Detection and Characterization of Rat Hepatic Stellate Cells in a 3-Dimensional, Perfused, Liver Bioreactor

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Abstract

One of the major challenges in liver research today lay in the understanding of the complex relationship between liver structure and function. The highly orchestrated events that take place in the liver to maintain homeostasis require the presence of all liver cell types. *In vivo* experiments offer only a snapshot of the liver, and usually involve perturbation of normal function through injury or experimental disease. The role of cell-cell interactions in maintaining normal liver function is far less understood than in pathological conditions. This may be because of the lack of methods in monitoring normal function *in vivo.* Culturing systems may capture pieces of the puzzle, but often capture only two cell types, and involve mediators presented to the cells in concentrations much higher than physiological values. In addition, the liver lobule contains a 3-dimensional metabolic zonation, and liver cell types comprise a heterogeneous population from the portal triad where the blood flows into the sinusoids to the central vein area where the blood flows out of the sinusoids. Liver cell types are dynamic responders to environmental cues from soluble factors to heterotypic cell interactions, to extracellular matrix proteins. Therefore, a system that serves to promote the health of all liver cell types through a 3-dimensional, perfused scaffold, and allows for self-organization of the liver cells in response to the engineered environment, would serve as a useful tool in understanding some of these complex, orchestrated events.

In the research presented here, methods were developed to detect and characterize the heterogeneous population that makes up the hepatic stellate cell population inside the liver bioreactor (Griffith et. al). This cell type, comprising a small percentage of total liver cells (approximately 5-10%), rapidly change their phenotype in response to liver injury, and, similarly, upon being taken out of the liver and cultured in 2-D on tissue culture plastic. This cell type plays a major role in relaying signals to and from both parenchymal and other nonparenchymal cells; stellate cells are also in charge of maintaining the components of the Space of Disse and are the key players in the pathology of liver fibrosis. They are found to be tightly complexed with sinusoidal endothelial cells and at the same time found to be tightly interacting with hepatocytes, sometimes even penetrating the hepatic plate. Stellate cell function, is therefore, highly dependent upon its interaction with other liver cells in maintaining the tightly knit structure-function relationship. For this reason, the liver bioreactor serves as a highly useful tool, in order to better understand the hepatic stellate cell's role in these complex situations.

In this dissertation, detection and characterization methods are developed with the goal of capturing the heterogeneous stellate cell population as a whole with a toolbox of characterization markers, as well as to learn more about their functionality and location within tissue structures. These tools can be used to detect and characterize the population at various timepoints during tissue formation inside the bioreactor, as well as after exposure to physiologically-relevant concentrations of toxins, viruses, pharmaceuticals, etc. In addition to increasing our basic understanding of the role of the hepatic stellate cell, the bioreactor may aid in the search for better therapeutic agents in the reversal of fibrosis and other liver diseases.

Thesis Supervisor: Linda Griffith

Title: Professor of Biological Engineering, Professor of Mechanical Engineering, Director of BPEC

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Table of Contents

Page Number

List of Figures

Page Number

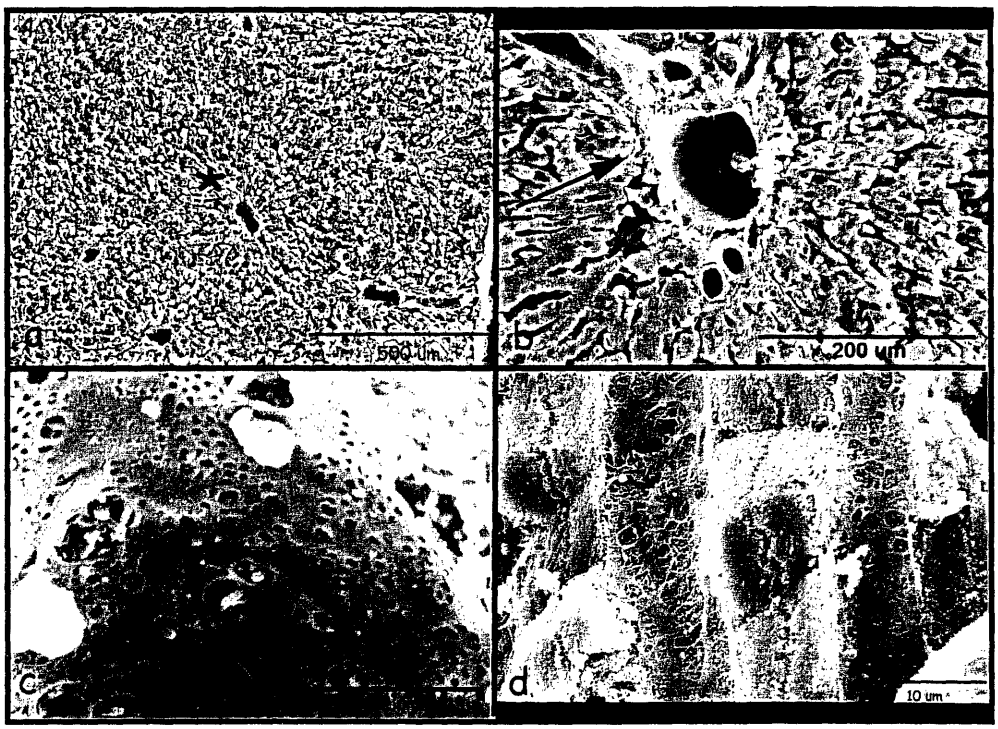
Background and Significance

Activated hepatic stellate cells have long been established as the primary source of ECM deposition during liver injury and fibrosis, independent of the underlying disease (1). As the complexity of this activation becomes apparent, new tools need to be developed in order to study these orchestrated events. Proteomics and gene arrays help to analyze these processes, and the liver bioreactor (2,3) may serve as an ideal environment to monitor an *in vivo*-like process.

Liver function is essentially inseparable from its function. For this reason, liver cells do not function as they do in vivo when cultured in 2-dimensions using traditional plating methods. In this introduction, the highly structured liver lobule will be dissected to reveal the hepatic stellate cell niche, the heterogeneity of the stellate population, and its role in tissue repair and disease. The stellate cell responds to different environments by changing its phenotype and functionality; this occurs during liver injury and during traditional culturing on plastic dishes. Therefore, it would be quite useful a need to study the stellate cell in a three-dimensional, liver-like model, based on this idea of structure/function correlation, in order to better understand how the stellate cell contributes to maintain homeostasis in the liver. This research aims to develop methods in order to use the 3-D dimensional liver bioreactor to detect and characterize hepatic stellate cell behavior in the remodeling of liver tissue and its interactions with other liver cell types.

Liver Background

Figure 1: Scanning Electron Micrographs of Adult Rat Liver



a. SEM of liver lobule, * points to central vein. b. SEM of portal triad, arrow pointing to the portal vein. c. SEM of a fenestrated endothelium in a sinusoid, with other liver cell processes appearing through. d. SEM depicting the alternating hepatic plate and sinusoid structure of the liver

The liver is the metabolic center of the body and is a highly, vascularized organ with a function that is inseparable from it's structure. The liver can be divided into hexagonal lobules (figure 1a) consisting of a central vein with single plates of hepatocytes, delineated by small, porous blood vessels or sinusoids (figure 1 d), radiating out to six portal triads (figure 1b) (the portal vein, hepatic artery, bile duct, and also lymphatic vessels and nerves). Each lobule is approximately 200-300 microns from central vein to portal triad, and the liver is composed of thousands of these lobules arranged further into liver lobes. There is a metabolic zonation from the central vein area

to the portal triad area of the liver, where there is differential glucose metabolism, vitamin A storage, production of proteins, metabolic enzyme expression, etc (4). The blood flows in the sinusoid from the portal triad into the central vein. Through coculturing of different liver types outside of the body, it has been realized that the many functions of the liver depend on the cooperation of nonparenchymal and parenchymal liver cells. Each liver cell contributes greatly to the structure/function relationship, and thus liver cells communicate closely with each other through three main mechanisms: gap junctions, paracrine signaling, and juxtracrine signaling (which requires close apposition of cell membranes) (4).

Sinusoidal Structure and Cell Types

The liver sinusoid contains three main cell types: the sinusoidal endothelial cell, the Kupffer Cell, and the Ito or stellate cell, all in close communication and not anchored down by a true basement membrane but rather situated next to a fluid area with extracellular matrix (ECM) components called the Space of Disse; the Space of Disse lays between the sinusoidal wall and the hepatocyte plate. The Kupffer cells are the resident macrophage of the liver and can be found inside the sinusoidal lumen. The sinusoidal endothelial cell is a specialized liver cell that contains numerous, dynamic fenestrations which control the direct communication between the blood and the hepatocytes (figure 1c). In the Space of Disse, and closely complexed with both the sinusoidal endothelial cell on one side and hepatocytes on the other end, lives the Hepatic Stellate Cell (HSC). This cell comprised 10-15% of the total number of liver cells (4,5).

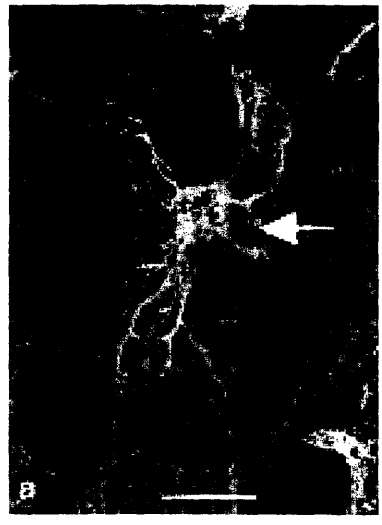
Hepatic Stellate Cells Background

Morphology and Ultrastructure

As mentioned above, HSC's are not embedded in a true basement membrane like pericytes in many tissues, but rather in a fluid space containing nonfibrous matrix proteins and glycoproteins called the Space of Disse. Stellate cells have many cytoplasmic extensions, those either complexed with the sinusoidal endothelial cells which encircle the sinusoid, and those which penetrate the Space of Disse and the hepatic plate and may even reach into a neighboring sinusoids (figure 2) (4). These processes can vary in range from 60-140μm (4,5). These processes contain an elaborate cytoskeleton and several organelles and allow one stellate cell to be able to contain more than one sinusoid and several hepatocytes at the same time. One characteristic feature of HSC's is the presence of large, membrane-bound lipid droplets, where serve as vitamin A storage composed of retinoids. These lipids droplets can reach up to 8 μm in diameter, but vary extensively depending on the location of the stellate cell in the liver lobule as well as the health and diet of the animal (4). The ultrastructure of a hepatic stellate cell consists of an oval nucleus, compressed with lipid droplets, and the presence of a large, extensive Golgi apparatus, few mitochondria and lysosomes, and a very well-developed and dynamic cytoskeleton, including both actin and intermediate filaments as well as bundles of microtubules. The number and kinds of filaments differ depending on the health of the organism and the organism species itself and include alpha smooth muscle actin, desmin, vimentin, and many proteins characteristic of neural origin, such as glial fibrillary acidic protein (GFAP) and synaptophysin. These proteins have been shown to be specific in the liver to stellate cells both *in vivo* and *in vitro* (4,5).

Figure 2: A Hepatic Stellate cell
This stellate cell in rat liver stained with a desmin antibody in green, contains the characteristic dendritic, cytoplasmic processes which extend into neighboring sinusoids. Projecting out of these processes, are hepatocyte-contacting spikes, where stellate cells penetrate into the hepatic plate.

Pictures courtesy of Oikawa et al., Journ of Gastroent and Hepatol (2002) 17. 861-872



In Vivo Functions

General Plasticity

In the liver, stellate cells exhibit a range of phenotypes ranging from a quiescent, fat-storing cell in normal liver, to a sustained activated, proliferative, myofibroblast-like phenotype during liver disease, importantly in liver fibrosis. Their phenotype highly depends on their environment and rapidly can change in reaction to liver injury and healing. This transformation from a quiescent to active phenotype which involves loss of lipid droplets and a very different protein expression profile, can be seen when stellate cells are isolated and cultured on plastic substrate. The cells typically show morphological signs of activation at approximately three days, followed by complete activation by one week in culture. Stellate cell activation is accompanied by a change in functionality as well (4).

Quiescent Functions

Quiescent stellate cells in normal liver have several essential roles in maintaining normal liver function. Approximately 70-80% of the body's vitamin A is stored in stellate cells in the form of lipid droplets, and they directly participate in vitamin A metabolism. HSC's also serve to modulate sinusoidal blood flow by contraction and relaxation and direct, close contact with sinusoidal endothelial cells. Stellate cells are also in charge of regulating the components of the Space of Disse by synthesizing and releasing ECM components and metalloproteinases, an important functions that are disrupted during liver fibrosis. HSC's are also involved in erythropoietin synthesis and synthesis of the plasminogen activation system. They maintain direct communication with both hepatocytes and sinusoidal cells and interact with them through both paracrine and juxtacrine mechanisms. Quiescent rat hepatic stellate cells are known to express many unique, intermediate filaments, glial fibrilliary acidic protein (GFAP) and desmin. However, desmin staining appears to occur in a zonal gradient (4). There is positive

staining around the periportal areas of the liver, with decreased staining in intermediate zones, followed by little or no staining in the centralobular regions. GFAP positive stellate cells are observed all throughout the sinusoids, excluding the large vessels; this level of staining could also be heterogeneous within the sinusoids, depending on the length of stellate cytoplasmic processes and degree of lipid storage (6).

Heterogeneity in the Liver Lobule

The normal liver also contains a very heterogeneous population of HSC's displaying phenotypical differences in a lobular gradient similar to that for hepatocytes. This ranges from a vitamin A-rich stellate cells with many, extensive cytoplasmic processes and little or no desmin expression, to those that are vitamin A-poor stellate cells, with few extensions and a high level of desmin expression, but all are located perisinusoidally, among the hepatocyte plates (4,6).

Hepatic stellate cells do not appear to be heterogeneous in their cell numbers and density in the different zones of the liver as shown by the gold chloride preparation (7). However, they do appear to be quite heterogeneous in their number and form of cytoplasmic processes, their extent of vitamin A-storing lipid droplets, and their expressed proteins, that seem to be an innate characteristics depending on their location in the liver lobule (7).

Activation of Stellate Cells *In Vivo*

Hepatic stellate cells exhibit a quiescent, fat-storing phenotype, as well as an activated, proliferative phenotype, characterized by a significant change in gene expression, including upregulation of extracellular matrix components, change of shape from dendritic to fibroblast-like, loss of lipid droplets (see Figure 3). Activation is also associated with an increased expression of fibrous extracellular matrix proteins, such as collagen I, as well as many other proteins. This activation occurs in response to liver tissue injury, change of environment such as when cultured outside of the liver, during liver regeneration after partial hepatectomy, is seen to be the cause of liver fibrosis during several disease states, and occurs in many types of liver cancer (8). Stellate cells are

suspected to at least partially activate during liver regeneration after partial hepatectomy to aid in tissue remodeling and angiogenesis. Despite the well-known characteristics of stellate cells in response to environmental change and after addition of certain growth factors and cytokines, the initiation events and potential reversibility of hepatic stellate cell activation are poorly understood (1,4,5).

