Review

Zonal liver cell heterogeneity: effects of oxygen on metabolic functions of hepatocytes

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Abstract—*The morphology and function of hepatocytes are known to vary with position along the liver sinusoids from the portal triad to the central vein. Functions such as urea synthesis and the activity of the cytochrome P-450 system are different, depending on whether the cell is located in a peri-portal zone or in a peri-venous zone. The factors determining this heterogeneous function are not fully understood. Possible explanations of this phenomenon include gradients in hormones, substrates, oxygen, pH, innervation and extracellular matrix composition. Past studies are reviewed, and some recent efforts are described that examine the potential role of oxygen in directly or indirectly inducing differences in hepatocyte function. Partial pressure of oxygen appears to have a role in urea synthesis, lipid metabolism, cytochrome P-450 activity and gluco-neogenesis, which corresponds to in vivo zonal localisation of these functions. Thus, as part of the overall design criteria for a bio-artificial liver device, oxygen distribution at the tissue surface should be included with the more standard criteria, such as optimisation of flow rate, cell dispersal and cell density within these systems.*

Keywords—*Bio-artificial liver, Hepatocyte, Heterogeneity, Oxygen*

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1 Introduction

THE MORPHOLOGY and function of hepatocytes are known to vary with position along the liver sinusoids from the portal triad to the central vein. For example, functions such as urea synthesis and the activity of the cytochrome P-450 system are different, depending on whether the cell is located in a peri-portal zone or in a peri-venous zone. Possible explanations of this phenomenon include gradients in hormones, substrates, oxygen, pH and extracellular matrix composition (HAUSSINGER, 1988; REID *et al.*, 1992; SUNGCHUL *et al.*, 1982; WOLFE *et al.*, 1983; 1985). However, the importance of each factor in causing heterogeneous function within the liver sinusoid has not been resolved. A quantitative evaluation of those external factors related to promoting heterogeneous function *in vivo* is likely to lead to an improved understanding of *in vivo* metabolism and the ability to control certain aspects of the *in vitro* function of hepatocytes in various bio-engineering applications.

The definition of the functional and structural unit of the liver is a matter of debate. The concept of the 'liver acinus' has been adopted by many investigators as the best compromise between patho-histological and microcirculatory findings (SASSE *et al.*, 1992; REID *et al.*, 1992; RAPPAPORT *et al.*, 1954; RAPPAPORT, 1976). The parenchyma in the region of the terminal afferent vessels forms the acinus, as seen in Fig. 1. Three different zones of tissue have been postulated, where the peri-portal zone is adjacent to the afferent vessels and the peri-venous zone receives blood that has already exchanged gases and metabolites with cells in zones 1 and 2.

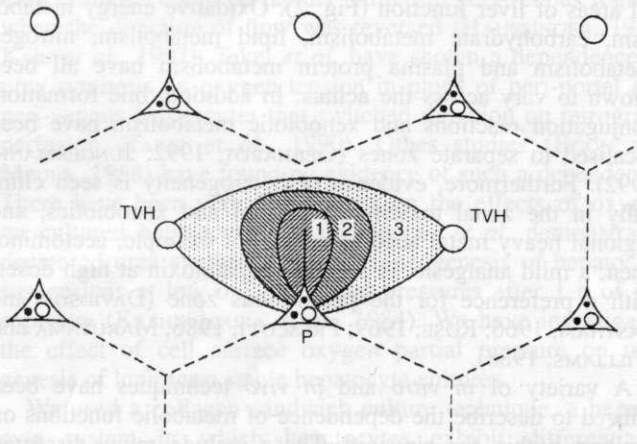

Fig. 1 *Schematic diagram of the liver acinus; the centre is formed by the terminal afferent vessels of the portal triad (P), the periphery of which is drained by terminal hepatic venules (TVH); there are three different acinar zones: (1) the peri-portal zone; (2) the intermediate zone; (3) the peri-venous zone (adapted from SASSE et al., 1992)*

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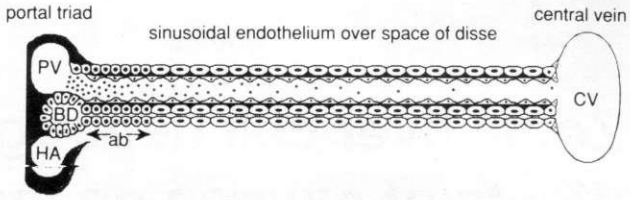


cell size	7-15μm	15-20μm	30-40μm	30-40μm
collagens	IV,V	IV,III	III,VI	III,I,VI
adhesion proteins	laminin	laminin, fibronectin	fibronectin	fibronectin
proteoglycans	HS-PG	HS-PG,HP-PG	HP-PG,HS-PG,CS-PG	HP-PG,DS-PG

zonation of liver function	
gluco-neogenesis, urea genesis, ß-oxidation	high ◄ ► low
liponeogenesis, c P450 (IAI)	low ◄ ► high
albumin, DNA	◄ uniform ►

Fig. 2 *Potential regulators of hepatocyte heterogeneity along a schematised sinusoid (modified from* Reid *et al., 1992) and zonation of metabolic functions (adapted from* Gebhardt, *1992); extracellular matrix for each category is listed in order of abundance: HS-PG = heparin sulphate-proteoglycan; HP-PG = heparin proteoglycan; CS = chondroitin sulphate; DS = dermatan sulphate; a = differentiation towards bile duct; b = differentiation towards hepatocyte; PV = portal vein; BD = bile duct; HA = hepatic artery; CV = central vein; ◇ = oxygen, hormones*

The heterogeneity of hepatocytes with respect to ultrastructure and enzyme activities along this axis results in different cellular functions within different zones of the liver acinus. Two types of heterogeneity have been described: gradients of gene expression and compartment-restricted expression. Most proteins are present in all hepatocytes across the lobules, although in different amounts. These gradients of gene expression are thought to be correlated with rates of transcription that can be dynamically shifted to other areas of the lobule under the proper stimulus (Gebhardt, 1992). In contrast, compartment-restricted expression has only been identified for ammonia-metabolising enzymes and is typically restricted to a subset of hepatocytes in a circumscribed area (Gebhardt and Mecke, 1984; Moorman *et al.*, 1989; 1990). The expression of this class of enzymes cannot be shifted to other areas of the lobule under most stimuli. These two patterns of heterogeneity indicate the complex interplay of regulatory signals in this system.

