in the elaboration of growth factors, and particularly in the elaboration of ECM.

## Interactions between subpopulations

The relations between non-parenchymal cells and hepatocytes, and the effect of the former in modulating the function of the latter, are many and complex. They include effects which reflect the gross architecture of the liver, cell-to-cell effects (which may be paracrine or dependent on cell-to-cell contact), and indirect effects dependent on matrix production.

Architecturally, sinusoidal lining cells constitute a functional unit at the borderline between the hepatocytes and the blood in which position they modulate the access of blood borne substances to hepatocytes.[4] The endothelial fenestrations control the size of macromolecules transferred into the Space of Disse. Both endothelial cells and Kupffer cells, being phagocytic, can prevent particulates such as bacteria and immune complexes in portal venous blood accessing hepatocytes. However, sinusoidal lining cells are not inevitably protective. Kupffer cells can initiate hepatocyte damage—for example, by the production of reactive oxygen intermediates, and in some toxic models Kupffer cell depletion prevents damage.[5]

Identifying the cell-to-cell influences of non-parenchymal cells on expression of normal hepatocyte function has depended largely on the use of primary cultures of liver cell subpopulations separated from rodent and more recently human liver by enzymatic perfusion and differential centrifugation or elutriation. Using conditioned medium from non-parenchymal cells from normal rat liver, Casteleijn *et al* demonstrated enhancement of hepatocyte glycogenolysis and modulation of the phosphorylation state of proteins secreted by hepatocytes, in each case attributed to prostaglandins.[6 7] Kupffer cell products from normal livers have also been reported to reduce protein synthesis and to depress cytochrome P-450 activity.[8] In inflammation, activation of Kupffer cells releases a variety of cytokines, leading to the well known modulation of hepatocyte protein synthesis that constitutes the acute phase response: upregulation of C-reactive protein, alpha-

**Abbreviations used in this paper**: ECM, extracellular matrix; EHS, Engelbreth Holm Swarm mouse sarcoma-derived matrix; RGD, arginine-glycine-aspartic acid; LETF, liver enriched transcription factors; HNF, hepatic nuclear factor; CEBP, CAAT enhancer binding protein. and editorial board.

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1-acid glycoprotein and fibrinogen production, and down-regulation of—for example, albumin and transferrin.[9] Indeed, it is worth noting that the approach of isolating subpopulations to investigate normal liver function can be confounded by the artefactual induction of an acute phase response, if the preparative procedure itself leads to modulation of cytokine secretion by Kupffer cells.

Co-cultures of different liver cell subpopulations can vividly illustrate interactions that occur between hepatocytes and non-parenchymal cells. Morin and Normand found that a pure population of hepatocytes demonstrated a progressive decrease in albumin production over a two week period, but when co-cultured with endothelial cells albumin production was maintained.[10] Many others have confirmed that co-cultures of hepatocytes and non-parenchymal cells both survive and function better than isolated hepatocytes. A graphic in vivo demonstration of this was the observation that although isolated purified hepatocytes do not survive long term on implantation into the relatively barren milieu of the peritoneum, a mixed population of hepatocytes and non-parenchymal cells survive long term, proliferate and maintain metabolic function.[11]

What is the mechanism of the beneficial effects of non-parenchymal cells on hepatocyte survival and proliferation? Despite the benefit demonstrated with conditioned medium and in co-cultures with cell-to-cell contact between different subpopulations, much evidence indicates that ECM is the most important factor.

**Extracellular matrix**

Extracellular matrix provides structural support to tissues, but it is a far more significant component than merely a scaffold. ECM affects the expression of function and the morphology of hepatocytes and other subpopulations. ECM is widely distributed in tisues. There are some chemical differences in matrix from different organs although there are broad overall similarities.[12] The major components of liver ECM are proteins and proteoglycans. The proteins include fibronectin, vitronectin, laminin and tenascin, the collagens (mainly type I and minor quantities of types III, IV and VI), and elastin; the proteoglycans are a heterogeneous group of proteins containing glycosaminoglycan (GAG) side chains. The components vary in distribution and in the Space of Disse, the prime site for affecting hepatocytes, fibronectin seems to be of primary importance. ECM can be secreted by most of the liver's subpopulations, but the stellate cell population is particularly active—a function upregulated in inflammation and cirrhosis.[13]

Although the ECM is only a small component of the liver, it plays a major role in the modulation of many biological processes of hepatocytes, including cell migration, differentiation, repair, and development. Hepatocytes are anchorage dependent cells, structurally and functionally polarised in vivo, and maintenance of polarisation involves both cell–cell and cell–matrix interactions.[14 15] Unlike other epithelial organs which have two well formed basement membranes and a substantial ECM between the endothelial and epithelial cells, the liver's configuration is unique, with a loose ECM intervening between fenestrated endothelial cells and the hepatocyte epithelium, and no basement membranes.