Figure 3: Sinusoidal Structure in Normal and Fibrotic Liver

Coloring: peach:hepatocyte plates, blue: stellate cells, pink:sinusoidal endothelial cells, green: biliary epithelium, purple: kupffer cells, yellow: deposition of extracellular depicting fibrotic areas.

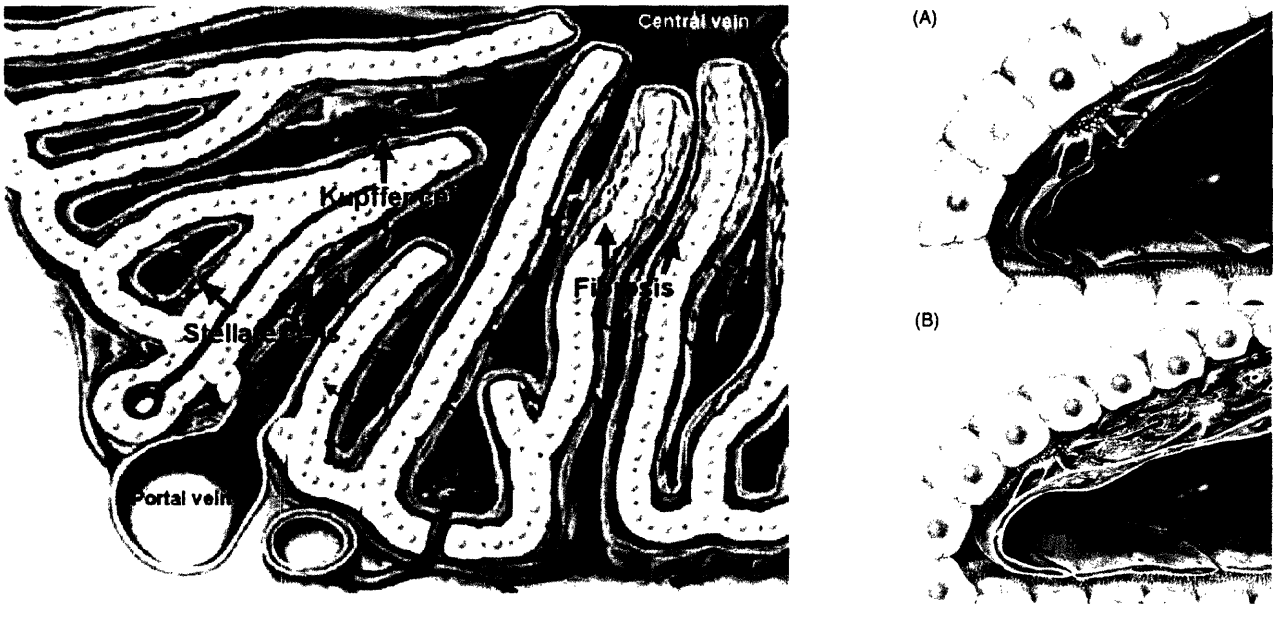


Figure 3a and 3b show the differences observed between a normal and fibrotic liver that contains activated stellate cells. This activation corresponds with loss of stellate lipid droplets, deposition of basement membrane proteins shown in yellow, stellate cell proliferation and expression of myofibroblastic proteins, loss of endothelial cell fenestrations, contraction of the sinusoid, and loss of hepatocyte microvilli.
Figure taken from Friedman et. al.

In Vitro Activation of Hepatic Stellate Cells

General Characteristics of *in vitro* Activation

When HSC's are taken out of the liver and cultured in tissue culture plastic, they lose their quiescent, vitamin A-storing, stellate phenotype and fully take on their activated, proliferative, myofibroblast-like phenotype after 7 days. This activation in rat HSC's includes loss of lipid droplets beginning after 24 hr. in culture, expression of alpha-smooth muscle actin (SMA), loss of expression of glial fibrilliary acidic protein, transient increased expression of desmin, and increased expression of N-CAM and synaptophysin, two neural markers. Activation of the total population of cultured stellate cells occurs approximately by seven days in culture, and this is characterized by loss of any GFAP or desmin staining, and complete expression of SMA in the population. This phenotype is similar to that seen in *in vivo* response to liver injury (1,4,5). However, because the stellate cells are no longer in a liver-like structure and are isolated from other liver cells types, one cannot learn about the effect of this activation on the liver as a whole, and the role of HSC's in liver remodeling vs. propagation of liver fibrosis. In addition, many of these conditions *in vivo* are a result of chronic conditions that take place over long periods of time, so a culture system which supports the long-term, differentiated cultured of all liver cell types, would be extremely useful in studying these long-term, complex events.

Differences Between *In Vivo and In Vitro* Activation

Kristensen, et al identified 43 proteins from the rat stellate cell proteome that showed altered expression during activation in vivo or in vitro, compared to freshly isolated, quiescent stellate cells. Although, 27 of these proteins showed similar patterns in injury-induced fibrotic livers compared to cultured, activated stellate cells (17 of which were novel), 16 proteins showed different expression levels (more than three-fold) between in vivo and in vitro, activated stellate cells. Such proteins included cofilin, actin variants, destrin, heat shock proteins, 5' nucleotidase, reticulocalbin, and RHO GDP-

dissociation inhibitor. These significant differences could be due to the 2-D culturing environment of the cells, and, therefore illustrate the need for a more in-vivo-like model of 3-D liver tissue, in order to study hepatic stellate cell activation events corresponding to those in the liver (9).

It has been shown that cultured, activated HSC's can partially restore their quiescent phenotype when cultured on a collagen gel or Matrigel, as seen by loss of the highly proliferative state, accompanied by the restoration of the long processes characteristic of the cells. There has also been evidence in vivo that shows reversibility of liver fibrosis, correlating with a loss of the activated markers of stellate cells (8).

Species Differences Concerning Hepatic Stellate Cells

There have been significant species differences noted in the literature that occur between rat hepatic stellate cells, mouse and other experimental animal stellate cells, and human hepatic stellate cells (4). For example, a subpopulation of quiescent, rat stellate cells are known to express desmin, whereas quiescent human stellate cells do not. Also, quiescent human stellate cells express smooth muscle actin (SMA), but only activated rat stellate cells express SMA. These and other differences in receptor expression should be taken into consideration, and provides a rationale for the use of human liver or stellate cells for study (4). In contrast to using human liver samples, animal models allow for controlled experimental situations, and therefore a model that supports the growth, differentiation, and manipulation of human liver cells over long periods of time could be quite useful in overcoming species differences.

Stellate Cells in Fibrosis

Cirrhosis has been defined as the end stage consequence of fibrosis of the hepatic parenchyma resulting in nodule formation. Both diseases affect at least one million patients in the United States and hundreds of millions worldwide. They result from a prolonged wound healing response to disease or chronic injury. Fibrosis occurs because of a sustained "activated" response of stellate cells, capillarization of sinusoids, and subsequently necrotic parenchyma (10).

Fibrosis can be caused by chronic injury (after months or years) resulting from a variety of causes, such as alcohol or drug abuse, hepatitis B and C, autoimmune disease, metabolic diseases, etc. There is much evidence that fibrosis is reversible (10,11), and therefore, it is important to study the initiation of these events, so therapies can be developed to reverse or slow the progression of disease. Restoring the normal ECM components of the Space of Disse is essential to restoring normal liver function, and this has illustrated the importance that all cellular and structural components might be needed when studying differentiated function *ex vivo* (11). Paradoxically, the role of cell-cell interactions under normal conditions is far less understood than in pathological ones (4).

Hepatic Stellate Cells comprise approximately 5-15% of all liver cells (4,5), and during the poorly understood initiation events, stellate cells seem to lose their characteristic cytoplasmic processes (which contact many other liver cells) and retinoids and become "activated." They deposit basement membrane-type ECM in the Space of Disse such as collagen I, increase their contractility, cause chemoattraction of white blood cells, etc. Through paracrine, and autocrine stimulation with cytokines and ECM, this "activated" phenotype is sustained. During recovery, after experimental or acute liver injury, the number of these activated stellate cells decrease (11). There are important questions about this process that have yet to be answered: can an activated stellate cell in the liver revert back to its quiescent phenotype? Alternatively, are activated stellate cells undergoing apoptosis and being replaced by new quiescent stellate cells, or is it a combination of reversion and apoptosis? An *in vitro* culturing system which captures in-

vivo-like liver function that can be monitored as activation events are occurring would be extremely valuable in answering these questions involving the hepatic stellate cell.

Hepatic stellate cell heterogeneity also matters in terms of liver fibrosis pathogenesis and therapy. It may be that only a subpopulation of these cells activates in injured liver tissue to produce ECM and cytokines which cause fibrosis to occur. It may also be that only a subpopulation of stellate cells can be targeted for therapy, such as apoptotic therapy. It is unknown whether all hepatic stellate cells have the same propensity to undergo activation, apoptosis, or proliferation, and unfortunately, these questions cannot be answered using traditional 2-dimensional culturing methods; these methods seem to select for one population of stellate cells. Also, isolation methods tend to select for a particular density range and therefore may only capture a vitamin A-rich population, for example, therefore, not capturing the richness in heterogeneity of HSC's that give this cell type it's characteristics and potential to change. Experiments to monitor and characterize the differential response of rat myofibroblasts and stellate cells during liver injury are needed to not only determine what population should be targeted for therapy, but to also better understand how this heterogeneity plays a role in maintaining normal liver structure and function (12).

The host immune response is also a possible target for antifibrotic therapies. Interactions between the fibrogenic cells of the liver and the immune system may amplify the activation of stellate cells through paracrine stimulation of ECM production or increased proliferation (12, 13).

Hepatic Stellate cells as a Putative Stromal Type after Liver Injury

Much less is known about a potential stem cell niche in adult liver than in other well-characterized systems, such as the hematopoietic system. The oval cell has been identified by many investigators (14,15,16) as a potential liver stem cell, located near the

terminary biliary ductules (where the bile duct meets the hepatocyte plate) of the adult liver. This cell has few differentiation markers and has been demonstrated to differentiate in vivo into both biliary epithelium and hepatocytes (14). The myofibroblast-like stromal cell of the liver has been identified to be the Ito or stellate cell population, which in itself, as mentioned above, appears to be a heterogeneous population in its fibroblast-like phenotype, its retinoid storage function, and as a smooth muscle-like cell. Most in vivo experimental systems, which explore putative liver cell proliferation, involve chemical injury which may be followed by partial hepatectomy; this chemical injury seems to be sufficient in inducing liver tissue regeneration involving possible, identifiable stem cells. Liver regeneration after partial hepatectomy without chemical injury is thought to be achieved by mature hepatocyte division, and it is not thought to be achieved through proliferation of a stem cell niche. Interestingly, in both cases, when oval cells are seen to be proliferating, or where mature hepatocytes are dividing to restore tissue structure, the newly dividing cells seem to be in close contact with an "activated" and sometimes proliferating stellate cell (14,17,15,16,18). It is difficult to then study any possible liver stem cell niche in normal tissue, when most studies involve liver injury and therefore perturbation of the "normal" stem/stromal microenvironment. A common injury model employed to induce oval cell proliferation involves 2-acetylaminofluorene (AAF) administration, which results in observable oval cell proliferation at the terminal duct (14, 17). Investigators also observe coinciding stellate cell proliferation and a very close connection between proliferating oval cells and stellate cell processes. This close contact is maintained up to ten days after injury (14), and yet little is known about the details of this oval/stellate cell relationship and how it might direct the regenerative process. Stellate cells secrete many paracrine factors that induce oval and hepatocyte proliferation, such as TGF-β. After 2-AAF administration, there seems to be a temporal relationship between TGF-beta 1 expression and proliferation/apoptosis of the oval cells. These oval cells emerge from the portal areas and migrated out with time along the periportal and midzonal hepatic parenchyma (17). Park et al. found colocalization during immunostaining of smooth-muscle actin, a marker of activated stellate cells, and TGF-β1; and this led to the observation that the increase of TGF-β 1 expression in stellate cells paralleled the proliferation of oval cells. Interestingly, after peak TGF-β1 expression in

stellate cells, oval cell apoptosis also reached a peak. The authors concluded that the hepatic stellate cells observed to be intimately connected with proliferating oval cells seem to play a large role in the activation and cessation of oval cell proliferation and the remodeling of liver parenchyma after AAF-induced liver regeneration. This intimate relationship observed by the close contact between the two cell types and the paracrine signaling occurring during tissue regeneration has been noted by several investigators using different injury models (17). Several other factors secreted by hepatic stellate cells seem to play a role in modulating oval cell behavior, such as HGF, TGF-α, aFGF, extracellular matrix proteins/proteases, etc. Stellate cells as well as oval cells are also known to express stem cell factor (SCF) as well as the SCF receptor, c-kit.

Besides the paracrine signaling observed in many stromal/stem cell pairs, the stellate/oval cell-cell contact seems to be a necessary part of the regenerative behavior (15). Nagai, et al cocultured oval-like cells (positive for alpha-fetoprotein, negative for albumin, CK19) from adult rats with rat hepatic stellate cells, in both transwell and same-well culturing systems. The investigators demonstrated differentiation of the oval cells into albumin(+), alpha 1-trypsin (+), transferrin(+) hepatocyte-like cells; from these experiments they demonstrated that both soluble factors secreted by the stellate cells, as well as direct cell-cell contact between stellate and oval cells was necessary to demonstrate this differentiated phenotype (15).

These findings again point to the usefulness of an in vitro, 3-D model of liver, where a putative, liver stem cell niche could be explored, and heterotypic interactions between different types of liver cells can be characterized and monitored in hopes to study these relationships in a more in-vivo-like niche.

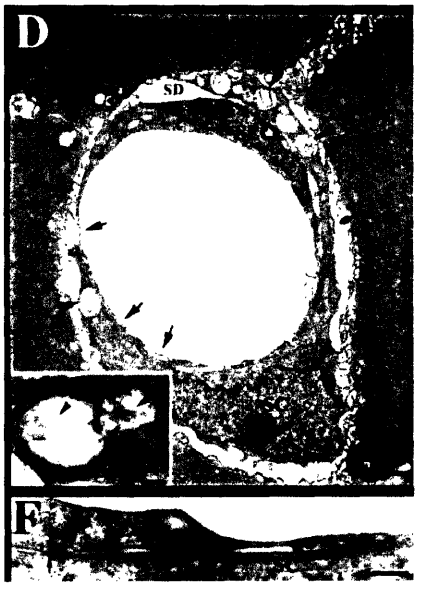
Stellate Cells in Liver Regeneration

Liver regeneration after partial hepatectomy is another case where stellate cells appear to activate in order to help orchestrate tissue remodeling; this is followed by a reversion back to a quiescent population of stellate cells. During liver regeneration,

mature hepatocytes begin cell division before sinusoidal cells, resulting in avascular islands of 12-15 hepatocytes in the newly forming tissue. After 48 hours and peaking during 72 hours post PHx, HSC's are seen enveloping sinusoidal endothelial cells, where they migrate into these avascular parenchymal islands during angiogenesis. ECM synthesis and degradation might play a large role in stellate cell activation and possible reversion back to a quiescent state, while also directing the synthesis and migration of liver cells during regeneration (8). The reversibility of the stellate cell activation in this case is questionable; it is also possible that the activated cells in these diseased states go through apoptosis and that new, quiescent stellate cells take their place. Because we can only capture a snapshot of the liver in these states by excising the tissue, we cannot easily answer these types of questions.