Zonal liver cell heterogeneity has been described in virtually all areas of liver function (Fig. 2). Oxidative energy metabolism, carbohydrate metabolism, lipid metabolism, nitrogen metabolism and plasma protein metabolism have all been shown to vary across the acinus. In addition, bile formation, conjugation reactions and xenobiotic metabolism have been localised to separate zones (Gebhardt, 1992; Jungermann, 1992). Furthermore, evidence of heterogeneity is seen clinically in the zonal toxicity of ethanol and xenobiotics, and regional heavy metal accumulation. For example, acetominophen, a mild analgesic, is a potent hepatotoxin at high doses, with a preference for the peri-venous zone (Davidson and Eastham, 1966; Rose, 1969; Prescott, 1986; Maruyama and Williams, 1988).

A variety of *in vitro* and *in vivo* techniques have been utilised to describe the dependence of metabolic functions on position within the sinusoid. These include micro-dissected liver tissue (Katz *et al.*, 1983), isolation of peri-portal or peri-venous cells from the liver (Poso *et al.*, 1986) and a membrane fragment culture system (Saad *et al.*, 1994). Studies of cultured cells enable the conditions to be better controlled, but most hepatocyte cultures provide stable function for only a few days. This time frame may not be sufficient to enable cells to acquire heterogeneity induced by variations in the controlled environment; therefore, long-term stable culture configurations may be useful in the study of hepatocyte heterogeneity *in vitro*.

The preservation of hepatocyte functions in general, and a heterogeneous hepatocyte population in particular, may be of special importance in bio-artificial liver (BAL) devices. These extracorporeal devices would utilise immobilised hepatocytes to perform their array of functions on the patient's blood or plasma. A variety of device configurations have been described, utilising both primary and transformed cells (Sussman *et al.*, 1992; Nyberg *et al.*, 1993). In general, hollow fibres are used as the vehicle of cell containment. Hepatocytes have been placed both in intraluminal and extraluminal compartments. In addition, several modifications have been reported, including hepatocyte adhesion to microcarriers and cells entrapped in collagen gels (Shatford *et al.*, 1992; Neuzil *et al.*, 1993).

It may be important to preserve heterogeneity of hepatocyte metabolism in these devices for at least three aspects of liver function: glucose regulation, nitrogen metabolism and drug metabolism. The zonal separation of glucose release and uptake has been implicated in the livers ability to operate as a 'glucostat' (Jungermann and Thurman, 1992). This is most clearly demonstrated in the predominance of gluco-neogenesis in the peri-portal zone and of glycolysis in the peri-venous zone (Gebhardt, 1992). These two zones of the liver interact via lactate transport in the circulation and both seem to be important in the appropriate storage and release of glucose and its precursors.

Another aspect of liver metabolism that may be important to mimic in a BAL device is the separation of ammonia degradation into urea synthesis peri-portally and glutamine synthesis peri-venously. This pathway seems to allow the decoupling of detoxification of ammonia from the role of urea in acid-base homeostasis (Haussinger, 1988). Finally, it may be important to preserve heterogeneous hepatocyte function with regard to xenobiotic metabolism in a BAL. This can be especially critical in liver failure patients who may need medication that will require hepatic biotransformation. Many constitutents of the drug-metabolising pathways have been

identified as heterogeneously distributed (Baron and Kawabata, 1983; Baron *et al.*, 1984; Baron, 1991; Thurman *et al.*, 1986; Traber *et al.*, 1988; Aanderson *et al.*, 1989).

For the reasons discussed above, it appears important to consider the preservation of hepatocyte heterogeneity in the design of BAL devices. Regulators of *in vivo* zonal heterogeneity are not sufficiently understood that we can easily mimic this phenomenon *in vitro*. Possible mediators (Fig. 2) include blood-borne hormones, oxygen, pH, extracellular matrix composition and innervation (Wolfle *et al.*, 1981; 1983, Wolfle and Jungermann, 1985; Sungchul *et al.*, 1982; Haussinger, 1988; Reid *et al.*, 1992). In addition, many of these regulators may be linked in a complex interplay. BALs based on current technology are limited in their ability to modulate many of these factors. For example, entrapped cells secrete extracellular matrix into the micro-environment over a period of days, thereby modifying any attempt at defining the long-term composition of the adhesive substratum. Similarly, hormonal and pH gradients are altered by the patients blood or plasma composition.

One factor that has the potential to be well regulated in a BAL is oxygen tension. Blood or plasma is easily oxygenated as part of the extracorporeal circuit. Furthermore, the oxygen uptake of hepatocytes could be utilised to modify oxygen distribution within the device. Given that oxygen is a potential regulator of hepatocyte heterogeneity and that its distribution may be controlled in a bio-artificial device, it is important to critically evaluate the potential role of oxygen to induce differences in hepatocyte function.

2 Role of oxygen tension

Peri-portal hepatocytes are exposed to greater oxygen tensions than their downstream peri-venous counterparts. In this paper, we investigate the ability of oxygen tension to modulate the observed *in vivo* heterogeneity of gene expression *in vitro*. Specifically, we present our long-term culture results, as well as those of other investigators, with regard to nitrogen metabolism, plasma protein production, xenobiotic metabolism, DNA content, lipid metabolism and carbohydrate metabolism.

2.1 *Estimation of oxygen tension*

In vitro diffusion of oxygen from the gas phase through static media or hydrogels to the cell surface causes gradients of partial pressure of oxygen. To correlate culture experiments with *in vivo* levels of oxygen, this effect must be quantified. Oxygen partial pressure at the cell level in culture systems has been estimated previously using a diffusion-reaction model (Rotem *et al.*, 1992; Yarmush *et al.*, 1992; Foy *et al.*, 1994). Fig. 3 shows the dependence of oxygen tension at the cell surface on ambient oxygen tension and hepatocyte density in a mono-layer configuration. This model has been extended to sandwich culture systems, where diffusion must also occur through a top layer of collagen gel. Using this model, gas phase oxygen partial pressures of 7.4, 37, 51, 74, 110 and 154 mm Hg discussed in the following long-term culture experiment correspond to oxygen partial pressures at the hepatocyte surface of 1.4, 21, 36, 57, 94 and 137 mm Hg, respectively. The physiological range is expected to be from 5 mm Hg to 90 mm Hg, depending on the proximity to the portal triad and hepatic arterioles and the length of each hepatic sinusoid (Kessler *et al.*, 1973; 1980; Yamamoto *et al.*, 1985; deGroot *et al.*, 1988). Another model (Nauck *et al.*, 1981) was not utilised in the sandwich system as it relies on sampling of the media in direct contact with the cells.

