Isolated hepatocytes: use in experimental and clinical hepatology

It is now over 25 years since Howard and Pesch first used collagenase to disaggregate adult liver tissue, with the resultant successful isolation of viable and functional hepatocytes. Before that, numerous mechanical and enzymatic ways of disrupting liver tissue had been used, with a limited degree of success only. Alternatives such as the use of tissue slices or explant organ cultures were also unsatisfactory and of only limited use. Then Berry and Friend introduced a major development when they described the first liver perfusion procedure. This led to a considerably improved viability of hepatocytes and a yield that was estimated to comprise up to 50% of the original tissue.

Although many investigators subsequently reported modifications, it is Seglen, in a series of meticulous studies, who is largely credited with substantially refining the technique and establishing the so-called two-step collagenase perfusion technique which has become the 'standard' protocol for many thousands of investigators. This technique involves perfusing the liver with a divalent cation free buffer, effectively loosening the desmosome and hemi-desmosome junctions between the adjacent cells and extracellular matrix. This is followed by proteolytic digestion of the matrix with collagenase, and careful maintenance of the temperature, pH, and oxygenation. This procedure results in complete disaggregation of the parenchymal tissue without the mechanical disruption which is so detrimental to the viability of hepatocytes. The capsule can then be removed and the hepatocytes simply shaken out into the medium, yielding cells with consistently high viability and purity.

This commentary does not aim to review the methodology, since this has been achieved in an excellent comprehensive monograph, but to discuss interesting recent developments and their impact on experimental and clinical hepatology. These developments now enable investigators to isolate hepatocytes from numerous species, including man, to isolate cells from small biopsy-sized fragments of liver, and to maintain them under various conditions in vitro for prolonged periods without loss of differentiated phenotype. These improvements have opened the way for extensive in vitro studies of liver growth, metabolism, and toxicology and exciting novel investigations relating to clinical liver disease, for example artificial liver support, hepatocyte transplantation, and gene therapy. The published reports cited are not intended to be a fully comprehensive list, and where appropriate, recent reviews are included.

Isolation of human hepatocytes

Although there have been methods of isolating hepatocytes from human liver for a number of years, it is the establishment of liver transplantation as a routine treatment for end-stage liver disease, with regular access to good quality tissue, that has led to appreciable progress in the field of human liver cell biology. In 1982, the isolation, in high yield and viability, of human hepatocytes from end-lobe wedges of tissue was reported. The method used was simply an adaptation of the two-step rat liver perfusion procedure. Exposed vessels on the cut surface are cannulated with three to five catheters and buffers and enzymes are perfused into the tissue and allowed to exude from the cut surface. To aid recirculation, vessels that have not been cannulated are sutured. Other variations include the use of a chelating agent (EGTA) to improve the efficiency of divalent cation removal or additional enzymes such as dispase, hyaluronidase, and DNAase. The latter is helpful since damaged cells can release DNA in large gelatinous strands which render cell separation and purification impossible.

Where whole lobes are unavailable or damage to the capsule prevents efficient recirculation, methods have been adapted and applied to biopsy fragments ranging from 2–50 g of tissue. On the whole these approaches are less efficient, with more variable yields and cellular viabilities, but nevertheless they sometimes offer the only alternative. To date there is no widely accepted and reliable method of isolating viable hepatocytes from needle biopsy sized fragments of liver. Even if successful, the small number of hepatocytes attainable would preclude most conventional cell biological studies. A recent development is the ability to isolate viable and responsive hepatocytes from end-stage diseased livers removed from patients at the time of orthotopic transplantation. To date, hepatocytes have been successfully isolated from the livers of patients with primary biliary cirrhosis and primary sclerosing cholangitis. In other diseases, including alpha-1 antitrypsin deficiency and cryptogenic cirrhosis, this has been less successful, partly because of the nodular cirrhotic nature of the tissue which prevents efficient sinusoidal perfusion and disaggregation.

