# HEPATOCYTES IN THE DEVELOPMENT OF LIVER SUPPORT SYSTEMS

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# HEPATOCYTES IN THE DEVELOPMENT OF LIVER SUPPORT SYSTEMS

HEPATOCYTEN IN DE ONTWIKKELING VAN
LEVER ONDERSTEUNENDE SYSTEMEN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR C.J. RIJNVOS
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.

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In memory of my father

For my mother

For Inez and Camilla

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CONTENTS

# CHAPTER 1

## Introduction

## General introduction

Advances in the management of chronic and acute hepatic disease have been made possible by the development of liver transplantation. The indications for liver function replacement can be divided into four main categories: i) end-stage chronic liver disease, ii) acute fulminant hepatic failure, iii) inborn errors of metabolism, and iv) tumors of the liver (76). The need for removal of the host liver, combined with orthotopic liver transplantation (OLT) has become widely accepted as the treatment of choice in the first, and largest category (75-77). The role of OLT is less straightforward in the treatment of fulminant hepatic failure and inborn errors of metabolism. In these areas, alternative therapeutic approaches to liver function replacement are being explored. The role of OLT in the treatment of hepatic tumors is beyond the scope of this thesis.

The term fulminant hepatic failure was originally introduced by Trey (84). It is usually defined as a potentially reversible, severe hepatic failure occurring in the absence of pre-existing liver disease, and complicated with hepatic encephalopathy within 8 weeks of the onset of symptoms attributable to hepatic dysfunction. The most common causes of fulminant hepatic failure are acute viral hepatitis, drug-induced toxicity, and poisoning. Viral infections account for 60-70% of all cases worldwide (46). The prognosis of this dramatic disease is poor: survival rates vary from ~10 to 30%, depending on the grade of encephalopathy, and the etiology involved (13,46). OLT has been described to increase survival in selected cases up to ~60% (13,76,94). However, the timing of the procedure is associated with considerable difficulty in this often very rapidly progressing disease, and restricted organ availability presents a major obstacle.

Inborn errors of liver metabolism are characterized by a genetic defect in a single hepatocellular pathway involving one enzymatic or receptor function. This defect generally leaves the anatomical and structural integrity of the affected liver undisturbed (74). Although varying in nature, many of these disorders give rise to profound metabolic and developmental disturbances in infancy or early adulthood, leading to early invalidation or death (33). Secondary liver damage (cirrhosis) may develop as a consequence of, or in association with several inborn errors. The majority of OLT recipients with inborn errors of metabolism have been transplanted because of the development of secondary chronic liver failure, but some anatomically normal livers have been replaced solely to correct an inborn error (75,76). However, one has to take into account the complexity of liver procurement, the difficult management of donor and recipient, and the high-risk surgical procedure, that render OLT

associated with considerable morbidity, mortality, and cost. Therefore, OLT seems a rather formidable enterprise for replacement of one single hepatocellular function.

In a first effort to reduce the amount of liver tissue used for replacement of liver function while obviating the need for host liver removal, the Rotterdam group displayed renewed interest in the heterotopic transplantation of an auxiliary partial liver (HLT) (81,82). Worldwide, a few cases of acute hepatic failure have been treated successfully by HLT, whereafter the host liver recovered spontaneously (9,60). Recently, it was shown that HLT resulted in sustained normalization of the metabolic defect in a dog model with an inborn error of purine metabolism (52). However, HLT is a technically demanding procedure, which shares some of the disadvantages of OLT.

This thesis focuses on the development of alternative strategies in the treatment of patients with acute fulminant hepatic failure and inborn errors of metabolism, using hepatocytes as the basis of liver support. When compared with transplantation of the liver as an organ, the following theoretical advantages may be attributed to hepatocyte-based liver support systems: i) reduction of mortality, morbidity, and cost, due to less surgical trauma, ii) recovery of the patient's own liver is not precluded (acute hepatic failure); unnecessary removal of the own liver is not mandatory (inborn errors), iii) increased efficiency in use of donor organs by possibility of storage, and iv) possibility of genetransfer. As will be discussed, acute liver failure may be best served by extracorporeal systems as a means of providing temporary support while waiting for either spontaneous regeneration to occur, or a suitable donor liver to become available for transplantation. By such a strategy of buying time, liver transplantation might be avoided altogether in those patients with acute liver failure in whom the liver regenerates (13,80). In contrast, inborn errors of liver metabolism may be treated most efficiently by means of hepatocyte transplantation (implantable systems), combined with gene-therapy procedures, aimed at permanently restoring the defunct enzymatic pathway. This is particularly true in those cases in which no other associated liver impairment has yet developed (38).

## *APPROACHES TO LIVER FUNCTION REPLACEMENT*

### *EXTRACORPOREAL SYSTEMS*

#### *(a) Artificial Systems*

In liver failure, all aspects of liver function appear to be compromized while the immediate cause of death is mostly related to complications of hepatic encephalopathy and/or portal hypertension (13,46). Early on, it was recognized that replacing all liver functions by artificial

means might be nearly impossible, so that one approach has focused on the liver's detoxifying ability. This approach includes hemodialysis (63), charcoal hemoperfusion (72), and immobilized enzymes (11), all aimed exclusively at removal of toxin build-up from the body. Although particularly charcoal hemoperfusion has been reported to result in improved cerebral status in encephalopathic patients, it does not seem to improve survival (62). Assuming that varying degrees of support beyond detoxification might be needed, other approaches have attempted to replace the entire complex of liver functions. Exchange transfusion and plasma exchange (99), xenogeneic extracorporeal perfusion (85), and cross hemodialysis, i.e. combined extracorporeal perfusion and hemodialysis (64), are among the many proposed strategies.

Although a functional activity of approximately 20% of that of normal liver has been estimated to be sufficient for correction of the 'milieu interne' in acute hepatic failure, none of the currently available methods of artificial liver support has attained this goal (34). This can be explained by the fact that these artificial systems cannot replace more than an estimated 0.03-3% of the liver's clearance capacity (34). Due to this lack of clearance capacity, today's available artificial liver support systems are unable to provide adequate correction of the milieu interne in the patient with acute hepatic failure.

*(b) Hepatocyte Systems*

Recognizing that a full complement of cellular function may be necessary in the treatment of liver failure, many investigators have focused on the use of isolated hepatocytes as the basis of bio-artificial liver support. The development in the late 60s and early 70s of reliable, reproducible techniques for the isolation of adequate numbers of viable hepatocytes has formed an essential contribution to this line of research (7,71). One approach to a hepatocyte-based support system is to design extracorporeal bioreactors containing functional hepatocytes. Such a bio-artificial device would support liver functions through an extracorporeal circuit connected to a patient's circulation. When comparing such systems with implantable ones, advantages of bioreactors may include i) more control of the cellular environment, particularly with respect to transport considerations (e.g. of macromolecules, oxygen), ii) more control over timing and the duration of use, i.e., no dependency on such processes as engraftment and vascularization of implantable systems, and iii) potential storage of the entire bioreactor, or its hepatocellullar component. Disadvantages of extracorporeal systems include i) the need for vascular access and associated potential for thromboembolic complications, ii) no possibility of direct contact with portal blood, iii) the need for repetitive treatment. These considerations imply that extracorporeal systems would theoretically be most suitable for rapid, on demand provision of

hepatic support, such as necessary in the case of acute fulminant hepatic failure, or as a bridge to organ transplantation.

Several types of bioreactors have been described, in which the hepatocytes have been used in a variety of configurations (80). In a preliminary clinical report, Matsumura et al. (59) showed a decline of bilirubin levels and improved mental status in a patient with liver failure by dialysis of his blood against a flowing suspension of cryopreserved rabbit hepatocytes. More recently, Arnaout et al. (4) demonstrated that microcarrier-attached hepatocytes situated in the outer chamber of a hollow fiber module produced bilirubin conjugates when the module was connected to conjugation-deficient Gunn rats. However, survival of the hepatocytes in this configuration was limited to ~4 hours. In another publication, Uchino et al. (86) describe the use of hepatocytes attached to stacked flat plates in a configuration similar to classical in vitro monolayer hepatocyte cultures. When a large-scale bioreactor, based on this design, was attached to anhepatic dogs, survival was increased and serum ammonia significantly reduced. Although promising, this design needs careful characterization and more studies with adequate control groups have to be awaited. In general, prior to clinical applicability, much work has yet to be devoted to improvement and characterization of bioreactor designs, improvement of hepatocellular survival, and adequate assessment of the fate of the hepatocytes before, during and after use in an extracorporeal support system. In addition, adequate storage procedures, such as cryopreservation have yet to see the light.

The feasibility of extracorporeal hepatocyte systems depends on the ability to maintain functional, differentiated hepatocytes. For this purpose, it is instructive to examine and use previous experience from attempts to maintain primary hepatocytes in long-term culture. Early efforts to culture adult hepatocytes invariably led to either overgrowth of contaminating cell types, or to de-differentiation or fetalization of the cultured hepatocytes (67). More recently, several novel culture conditions have been explored, including hormonally defined media (23), dimethyl sulfoxide containing media (43), coculture with other cell types (36), and culture on complex matrices (8). In these systems, liver functions have been maintained for periods ranging from 2 - 7 weeks. On the other hand, most of these culture conditions have clear disadvantages, such as toxicity, lack of adequate characterization or simplicity, or the presence of tumor-derived substances. The culture configuration in these systems generally consists of a monolayer of hepatocytes attached to either plastic, or extracellular matrix protein. However, unlike most epithelial cells, hepatocytes have a distinct polarity, with a belt of apical (bile-canalicular) surface surrounding each cell and dividing two basolateral (sinusoidal) surfaces, each of which is in contact with extracellular matrix. These surfaces have different functional properties; for instance, protein secretion takes place across the basolateral surfaces, whereas

bile salts are secreted across the apical surface. In an attempt to restore this cellular polarity reminiscent of the in vivo state, Dunn et al. (25) have developed a "double gel" , or "sandwich" culture configuration, in which the hepatocytes are cultured in between two layers of hydrated rat tail collagen (Fig. 1). While hepatocytes in classical monolayer cultures ("single gel" configuration) lose their adult liver phenotype within 1-2 weeks of incubation, hepatocytes in the double gel system retain their normal morphology and function for at least 6 - 8 weeks (25,26). Stable, long-term expression of protein synthesis and secretion, bile salt secretion, urea production, and detoxification were documented in the double gel system. In addition to these biochemical activities, extracellular matrix production, cytoskeletal organization, and membrane sorting patterns similar to intact liver have been described (26). Recently, evidence has been obtained which suggests that transcription may be responsible for the observed differences in albumin secretion between the single gel and double gel systems (27). Translational activity increases over time in both culture configurations.

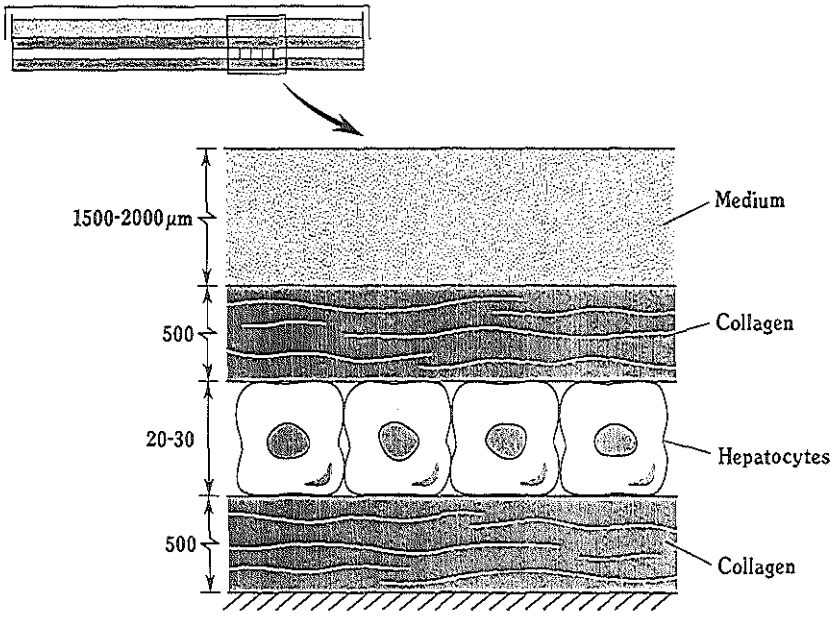


**Figure 1.** Schematic representation of the double gel, or sandwich, culture technique for hepatocytes. Dimensions are approximates and are given in μm.

*IMPLANTABLE HEPATOCYTE SYSTEMS*

Hepatocyte-based support may also be accomplished by implanting functional hepatocytes into the affected individual. Similar to the bioreactors, the implanted hepatocytes may be in cell-supension, attached to matrices substrates (e.g. microcarriers), or micro-encapsulated. The implantable 'systems' offer the advantages of i) technical simplicity, ii) possibility of permanent therapeutic effect obviating the need for repetitive treatment, and iii) exposure of the cells to the natural environment provided by the host. For example, transplanted cells may be able to increase cell mass by responding to regenerative signals. Disadvantages may include i) lack of control on the cellular microenvironment after implantation, ii) difficulty of attributing functional activity to the graft, and iii) transport limitations due to the lack of early vascular supply to the engrafted cells.

The first description of hepatocyte transplantation for acute liver failure was by Sutherland et al. (79), who demonstrated that intraportal and intraperitoneal injection of isolated hepatocytes resulted in significant increase in survival rates of rats with dimethylnitrosamine-induced liver failure. However, histological proof of surviving hepatocytes post-transplantation could not be obtained. Similar results on intraperitoneal hepatocyte transplantation in various other models of acute liver failure have been reported, but histological evidence of surviving cells was not provided (53,61). Intrasplenic hepatocyte transplantation was shown to be beneficial for survival in galactosamine-induced liver failure in rats, and surviving hepatocytes were observed in the spleen (73). Makowka et al. (54,55) demonstrated that not only transplantation of syngeneic, but also of allogeneic and xenogeneic hepatocytes resulted in significantly reduced mortality rates in galactosamine poisoned rats. These findings were further extended by several investigators who reported similar results with transplantation of liver cytosol extracts, hepatocyte fragments, and even cell-free culture supernatants (6,50,56). These results indicate that transplantation of intact, metabolically active hepatocytes does not account for improved cure rates in acute liver failure, and that other, supposedly trophic factors may be involved. In addition, although Bosman et al. (10) documented a delay in the onset of hepatic encephalopathy by intraperitoneal transplantation of microcarrier-attached hepatocytes, this technique does not seem to improve survival rates (10,22). Interestingly, some survival benefit was observed when the microcarriers had been implanted several days prior to the induction of acute liver failure (22). This seems to suggest that it is necessary to allow time for the vascularization of the implanted microcarriers to occur. For the acutely ill patient with fulminant hepatic failure, the usefulness of hepatocyte transplantation remains, therefore, to be determined.

As indicated above, hepatocyte transplantation may be the solution for treatment of inborn errors of liver metabolism, particularly when combined with gene-therapeutic approaches to correct the genetic defect prior to (re)implantation of the (patient's own) hepatocytes. Ever since the development of suitable isolation procedures (7,71), hepatocyte transplantation has been extensively studied for its capability to correct genetic metabolic disorders. Rugstad et al. (70) reported decline of bilirubin levels in the serum of hyperbilirubinemic Gunn rats, deficient in uridyl-di-phospho-glucuronyl-transferase, after subcutaneous implantation of rat hepatoma cells. Some seven years later, Sutherland et al. (78), Matas et al. (58), and Groth et al. (35) independently described temporary improvement of hyperbilirubinemia by intraportal transplantation of non-deficient hepatocytes. Other investigators reported that the intrasplenic injection of suspended hepatocytes produced similar metabolic effects (14,57), while this technique did not lead to mortality from portal thrombosis as seen after intraportal infusion (32,35). Although Woods et al. (98) attributed the decrease of serum bilirubin in Gunn rats after hepatocyte transplantation to RES-blockade with subsequent prolongation of erythrocyte survival, Vroemen et al. (91,92) were finally able to provide direct evidence of metabolic activity of intrasplenically transplanted hepatocytes by showing the appearance of de novo formed bilirubin conjugates in the bile of these conjugation-deficient rats. Similar findings were described by Demetriou et al.(19-21) who used intraperitoneal deposition of microcarrier-attached hepatocytes to partially overcome the genetic defect in both the Gunn rat, and the analbuminemic NAR rat. Many other transplantation techniques have since been explored, including intrapancreatic injection (90), interscapular fat pad injection (45), intraperitoneal implantation of hepatocytes in free suspension (40), on bioabsorbable polymers (87), or in microcapsules (24). The success of these endeavours has been limited.

The fate of transplanted hepatocytes depends, in part, on the transplantation technique used. Until recently, intraportally infused hepatocytes were undetectable, since they could not be distinguished from the host's hepatocytes. Intrasplenically engrafted syngeneic hepatocytes seem to survive indefinitely while retaining their normal morphological features (16,49). More recently, experiments with hepatocytes from transgenic mice revealed that up to more than half of intrasplenically injected hepatocytes translocate into the liver and permanently engraft there (37,65). The fate of hepatocytes transplanted into the peritoneal cavity is somewhat less clear. Microencapsulated hepatocytes may be protected against immunological rejection, but their survival inside the capsules after intraperitoneal implantation seems restricted (24). Survival of microcarrier-attached hepatocytes after intraperitoneal implantation was demonstrated by several groups (10,21,93), whereas others observed the cells to be subject to rapid necrosis (40). Since it is known that hepatocytes are anchorage-dependent cells, it is not surprising that

their intraperitoneal survival is extremely limited when they are injected as free suspension, without the opportunity to attach to a supporting matrix (40,87). The recent description by Thompson et al. (83) concerning the formation of neoorganoid structures in the peritoneal cavity by attaching hepatocytes to prevascularized supports consisting of collagen coated, non-biodegradable synthetic fibers is interesting, but awaits further investigation. It may be concluded that, despite its technical drawbacks, the intrasplenic technique remains the golden standard of hepatocyte transplantation techniques, while innovative approaches are being developed and tested.

Most of the above cited publications have documented temporary metabolic support when other than autologous hepatocytes were used. Since allogeneic hepatocytes have been shown to be rejected (12,15,28), much effort has been devoted to abrogation of the immune response. Host immunosuppression with cyclosporine has shown limited success in preventing rejection of allogeneic hepatocytes (17,57). Alternative strategies, such as ultraviolet irradiation (18) and cell culture prior to transplantation (66) seem to be encouraging, but need to be examined in greater detail and in various species. In view of the above, one approach in the treatment of genetic enzyme deficiencies seen in inborn errors of liver metabolism would involve removing hepatocytes from the affected individual, introducing the defective gene in vitro, and reimplanting the autologous cells into a receptive locus. Apart from adequate transplantation methods, this approach (commonly referred to as ex-vivo gene therapy) requires stable integration and expression of the introduced gene. Targeting hepatocytes for gene therapy is attractive, since many metabolic processes are dependent on proteins preferentially expressed in the liver, and because hepatocytes can sustain high levels of expression of a larger number of genes than any other cell type. Several groups have recently accomplished efficient expression of a variety of genes introduced into hepatocytes in vitro by means of retroviral vectors (3,39,51,97). Currently, the first descriptions of the feasibility of ex-vivo gene therapy are being reported. Anderson et al. (2) were the first to publish preliminary data on the expression of NeoR gene, introduced through retrovirus-mediated gene transfer into hepatocytes, that were subsequently implanted intraperitoneally on floating collagen supports. More recently, it has been demonstrated that introduction of the LDLR gene into allogeneic (95,96), and autologous (68) hepatocytes of LDLR deficient Watanabe rabbits, followed by intrasplenic transplantation, resulted in temporary, and prolonged expression of this gene in the recipient rabbit, respectively. This approach caused partial correction of the genetic hypercholesterolemia in these rabbits. Notwithstanding these encouraging results, it needs to be emphasized that the transfection efficiencies reported are still low, and the mechanisms involved in the gene transfer have only begun to be elucidated. Insight into such mechanisms will provide valuable information to be used in further studies along this route. Although in vivo transfection of

regenerating liver by amphotropic retroviruses has recently been accomplished (29,69), more experimental work needs to be done before such an approach will prove useful.

**Objectives and rationale of the experimental work.**

From the above review it becomes clear that, although substantial progress has been made over the past two decades, numerous questions have yet to be answered before the clinical application of these novel approaches to liver function replacement becomes reality. This thesis addresses some of these questions related to the use of hepatocytes for either extracorporeal liver support systems, or for gene transfer and transplantation as part of the concept of ex-vivo gene therapy. The thesis is based on experimental studies using rat hepatocytes, and can be divided into three areas of investigation.

The first series of experiments focuses on hepatocyte-based extracorporeal liver support systems. Its *objective* was to obtain insight into the behavior of hepatocytes under conditions that may be encountered during extracorporeal support. Such conditions include i) plasma perfusion through an extracorporeal circuit containing a plasmaseparator, and ii) exposure to mediators of the acute phase response, since connection of animals or patients to artificial surfaces has been shown to rapidly evoke an acute phase response through elevation of defined cytokine levels (41). The effects of exposure to such 'in vivo' conditions were studied in stabilized, i.e. more than 1 week old, double gel cultures of rat hepatocytes. The *rationale* for these experiments was based on the fact that almost all prior in vitro studies on hepatocellular acute phase response have been performed using either transformed cell lines, or short-term conventional cultures of unstable primary hepatocytes (5,30). These studies may, therefore, not have accurately reflected the in vivo situation. Furthermore, no studies have addressed the resolution of the response upon withdrawal of the stimulus. In addition, one of the very few reports on the viability of hepatocytes inside a bioreactor describes a rapid decline of cell viability within the first 4 hours (4). The discovery of culture techniques, such as the double gel configuration, that enable hepatocytes to maintain phenotypic stability over prolonged periods of time (25,26), has paved the way for further evaluation of the feasibility of hepatocyte-based liver support systems.

The second area of investigation concerns hepatocyte preservation. The *objective* of this part was to define optimal freezing conditions required for long-term hepatospecific function following a freeze-thaw cycle of stable hepatocytes in double gel culture. For this purpose, a stepwise analysis was performed of the possibly deleterious effects of the major steps involved

in cryopreservation protocols on sandwiched hepatocytes. The *rationale* for these experiments was based on the fact that, despite representing a major step forward in the development of bio-artificial liver support systems, the discovery of stable long-term culture systems with a 6-8 week culture duration cannot ensure a continuous supply of cultured hepatocytes for on demand use. The necessity of storage, most likely by means of cryopreservation, is all the more obvious when one considers the problem of limited donor availability. Previous studies regarding hepatocyte cryopreservation have mostly dealt with isolated hepatocytes. Their results are often contradictory, and seem to be related to the viability assays used. Furthermore, selection of surviving hepatocytes, e.g. by means of centrifugation over Percoll, makes interpretation of the results of many reports difficult. Moreover, recovery of long-term hepatospecific function after hepatocyte cryopreservation has never been demonstrated. One could speculate that hepatocytes, cultured in a stable configuration, may be less vulnerable to freezing stresses than isolated cells. Indeed, it has been shown that while water permeability and nucleation parameters of cultured hepatocytes approach values found for other mammalian cells, they are in striking contrast with those of freshly isolated hepatocytes (42,100). Freezing of cultured hepatocytes has thusfar received no attention, although it might be attractive from a practical point of view. Therefore, an initial study regarding the feasibility of cryopreservation of hepatocytes that had been cultured in the double gel configuration was recently carried out in our laboratory with encouraging preliminary results (48).

The third area of investigation involves the concept of ex-vivo gene therapy. Its *objective* was twofold: first, to obtain further insight into the mechanisms involved in retrovirus infection of hepatocytes, and second, to evaluate a novel technique of hepatocyte transplantation into so-called solid supports as an alternative to the classic intrasplenic injection. The *rationale* of the first objective was based on the fact that insight into the mechanisms involved in retrovirus-mediated gene tranfer is limited, while the efficiency of gene transfer is still low. Retrovirus-mediated gene transfer relies on the production of replication-defective retroviral vectors by packaging cells, which supply the vector with viral proteins (88). By providing the viral proteins, including the envelope proteins of the virus, these packaging cells define the host range or tropism of retroviruses. Thus, ecotropic viruses can only infect mouse and rat cells, but not cells of any other species, whereas amphotropic viruses have a broad host range including monkey and man. Both ecotropic, and amphotropic viruses have been reported to infect cultured hepatocytes or hepatoma cells, with varying degrees of efficiency (1,39,44,51,97). In contrast, in vivo infection of normal murine liver with ecotropic virus could not be accomplished, while induction of regeneration through partial hepatectomy resulted in increased susceptibilty of the regenerating liver cells to amphotropic, but not ecotropic virus infection (29,69). Apparently, cell proliferation is not the only factor that

determines the infectibility of hepatocytes by retroviruses (31). The recent identification of a cation amino acid transporter (CAT-1), that serves as ecotropic virus receptor (1,47) represents a major step forward in the understanding of the mechanisms involved in this technology.

Ex-vivo gene therapy does not only require insight into the gene therapy aspects of this concept, but also necessitates optimization of hepatocyte transplantation techniques. The intrasplenic route has been most widely studied, but is associated with two disadvantages. First, the number of hepatocytes that can be accomodated in the spleen is limited. In spite of the recently demonstrated translocation of hepatocytes from the spleen to the host liver (37,65), it remains to be seen whether this phenomenon is sufficient to resolve this numeric drawback of intrasplenic transplantation. Second, potential complications, including hemorrhage, spleen and liver infarction, and shock may become particularly hazardous in large animal and human recipients. Intraperitoneal transplantation is not associated with these disadvantages, but survival after intraperitoneal transplantation is controversial, and could be limited because of lack of adequate vascularization, and thus, nutrition and oxygenation of the transplanted cells. In addition, it is known that hepatocytes require a matrix to which they can adhere in order to survive intraperitoneal implantation. The recent description of neovascularization of non-biodegradable polytetrafluoroethylene (PTFE) solid supports is promising, since this would circumvent problems related to the initial vascular supply of the hepatocellular graft (83).

In summary, the questions that have been addressed in the following chapters are:

1. Are hepatocytes in stable double gel culture configuration capable of responding to mediators of the acute phase response in a physiological way, including a return to homeostasis upon withdrawal of the mediator?
2. What are the effects of connection of hepatocytes in double gel to a rat through an extracorporeal circuit on morphology and long-term function?
3. Is it possible to expose hepatocytes in double gel culture to the multiple steps involved in a freeze thaw protocol, and what are the effects of these steps on long-term function, morphology, and cytoskeletal organization?
4. Is ecotropic retroviral infectivity of hepatocytes (in vivo and in vitro) correlated with expression of the ecotropic receptor (CAT-1), and does CAT-2 represent the amphotropic receptor?
5. What are the functional, morphological, and proliferative characteristics of hepatocytes transplanted into PTFE solid supports, and how do these features compare to those of intrasplenically transplanted hepatocytes ?