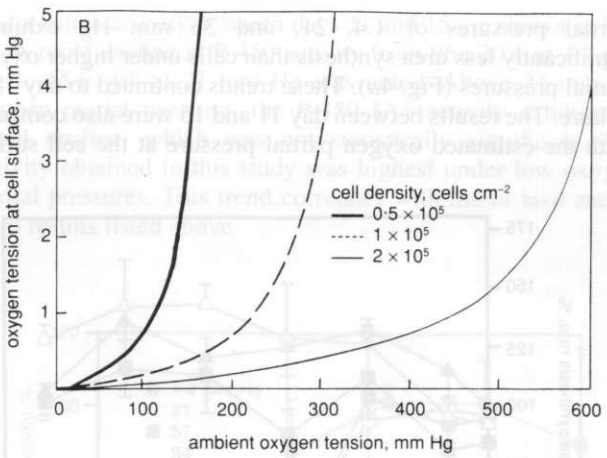


Fig. 3 *Calculated cell-surface oxygen versus gas-liquid oxygen tension based on a diffusion-reaction model of transport through media to hepatocyte monolayer (Yarmush et al., 1992); diffusive transport of oxygen is assumed to proceed with a diffusion coefficient of 2×10^{-5} cm^2 s^{-1}; and the oxygen uptake rate of the hepatocytes follows Michaelis-Menten kinetics; this model is later adapted to sandwich culture, where a collagen gel of thickness 0.23 mm is placed on top of the cells, followed by a layer of media that was not considered to be well mixed, producing an effective diffusion distance of 1.14 mm from the top of the medium to the cell surface*

2.2 *Effect of O_2 on nitrogen metabolism (urea synthesis)*

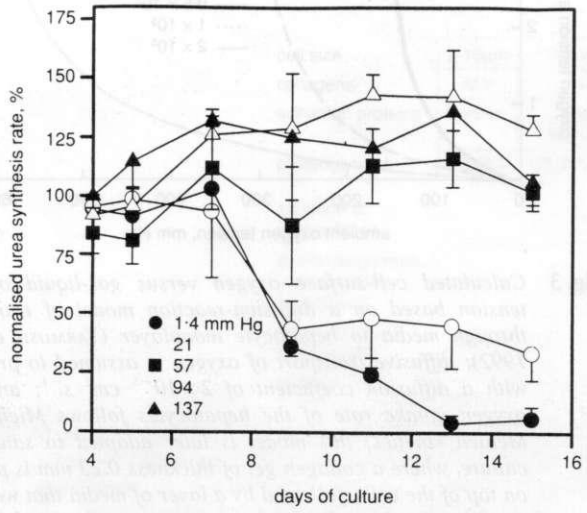
Urea synthesis is known to occur primarily in peri-portal hepatocytes. A variety of enzymes of the urea biosynthetic pathway have been localised to the peri-portal region using immuno-histochemical techniques. These include carbamoyl-phosphate synthetase (Gaasbeek *et al.*, 1984*a;b;* Smith and Campbell, 1988), arginosuccinate synthase and arginase (Saheki *et al.*, 1983). In addition, urea genesis was found to be two to three times higher in peri-portal cells than in peri-venous cells using separate cultured cell populations (Quistorff *et al.*, 1986; Poso *et al.*, 1986).

The potential of oxygen to mediate this aspect of liver zonal heterogeneity has been investigated both *in vivo* and *in vitro*. *In vivo* perfusion studies of the liver lobule with a buffer of defined oxygen content showed that antegrade perfusion led to a urea synthesis rate that was three times higher in peri-portal cells than in peri-venous cells. Furthermore, the primary site of urea synthesis could be rapidly shifted to peri-venous regions when the direction of flow was reversed (Haussinger, 1983; Kari *et al.*, 1987). Takei *et al.* have shown a dependence of urea synthesis on oxygen tension in plugs of peri-portal and peri-venous hepatocytes that switched direction on retrograde perfusion (Takei *et al.*, 1990). Other studies (Boon and Meijer, 1988) have found no evidence of such a dependence. There have been very few studies on the effects of oxygen on cultured hepatocytes. Kashiwagura *et al.* demonstrated decreased urea synthesis and gluco-neogenesis of hepatocyte suspensions at low oxygen partial pressures after 1 h of gas exposure (Kashiwagura *et al.*, 1984). We have investigated the effect of cell surface oxygen partial pressure on urea genesis of long-term stable hepatocyte cultures.

We used a collagen sandwich culture technique, a hepatocyte system in which hepatocytes exhibit differentiated function for several weeks (Dunn *et al.*, 1989), to allow sufficient time for the cells to adapt to, and be influenced by, various oxygen tensions. Long-term cultures of an initially heterogeneous hepatocyte population showed differences in urea synthesis after five days of switching to altered oxygen environments. Hepatocytes exposed to cell surface oxygen

partial pressures of 1.4, 21, and 36 mm Hg exhibited significantly less urea synthesis than cells under higher oxygen partial pressures (Fig. 4*a*). These trends continued to day 15 of culture. The results between day 11 and 15 were also compared with the estimated oxygen partial pressure at the cell surface (Fig. 4*b*). As the oxygen partial pressure at the cell surface increased from 1.4 to 57 mm Hg, the urea production increased from 12% to 112%; however, further elevations in oxygen partial pressure did not produce statistically* significant differences in urea production.

Thus, urea synthesis, which predominates in the oxygen-rich peri-portal region of the acinus, is also increased in both long-term hepatocyte cultures and perfusion experiments under greater oxygen tension.