Cryopreservation

The ability to freeze and store cells would greatly facilitate studies with primary hepatocytes, particularly when yields of human hepatocytes are often greater than 10^6 cells – well in excess of the number required even for large scale experiments. However, cryopreservation is notoriously difficult with hepatocytes. While numerous published methods are available, a considerable loss of viability always occurs after thawing. This is associated with much lower attachment
efficiencies, and morphologically the appearance of cells of inferior quality. Despite this, the indication is that these cells do retain sufficient functional integrity to be of use in some investigations. Whether cryopreserved hepatocytes stored for prolonged periods can provide reliable and consistent data is still controversial. One attempt to overcome the problem involves freezing of cells which have first been allowed to attach to microcarrier beads. This procedure has suggested better reproducibility. Further improvements are desirable.

Non-enzymatic isolation
Because of the potential damage to cell surface plasma membrane receptors, methods which avoid the use of proteases have been described. These rely on perfusion with a chelating agent (EDTA or EGTA) followed by disruption and purification of cells on Percoll gradients. However, the yield and quality of the resulting cell preparation is inferior and it is doubtful if this method should replace enzymatic ones. Moreover, it is probable that if plasma membrane receptors are damaged during isolation, they will be repaired by the cell's own protein synthetic machinery after a period of 'recovery' in culture.

Hepatocyte heterogeneity
Another topic of recent interest is that of the functional heterogeneity of hepatocytes across the liver acinus. Oxidative metabolism, gluconeogenesis, and urea synthesis are predominantly periportal functions whereas glutamine synthesis, ketogenesis, and xenobiotic metabolism are perivenous activities. This has led to the development of techniques designed to isolate preferentially cells from the periportal or pericentral zones of the liver. Digitonin is used to destroy selectively cells around the central vein if perfused via the vena cava, or conversely, in the periportal region if the portal vein is cannulated. This is followed by conventional collagenase digestion. This method suffers the disadvantage that limiting the extent of the toxicity can be difficult. Separation can also be achieved by exploiting differences in cell size. By careful morphometric analysis, a gradation in diameter of hepatocytes seems to exist across the hepatic acinus. Thus, isolation of subpopulations using gradient centrifugation techniques is also feasible. Centrifuge elutriation, a sophisticated counter-flow/centrifugation separation procedure, is particularly amenable to the production of high yields of zonally derived cells.

Maintenance of hepatocytes in vitro
In common with other primary epithelial cell types, hepatocytes remain difficult cells with which to work in vitro and functional activity deteriorates rapidly after isolation. Three basic experimental approaches have been developed in an effort to overcome this problem and yield long term phenotype-stable primary hepatocyte culture systems: (i) coculture of hepatocytes with other cell types; (ii) addition of various soluble agents; and (iii) use of extracellular matrix components.

By coculturing with an epithelial cell line isolated from neonatal rat liver, the enhanced survival of primary rat hepatocytes and their ability to express albumin was reported. The mechanism seemed to require direct cell contact since conditioned medium from the cell line alone was insufficient. Although initially thought to be highly specific to the liver epithelial cell line first described, subsequent work has shown that other cells can support hepatocyte functional activity. The mechanism is not fully understood. Gap junctional communication is thought not to be necessary and the presence of a novel protein present on the plasma membrane of cells has been described. While this method represents a major improvement in culture conditions, it suffers the disadvantage that mixed cell populations are at times difficult to work with, particularly analytically.

A variety of soluble compounds has been used to enhance the longevity of hepatocytes. In one of the first of these studies an agent known to induce differentiation in other cells (and used as a cryoprotectant), namely dimethyl sulphoxide (DMSO), was used. Surprisingly, chronic exposure of hepatocytes to as much as 2% DMSO was shown to maintain liver specific gene expression at a high level. This technique has been exploited for many long term investigations of transcriptional and translational control. Other soluble agents, such as the drugs phenobarbitol and metyrapone, have also been used in studies, especially of cytochrome P450 isozymes, although the pattern of constitutive enzyme expression often changes and inducible enzymes may become prominent. Thus, this method may not be ideal, especially if basic cell physiological mechanisms are under investigation.