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# PART I

## Extracorporeal liver support

## CHAPTER 2

# A stable long-term hepatocyte culture system for studies of physiologic processes: cytokine stimulation of the acute phase response in rat and human hepatocytes

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**Abstract**

Prior studies on the *in vitro* hepatic acute phase response have either involved hepatoma cell lines, or conventional short-term cultures of primary hepatocytes. No data is available on the response of primary hepatocytes in stable long-term culture systems. In this study, the acute phase response of rat and human hepatocytes in a new long-term culture system was examined in response to IL-6, IL-1ß, and TNF-α. The cultured cells were sandwiched between two layers of collagen in a (double gel) configuration which has been shown to preserve both hepatocyte function and morphology over prolonged periods of time. The stability of this culture configuration enabled us to investigate, for the first time, the temporal aspects of the response in addition to the effects of the mediators on protein secretion. Exposure of rat hepatocytes to IL-6 after culture for 16 days resulted in a two-fold reduction of albumin secretion, and a 15-fold increase in the secretion rates of fibrinogen and α2-macroglobulin. In all instances, the peak response occurred at 48 hours after IL-6 exposure, and all protein secretion rates returned to pretreatment values within 5 days post-treatment. Changes in the mRNA levels of these proteins in response to IL-6 corresponded with those changes seen with the secreted products, indicating pretranslational regulation. Administration of IL-1ß to rat hepatocytes produced a similar decline of albumin secretion and a 5-fold increase of fibrinogen secretion, whereas α2-macroglobulin secretion remained undisturbed. In contrast, TNF-α did not affect the secretion of any protein examined. Human hepatocytes in double gel culture configuration reacted to IL-6 and IL-1ß with an approximate 40-fold increase in serum amyloid A secretion, peaking at day 3 post-treatment. Both the secretion pattern and temporal response of these cultured hepatocytes to cytokines appear to closely mimic the *in vivo* hepatocellular response. The double gel culture system is a stable, attractive tool for further investigation of the acute phase, and other hepatocellular responses to physiologic stimuli.

## Introduction

The hepatic acute phase response is a reaction to trauma, injury, or inflammation (6, 22). This response leads to an alteration in the liver's protein synthesis, characterized by an increased production of "positive" acute phase proteins, and a down regulation of "negative" acute phase proteins (6,22,28). Predominant positive acute phase proteins in the rat are fibrinogen and $\alpha 2$-macroglobulin. In humans they include serum amyloid A (SAA) and C-reactive protein. Albumin is a negative acute phase protein for both species. Comprehensive reviews on the acute phase proteins have been published (22,28). The principal mediators of the hepatic acute phase response include IL-6, IL-1, and TNF-$\alpha$ (1,6,8,22,28). The most important sources for these hormones are activated macrophages, monocytes, fibroblasts, and endothelial cells (1,22,26,28).

In view of the pleiotropic *in vivo* response, many investigators have used *in vitro* systems consisting of hepatoma cell lines in order to study the underlying regulatory mechanisms of the acute phase response (2,7-9,23,24,28,37,44). Although these studies have offered great insight in several aspects of the response, they provide information which may be applicable only to the particular cell line under study (8,28). For a more realistic evaluation it is, therefore, necessary to test the *in vitro* response of normal hepatocytes. However, most studies with cultured primary hepatocytes have used conventional monolayer culture systems, which limit hepatocyte survival to 1-2 weeks (3,4,11,12,25,26,33,34,40,41). This short culture duration confines these studies to the initial events of the acute phase protein response, and precludes more long-term studies. In addition, the cells generally do not maintain phenotypic stability and tend to begin de-differentiating within 1-2 days in conventional culture, which can make interpretation of results problematic, especially with regard to their *in vivo* relevance.

Recently our laboratory has developed and characterized a new, long-term, phenotypically stable hepatocyte culture system, which consists of parenchymal liver cells sandwiched between two layers of collagen. This "double gel" configuration appears to establish the normal cellular polarity seen in intact liver and enables the cells to maintain normal morphology and stable function for 6-8 weeks (17-19,54).

The purpose of this study was to determine whether a physiological response to cytokines could be seen in rat and human hepatocytes, whose phenotype had been stabilized by the double gel configuration. Hepatocytes were exposed to varying concentrations of IL-6, IL-1ß, and TNF-$\alpha$ and the concentrations of several secreted acute phase proteins were examined, and

followed over time. The results indicate that the double gel culture system provides an attractive *in vitro* model for the study of the acute phase protein response.

## Materials and Methods

*Rat hepatocyte isolation.* Female Lewis rats, weighing 180 - 220 grams, were used as the source for rat hepatocytes (Charles River Laboratories, Boston, MA). Cells were isolated as previously described in detail (17-19). This technique is a modification of the two-step collagenase perfusion technique originally described by Seglen et al. (51), and includes purification of the cell suspension by means of Percoll, as reported by Kraemer et al. (35). In average, 200-300 million hepatocytes were harvested per isolation, with viabilities ranging from 90-98% as assessed by trypan blue exclusion.

*Human hepatocyte isolation.* Human hepatocytes were obtained from patients undergoing hepatic resection for malignancy. Biopsies of normal liver tissue, weighing 4.3-19 g, were transferred to calcium-free Krebs Ringer buffer (KRB) at 4°C. The portal and hepatic veins of the liver piece were cannulated using a modification of the procedure described by Reese and Byard (48). The specimen was sequentially perfused with KRB for 10 min, followed by KRB containing 0.5 mg/ml of collagenase (type I, Sigma Chemical Corp., St. Louis, MO), and 5 mM $CaCl_2$, for another 10 min. All solutions were equilibrated with 95% $O_2$ and 5% $CO_2$ at 37°C. The perfusion rate was continuously adjusted to maintain a pressure of 102 ± 10 mmHg. After perfusion, the cells were dispersed, filtered, and washed twice with cold KRB. Purification of the cell suspension was achieved using Percoll. Hepatocyte viability varied from 82 - 95% (trypan blue exclusion). Per gram of liver, 32 - 50 million viable hepatocytes were obtained.

*Hepatocyte cultures.* Type I collagen was prepared from rat tails as described previously by Elsdale and Bard (20). In order to calculate the collagen concentration, the absorbance was measured at 280 nm. A final concentration of 1.11 mg/ml was used in all experiments. Rat hepatocytes were suspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Gaithersburg, MD), supplemented with 1.07 ng/ml ($3x10^{-4}\mu M$) glucagon (Lilly, Indianapolis, IN), 0.25 $\mu$g/ml (0.6$\mu$M) hydrocortisone (Upjohn, Kalamazoo, MI), 0.133 mU/ml ($1.85x10^{-5}\mu M$) insulin, (Squibb, Princeton, NJ), 200 U/ml penicillin, 200 $\mu$g/ml streptomycin, and 10% fetal bovine serum (Hazelton, Lenexa, KA). These hormone concentrations are considerably lower than those used in previous studies (17,19,54) and approached more physiological levels (36) in an attempt to minimize the influence of pharmacologic hormone concentrations on the

acute phase response (22). Cells were seeded at a density of 2 million cells per 60x15 mm dish (Falcon™, Lincoln Park, NJ). At least 30 min prior to plating, dishes were coated with collagen. Twenty-four hours later the media was aspirated, and the second layer of collagen was applied. After a gellation period of 30 min, media was added. Spent media was replaced by fresh media daily thereafter. Culture supernatants were collected until the 26th day of incubation.

Human hepatocytes were cultured in DMEM with 0.5 U/ml ($6.96x10^{-2}\mu M$) insulin, 7 ng/ml ($2x10^{-3}\mu M$) glucagon, 7.5 $\mu$g/ml (20.7$\mu$M) hydrocortisone, and 0.02 $\mu$g/ml epidermal growth factor (EGF; Collaborative Research, Lexington, MA). This standard hormone composition was used for the human cells, following pilot experiments with rat hepatocytes that failed to show a significant difference in acute phase response between the reported physiological, and the standard hormone concentrations. The concentrations for the antibiotics and fetal bovine serum added were similar to the ones used for rat hepatocytes, as was the procedure for assembling the double gel configuration. In general, human cells were maintained in culture for up to 21 days.

*Protein analysis.* Culture supernatants were stored at 4°C prior to analysis by enzyme-linked immunosorbant assays (ELISA). Analysis included assays for albumin, fibrinogen, α2-macroglobulin, and SAA. Chromatographically purified albumin was purchased from Cappel (Cochranville, PA), and fibrinogen was purchased from Sigma. Antibodies to these two proteins were purchased from Cappel. α2-Macroglobulin and its antibody were kindly provided by D.D. McGabe of Du Pont (Wilmington, DE), and Dr. P.C. Heinrich (University of Aachen, Germany), respectively. Human SAA and its antibody were purchased from Calbiochem (San Diego, CA). The ELISA assay is described elsewhere in detail (17). The data are given as mean values ± standard deviations. For statistical comparison of results, the student's t-test was used.

*Cytokines.* IL-6 was purchased from Collaborative Research Inc., Lexington, MA. IL-1ß and TNF-α were kindly provided by Drs. C. Dinarello and J. Gelfand of New England Medical Center (Boston, MA). Doses are given in pg/ml, with the following activities: $1x10^9$ U/mg IL-6, $5x10^8$ U/mg IL-1ß, and $1x10^7$ U/mg TNF-α. The cytokines were kept as stock solutions at -70°C, dissolved in 50-150 $\mu$l of PBS immediately before use, and subsequently added to the supernatant culture medium. Hepatocytes were exposed to cytokines on the 16th and 9th day of culture, respectively, for rat and human hepatocytes, i.e., when the stable plateau of protein secretion levels had been reached. Control dishes received an equal volume of PBS only. Twenty-four hours later the supernatant was replaced by culture medium without cytokines.

*RNA assays (Northern analyses).* Total RNA was prepared by lysing the cells in guanidinium-isothiocyanate buffer and centrifugation of the lysates through a CsCl cushion (13). Ten $\mu$g of total RNA per lane were electrophoresed through 1% agarose formaldehyde gels and transferred to nylon membranes (38). cDNA probes were labeled with $^{32}$P dCTP using the random primer method (21). Hybridization was performed in 50% formamide, 1M NaCl, 5% dextran sulfate, 1% SDS, 250 $\mu$g/ml salmon sperm DNA, and $10^6$ cpm/ml $^{32}$P labeled probe for 18 h at 42°C. The membranes were subsequently washed in 2x SSC (1x SSC= 0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS, 3 x 10 min at room temperature, and 2 x 30 min at 65 °C, and exposed to XAR film (Eastman Kodak Co., Rochester, NY) at -70°C, using intensifier screens. Exposure times ranged from 2 hours to 3 days. The following cDNA probes were used: 600 bp PstI fragment of pBR alb 149 (coding sequence of rat albumin) (55); 1.5 kb PstI fragment of pßfib (cDNA of rat fibrinogen) (15); 560 bp PstI fragment of p680.8 (coding, and part of 3' noncoding sequence of human ferritin) (16). For detection of rat $\alpha_2$-macroglobulin mRNA a $^{32}$P 5'endlabeled, 30-mer antisense oligonucleotide from the sequence reported by Gehring et al. (29) was used. Hybridization took place in 6x SSC, 5x Denhardt's (0.1% polyvinylpyrolidon, 0.1% Ficoll, 0.1% BSA), 0.5% SDS, 0.1 mg/ml salmon sperm DNA, and $10^6$ cpm/ml $^{32}$P labeled probe at 42°C for 18 h. The membranes were washed in 6x SSC, 0.1% SDS, 2 x 15 min at 60°C. After each hybridization, dehybridization was performed by incubation in 50% formamide, 2x SSC, 1% SDS, for 60 min at 65°C. RNA-extraction took place on days 16-18, 22, and 23 of culture, corresponding to the 1st, 2nd, 6th, and 7th day after induction of acute phase response. Negative controls included non-stimulated cell cultures that were submitted to identical extraction procedures. L-Ferritin mRNA was used to control for the amount of RNA on the membrane, since it has been shown to remain unaltered during acute phase response of cultured cells (49).

## Results

*Pattern of albumin secretion in double vs. single gel cultures.* In order to determine the time necessary for rat hepatocytes in double gel configuration to stabilize, the albumin secretion in these cultures was followed, and compared with that of hepatocytes in single gel cultures. As seen in Fig. 1, hepatocytes in the double gel showed a steady increase in albumin secretion after the third day of incubation, and reached a plateau level around day 15; the secretion rate remained at this level until the end of the experiment at day 26. In the single gel cultures, relatively small amounts of albumin were detectable beyond one week of incubation.

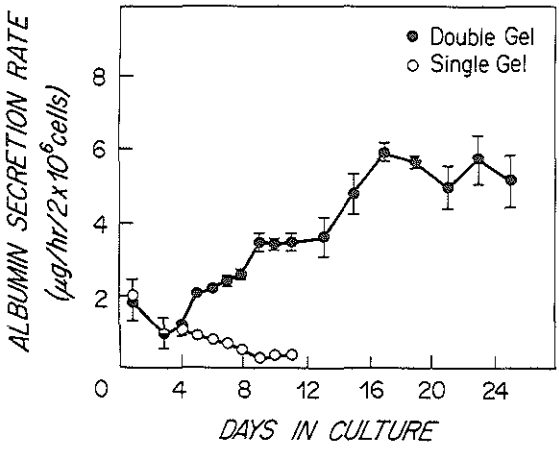


**Figure 1**: Albumin secretion in rat hepatocyte cultures in single gel vs. double gel configuration (mean±SD). Media was collected every 24 h for protein analyses.

*Response to IL-6.* Because IL-6 has been identified as the major mediator involved in the regulation of acute phase proteins in rats and humans (12,27,50), initial experiments were focussed on stimulation with this cytokine. In order to find the optimal stimulatory concentration of IL-6, dose response curves were established (Fig. 2).

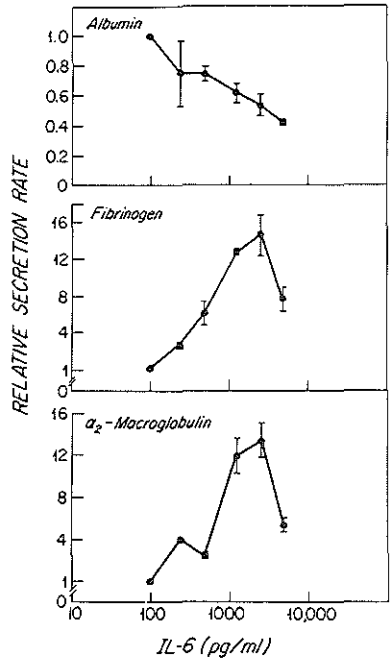


**Figure 2**: Dose response curves for the secretion of albumin (top), fibrinogen (middle), and $\alpha_2$-macroglobulin (bottom) to a single administration of IL-6. Human recombinant IL-6 was administered to rat hepatocyte cultures in double gel configuration on the 16th day of culture. After 24 h the media was changed and no further cytokines added. The results are peak concentrations obtained 2 days after cytokine exposure. The protein secretion rates were normalized to values measured on day 16. All data points represent duplicate measurements of at least two experiments.

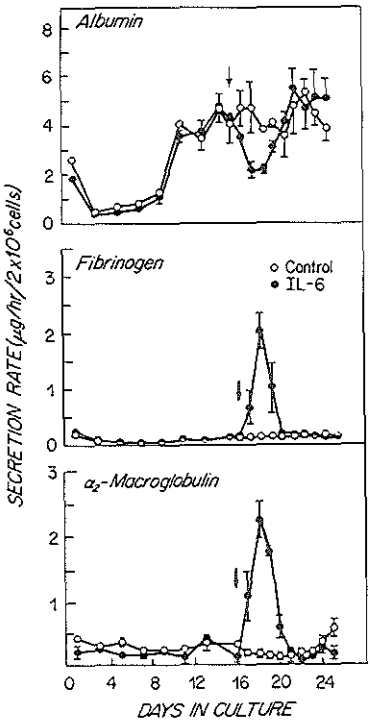


**Figure 3.** Response of rat hepatocytes in double gel culture to IL-6. Cultures were exposed for 24 h to a single dose of 2500 pg/ml of IL-6 on the 16th day of incubation (arrows). Secretion rates of albumin (top), fibrinogen (middle), and $\alpha_2$-macroglobulin (bottom) are expressed as $\mu$g/h/2x$10^6$ cells (mean $\pm$ SD). Data were obtained from a minimum of 2 experiments (duplicate measurements per experiment).

Cultures were stimulated on day 16 with IL-6 doses increasing from 25 to 5000 pg/ml, and protein secretion rates were followed for 3 consecutive days. Peak protein concentrations were invariably seen at 2 days after exposure to IL-6. A maximal (i.e., 2-fold) decrease in albumin secretion was observed at an IL-6 dose of 5000 pg/ml. Maximal stimulation of fibrinogen secretion (15-fold) and $\alpha_2$-macroglobulin secretion (13-fold) was obtained with 2500 pg/ml of IL-6. Further experiments were performed with IL-6 doses of either 2500, or 5000 pg/ml.

Fig. 3 shows the temporal response of hepatocytes in the double gel system to 2500 pg/ml of IL-6 on the 16th day of culture. At 2 days post-exposure albumin secretion had decreased from its pretreatment value of 4.29 $\pm$ 0.82 to 2.1 $\pm$ 0.35 $\mu$g/hr ($p<0.01$). It remained at this low level for one more day and returned to its baseline value within 4-5 days after exposure. Concomitantly, fibrinogen secretion peaked at 2.02 $\pm$ 0.34 $\mu$g/hr ($p<0.001$) on the second day after exposure to IL-6 (a 15.8-fold increase), and returned to its pretreatment value by the end of the next 2 days. A virtually identical picture was seen with regard to the $\alpha_2$-macroglobulin secretion, which peaked at 2.26 $\pm$ 0.29 $\mu$g/hr on day 2 after exposure (14-fold increase; $p<0.001$), and returned to its pretreatment value 4-5 days after the initial stimulus.

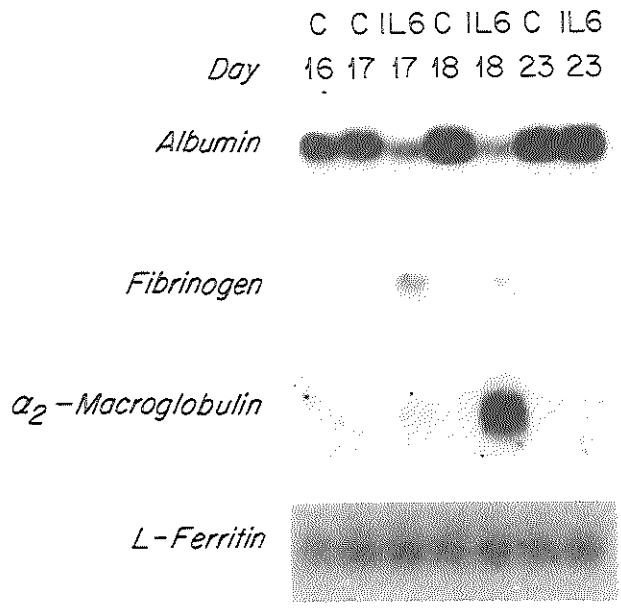


**Figure 4.** mRNA levels in rat hepatocytes in response to 5000 pg/ml of IL-6, administered on day 16 of culture. mRNA's for albumin, fibrinogen, $\alpha_2$-macroglobulin, and L-ferritin are shown. Total RNA was extracted from non-treated control cells (C), as well as from IL-6 treated cells (IL-6) on days 17, 18, and 23 of culture (i.e., days 1, 2, and 7 post-treatment). Total RNA was separated on a denaturing agarose gel, blotted on a nylon membrane, and hybridized with cDNA probes for albumin, fibrinogen, $\alpha_2$-macroglobulin, and L-ferritin. The hybridization with the indicated probes was carried out sequentially on the same membrane.

*mRNA Analyses.* Northern blot analyses were performed to investigate whether the observed alterations in protein secretion rates were reflected in the mRNA levels of the respective proteins. A single dose of 5000 pg/ml of IL-6 was administered to rat hepatocytes on the 16th day of culture. mRNA levels were estimated from parallel cultures 1, 2, and 7 days after IL-6 treatment, as well as from untreated control cultures. The expression of the albumin transcript was markedly decreased 24 and 48 hours after IL-6 treatment (Fig. 4). By the 7th post-exposure day, the albumin mRNA level had returned to control levels. The expression of the mRNAs encoding fibrinogen and $\alpha_2$-macroglobulin was elevated 1 and 2 days after exposure to IL-6, and also returned to control levels by day 7 (Fig. 4). In order to ensure that these changes were specific to these proteins (and to ensure that identical amounts of RNA were blotted), hybridization with an L-ferritin probe was performed. The same amount of L-ferritin mRNA was found in each lane (days 1, 2, and 7) of the Northern blots.

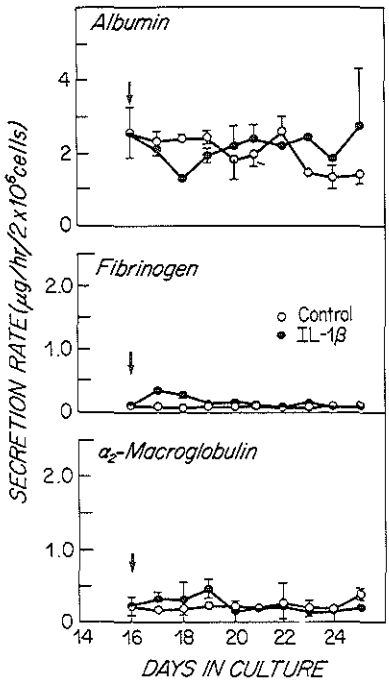


**Figure 5.** Response of rat hepatocytes in double gel culture to IL-1ß. A single dose of 10,000 pg/ml of IL-1ß was administered on day 16 of culture (arrows). Further details on exposure are identical to those given for IL-6 (see Fig. 3).

*Response to IL-1 and TNF.* IL-1ß was administered at concentrations ranging from 500 to 25,000 pg/ml on day 16 of culture. IL-1ß at concentrations below 5000 pg/ml did not stimulate the proteins measured. At 10,000 pg/ml a twofold reduction of albumin secretion was observed after 48 h, followed by a rapid return to pretreatment levels (Fig. 5). The same IL-1ß concentration evoked a peak in fibrinogen secretion of $0.327 \pm 0.04$ $\mu$g/h, which represents a 5-fold increase when compared to pretreatment values. No additional effect was obtained by further increasing the dose of IL-1ß (data not shown). α2-Macroglobulin secretion was not significantly increased by IL-1ß, irrespective of the dose (Fig. 5). TNF-α, at doses ranging from 125 to 5000 pg/ml, did not affect the secretion rates of any of the proteins evaluated (data not shown).

*Human hepatocytes.* In order to demonstrate the physiological responsiveness of human hepatocytes in the double gel configuration, the effects of cytokine stimulation on the secretion rates of albumin and SAA were measured. In human hepatocyte cultures a plateau phase of albumin secretion was generally seen between days 9 and 12, followed by a gradual decline during the final week of incubation (Fig. 6A and C). Exposure to 5000 pg/ml of IL-6 on day 9

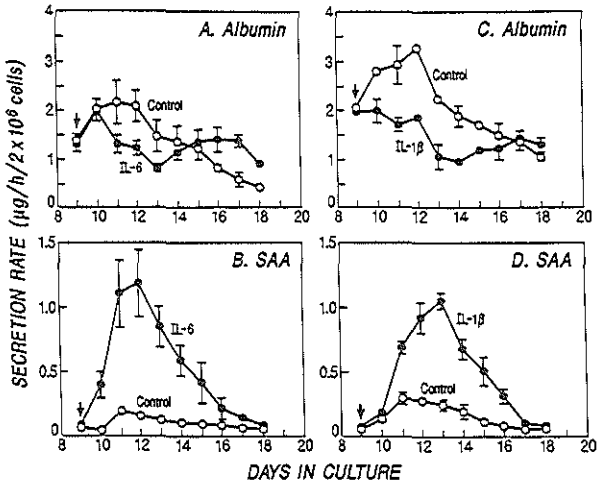


**Figure 6.** Response of human hepatocytes in double gel culture to IL-6 (A and B) and IL-1ß (C and D). The secretion rates of albumin (A and C) and SAA (B and D) are depicted. Cell density was similar to rat hepatocyte cultures ($2x10^6$ cells per dish). Cytokine exposure took place on the 9th day of culture (arrows). The doses used were 5000 pg/ml for IL-6, and 10,000 pg/ml for IL-1ß. Results are depicted as $\mu g/h/2x10^6$ cells (mean±SD), and are the outcome of duplicate measurements of 2 experiments.

of culture resulted in a decrease in albumin secretion to significantly lower values ($1.22 \pm 0.22$ $\mu g/h$) vs. control cultures ($2.16 \pm 0.41$ $\mu g/h$) between the 2nd and the 4th day after treatment ($p<0.05$). On the 5th post-exposure day the albumin concentration was comparable to that of control cultures (Fig. 6A). In addition, IL-6 evoked a peak increase in the secretion of the human positive acute phase protein SAA from baseline rates of $0.03 \pm 0.01$ to $1.19 \pm 0.3$ $\mu g/h$ at 3 days post-exposure ($p<0.001$), followed by a return to pretreatment values in the next week (Fig. 6B). A single dose of IL-1ß (10,000 pg/ml) resulted in significantly decreased albumin secretion levels between days 1 and 6 after induction, when compared to controls ($p<0.001$; Fig. 6C). IL-1ß exerted a stimulatory effect on the SAA secretion, which was comparable to that of IL-6 (Fig. 6D). TNF-α did not significantly change the secretion rates of the proteins examined (data not shown).

## Discussion

In previous reports we have shown that primary rat and human hepatocytes cultured in double gel configuration establish a stable rate of protein secretion for prolonged periods of time (17-19,54). In this study we have demonstrated that this culture system can be used for studies involving physiological responses, such as the hepatic acute phase response. Not only were certain cytokines shown to give rise to an acute phase response in double gel cultures of both rat and human hepatocytes, but also the response was shown to return to a pre-treatment baseline level within a few days after the treatment.