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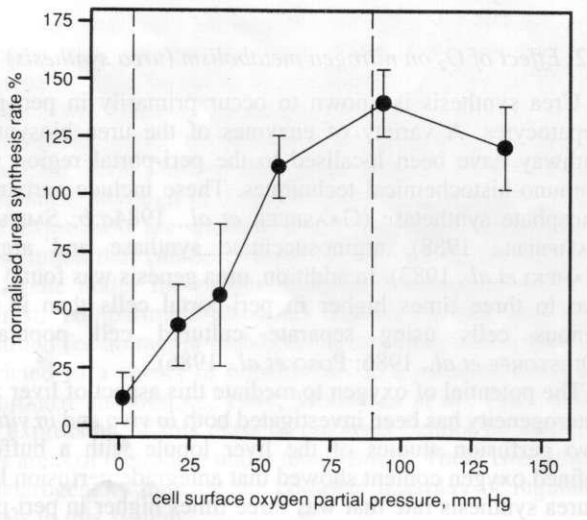

b

Fig. 4 *(a) Normalised urea synthesis rates versus days of culture with the cell surface oxygen partial pressure (see fig. 3 for details of model estimation); each set of data is normalised by setting the functional activity for a certain time point and condition to 100% for each isolation; values of functional activity were normalised to the DNA content at each time point for all conditions; 100% = a urea production rate on day 4 of culture for 137 mm Hg at the cell surface level of 267.2 ± 28 and 146.8 ± 44 ng urea nitrogen μg^{-1} DNA h^{-1} for each of two isolations: (b) effect of cell surface oxygen partial pressure on the average normalised urea synthesis rate between day 11 and day 15 of culture; dashed lines = estimated physiological range of oxygen partial pressures between 5 and 90 mm Hg; urea concentration was analysed in collected media using a BUN-urea nitrogen kit (Sigma)* (DNA content determined as described in fig. 7)*

2.3 *Effect of O_2 on production of plasma proteins (albumin)*

Early immuno-histochemical investigations of albumin and other plasma proteins showed an artificial mosaic distribution of these compounds due to non-specific random protein uptake in hepatocytes or inhyomogeneous penetration of antibodies (Guillouzo *et al.*, 1978; LeBouton and Peters Masse, 1980*a;b*; Pignal *et al.*, 1982; Cazier *et al.*, 1987; Gebhardt, 1992). Re-evaluation of this issue via *in situ* hybridisation has revealed that the synthesis of albumin mRNA and protein occurs to a similar extent in hepatocytes throughout the sinusoid without zonal variations (LeBouton and Peters Masse, 1980*a;b*; Yokata and Fahimi, 1981; Guillouzo *et al.*, 1982; Bernau *et al.*, 1985; Tuczek *et al.*, 1985; Shroyer and Nakane, 1987; Saber *et al.*, 1990).

The effects of oxygen tension at the cell surface on albumin secretion have not been widely investigated *in vitro* or *in vivo*. We have studied the potential of oxygen tension to affect rates of albumin secretion in long-term hepatocyte cultures. Under different partial pressures of oxygen, the albumin production rate, normalised to DNA content, increased from day 5 to day 9 as the hepatocytes stabilised in the culture (Fig. 5*a*). Average albumin secretion over days 11–15 shows that the albumin secretion rate was unaffected by cell surface oxygen partial pressures between 21 and 94 mm Hg (Fig. 5*b*, $p<0.05$). Therefore, as seen *in vivo*, variations in oxygen tension at the cell surface within the physiological range do not seem to modulate albumin secretion rate in hepatocyte cultures.

2.4 *Effect of O_2 on xenobiotic metabolism (cytochrome P-450 IA1 mono-oxygenase)*

Cytochrome P-450 mono-oxygenases catalyse phase I reactions in xenobiotic biotransformation. One of the isozymes, P-450 IA1, has been the subject of previous studies. In general, immuno-histochemical studies of uninduced rat liver show peri-venous localisation of P-450 IA1 (Baron *et al.*, 1978; 1981; 1982; 1984; Moody *et al.*, 1986; 1988; Smith *et al.*, 1990). Although some researchers have reported conflicting results (Wolf *et al.*, 1984; Tsudkidate *et al.*, 1989), total P450 content has also been reported to be increased in the peri-venous region (Gooding *et al.*, 1978; Smith and Wills, 1981; Steinberg *et al.*, 1988; Watanabe *et al.*, 1989; Seibert *et al.*, 1989; Kanai *et al.*, 1990). Upon induction with phenobarbitol, both an increase in cytochrome P450 content and a proliferation of endoplasmic reticulum have been shown peri-venously (Kanai *et al.*, 1986; 1990).

One short-term *in vitro* study (Saad *et al.*, 1994) has demonstrated the differential effect of oxygen tension on the preservation and inducibility of P-450 iso-enzymes. Most iso-enzymes were increased at greater oxygen tensions in this short-term culture system; however P-450 IA1 activity was seen to increase with decreasing oxygen tension (correspond-

* For stable long-term cultures, 2 million viable hepatocytes were seeded between two layers of type I collagen as described previously (Dunn et al., 1989). Cultures were incubated for 4 days in 2 ml of medium in room air supplemented with 10% CO_2 to stabilise hepatocytes after isolation. Culture dishes were then placed within a larger container on a rotary shaker into which gas of specified oxygen partial pressure was continuously purged at 0.5 l min^{-1} (Rotem *et al.*, 1994)

*All experiments were repeated with cells from at least two separate isolations with three culture dishes per condition per isolation. All values are expressed as mean ± SD. For statistical comparisons, a students *t*-test was performed at a confidence level of 5%.

ing to the peri-venous zone *in vivo*).

The ability of oxygen tension to directly or indirectly create variations in P-450 activity has not been extensively investigated in stable long-term cultures *in vitro*. Using a sandwich culture system under varying oxygen tensions, we have demonstrated an increase in cytochrome P-450 IA1 activity between days 5 and 11 of culture (Fig. 6*a*). Hepatocytes cultured with the lowest cell surface oxygen partial pressure of 1.4 mm Hg exhibited significantly greater P-450 IA1 activity than hepatocytes cultured at any other oxygen partial pressure.