Regenerating livers from partial hepatectomized rats were analyzed for sinusoidal structure using microscopy methods (18). Interestingly, 72 hr. after 2/3 partial hepatectomy (PHx), sinusoids were seen to be compressed, and sinusoidal endothelial cells were seen to be enveloped by a stellate-like cell. These cells did not, however, possess lipid droplets characteristic of quiescent hepatic stellate cells, but were observed to contain inclusions, some open to their basolateral sign (the Space of Disse), containing fibrous or flocculent material (Figure 4). This could represent accumulation and deposition of extracellular matrix components, similar to what is seen during liver injury. This cell could represent a stellate cell at an intermediate stage between quiescence and activation, but since liver regeneration does not result in fibrosis, it would be interesting to study the stellate cell population during liver regeneration in order to understand how the population reverts back to a quiescent one. Stellate cells do proliferate 48 hours post-PHx, so this may induce loss of lipid droplets and activated state, and the ratio of stellate cells to endothelial cells is greatest at 72 hours, allowing for this observable close interaction between the two cell types (18). The tools developed here will aid in detecting and characterizing the journey of the stellate cell population through phenotypical stages similar to these.

Figure 4: Liver regeneration 72hr., post 2/3rd partial hepatectomy



SC=Stellate Cell, SD=Space of Disse, SEC=Sinusoidal Endothelial Cell, Arrows: fenestrated endothelium, Arrowheads, fibrous vesicles inside stellate cells and opening into the Space of Disse. (Wack et al., Hepatol 2001 Feb; 33(2) 363-78)

Stellate Cells and Their Neural Markers

Hepatic stellate cells display neural/endocrine characteristics such as their reaction to gold chloride staining, GFAP, synaptophysin, and N-CAM expression (19,20), morphology, etc. It is possible that stellate cells originate from the neural crest and, therefore, may share some of the same properties of analogous, adult nerve cells. Typically, the expression of neurotrophins and their receptors in non-neural tissue seems to be related to tissue remodeling, differentiation, proliferation, etc. Immunoreactivity to antibodies to NGF, BDNF, and other neurotrophins was found in hepatic stellate cells of both rat and human normal and pathological livers. This staining was localized to stellate cells only as seen by colocalization with desmin in rat livers and SMA in human livers. Hepatic stellate cells seem to be a source of both neurotrophins in the liver and also

express neurotrophin receptors. These findings correlate with the large role that HSC's play in liver tissue remodeling and the pathophysiology of liver disease (21).

Similarities between Astrocytes and HSC's

Rat astroglial primary cultures have been compared to rat hepatic stellate cells, through immunostaining and morphological studies. Both desmin and GFAP showed colocalization in the astrocyte populations. In the primary astrocyte populations, there were two main morphologies: cytoplasmic process-bearing cells and process-lacking cells. Process-lacking cells stained for filamentous desmin, where process-bearing cells only stained diffusely and faintly for desmin; GFAP was found strongly staining process-bearing cells and was found to be completely absent in very filamentous cells. These staining patterns are strikingly similar to those found in the liver lobule in hepatic stellate cells; this heterogeneity in phenotype is thought to possibly be due to transitional stages in differentiation in both the astrocytes and stellate cells; therefore, these cells might be receiving similar differentiation cues. In addition, both astrocytes and stellate cells share a stellate morphology, share a juxtaposition to blood vessels, have direct contact with nerve fibers, respond to tissue injury in similar ways, etc (22). It is possible then to learn more about the function and plasticity of glial and hepatic stellate cells through comparison.

Astrocytes are known to contribute to the barrier features of the blood-tissue surface, and this same functionality was explored in rat hepatic stellate cells by staining for expression of metallothionein protein (MT), a functional marker of blood-brain barrier. In the early liver cultures, stellate cells did not stain for MT, but did show strong GFAP staining; as the stellate cells showed typical activation and proliferation, GFAP expression disappeared, and MT expression became present, especially when the stellate cells became positive for SMA. Inversely, astrocytes stained positive for MT in early culture, but in later timepoints, lost MT and SMA staining, while gaining GFAP staining. The acquisition of myofibroblastic features in hepatic stellate cells might then help them to establish a strong blood-tissue barrier, seen in diseased liver. GFAP expression is seen in senile and diseased brain where there is the blood-brain barrier is disturbed; GFAP-

positive stellate cells in normal liver are adjacent to sinusoids or small blood vessels of high permeability. During fibrosis, this permeability is greatly decreased and capillarization occurs (endothelial cells lose their fenestrations); this correlates with sustained stellate cell activation and loss of GFAP expression. HSC's activation could possibly play a role in enhancing a tissue-blood barrier in the liver during disease and remodeling (23).

Synaptophysin

Functionally, synaptophysin is probably involved in membrane fusion leading to exocytosis. Normal rat and human hepatic stellate cells have been shown to express synaptophysin, and this expression is greatly increased in activated stellate cells from diseased liver (24). Close contacts between nerve endings and HSC's have been observed, and some have gone as far to say that possible the central nervous system could be influencing liver tissue repair in regeneration/liver disease through the stellate cells (24). This could result in paracrine stimulation of HSC's, resulting in change of ECM deposition, contraction of HSC and subsequently sinusoid diameter, etc. This is implicating that perhaps stellate cells could function as end organ effector cells of the CNS. This hypothesis seems to be supported by the observed synaptophysin expression, which is seen on neurotransmitter vesicles in nerve cells (24).

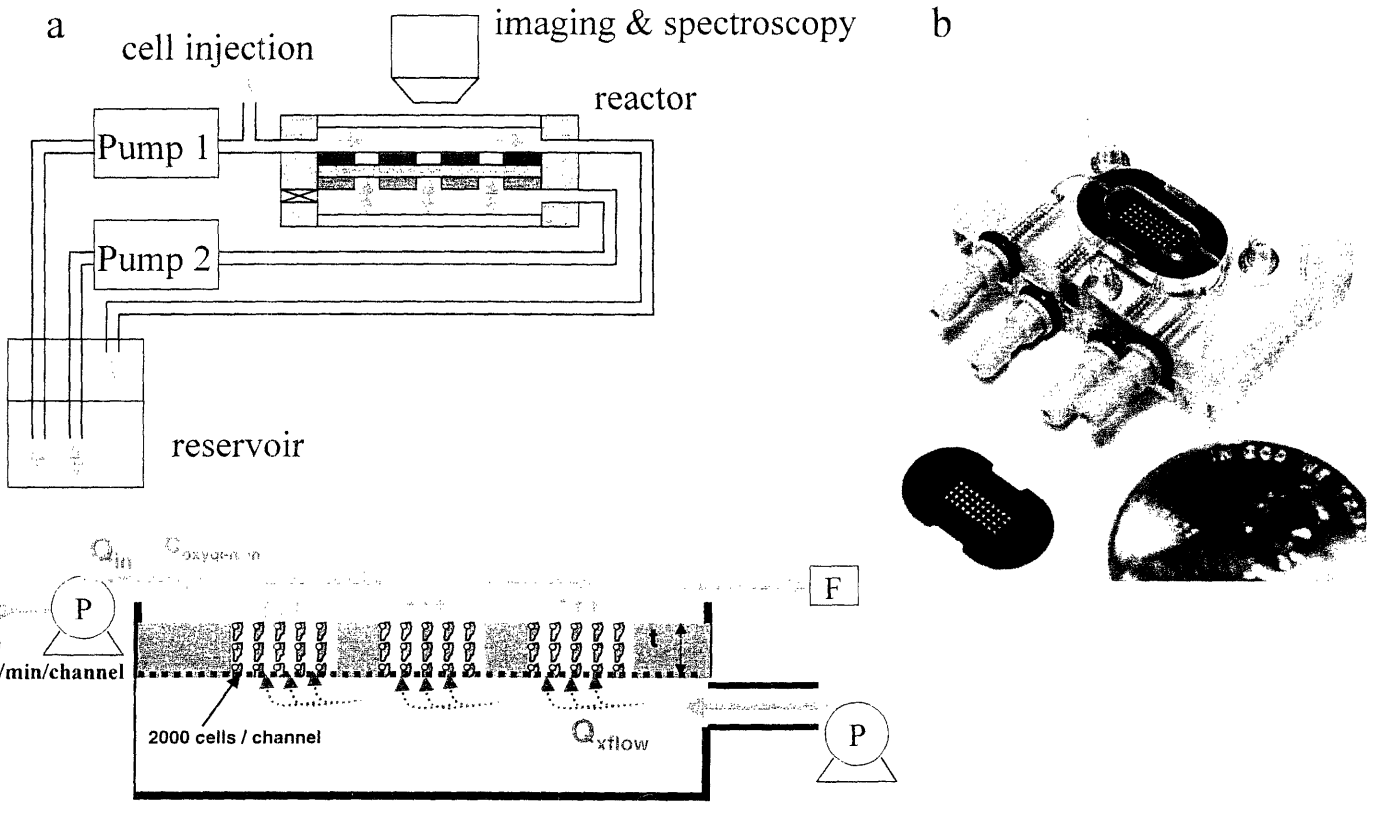
The human liver shows intralobular innervation, and this pattern is similar in several species. In the portal and centrolobular spaces, single axon and axon bundles, with or without Schwann sheaths, are seen close to fibroblasts and myofibroblasts, and in the intralobular spaces are visible quite close to the hepatic stellate cell. Nerve endings with vesicles containing neurotransmitters, such as substance P and vasoactive intestinal peptide, are often observed very close to HSC's (5). It is possible then, because of these observations and evidence pointing to a possible neural crest origin for stellate cells, that HSC's are receiving and propagating signals from the nervous system, and therefore, may share many more functional characteristics with some neural cell types. Other neural markers will be explored here as potential candidates for HSC characterization tools, in order to learn more about the functionality of HSC's under various conditions.

The 3-Dimensional, Perfused Liver Bioreactor

General Characteristics

The 3-dimensional liver bioreactor (Figure 5b) described previously supports a 3-D, perfused (Figure 5c) culture of primary liver cells, and provides an environment for tissue-like structures to form. This bioreactor supports a high level of differentiated liver function, such as p450 enzyme activities, albumin and urea secretion, and in vivo-like morphologies, measured up to 3 weeks in culture. Primary liver cells are isolated from adult rat livers, seeded into a spinner flask with Hepatocyte-Growth Medium (HGM), and allowed to form spheroid structures for 3 days. At this point, spheroids are filtered down, and a population with diameters ranging from 100-300um are seeded by a syringe into the liver bioreactor (Figure 5a). The spheroids settle into collagen I coated channels in a scaffold support, where they adhere and spread to form tissue structures under constant perfused flow (2,3).

Figure 5: Schematic of Liver Bioreactor



Other Liver Cell Types in the Bioreactor

Up until this point, a population consisting of approximately 95% hepatocytes was used to seed the spinner flasks. Interestingly, fenestrated endothelium and possible Kupffer cells, as well as a smooth cell type, have been observed in scanning electron micrographs of spheroids, as well as bioreactors after 10 days in culture (work not yet published). This provides evidence that the bioreactor supports the culture of other liver cell types, and that they seem to be reorganizing to contribute to the formation of tissue structures (1). Here, I begin on the road for deliberate addition of hepatic stellate cells, detection, and characterization of these cells and their heterotypic interactions within the bioreactor.

Research Goals

The functional morphology or phenotype of hepatic stellate cells seems to be regulated by growth factors, cytokines, etc. through paracrine communication with other liver and immune cell types as well as autocrine signaling which serves to amplify the response *in vitro* as well as *in vivo*. Recent findings suggest that stellate cells have a neural crest origin and have gene expression patterns similar to neural cell types and/or myofibroblastic cell types (8). Because stellate cells seem to change their phenotype accordingly after partial hepatectomy during tissue remodeling, liver regeneration may be an excellent system to study in order to clarify the hepatic stellate cell (HSC) function during complex reorganization of liver tissue through cell-cell interaction and cell-matrix interactions. However, because we cannot monitor liver regeneration in real-time, a liver model is needed that demonstrates tissue reorganization and that allows for visualization using microscopy and other tools. This is an excellent application for the liver bioreactor, as the optical window and 2-photon microscopy allow for live-cell imaging, and therefore, real-time monitoring of events.

The goals here are to develop tools to characterize a heterogeneous population of primary liver cells and their heterotypic interactions in a three-dimensional culture system, namely the liver bioreactor. This work aims to use and manipulate biological tools to characterize the behavior of cell types in an engineered environment. The long-term goal is to look for *in vivo*-like or disease-state relationships and to begin to be able to analyze the normal and pathological cross-talk between liver cells that comprises the liver's inseparable structure/function relationship. Cell-cell contact, ECM distribution, cell health and differentiation, as well as protein expression patterns can be monitored inside the liver bioreactor, with the work completed here and research presently being performed in the Griffith lab. In the near future, we can use these tools to not only characterize the bioreactor system in a "normal" state, but after perturbation, such as

injury and introduction of disease. This work aims to develop tools to apply to the liver bioreactor to begin to understand the structure/function relationship between liver cells and how the liver maintains homeostasis, in hopes that this "normal" function can then be restored in injured livers through the future development of new therapeutic targets. In addition, the liver bioreactor then has the potential to act as a tool to increase our basic understanding of how hepatic cells work together to orchestrate the complex functions of the liver, including self-organization into tissue structures in an engineered, 3-dimensional, perfused system, modeled after *in vivo* conditions.

Research Design and Rationale

Imunostaining Characterization of the Plasticity of the Hepatic Stellate Cell Phenotype

Introduction

The hepatic stellate cell population comprises a heterogeneous population of cells in normal liver, and this population changes patterns of protein expression in response to liver injury or change of environment, such as prolonged culture on plastic. Because of this heterogeneity and plasticity of phenotype, no method has yet been developed to identify the stellate cell population using only one or two protein markers. The heterogeneity of normal liver, It seems that a toolbox of markers may be needed in order to detect the whole, heterogeneous stellate cell population in the liver, as well as begin characterize it *in vitro*. When these cells receive signals which cause activation that can result in tissue remodeling or fibrosis, it is unknown whether or not the stellate population as a whole responds in a similar manner. In order to study questions such as these, one must first detect and characterize the whole population one is studying. Only then can this population be fully characterized during and after experimental perturbation. For this reason, a toolbox of six antibodies, comprised of some traditional markers described in literature, and some novel characterization markers, were evaluated as tools to aid in detection and characterization of a hepatic stellate cell population exposed to various conditions.

Problems with Traditional Markers of Quiescent HSC's in the Liver Lobule

Using 3-D confocal laser microscopy, Tanikawa, et al stained whole stellate cells *in vivo* (with cytoplasmic processes up to 140um) with gfap and desmin, and imaged them. In the normal rat liver, antibodies against desmin have been used as a marker of hepatic stellate cells from both normal and injured (quiescent and activated) liver

samples. Immunostaining with desmin reveals no HSC's in pericentral regions of the normal rat liver. However, immunostaining with a GFAP antibody reveals a somewhat even distribution of HSC's in the liver lobule (6).