Hepatocytes cultured on plastic attach poorly, function badly, and soon die. Survival and function can be prolonged by culturing on the readily available source of ECM, a thin layer of type I collagen from rat tails, but the cells remain relatively flat, with rapid diminution in hepatocyte specific functions such as albumin synthesis. However, culture on complex ECMs leads to prolonged survival, continued expression of differentiated function, and preservation of a more normal, near-cuboidal cell shape.[16] These survival and performance advantages can be provided by a variety of extracellular supports: thick gels of collagen, laminins extracted from pig liver, and in many experimental studies the Engelbreth Holm Swarm mouse sarcoma-derived matrix (EHS) which contains laminin, type IV collagen, heparan sulphate proteoglycan, entactin, and other components.[17] EHS will maintain albumin secretion at normal levels for weeks, and sustains the activity of cytochrome P-450. Complex matrix re-establishes the polarity of the cell membrane, cytoskeleton and cytoplasmic organelles of hepatocytes. In contrast to the flattened cells with prominent intracellular microfilaments on thin collagen, on EHS hepatocytes cluster in multicellular aggregates maintaining a rounded shape and exhibiting prominent endoplasmic reticulum.[18] Such observations indicate that the similarly constituted ECM in the Space of Disse is biologically active, playing a key role in hepatocyte differentiation and polarity.

How does ECM exert the functions of inducing attachment, imposing polarity and maintaining differentiated function of attached hepatocytes? Surface integrins, which constitute a large family, provide a series of receptors for the molecules of the ECM. Typically these receptors bind specific repetitive amino acid motifs found in matrix proteins, such as the RGD (arginine-glycine-aspartic acid) tripeptide.[19] Via these integrins, ECM affects many cell functions such as organisation of the actin cytoskeleton, cell adhesion, migration and invasion, intracellular signalling pathways, and changes in gene expression.

Various alterations in the integrin receptors, including receptor clustering as well as ligand occupancy, with or without tyrosine phosphorylation, add a further level of control.[20] More than 20 signal transduction molecules are recruited depending on these alterations, which provide ample scope for the diversity of molecular responses initiated by integrin–ECM interactions. For example, aggregation of integrins without ligand binding leads to rapid transmembrane accumulation of a multitude of signal transduction molecules, but only one cytoskeletal molecule (tensin). Integrin aggregation and ligand occupancy without phosphorylation affect disposition of some cytoskeletal proteins like tensin, vinculin, talin, and alpha actinin, whereas aggregation, occupancy and tyrosine phosphorylation are required for aggregation of other cytoskeletal molecules such as F-actin, paxillin and filamin.

Although those interactions involving receptor clustering, occupancy and phosphorylation are conventional, a novel type of regulation leading to altered gene expression is emerging for the ECM–integrin axis, with the demonstration that mechanical stress can directly affect intracellular molecular events leading to protein synthesis.[21] It has been shown in human umbilical endothelial cells that mechanical stress leads within a few minutes to recruitment of mRNA and ribosomes to a focal adhesion complex composed of β1-integrin, talin, actin, and vinculin. This recruitment can be mimicked with RGD peptides. Although not yet fully explored, in hepatocytes calcium signalling can be modulated when shear pressure is induced—presumably on the cytoskeleton—by binding monoclonal antibody to an integrin and mechanical loading in vitro.[22] Such experiments may offer clues to an unappreciated rationale for the high rates of blood flow that occur through the intact liver in vivo.

Earlier discussion emphasised that non-parenchymal cells largely provide the ECM scaffold for hepatocytes. How far does that mechanism account for the effectiveness of non-parenchymal cells in maintaining differentiated hepatocyte function? Bader *et al* designed three dimen-

sional models, based on matrix overlaid hepatocytes, into which hepatic non-parenchymal cells can be incorporated mimicking the plate organisation of the liver.[23] In such models, the contribution of the matrix appeared paramount, and addition of non-parenchymal cells had no additional or synergistic effect. Such models, however, offer the potential to investigate communication between hepatocytes and non-parenchymal cells in the liver, and may be particularly valuable in exploring the consequences of activation of non-parenchymal cells in inflammatory states.