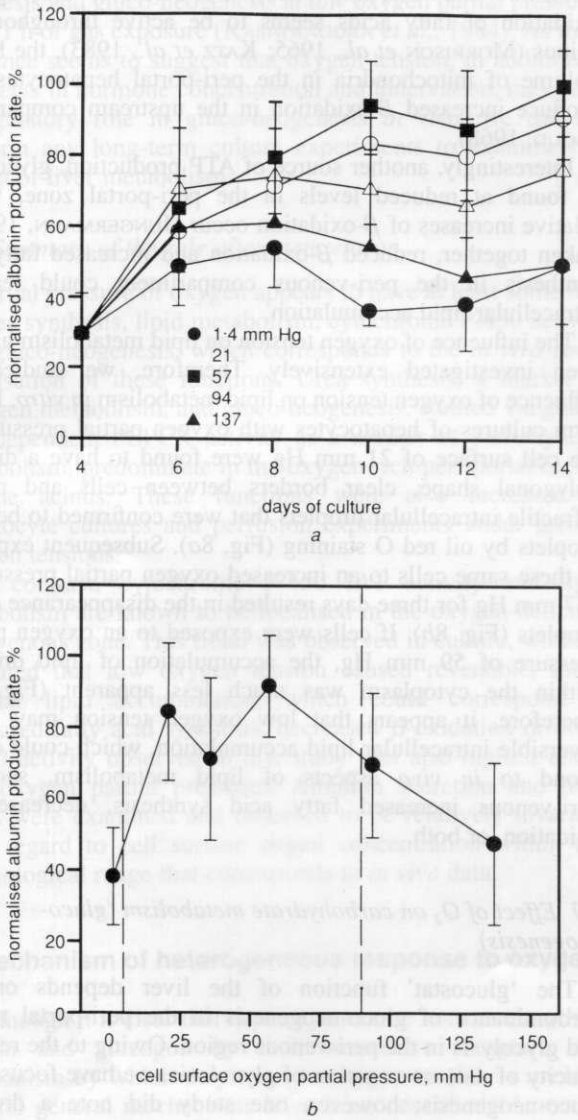

Fig. 5 *(a) Normalised albumin secretion rates versus days of culture with the cell surface oxygen partial pressure (see fig. 3 for details of model estimation); each set of data is normalised by setting the functional activity for a certain time point and condition to 100% for each isolation; values of functional activity were normalised to the DNA content at each time point for all conditions; 100% = albumin secretion rate on day 14 of culture for the cell surface oxygen partial pressure of 57 mm Hg, and was 1.1 and 0.8 μg albumin μg^{-1} DNA h^{-1} for each of two isolations: (b) effect of cell surface oxygen partial pressure on the average, normalised albumin production rate between day 11 and day 15 of culture; dashed lines = estimated physiological range of oxygen partial pressures between 5 and 90 mm Hg (cell culture techniques described in fig. 4); albumin content was analysed in collected media using an enzyme-linked immunosorbent assay (Dunn et al., 1989) (DNA content determined as described in fig. 7)*

The average activity between days 11 and 15 are shown in Fig. 6*b*. A rapid decline in P-450 activity from 96±20% at 1.4 mm Hg to 65±14% at 21 mm Hg was noted. Above 21 mm Hg oxygen partial pressure, the P-450 IA1 activity exhibited a small decline, which was not statistically significant. The activity obtained in this study was highest under low oxygen partial pressures. This trend correlates with the *in vivo* and *in vitro* results listed above.

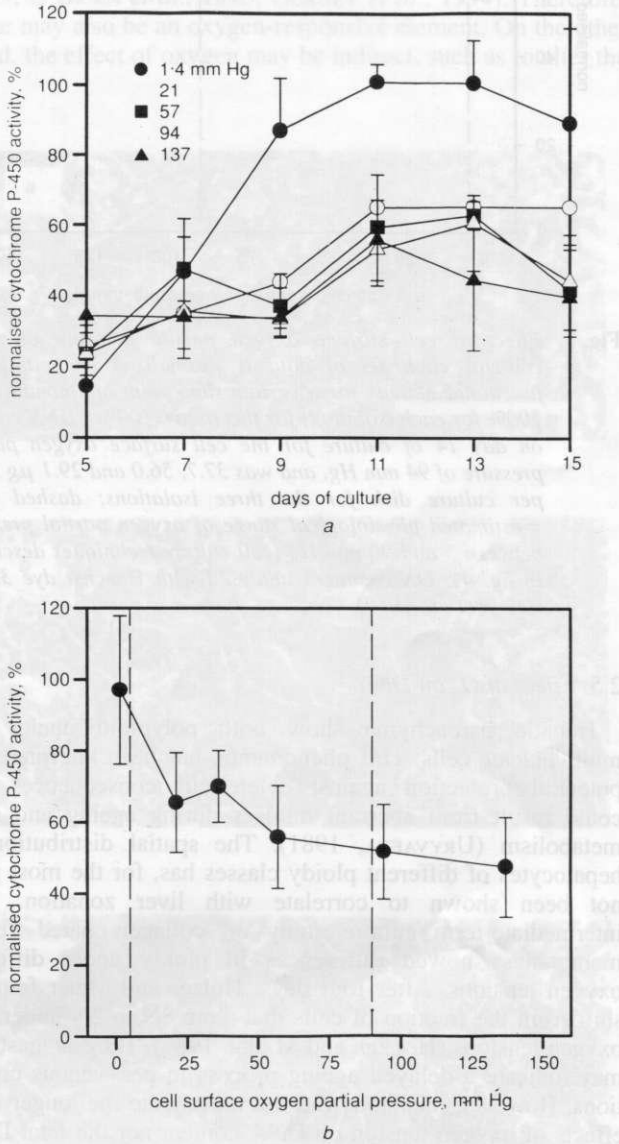

Fig. 6 *Normalised cytochrome P-450 IA1 activity versus day of culture with the cell surface oxygen partial pressure (see fig. 3 for details of model estimation); each set of data is normalised by setting the functional activity for a certain time point and condition to 100% for each isolation; values of functional activity were normalised to the DNA content at each time point for all conditions; 100% = activity on day 11 of culture for the estimated cell level oxygen partial pressure of 1.4 mm Hg, and was 148±7 and 280±12 nmoles resorufin μg^{-1} DNA h^{-1} for each of two isolations, respectively: (b) effect of cell surface oxygen partial pressure on the average normalised cytochrome P-450 between day 11 and day 15 of culture; dashed lines = estimated physiological range of oxygen partial pressures between 5 and 90 mm Hg (cell culture techniques described in fig. 4); activity of cytochrome P-450 IA1 measured according to a non-invasive assay based on the conversion of ethoxyresorufin to fluorescent resorufin by the P-450 IA1 enzyme system (Rotem et al., 1994) (DNA content determined as described in fig. 7)*

