Leading article – Hepatology series

Isolation and culture of biliary epithelial cells

The epithelial cell lining of the biliary tract is a target in a number of human diseases including primary sclerosing cholangitis, biliary atresia, chronic allograft rejection (vanishing bile duct syndrome), graft *versus* host disease, and primary biliary cirrhosis.¹ In some conditions both the intrahepatic and extrahepatic biliary tree may be damaged while in others specific regions of intrahepatic biliary epithelium are involved. Hepatocytes may be isolated as homogenous populations for *in vitro* studies with relative ease as they form the major cellular compartment of liver. The functions of non-parenchymal cells are less well understood than those of hepatocytes, and research has been hampered by lack of availability of specific non-parenchymal cell types in sufficiently pure form. The study of normal biology and pathobiology of human biliary epithelium has been largely restricted to observations of cells in histological sections. However, difficulties arise in interpreting significance from subjective observations on the small number of cells in sections because they may not be representative of the total cells in the organ as a whole. During the past two decades advances have been made in isolating and culturing both extrahepatic and intrahepatic biliary epithelial cells from a range of mammalian species. Much of the methodology in current use was developed in rats and this remains the model of choice. More recently significant advances have been made in the culture of human biliary epithelial cells.

Owing to the lack of availability of human tissue for research, there was until recently little potential for isolating human biliary epithelial cells. However, advances in organ allografting have improved access to human tissue, and during the past six years methods have been described for isolating human biliary epithelial cells from extrahepatic ducts,^{2,3} gall bladder,⁴ and the intrahepatic biliary tree.^{5,6} Relatively large numbers of extrahepatic biliary epithelial cells, which require little further purification, can be generated after collagenase treatment of the luminal surface of extrahepatic ducts or gall bladder. An *in vitro* system for studying interactions between normal human common bile duct epithelium and lymphocytes was established for studying mechanisms of liver allograft rejection.³ The suitability of using cells derived from extrahepatic ducts for the study of conditions in which intrahepatic ducts are selectively involved, however, is unclear. Increasing evidence shows morphological, phenotypic, and possibly functional heterogeneity between cells in different regions of the biliary tree.⁷⁻⁹ In primary biliary cirrhosis the characteristic lesion is restricted to the epithelium of medium sized interlobular ducts.¹⁰ Thus, for the study of some aspects of biliary epithelial cell biology and pathology there may be no substitute for human intrahepatic biliary epithelium.

Isolation of intrahepatic biliary epithelial cells

Intrahepatic biliary epithelial cells represent only about 5% of total cells in liver.^{11,12} This presents a major problem with isolating adequate numbers of pure intrahepatic biliary epithelial cells for study. One approach to overcome this problem involved inducing biliary epithelial cells to

proliferate *in vivo* before their isolation. Induction of compensatory proliferated bile ductules by bile duct ligation of rat liver can expand the biliary epithelial cell population by up to 100 fold^{13,14} and has been utilised extensively to generate sufficient biliary epithelial cells for study.^{14,15} The hyperplastic biliary epithelial cells form well differentiated tubules after six or more weeks of ligation and retain many characteristics of normal adult rat biliary epithelial cells.¹⁶ However, it is debatable whether hyperplastic cells represent an acceptable model for the study of normal cells as differences in specific phenotypic features have been shown.¹⁷ Even in normal liver, immunological differences exist between the biliary epithelium of bile ductules and the lining of interlobular and septal ducts.^{7-9,17-19} Preparations of hyperplastic biliary epithelial cells contain a relatively decreased contribution by biliary epithelial cells from larger ducts. This could be an important consideration if the property of interest is differentially expressed on bile duct epithelial cells of larger ducts. Methods are available for isolating biliary epithelial cells with high purity from normal rat liver^{20,21} but obtaining adequate yield is problematic. Despite uncertainty about the suitability of hyperplastic biliary epithelial cells as a model for normal biliary epithelium, the study of normal and hyperplastic rat biliary epithelial cells has significantly advanced our knowledge of biliary epithelial cell biology, particularly in the areas of hepatocarcinogenesis²² and biliary cell plasticity.²³

Several techniques which enable the separation of intrahepatic biliary epithelial cells from the surrounding parenchyma have been devised. Perfusion of rat liver *in situ* with digestive enzymes such as collagenase²⁴ generates a heterogeneous mixture of cell types which requires further purification to enrich the proportion of the cell type of interest. Historically, several techniques including differential density gradient centrifugation,^{21,22} centrifugal elutriation,²⁵ fluorescence activated cell sorting,²⁶ and immunological selection methods^{6,20} have been used either singly or in combination to produce a population enriched with the cell type required. The degree of enrichment for different procedures is variable ranging from less than 75%²⁷ to greater than 95%.⁶ Density gradient centrifugation can generate greater than 90% rat biliary epithelial cells²⁸ but similar procedures applied to human cells yield only 10% biliary epithelial cells and further purification is required.⁶