*Comparison with in vivo acute phase response.* After turpentine injection, the peak responses at protein level *in vivo* have been observed between 2 and 3 days following the stimulus (30,43,45,50). Generally, these changes are followed by a return to pre-injection values within the next week (50). The changes in the mRNA levels of the respective proteins seem to occur earlier, i.e. between 12 and 36 hours after turpentine injection. In the case of *in vivo* administration of recombinant IL-6 or IL-1, maximal mRNA levels of responsive positive acute phase proteins in mice and rats occur earlier, i.e., roughly between 6 and 24 hours (30,32,39,40,46,47,53), while albumin mRNA decreases steadily during the first 24 hours (30,32,39,46,47). At protein level, maxima occur around 24 to 48 hours (10,30,32,39,42,46). The presented response kinetics in double gel hepatocyte cultures closely resemble these *in vivo* patterns.

*Comparison with other in vitro studies.* Prior *in vitro* studies have either used hepatoma cell lines (2,6-9,31), or primary hepatocytes in conventional culture (3,12,25,33,34,41). Although, as a group, the former follows the *in vivo* response patterns, the phenotypic differences between individual cell lines, as illustrated by Ganapathi et al. (23), make interpretation of results difficult. On the other hand, the studies on primary cells, which display normal phenotype, have thusfar been hampered by decreasing baseline protein secretion rates due to lack of culture stability. In combining normal phenotype of the primary hepatocyte with long-term stability, our double gel culture system offers an attractive alternative to the approaches mentioned. The only other description of acute phase response in a long term culture system is by Conner et al. (14), who used co-cultures with rat liver epithelial cells, and repeatedly exposed the cells to conditioned medium from activated monocytes. This system is more complicated than ours, since it requires a second cell type. Nonetheless, the reported return to pretreatment levels in 4 days following the withdrawal of the stimulus is comparable to our observations.

*IL-6 and TNF-α stimulation.* Among the three cytokines, IL-6 elicited, in a dose-dependent fashion, the most pronounced response in the synthesis of both positive and negative acute phase proteins. This is in agreement with the findings of others in primary rat (3,26-28), and human hepatocytes (11,12,41). It is further supported by the recent findings of Sonne et al. (52), who showed that IL-6 has a much higher binding affinity to primary rat hepatocytes than IL-1ß or TNF-α. In addition, the extent of the response to recombinant IL-6 we found is comparable to previous observations *in vivo* (22,30,39,47), and *in vitro* (3,26). The concomitant changes at the respective mRNA levels of the proteins examined after IL-6 induction of rat hepatocytes lend additional support to the notion that the acute phase response is primarily regulated at the pretranslational level (1,6,12,50). Previous observations on TNF-α (3,4,5,9) are also supported by the data presented here, confirming its lack of effect on the acute phase response in cultured primary hepatocytes.

*IL-1 stimulation. In vivo* administration of recombinant IL-1 in the rat has recently been demonstrated to stimulate the expression of fibrinogen, α1-acid glycoprotein, and cystein proteinase inhibitor, and to reduce that of albumin (32). The observed effects of recombinant IL-1ß in double gel cultures of rat hepatocytes resemble this *in vivo* response. It is of note that our data on fibrinogen induction by IL-1 are in contrast to the results of several studies on hepatoma cells (2,6,8,37). This difference may be explained by the phenotypic differences that exist among the cell lines examined. On the other hand, since it is known that non-parenchymal cells (e.g. Kupffer cells, fibroblasts, and endothelial cells) are able to secrete IL-6 in response to IL-1, it is also conceivable that the IL-1 stimulation of fibrinogen in our system may be a result of indirect stimulation through IL-6. However, non-parenchymal cell contamination in our cultures was consistently lower than 1%.

*Human hepatocyte response.* Most studies concerning the human hepatic acute phase response *in vitro* have been performed on hepatoma cell lines (9,23,24,37,44,47). Castell et al. (11,12) have recently reported on IL-6 stimulation in conventional short-term cultures of primary human hepatocytes. In contrast to our observations, they reported that IL-1ß did not enhance SAA secretion. These results may not be contradictory with ours, given the fact that their investigations were confined to an observation period of 24 hours after the stimulation while our findings indicate that the increase in SAA secretion is not pronounced until the second day after treatment with IL-1ß, and peaks at days 3 and 4 (Fig. 6D). In a preliminary report on primary cultures of frozen-thawed human hepatocytes, Moshage et al. (41) found that both recombinant IL-1 and IL-6 enhanced SAA and C reactive protein secretion, reaching maximal values on day 3.

In summary, we have documented the capacity of our stable long-term hepatocyte culture system to display a classical acute phase protein response, which resembles the *in vivo* response, and is followed by a return to pretreatment protein secretion rates and patterns. Our findings confirm the importance of IL-6 as a mediator of the hepatic acute phase response. The double gel culture system provides a useful new tool for further investigations into the mechanisms involved in the acute phase response, especially studies on chronic cytokine exposure and the events involved with the resolution of the response. In addition, this system may be used to follow various aspects of hepatocellular metabolism during the response in order to more fully appreciate the mechanisms by which cells modulate protein expression.

**Acknowledgement**

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## CHAPTER 3

# An extracorporeal microscopy perfusion chamber for on-line studies of environmental effects on cultured hepatocytes

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Submitted for publication

**Abstract**

The development of bioartificial organ support systems is hampered by the lack of knowledge on the effects of different (in vivo) environments on cells during extracorporeal perfusion. For the present study, a perfusion chamber was designed for continuous monitoring of cultured cells during perfusion with media, as well as during plasma perfusion in an extracorporeal circuit. The chamber was characterized, and shown to provide satisfactory thermal and perfusion profiles, and no major pH fluctuations. Further testing of the chamber was performed with hepatocytes that were cultured in between two collagen layers (sandwich configuration), which was previously shown to preserve hepatocyte morphology and function for over 6 weeks of culture. Perfusion of the hepatocytes with culture media did not adversely affect cell morphology, quantitative cell shape indices, and function, provided the perfusion time was $\leq$48h. Perfusion of the cultures during connection of the chamber to an extracorporeal circuit involving normal rats for 6h resulted in reversible cytoplasmic changes, unaltered cell shape indices, and a 40% increase in albumin secretion rate during the first post-perfusion day, followed by a return to stable control levels. We conclude that this chamber is a valuable asset for on-line studies of cells under (extracorporeal) perfusion conditions, and could be used for a large variety of applications, including studies on regeneration, reperfusion damage, and detoxification.

## Introduction

Dynamic observations of cellular responses to environmental perturbations are of major interest in various fields of experimental cell research, in particular in the development of bioartificial organs. Although several in vitro perfusion systems have been described that allow monitoring of cell function and/or morphology for several days (4,8,9), these systems cannot be used in connection with an extracorporeal perfusion system as would be neccessary in bioartificial organ support devices. With regard to bioartificial liver support, relatively little progress has been made in recent years (10,12,13). In addition, the fate of hepatocytes during, and after extracorporeal perfusion is largely unknown. In a recent report, Arnaout et al. (1) described a rapid decrease in viability of microcarrier attached hepatocytes during perfusion in connection with an extracorporeal circuit. The nature of the cell damage, however, remains unclear.

The present study was undertaken to design and test a system that would enable investigators to continuously monitor cellular behavior under different conditions, including exposure to an in vivo environment during extracorporeal perfusion. Further requirements of the system involved acceptable thermal and perfusion characteristics, and the possibilty of functional and morphological follow up of the cells after perfusion. The system was tested using rat hepatocytes cultured in a collagen sandwich configuration, which has been shown by our group to enable hepatocytes to express stable phenotype (5,6) and physiological responsiveness (2) over the long term.

## Materials and Methods

*Animals and hepatocyte isolation.* Adult female Lewis rats (Charles River Laboratories, Boston, MA), weighing 150-200 g, were used for connection to the perfusion system, and as donors of hepatocytes, blood and plasma. Hepatocytes were isolated according to the two-step collagenase perfusion technique as originally described by Seglen (11), and modified as reported previously by our group (5,6). Per isolation, ~250-350x$10^6$ hepatocytes could be retrieved from one donor liver, with viabilities ranging from 90-98% (trypan blue exclusion).

*Hepatocyte sandwich cultures.* Hepatocytes were cultured on 12x12 mm No. 1 cover glasses (Bradford Scientific, Inc., Epping, NH), which had been sterilized prior to use. Seven cover glasses were distributed on the bottom of a 100 mm plastic tissue culture dish (Falcon™, Lincoln Park, NJ), and covered with 2.8 ml of type I collagen, prepared from rat tail tendons

in a procedure outlined elsewhere (5,6). The collagen was allowed to gel at 37°C for 60 min before cell seeding. Eight million viable hepatocytes were seeded per tissue culture dish, and incubated in 10% $CO_2$ at 37°C for 24 h. Then, the medium was aspirated, and a second layer of collagen was spread over the cultures in order to establish the sandwich configuration. After incubation in 37°C for another 60 min to allow the collagen to gel, new medium was added to the cultures, and replaced daily thereafter. The culture media used throughout these studies consisted of Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Gaithersburg, MD), supplemented with 7 ng/ml glucagon (Lilly, Indianapolis, IN), 7.5 $\mu$g/ml hydrocortisone (Upjohn, Kalamazoo, MI), 0.5 U/ml insulin (Squibb, Princeton, NJ), 20 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA), 200 U/ml penicillin, 200 $\mu$g/ml streptomycin, and 10% fetal bovine serum (J.R. Scientific, Woodland, CA). On the 5th day of culture, the cover glasses were cut out from the collagen and each of these sandwich cultures on cover glass was transferred to a 35 mm tissue culture dish.

*Microscopy perfusion chamber.* A thermally controlled microscopy chamber was designed to observe hepatocytes during continuous perfusion with media and plasma as part of an extracorporeal unit. The chamber was comprised of two separate rectangular pieces (90x125 $mm^2$) of polysulfone (Amoco Performance Products, Inc., Ridgefield, CT) as shown in Fig. 1A. Both pieces had coinciding rectangular holes (16x16 $mm^2$) in the center for optical observations. The top piece was 2 mm in thickness and a ridge was created around the center opening to mount a cover glass. In addition, the top piece had two small holes (2 mm in diameter) to which inlet and outlet tubes (Masterflex, Cole-Palmer Instrument Co., Chicago, IL) were connected. A 2 mm deep channel bed was milled into the bottom piece to permit the flow of the perfusate. The channel, centrally located along the longitudinal axis, gradually expanded in width from 3 mm at the inlet and outlet to 16 mm where it reached the window area (Fig. 1A). This expansion was included to achieve uniform flow conditions. The heater window was mounted onto the ridge created around the opening in the bottom piece (4 mm in thickness). The heater window was composed of two layers of glass. The bottom surface of the top layer was covered with a transparent, electrically conductive tin oxide coating serving as a resistance heater (50 Ω). Power leads were soldered to two parallel strips of additionally deposited gold coatings located 10 mm apart. The electrically conductive surface was insulated by a $SiO_2$ layer. A copper-constantan foil thermocouple (5 $\mu$m thick) (CO2-T, Omega, Stamford, CT) was located on top of the bottom glass layer (100 $\mu$m). A thin ridge of ~2 mm silicone RTV (GE Silicones, Waterford, NY) was placed on the bottom piece of the chamber along the channel to prevent leakage from the chamber when the two pieces were connected.

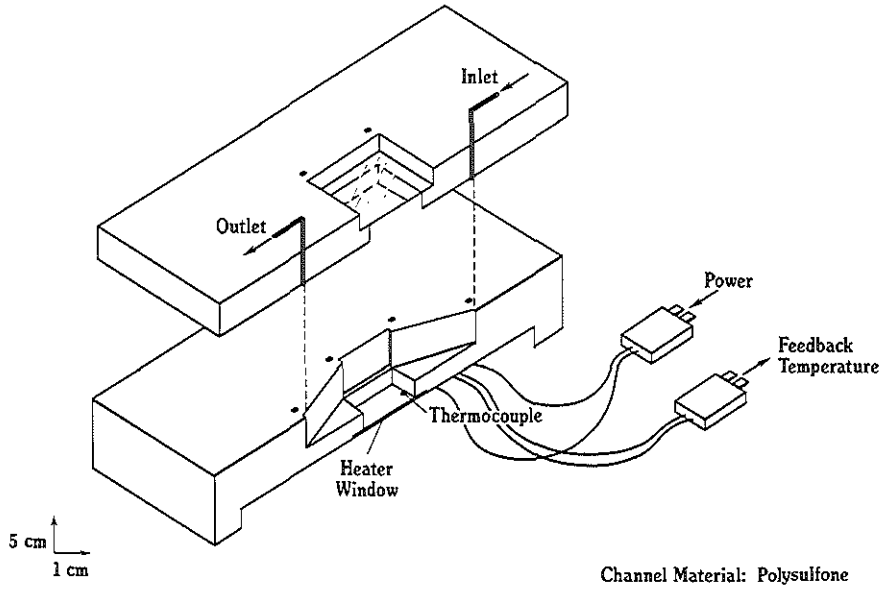


**Figure 1A.** Schematic drawing of the perfusion chamber used in this study.

*Perfusion system.* Fig. 1B gives an overview of the perfusion system components during extracorporeal rat perfusion. The perfusion chamber, containing the sandwiched hepatocytes, was positioned on the table of a light microscope (Olympus CK2), on which a video camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) was mounted. The camera was connected to a time lapse video cassette recorder and monitor through an IBM™ compatible computer. This computer also regulated the temperature controller unit (Interface Techniques Company, Cambridge, MA), which maintained the desired temperature in the chamber using feedback from the thermocouple on the heater window in the perfusion chamber. Based on this feedback, the power setting to the heater window was continually adjusted to obtain the setpoint temperature (i.e., 37°C) entered into the computer (3). Arterial rat blood was pumped at ~1 ml/min through size 14 Masterflex pump tubing, situated in a peristaltic pump (Pharmacia-3, Sweden). From there, it entered a plasma separator (courtesy of Mitsubishi Rayon Co., Ltd, Tokyo, Japan), which consisted of a polyolefin microporous hollow fiber

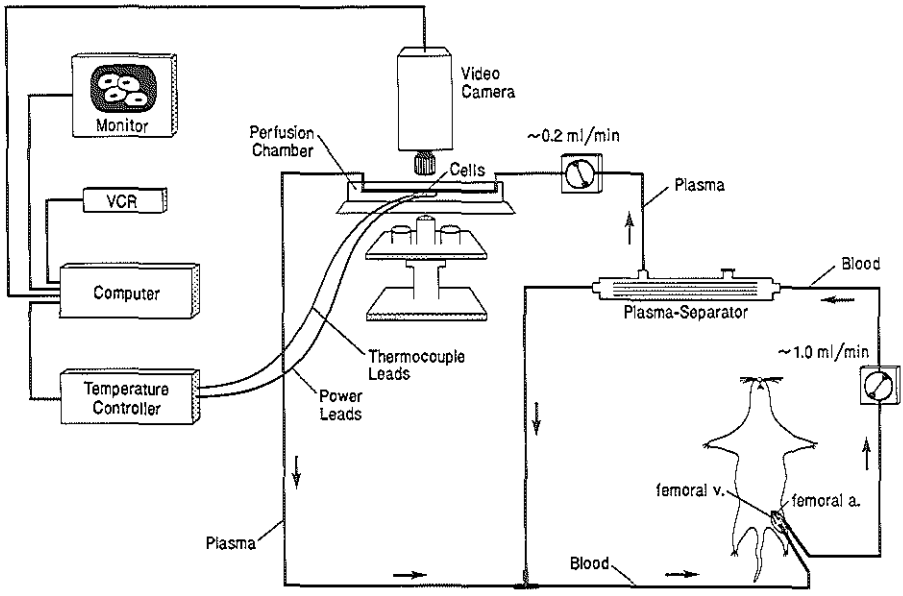


**Figure 1B.** Overview of the perfusion system components. Shown is the situation during extracorporeal perfusion of a rat.

membrane module (EHF270W), with an average slit width of ~0.28 $\mu$m. Separated plasma, collected in the extracapillary space of the hollow fiber module, was pumped through the perfusion chamber by means of a second pump (containing size 13 pump tubing) at a flow rate of ~0.2 ml/min. The separated plasma and remaining blood components were reunited before entering the rat circulation through the venous canule (see below). All tubing outside the peristaltic pumps consisted of PE-50 polyethylene (Becton Dickinson&Co, Parsippany, NJ). In this configuration, the dead volume of the entire perfusion system was 5.04 ml, of which 2.38 ml was accounted for by the chamber.

When experiments were performed using culture media as the perfusate, the media would routinely be kept in a small sterile flask, which was used as a reservoir, and would be flowing through a gas exchanger to equilibrate with 10% $CO_2$ and 21% $O_2$ before entering the perfusion stage. The gas exchanger contained 2.5 m of gas permeable silastic tubing (o.d. 0.077in., Dow

Corning, Midland, MI). Prior to use the entire system was gas sterilized. The chamber was kept in sterile environment until ready for use. The remainder of the system was rinsed extensively with sterile saline for ~24 h prior to perfusion, following prewetting of the plasmaseparator with 100% ethanol.

*Experimental design.* Since previous work has shown that sandwiched hepatocytes need the first week of culture to reach stable phenotype (6), experiments were carried out with 7-10 days old cultures. Prior to connecting the chamber to the entire perfusion system, the latter was primed with either media, or with heparinized syngeneic blood. At this stage, special care was taken to remove all air bubbles from the sytem, particularly from the plasma separator.

For the experiments involving extracorporeal circulation, an adult Lewis rat (~150-200 g, n=5) was simultaneously prepared. General anesthesia was established with 125 mg/kg Ketamine i.p. (Parke-Davis, Morris Plains, NJ). The left femoral artery and vein were exposed through an inguinal incision, dissected free, and occluded distally by a 6-0 ethilon tie. The femoral artery and vein were subsequently opened, and cannulated with PE-50 tubing, prefilled with heparinized saline. The cannules were secured into place with two additional ties. The rats were systemically heparinized (350 IU/kg body wt) prior to attachment to the perfusion system.

A cover glass containing sandwiched hepatocytes was then placed on the heater window of the perfusion chamber under sterile conditions, and the chamber was closed. Subsequently, the chamber was primed with either gas-equilibrated media, or syngeneic plasma, of 37°C. The chamber was placed on the microscope stage, connected to the temperature controller, brought to 37°C, and attached to the perfusion system. When unobstructed perfusion was established, the rat was attached to the perfusion system through connection with the arterial and venous cannula, respectively. The sandwiched hepatocytes on the cover glass were continually observed during each perfusion experiment; these observations were recorded using time-lapse video. Upon termination of the perfusion, the chamber was disconnected from the system, and opened under sterile conditions. The cover glass culture was removed, washed extensively (see below) with warm media, transferred to a new 35 mm dish, and incubated at 37°C for at least 4 days after perfusion. Media was collected daily and kept at 4°C.

*Evaluation.* Apart from qualitative evaluation, the morphological data recorded with time lapse video microscopy were also analyzed in a semi-quantitative way using an Argus 10 image processor (Hamamatsu). Hepatocyte perimeter and surface area were documented for a minimum of 8 cells per field. For adequate comparison, at least 5 fields were evaluated for each time point. Albumin secretion rates were determined by ELISA, as reported previously (6).

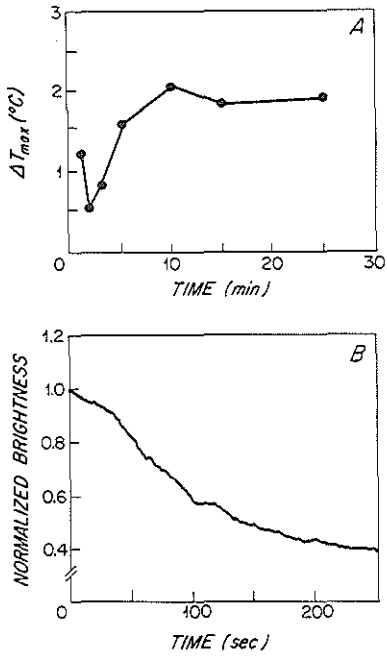


**Figure 2.** Characteristics of the perfusion chamber at a typical flow rate of 0.2 ml/min. (A) Maximal temperature difference across the perfusion chamber, as measured by thermocouples at inlet and outlet edges of a No. 1 cover glass. (B) Flow characteristics as judged by the disappearance of brightness after introduction of dark dye into the perfusion system, as a function of time. Brightness is normalized to the initial brightness.

Values are expressed as mean±SD. The student's t-test was used for statistical comparison.

## Results and Discussion

In a series of experiments we determined the thermal and perfusion characteristics of the microscopy chamber. In order to ascertain that the thermal gradients on the heater layer were within acceptable limits, the temperatures at the inlet and outlet edge of a glass cover slip were measured using copper constantan thermocouples. Fig. 2A shows the maximal temperature differences as a function of time of exposure to a typical flow rate of 0.2 ml/min. For the glass coverslips, maximal gradients were found to occur within the first 10 min of perfusion, whereafter no further temperature fluctuation was observed, and the maximum temperature difference across the cover glass was <2°C . In order to ensure that the cells in the chamber were exposed to the same perfusion characteristics, we measured the disappearance of brightness over time after introduction of dark dye into the chamber inlet. Fig. 2B shows that perfusion at 0.2 ml/min caused a rapid decrease in brightness down to a stable value, suggesting uniform perfusion. In addition, there were no major shifts in pH during perfusion (<0.15), as judged by pH measurements at the inlet and outlet ends of the chamber (data not

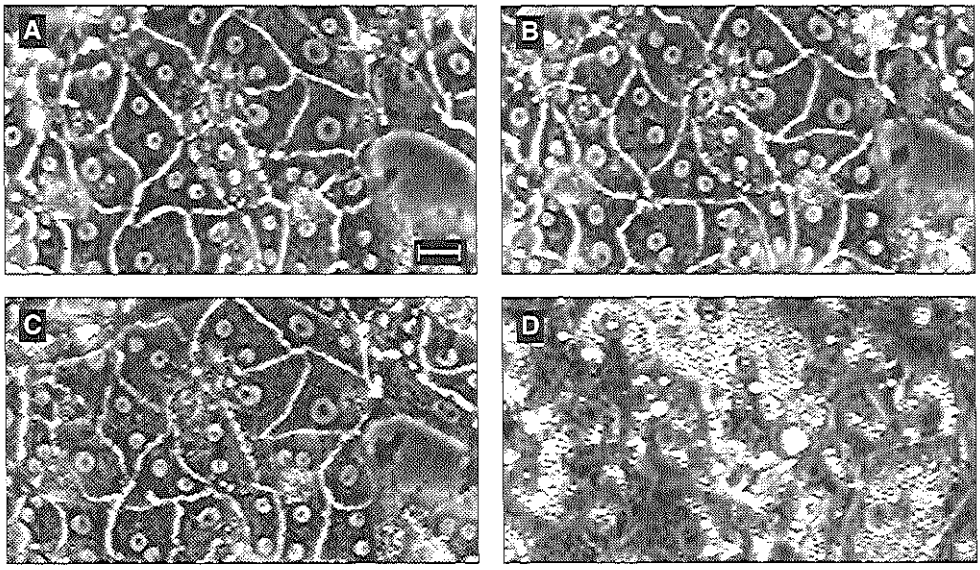


**Figure 3.** Morphological effects of prolonged perfusion with culture media on hepatocyte sandwich cultures in the perfusion chamber as monitored by time lapse video microscopy. (A) Initial aspect on day 8 of culture (immediately before perfusion); (B) same field as in A, after 24 h of perfusion; (C) at 48 h; (D) different field of the same culture at 96 h. Scale bar represents 35 $\mu$m.

shown). These results indicate that the perfusion chamber provides uniform cell perfusion under stable chemical and thermal conditions.

Morphological changes induced by perfusion of sandwiched hepatocytes in this system were studied by real time video microscopy. In an attempt to define the limits of study, the effects of long-term perfusion with culture media were studied (Fig. 3). During the first 48 h of perfusion no significant changes were observed (Fig. 3A-C). Beyond 48 h of perfusion, degenerative changes started to occur, most notably a stark increase in the granularity of the cytoplasm, including an increasing number of fatty droplets, and a decreasing intensity of the cell-cell boundaries. After ~96 h most cell boundaries had vanished suggesting that many hepatocytes had died, and those cells that remained visible were filled with granules and vacuoles (Fig. 3D). These degenerative changes may be due to the fact that media was not changed during these long-term experiments. In addition, the prolonged exposure to flow conditions may have direct, adverse effects on the cells. Another possible factor includes the

**Table 1.** Effects of 48 h perfusion with medium on cell shape indices of sandwiched hepatocytes

| Time (h) | 0 | 6 | 24 | 48 | Day +6 |
|---|---|---|---|---|---|
| P ($\mu$m) | 168±21 | 171±25 | 170±30 | 165±19 | 169±22 |
| SA ($\mu m^2$) | 1530±309 | 1591±268 | 1541±327 | 1534±346 | 1595±311 |

P: perimeter, SA: surface area, mean±SD, n=5. Perfusion was initiated on day 7 of culture (=time 0). Day+6 represents values at 6 days post-perfusion

denaturation of proteins (e.g. from the serum in the media) after prolonged interaction with artificial surfaces (7).

Further studies with media as the perfusate were limited to 48 h of perfusion. In order to provide more quantitative morphological data, the average cell perimeter, and surface area were documented before, during, and after 48 h medium perfusion (n=5). Table 1 shows that neither cell shape index is influenced significantly by this regimen for up to 6 days after perfusion. As a measure of hepatospecific function, albumin secretion was measured before, during and after perfusion, and found to remain constant (Fig. 4). These data indicate that 48 h of perfusion of stabilized sandwiched hepatocytes in the perfusion chamber under the conditions mentioned does not lead to significant changes in hepatocellular morphology and function, either during the perfusion, or in the follow up period of 6 days.

Connection to the extracorporeal circuit, and perfusion of the cultured hepatocytes in the attached chamber was carried out in 5 rats. For practical reasons, 6 h was chosen as perfusion time. Although the cell shape indices (perimeter and surface area) did not change significantly during the perfusion (Table 2), the cytoplasm contained slightly more fatty deposits and revealed a somewhat more granular aspect towards the end of the perfusion (6 h), when compared to its initial aspect (Fig. 5A-B). These changes were reversed within the first day of post-perfusion culture under static conditions. On day 4 after perfusion through the extracorporeal circuit, the perfused hepatocytes revealed morphological features identical to those of undisturbed control cultures (Fig. 5C-D, Table 2).