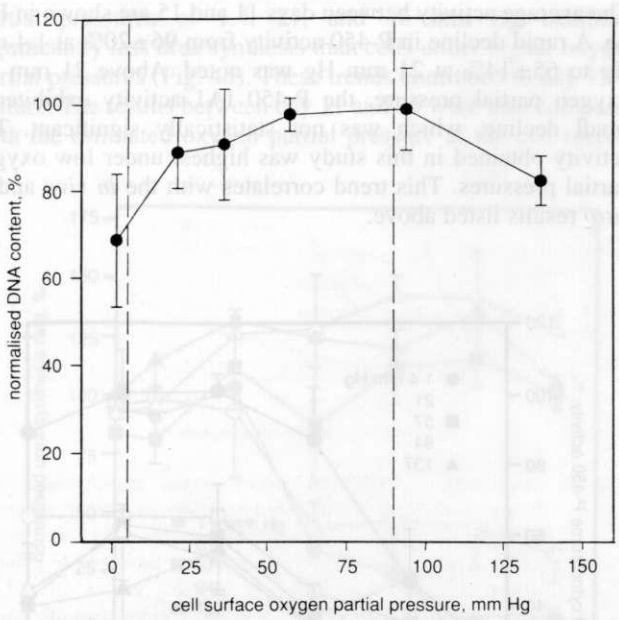

Fig. 7 *Effect of cell surface oxygen partial pressure on DNA content; each set of data is normalised by setting the functional activity for a certain time point and condition to 100% for each isolation; for this assay, 100% = DNA content on day 14 of culture for the cell surface oxygen partial pressure of 94 mm Hg, and was 37.7, 56.0 and 29.1 μg DNA per culture dish for the three isolations; dashed lines = estimated physiological range of oxygen partial pressure between 5 and 90 mm Hg (cell culture techniques described in fig. 4); DNA content analysed with Hoechst dye 33258 (Dunn et al., 1989)*

2.5 *Effect of O_2 on DNA*

Hepatic parenchyma show both polyploid nuclei and multinucleate cells. This phenomenon has been interpreted as potential protection against deleterious consequences that could result from aberrant mitoses during ageing and drug metabolism (Uryvaeva, 1981). The spatial distribution of hepatocytes of different ploidy classes has, for the most part, not been shown to correlate with liver zonation. One intermediate-term culture study on collagen-coated Teflon membranes showed differences in ploidy under different oxygen tensions. After four days, Holzer and Maier found a shift from the fraction of cells that were 8N to 2N under low oxygen tensions (Holzer and Maier, 1987). They suggest this may indicate a delayed ageing process in peri-venous conditions. However, this study did not investigate the longer term effects of oxygen tension on DNA content nor the total DNA content of hepatocyte cultures.

We have examined the total DNA content of stable hepatocyte cultures under different oxygen tensions (Fig. 7). Cells exposed to oxygen partial pressures between 21 and 94 mm Hg (physiological levels) were found to have no statistically significant difference in the levels of DNA, whereas lower (1.4 mm Hg) and higher (137 mmHg) oxygen partial pressure at the cell surface correlated with statistically significant reduced DNA levels. The decline in DNA content at low O_2 levels is consistent with studies that suggest that hepatocyte respiration is impaired at oxygen partial pressures below 1–2 mm Hg (deGroot *et al.*, 1988). The loss of DNA at elevated partial pressures may be due to increased free radical damage derived directly from the elevated oxygen levels. Therefore, as seen *in vivo*, it appears that *in vitro* variations in oxygen tension within physiological limits of cell surface oxygen concentration do not result in large variations in total DNA or ploidy.

2.6 *Effect of O_2 on lipid metabolism*

The zonation of lipid metabolism is a matter of debate (Gebhardt, 1992). In addition, there are considerable sex differences that are not well understood. Using micro-dissection or zonal liver injury techniques, two enzymes involved with lipo-neogenesis (ATP-dependent citrate lyase and acetyl-CoA carboxylase (ACC)) were found to be slightly elevated peri-venously, especially in the female rat (Morrison *et al.*, 1965; Katz *et al.*, 1983; 1986; 1989; Witters *et al.*, 1988). ACC and the rate of fatty acid synthesis were found to be higher in isolated peri-venous cells than in peri-portal cells (Guzman and Castro, 1989). Furthermore, although β-oxidation of fatty acids seems to be active throughout the acinus (Morrison *et al.*, 1965; Katz *et al.*, 1983), the higher volume of mitochondria in the peri-portal hepatocytes may produce increased β-oxidation in the upstream compartment (Loud, 1968).

Interestingly, another source of ATP production, glycolosis, is found at reduced levels in the peri-portal zone, where relative increases of β-oxidation occur (Jungermann, 1986*a*). Taken together, reduced β-oxidation and increased fatty acid synthesis in the peri-venous compartment could lead to intracellular lipid accumulation.

The influence of oxygen tension on lipid metabolism has not been investigated extensively. Therefore, we studied the influence of oxygen tension on lipid metabolism *in vitro*. Long-term cultures of hepatocytes with oxygen partial pressures at the cell surface of 21 mm Hg were found to have a distinct polygonal shape, clear borders between cells and round, refractile intracellular droplets that were confirmed to be lipid droplets by oil red O staining (Fig. 8*a*). Subsequent exposure of these same cells to an increased oxygen partial pressure of 137 mm Hg for three days resulted in the disappearance of the droplets (Fig. 8*b*). If cells were exposed to an oxygen partial pressure of 59 mm Hg, the accumulation of lipid droplets within the cytoplasm was much less apparent (Fig. 8*c*). Therefore, it appears that low oxygen tension may cause reversible intracellular lipid accumulation, which could correspond to *in vivo* aspects of lipid metabolism, such as peri-venous increased fatty acid synthesis, decreased β-oxidation, or both.