In this laboratory improvement in biliary epithelial cell purity has been achieved using an immunological rationale. Intrahepatic biliary epithelial cells are positively selected from a semi-pure differential density centrifugation product using a monoclonal antibody which recognises an antigen on the surface of the cells. This approach has also been taken by others for isolating biliary epithelial cells from normal rat liver.²⁰ While this method has the advantage of generating a high purity of isolate (greater than 95%), the yield of cells is relatively low when normal rat liver or smaller amounts of human liver are used. Demetris *et al*⁵ were able to generate larger numbers of cells by taking advantage of the large mass of human liver. The entire biliary tree of whole livers or large

segments was perfused with collagenase generating large numbers of cells. However, whole human livers or very large segments are available for research infrequently only and thus this method is impractical for the routine preparation of biliary epithelial cells.³ Immunological separation methods, while yielding small numbers of cells, have the advantage that they can be applied to smaller quantities of tissue. Furthermore, problems of small yield are being overcome by improvements in culture of biliary epithelial cells so that the number of cells available for study can be increased in vitro. In our hands as little as 5 g of normal liver can generate sufficient cells for culture.

Culture of biliary epithelial cells

Routine expansion of the cell number in vitro is now possible for a variety of primary human cell types. Historically, epithelia have proved more difficult to culture than many other cell types, and only very limited culture was possible.²⁹ This is partly due to the propensity of epithelia to differentiate losing their proliferative capacity.³⁰ Improvements in methods for isolating biliary epithelial cells have not generally been accompanied by parallel advances in long term survival of these cells in culture. A number of reports show that intrahepatic biliary epithelial cells from rat liver^{20 31 32} and human liver^{5 6}; human^{2 3} and bovine extrahepatic duct³³; and gall bladder from human,⁴ guinea pig,³⁴ and dog³⁵ can be maintained in culture for short periods only (three to four weeks maximum). Little proliferation is seen and the cells quickly differentiate and deteriorate. A range of growth factor, hormone, and amino acid additives to culture medium, tested in an attempt to induce proliferation by biliary epithelial cells in vitro, had little effect either on DNA synthesis or in extending survival in culture. Additives included endothelial cell growth factor,⁵ tri-iodothyronine, transferrin, cholera toxin, adenine,⁶ L-proline,³⁶ and lithocholic acid.³⁷

Epidermal growth factor receptors are present on the plasma membrane of normal rat biliary epithelial cells both in situ and after 24 hours in culture.³⁸ Moreover, epidermal growth factor induces an increase in DNA synthesis by hyperplastic rat biliary epithelial cells in vitro.³² One recent report describes a method for generating long term proliferating cultures of intrahepatic biliary epithelial cells from normal rat liver in serum free, defined medium.³⁹ Critical soluble factors were epidermal growth factor and forskolin. Of equal importance for the establishment of long term cultures was the use of collagen gel as the substrate for adherence.

We have found that epidermal growth factor and cholera toxin have little effect on human biliary epithelial cells unless they are co-cultured with growth arrested fibroblasts.⁶ The culture of epithelial cell types with growth arrested fibroblasts has been practised extensively in the culture of a range of different epithelia.⁴⁰⁻⁴² When biliary epithelial cells are co-cultured with irradiated mouse 3T3 fibroblasts, survival can be extended from one week to four weeks, with a significant increase in the cell number.

The fibroblast derived factor responsible for proliferation and maintenance of epithelia in vitro has not been determined but recently a new family of fibroblast derived growth factors which are mitogenic for epithelia has been described.⁴³⁻⁴⁵ These include keratinocyte growth factor,⁴³ scatter factor,⁴⁴ and hepatocyte growth factor.⁴⁵ Hepatocyte growth factor has been shown to be a potent mitogen in vitro for epithelia from a range of tissues including kidney, breast, lung, and skin⁴⁶⁻⁴⁸; it also induces proliferation of human intrahepatic biliary epithelial cells in vitro.⁴⁹ Proliferation was sustained for between three and five months and the cell number increased more than a

million fold.⁵⁰ Cells could be serially subcultured through between seven and nine passages and were cryopreserved in liquid nitrogen with minimal loss of viability upon retrieval. The response of biliary epithelial cells to hepatocyte growth factor is consistent with the distribution of the hepatocyte growth factor receptor (c-Met) which is expressed on the epithelium of major bile ducts in normal human liver.⁵¹ In addition, immunoreactivity of anti-hepatocyte growth factor is localised in bile duct epithelium in human liver and gall bladder.⁵²

Characterisations

The development of defined culture conditions which allow long term culture of biliary epithelial cells allows generation of larger numbers of cells for further studies. However, a problem associated with cultivating cells for prolonged periods is uncertainty about changes that may occur as a result of extended culture. Clonal expansion of a small fraction of the cells in the initial isolate could, after several generations, yield a population of cells with properties different to the major original cell type. For this reason it is important to monitor the purity of biliary epithelial cells both in original isolates and during extended culture using a range of specific markers which distinguish biliary epithelial cells from other cell types of liver.