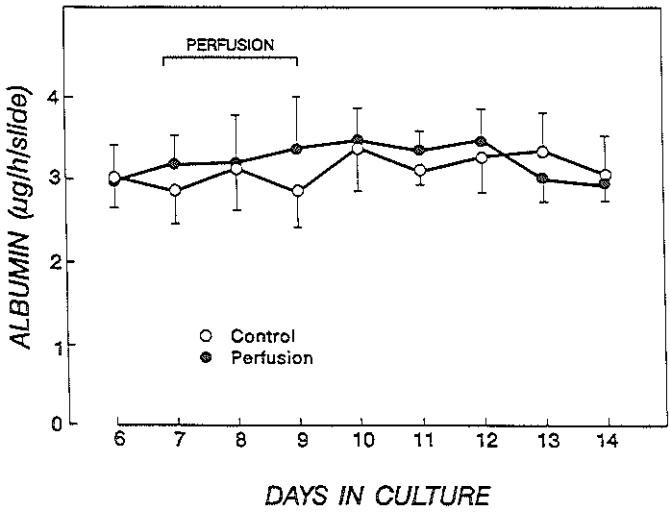


**Figure 4.** Albumin secretion rates before, during, and following perfusion of sandwiched hepatocytes with culture medium in the perfusion chamber. Values are expressed as mean±SD. Perfusion period is indicated.

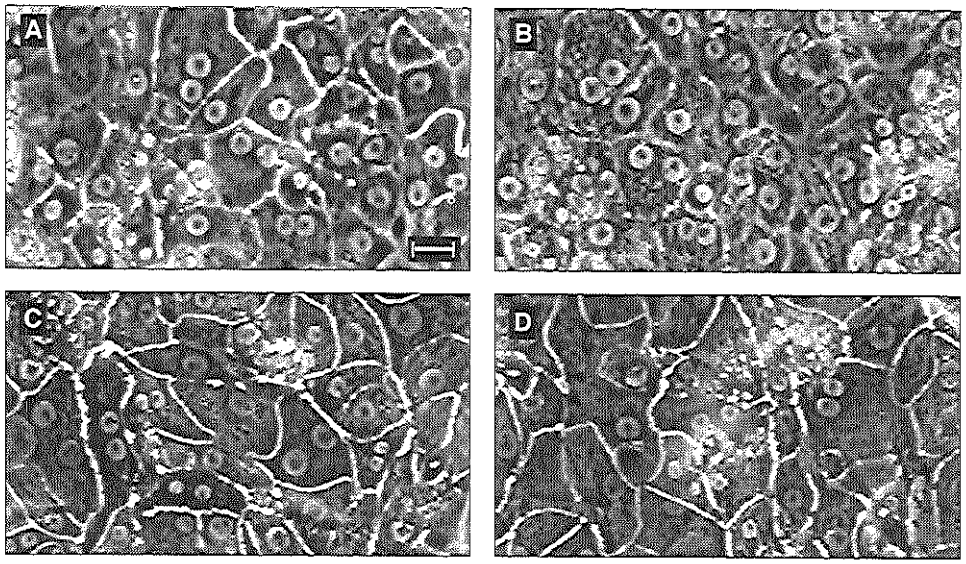


**Figure 5.** Morphological aspect of sandwiched hepatocytes before, during, and after 6h perfusion with plasma through the extracorporeal circuit. (A) Initial aspect on day 7 of culture (i.e., immediately before perfusion); (B) same field as in A, at the end of the perfusion; (C) different field of the same culture, 4 days after perfusion; (D) control culture at the same total culture time as (C). Scale bar represents 35 $\mu$m.

**Table 2.** Effects of 6 h of perfusion with plasma in the extracorporeal rat circulation system on cell shape indices of sandwiched hepatocytes

| Time (h) | 0 | 6 | Day +4 |
|---|---|---|---|
| P ($\mu$m) | 166 ± 24 | 162 ± 24 | 165 ± 22 |
| SA ($\mu m^2$) | 1529 ± 302 | 1531 ± 310 | 1493 ± 311 |

P: perimeter, SA: surface area, mean±SD, n=5. Perfusion was performed on day 7 of culture (time 0). Day+4 represents values at 4 days post-perfusion

In addition to these morphological observations, albumin secretion was documented before and after extracorporeal perfusion. In contrast to media studies, no albumin data could be obtained during plasma perfusion due to the presence of large quantities of albumin in the plasma. As can be seen from Fig. 6, the initial albumin secretion rate was comparable to that of control cultures. During the first post-perfusion day albumin secretion increased with ~40%, whereafter it returned to control levels. Stable albumin secretion was maintained for another 5 days until the end of the observation period. In order to ensure that this rise in albumin secretion rate was not due to rat plasma-albumin trapped in the collagen sandwich, cover glass cultures were washed 8 times with 4ml of media at 37°C for 10 min upon termination of the perfusion. Judging from the albumin content of the wash-media, this procedure allowed for adequate removal of the serum-albumin (Fig.6-*insert*). In short, attachment of sandwiched hepatocytes in our chamber to an extracorporeal circuit for 6 h resulted in minimal, reversible morphlogical changes, unaltered cell shape indices, and a temporary increase in albumin secretion during the first post-perfusion day. This suggests that, under the presented circumstances, the cells may be used more than once for extracorporeal perfusion purposes. These observations are in contrast to the findings of Arnaout et al. (1), who noted that microcarrier-attached hepatocytes, inoculated in the extracapillary space of a hollow fiber module, could only retain viability for up to 4h during connection to a similar extracorporeal circuit, beyond which time they rapidly died. This difference may be due to the fact that our system combines a stable culture configuration (5,6) with the required thermal, and perfusion characteristics.

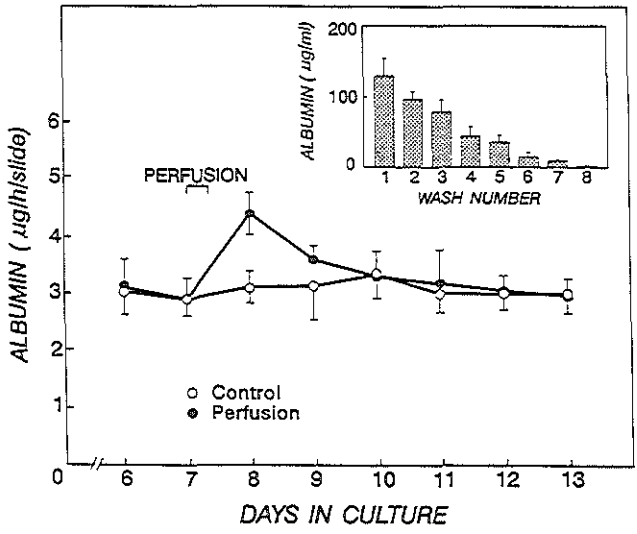


**Figure 6.** Albumin secretion rates of sandwiched hepatocytes before and after perfusion in the extracorporeal rat circulation system. Perfusion was performed on day 7 of culture (as indicated), and lasted for 6 h. Values are expressed as mean±SD. *Insert:* Albumin content of the wash-media from 8 consecutive washes (4ml of media, 10 min at 37°C, for each wash) of cultures immediately following perfusion. Note that albumin is given as wash-medium *content* instead of secretion rate.

This study offers the first description of a system with which the effects of extracorporeal circulation and perfusion on cultured hepatocytes can be observed continuously. It enables the monitoring of dynamic changes in cell morphology during extracorporeal perfusion, while it does not preclude functional follow-up. In addition, functional activity (e.g. cytochrome P-450 activity, mitochondrial respiration) and cell viability could be assessed 'on-line' by the use of vital staining and fluorescent labeling techniques during perfusion. Future studies could also include the effects of manipulations in the in vivo environment, e.g. liver resection or chemically induced hepatic failure, on the hepatocytes in the chamber. Apart from regeneration studies, the system could be used for investigations regarding reperfusion injury and cell repair mechanisms, dynamic observations on the role of secondary messengers, and detoxification under conditions that closely mimic the in vivo state. In addition, environmental effects on other cell types, including endothelial cells, may be investigated with this system. We believe that the system is a valuable asset in the studies of cellular responses to different environmental

conditions, and may prove particularly useful in the characterization of bioartificial organ support systems.

**Acknowledgement**

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# PART II

## CRYOPRESERVATION

## CHAPTER 4

# Effects of dimethyl sulfoxide on cultured rat hepatocytes in sandwich configuration

I.H.M. Borel Rinkes, M. Toner, R.M. Ezzell, R.G. Tompkins, M.L. Yarmush

Cryobiology 1992; 29:443-453

**Abstract**

A recently developed sandwich culture system, in which hepatocytes are sandwiched between two layers of collagen, has been shown to be capable of maintaining long-term expression of hepatocellular function (Dunn, J.C.Y., et al. *Biotechnol Prog* 1991;7:237-245). The development of an adequate technique for the cryopreservation of hepatocytes in such a stable culture configuration would ensure a ready supply of hepatocytes for use in bioreactors or bioartificial liver support devices. This report describes the effects of exposing hepatocytes in sandwich culture to different concentrations of the cryoprotectant dimethyl sulfoxide ($Me_2SO$) at 22°C on day 7 of culture. Cell function, morphology, and cytoskeletal organization were followed for 14 days after exposure. Hepatocellular morphology and albumin secretion remained normal when cultures were exposed for up to 120 min to predicted final $Me_2SO$ concentrations of up to 1.33 M. Exposure for less than 60 min to equilibrium concentrations of up to 3.33 M $Me_2SO$ did not adversely affect cell morphology or albumin secretion rate, but at the highest concentration (3.33 M), increase of the exposure time to 60 or 120 min resulted in dramatic, irreversible cell damage and loss of function. Actin filament organization was shown to be undisturbed when the cells were exposed to 1.33 M $Me_2SO$ for 60 min, but was irreversibly disrupted by exposure to 3.33 M for 120 min. Based on these results, a simple and safe procedure is suggested for the addition of $Me_2SO$ to hepatocytes in a sandwich culture configuration and its subsequent removal, which will be valuable for studies on hepatocyte cryopreservation.

## Introduction

Successful means for storage of liver cells would provide a valuable source of material needed for extracorporeal liver support devices and for hepatocellular transplantation. To date, however, most studies on hepatocyte cryopreservation have been associated with massive loss of cell function and viability (5,11,12,14,15,18,20,21). These reports are primarily based on studies on freshly isolated rat liver cells in free suspension. Apart from a reduction in hepatocellular function, including protein synthesis (5,11,12,14,20), cryopreservation also results in decreased attachment of the hepatocytes to culture plates following a freeze-thaw cycle (15,21). Only a few reports have appeared on the cryopreservation of cultured hepatocytes and they have not addressed the preservation of hepatospecific function (4,13). Recently, our group demonstrated significant long-term hepatocellular function following a freeze-thaw cycle for cultured cells in a collagen sandwich configuration (17). Although a significant level of function was preserved, overall viability was estimated at only 20% of control cultures. Clearly, a better understanding of the physicochemical phenomena involved would help define more optimal freeze-thaw conditions.

Studies on the cryopreservation of cultured hepatocytes have not addressed the deleterious effects of cryoprotective agents (CPA). Potentially, the CPA can be lethal because their addition and removal are associated with changes in the cell volume related to the permeabilty of cell membranes to penetrating CPA (19,26). Following the addition of the CPA there is a sharp initial decrease in cell volume caused by the flux of water from the cell to the hyperosmotic extracellular fluid. In the later stages of volume response, the continuous slow inflow of the CPA will result in the entry of water back into the cell in compensation for the osmotic imbalance. The opposite is encountered upon removal of CPA; an initial, rapid increase in cell volume is followed by a subsequent return to osmotic equilibrium. The magnitude of these osmotic changes for a given cell type correlates directly with the CPA concentration (24). These phenomena can lead to serious damage to cells on single exposure to high concentrations of penetrating CPA. Although excessive volumetric changes can be limited by adding and removing the CPA in a step-wise fashion, the prolonged exposure of cells to CPA under non-frozen conditions can result in toxic cell damage most likely due to protein denaturation (1). Hence, it is important to minimize the time during which the cells are exposed to high concentrations of CPA, while allowing enough time for complete equilibration.

This report represents an effort to study the effects of addition and removal of $Me_2SO$ (as a model penetrating CPA) on the function, morphology, and cytoskeletal organization of

hepatocytes in sandwich culture configuration in relation to time of exposure to $Me_2SO$, and concentration of $Me_2SO$.

## Materials and Methods

*Preparation and culture of hepatocytes.* Hepatocytes were isolated from 2 months old female Lewis rats (ca. 200 g, Charles River Laboratories, Boston, MA) using a modification of Seglen's procedure (25), as described in detail elsewhere (7,8). Approximately $2\text{-}3x10^8$ hepatocytes were harvested per isolation, with viabilities ranging from 90 to 98%, as determined by trypan blue exclusion. Type I collagen was prepared from rat tail tendons as described by Elsdale and Bard (9). At least 30 min prior to plating, tissue culture dishes (60 mm; Falcon™, Lincoln Park, NJ) were coated with 1 ml of collagen (1.11 mg/ml). Hepatocytes were suspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Gaithersburg, MD), supplemented with 7 ng/ml glucagon (Lilly, Indianapolis, IN), 7.5 $\mu$g/ml hydrocortisone (Upjohn, Kalamazoo, MI), 0.5 U/ml insulin (Squibb, Princeton, NJ), 20 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA), 200 U/ml penicillin, 200 $\mu$g/ml streptomycin, and 10% fetal bovine serum (Hazelton, Lenexa, KA). The cells were plated at a seeding density of $10^5$ /cm$^2$. Sandwich cultures were established by spreading a second layer of collagen over the cells after 24 h of culture. This was followed by incubation for 30 min at 37°C, to allow the collagen to gel. Then, new culture media was added, to be replaced with fresh media (4 ml/dish) daily thereafter. Culture supernatants were collected daily for protein analysis. The average thickness of each collagen layer is estimated to be ~500 $\mu$m.

*$Me_2SO$ experiments.* Hepatocyte cultures were kept under standard conditions until day 7. On day 7 the cultures were exposed to $Me_2SO$ (Fisher Scientific, Fair Lawn, NJ), using a one-step addition at 22°C. Specifically, culture media was aspirated, and replaced by 4 ml of media to which $Me_2SO$ had been added in various concentrations. The cultures were then left at room temperature (22°C) for varying periods of time. At the end of the exposure time the $Me_2SO$-containing media was removed at once, and the cultures were washed twice with normal media, allowing a pause of 10 min between washes. Thereafter, the cultures were treated as before the exposure. All procedures were performed under sterile conditions. Initial $Me_2SO$ concentrations added were 0.5 M, 1.0 M, 2.0 M, and 5.0 M, resulting in predicted final equilibrium concentrations of 0.33 M, 0.66 M, 1.33 M, and 3.33 M, respectively. Unless stated otherwise, concentrations referred to in the text are post-equilibration concentrations. Exposure times varied between 15 and 120 min. Control cultures were treated with media

alone, instead of media plus $Me_2SO$. All cultures were followed for 2 weeks after exposure (i.e., a total of 21 days of culture).

*Protein assays.* Collected media were analyzed for rat serum albumin content by enzyme-linked immunoassay (ELISA) as previously described (7,8). Rat albumin standard, and peroxidase-conjugated antibody to rat albumin were purchased from Cappel (Cochranville, PA). The absorbance was measured at 490 nm with a Dynatech MR600 plate reader (Chantilly, VA). A minimum of 3 experiments was carried out for each different condition, and the data from each experiment were obtained from at least 2 cultures per condition. Per sample, duplicate measurements were performed. Data are given as mean values ± standard deviation.

*Actin localization.* For analysis of the organization of the actin cytoskeleton, a modification of the Rhodamine-Phalloidin staining technique as previously reported (3,10) was used. Hepatocytes were cultured on No. 1 cover glasses (24x60 mm; Baxter Healthcare Corp., McGaw Park, IL). The cover glasses were prepared with an edge of silicone rubber (GE Silicones, Waterford, NY) to prevent the collagen sandwich from sliding off, and sterilized prior to use. Two cover glasses were placed in a 100 mm plastic tissue culture dish and covered with a layer of collagen. The procedures for cell seeding and the addition of the top layer of collagen were as described above. On the 3rd day of culture the cover glasses were cut from the collagen, and transferred to rectangular 4-well polystyrene culture dishes (Nunc, Inc., Naperville, IL). At different time points following the exposure to $Me_2SO$, the cultures were fixed for 20 min in 4% (w/v) paraformaldehyde in PBS at ambient temperature. After rinsing 3 times in PBS, the cells were permeabilized with 0.1% (v/v) Triton X-100 (Sigma Chemical Company, St Louis, MO) for 5 min at ambient temperature, and subsequently washed in PBS for 10 min on a rotating platform. The specimens were then covered with Rhodamine-Phalloidin (3.3 $\mu$M in methanol, Molecular Probes, Inc., Eugene, OR) diluted 1:20 with PBS, and incubated for 1 h at 37°C. Coverslip cultures were washed 3 times (5 min each) with PBS, placed face up on a 1x3 inch glass slide, and covered with a second cover glass, using N-propylgalate mounting medium to reduce photobleaching. Slides were examined using a MRC 600 scanning laser confocal imaging system attached to a Zeiss axiovert microscope. Image-1 software (Universal Imaging Corp., West Chester, PA) was used to obtain three-dimensional (3-D) intensity profiles of F-actin staining.

## Results

*Diffusion of $Me_2SO$.* In order to determine the diffusion of $Me_2SO$ in the sandwich culture system, the disappearance of 5 M $Me_2SO$ in the supernatant medium was measured using an osmometer (Model 3L, Advanced Instruments, Inc., Needham Heights, MA). The addition of an initial concentration of 5 M $Me_2SO$ (4 ml) to the sandwich configuration (2 ml) resulted in a rapid disappearance of $Me_2SO$ from the medium reaching a final normalized equilibrium concentration ($C/C_o$) of 0.68 without cells and 0.65 in the presence of cells (Fig. 1). The decay time constants for the sandwich configuration, both with and without cells, were 3.56 and 4.36 min, respectively. These decay times were obtained from an exponential curve-fit to data points of the form

$$\langle \bar{C}_m \rangle = \bar{C}_f + (1-\bar{C}_f) \exp[-t/\tau] \qquad [1]$$

where $\langle \bar{C}_m \rangle$ is the average $Me_2SO$ concentration in the supernatant medium normalized to the initial $Me_2SO$ concentration ($\langle C_m \rangle / C_o$), $\bar{C}_f$ is the normalized final equilibrium concentration ($C_f/C_o$), and $\tau$ is the time constant (curves not shown). These results strongly suggest that the presence of hepatocytes, sandwiched in between the two collagen layers at a confluency of approximately 50%, does not significantly alter the diffusional behavior of $Me_2SO$. In addition to the experimental measurements, a simple one-dimensional diffusion model was used to predict both the disappearance of $Me_2SO$ from the medium and the $Me_2SO$ concentration at the cell level in the collagen sandwich as a function of time. By assuming that $Me_2SO$ diffusivities in the medium and the collagen gel are equal, the time-dependent one-dimensional diffusion equation subject to the initial and boundary conditions depicted in Fig. 1 (see insert) can be solved as follows (6):

$$\bar{C}(x,t) = \frac{C}{C_o} = \left\{ \frac{h}{l} + \frac{2}{\pi} \sum_{n=1}^{\infty} \frac{1}{n} \sin \frac{n\pi h}{l} \exp[-\pi^2 n^2 D t / l^2] \cos \frac{n\pi x}{l} \right\} \qquad [2]$$

where h and l are distances as defined in Fig. 1, D is the diffusion coefficient of $Me_2SO$ in water, and Co is the initial $Me_2SO$ concentration. As shown in Fig. 1, the average normalized $Me_2SO$ concentration in the supernatant medium can be estimated from

$$\langle \bar{C}_m \rangle = \frac{1}{h} \int_0^h \bar{C}(x,t)\, dx \qquad [3]$$

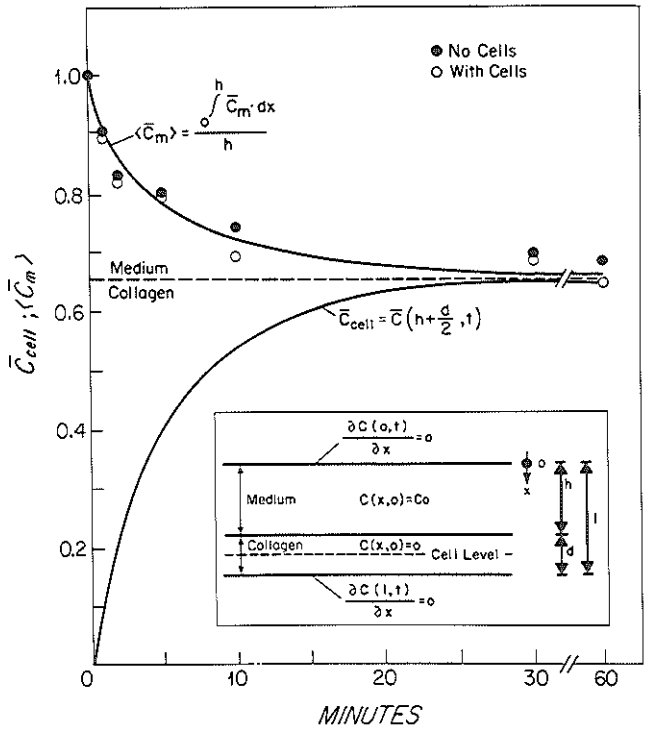


**Figure 1.** Diffusion kinetics of 5.0 M $Me_2SO$ in the sandwich culture system. Normalized measured concentrations in the supernatant medium with (open circles) and without (filled circles) cells in the system. The curves represent predicted $Me_2SO$ concentrations based on the diffusion model described. $\overline{C}_{cell}$ is normalized concentration at cell level, $\langle \overline{C}_m \rangle$ is normalized average concentration in the supernatant medium. Initial and boundary conditions for the diffusion equation are given in the insert, together with a definition of the distances used.

The theoretical curve is in reasonable agreement with the experiments given a value for D of $1x10^{-5}$ $cm^2/s$ as estimated from Baker and Jonas (2). The model can also be used to predict the $Me_2SO$ concentration at the cell level, $\overline{C}_{cell}$, by evaluating Eq. [2] at x = h+(d/2). As can be seen from Fig. 1, the normalized $Me_2SO$ concentration at cell level increased gradually from 0 (i.e., 0 M) at t=0 to a final value of 0.67 (i.e., 3.33 M) with an approximate time constant (i.e., 63% equilibration) of 5 min at 22°C. In order to ensure that cells were completely equilibrated with $Me_2SO$, all experiments involved at least 15 min exposures to $Me_2SO$.

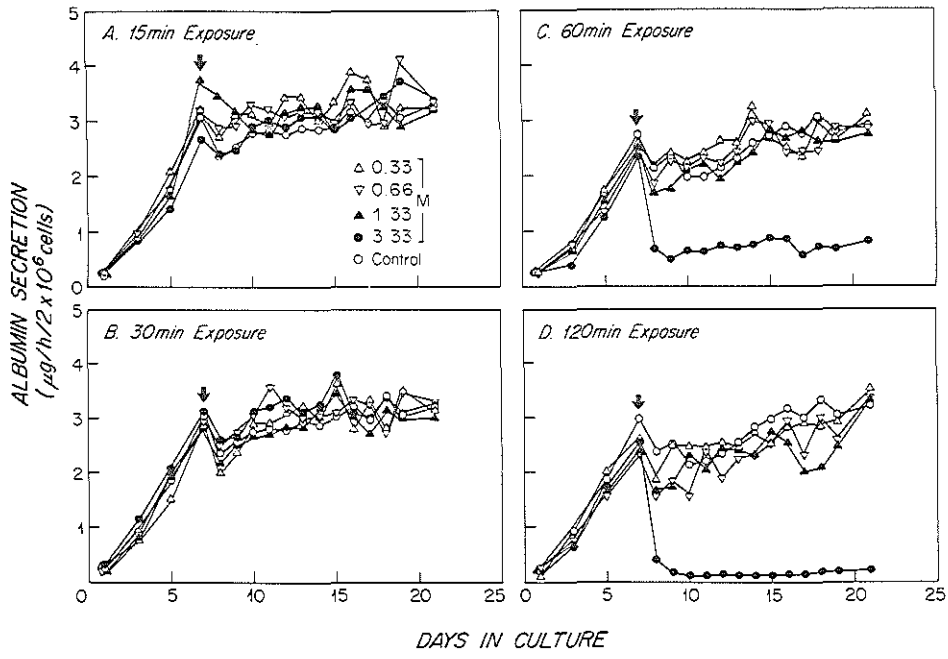


**Figure 2.** Effect of one-step addition of $Me_2SO$ on the albumin secretion rate of adult rat hepatocytes, cultured in between two layers of collagen. The exposure to $Me_2SO$ took place at 22°C on day 7 of culture (arrow). $Me_2SO$ concentrations varied from 0.33 M to 3.33 M. These concentrations refer to predicted final equilibrium concentrations. Respective control cultures were treated identically, but without the presence of $Me_2SO$. The exposure times used were 15 min (A), 30 min (B), 60 min (C), and 120 min (D). Data from a typical experiment (with cells from one isolation) are given. Data points represent duplicate measurements of 3 culture dishes per experimental condition (mean values). For reasons of clarity, error bars are omitted. In all cases, standard deviations did not exceed 0.5 $\mu$g/h/2 x $10^6$ cells.

*$Me_2SO$ effect on albumin secretion.* The effect of a one-step addition and removal of $Me_2SO$ to hepatocytes in sandwich culture configuration is shown in Fig. 2. The results depicted are representative of one experiment; similar patterns were consistently found in at least 2 other experiments. Exposure of the cells after 7 days of culture to the various concentrations of $Me_2SO$ for 15 or 30 min did not adversely affect their albumin secretion rate in the following 2-week period (Figs. 2A and B). When the exposure time was increased to 60 min, exposure to 3.33 M $Me_2SO$ (final equilibrium concentration) resulted in an approximate 70% reduction of the albumin secretion rate, while final concentrations of 1.33 M, 0.66 M, and 0.33 M had no effect (Fig. 2C). Sandwiched hepatocytes responded to one-step addition of 5.0 M $Me_2SO$

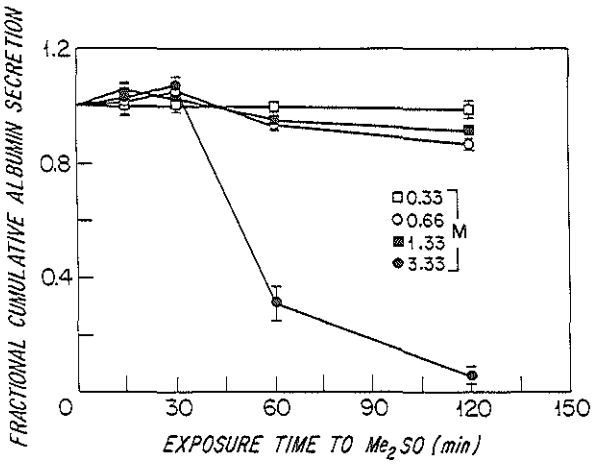


**Figure 3.** The fractional cumulative albumin secretion of hepatocytes in sandwich culture, expressed as a function of the exposure time to the depicted final equilibrium concentrations of $Me_2SO$. The fractional cumulative albumin secretion is defined as the albumin secretion over the 14 days following the exposure to $Me_2SO$, relative to the value of the respective control experiment (no $Me_2SO$ exposure). Each data point represents the results of all experiments performed (mean±SD, n=3-5).