Ballardini and Ramm have both identified a group of desmin-negative stellate cells in rat that, upon isolation and culture on plastic, become activated and induce expression of both desmin and smooth muscle actin. These studies show that desmin alone is not a reliable marker to identify the whole HSC population in a normal rat liver (25). However, it is possible that both GFAP and desmin used together may capture a large portion of the whole population of quiescent hepatic stellate cells. Smooth muscle actin was also chosen as an activation marker for stellate cells, but will be used along with other functional markers discussed below in order to learn more about the activation process and whether or not it affects the HSC population as a whole.

Selection of Putative Neural Markers to Characterize HSC's

As mentioned here, in the literature, it has been shown that hepatic stellate cells express the intermediate filament, GFAP, and the synaptic vesicle protein, synaptophysin, both characteristic of neural cells. GFAP seems to stain sinusoidal, quiescent stellate cells only, and synaptophysin shows increased positive staining in activated stellate cells. In addition to traditional stellate cell markers of both quiescence and activation (desmin and SMA), GFAP, synaptophysin, and the neural proteins described below were evaluated as characterization markers in the research completed here. These markers were selected as a part of a toolbox used in order to capture more of the heterogeneous stellate cell population, as well as to gain more information about their functionality in the 3-D, liver bioreactor.

CNPase

In the neural system, CNPase is a traditionally used marker of oligendrocytes, one of the glial, neuromuscular cell types (26). The morphology of this cell type very closely resembles the hepatic stellate cell dendritic shape. Oligodendrocytes are found to be tightly interacting and communicating with neuronal cells, similarly to stellate cells and hepatocytes. It is also possible that CNPase plays a role in other neural cells besides oligendrocytes. 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase), a myelin-associated enzyme, activity was found to be higher in rat brain oligendrocytes than neurons, but not appreciably different from that in astrocytes (26). CNPase activity has been observed in tissues other nervous tissue, such as the spleen. CNPase activity was found in all major fractions of a liver homogenate, with the highest activities in the microsomal and nuclear fractions. It is not known, however, what cell type(s), if any in the liver, express CNPase. CNPase activity seems to be highest in tissues that contain cells with membranes capable of undergoing transformation and elaboration (27). Therefore, CNPase expression was explored here in liver, as well as in a stellate cell line in three different culture settings.

Beta-Tubulin

Beta-tubulin is found to be highly expressed in neuronal cells, but not exclusively (28). Cod hepatic stellate cells contain many typical stellate filaments; as shown by immunoelectron microscopy, cod stellate cells in normal liver express smooth muscle actin, desmin, and beta-tubulin (but not GFAP or vimentin which is found in normal rat liver) (28). It is possible then that quiescent or activated rat hepatic stellate cells may also express this protein, and therefore a beta-tubulin antibody was also used to explore the phenotype of hepatic stellate cells in different environments.

From previous studies mentioned here, neural markers characteristic of astrocytes and other neural cell types, such as GFAP, as well as some neurotrophins and immature

neural markers have been identified in hepatic stellate cells. It is possible that we can learn more about the functionality and origin of hepatic stellate cells by exploring these similarities and differences.

Rationale for the Selection of a Rat Stellate Cell Line as a Positive Control

Challenges in Isolating Primary Rat Stellate Cells

Hepatic stellate cells comprise approximately 10-15% of the total number of liver cells, although during liver injury this percentage can be much greater (4,5). Because stellate cells comprise a heterogeneous population with varying densities depending greatly on the degree of lipid content, many isolation methods using density gradients (29,30,31) select for only a subpopulation of cells. Some methods include purification of stellate cells through side-scatter cell sorting, but these methods can be costly and time-consuming (32). Assessment of viability and purity is difficult as well, as markers such as desmin and smooth muscle actin are absent on some freshly isolated stellate cells. Because of these isolation and detection difficulties, it is necessary to develop a toolbox of markers for detection and characterization, and some sort of positive control to develop characterization protocols that can later be applied to primary stellate cells, no matter how few they are and what phenotype they are expressing.

Rationale for Selecting the HSC-T6 Rat Hepatic Stellate Cell Line

The Friedman group generated an immortalized rat hepatic stellate cell line (HSC-T6) from a normal adult rat liver. Stellate cells were isolated from normal Sprage-Dawley rat livers, cultured for 15 days on plastic culture dishes in the presence of 10% FBS, and subsequently transfected with lipofectamine containing cDNA in which the expression of the large T-antigen of SV40 is driven by the Rous sarcoma virus promoter. After 5-7 days, clones were isolated and amplified. One of the many clones, named HSC-T6 displayed a phenotype most closely resembling that of primary hepatic stellate cells. A stable phenotype has been established for over 40 passages. Unlike other stellate cell lines, these cells, although displaying an activated, proliferative phenotype in 2-D culture, retain the retinoid uptake and processing capability characteristic of quiescent rat hepatic stellate cells (33). These cells can be quite useful in studying the phenotypical changes that occur in hepatic stellate cells, as well as their communication with hepatocytes in culture.

HSC-T6 cells express in 2-D culture both myogenic and neural crest markers characteristic of both activated and nonactivated hepatic stellate cells. Specifically, this cell line express cytoskeletal proteins including desmin, alpha smooth muscle actin, glial acidic fibrillary protein, and vimentin. The display a fibroblastic shape and rapid proliferation characteristic of an activated phenotype. The cells also express all six retinoid nuclear receptors and retain the ability to store fat as retinoids (33).

These cells, therefore serve as an excellent positive control evaluating detection and phenotype characteristics of stellate cells in different culturing systems, including coculture with hepatocytes inside the tissue structures of the three-dimensional liver bioreactor. This cell line was therefore selected for characterization in two-dimensional culture (see Appendix) and three-dimensional spheroids, as a positive control for detection and characterization protocol development.

Rationale Behind Coculture Spheroid Formation

Spheroids were formed as previously described (2,3), but with slight modification. 10 million HSC-T6 cells were cocultured with 10 million cells from a hepatoctye isolate in a spinner flask containing 50% Hepatocyte Growth Medium and 50% HSC-T6 medium (in order to strive to deliver nutrients that both cell types may need).

The purpose of making coculture spheroids using the HSC-T6 cell line combined with an isolated fraction of primary hepatocytes served to test the detection and characterization capabilities developed here and in addition to electron microscopy, so that in the near future, primary stellate cells may be deliberately added and detected in 3-D spheroids. For this reason, a large ratio of HSC-T6 cells was added to the spinner flask to ensure incorporation into the spheroids, and to test characterization methods. In the future, the methods developed here can be used to detect primary stellate cells, even in small numbers.

HSC-T6 cells were cocultured in a spinner flask with isolated, primary hepatocytes. Hepatocytes were isolated as explained previously (2,3) which typically results in a 95% pure hepatocyte fraction. It is possible then, that nonparenchymal cells may be incorporated into these spheroids, and it is also possible that these primary nonparenchymal cells may repopulate inside of the spheroid. This possibility must be taken into account when analyzing the staining patterns of HSC-T6 cells in the coculture spheroids; it is possible that some staining may be due to primary stellate cells and this must be considered. This is also why a very large proportion of HSC-T6 cells was seeded into the spinner flask in these experiments.

Rationale Behind Coculture Bioreactor Seeding and Maintenance

Bioreactors were seeded with the described, coculture, 3-day spheroids, in order to obtain some initial detection and characterization results, using the HSC-T6 cell line. Spheroids were seeded into the millireactor F model as described by Powers et. al (2), but

with the slight modification of supplementing the medium with 50% HSC-T6 medium containing 10% serum. The rationale behind this addition was to ensure HSC-T6 cell health inside the bioreactor in order to initially test the detection methods developed here. In addition, in place of the previously used silicon scaffold, a polycarbonate scaffold was used, because of it's ability to be embedded and sectioned for transmission electron microscopy; in addition, in the near future, it will be possible to section these scaffolds for histology and immunostaining purposes as well. A total of six bioreactors cultures under constant perfused flow were monitored and imaged daily using light microscopy; their medium was changed every three days, and samples were taken down for analysis on day 7 post-seeding, which corresponds to day 10 post-isolation.

Development of Immunostaining Protocols Using Toolbox of Antibodies

Table 1: Antibody Information

Antibody Name	Company	Product Number	Dilution	Incubation Time, Temp (^{0}C)
Mouse anti-desmin	BD Biosciences	550626	1:50	Overnight, (4 ^{0}C)
Mouse anti-GFAP	BD Biosciences	556327	1:100	Overnight, (4 ^{0}C)
Anti-α SMA:FITC	Serotec	F3777	1:50	2hr., room temp.
Rabbit anti-GFAP	Chemicon	AB5804	1:100	Overnight, (4 ^{0}C)
Rabbit anti-desmin	Lab Vision	Rb-9014-R7	1:100	Overnight, (4 ^{0}C)
Mouse anti-synpaptophysin	Chemicon	MAB368	1:50	Overnight, (4 ^{0}C)

Mouse anti-β-Tubulin	Chemicon	MAB1637	1:50	Overnight, (4 ^{0}C)
Mouse anti-CNPase	Chemicon	MAB326R	1:100	Overnight, (4 ^{0}C)
Goat anti-rabbit TRITC	Sigma	T6778	1:100	1 hr., room temp.
Goat anti-mouse cy3	Jackson Immunoresearch	115-165-166	1:100	1 hr., room temp,

Rationale behind Immunostaining in Liver

It is important to not only characteristic the HSC-T6 stellate cell line in 2-D cultures using the toolbox of antibodies developed here, but to also use this toolbox to characterize sections of normal, adult rat liver. Therefore, each antibody (table1) was used to immunostain liver in order to later compare characterization of the liver bioreactor with *in vivo* results using the same markers.

Development of Immunostaining Protocols for Coculture Spheroids

Samples of 3-day spheroids were collected and spun down in a centrifuge at 50g for three minutes. These samples were then either fixed in 2% Paraformaldehyde for 1 hr. for immunostaining, or in 2.5% gluteraldehyde for approximately 1 hr. for electron microscopy (appendix)

For immunostaining purposes, the spheroids were embedded in 1% agarose, and sectioned using a cryostat to produce 5 μm sections of coculture spheroids. These sections were then immunostained for characterization using the toolbox of antibodies

described here. Approximately fifty random spheroids for each antibody were studied, in order to gain a sense of staining throughout the depth of the spheroids and to uncover some staining patterns.

Development of Immunostaining Protocols for Liver Bioreactor

Presently, work is being performed in the Griffith lab to develop methods of sectioning and immunostaining the plastic scaffold from the liver bioreactor. This will allow for characterization throughout the depth of the bioreactor channel, and will provide interesting information as to the reorganization of different types of liver cells into functional tissue units. The work performed here will allow for detection and characterization using immunostaining once these sectioning methods are completed in the near future. Here, bioreactors were seeded (2) with the previously described, 3-day, coculture spheroids, allowed to form tissue structures, and cultured under constant perfused flow for 7 days. Polycarbonate scaffolds were used to house the cells, as these scaffolds are sectionable after embedding in hard plastic for TEM. Reactors after day 7 were taken down, scaffolds were fixed in both paraformaldehyde for initial immunostaining studies and gluteraldehyde for TEM and SEM. One whole polycarbonate scaffold was permeabilized (appendix) and stained with one putative stellate cell marker, in order to gain some initial information about the possible detection of stellate cells inside the reactor. This sample does not provide information as to the spatial arrangement or phenotype of HSC-T6 cells inside the bioreactor, but gives some initial information about detection capabilities, which can be complemented with electron microscopy characterization and, in the future, immunostaining of sectioned bioreactor scaffolds.

Development of Electron Microscopy Protocols

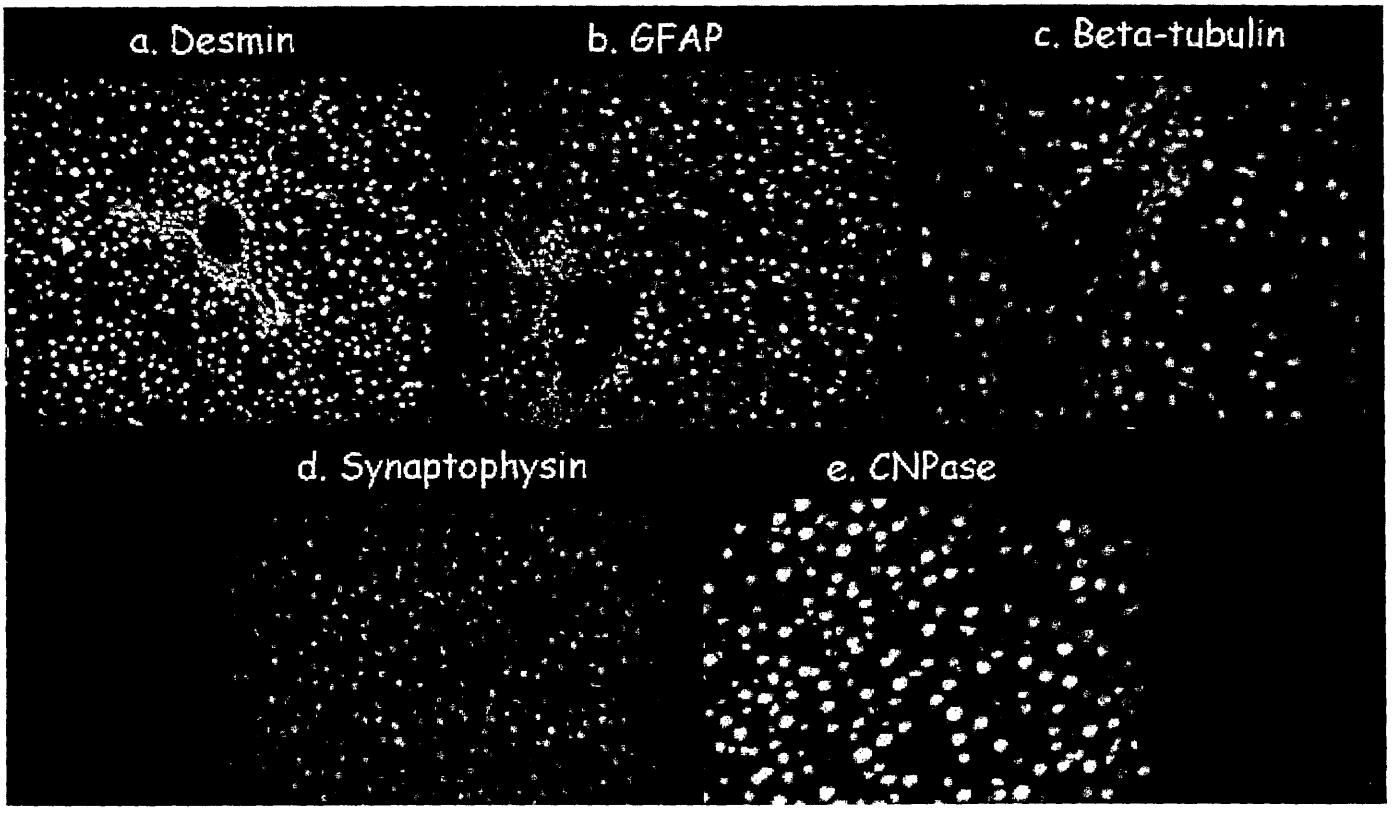
Electron microscopy characterization can be quite helpful in characterizing the microstructure of three-dimensionally cultured cells, especially through comparison with normal liver tissue. Based upon the high structure/function relationship of the liver, it is possible to identify many characteristics of highly differentiated liver cells, such as the presence of in-vivo-like homotypic and heterotypic interactions between cells, bile canaliculi, extracellular matrix, presence of metabolic organelles, degree of lipid storage, etc. It is also possible to obtain information about how cells are oriented or interacting together as well as to get an idea of the health of the cells. Scanning electron microscopy as well as Transmission Electron Microscopy techniques (2) were adapted (appendix) to the 3-D culturing systems used here, in collaboration with the Stolz Lab at the University of Pittsburgh, in order to complement the immunostaining characterization, as well as to provide a closer look into the microarchitecture of the samples.