**"Spheroidal cultures"**

A major stimulus to the understanding of the maintenance of differentiated function is the wish to develop functional ex vivo liver tissue for use as a bio-artificial liver. Cultured hepatocytes can be induced to form "spheroids" in vitro, either by microgravity or elliptical shaking, or by the process of cell encapsulation which in itself imposes geometric structure in cells. Such "spheroids" demonstrate improved liver specific functions.[24 25] Alginate is one of several substances used for cell encapsulation, and data from our laboratory using cell lines, and from others using primary cells, indicate that this inert substance has notable effects on maintenance of cell function, reminiscent of the effect of exogenous ECM.[25] It remains to be seen whether alginate encapsulation is effective because it imposes physical restraints in which cells adopt a morphology similar to that seen in vivo, or whether encapsulation works by allowing ECM secreted by the cells—for hepatocytes themselves can secrete matrix[26]—to be retained in the microenvironment of the cells and to exert its effect on the resulting "spheroids".

**Transcription factors**

It seems clear that maintenance of near cuboidal hepatocyte morphology, appropriate ECM support, and cell-to-cell communication combine to maintain differentiated hepatocyte function.The processes of signal transduction initiated by ECM–integrin interactions are beginning to be unravelled. At the nuclear level, the processes that control hepatocyte specific functions are also becoming clearer, even though the link from the signal transduction pathways remains largely uncharted.

The promoter sequence of each gene is unique and contains a number of recognisable motifs which allow regulation of expression of that gene by a group of transcription factor molecules. These protein messengers bind to their complementary promoter sequences of genes. The factors may be sequestered in an inactive form in the cells, and released to access the promoter sequence when activation is required. There are several classes of transcription factors, but they all act via one of two basic mechanisms: they bind to a consensus sequence to promote transcription of the DNA into RNA (i.e. activation) or they bind to inhibit transcription of that particular gene (i.e. extinction). Some transcription factors are ubiquitous and those often bind to sequences within a promoter region such as the TATA box which is found in most (but not all) gene promoters. Others, however, are enriched in a particular cell such as the hepatocyte and these were originally thought to be all that was required to confer liver specific function. It is now known that none of the transcription factors described to date is entirely tissue specific, although they are often found in only one or two tissues.

Transcription factors at high levels in the liver are referred to as liver enriched transcription factors (LETFs), although their names often reflect the original supposition that those factors were unique to the liver—for example, hepatic nuclear factors 1–6 (HNF1–6).[27 28] These HNFs, and the CAAT enhancer binding proteins (CEBP α, β, δ, and γ), another class of transcription factors, combine to regulate liver specific function.

A number of target genes for the LETFs have been described reflecting a wide spectrum of liver specific differentiated function. As an example HNF3 target genes include transthyretin, albumin, α-fetoprotein, ApoB, ApoA1, transferrin, α-1-antitrypsin, tyrosine aminotransferase, cholesterol-7α-hydroxylase, phospho-enol-pyruvate carboxy kinase, phopshofructokinase 2, aldolase B, and cytochrome P-450 2c6. HNF3's role is exemplified by hepatoma cells expressing a non-functional HNF3 mutant which cease to express many of these HNF3 target genes. Conversely, transfection of the HNF4 gene into previously de-differentiated hepatoma cells which do not express HNF4 cells causes an increase in differentiated function.[29] At a more clinical level there is a case report of an individual with severe factor VII deficiency whose phenotype seems to be due to a single nucleotide transposition in the gene promoter in the region that should bind HNF4, leading to a 93% decrease in promoter activity in vitro.[30] Transcription factor activity is, however, very complex, and there is evidence of cross regulation of gene expression by different members of the HNF family on one another—for example, HNF1 and HNF4 regulate the action of one another's promoter, and HNF6 regulates the transcription of liver specific genes partly by its action on the HNF3 gene.[27] As would be anticipated, a change from a non-proliferating to a proliferating state, induced by hepatic resection for example, is associated with striking changes in LETF activity modulating changes in gene expression.[31] CEBP-β has also been implicated in the control of the acute phase response and more recently there is evidence that it plays a role in cell cycle control.[32]

**Summary**

The conventional physiological requirements of fluid flow, oxygenation, nutrition, and removal of waste products are only the tip of the iceberg of the requirements for maintaining differentiated hepatocyte function in vivo or replicating it in vitro. Maintenance of near-normal cell morphology and an extracellular support acting not merely mechanically but by specific molecular interactions, are required for maintenance of function in the resting liver. Response to change, be it the presence of inflammation or the necessity for growth, induces a sequence of events to which the functional repertoire adapts, and those processes are clearly dependent on cooperative interactions among the different cell populations in the liver.

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