The third technique, and arguably the most physiologically appropriate, is to culture hepatocytes on or in some form of basement membrane or extracellular matrix material. Several options are available, most of which induce some form of three dimensional cellular structure. Bissell et al were the first to show that a basement membrane gel extracted from a murine transplantable sarcoma (Matrigel) was capable of maintaining a high level of liver specific gene expression in isolated hepatocytes. Convincing evidence indicates that hepatocytes retain or rather regain membrane polarity when cultured on basement membrane matrix, thus providing a rationale for the improved stability of differentiated phenotype. In addition, the use of a liver derived biomatrix, although more complex, was considered equally effective. While various collagen gel and floating collagen membrane systems have been used, the collagen sandwich technique recently described has proved the most practicable. Finally, it has been suggested that hepatocyte spheroids or organoids also retain functional integrity. All the above models may show some limiting factors, and, clearly, although substantial improvements have been made, optimal culture conditions for hepatocytes have not yet been achieved.

The ability, using increasingly sophisticated techniques, to isolate and maintain hepatocytes in vitro provides the opportunity of creating models to exploit many new experimental initiatives. For example, a viral infectivity model recently described for hepatitis B virus introduces the possibility of a reliable bioassay with which to test anti-viral agents. Other areas include the use of hepatocyte cultures for investigations of the hepatic stage of the life cycle of the malarial parasite and the regulation by cytokines of acute phase protein expression.

Hepatocyte couplets
Although hepatocytes in monolayers seem to develop bile canaliculi, this model is not amenable to the study of bile synthesis and secretion. The hepatocyte couplet now offers a worthwhile viable alternative. Couplets are generated by limited collagenase digestion. This preserves the canicular space and bile is secreted into the (sealed) vacuole between hepatocytes. Couplets will remain functionally active for several hours, sequentially pumping and collapsing the vacuole. The influence of drugs and toxins can then be monitored directly with obvious advantages. In addition, it is possible, using centrifugal elutriation, to enrich the population of couplets yielding a purity of up to 80% and to isolate...
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Enriched populations of periportal or perivenous derived couplets.31

Liver slices
With the advent of a new precision slicing apparatus, the use of liver slices has received fresh impetus. Past efforts were unsatisfactory because of the unavoidable variability in the size and thickness of slices, which often lead to unacceptable irreproducibility. Liver tissue can now be processed to give highly consistent and uniform slices32 of a preselected thickness (usually 200–300 μm). This technique may be particularly appropriate where the nature of the tissue renders it impossible to perfuse and makes it difficult therefore to prepare high enough yields of isolated cells, as may be the case with human liver tissue. It offers the potential advantage that cellular architecture is to some extent preserved with the consequence that cell-cell and cell-matrix interactions may help maintain the functional integrity. It may also be a useful approach with which to generate comparative data across different species.33 To date its use has been limited largely to toxicology and drug metabolic studies. However, disadvantages include the relatively short term viability, the leaching into the medium of products of dead and damaged cells and limited diffusion of nutrients, electrolytes, and O₂ into the slices.

Clinical applications
The ability to isolate viable and functional hepatocytes and to manipulate them in vitro, has brought closer to reality the possibility of alternatives to orthotopic liver transplantation for the treatment of acute or chronic liver failure and the concept of gene therapy as a treatment for genetic disorders. Remarkable progress with various experimental approaches has been made within the past two years, to the extent that approved clinical trials for the use of ‘artificial liver’ support systems and for the treatment of genetic defects are underway.