Results

Liver Immunostaining using Toolbox of Antibodies

Liver sections from normal rat liver were immunostained for each selected marker in order to characterize the phenotype of normal, in-vivo hepatic stellate cells in the liver lobule and to get some general indication of zonal differences. As shown in figure 6a, the liver showed zonal staining of desmin, with large positive areas around the portal triad. GFAP-positive stellate cells shown in figure 6b in red were observed approximately evenly distributed through the sinusoids, with minimal staining seen in the large vessels. Beta-tubulin staining in figure 6c shown in red showed minimal staining with positive areas around large vessels. Immunostaining with synaptophysin shown in figure 6d showed faint scattered staining in stellate-like morphologies. Finally, CNPase staining shown in figure 6e in green showed minimal, faint staining throughout the sinusoid, but it was unclear whether this staining was localized to stellate cells. In the future, combinations of these six antibodies will be employed to attempt to show possible colocalization patterns.

Figure 6: Immunostaining Characterization of Normal Rat Liver

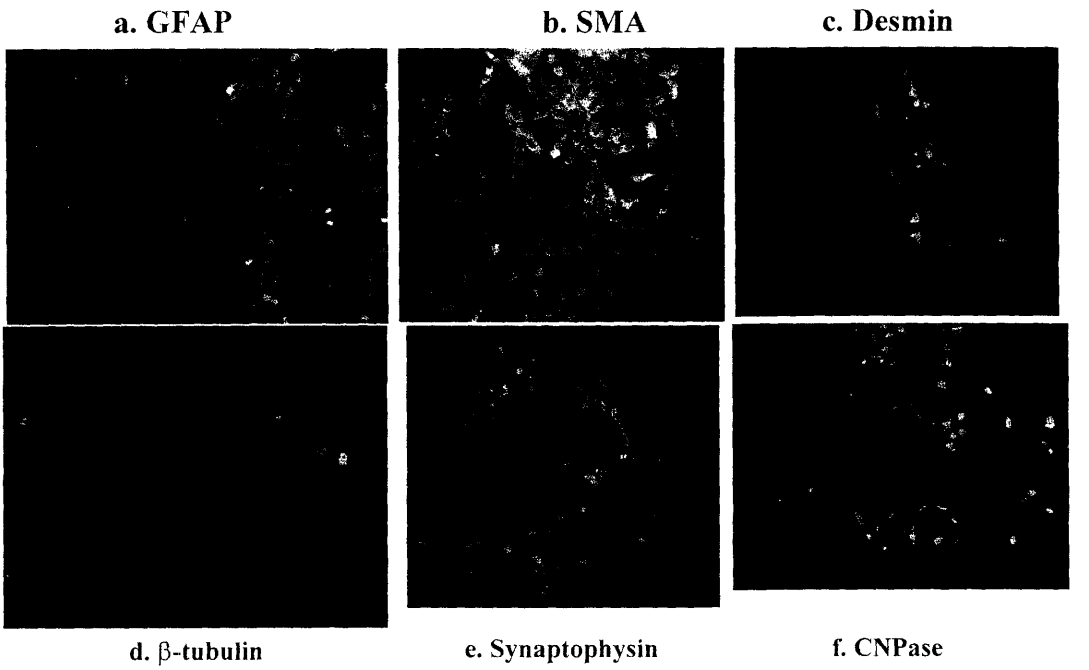


Immunostaining Characterization of HSC-T6 Cell Line in 2-D Cultures

Results from 2-D characterization of the HSC-T6 cell line using the toolbox of six antibodies showed interesting, positive results. The cell line stained largely positive for desmin (figure 7c), smooth muscle actin (figure 7b), CNPase (figure 7f), and beta-tubulin (figure 7d). Areas of positive GFAP staining were also observed (figure 7a), although only approximately 20% of the cells were positive; similarly, areas on the periphery of confluent growth of cells stained positive for synaptophysin, indicating that, possibly, as

the cell line reaches confluence, interesting heterogeneity of protein expression may occur. This could be the result of cell-cell contact and nutrient gradient effects.

Figure 7: Immunostaining Characterization of HSC-T6 Cell Line in 2-D Cultures



Characterization of 3-D Coculture Spheroids

Immunostaining Characterization

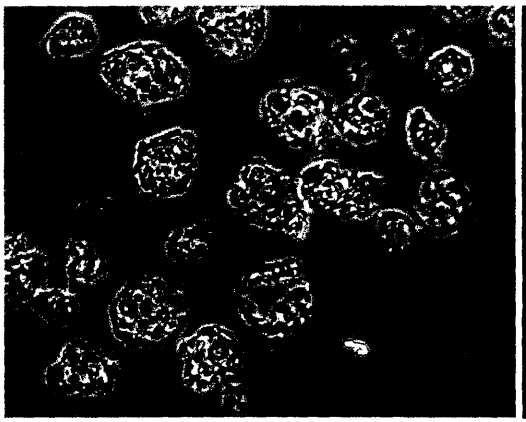
General Observations

In two independent experiments, three-day, coculture (1:1 HSC-T6: Hepatocyte fraction) spheroids were fixed, sectioned, and analyzed for overall morphology, using fluorescent and electron microscopy (EM) and characterization using the toolbox of antibodies described here. In general, spheroids ranged from 40-100 μm (figure 8a). Some spheroids appeared to contain mostly HSC-T6 cells, while others appeared to

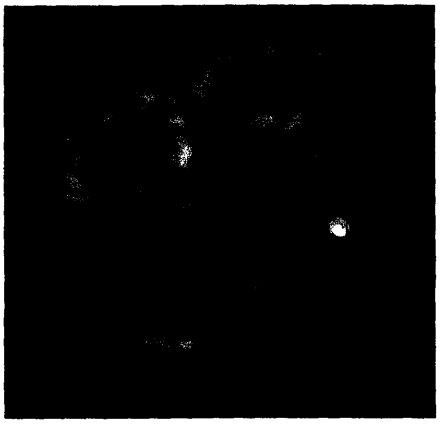
contain both hepatocytes and HSC-T6 cells as noted by green hepatocyte autofluorescence (figure 8d) and electron microscopy identification of hepatocyte-like morphology; many of these hepatocytes appeared to be in poor health as noted by blebing and necrotic areas in electron micrographs (figure 17b and 17c *), as well as a bright diffuse, red and green, autofluorescence; a positive hepatocyte antibody may be used in the future to definitely identify hepatocytes in coculture with other liver cells, but here we focus on stellate cell detection and characterization. Commonly, HSC-T6 cells seemed to appear as a layer around the outside of the spheroid as seen by both immunofluorescence and EM, although HSC-T6 cells were also seen towards the interior of some spheroids. Lastly, spaces possibly devoid of cells were sometimes observed in the interior of spheroids, giving interior section of the spheroid a "donut" shape (figure 8b).

Figure 8: Immunofluorescence Observations of General Coculture Spheroid Morphologies

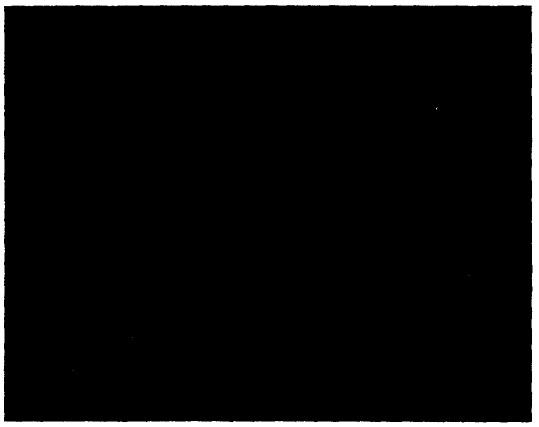
a. Light micrograph, showing sample of a section of spheroids



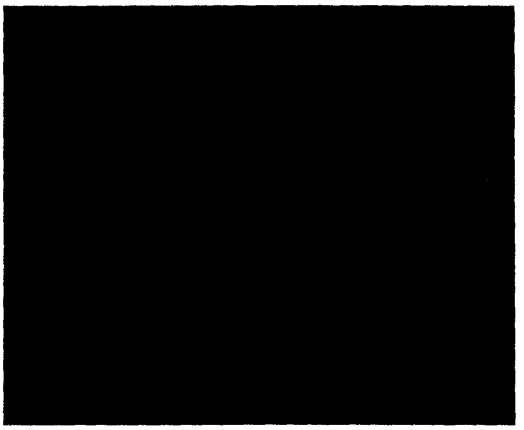
b. Spheroids stained with Hoescht's Dye, illustrating "donut" shape.



c control showing cy 3 secondary only



d. Control showing green hepatocyte autofluorescence



Characterization of Spheroids UsingToolbox of Antibodies

In addition to characterizing overall morphology, a differential pattern of HSC-T6 protein expression was observed using the same toolbox of antibodies developed in 2-D HSC-T6 cultures. These results show that not only do the HSC-T6 cells show a different pattern of protein expression in 3-D spheroids, compared to 2-D culture, but antibodies showing positive expression seemed to capture different expression patterns, indicating that possibly, the HSC-T6 cells were becoming a heterogeneous population in the coculture spheroids. In contrast to 2-dimensional cultures of HSC-T6 cells, when cocultured with primary hepatocytes in 3-dimensional spheroids, there seem to be only a small population of desmin(+) cells (figure 9a,b). These cells could be HSC-T6 cells, or since the hepatocyte fraction is not absolutely pure, they could be primary stellate cells or myofibroblasts.

Figure 9: HSC-T6/Hepatocyte Spheroids stained with anti desmin and cy3 (red).

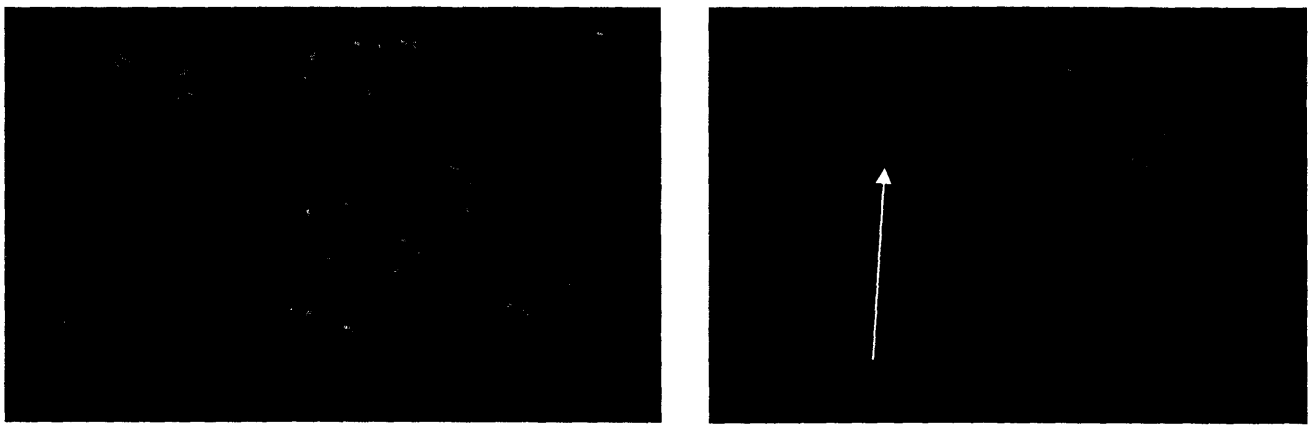
a. red desmin positive stellate on outside of green autofluorescent hepatocyte

b. section showing bright desmin positive spheroid, and faint red fluorescence of possible dead hep.

c. red and green autofluorescence of hepatocytes.

In addition, there was no positive smooth muscle actin cells observed, and this was also in contrast to positive HSC-T6 cells seen in 2-D culture (figure 10, arrow points to hepatocyte autofluorescence). As described here and in the literature, smooth muscle actin staining in stellate cells is associated with the activated phenotype seen *in vivo* and *in vitro*, but is not observed here in spheroids. This may indicated that the population of stellate cells in these spheroids are not fully activated.

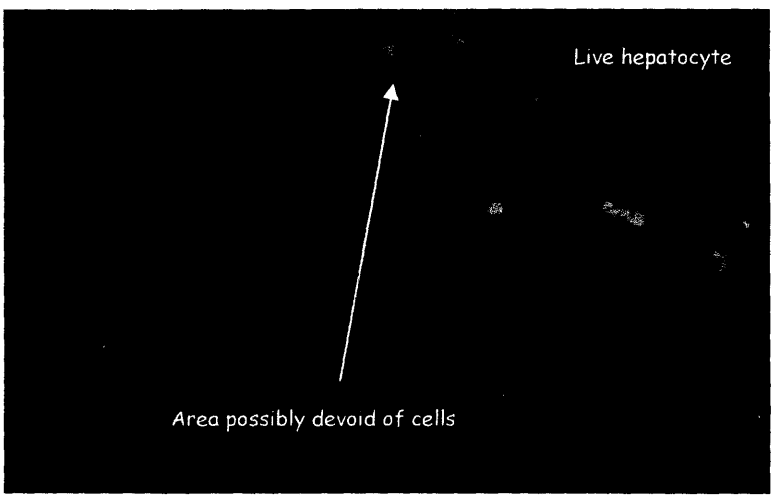
Figure 10: HSC-T6/Hepatocyte Spheroids stained with anti-SMA:fitc (green)



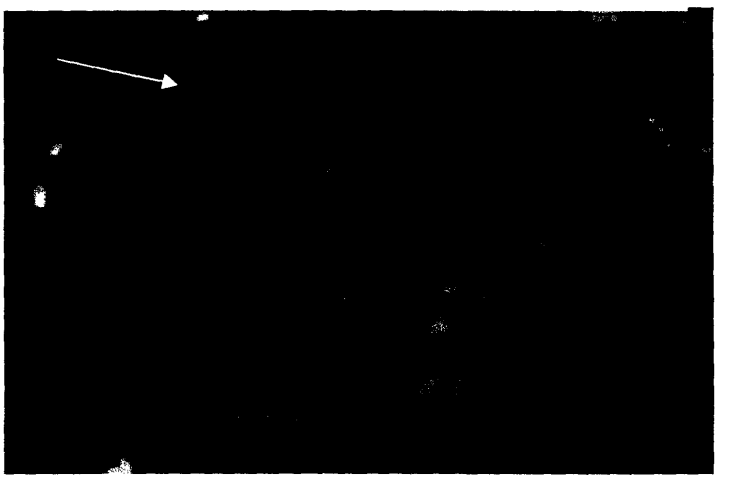
Also in contrast to protein expression seen in 2-dimensional cultures, there was widespread GFAP staining observed throughout many spheroids, both on the exterior and interior portion of the spheroids (figure 11a, b). This antibody showed the most widespread staining patterns than any of the other six antibodies employed for characterization. As described here, GFAP staining is widespread in normal, rat liver in sinusoidal quiescent stellate cells.