(equilibrium concentration 3.33 M) followed by an exposure of 120 min with an immediate, and irreversible loss of albumin secretion (Fig. 2D). The albumin secretion rates of cells that were exposed for two hours to 1.33 M, 0.66 M, or 0.33 M $Me_2SO$ (initial concentrations added were 2.0, 1.0, and 0.5 M, respectively) decreased temporarily, but recovered within the next 4 days and remained at values comparable to those of the control cultures (Fig. 2D).

For comparative purposes, cumulative albumin secretion of all experimental cultures during the two weeks following exposure to $Me_2SO$ can be expressed as a fraction of the values obtained from the control cultures. To obtain these values, the areas underneath each albumin secretion rate graph between days 7 and 21 of culture (normalized to the respective control values) were determined, and plotted as a function of the exposure time. As shown in Fig. 3 the cumulative albumin secretion of hepatocytes that were exposed to 3.33 M $Me_2SO$ (final equilibrium concentration) for 60 minutes or more, decreased dramatically. However, when final $Me_2SO$ concentrations of 1.33 M or lower were used, albumin secretion remained close to normal, irrespective of the exposure time (Fig. 3) and consistent with the morphological changes described below.

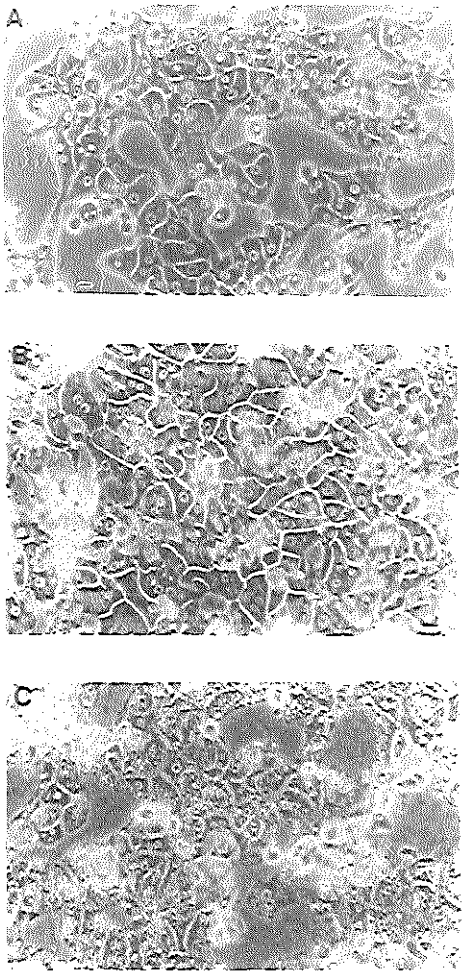


**Figure 4.** Typical morphological appearance of hepatocytes in sandwich culture on day 21 of culture, i.e. 14 days after one-step exposure to $Me_2SO$ at 22°C. (A) control hepatocytes, (B) hepatocytes that were exposed to 1.33 M $Me_2SO$ for 60 min, (C) hepatocytes that were exposed to 3.33 M $Me_2SO$ for 120 min (phase contrast microscopy, scale bar represents 20 $\mu$m). $Me_2SO$ concentrations refer to final predicted concentrations after equilibration.

*$Me_2SO$ effect on cell morphology and cytoskeletal structures.* Standard light microscopy was used to qualitatively evaluate the morphological characteristics of cells exposed to $Me_2SO$. Pictures taken on the 21st day of culture (2 weeks after exposure) are shown in Fig. 4. One-step exposure of the cells to 0.33 M, 0.66 M, or 1.33 M $Me_2SO$ for up to 120 min did not lead to apparent alterations in cell morphology, when compared to control cultures (Figs. 4A and B). In contrast, those cells that had been exposed to 3.33 M $Me_2SO$ for 60 min or more showed marked signs of degeneration with increased cytoplasmic granularity and loss of membrane integrity (Fig. 4C).

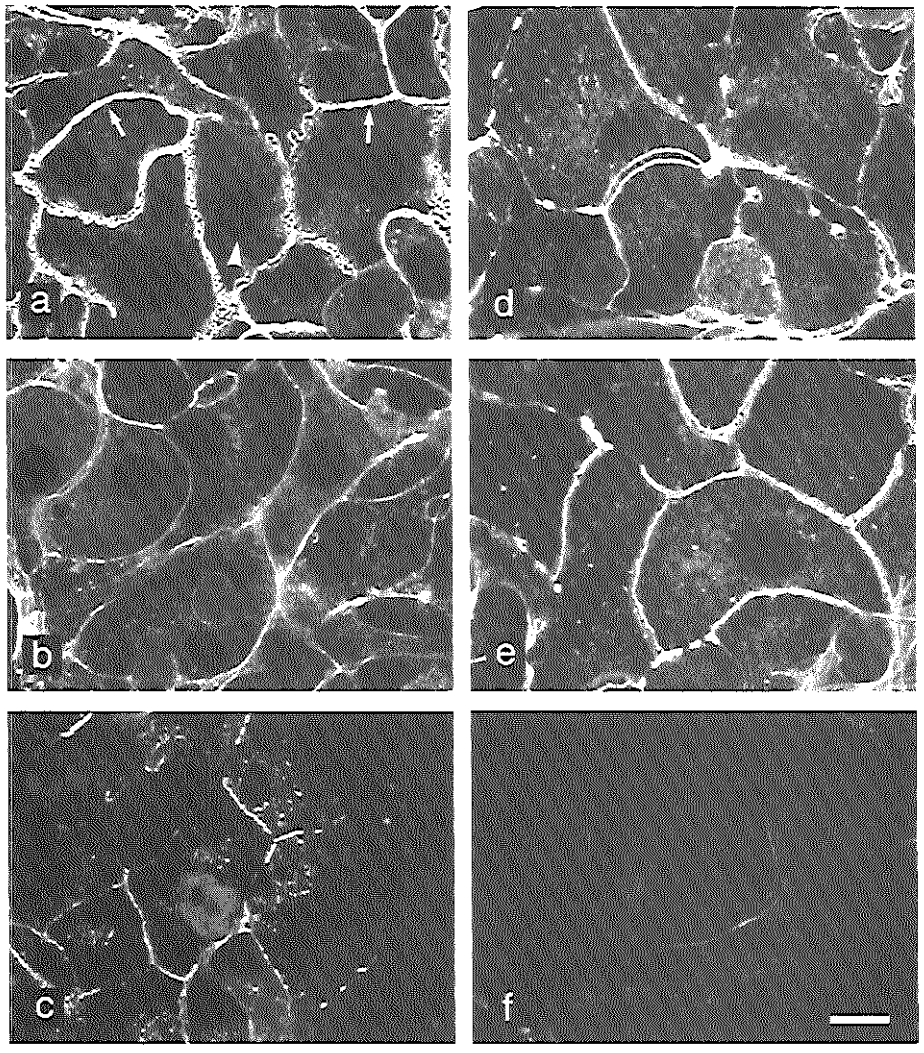


**Figure 5.** Micrographs taken on a confocal microscope showing the distribution of filamentous (F-) actin by the binding of rhodamine-phalloidin. A-C: micrographs obtained on day 7 of culture, i.e., directly following $Me_2SO$ exposure. D-F: taken on day 14 of culture, 1 week post-exposure to $Me_2SO$. Panel A and D are control hepatocytes in sandwich collagen culture configuration. Arrows indicate F-actin, arrowhead points to nucleus. Panel B and E are sandwiched hepatocytes after exposure to 1.33 M $Me_2SO$ (final concentration) for 60 min on day 7 of culture. Panel C and F are cultures exposed to 3.33 M $Me_2SO$ for 120 min. Scale bar represents 20 $\mu$m. Results from one representative experiment are depicted. Similar results were obtained in different hepatocyte populations.

To examine the effect of $Me_2SO$ on the organization of the actin cytoskeleton, the distribution of filamentous (F-) actin was localized by staining with rhodamine-phalloidin. After 7 days in sandwich culture configuration, F-actin was shown to be predominantly associated with the plasma membrane (Fig. 5A), a pattern also seen in normal liver (8). Exposure to $Me_2SO$ concentrations of up to 1.33 M for 60 min did not cause any change in the pattern of actin distribution seen in control cultures (Fig. 5B). However, upon exposure to 3.33 M $Me_2SO$ for 120 min, gross changes in the cytoskeletal organization could be seen, most specifically involving the disruption of the association of actin filaments with the plasma membrane (Fig. 5C). Seven days after the exposure to $Me_2SO$ the observed changes in F-actin were even more pronounced. Normal patterns of F-actin distribution were present in both the control cultures and those exposed to 1.33 M $Me_2SO$ for 60 min (Figs. 5D and E). In contrast, exposure to 3.33 M $Me_2SO$ for 120 min had led to complete destruction of the actin filament organization, with virtually no actin visible in the typical close association with the plasma membrane (Fig. 5F). Quantitative comparison of 3-D intensity profiles from the fields shown in Fig. 5 revealed equal staining intensity in panels A, B, D, and E, a loss of intensity in panel C, and virtually no staining discernable in panel F (data not shown).

## Discussion

These studies are the first to describe an analysis of the morphological, cytoskeletal, and functional consequences of $Me_2SO$ exposure on rat hepatocytes in a sandwich culture configuration. The results indicate that the one-step addition and removal of $Me_2SO$ at 22°C in initial concentrations of up to 5.0 M (equilibrium concentration 3.33 M) to hepatocytes in sandwich culture disturbs neither cell morphology nor function (as measured by albumin secretion), provided that the exposure time is less than 60 min. When longer exposure times are used, 3.33 M $Me_2SO$ causes irreversible damage, whereas concentrations of 1.33 M and lower do not. The organization of actin filaments was also shown to be unaffected by exposure to 1.33 M for 60 min, but was irreversibly damaged by exposure to 3.33 M $Me_2SO$ for 120 min.

Prior studies that have investigated the effects of addition and removal of $Me_2SO$ to hepatocytes have been performed primarily on freshly isolated hepatocytes in suspension. De Loecker et al. (20) reported a rapid decline in cell viability after the addition of 1.8 M $Me_2SO$ to fresh hepatocytes as judged by trypan blue exclusion. Similarly, Fuller et al. (11) showed that the recovery of hepatocellular protein synthesis was greatly reduced after brief exposure to $Me_2SO$ concentrations of more than 1.0 M. These authors have also emphasized the

importance of evaluating protein synthesis in the assessment of cell viability. In conventional monolayer cultures of primary hepatocytes, very low concentrations (2%) of $Me_2SO$ have been demonstrated to enhance hepatocyte survival and differentiated function (16,22,29), possibly caused by an increase in intracellular free ionized calcium (29). However, higher $Me_2SO$ concentrations, as would be necessary in cryopreservation, are damaging to such hepatocyte cultures. This was illustrated by Beecherl et al. (4), who reported a more than 50% loss of dye excluding cells after 5 min exposure of short term hepatocyte cultures to more than 1.0 M $Me_2SO$. With regard to protein synthesis, our group has previously found comparable damage to both freshly isolated hepatocytes and to cells in short term hepatocyte cultures after brief (15 min) exposure to $Me_2SO$ concentrations between 1 and 2 M (17). In contrast, the results presented in this study show that hepatocytes cultured in sandwich configuration can safely be exposed to 1.33 M of $Me_2SO$ for up to 2 hours, and 3.33 M for up to 30 min, without any loss of long-term protein synthesis capability.

The beneficial effect of the sandwich culture configuration on the survival and function of hepatocytes following $Me_2SO$ exposure may be explained by a combination of two mechanisms. First, the presence of the top layer of collagen may result in a more gradual exposure of the cells to the solute as opposed to the abrupt, total exposure that is seen in the case of a free cell suspension. This slower exposure to $Me_2SO$ may minimize cellular damage resulting from excessive osmotic stresses (19,26). Second, hepatocytes in collagen sandwich may be less vulnerable to the toxic effects of $Me_2SO$ due to their greater stability and/or decreased sensitivity in this physiological, polarized configuration. The latter reasoning may explain the retention of viability even at very long exposure times (60-120 min). Our previous observations (i) that it takes about a week for hepatocytes to polarize themselves in sandwich culture (8), and (ii) that hepatocytes completely lost protein synthesis capacity when frozen before one week of culture in sandwich configuration (17), support this hypothesis.

The mechanism of toxicity of high concentrations of $Me_2SO$ during prolonged exposure at above zero temperatures is not fully understood. In a recent hypothesis, Arakawa et al. (1) propose that hydrophobic interactions of the solvent with proteins may play a key role by causing protein denaturation. Sites of damage may include the plasma membrane and the cytoskeleton, in the form of the organization of filamentous actin. Our results show that the association of F-actin with the plasma membrane was completely disrupted in hepatocytes exposed to 3.33 M $Me_2SO$ for 2 hours, whereas concentrations of up to 1.33 M did not affect the F-actin organization. In addition, our results confirm that the actin distribution pattern correlates well with hepatocellular function, as has previously been found for normal, unperturbed hepatocytes in sandwich configuration (8). Interestingly, Yamamoto (29) found

that very low concentrations of $Me_2SO$ caused a similar rearrangement of the F-actin organization in conventional short-term monolayer hepatocyte cultures.

The observed alteration and subsequent disruption of the F-actin organization in stabilized, sandwiched hepatocytes by high concentrations of $Me_2SO$ are also consistent with observations in other cell types. Although less dramatic than 1,2 propanediol, $Me_2SO$ has been demonstrated to have a depolymerizing effect on *in vitro* actin microfilaments (27). In fibroblasts, $Me_2SO$ caused actin stress fibers to disappear from the cytoplasm while actin bundles formed in the nucleus (23). Reorganization of the actin network associated with the plasma membrane has also been described in $Me_2SO$ treated *Dictyostelium* (30). Vincent et al. (28) recently showed $Me_2SO$ to cause dramatic, partly reversible changes in the actin distribution in mouse oocytes in a dose-, and temperature-dependent fashion. These changes included disruption of actin organization and development of microvilli. The mechanism by which $Me_2SO$ disrupts the cellular actin organization may include dissociation of the subcortical microfilaments with secondary damage to the plasma membrane (1,28,30). Alternatively, the osmotic effects of the addition and removal of the cryoprotectant initially may have damaged the plasma membrane and disrupted the actin cytoskeleton. More work is needed to further clarify the exact mechanisms underlying the described phenomena.

In conclusion, this study suggests that one-step addition at 22°C of 2.0 M $Me_2SO$ (initial concentration in medium) to hepatocytes in sandwich culture configuration, followed by 60 to 120 min exposure before the actual freezing is commenced, will neither adversely affect the protein secretion rate of the cells, nor disturb their morphology and cytoskeletal organization, while allowing for equilibration of the cryoprotectant. We are currently extending these studies to involve actual cryopreservation of sandwiched hepatocytes.

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## CHAPTER 5

# A controlled rate freezing device for cryopreservation of biological tissue

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Cryo-Lett 1992 (in press)

**Abstract**

A controlled rate freezing device was designed and characterized for the cryopreservation of biological tissue. Thermal control of the specimen was achieved by balancing the convective cooling power of a cold gaseous nitrogen stream with electrical power dissipation on the heater layer. The device was designed to: (i) control the cooling and warming rates over several orders-of-magnitude up to 100°C/min; (ii) achieve a uniform temperature on the surface of the heater layer between 0° and -80°C; (iii) contain sufficient surface area (4.7 x 7.5 $cm^2$) for tissue specimens or cultured cells; and (iiii) have multi-step protocol ability including constant temperature holding periods. A specific example involving the cryopreservation of cultured hepatocytes is illustrated, and demonstrates that the controlled rate freezing device could be used for any tissue type provided it fits on the heater layer surface area. For larger size tissues, cooling channels and heater layers with appropriate sizes could easily be built.

## Introduction

Low temperature preservation of biological materials has been the subject of intense scientific research in the fields of medicine, agriculture, nutrition, and biology (5). The ability to store biomaterials in the frozen state would offer several advantages including establishing a large supply of tissues for transplantation, thus allowing enough time for tissue matching and required tasks. The biomaterials of interest include individual cells such as erythrocytes and bone marrow cells, tissues such as skin and cornea, and tissue-derived structures such as cultured hepatocytes and pancreatic islets. Of the many factors that have been documented to affect the survival of biomaterials exposed to a freeze-thaw cycle, the cooling and warming rates have been shown to be major critical parameters for survival (5). Although several commercially available systems for controlled freezing of cells and tissues exist, most of these systems are expensive and are primarily designed for a specific application with a narrow range of controllable rates.

For the present study we designed and characterized a simple controlled rate freezing device for the purpose of reversible cryopreservation of biomaterials. The new design was based on the following requirements: (i) controlled cooling and warming rates over several orders-of-magnitude up to 100°C/min for temperatures as low as -80°C; (ii) minimal lateral thermal gradients in order to expose all components of the tissue specimen to an identical thermal history; (iii) simple programming which would allow multi-step freezing protocols with several holding periods; and (iiii) easy access to the specimen for seeding of external ice and for removal of the specimen upon completion of a freeze-thaw protocol.

## Design of the controlled rate freezing device

A schematic overview of the controlled rate freezing device is shown in Fig. 1. The system elements responsible for the temperature control function include the cooling source, the heater layer, and the temperature controller. Briefly, gaseous nitrogen ($N_2$), chilled by passage through a copper coil immersed in liquid $N_2$, flows through a channel where it comes in contact with the heater layer on which the specimen is located. Thermal control of the specimen is achieved by balancing the convective cooling power of the gaseous nitrogen stream with the electrical power dissipation on the heater layer in a manner similar to our previous cryomicroscope system design (4). The sample temperature is measured by a thermocouple located on the heater layer immediately beneath the specimen. The temperature controller (Interface Techniques Company, Cambridge, MA) provides the heater layer with the power needed to counterbalance the refrigerant and thus maintain the desired sample temperature. The desired freeze-thaw protocol is

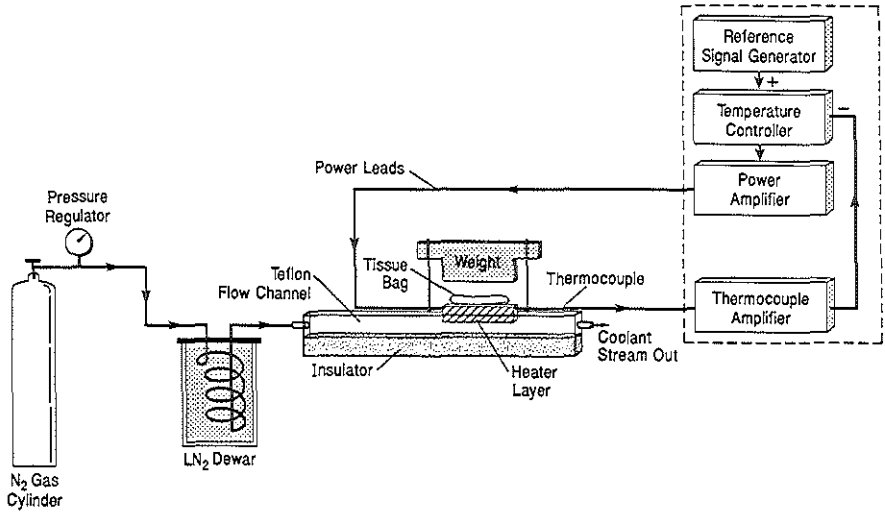


**Figure 1.** Schematic overview of the controlled rate freezer unit.

entered into an IBM™ compatible computer. From this input, the program executes the protocol by generating the appropriate set point (4). The set point is converted to an analog voltage and sent to the temperature controller. Depending on the offset between the setpoint and the actual temperature, the temperature controller adjusts the power to the heating layer to precisely follow the desired protocol. To ensure maximal thermal contact between the heater layer and the specimen -which must be placed in a plastic bag-, a simple mechanism was designed to uniformly press a weight onto the bag. The overall performance of the freezer device is determined by the thermal properties of the flow channel and the heater layer.

**Flow Channel Design.** Fig. 2 shows the details of the mechanical design of the refrigerant flow channel. The flow channel was made from the polymer polypenco acetal (Delrin, DuPont, Wilmington, DE). At the location of the heater layer, the channel was rectangular with a height of 1.4 cm and a width of 7.0 cm. In order to prevent edge effects and boundary layer separation, the channel was designed such that it gradually widened with an opening angle of 10° and an entrance length of 22.5 cm. The channel was machined into the bottom of the polypenco acetal, and then a top layer of 0.5 cm thick was glued onto it using silicone RTV (GE Silicones,

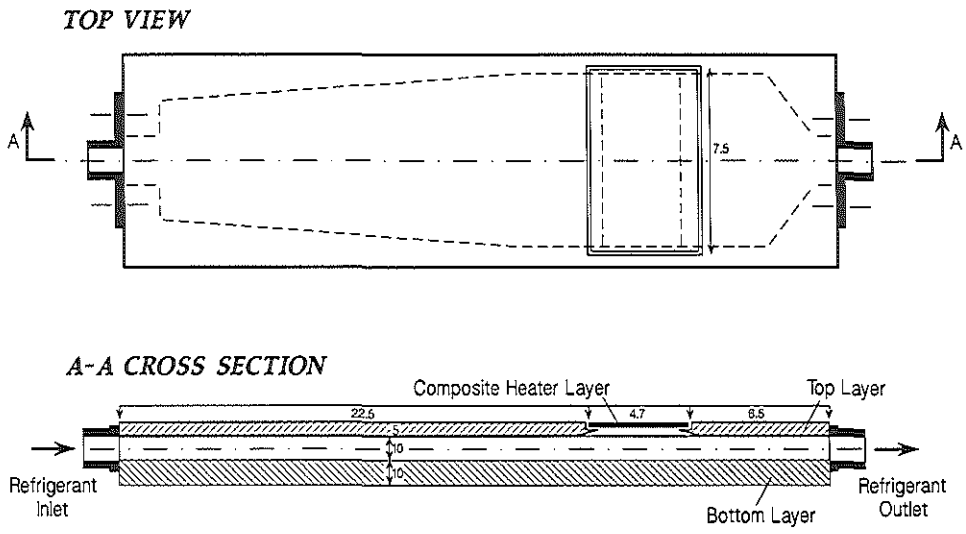


**Figure 2.** Technical drawing of the stream flow channel and the heater layer. Vertical dimensions on cross section are given in mm, all other distances in cm.

Waterford, NY). The top layer of the channel had an opening of 7.5 x 4.7 cm for placement of the heater layer described in detail below (see Fig. 2). The channel was insulated from the environment to prevent heat losses using 2 cm thick cork as a bottom layer and 1 cm thick styrofoam to minimize the heat losses from the side and top surfaces. Typical flow conditions used were between 1,000 and 10,000 $cm^3$/min with stream temperatures as low as ~ -150°C as measured by a flowmeter (Gilmont, Inc.) at the outlet stream of the channel. The Reynolds number (Re) at the location of the heater layer under these flow conditions can be estimated from

$$Re = \frac{\rho V L}{\mu} \qquad [1]$$

where $\rho$ ( = 1.71 kg/$m^3$ at 200K) is the density of cold nitrogen stream; V is the linear flow velocity (m/s); L is the characteristic length (m); and $\mu$ ( = $1.29 \times 10^{-5}$ Pa-s at 200K) is the kinematic viscosity of the coolant gas. The characteristic length can be estimated from L = 4A/P where A is the surface area of the channel and P is the perimeter of the channel at the location of the heater layer. Re can be shown to be less than 500 for flow rates of less than 10,000 $cm^3$/min, which are typical of those used in this study. Therefore, the coolant stream in the channel can, in

general, be considered to be laminar. We have also used helium as the coolant source to achieve rapid cooling rates between 500 and 1,000 °C/min. Under these conditions, Re was higher than 2,000, and the flow was turbulent.

**Heater Layer.** The heater was composed of 3 layers as shown in Fig. 3: 1) the bottom aluminum plate; 2) the kapton insulated copper electrical heater layer; and 3) the top aluminum plate. These 3 layers were held together with silicon RTV. The average thicknesses of ~ 300 μm for RTV layers were estimated from the total thickness of the entire heater layer as measured by a micrometer. The size of the heater layer was 7.5 x 4.7 cm. Fig. 3 shows the enlarged cross-section and the dimensions of the heater. A copper-constantan foil thermocouple (CO2-T, Omega, Stamford, CT) was attached to the top aluminum layer for the feedback to the temperature controller. The 100 μm thick kapton heater layer had a resistance of 230 Ω (HK-TBA, Minco Products, Inc., Minneapolis, MN). Power leads from the power amplifier in the temperature controller were connected to the heater layer.

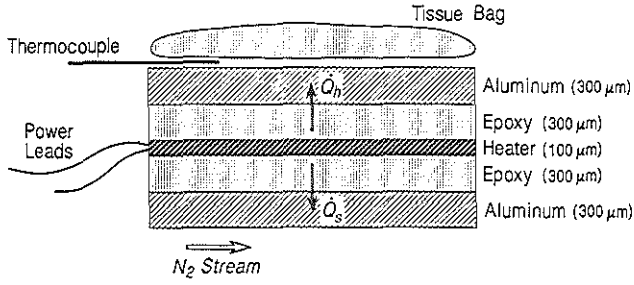


**Figure 3.** Construction of the heater layer with its corresponding approximate thicknesses. $\dot{Q}_h$ is the heater power and $\dot{Q}_s$ is the coolant power. Not shown is the weight which was routinely placed on top of the specimen-containing tissue bag to achieve maximal thermal contact between the heater layer and the specimen.

## Thermal Behavior

The ultimate performance of the system is determined by the thermal characteristics of the heater layer. Thermal performance of the heater layer was characterized by evaluating the steady-state power requirements of the heater, maximum cooling rates attainable, and temperature gradients on the heater surface.