2.7 *Effect of O_2 on carbohydrate metabolism (gluco-neogenesis)*

The 'glucostat' function of the liver depends on the predominance of gluco-neogenesis in the peri-portal region and glycolysis in the peri-venous region. Owing to the relative paucity of data on zonation of glycolosis, we have focused on gluco-neogenesis; however, one study did note a drop in glycolosis in the peri-portal zone (Jungermann, 1986*a*). In contrast, many of the gluco-neogenic enzymes, such as phospho-enolpyruvate carboxykinase (PEPCK) have been localised in the peri-portal area (Jungermann and Katz, 1989; Gebhardt, 1992; Giffin *et al.*, 1993). Perfused rat livers have shown gluco-neogenesis primarily in the peri-portal area (Matsumara *et al.*, 1984; Bartels *et al.*, 1987; 1988). Isolated and cultured peri-portal hepatocytes have shown elevated capacities for gluco-neogenesis (Lindros and Pentilla, 1985; Quistorrf, 1985; Quistorrf *et al.*, 1986; Chen and Katz, 1988).

The effects of varying oxygen levels on carbohydrate metabolism have been examined by several investigators. There is considerable controversy about the ability of oxygen to have a short-term effect on carbohydrate metabolism as analysed by liver perfusion and stopped-flow oxygen measurements (Jungermann and Thurman, 1992). However, in

short-term static culture, oxygen tension has been shown to modulate the glucagon-dependent activation of PEPCK by both increased synthesis and activity under arterial oxygen tensions (HELLKAMP *et al.*, 1991). Glucagon-dependent PEPCK gene expression seems to be sigmoidaly dependent on oxygen concentration (NAUCK *et al.*, 1981). The role of O_2 in metabolic regulation is also supported by 48 h cultures where hepatocytes contained higher levels of PEPCK at arterial O_2 tensions and arterial glucagon levels (WOLFE *et al.*, 1985). Romero *et al.* also reported a dependence of gluco-neogenesis on oxygen tension in isolated hepatocytes (ROMERO *et al.*, 1987). Kashiwagura *et al.* have demonstrated decreased urea synthesis and gluco-neogenesis at *low* oxygen partial pressures after 1 h of gas exposure (KASHIWAGURA *et al.*, 1984). As most evidence seems to suggest that oxygen tension, in addition to gradients in hormone concentration and innervation, may have a regulatory role in gluco-neogenesis *in vivo*, we did not perform any long-term culture experiments to examine this aspect of liver metabolism.

2.8 *Summary of the role of oxygen tension*

Partial pressure of oxygen appears to have at least some role in urea synthesis, lipid metabolism, cytochrome P-450 activity and gluco-neogenesis, which corresponds to the *in vivo* zonal localisation of these functions. Urea synthesis, a marker of nitrogen metabolism, and gluco-neogenesis, studied via glucagon-dependent PEPCK activity as a marker of carbohydrate metabolism, predominate in the oxygen-rich peri-portal region of the acinus. These functions were also increased in hepatocyte cultures and perfusion experiments under greater oxygen tensions.

In contrast, cytochrome P-450 IA1 activity and lipid metabolism are known to be localised in the oxygen-deficient peri-venous zone. This trend was observed in culture, where it appeared that low oxygen tension caused reversible, intracellular lipid accumulation, which could correspond to increased fatty acid synthesis, decreased β-oxidation or both. P-450 activity observed in this study was also highest under low oxygen partial pressures. Albumin secretion and total DNA were examined and observed to be relatively invariant with regard to cell surface oxgen concentration within the physiological range that corresponds to *in vivo* data.

3 Mechanism of heterogeneous response to oxygen

Although it is clear that hepatocytes sense hormones such as insulin and glucagon by their respective receptors, the mechanism by which they react to oxygen tension is unclear. Several general mechanisms have been described (ACKER *et al.*, 1988). The purported 'oxygen sensor' may be cytochrome c in the respiratory chain, the ratio of oxidised to reduced glutathione or that of disulphide to sulph-hydryl groups, lactate dehydrogenase iso-enzymes or several other enzymes with appropriate K_m values (JUNGERMANN and THURMAN, 1992). Another proposal was based on oxygen 'sensing' via an oxygen-binding heme protein, which would act directly or indirectly (i.e. as a protein kinase) as a transcription factor (GOLDBERG *et al.*, 1988; GILLEZ-GONZALES *et al.*, 1991). Evidence in support of this hypothesis is given in a recent study (KIETZMANN *et al.*, 1993).

Increased glucagon-dependent activation of PEPCK (normally observed peri-portally) by use of carbon monoxide, which is known to lock heme in the oxy-conformation, has been demonstrated under peri-venous oxygen tensions. In contrast, uncoupling of the respiratory chain with dinitrophenol did not have an effect on the modulation of PEPCK gene activation by oxygen. These experiments suggest a primary role for an oxygen-binding, carbon monoxide-sensitive heme protein. Chimeric genes of PEPCK and bovine growth hormone (BGH) have been utilised to show BGH expression in the peri-portal region of mouse liver. This indicated that the first 460 bp of the 5′-flanking region of the PEPCK gene are sufficient for zone-specific expression (MCGRANE *et al.*, 1990). Multiple protein binding domains have been identified in the promoter-regulatory region of this gene, including glucagon (via cAMP) and insulin-responsive elements (ROESLER *et al.*, 1989; O'BRIEN *et al.*, 1990; GURNEY *et al.*, 1994). Therefore, there may also be an oxygen-responsive element. On the other hand, the effect of oxygen may be indirect, such as to alter the