Figure 11: HSC-T6/Hepatocyte Spheroids stained with anti GFAP and cy3 (red).

a. Bright positive GFAP staining, area with no nuclei staining present.



b. Area with large, round nuclei, possible live hepatocytes



β-Tubulin (figure 12) and synaptophysin expression (figure 13) were not detected in spheroids, again in contrast to HSC-T6 2-D staining. Negative staining of SMA, beta-tubulin, and synaptophysin compared with 2-D cultures may be indicative of a change in HSC-T6 phenotype in response to a change in environment from 2-D to 3-D spheroid culture.

Figure 12: HSC-T6/Hepatocyte Spheroids stained with anti β-Tubulin and cy3 (red).

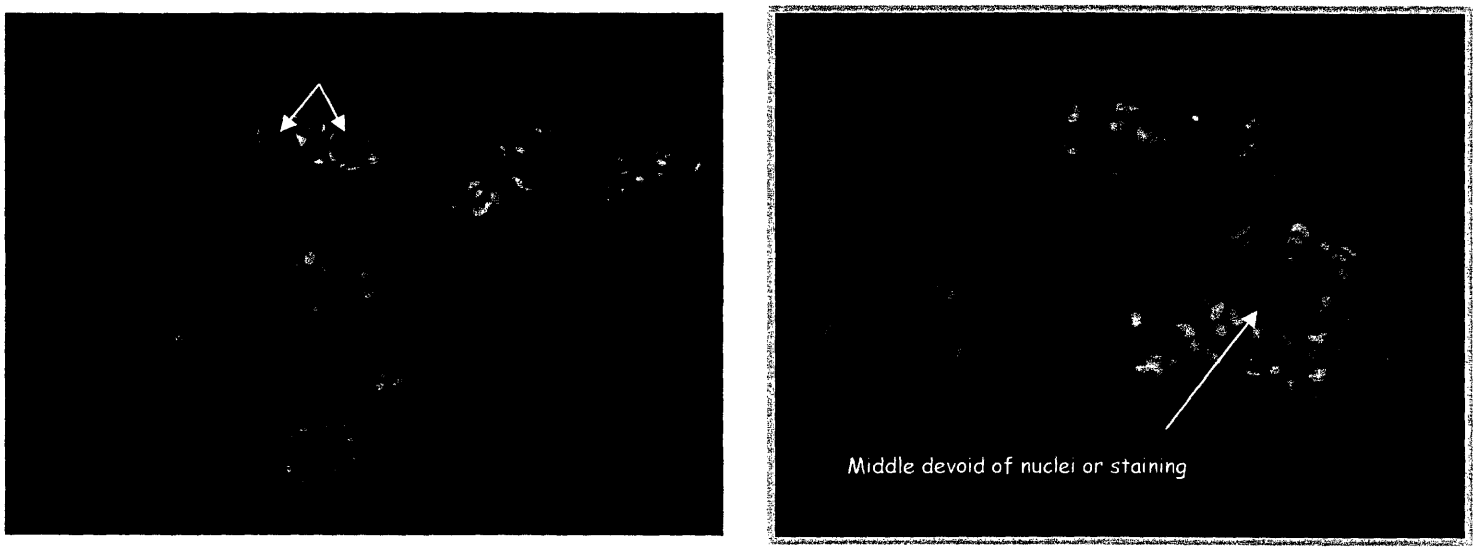
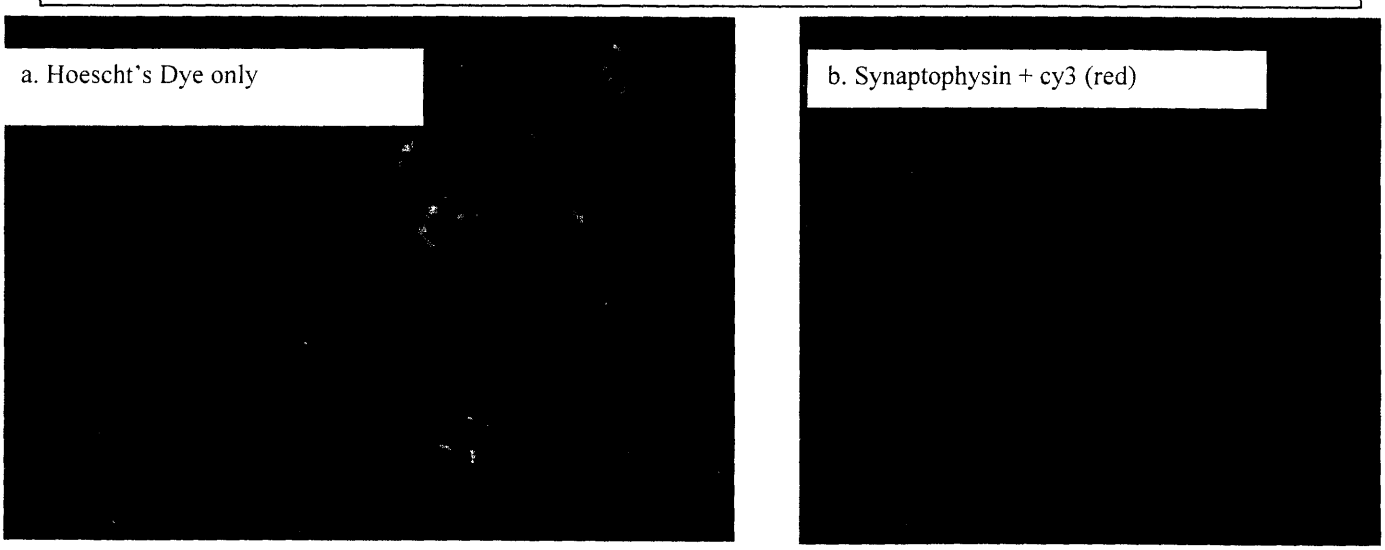
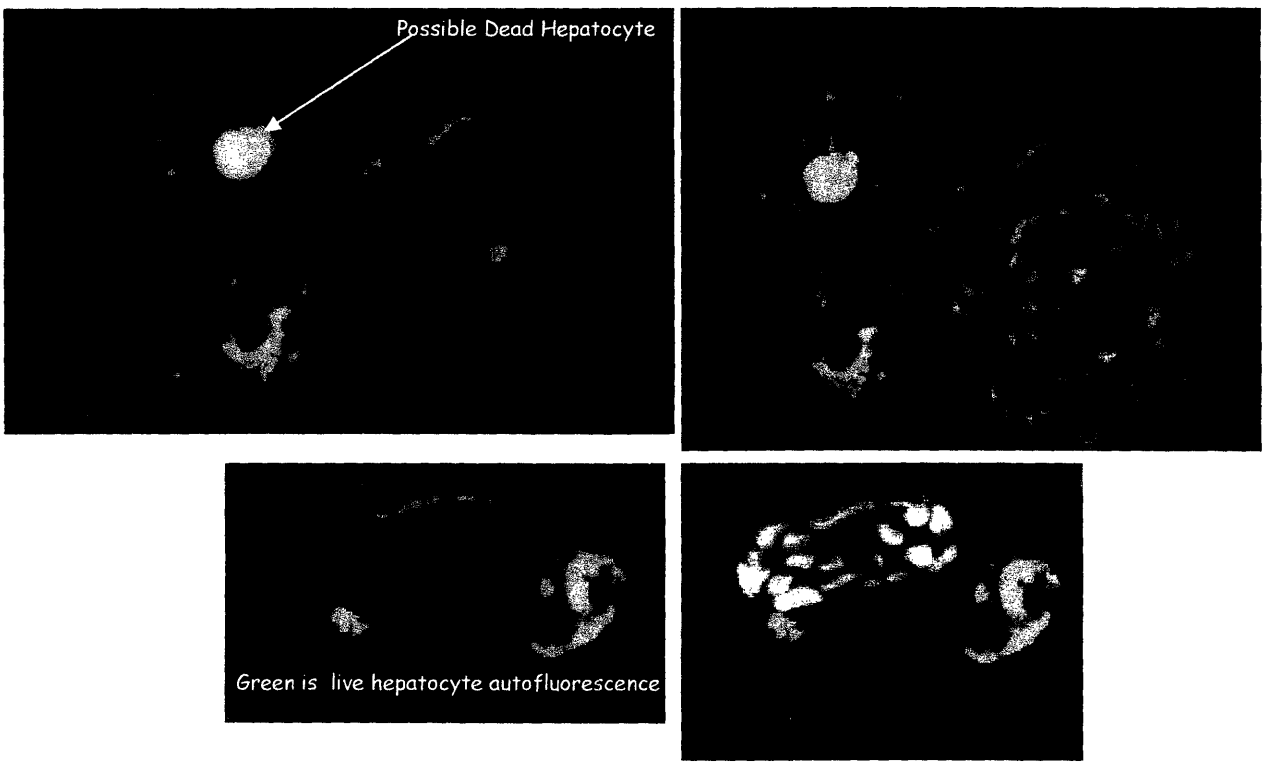


Figure 13: HSC-T6/Hepatocyte Spheroids stained with anti synap and cy3 (red).



Interestingly, like in 2-D cultures, CNPase positive cells were widely seen, but not as much throughout the spheroids. Highly CNPase-positive cells were often detected on the periphery of the spheroids (figure 14), possibly comprising the outside layer of HSC-T6 cells often observed in electron micrographs. This differential patter of staining is interesting and may be something to look for in future characterization of stellate cells in the bioreactor.

Figure 14: HSC-T6/Hepatocyte Spheroids stained with anti CNPase and cy3 (red).



Comparison of Immunostaining Results in Liver, 2-D,and 3-D Coculture Spheroids

Protein expressions concerning the toolbox of antibody markers used are summarized in figure 15; through literature findings and research performed here, protein expression comparisons of stellate cells can be analyzed to provide clues about stellate cell phenotypical changes in response to change in environment. These methods can be now be applied to the 3-D bioreactor for detection and characterization of primary stellate cells. Using the HSC-T6 cell line as a positive control for development of detection and characterization methods has been successful and has already provided some interesting results in different culture settings.

Figure 15: Summary of Immunostaining Characterization Results

	Normal Liver (Quiescent Stellate Cells)*	**Activated Stellate Cells***	**HSC-T6 Cells in 2-D culture**	**HSC-T6 Cells in 3-D Coculture Spheroids**
Desmin	Variable staining, mostly process-lacking, vitamin A poor, stellate cells	Increases, then eventually decreases upon expression of SMA	Positive	Partially positive
Smooth Muscle Actin	Negative	Positive	Positive	Negative
GFAP	Positive perisinusoidal staining	Negative	20% positive	Positive
Synaptophysin	Minimal, scattered staining, mostly around lg. vessels	Increased staining in stellate cells	Positive in periphery of colonies	Negative
β-Tubulin	Found in freshly isolated rat HSC's, and positive in normal cod liver stellate cells	Unknown	Positive	Negative

CNPase	CNPase activity found in all liver homogenate fractions	Unknown	Positive	Positive, mostly on the outside layer of spheroids

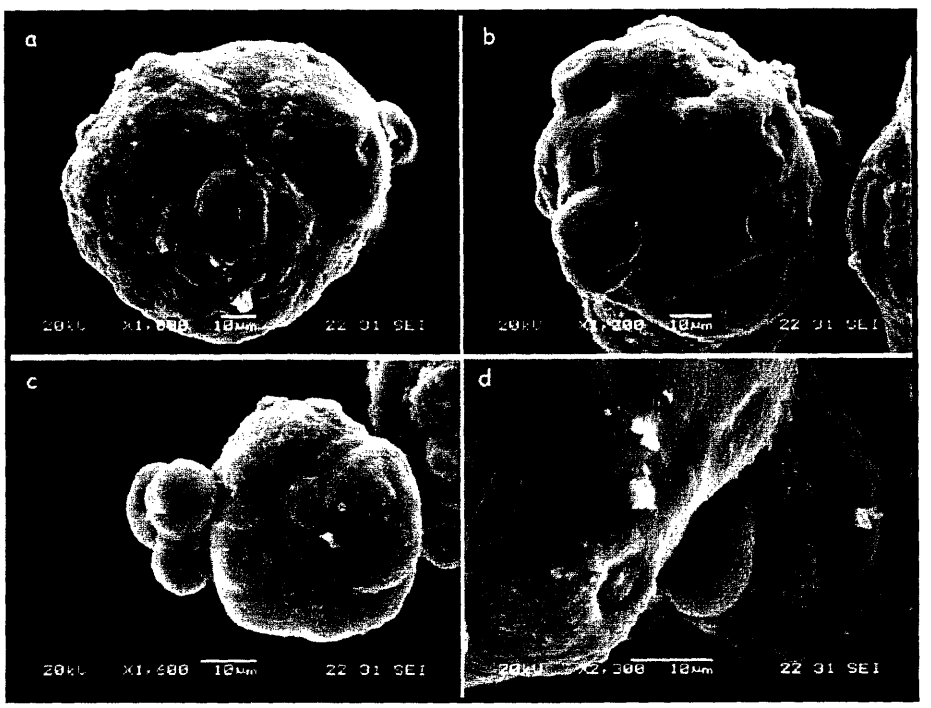
* Data taken from a combination of research presented here and literature findings.

Electron Microscopy Results

Scanning Electron Microscopy

Scanning electron micrographs of coculture spheroids shows the presence of two cell types, one smooth (possibly HSC-T6 cells), and one with microvilli, possible being hepatocytes (center of figure 16d). Cells show a typical spheroidal structure with cell-cell junctions apparent.