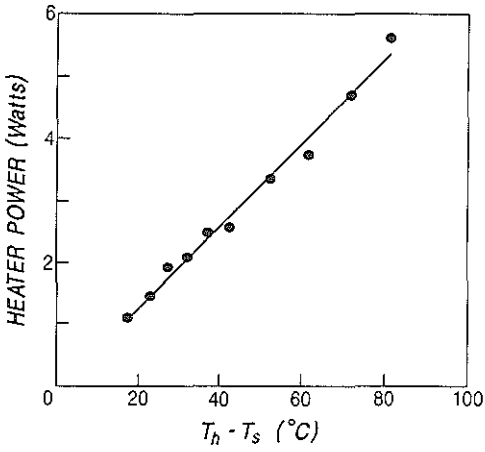


**Figure 4.** Heater power as a function of the temperature difference between the heater and stream temperatures. Measurements were carried out under steady-state conditions. The flow rate and the temperature of the stream were 4000 $cm^3$/min and -62°C, respectively. The equation for the least-squares line fit to experimental data was y = 0.00942 + 0.0655 x ($r^2$ = 0.993).

**Power Requirements.** The power requirements for the heater layer were measured under steady-state operating conditions for a range of heater temperatures between 20° and -80°C. Under steady-state conditions, the power required to keep the temperature of the heater layer at a desired level is equal to the convective cooling power of the refrigerant stream:

$$\dot{Q}_h = \dot{Q}_s = hA\,(T_h - T_s) \qquad [2]$$

where $\dot{Q}_h$ is the heater power; $\dot{Q}_s$ is the convective cooling power; h is the convective heat transfer coefficient; A is the surface area of the heater; $T_h$ and $T_s$ are the steady-state heater and stream temperatures, respectively. The heater power required to keep the temperature of the heater layer at a desired value was determined through direct measurement of the voltage across the heater layer. Heater power was estimated from $Q_h = v_h^2 / R_h$; where $v_h$ is the measured voltage and $R_h$ is the resistance of the heater layer (230 Ω). Results are shown in Fig. 4 for a typical stream flow rate of 4000 $cm^3$/min and a stream temperature of -62°C. As expected from Eq. [2], there is a linear relationship between the power requirements for the heater layer and the difference between the heater and stream temperatures. The convective heat transfer coefficient was determined to be 18.6 x $10^{-4}$W/$cm^2$-K directly from the slope of the figure using least-squares fit to the experimental data and assuming an effective area of 35.25 $cm^2$ (4.7 x 7.5 cm) for the given flow conditions (i.e., 4000 $cm^3$/min and -62°C). The current system has a capacity of up to 25 Watts and allows using lower stream temperatures and faster stream flow rates.

**Maximum Controlled Cooling Rate Capacity.** One of the most important thermal parameters of the freezing device is its ability to control the change of temperature. This could easily be measured by determining the slew rate capability of the heater layer. The slew rate is defined as the maximum cooling rate that can be obtained when the heater power is turned off (3). Assuming first order response, the rate of change of the heater layer temperature depends on the convective cooling capacity when the electrical power dissipation is zero. The governing equation for a bulk system can be written as follows:

$$M_T \frac{dT_h}{dt} = - hA (T_h - T_s) \qquad [3]$$

where $M_T$ is the total thermal mass of the heater layer. Table 1 summarizes the estimation of the total thermal mass of the heater layer.

**Table 1.** Estimation of composite heater layer thermal mass. The thicknesses of each individual layer of the heater are given in Fig. 3. The volume of the individual layers was calculated from the heater surface area (4.7x7.5 $cm^2$) and the corresponding thickness. The thermal mass was then estimated from ($\rho CV$).

| Layer | V $10^{+6}$ ($m^3$) | $\rho$ ($kg/m^3$) | C (J/kgK) | $M_T$ (J/K) |
|---|---|---|---|---|
| Aluminum | 1.1 | 2773 | 1004 | 3.06 |
| Epoxy | 1.1 | 1410 | 1255 | 1.94 |
| Kapton | 0.35 | 4000 | 1297 | 1.81 |
| Epoxy | 1.1 | 1410 | 1255 | 1.94 |
| Aluminum | 1.1 | 2773 | 1004 | 3.06 |
| TOTAL | | | | 11.8 |

Eq. [3] is subject to the initial condition of uniform temperature, $T_h^{init}$, prior to turning off the electrical power. The solution of the heater layer temperature from Eq. [3] is:

$$\theta = \frac{T_h - T_s}{T_h^{init} - T_s} = e^{-t/\tau} \qquad [4]$$

where $\theta$ is the normalized temperature (i.e., fraction of heat remaining in the heater layer), $\tau$ is the time constant for the heater layer and given by the ratio of the thermal mass, $M_T$, to the heat

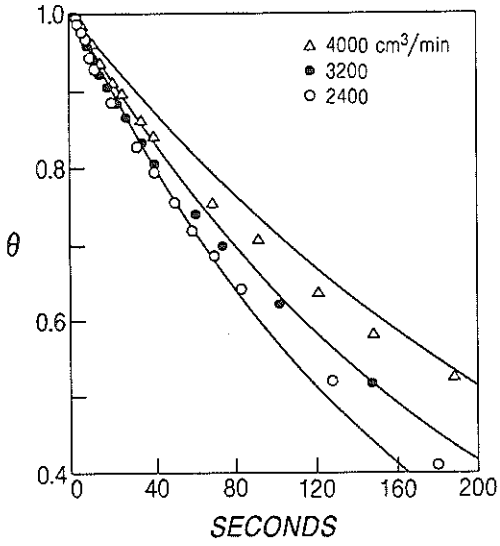


**Figure 5.** Experimental, and predicted (solid lines) temperature history of the heater layer after the heater power was turned off, as a function of three different flow conditions. The measured stream temperatures were -62°, -52°C, and -41°C for flow rates of 4000, 3200, and 2400 $cm^3$/min, respectively. θ is normalized heater layer temperature

transfer coefficient area product, hA. The time constant for the heater layer can then be estimated to be ~ 179 sec using the measured convective heat transfer coefficient of 18.6 x $10^{-4}$W/$cm^2$-K at a stream flow rate of 4000 $cm^3$/min. Assuming that the effect of changing the stream flow rate affects only the Re, the convective heat transfer coefficient at different stream flow rates can be estimated from $Nu/Nu_o \sim (Re/Re_o)^{1/3}$, where Nu (= $hL/k_s$) is the Nusselt number, L is the characteristic length, $k_s$ is the thermal conductivity of the stream, and the subscript "o" refers to the measured Nu and Re numbers at 4000 $cm^3$/min (2). Since the time constant of the heater layer is inversely proportional to the convective heat transfer coefficient, the heater layer time constant could be approximated from the stream flow rate. Fig. 5 shows both experimental and predicted (Eq. [4]) temperatures as a function of time after the heater power is turned off for 3 different stream flow rates. The initial temperature for all of these experiments was 0°C. The agreement between the predicted values from the simple first order lumped model and the experimentally measured values appears good.

The maximum cooling rate between the initial temperature and a desired final temperature above the stream temperature can be calculated from:

$$B_{max} = \frac{T_h^{init} - T_h^f}{t_{min}} \qquad [5]$$

where $t_{min}$ (which can be predicted from Eq.[4]) is the minimum time to reach the final temperature ($T_h^f$). Values for the maximum cooling rate at different stream flow rates, which were obtained by solving Eqs. [4] and [5], are shown in Fig. 6 as a function of the difference between the initial heater layer temperature and stream temperature. The difference between the initial heater layer temperature and stream temperature can be regarded as the driving force for cooling. Substantial cooling rates can be achieved for larger driving forces (i.e., larger temperature differences), higher stream flow rates, and higher desired final temperatures. For example, for a stream flow rate of 8000 $cm^3$/min and a typical stream temperature of -100°C, the maximum cooling rates that could be achieved are ~ 180°C/min and ~120°C/min for final cooling temperatures of -40°C and -80°C, respectively. Although faster cooling rates could be obtained by increasing the stream flow rate, for most of the typical applications 100°C/min is sufficient.

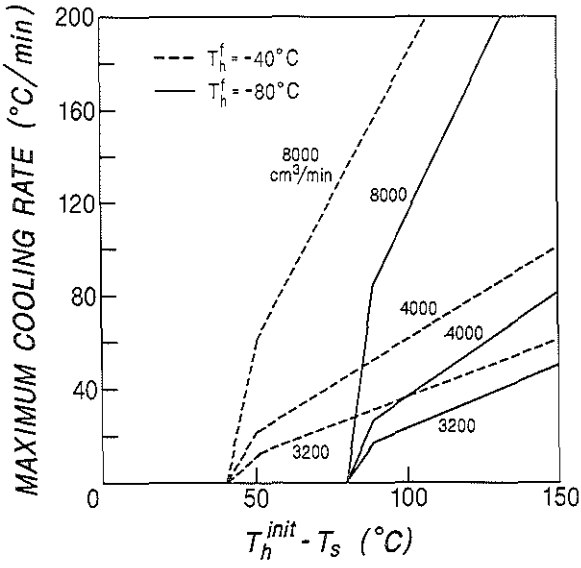


**Figure 6.** Maximum cooling rate capacity of the heater layer as a function of the difference between initial heater layer temperature and the stream temperature for three different stream flow rates. The results are also plotted for two different final temperatures.

**Temperature Gradients.** For an ideal freezer, it is desirable to have a uniform surface temperature so that the tissue is exposed to the same thermal profile. Even though the heater layer may be assumed to have a uniform electrical heat input and convective heat removal, there may be large temperature gradients due to the fact that the outer edge of the heater layer is in direct contact with the cold polypenco acetal channel. In order to estimate these gradients, temperatures at several different locations on the heater layer were measured with copper-constantan thermocouples. The location of these thermocouples are shown in Fig. 7 together with measured temperatures at different steady-state heater layer temperatures between 0° and -80°C. As can be seen from this figure, the temperature field on the heater layer was uniform with maximal differences being between ± 2°C.

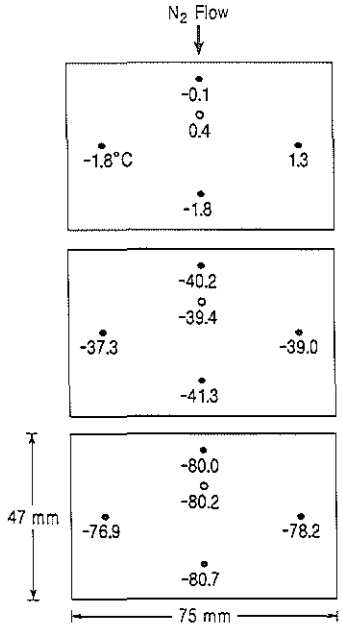


**Figure 7.** Temperature measurements on the surface of the heater layer between 0 and -80°C. The figure is drawn to scale and the correct locations of the thermocouples are marked on the figure with filled circles. The open circles refer to the location of the feedback thermocouple.

## Applications

The controlled rate freezing device described in this study has many potential applications in cryopreservation of cells and tissues. In our laboratory, we have recently used this system to freeze cultured hepatocytes which are sandwiched between two layers of collagen (1). In these

experiments, the effects of cooling rate, warming rate, and final cooling temperature were evaluated using the controlled-rate freezing device. Fig. 8 shows the temperature-time charts for three different cryopreservation protocols. The cooling protocol has 8 separate controlled steps: 1) cooling from room temperature to -12°C at a rate of 10°C/min; 2) holding at -12°C for 3 minutes to allow thermal equilibration; 3) rapid cooling by turning off the power to the heater layer; 4) spontaneous formation of extracellular ice and subsequent increase in temperature due to the released latent heat of fusion; 5) turning on the heater power to keep the temperature constant at -12°C for 15 minutes to allow cellular dehydration of hepatocytes; 6) controlled cooling rate at 5°C/min to -40°C; 7) holding at the final temperature; 8) warming at a given rate. In addition, Fig. 8 depicts the long-term albumin secretion rate of the cryopreserved hepatocytes. As can be seen from this figure, the protein secretion of hepatocytes was optimal at a cooling rate of 5°C/min (1). Although the seeding of external ice (i.e., steps 3-4) was achieved by rapid cooling and spontaneous nucleation of the external solution at ~ -25°C, the current design of the device is suitable for controlled seeding of the external ice by touching the edge of the tissue bag with a chilled needle or spatula. The device is also convenient for controlled rate freezing of cell suspensions and/or skin grafts for transplantation and experimental purposes.

**Acknowledgements**

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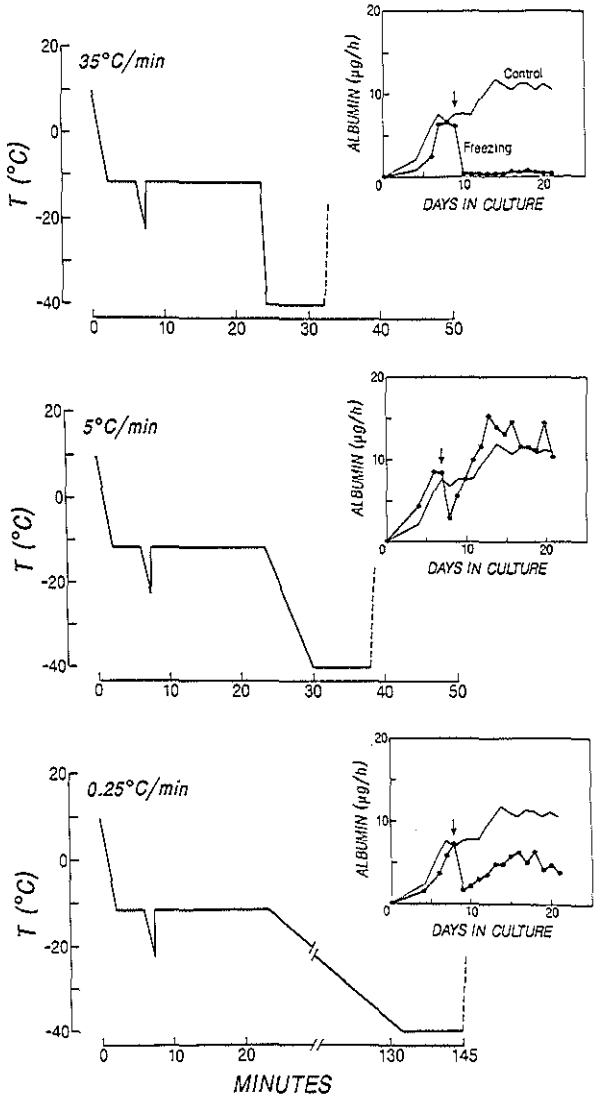


**Figure 8.** Temperature-time charts for three different cooling rates in a cryopreservation protocol for cultured hepatocytes. The corresponding recovery of long-term albumin secretion after freezing on day 7 of culture is shown as insert next to each chart.

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## CHAPTER 6

# Long-term functional recovery after cryopreservation of hepatocytes in a three-dimensional collagen culture configuration

I.H.M. Borel Rinkes, M. Toner, S.J. Sheehan, R.G. Tompkins, M.L. Yarmush

Cell Transplantation 1992; 1:281-292

**Abstract**

Hepatocyte cryopreservation is essential to ensure a ready supply of cells for use in transplantation or as part of an extracorporeal liver assist device to provide on-demand liver support. To date, most of the work on hepatocyte cryopreservation has been performed on isolated hepatocytes, and has generally yielded cells which display low viability and greatly reduced, short-term function. This report presents the development of a freezing procedure for hepatocytes cultured in a sandwich configuration. A specially designed freezing unit was used to provide controlled temperatures throughout the freeze-thaw cycle. Cooling rate, warming rate, and final freezing temperature were evaluated as to their effect on hepatocyte function as judged by albumin secretion. Under optimized conditions (cooling at 5°C/min and warming at ≥400°C/min), freezing to -40°C resulted in full recovery of albumin secretion within 2-3 days post-freezing, whereafter albumin secretion levels remained normal for the duration of the experiments (2 weeks). Freezing to -80°C led to an approximate 70% recovery of long-term protein secretion when compared to control cultures. In addition, the overall hepatocyte morphology as judged by light microscopy, closely followed the functional results. The sandwich culture configuration, thus, enables hepatocytes to maintain a satisfactory level of long-term protein secretion after a freeze-thaw cycle under optimized conditions, and offers an attractive tool for further studies into the mechanisms of freezing injury and subsequent hepatocellular recovery. These results are a promising step in the development of satisfactory storage procedures for hepatocytes.

## Introduction

The use of hepatocytes in providing temporary or permanent liver support would serve as an excellent alternative to orthotopic liver transplantation, which is currently the only available treatment for various forms of liver failure (45). During the past two decades, several studies have shown experimental hepatocyte transplantation to be effective in partially overcoming metabolic defects due to inborn errors of metabolism (4,10,41,47,52), and in the management of acute hepatic failure (32,44). Another promising approach to conditions requiring temporary liver support involves the use of cultured hepatocytes in extracorporeal bioartificial liver support systems, including hollow fiber devices (2,33,48,53). Whether hepatocytes are used for transplantation or within extracorporeal bioreactors, the ultimate success of such treatments relies on a readily available supply of large numbers of hepatocytes when the necessity for liver support arises. Since hepatocytes do not readily proliferate in culture, cryopreservation is critical for providing such a stable supply of hepatocytes either from single or multiple sources so that sufficient numbers of cells may accumulate over time.

The majority of studies on hepatocyte cryopreservation have involved the use of freshly isolated rat hepatocytes in suspension. In many cases, the results (which are generally short-term) are contradictory, and depend largely on the methods used for the assessment of viability. The post-freeze cell viability, as judged by trypan blue exclusion, has been reported to range from ~ 30 to 80% directly after freezing (6,818,23,28,29,38), but tends to decrease drastically within several hours (9,24). While cellular attachment immediately following cryopreservation was found to be well over 50% by some investigators (29,42), others observed dramatic impairment of hepatocyte attachment to culture dishes despite high trypan blue exclusion ratios (6,23). More importantly, short-term rates of protein synthesis and gluconeogenesis after cryopreservation of isolated hepatocytes were documented to be near normal by some investigators (19,42,46), whereas others observed a reduction of such functions of up to 90% (6,9,18,28). Furthermore, Gomez-Lechon et al.(19) found no damage to the hepatocellular ultrastructure as a result of cryopreservation, while other groups reported significant alterations (16,28,42).

Although difficult to quantify, successful intrasplenic transplantation of single hepatocytes has been used as long-term measure of post-freezing viability. Several investigators observed adequate functional and morphological recovery of at least part of the population of initially transplanted cells (31,37). In contrast, Fuller et al. (17), and Kusano et al. (27) demonstrated that only a small number of cryopreserved cells remained at 1 month postoperatively. A further

complication to the picture is the use of post-freeze centrifugation (e.g. with Percoll) as a step prior to viability assessment of frozen-thawed isolated hepatocytes (6,23,24,38,46). Such steps obviously complicate comparisons between the various freezing studies.

Reports concerning cryopreservation of cultured hepatocytes are few and mostly preliminary in nature (3,22). Recently, our laboratory has described a sandwich culture configuration, consisting of two layers of collagen in between which hepatocytes are cultured (11,12,13). This culture geometry has been shown to sustain a stable hepatocellular phenotype for at least 7 weeks. Theoretically, this sandwich culture configuration provides an attractive alternative for the study and evaluation of hepatocyte cryopreservation, because it contains a fixed number of stabilized hepatocytes, expressing differentiated long-term function. In an initial study, designed to assess the feasibility of freezing double gel cultures, an approximate 26% recovery of albumin secretion was observed (25). More recently, a procedure for the addition and removal of cryoprotectant dimethyl sulfoxide ($Me_2SO$) to sandwich hepatocyte cultures was reported, which does not lead to apparent loss of long-term hepatocyte function or cytoskeletal organization (5).

The objective of this study was to define optimal freezing conditions required for long-term function following a freeze-thaw cycle of hepatocytes in sandwich culture configuration. For this purpose, a controlled rate freezing unit was designed and used to ensure accurate thermal control. The results show that, using optimal freezing conditions, an approximate 70% recovery of long-term protein secretion can be obtained for at least 2 weeks following freezing at -80°C.

## Materials and Methods

*Hepatocyte isolation.* Hepatocytes were isolated from 2-3 month old female Lewis rats (Charles River Laboratories, Boston, MA), weighing 180-220 g, by a modification of the two-step collagenase perfusion method originally reported by Seglen (43). This modification has been described in detail elsewhere (11,12), and includes purification of the cell suspension by means of centrifugation over Percoll, as reported by Kraemer et al. (26). Each isolation yielded $250\text{-}350 \times 10^6$ hepatocytes. Cell viability ranged from 90 to 98 %, as judged by trypan blue exclusion. Cells were stored on ice (0°C) prior to use.

*Hepatocyte sandwich cultures.* This culture configuration consists of two layers of collagen in between which the hepatocytes are sandwiched. For the freezing experiments, a modification

of the methods previously described (5,12,25) was used to establish sandwich culture configuration. Hepatocytes were cultured on No. 1 cover glasses (24x60 mm; Baxter Healthcare Corp., McGaw Park, IL). The cover glasses were prepared with an edge of silicone rubber (GE Silicones, Waterford, NY) to prevent the collagen sandwich from sliding off, and sterilized prior to use. Two cover glasses were placed in a 100 mm plastic tissue culture dish (Falcon™, Lincoln Park, NJ). At least 60 min prior to cell seeding these dishes were coated with the first layer of 3 ml of type I collagen (1.11 mg/ml), which had been prepared as described by Elsdale and Bard (14). After coating, the dishes were placed in a 37°C incubator for adequate gellation of the collagen. Nine million viable hepatocytes were seeded per dish, taking special care to ensure equal distribution of the cells, and incubated for 24 h at 37°C in the presence of 10% $CO_2$. After aspiration of the supernatant medium and non-attached cells, the second layer of collagen was applied over the hepatocytes in the same fashion as the first layer. This was followed by incubation at 37°C for 60 min to allow gellation of the collagen, before the addition of fresh culture media. The culture media used throughout these studies consisted of Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Gaithersburg, MD), supplemented with 7 ng/ml glucagon (Lilly, Indianapolis, IN), 7.5 $\mu$g/ml hydrocortisone (Upjohn, Kalamazoo, MI), 0.5 U/ml insulin (Squibb, Princeton, NJ), 20 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA), 200 U/ml penicillin, 200 $\mu$g/ml streptomycin, and 10% fetal bovine serum (J.R. Scientific, Woodland, CA). Twenty-four hours after administration of the second layer of collagen the cover glasses were cut out from the collagen, and each cover glass was transferred to a new culture dish (Fig. 1). Culture media was changed daily thereafter, and culture supernatants were collected for protein analysis.

*Freezing unit.* A controlled-cooling apparatus was designed for the freezing of sandwiched hepatocytes. Gaseous nitrogen, cooled by passage through a coil immersed in liquid nitrogen, flowed through an insulated rectangular channel (~7.0x1.4 cm) which housed the cryostage plate. The rectangular channel was made out of polypenco acetal (Delrin, DuPont, Wilmington, DE). The cryostage plate heater was located on the top surface of the channel and measured 7.5x4.7 cm. The plate was composed of 3 layers, held together by epoxy (Devcon Corp., Danvers, MA): 1) a 300 $\mu$m thick bottom aluminum layer, 2) a 100 $\mu$m thick Kapton heater layer (230 Ω, 10W, HK-TBA, Minco Products, Inc., Minneapolis, MN) , and 3) a top aluminum plate (300 $\mu$m thick). Power was supplied to the resistance heater layer with electric leads attached to the contact strips. The sample was located on top of the cryostage plate heater and the sample temperature was measured by a copper-constantan foil thermocouple (CO2-T, Omega, Stamford, CT) at the center of the heated cryostage plate. The temperature controller used feedback from the sample thermocouple to adjust the power to the heating element to counteract the refrigerant. This approach kept the sample at a specified setpoint temperature (7).

The desired cooling/warming rates were entered into an IBM™ compatible computer. From this input, the program executed the protocol by mathematically generating the appropriate time-varying setpoint. The setpoint was converted to an analog voltage and sent to the temperature controller. Depending on the offset between the setpoint and the actual temperature, the temperature controller adjusted the power to the heating element to precisely achieve the desired temperature. The error in the desired temperature at the center thermocouple was usually less than 0.1°C. The maximum cooling rate which was achieved in the presence of a sample bag containing the sandwiched hepatocytes was ~ 20°C/min. For the faster cooling rate of 35°C/min used in this study, the glass slide with sandwiched hepatocytes was directly placed onto the cryostage to minimize the thermal mass.