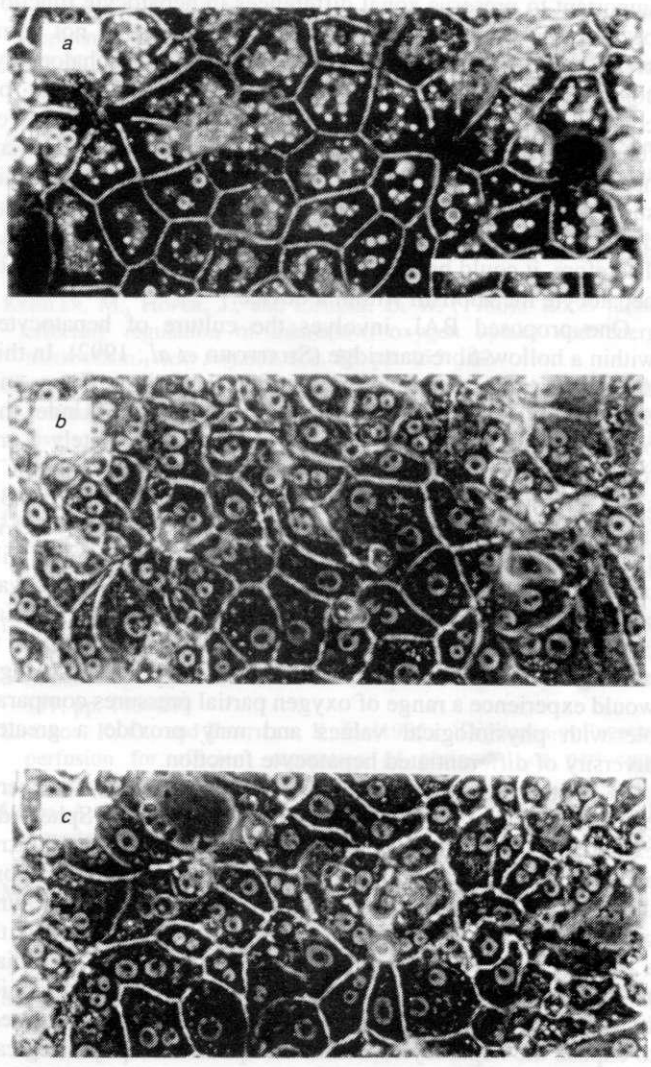

Fig. 8 *Effect of cell surface oxygen partial pressure on hepatocyte lipid accumulation: (a) hepatocytes that experienced an estimated cell surface oxygen partial pressure of 21 mm Hg between day 4 and day 14 of culture; photomicrograph taken on day 14; (b) hepatocytes in (a) after an additional three days of culture at an estimated cell surface oxygen partial pressure of 137 mm Hg, showing disappearance of lipid droplets; (c) hepatocytes that experienced an estimated cell surface oxygen partial pressure of 57 mm Hg between day 4 and day 14 of culture; 400× magnification (cell culture techniques described in fig. 4); round refractile droplets within the cells were confirmed to be lipid droplets by oil red O staining; hepatocytes were washed with 12 ml tap water at RT, fixed with 3% formaldehyde, permeabilised with 60% isopropanol, stained overnight with 3 ml of oil red O solution (Sigma) and rinsed with tap water (modified from* RAMIREZ ZACARIS *et al., 1986)*

extracellular matrix secreted by the cell, which in turn alters cellular function through other cell signalling pathways.

4 Implications in BAL design

One potential application for these findings concerns engineering a BAL device based on cultured hepatocytes. These findings demonstrate that exposing hepatocytes within the BAL to a range of oxygen partial pressures may help produce a BAL with a wider variety of complementary differentiated functions. It may be important to preserve heterogeneity of hepatocyte metabolism for at least three known aspects of liver function: glucose regulation, nitrogen metabolism and drug metabolism. In addition, it may be important to preserve zonal differences in hepatocyte function because the clinical insult from hepatic failure is not completely understood. For example, hepatic encephalopathy, common in end-stage liver disease, is not known to be consistently linked to abnormalities in any single liver function. Thus, to reverse this clinical problem, BALs may need to mimic more than one zone of the liver. As seen in this study, oxygen tension is one potential mediator of zonal differences in function that can be well regulated in a BAL. Therefore, it could be manipulated to promote heterogeneity in hepatocyte metabolism within a device.

One proposed BAL involves the culture of hepatocytes within a hollow-fibre cartridge (SHATFORD *et al.*, 1992). In this design, hepatocytes are cultured within the hollow fibres, and the primary perfusate flow occurs on the shell side. Under the proposed conditions (4×10^6 cells ml^{-1}, approximately 8 ml total volume within fibres, 19 cm cartridge length, 30 ml min^{-1} shell side flow rate, and an oxygen consumption rate of 0.32 pmole cell^{-1} h^{-1}), if the oxygen partial pressure at the inlet is 100 mm Hg, then the oxygen partial pressure at the outlet will only have dropped to 95 mm Hg. However, if the flow rate was reduced to 1.5 ml min^{-1}, then for the same inlet oxygen partial pressure the oxygen partial pressure at the outlet would be 9 mm Hg. In this case, the cells within the hollow-fibre cartridge would experience a range of oxygen partial pressures comparable with physiological values and may provide a greater diversity of differentiated hepatocyte function.

In another study, multicellular hepatocyte spheroids were entrapped in a hollow-fibre device (WU *et al.*, 1995). Spheroids exhibit a relatively uniform diameter of approximately 100 μm and are 6–8 cell layers thick. If we model the oxygen transport through a homogeneous sphere, the steady-state minimum oxygen concentration occurs at the centre of the spheroid, with a gradient to the surface that depends on the square of radial position. Therefore, by optimising flow rate, and thus bulk fluid oxygen partial pressure, this BAL design could be configured to expose the hepatocytes within the spheroid to physiological radial gradients of oxygen partial pressures and could induce heterogeneous function.

5 Conclusions

We have reviewed the potential for oxygen tension to act as a direct or indirect regulator of zonal hepatocyte heterogeneity. Partial pressure of oxygen appears to have at least some role in urea synthesis, lipid metabolism, cytochrome P-450 activity and gluco-neogenesis, which corresponds to *in vivo* zonal localisation of these functions. Given that oxygen is a potential regulator of hepatocyte heterogeneity and that its spatial distribution may be controlled in a bio-artificial liver device, it seems reasonable to consider the potential distribution of oxygen at the cell surface in the overall design of devices and the optimisation of flow rate, cell dispersal, and cell density within these systems.

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