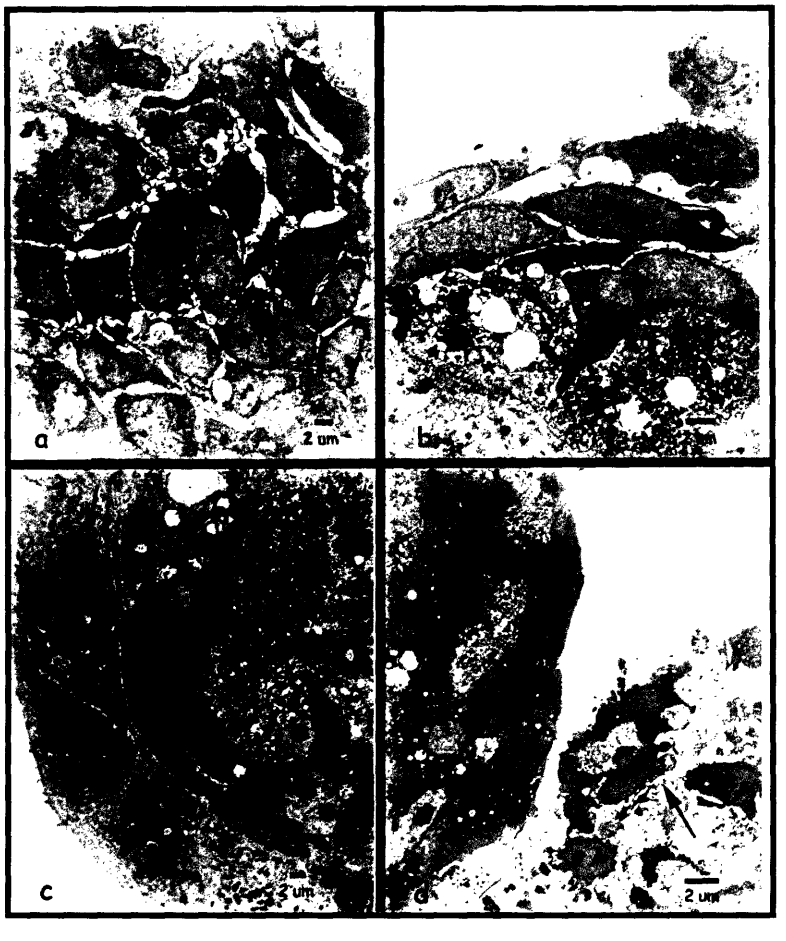
Figure 16: Scanning Electron Micrographs of Coculture Spheroids



Transmission Electron Microscopy

Transmission electron micrographs of the HSC-T6/Hepatocyte fraction (1:1), 3-day spheroids show the presence of two cell types, most likely hepatocytes and HSC-T6's. The micrographs show the presence of many necrotic or apoptotic hepatocytes (figure 17b,c), corresponding with the largely autofluorescent cells shown in immunostained sections and unhealthy-appearing groups of cells in SEM images. The HSC-T6's, although occasionally seen amongst the hepatocytes, are largely seen to cover the outside section of the spheroid, showing the presence of large lipid droplets (could in the future be stained with Oil Red O, see appendix for protocol), an oval nucleus, and many cytoplasmic extensions. Some spheroids appeared to consist largely of hepatocytes only, some consisted of only HSC-T6 cells, while others appeared to contain both types of cells.

Figure 17: Transmission Electron Micrographs of 3-Day, 1:1 HSC-T6: Hepatocyte Fraction, Coculture Spheroids



Some possible endothelial fenestrations are observed and warrant further exploration (with immunostaining, DiI-Ac-LDL uptake, etc.). HSC-T6 and Hepatocytes membranes seem to be closely interacting through possible gap junctions, while layers of HSC-T6 cells seem to be loosely separated at some points by something similar to what is seen in the Space of Disse; this could possibly be microvilli, cytoplasmic extensions, extracellular matrix deposits, etc. In figure 17b, a hepatocyte-containing process or spike similar to that seen in vivo (4,5) can be seen on an HSC-T6 cell between two dead or dying hepatocytes. This process contains numerous Golgi bodies, characteristic of the hepatic stellate cell.

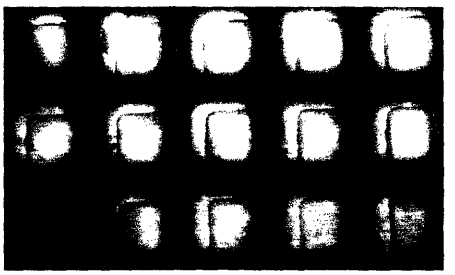
Characterization of 3-D Coculture Bioreactors

Overall Morphology Using Light Microscopy

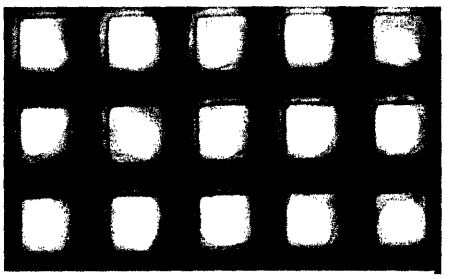
Tissue structures in coculture reactors appeared to form by 24 hr. post seeding, and tissue structures seem to increase in size until 7 days post seeding (10 days post-isolation) where some structures where observed to be growing out of the channels and into the filters (see immunostaining results). At day seven, novel morphologies (figure 18 d, e) were observed using light microscopy. Several crater-like and long channel-like morphologies were visible and these will be something to explore further using electron microscopy characterization.

Figure 18: Progression of Tissue Formation in a Coculture, HSC-T6: Hepatocyte Fraction Bioreactor

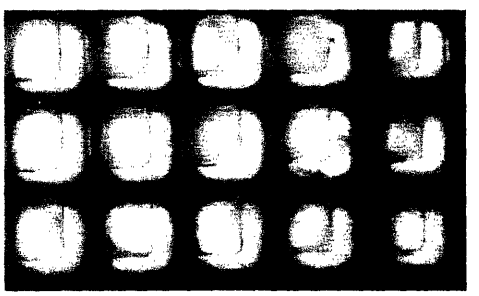
a. 24 hr. pre-reversal of cross-flow pump



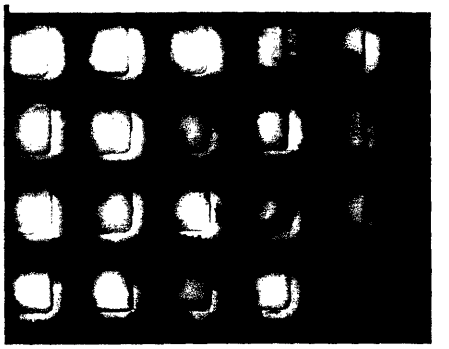
b. 3-Days (6 days post isolation)



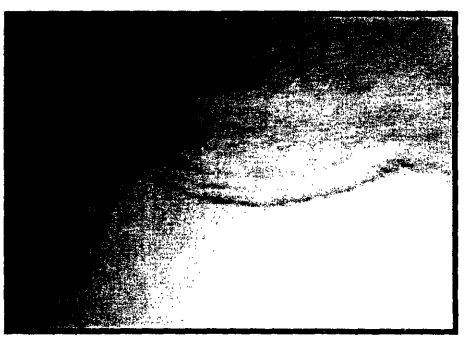
c. 5 Days



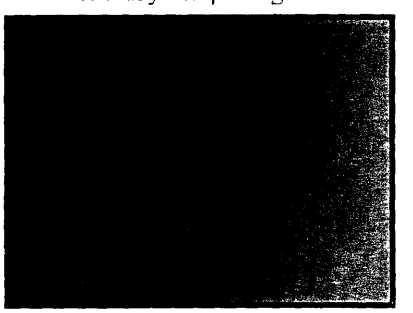
d. 7 days



d. 7 day morphologies



e. 7 day morphologies

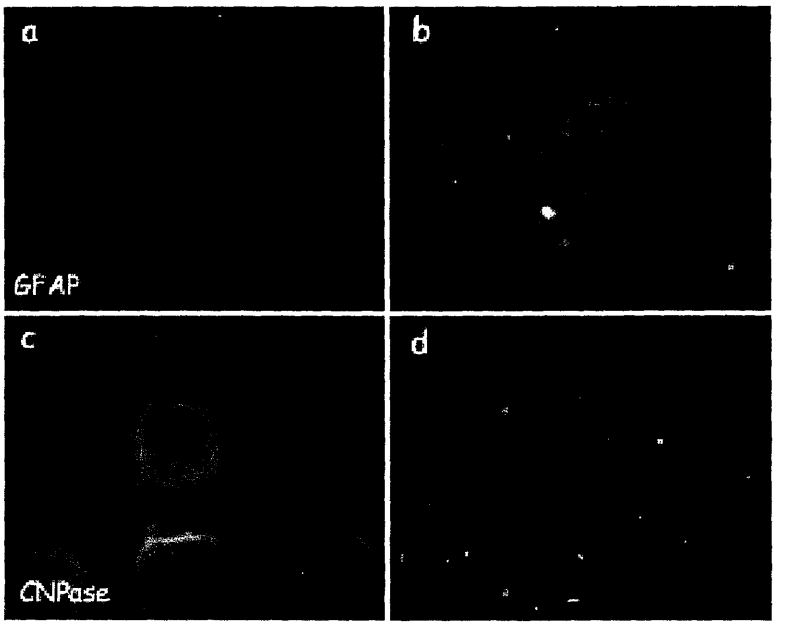


Initial Immunostaining Results

Results Immunostaining Coculture Rxr's

When viewing 7day (10-day post isolation) bioreactors, seeded with coculture spheroids, many tissue structures were found to be stuck to and possibly growing into the support filter. Upon closer inspection, many of these cells were gfap-positive HSC-T6 cells (figure 20a,b). A population of CNPase-positive cells was also observed (figure 20c,d). In the near future, after bioreactor scaffold sectioning protocols are complete, the immunostaining detection and characterization methods developed here will be directly applicable for use in the 3-D, perfused liver bioreactor.

Figure 19: Immunostaining Results of the Filter from a 7 day Coculture Bioreactor



Characterization Using Electron Microscopy

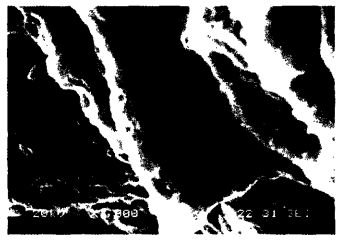
Scanning Electron Microscopy

Initial scanning electron micrographs of a tissue structure extracted from the scaffold of a 7-day, coculture reactor, show interesting morphologies. Figure 21a and 21d show the presence of fenestrated endothelium in a section of tissue from a reactor channel (whole section shown in figure 21b, amongst spheroids). In figure 21c, a smooth cell type, possibly a population of stellate cells, was observed lining the pores in the tissue structure; this is similar to what has been occasionally observed in the past in bioreactor tissue structures. It would be interesting to determine whether endogenous stellate cells can repopulate the tissue structures beginning with small populations contaminating the isolated hepatocyte fraction.

Figure 20: Scanning Electron Micrographs of 7 day, Coculture Bioreactor Tissue Ultrastructure

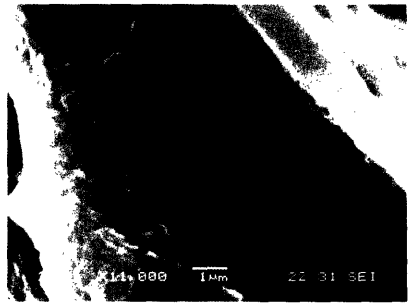
a. Extracted tissue section of a tissue structure from a 7-day bioreactor

b. Fenestrated endothelium



c. Smooth cell type lining a pore on the outside of the tissue structure

d. Possible endothelial cell junctions with few fenestrations

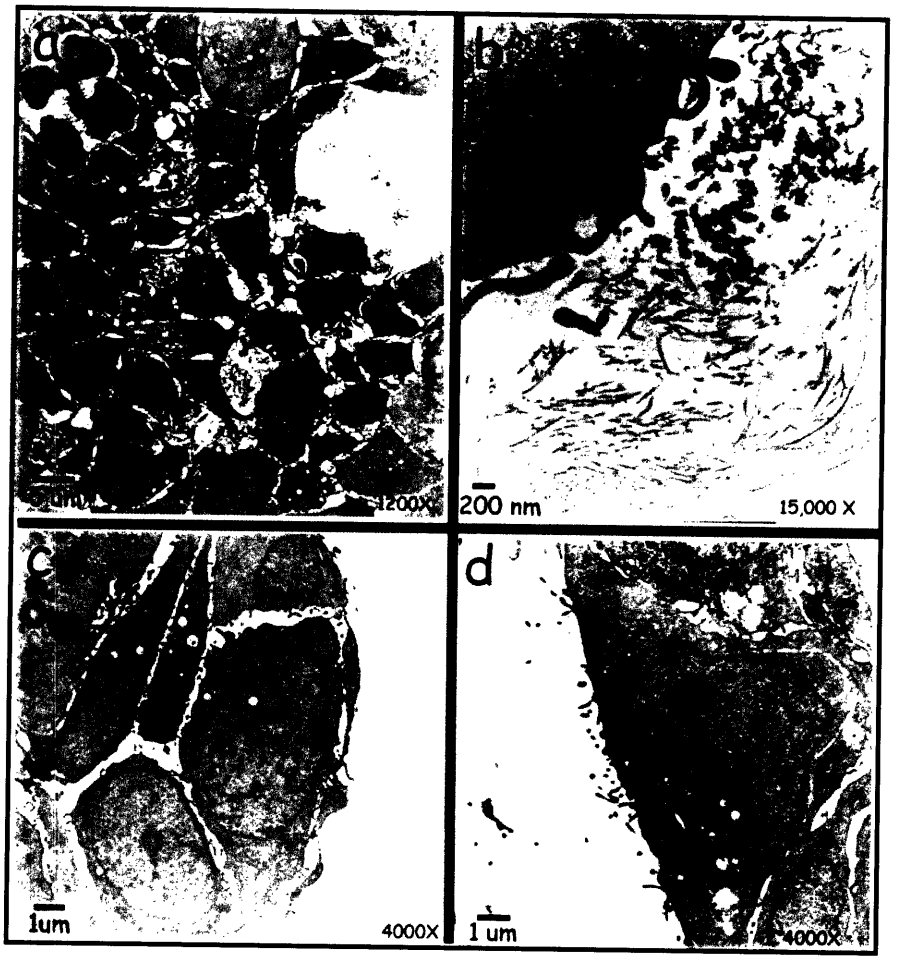


Transmission Electron Microscopy

After 7 days in the reactor, chips were fixed and processed for TEM. Some channels were seen to be overgrown with cells, possibly proliferating HSC-T6 cells. At this point, few hepatocytes were seen in the channels, and HSC-T6 cells formed a large part of the tissue structures; these cells contained numerous lipid droplets and mitochondria, as well as numerous cytoplasmic processes characteristic of stellate cells *in vivo* (4).

Figure 7d shows vesicular structures in HSC-T6's containing fibrous or flocculent material, often opening to spaces between cells; these structures are strikingly similar to those seen in stellate cells enveloping sinusoidal endothelial cells in regenerating livers, 72hr post PHx (2/3 partial hepatectomy) (Wack et. al, Hepatology 2001). Open circular spaces surrounded by HSC-T6's show large deposition of collagen I fibers, and possibly other extracellular matrix proteins (figure 22b). Spaces between each HSC-T6 show numerous cytoplasmic processes (figure 22a,c), typical of that seen in the Space of Disse in normal liver by Braet and Wisse. Because of large deposition of collagen I fibrils and possibly other extracellular matrix proteins, it would be useful in future experiments in the bioreactor to immunostain for extracellular matrix components found in the Space of Disse of normal liver and those found during basement membrane deposition in fibrosis.

Figure 21: Transmission Electron Micrographs of 7-day Coculture Bioreactor Tissue Microstructure



General Discussion

The research completed here aims to develop methods for detection and characterization of hepatic stellate inside the liver bioreactor, so that this culture system can be utilized as a tool to better understand stellate cell function and relationships with other liver cells in response to environmental cues. Using the HSC-T6 cell line, it has been show here that stellate cells can be identified and characterized using a toolbox of antibody markers in 3-D cultures, and compared with characterization results from normal and diseased liver. Immunofluorescence microscopy, as well as electron microscopy provides for a directional map of how liver cells interact in the engineered environment of the 3-D liver bioreactor. This map can give us essential information as to the roles of stellate cells in the remodeling of liver tissue and the propagation of liver disease. These methods can now be applied to the detection and characterization of primary stellate cells in relation to the parenchymal and other nonparenchymal liver cells. The development of a toolbox of antibody markers serves to provide a greater depth of knowledge concerning the heterogeneous population that comprises the hepatic stellate cell population.