*Freezing protocol.* Fig. 1 illustrates the various steps involved in establishing sandwich hepatocyte cultures on a coverglass, and subjecting these cultures to the freeze-thaw cycles used. Sandwich cultures of hepatocytes on cover glasses were subjected to freezing between days 7 and 9 of culture, i.e., once phenotypic stability has been reached (12). One hour prior to freezing, a solution of DMEM containing 2.0 M $Me_2SO$ (Sigma Chemical Company, St. Louis, MO) was added to the hepatocyte cultures at ambient temperature, as described previously (5). Five min before freezing the cover-glass was transferred under sterile conditions to a 50$\mu$m thick, heat sealable, polyester pouch (Kapak Corp., Minneapolis, MN) filled with a given volume (3 or 5ml) of the $Me_2SO$ solution. The pouch was sealed using a Scotchpak Pouch Sealer (Kapak), and immediately transferred to the programmable freezing unit described above. In order to monitor the vertical temperature gradient in the pouch, a foil thermocouple (SA1-T, Omega) was placed on top of the pouch and connected to a 7100 BM stripchart recorder (Hewlett Packard, Temperature Module, model 17502A). Although different protocols were tested in the course of these studies, a typical freezing protocol is shown in Fig. 2. Both the temperature of the controller on the heater layer, and the temperature on top of the filled pouch are depicted. Briefly, each experiment was initiated by cooling the pouch at 10°C/min from 20°C to -12°C. After thermal equilibration at this holding temperature for at least 3 min, the power of the freezing controller was shut off, which resulted in the rapid cooling of the pouch and its contents, until the extracellular ice seeded spontaneously. This release of latent heat in the system was indicated by a rise in temperature. Upon this event, the power of the freezing controller was immediately turned back on, so that the holding temperature was set to -12°C. The average extracellular ice formation temperature ($T_{seed}$) was -22.8±2.3°C on the bottom surface of the pouch, as measured by the feedback thermocouple on the cryostage plate. Due to the vertical temperature gradient in the pouch, the average extracellular ice formation temperature on top of the pouch was -18.5±2.3°C. Once the heater power of the freezing unit was turned back on, the feedback temperature promptly returned to

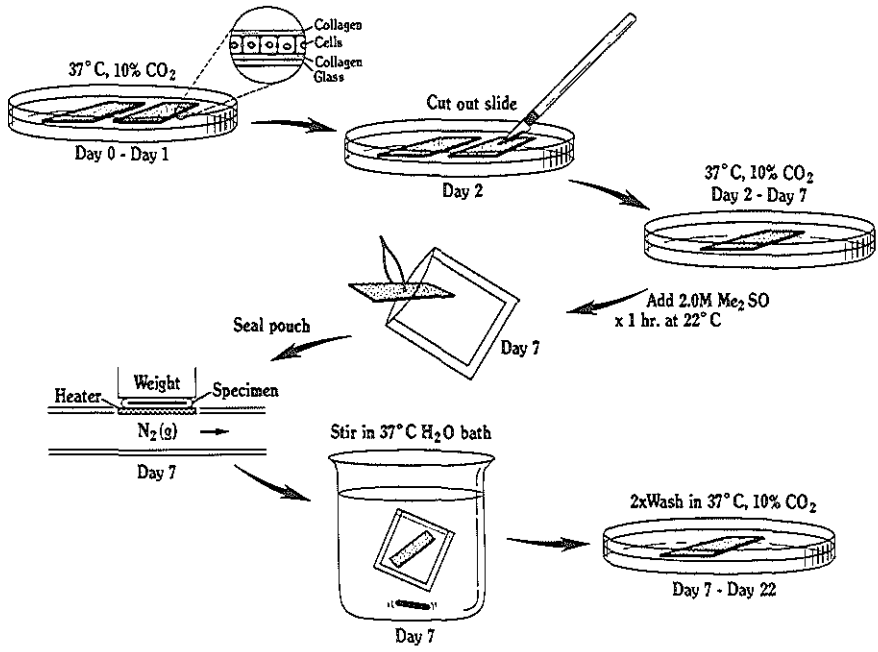


**Figure 1.** Schematic diagram of the steps involved in establishing sandwich culture on glass coverslips, and subjecting these cultures to the typical freeze-thaw protocol used in this study.

the holding temperature. However, the temperature on top of the pouch tended to slightly 'overshoot' (-10.1±0.9°C) before returning to the holding temperature. The system was held at -12°C for 15 min to ensure cellular dehydration prior to the controlled-rate cooling to the desired final temperature. Generally, there was ~1 min lag time between the temperatures on both sides (i.e., top and bottom) of the pouch. However, this small difference did not affect the cooling rate. The cultures were held at the desired final temperature between -40 and -80°C for a minimum of 5 min to allow for thermal equilibration. They were then thawed at one of three warming rates: rapid, intermediate, or slow. Rapid and intermediate warming rates were established by immersing the frozen pouch immediately in a bath containing sterile water at 37°C. Intermediate warming rates averaged 150 °C/min in a pouch containing 5 ml of solution. By reducing the fluid volume inside the pouch to 3 ml, and slowly stirring the water in the bath, more rapid warming rates of ≥ 400 °C/min were obtained. Slow warming was established by keeping the pouch on the freezer unit and programming a 5 °C/min warming

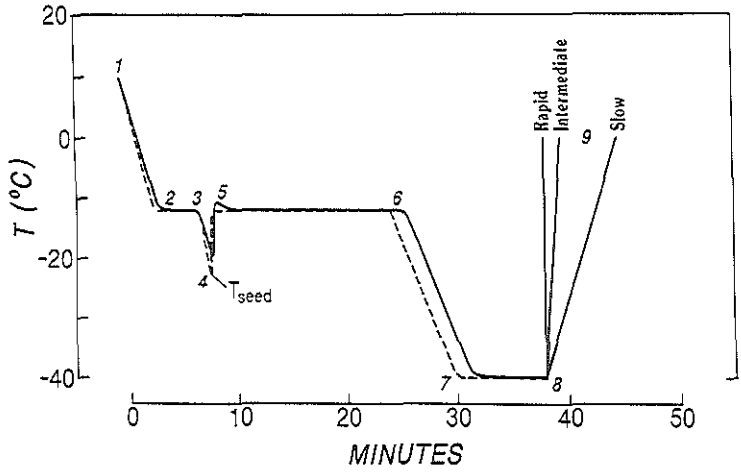


**Figure 2.** Typical freeze-thaw protocol. Temperature is depicted as a function of time. In the diagram a cooling rate of 5 °C/min and a final temperature of -40°C are illustrated in combination with the three warming rates used. Drawn line shows temperature on top of the polyester freezing pouch, as measured by foil thermocouple. When different from this temperature, dashed line shows actual controller temperature on the freezing stage, i.e., approximating the temperature at cell level. 1-2: cooling at 10°C/min to -12°C. 2-3: equilibration at -12°C. 3: power off. 4: $T_{seed}$ is the average extracellular ice formation temperature on the bottom of the pouch, as measured by the feedback thermocouple on the cryostage plate. 4-5: release of latent heat, and power back on. 5-6: 15 min dehydration at holding temperature of -12°C. 6: start controlled cooling to final temperature. 7-8: holding at final temperature. 8-9: thawing at various rates.

rate. Warming was discontinued at 0°C. Subsequently, the pouches were reopened and the coverglass culture was carefully removed from the pouch using sterile techniques. The coverglass with sandwiched hepatocytes was then transferred to a tissue culture dish and fresh culture media (prewarmed to 37°C) was added. Thirty min later the media was changed to wash out the remaining $Me_2SO$. Cultures were followed for 2 weeks after freezing.

*Protein assays and data analysis.* Collected media were analyzed for rat serum albumin content by enzyme-linked immunoassay (ELISA) as previously described (12). Rat albumin standard, and peroxidase-conjugated antibody to rat albumin were purchased from Cappel (Cochranville, PA). The absorbance was measured at 490 nm with a Dynatech MR600 plate reader (Chantilly, VA). The albumin secretion rates shown in Figs. 3 and 6 are the outcome of experiments with cells from one single source. Each data point represents the result (mean ± SD) from a

minimum of 2 cover glass cultures per experimental condition. All ELISA measurements were performed in duplicate. Each experiment was repeated at least once using hepatocytes from different donor animals, which resulted in similar findings. The overall response from all experiments under the various freeze-thaw protocols is summarized in Figs. 4 and 7. The average survival rates were calculated from the cumulative albumin secretion of each experiment during the second week of culture following a freeze-thaw cycle (i.e., between days 14 and 21), normalized to the respective control experiments. Control experiments represent cultures which did not undergo any freezing treatment. In a previous study we have recently shown that the exposure to similar concentrations of $Me_2SO$ for up to 2 hours does not induce any damage to sandwiched hepatocytes (5). Error bars represent standard deviation of the mean.

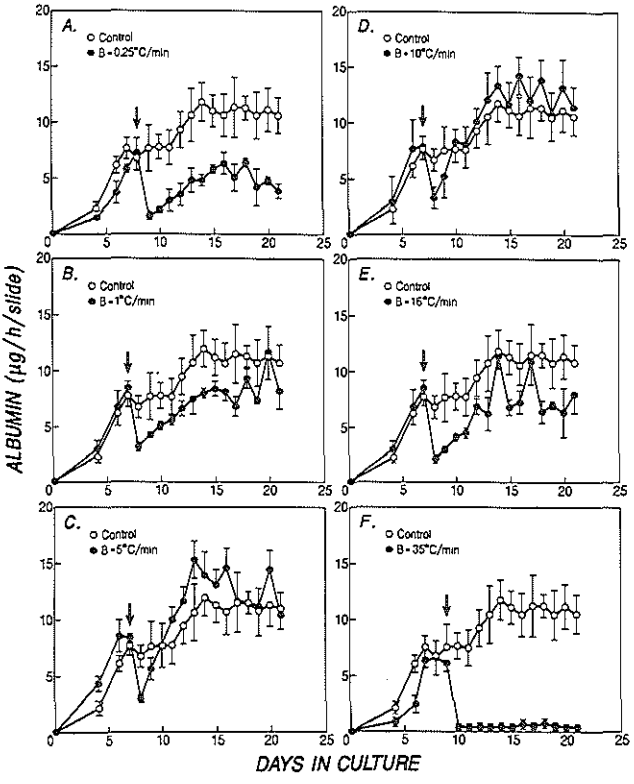


**Figure 3.** Effect of cooling rate to -40°C on albumin secretion of hepatocytes in sandwich configuration after freezing on day 7 of culture (arrows). The results of typical experiments from one representative cell batch are shown (mean±SD). Cooling rate is expressed as *B* in °C/min. Albumin secretion rate is expressed as $\mu g$ per hour per slide, i.e., ~$2x10^6$ hepatocytes.

## Results

*Effect of cooling rate to -40°C.* Initial experiments analyzed the effect of different cooling rates *(B)* on long-term albumin secretion of sandwich hepatocyte cultures after freezing to -40°C. Results of a set of typical experiments with one cell batch are shown in Fig. 3. Similar findings were obtained using different cell batches. Very slow cooling ($B$=0.25 °C/min) resulted in drastically reduced albumin secretion immediately following the freeze-thaw cycle, which recovered slightly during the following 2-week-period to approximately 40% of control levels (Fig. 3A). Cooling at $B$=1 °C/min resulted in a similar dip in albumin secretion followed by a gradual return to ~ 80% of control levels (Fig. 3B). In contrast, cooling to -40°C at $B$=5 °C/min resulted in an initial reduction of albumin secretion, which was followed by a complete recovery within the next 2 days (Fig. 3C). A similar pattern was observed for $B$=10 °C/min (Fig. 3D). However, when cultures were cooled at 16 °C/min, recovery of protein secretion was only partial (Fig. 3E), and a further increase in cooling rate ($B$=35 °C/min) led to irreversible cell damage and a complete loss of albumin secretion (Fig. 3F). Neither the seeding of the extracellular ice, nor the holding at -12°C for 15 min in the presence of extracellular ice had any deleterious effect on long-term albumin secretion (data not shown).

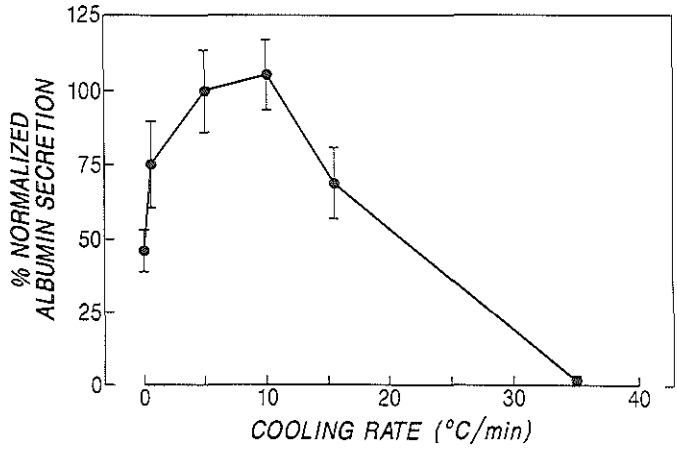


**Figure 4.** Normalized albumin secretion of hepatocytes in sandwich culture after freezing to -40°C as a function of cooling rate. Normalized albumin secretion is defined as cumulative albumin secretion in all experiments during the second week following freezing, i.e. between days 14 - 21 of culture, normalized to the respective control value of each experiment. Freezing took place on day 7 of culture. Data points represent a minimum of 4 experiments per condition (mean±SD).

For comparison purposes, the cumulative albumin secretion of each experiment during the second week of culture following freezing (i.e., between days 14 and 21) was normalized to the respective control value of that experiment. Fig. 4 shows these normalized values of all experiments as a function of the cooling rate to -40°C. Cooling rates of 5 to 10°C/min appear to yield maximal function. In addition to albumin secretion measurements, success was also assessed by visual examination of the cells post-freezing. Micrographs taken on day 21 of hepatocyte sandwich culture, i.e. 14 days post-freezing, using standard light microscopy, are shown in Fig. 5. Freezing at 5 °C/min did not lead to apparent changes in cell morphology when compared to control cultures (Figs. 5A and B). However, very slow freezing ($B$=0.25°C/min) resulted in increased cytoplasmic granularity and cell lysis (Fig. 5C). Very rapid freezing ($B$=35°C/min) led to nearly complete destruction of the normal hepatocellular morphology (Fig. 5D). Based on all of our results up to this point (and taking practical considerations into account), a $B$ value of 5 °C/min was chosen for further experiments.

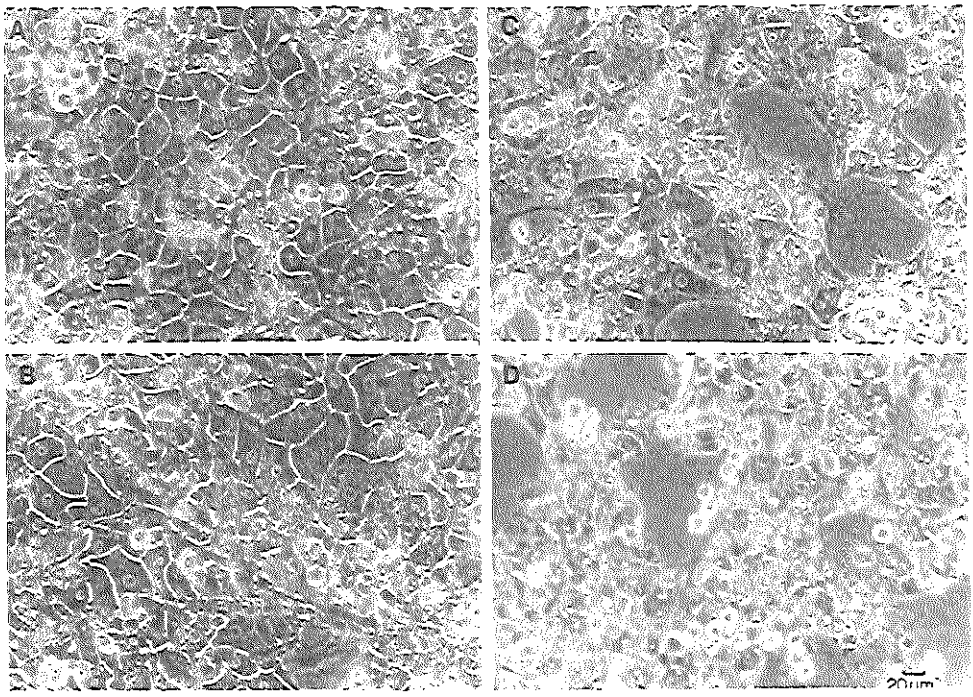


**Figure 5.** Typical morphological appearance on phase contrast microscopy (20x obj.; Olympus CK2, Japan) of hepatocytes in sandwich culture configuration on day 21 of culture, i.e.14 days after freezing. A) control culture; B) hepatocytes that were frozen to -40°C at $B$=5°C/min; C) $B$=0.25°C/min; D) $B$=35°C/min.

*Effects of warming rate and final cooling temperature.* In the next set of experiments, the effect of warming rate on post-freezing long-term protein secretion was analyzed in relation to the final freezing temperature. When hepatocytes in sandwich culture were frozen to a final temperature of -40°C, no dramatic effect of warming rate on long-term function was observed, as judged by albumin secretion during the second week post-freezing (Figs. 6A-C). In contrast, when the cultures were frozen to a final temperature of -80°C, warming rate greatly affected the functional recovery. Rapid warming resulted in gradual restoration of protein secretion to near normal values, intermediate warming led to only partial functional recovery, and slow warming induced a total loss of protein secretory function (Figs. 6D-F).

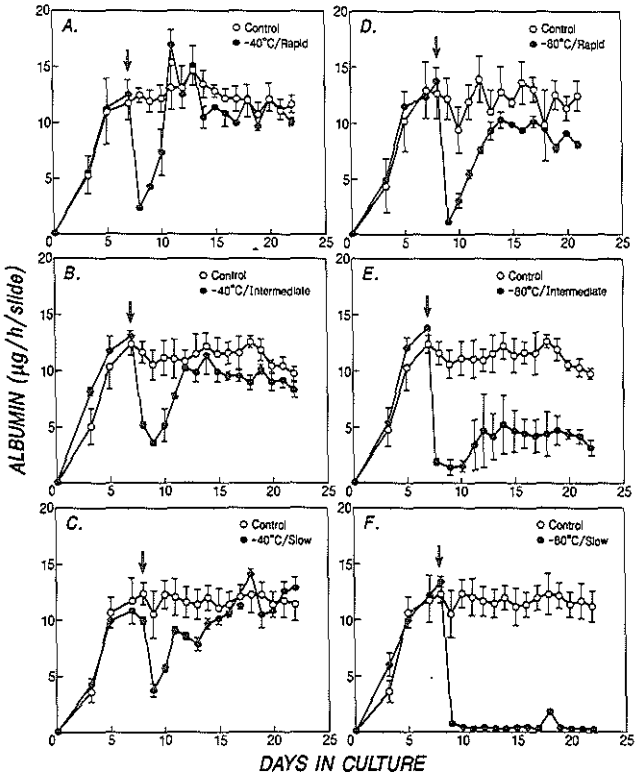


**Figure 6.** Albumin secretion rate of hepatocytes in sandwich culture after freezing to -40°C (A-C), or to -80°C (D-F), as a function of warming rate. Cultures were frozen on day 7-8 of culture (arrows). Secretion rate is expressed as µg per hour per slide, i.e., ~$2x10^6$ hepatocytes. Experimental results from representative cell batches are shown, but similar findings were obtained from different cell batches. A and D) rapid warming (≥400°C/min); B and E) intermediate warming (~150°C/min); C and F) slow warming (5°C/min).

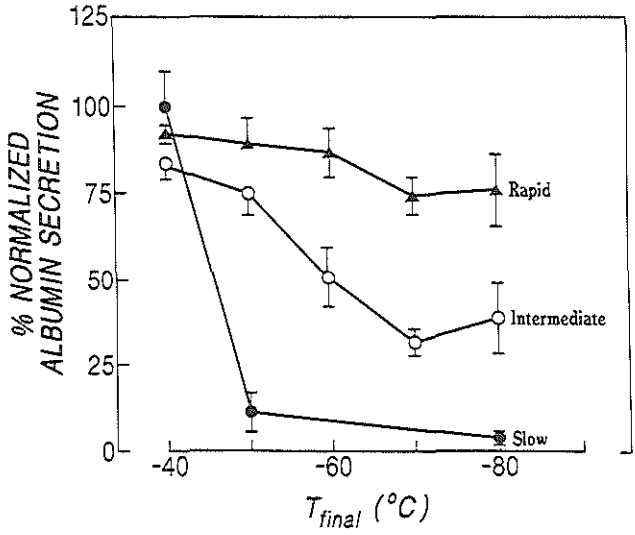


**Figure 7.** Normalized albumin secretion as a function of final freezing temperature and warming rate. Data points represent the outcome of all experiments performed (mean±SD; n=2-6 per condition). Definitions of warming rates are identical to those given in Fig. 6.

The aforementioned results were quantified by expressing the normalized cumulative albumin secretion during the second week post-freezing as a function of final freezing temperature. As shown in Fig. 7 the warming rate is crucial to long-term hepatocellular function for final temperatures below -40°C. Rapid warming resulted in an overall restoration of function of 73.9±10.9% after freezing at -80°C, as opposed to 38.3±10% for intermediate warming, and 2.9±1.3% for slow warming rates (Fig. 7).

The micrographs in Fig. 8 illustrate the morphological changes that accompanied the above observations. Two weeks after freezing to -80°C with slow warming, the cells showed a strong increase in cytoplasmic vacuoles, and partial loss of membrane integrity (Fig. 8A). In contrast, the majority of those hepatocytes that were exposed to the same temperature, but thawed rapidly, displayed the characteristic architecture and morphological appearance observed in control cultures (Fig. 8B).

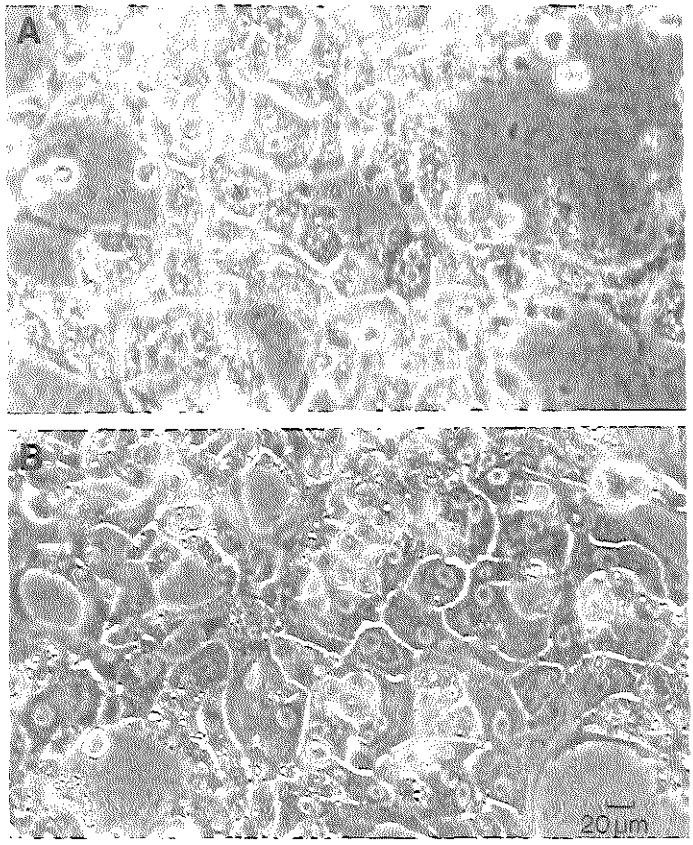


**Figure 8.** Effect of warming rate on morphological appearance on phase contrast microscopy of hepatocytes in sandwich culture configuration on day 21 of culture, i.e.14 days after freezing at -80°C. A) slow warming (5°C/min); B) rapid warming (≥400°C/min).

## Discussion

This study is the first to introduce an analysis of the damaging effects of several critical freezing parameters on long-term protein secretion and morphology of a population of phenotypically stable cultured hepatocytes. The freezing unit, designed for this purpose, achieved precise temperature control within a planar geometry without significant thermal gradients. The results indicate that cooling rate, warming rate, and final temperature are critical

factors determining post-freeze survival. Under optimized conditions, i.e., a cooling rate of 5°C/min and a rapid thawing rate of ≥400°C/min, hepatocytes in sandwich culture were able to recover, functionally and morphologically, from the damage inflicted by freezing to -40°C within 2-3 days. Freezing to -80°C led to an approximate 30% loss of long-term protein secretion, while, for the majority of hepatocytes, the characteristic morphology was preserved.

The major challenge to cells during freezing is not their ability to endure storage at very low temperatures, but rather, their ability to overcome an intermediate temperature range (~-15 to -60 °C) that a cell has to traverse during cooling and warming. For a cell subjected to subzero temperatures, ice preferentially forms outside the cell in the suspending solution. Due to the precipitation of ice, i.e., pure water, the partially frozen extracellular solution is more concentrated than the intracellular compartment. As a consequence, the cell will dehydrate in an attempt to restore osmotic equilibrium. If cooling rates are too high, intracellular water can not be removed from the cell fast enough, and thus intracellular ice formation may occur (51). It is well known that intracellular ice formation is associated with lethal cell damage (34). A second mechanism for cell damage during freezing involves recrystallization. This is defined as the tendency of very small, and presumably innocuous ice crystals that may have formed intracellularly during the cooling phase, to increase in size during the warming stage (34). Under such circumstances, slow warming rates may be harmful since they allow time for recrystallization to occur. On the other hand, slow freezing may also be injurious, since the shrinking cells will undergo prolonged exposure to a residual unfrozen medium that forms channels of strongly increasing solute concentration and decreasing size (30,34). Thus, optimization of cooling and warming rates for the cell type under study are key components for a successful freeze-thaw process.

Given the aforementioned cellular responses to freezing, a number of practical issues need to be considered. First, a dehydration period at relatively high subzero temperatures in the presence of external ice should be chosen, because it reduces the likelihood of intracellular ice formation (35,49,50) during the subsequent cooling step. This has been advocated by some for the freezing of isolated hepatocytes as well (9,18,23). For minimal cell damage it is preferable to dehydrate the cells at the lowest possible temperature without intracellular ice formation. Based on theoretical predictions as well as on prior cryomicroscopic observations with both isolated (20,50) and cultured hepatocytes (21) in the presence of $Me_2SO$, a dehydration temperature of -12°C was chosen for this study. Dehydration at this temperature showed no deleterious effects on either hepatocyte morphology, or long-term albumin secretion.

The graphic representation of normalized function as a function of cooling rate (*cf.* Fig. 4) resembles an "inverted U-shape" with a distinct optimal cooling rate range between 5 and 10°C/min. This pattern is in accordance with the theory presented above and has been reported for many other cell types (for review see 34). This range of optimal cooling rates is supported by the cryomicroscopic findings on intracellular ice formation in cultured hepatocytes in sandwich configuration (21). Despite the use of what can be considered as an optimal cooling rate, long-term protein secretion was significantly reduced when final freezing temperatures were below -40°C. Possible explanations for these observations might include intracellular ice formation during cooling, and/or the recrystallization of small intracellular ice crystals during warming. To determine the deleterious step, we varied the warming rate for subsequent experiments. The results show that rapid warming may, indeed, increase cell survival, most likely by not allowing sufficient time for recrystallization to take place. Similar observations have been reported for mouse embryos (34,39,40), and for hamster lung fibroblasts (15,36).

The optimal freezing protocol used in this study is quite comparable to procedures described for isolated cells (6,8,18,42). However, our results regarding viability, particularly post-freeze protein secretion, compare favorably to those reported for isolated hepatocytes (6,9,18,42). Although we have no direct insight into the specific reasons for this difference, it is conceivable that the presence of an extracellular matrix and a physiologically organized cytoskeleton, as encountered in the sandwich system, may stabilize the plasma membrane and cell organelles against freezing stresses. This hypothesis is supported by the fact that the estimated water permeability and nucleation parameters of cultured hepatocytes approach values found for other mammalian cells, and are in striking contrast with those of isolated or digested hepatocytes (21,50,53). In addition, the presence of a collagen gel in the intact matrix may alter the structure of the external ice and, subsequently, the interaction of the external ice with the plasma membrane (1). Finally, our previous cryomicroscopic observations with freshly isolated hepatocytes (20,50) revealed a threshold cooling rate range for the formation of intracellular ice (~75-150°C/min) which is much higher than the cooling rates used in most studies to freeze isolated cells (6,8,18,42). Thus, the lack of success in freezing isolated hepatocytes may be partially attributed to the fact that suboptimal cooling rates were used in those studies.