Artificial liver support
Although the idea of artificial liver support is not new, the functional complexity of the liver has until recently precluded all but the most simplistic approaches, primarily involving blood detoxification.34 These have been of limited success. Now, several groups have developed systems that use extracorporeal columns of isolated, intact hepatocytes and which have indicated more promise. The system of choice uses cylinders filled with porous hollow fibres. Hepatocytes from dogs, pigs, or rats are immobilised within these hollow fibre cartridges, usually attached to microcarrier beads or in a collagen gel matrix.35 Cells within these structures seem to remain functionally viable for up to seven days when perfused continually with physiological fluids.36 Animals with acute liver failure are connected to a complex recirculation network involving plasmapheresis, and plasma is perfused through the ‘bioreactor’ columns containing hepatocytes. Using this sophisticated technology, dogs in hepatic failure can be maintained stably for at least six hours.37 Interestingly, in separate experiments, both allogeneic and xenogeneic (pig) hepatocytes were used to successfully maintain the metabolism of beagle dogs.38 Since an adequate supply of human cells is likely to be a major limiting factor, this is a very important and encouraging observation.

While the results from hollow fibre hepatocyte columns are encouraging, there is a question concerning the functional role of the non-parenchymal cells which constitute the liver. There is increasing awareness of the functional importance of sinusoidal cells in vivo and the concept that cells interact with one another within tissues is now widely recognised and is considered an integral part of normal physiological processes. Should the ultimate artificial liver system take these factors into account then? Is it necessary to consider the addition, in the correct proportions, of Kupffer cells, lipocytes, or even sinusoidal endothelial cells, and if so would the functional lifetime of the bioartificial liver be extended? Unfortunately, the degree of complexity which would ensue may render this approach impracticable. Nevertheless relatively short term support, which may offer a critically important additional period of time to allow subsequent spontaneous recovery or to enable the procurement of a suitable donor organ for transplantation, does suggest a very worthwhile advantage. Whether it is applicable to the clinical situation remains to be established but initial reports seems to be favourable.39

Hepatocyte transplantation
In an effort to provide temporary support to animals whose liver had been severely damaged by toxin administration, intact hepatocytes have been injected by various routes including intraperitoneally, intrasplenically, or intraperitoneally (reviewed in 3). Cells were either administered as suspensions or attached to some substrate such as micro-carrier beads or synthetic fibres. While many successes were reported, several problems in interpreting the data arose.

Firstly, determining the fate of transplanted hepatocytes and identifying their ultimate localisation was difficult. This problem has now been overcome with the advent of the elegant technique of genetically marking cells, enabling the fate of transplanted hepatocytes to be accurately monitored within the host. The simplest is the use of a DNA vector incorporating the bacterial beta-galactosidase gene. Transplanted hepatocytes can then be identified unequivocally by histochemical staining for active enzyme generating easily visualised blue stained cells in tissue sections.40 Secondly, the efficacy of the hepatocyte transplantation technique was not always directly attributable to the viability of the cells inoculated. Indeed there were experimental situations where administration of broken cell preparations or liver cell cytosols was sufficient to reverse the induced hepatic failure in some situations.41

However, there is no doubt that survival and functional activity of transplanted cells in vivo is a reality.42 This is especially so when cells are attached to some form of artificial polymer or fibre network transplanted into the mesenteric bed.43 Under these circumstances, the implants become vascularised and therefore form the basis of a model which may remain viable and therefore functional long term. These three dimensional structures could even be impregnated with pertinent growth or angiogenic factors or other types of liver cells, or both, in the hope that this may further improve the functional integrity.

Use of hepatocytes for gene therapy
The experimental animal studies described above have paved the way for gene therapy experiments in man with the inoculation of genetically altered heterologous or autologous hepatocytes (reviewed in 44–46). An ambitious clinical protocol for the treatment of the condition familial hypercholesterolemia is underway.47 This calls for a partial liver resection from a high risk patient, isolation of hepatocytes from the tissue and the introduction of the normal low density lipoprotein (LDL) receptor gene using a retroviral vector into the cells. Experimental studies using a genetically LDL receptor-defective rabbit model provided the evidence that this was a worthwhile approach,48 thus receiving ethical approval and the success in transfecting human hepatocytes with the LDL receptor gene.49 Some doubt remains as to
whether this will result in sufficient lowering of plasma cholesterol concentrations and the likely duration of the effect. Many other defects including methylymalony-CoA mutase, glucuronosyl transferase, and adenosine deaminase deficiencies are also targeted as treatable findings.


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