Conclusions and Recommendations

General Conclusions

The hepatic stellate cell plays an essential role in the remodeling and growth of liver tissue after injury, as well as the propagation of liver disease to result in fibrosis of the liver. This heterogeneous population is small in numbers and changes its protein expression markers and functionality rapidly in response to changes in its environments. A toolbox of characterization markers was developed in three-dimensional cultures here, using a rat hepatic stellate cell line, HSC-T6, as a positive control. Immunofluorescence and electron microscopy methods were adapted to allow for in depth characterization of cell morphology and functionality, as well as to allow for observation of homo-and heterotypic cell interactions. Through this work, it is now possible to monitor stellate cell interactions in the liver bioreactor and compare them to *in vivo* results.

Work in Progress

The present research being completed in the Griffith Lab will allow for sectioning and immunostaining of the liver bioreactor; other researchers in the lab are optimizing detection methods for sinusoidal endothelial cells and Kupffer cells as well. Therefore, the methods developed here can then directly be used to detect monitor primary stellate cell functionality, reorganization, and interaction with other primary liver cell types inside the three-dimensional, perfused bioreactor.

Recommendations

Throughout the work performed here, it was observed that a positive hepatocyte marker would be helpful in identifying parenchyma and monitoring its interactions with nonparenchymal cells. Future work should concern the deliberate addition of primary

nonparenchymal cells into the bioreactor, using the detection and characterization methods developed here and by other researchers in the lab, to monitor and characterize tissue structure formation and liver-like interactions inside the bioreactor.

Significance

In the literature it has been observed that tens of biologically active factors are synthesized and released by liver cells that exert profound autocrine and paracrine effects. It has also been demonstrated that cell-cell interactions, both homo- heterotypic, are necessary to maintain the intricate liver structure/function relationship, and all of these factors contribute to the maintenance of normal liver homeostasis; when this homeostasis is disrupted through injury, infection, or other extrinsic factors, liver disease results (4,5). Interestingly, the role of cell-cell interactions and the cross-talk between various types of liver cells is better understood during pathological situations than in normal liver function. One possible reason for this is the lack of methods to study normal function *in vivo* in real-time, using human liver cells to avoid species differences. The 3-dimensional liver bioreactor developed in the Griffith lab is modeled after *in vivo* properties, such as constant, perfused flow and a three-dimensional scaffolding. Liver cells appear to self-organized into highly functional tissue structures inside the bioreactor, which supports long-term culture (2,3). It has become apparent that all liver types are important in maintaining liver structure and function. The research completed here will allow for detection and characterization of hepatic stellate cells, in order to better understand their role in liver health and disease.

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Appendix

I. Immunostaining Information

Table 1: Antibody Information

Antibody Name	Company	Product Number	Dilution	Incubation Time, Temp (^{0}C)
Mouse anti-desmin	BD Biosciences	550626	1:50	Overnight, (4 ^{0}C)
Mouse anti-GFAP	BD Biosciences	556327	1:100	Overnight, (4 ^{0}C)
Anti-α SMA:FITC	Serotec	F3777	1:50	2hr., room temp.
Rabbit anti-GFAP	Chemicon	AB5804	1:100	Overnight, (4 ^{0}C)
Rabbit anti-desmin	Lab Vision	Rb-9014-R7	1:100	Overnight, 4 ^{0}C)
Mouse anti-synpaptophysin	Chemicon	MAB368	1:50	Overnight, (4 ^{0}C)
Mouse anti-β-Tubulin	Chemicon	MAB1637	1:50	Overnight, 4 ^{0}C)
Mouse anti-CNPase	Chemicon	MAB326R	1:100	Overnight, (4 ^{0}C)
Goat anti-rabbit TRITC	Sigma	T6778	1:100	1 hr., room temp.
Goat anti-mouse cy3	Jackson Immunoresearch	115-165-166	1:100	1 hr., room temp,

Immunostaining Protocols

1) Fix samples in 4% Paraformaldehyde in PBS for 20 min for 2d cultures and sections, and 1-2 hr. for reactor scaffolds in petri dishes, at room temp.

2) Wash samples in PBS three times for 15 min. each at room temp.

3) If sample is not sectioned (i.e., whole cells in Nunc wells), trypsinize the samples using 0.1% Triton-X in PBS for 20 min.

4) Wash samples in PBS three times for 15 min. each at room temp.

5) Wash the sample in PBG (PBS with 0.5% BSA, 0.15% glycine) three times for 15 min. each at room temp.

6) Block the samples with 5% Goat Serum in PBG for 30 min. at room temp.

7) Wash three times with PBG for 15 min. each at room temp.

8) Dilute primary antibodies according to Table 1 in PBG and incubate for the desired times (Table1).

9) Wash samples in PBS three times at room temp.

10) Dilute secondary antibodies according to Table 1 in PBG and incubate for 1 hr. at room temp.

11) Wash three times with PBG.

12) Wash three times with PBS.

13) Stain with a nuclear stain such as 1/1000 diluted Hoescht's Dye for 1min.

14) Washing immediately with PBS, three time for 15min. each.

15) Mount on slide with mounting medium (Gelvatol kept at 4 degrees C) and place a coverslip on top of mounted sample. Let your sample solidify overnight if possible before viewing.

II. Electron Microscopy

SEM for Spheroids, Bioreactor

The polycarbonate chip and filter were carefully removed at appropriate timepoints from the reactor and immediately fixed in 2.5% gluteraldehyde in PBS for 2 hours. For spheroids, the total sample of 100ml was removed from the spinner flask after 3 days, spun down at 40g for 3min at 4^0C., and resuspended in 2.5% gluteraldehyde for 2 hours. All samples were then washed with PBS three times for 15 minutes each. The samples were then post-fixed with a 1% solution of Osmium tetraoxide in PBS for 2 hours at room temp, followed by three PBS washes for 10 min. each. In order to increase the tissue structure stability, the samples were then immersed in a 1% percent aqueous solution of thiocarbohydrazide for 30 min, followed by three more 10 min. PBS washes, and then post-fixed again in the Osmium tetraoxide solution for 1 hr. This process was repeated once more to result in three cross-linked osmium layers. After washing three times again with PBS, the samples were then dehydrated using a graded ethanol series (30%, 50%, 70%, 95%, 100% X 3), where they were immersed in each solution for 10 min. The chips were then dried using a series of hexamethyldisilazane (HMDS)/ethanol solutions (3:1 EtOH/HMDS, 1:1, 1:3 EtOH/HMDS), where the samples were immersed for 15 min. in each solution. After two washes with 100% HMDS in covered petri dishes, the samples were then exposed to air and allowed to dry. Immediately following this, the samples were then mounted and sputter-coated with gold/palladium for 2min. using an Anatech Ltd. Hummer 6.2 sputter coater. The samples were then viewed in a JEOL JSM-5600 LV scanning electron microscope. Both low and high magnification images were captured so that both macro-and micro- tissue structures could be characterized.

TEM for Spheroids, Bioreactor

Modified from: **Powers, M.P., Janigian, D.M., Wack, K.E., Baker, C.S., Stolz, D.B., and Griffith, L.G.** ***Functional Behavior of Primary Rat Liver Cells in a Three-Dimensional Perfused Microarray Bioreactor.*** **Tissue Eng 2002;8(3):499-513.**

Scaffolds were fixed in 2.5% glutaraldehyde in PBS for 24 h, washed three times with PBS, and post-fixed for 1 h with aqueous 1% osmium tetroxide. After three PBS washes, scaffolds were dehydrated through a graded ethanol series, and then further dehydrated in four 15-min changes of 100% ethanol. Scaffolds were subjected to two 10-min incubations in propylene oxide, and then preembedded with a 1:1 mix of propylene oxide: Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1 h. Scaffolds were then incubated in 100% Polybed overnight at 4°C. The following day, resin was changed four times before em-bedding chips in a thin layer of resin, just enough to fill the channels, and curing at 37°C overnight and then at 65°C for an additional 2 days. Blocks of cells from individual channels were removed from the scaffold and reembedded in rubber molds and cross-sectioned perpendicular to channel flow. Thin sections (60 nm) were collected on copper grids and stained with 4% uranyl acetate in 50% methanol for 7 min and with 1% lead citrate for 10 min. Cells were viewed with a JEOL (Tokyo, Japan) JEM 1210 transmission electron microscope (TEM) at 80 kV.

III. Stellate Cell Isolation using Nycodenz Gradients

Materials to Prepare in advance:

28.7% Nycodenz in HBSS

-Sterilize in autoclave for 15 minutes liquid cycle

HBSS-BSA

HBSS with 3g/L BSA

Protocol:

1.Pellet NPC fraction in centrifuge- 400gx 10 min

2. Resuspend in 19ml HBSS (note protocol in paper called for GBSS) with 3gm/L BSA (HBSS-BSA)

3. Mix with 16ml of 28.7% (w/v=gms/100ml) Nycodenz in HBSS (note protocol says GBSS w/o NaCl)

4. Put 6 ml of HBSS-BSA into two 50ml centrifuge tubes

5. Put 17.5 ml HBSS-BSA-Nycodenz with cells underneath

6. Centrifuge 1,400 g for 20 minutes at 4 C

7. Take layer at edge of Nycodenz and resuspend in 50 ml HBSS

8. Centrifuge 400g 10 minutes

9. Resuspend pellet in culture media.

10. Plate cells on tissue culture-treated plastic.

Protocol from Hepatology 1987 vol 7 #4 pp680-687
"The synthesis of Proteoglycans in Fat-Storing Cells of Rat Liver", Stephen Schafer, Ottwin Zerbe, Axel Gressner

IV. Protocol for Oil Red O stain

(Koopman, Rene. Schaart, Gert. Hesselink, Matthijis KC; Histochem Cell Biol; 2001; 116;63-68)

Materials:

3.7% formaldehyde solution in deionised water
Oil Red O stock solution
500 mg oil red O dissolved in 60% triethyl-phosphate
Oil Red O working solution
36% triethyl-phosphate solution - containing 12 ml oil red O stock solution and 8 ml deionized water. Filter through Whatman paper number 42 to remove crystalized oil red O.
(Note Whatman paper 42 has pore size 2.5 um (Particle retention))
PBS (buffer for dilution of Triton X-100, glycerol and antibodies)

Oil red O staining:

1. Chemically fix the cell using 3.7% formaldehyde for 1 hr
2. Wash off excess formaldehyde with three washes of deionized water for 30 seconds each
3. Immerse samples in oil red O for 30 minutes
4. Wash sections with three washes of deionized water for 30 seconds each
5. If desired counterstain to visualize nuclie
6. Rinse for 10 min in running tap water
7. Cover with a coverslip using 10% glycerol in PBS

Combined oil red O and immunofluorescence staining:

1. Chemically fix the cell using 3.7% formaldehyde for 1 hr
2. Wash off excess formaldehyde with three washes of deionized water for 30 seconds each
3. Treat samples with .5% Triton X-100 in PBS for 5 min
4. Wash with three exchanges of PBS for 5 minutes each
5. Incubate for 30 min at room temp with primary antibody in appropriate dilution.
6. Wash with three exchanges of PBS for 5 minutes each
7. Apply the secondary (FITC/TRITC conjugated) antibody for 30 minutes at room temp
8. Wash with three exchanges of PBS for 5 minutes each
9. Immerse samples in oil red O for 30 minutes
10. Wash sections with three washes of deionized water for 30 seconds each
11. If desired counterstain to visualize nuclie

12. Rinse for 10 min in running tap water a
13. Cover with a coverslip using 10% glycerol in PBS containing DAPI to visualize nuclei.

Notes on this protocol- This may help assess how well stellate cells in spheriods or the bioreactor uptake and store vitamin A and lipids, or how quiescent a phenotype the cell line is.

V. HSC-T6 Medium and Passaging Information

Medium Recipe:

-500 ml, sterile DMEM with low glucose and added sodium pyruvate

-10% fetal bovine serum

-5 ml of a penicillin streptomycin solution (same solution used in HGM)

A vial of HSC-T6 cells was taken out of storage in liquid nitrogen and thawed quickly in a warm water bath. The solution was then combined with 45 ml of fresh HSC-T6 medium and spun down at 400g for 5min. After resuspension in 10ml of HSC-T6 medium, the cells were plated and culture in T-75 flasks. The medium was changed every other day, and after the cells reached 75% confluence, they were trypsinized using 5ml of trypsin solution for 5 min. in the incubator. The cells were then washed with medium, spun down at 400g for 5 min., resuspended in 40 ml of HSC-T6 medium, and plated in four, new T-75 flasks. Cells were trypsinized and used in experiments as needed.

VI. Spheroid and Reactor Seeding

Modified from: **Powers, M.P., Janigian, D.M., Wack, K.E., Baker, C.S., Stolz, D.B., and Griffith, L.G.** ***Functional Behavior of Primary Rat Liver Cells in a Three-Dimensional Perfused Microarray Bioreactor.*** **Tissue Eng 2002;8(3):499-513.**

Hepatocytes were isolated from 150 to 230-g male Fischer rats by a modification of Seglen's two-step collagenase perfusion procedure 18 as described previously. The resulting cell suspension is centrifuged three consecutive times at 50g (2 min each). After the final centrifugation the pellet is resuspended in hepatocyte growth medium (HGM), similar to that described by Block and co-workers but without hepatocyte growth factor. Cell viability before cell seeding was 90–95% as determined by trypan blue exclusion. The fraction of nonparenchymal cells that is typically observed in this suspension is approximately 5%. Spheroids were formed in suspension culture similar to the methods of Wu and coworkers but modified as described here to include HSC-T6 cells as well as hepatocytes. One hundred milliliters of the cell suspension was then added to a 250-mL spinner flask (Bellco Glass, Vineland, NJ), which was stirred at 85 rpm for up to 72 h. Spheroids were resuspended in 25 ml of rinse medium and centrifuged at 40 3 g for 3 min, and resuspended in 30 ml of 50% HGM, 50% HSC-T6 medium.

The heart of the reactor is a 230-mm-thick polycarbonate scaffold containing an array of 100 round-edge square channels, each of cross-sectional dimension 300 by 300 mm. A microporous filter and supporting scaffold beneath the cell scaffold provide for initial retention of cells in the channels under perfusion flow through the cell mass; cell retention at times greater than 1 day is governed by cell adhesion to the channel walls. Reactors were primed with rinse solution to passivate the reactor, connector, and tubing surfaces, and to remove bubbles from the flow paths. The rinse solution comprised phenol red-free Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate (110 mg/ml) and glucose (1 g/L) (Life Technologies, Rockville, MD) supplemented with bovine serum albumin (2 g/L; Sigma, St. Louis, MO) and penicillin–streptomycin (100

U/ml). Immediately before seeding, reservoir bottles were aspirated and re-filled with the medium described above. Cross-flow was reversed after 24 hours to remove debris in the channel and the cross-flow was maintained this way for the remainder of the culturing period. The medium reservoir was replenished with fresh medium every other day. The reactors were kept in an incubator set at 37 degrees C and 8.5% oxygen.