In vivo biliary epithelial cells are cuboidal or columnar cells which demonstrate polarity having numerous microvilli at the apical pole and nucleus toward the basal region. Junctional complexes and intercellular lacunae are present between adjacent cells.⁵³ Freshly isolated biliary epithelial cells have ultrastructural characteristics consistent with their appearance in vivo.^{5 54} However, in long term monolayer culture the cells become flattened, intercellular connections break down, and polarity is lost. Specialised substrata for anchorage, for example gels of collagen,³⁹ laminin rich extracellular matrix (matrigel,³²), or permeable supports (Joplin, unpublished data) are effective in maintaining polarity for longer. In collagen gel, long term cultures of biliary epithelial cells maintained epithelial morphology and formed three dimensional ductular structures with discernable lumen.³⁹

Clearly, the local environment with which a cell is in contact is important for maintaining a well differentiated morphology. Furthermore, the growing surface may also be important in regulating phenotype. The phenotypic profile of biliary epithelial cells includes cytokeratins 7 and 19, gamma glutamyl transferase (not mouse), and human epithelial antigen, glycoprotein 34, (Egp34, human only,⁵⁵). Markers for other liver cells which are not expressed by biliary epithelial cells, include albumin, desmin, factor VIII related antigen, and asialoglycoprotein receptors and are useful as controls. Culture of biliary epithelial cells as monolayers results in some loss of phenotypic stability after several weeks of culture.⁵⁰ Greater phenotypic stability was achieved when cells were cultured in collagen gel.³⁹

Biliary epithelial cells can be distinguished histochemically from other cells in liver as they lack cytochrome P-450-dependent mono-oxygenase activity but stain positively for gamma glutamyl transferase at the apical pole.⁵⁶ Isolated normal and hyperplastic rat biliary epithelial cells and dog gall bladder epithelial cells show strong gamma glutamyl transferase activity in both short and long term culture.^{35 39 57 58} However, little activity is seen in mouse biliary epithelial cells.⁵⁹ In adult human liver, gamma glutamyl transferase activity is strong in biliary epithelial cells but not in hepatocytes.⁶⁰ Demetris *et al*⁶ reported weak gamma glutamyl transferase activity in intrahepatic biliary epithelial cells cultured short term, and in this laboratory more than 95% of biliary epithelial cells

induced to proliferate in long term culture retained gamma glutamyl transferase activity after up to five months of culture.⁵⁰

Other properties of biliary epithelial cells which have been used to study their characteristics in vitro include functional aspects such as mucous production by gall bladder epithelium⁴ and transport mechanisms important in regulation of intracellular pH of hyperplastic rat liver biliary epithelial cells.⁶¹

Improvement in culturing biliary epithelial cells without loss of the properties which characterise them in vivo suggest that these cultivated cells are an acceptable model for the study of both normal biology and pathophysiological mechanisms. Major histocompatibility complex (MHC) class II antigens (DR, DP, DQ) are expressed de novo on the intrahepatic biliary epithelial cells of patients with liver allograft rejection, PSC, and PBC.¹⁹ Class I and class II antigens are upregulated on biliary epithelial cells in vitro in response to certain cytokines, including interleukin I and gamma interferon.^{62 63} The inducibility of MHC antigens on biliary epithelial cells has led to novel experiments regarding mechanisms which may operate in allograft rejection.³ Medium conditioned by lymphocytes infiltrating liver allografts was able to induce class II expression on normal cultured human extrahepatic biliary epithelial cells. Moreover Ayres *et al*⁶³ have shown induction of intercellular adhesion molecule 1 on human intrahepatic biliary epithelial cells in response to certain cytokines.

In other functional studies, secretin was found to stimulate exocytosis by rat biliary epithelial cells through a cyclic AMP-mediated mechanism.⁶⁴ Also the cytotoxic effect of a range of bile acids on human intrahepatic biliary epithelial cells has been studied using a chromium release assay.⁶⁵ Finally, isolated biliary epithelial cells have been used to study the subcellular distribution of certain antigens.⁶⁶ Previously such studies required immuno-electron microscopy with its inherent technical problems including that of limited sampling. Use of purified biliary epithelial cells permitted sampling of a larger number of cells from representative wedges of tissue and thus in this respect represent a significant advance over previous methodologies.

Summary

At one time it was thought that biliary epithelial cells simply formed the lining to the tubular conduits which constitute the biliary tract. Development of in vitro systems for culturing biliary epithelial cells has enabled functional studies which increasingly show that this is far from true, and that biliary epithelial cells do have important functional roles. Disruption of these functions may be involved in the generation of pathology. Most functional studies to date have utilised cells isolated from rat liver. Increasingly, variations are being found between human and animal cells both in terms of function and phenotype. The relevance of animal cells in the study of human disease therefore remains obscure. Human biliary tract disease has to date been studied almost exclusively by examination of histological sections. The development of improved methods for isolating highly pure biliary epithelial cells from human liver provides a new technology with which to investigate directly the dynamics of human biliary epithelial cell biology and pathobiology. It is predicted that further progress will now be made in dissecting the biology and physiology of human biliary epithelium.

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