The rationale for the timing of the freezing experiments between days 7 and 10 of culture was based on several prior observations. First, it takes about a week for hepatocytes in sandwich culture to reach steady state function (12,13). Hence, they may not be considered phenotypically stable before that time. Second, hepatocytes that have been in sandwich culture for less than 7 days have been shown to be considerably more susceptible to the addition of

cryoprotectant as well as to freezing (25). Third, recent cryomicroscopy studies have revealed that 1 day old cultures are more likely to develop intracellular ice formation, at higher temperatures, than 7 day old cultures (21). The latter group may, therefore, be expected to exhibit greater tolerance to the freeze-thaw cycle, and the data presented here support this assumption. Our results are also of note, because they suggest that short term functional assays used in the assessment of cryopreserved isolated hepatocytes may be misleading.

In summary, our results have shown that hepatocyte sandwich cultures, which have been exposed to subzero temperatures in a fully controlled fashion, can regain satisfactory levels of long-term differentiated function and structure. This represents a promising step towards the realization of satisfactory hepatocyte cryopreservation, which would be invaluable for the provision of viable hepatocytes either for transplantation purposes, or for use in bioartificial liver support systems, e.g. hollow fiber devices.

**Acknowledgement**

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# PART III

## Ex-vivo Gene Therapy

# CHAPTER 7

## Retroviral infection and the expression of cationic amino acid transporters in rodent hepatocytes

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Submitted for publication

**Abstract**

The susceptibility of rodent hepatocytes to ecotropic murine leukemia viruses (MuLV) infection was examined in vivo and in vitro and compared to the expression of two hydrophobic membrane proteins that are transporters for cationic amino acids (CAT-1 and CAT-2). CAT-1 confers susceptibility to ecotropic infection when expressed in nonpermissive mammalian cells and it has been suggested that CAT-2 could be the amphotropic MuLV receptor. In the present study CAT-1 was found to be expressed in hepatoma derived cells and in primary hepatocytes in vitro, both of which were infectible by ecotropic MuLV. In contrast, CAT-1 expression was not found in normal or regenerating adult liver in vivo, which were not susceptible to ecotropic infection. CAT-2 was expressed in normal and regenerating adult liver in vivo as well as in Hepa1 cells and primary hepatocytes in vitro. Expression of CAT-2 in a nonpermissive Chinese hamster cell line was not sufficient to confer amphotropic infectivity using a protocol that could easily demonstrate the capacity of CAT-1 to confer susceptibility to ecotropic infection. Our data establish CAT-1 expression as a major determinant for ecotropic tissue tropism and suggest that CAT-2 is not the amphotropic receptor.

## Introduction

The development of retroviruses as vectors has increased interest in understanding the restrictions to infection of specific tissues by murine leukemia viruses (MuLV). In particular, there is a great interest in the use of retroviral vectors to introduce genes into the liver, an important target organ for the correction of several metabolic disorders (14). Jaenisch and Hoffmann (17) reported the failure of hepatocyte infection by ecotropic MuLV in viremic mice. Subsequently, several laboratories have confirmed this finding, but have achieved amphotropic and ecotropic MuLV infection of fetal liver, hepatoma-derived cell lines, and hepatocytes propagated in cell culture (2,15,16,21,24,34,36). It has been suggested that the failure to infect adult hepatocytes in vivo, but not in vitro, may be caused by factors associated with cell proliferation and required for virus replication. Indeed, it has recently been reported that partial hepatectomy permits infection of the regenerating hepatic remnant by amphotropic MuLV (13,27). However, induction of cell proliferation by partial hepatectomy is not sufficient to render hepatocytes infectible by ecotropic MuLV (17).

An important determinant of ecotropic MuLV infection is the expression of rodent CAT-1, a transporter for cationic amino acids (1,18,31). This hydrophobic membrane protein contains an extracellular domain that serves as a virus envelope binding site. Mouse CAT-1 confers susceptibility to infection when introduced into nonpermissive mammalian cells (1). Previously, we have observed that CAT-1 is not expressed in the liver of adult rodents, consistent with the absence of hepatocyte infection by ecotropic MuLV in viremic mice (18). However, the expression of CAT-1 has not been investigated in cultured hepatocytes.

A second transporter of cationic amino acids (CAT-2), which is related to CAT-1, has recently been shown to be expressed in adult mouse liver (7). CAT-2 is encoded by a gene (Tea = Tcell early activation gene) that was originally identified in MuLV-induced lymphomas (22). The sequence of the Tea- and CAT-2-cDNAs are identical except for the 5' end and a stretch of 120 nucleotide residues in the middle of the open reading frame. These differences are most likely due to alternative splicing of the Tea transcripts in hepatocytes and lymphocytes. Chromosome mapping studies have identified synteny between the CAT-2 gene and the susceptibility to amphotropic MuLV infection on chromosome 8 in mice (22). These studies and the structural similarity between CAT-2 and CAT-1 have led to the suggestion that CAT-2, or the related Tea protein that is expressed in lymphocytes, might represent the amphotropic MuLV receptor (22,30).

In the present study, we have investigated the role of CAT-1 and CAT-2 expression in MuLV infection. The results demonstrate the importance of rodent CAT-1 expression in the tropism of ecotropic MuLV for hepatocytes. In addition, they show that expression of the CAT-2, or the Tea protein, is not sufficient for infection by amphotropic MuLV.

## Materials and Methods

*Cells and Viruses.* Replication-defective ecotropic or amphotropic BAG virus-producing cell lines were provided by Dr. C. Cepko. BAG virus encodes E. coli ß-galactosidase under the control of the Moloney MuLV long terminal repeat (25). Hepa1 cells were provided by Dr. G. Darlington. Both virus producing and Hepa1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Gaithersburg, MD), supplemented with 10% calf serum (Hazelton, Lenexa, KS). CHO-K1 cells (American Type Tissue Culture Collection) were grown in F12 nutrient mix (Gibco, BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum.

*Virus Assay.* Supernatants from confluent plates of virus producing cells were harvested every 12 hrs, filtered through a 0.45 $\mu$m filter and supplemented with 8 $\mu$g /ml polybrene (Sigma, St. Louis, MO). Cells were exposed to virus-containing medium (0.5-1 x $10^5$ infectious particles / ml) for 12 hrs. Cells infected with BAG virus were identified two days after exposure to virus by incubation with the substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), which forms a blue preciptate in cells expressing β-galactosidase (25).

*Isolation and Culture of Hepatocytes.* Hepatocytes were isolated from 4 week old female Lewis rats (40-50 g, Charles River Laboratories, Boston, MA) using a protocol described earlier (8,9), but modified to compensate for the smaller size of the animals (50% of the original perfusate volume, 67% reduction in flow rate). Hepatocytes (1.4-1.9 x $10^8$) were harvested after fractionation over a Percoll gradient (19). Cell viability was routinely 90-98% as determined by trypan blue exclusion. At least 30 min prior to plating, tissue culture dishes (60mm; Falcon™, Lincoln Park, NJ) were coated with 1 ml of type I collagen (1 mg/ml DMEM) prepared from rat tail tendons (10). Hepatocytes were plated at a seeding density of $10^5/cm^2$ in either SUM medium (11), or in DMEM supplemented with 200 U/ml penicillin, 200 $\mu$g/ml streptomycin, 10% fetal bovine serum, and: **A**) high growth factor concentration: 7 ng/ml glucagon (Lilly, Indianapolis, IN), 7.5 $\mu$g/ml hydrocortisone (Upjohn, Kalamazoo, MI), 0.5 U/ml insulin (Squibb, Princeton, NJ), 20 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA), or **B**) low growth factor concentration: 0.7 ng/ml glucagon, 0.75 $\mu$g/ml hydrocortisone and 0.5 mU/ml insulin. In some experiments, a second

layer of collagen was applied over the adherent hepatocytes after 24 hours (double gel cultures). Medium was replaced every day and the supernatant examined for albumin production by ELISA (8).

*In Vivo Infection.* In four week old Lewis rats, the portal vein was exposed by midline laparotomy under ether anesthesia and injected with one ml of either PBS, or ecotropic BAG-virus containing medium ($1x10^5$ virus particles/ml) supplemented with polybrene (8 $\mu$g/ml). The rats were sacrificed three days later, and the inferior vena cava was perfused with 30 ml of PBS, followed by 4% formaldehyde in PBS for tissue fixation. The fixed liver and spleen were cut into segments of about 0.5 $cm^3$. The segments were rinsed three times in PBS and then incubated in X-Gal substrate for 12 hrs at 37°C. Subsequently, 30 $\mu$m frozen sections were prepared.

*Regenerating Liver.* A 70% hepatectomy was performed by removal of the left lateral and median lobes from four week old Lewis rats through a midline laparotomy under ether anesthesia. 12, 24, 36 or 48 hours later, the animals were sacrificed and induction of regeneration in the hepatic remnant was verified by visual inspection prior to homogenization and isolation of RNA.

*Northern Blots.* Total RNA was isolated by homogenizing cells or tissues in guanidinium isothiocyanate and centrifugation through a CsCl cushion (6). Poly $A^+$-enriched RNA was prepared by oligo dT cellulose chromatography (28). The RNA was separated by electrophoresis on 1% agarose-formaldehyde gels and transferred to nylon membranes (Gene Sreen, Dupont). cDNA probes for mouse CAT-1 or -2 (MCAT-1 or -2) were labeled with $^{32}$P-dCTP using the random primer method (12). The MCAT-2 cDNA probe used does not distinguish between CAT-2 and Tea transcripts. Hybridization was performed in 40% formamide, 1M NaCl, 5% dextran sulfate, 1% SDS, 250 $\mu$g / ml fish DNA and $10^6$ cpm/ml $^{32}$P-labelled probe for 18 hrs at 42°C. The membranes were subsequently washed in 2 X SSC (1 X SSC= 0.15 M NaCL, 15 mM sodium citrate), 0.1% SDS, 3 x 10 min at RT and 2 x 30 min at 65°C and exposed to XAR film (Eastman Kodak Co, Rochester, New York) at -70°C, using intensifier screens (Dupont Lightening plus). Exposure times ranged from 1 to 5 days. Hybridization probe was removed from filters by incubation in 50% formamide, 2 X SSC, 1% SDS, for 60 min at 65 °C.

*Transfection and Stable Cell Lines.* MCAT-2 and MCAT-2/Tea (= MCAT-2 cDNA with the Tcell specific exon) cDNAs were subcloned into the mammalian expression vector, pJAY3 (20). CHO-K1 cells were cotransfected with 10 $\mu$g of expression plasmid and 0.5 $\mu$g of pHyg

using the lypoamine DOGS (4). pHyg is a mammalian expression vector encoding the E.coli hygromycin B phosphotransferase (29). Transfected cells were selected for two weeks in media containing 400 $\mu$g / ml hygromycin (Boehringer Mannheim, Indianapolis, IN). Hygromycin-resistant cell clones were isolated with cloning cylinders and CAT-2 expression was verified by western blots.

*Western Blots.* Cells (5 x $10^6$) were scraped from a confluent culture plate, washed in PBS and lysed in 50 $\mu$l lysis buffer (10 mM Tris pH 7.5, 10mM NaCl, 1 mM $MgCl_2$, 5% NP-40, 0.1 mM PMSF). Nuclei were pelleted by centrifugation at 2000 g for10 min. An equal volume sample buffer (50 mM Tris pH 6.8, 2mM EDTA, 4% SDS, 8% urea, 1% 2-mercaptoethanol) was added to the supernatants. 10 $\mu$l of the lysate was separated on a 8% SDS-acrylamide gel and transferred to a nitrocellulose membrane. Immunostaining of the membranes was performed by serial incubation in blotto (50mM Tris pH 8, 2mM $CaCl_2$, 5 % dry non fat milk), and 10% goat serum (1hr, RT), 1:500 dilution of rabbit antiserum directed against the C-terminus of MCAT-2 (7) in 0.4% BSA, 0.08% $NaNO_3$, 0.4% Tween 20 (ON, 4°C); 3 times in blotto (15 min, RT); 1: 1000 dilution of a $^{125}$I-goat anti-rabbit IgG (ICN Biomedicals Inc., Costa Mesa CA) in blotto (2 hr, 4°C), 6 times in TBST (100 mM Tris pH 8, 1.5 M NaCl, 0,5% Tween 20) (15 min, RT). Membranes were exposed overnight to XAR film at -70°C.

## Results

*Expression of CAT-1 and susceptibility to infection with ecotropic BAG virus*

To determine the susceptibility of hepatocytes to in vivo infection by ecotropic MuLV, either replication-defective BAG virus (1 x $10^5$ IU), or saline was injected through the portal vein into the liver and spleen of four week old Lewis rats. Forty-eight hours later, β-galactosidase activity was observed in the spleen (Fig.1A, top, left), but not in the liver (Fig.1A, top, right). Examination of 30 $\mu$m cryostat sections by stereomicroscopy demonstrated that approximately 1/1000 spleen cells, but no hepatocytes, were infected (data not shown). Spleen and liver from PBS injected rats did not contain blue cells after incubation in X-gal (Fig. 1A, bottom). Despite the failure to infect hepatocytes in vivo, Hepa1, a cell line derived from a murine hepatoma, was easily infected by ecotropic BAG. The frequency of Hepa1 infection was 1-10% of that observed for murine NIH3T3 fibroblasts (Fig.1B and D).

The susceptibility of cultured primary hepatocytes to ecotropic MuLV infection was examined by exposing the cells to BAG virus 24-48 hours after plating and staining with X-Gal substrate

two days later. Aquired ß-galactosidase activity could be seen in hepatocytes from 4 weeks old rats exposed to ecotropic BAG virus, but not in controls incubated with polybrene containing medium (Fig.2).

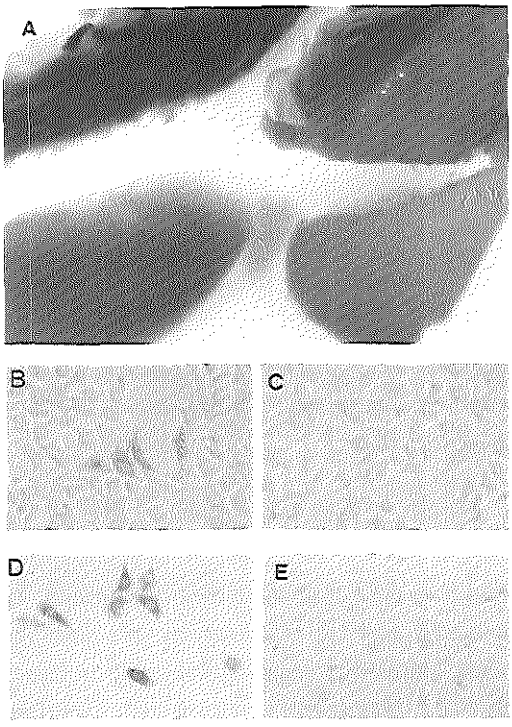


**Figure 1.** ß-Galactosidase activity in tissues and cells assayed two days after exposure to ecotropic BAG virus, a recombinant MuLV, that contains the ß-Gal gene in its genome. A: spleen (left) and liver (right) from rats injected with ecotropic BAG (top) or from PBS-injected control animals (bottom). B,C: Hepa1 mouse hepatoma cells. D,E: NIH3T3 mouse fibroblasts. B and D: cells exposed to ecotropic BAG virus. C and E: cells exposed to polybrene containing media.

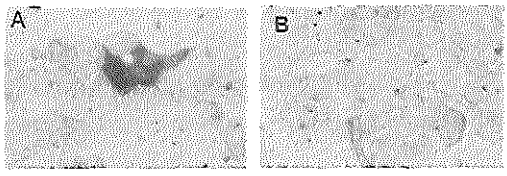


**Figure 2.** Hepatocytes isolated from 4 week old rats and grown for one day on a single collagen layer in SUM media were exposed to A) ecotropic BAG virus, or B) polybrene containing media. Two days later the cells were stained with X-Gal.

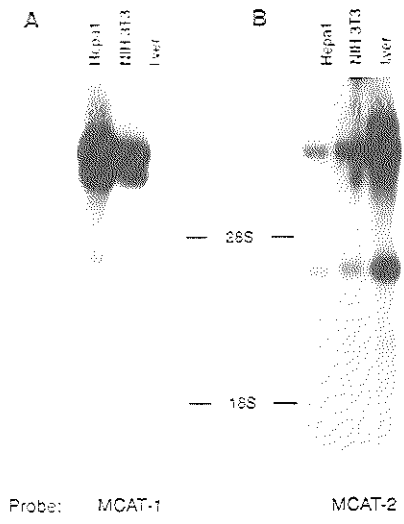


**Figure 3.** Northern blot analysis with poly A$^+$-enriched RNA (5 $\mu$g per lane) from mouse hepatoma cells (Hepa1), mouse fibroblasts (NIH3T3) and adult mouse liver. The blot was (A) hybridized with a MCAT-1 cDNA probe, (B) deprobed and hybridized with a MCAT-2 cDNA probe.

In order to investigate the role of ecotropic MuLV receptor expression in BAG infection, we measured CAT-1 mRNA levels in hepatocytes by northern blot analyses. We observed comparable, steady state levels of the 7.9 kb and 7 kb CAT-1-encoding transcripts in Hepa1 cells and NIH3T3 fibroblasts, but did not detect CAT-1 transcripts in normal adult liver (Fig. 3A). Expression of CAT-1 was also absent from primary hepatocytes investigated directly after isolation, but was induced in these cells when they had been cultured for 2 days (Fig. 4A, lanes 5 and 6). In contrast, induction of expression was not observed in regenerating liver tissue examined between 12 and 48 hours after partial hepatectomy (Fig. 4A, lanes 1-4).

Hepatocytes plated on a single collagen matrix lost viability within four days after plating. To prevent their deterioration, we applied an additional collagen layer over the cells to create a monolayer of cells between two layers of collagen (double gel culture). In contrast to the

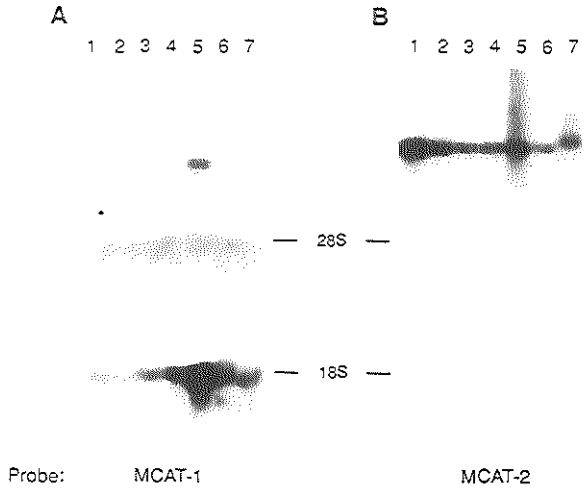


**Figure 4.** Northern blot analysis using total RNA (20 $\mu$g/lane) and hybridized as described in Fig. 3: (A) with MCAT-1 probe, (B) with MCAT-2 probe. RNA was isolated (lanes 1-4) from regenerating rat liver (1) 12 h; (2) 24 h; (3) 36 h; (4) 48 h after partial hepatectomy; (lanes 5-6) from primary rat hepatocytes (5) after 2 days in culture; (6) immediately after isolation of the cells from adult liver; (lane 7) from normal adult mouse liver.

conventional single gel configuration, this double gel technique permits hepatocytes to remain viable for at least several weeks. In previously reported studies, cells treated in this fashion were shown to recover their capacity to secrete plasma proteins, such as albumin and fibrinogen, and to respond to cytokines, both established properties of differentiated hepatocytes (3,8,9). Indeed, hepatocytes prepared from four week old Lewis rats, grown in a double gel in SUM media resumed albumin secretion within seven days after plating and maintained a stable rate of production over the following two weeks (Fig. 5).

We hypothesized that establishment of double gel culture might prevent the induction of CAT-1 expression in hepatocytes. However, despite recovery of albumin production (Fig. 5), CAT-1 mRNA remained present for up to 23 days in cells from double gel cultures (Fig. 6 A). In addition, CAT-1 expression was not influenced by the addition of fetal bovine serum (Fig. 6

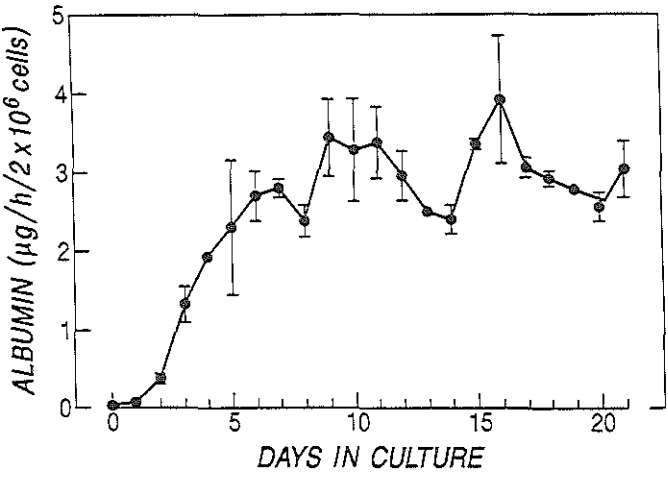


**Figure 5.** Albumin secretion of hepatocytes from 4 week old rats, cultured in a double collagen gel configuration. Data points represent duplicate measurements of 4 culture dishes from one particular cell batch (mean ± SD). Similar results have been obtained using other cell preparations.

B), the presence of a second collagen layer (Fig. 6C), or by a reduction of the concentration of growth factors in the medium (Fig. 6 A, lane 8). The infectivity of these cells could not be examined, due to the mechanical barrier between virus and cells created by the top collagen layer (data not shown).

*Expression of CAT-2 and susceptibility to infection with amphotropic BAG virus*

We examined the expression of CAT-2 by hybridization of the blots in Figs. 3A, 4A and 6C with $^{32}$P-MCAT-2, after stripping of the MCAT-1 probe. The 8.5 and 4 kb transcripts that encode CAT-2 were detected in RNA from liver in vivo as well as in Hepa1 and NIH3T3 cells in vitro (Fig. 3B). Furthermore, comparable, steady state levels of CAT-2 RNA were encountered in regenerating liver obtained 12-48 h post-hepatectomy (Fig. 4B lanes 1-4), and in cultured primary hepatocytes, maintained in a variety of culture conditions (Fig. 4B, lane 5 and Fig. 6D). Unlike CAT-1, CAT-2 expression in cultured hepatocytes was not significantly different from that observed in intact liver (Fig. 4B, lane 7).

Others have previously demonstrated successful infection of cultured hepatocytes and regenerating liver by amphotropic MuLV (13,27). However, normal adult liver cannot be infected by viruses from this MuLV subgroup. This finding does not exclude the possibility

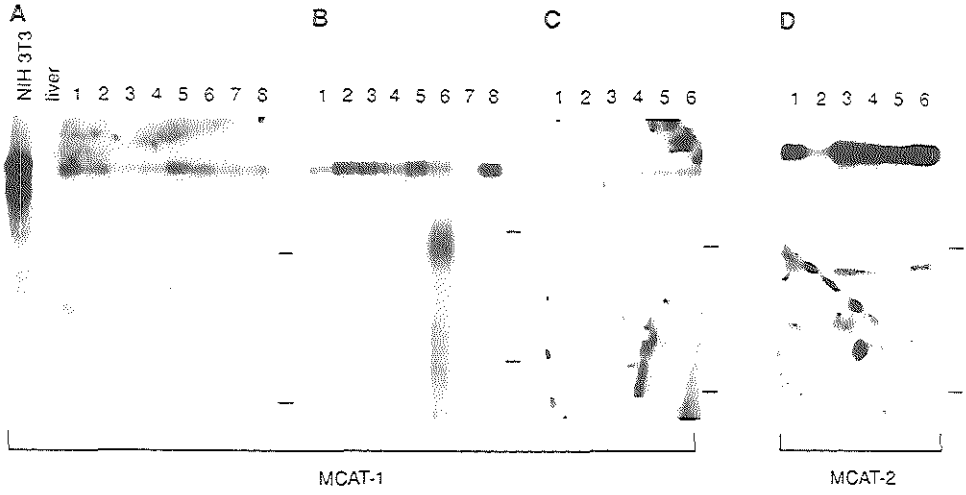


**Figure 6.** Northern blot analysis with total RNA (20 $\mu$g per lane) hybridized (A-C) with a MCAT-1 cDNA probe and (D) with a MCAT-2 cDNA probe. RNA was isolated from rat hepatocytes grown in culture for different time periods and under different culture conditions. (A) RNA from NIH3T3 cells, mouse liver and from hepatocytes isolated from 2 month old rats and cultured in DMEM with high growth factor concentration; (1 + 2) single gel cultures grown for (1) 1 day and (2) 7 days; (3 - 5) double gel cultures, grown for (3) 3 days, (4) 7 days and (5) 14 days; (6 + 7) single gel culture with a second collagen layer added on day 6, grown for (6) 7 days and (7) 14 days; (8) double gel culture in DMEM with low growth factor concentration, grown for 23 days. (B) RNA from hepatocytes isolated from 4 week old rats and grown in SUM media: (1-3) cells grown on a single collagen gel, media containing 10% fetal calf serum; (4-6) cells grown without a collagen gel, media containing 10% fetal calf serum; (7+8) cells grown on a single collagen gel, media containing no serum; culture periods were (1, 4 + 7) 1 day; (2, 5 + 8) 2 days; (3 + 6) 4 days. (C) RNA from hepatocytes isolated from 2 month old rats and cultured in DMEM with high growth factor concentration on a single collagen gel; culture periods were (1) 1 day; (2) 2 days; (3) 3 days; (4) 4 days; (5) 5 days; (6) 7 days. (D) Same RNAs as in (C) but hybridized with a MCAT-2 probe.

that CAT-2 might be a receptor for amphotropic MuLV, because successful infection may require additional factors not expressed in differentiated hepatocytes in vivo. To examine a possible role for CAT-2 in amphotropic MuLV infection directly, CHO-K1, a Chinese hamster-derived cell line that is neither susceptible to amphotropic nor to ecotropic MuLV infection, was transfected with expression plasmids containing CAT-1, CAT-2, or CAT-2/Tea, respectively. Western blots using a polyclonal antiserum directed against the C-terminus shared by both CAT-2 and Tea verified expression in the transfected cells (data not shown). Clones of CHO-K1 cells that expressed CAT-1 or either version of CAT-2 were exposed to amphotropic or ecotropic BAG virus and two days later, infected cells were identified by X-Gal staining. As expected, CHO-K1 cells that expressed CAT-1 had acquired susceptibility to infection by ecotropic BAG (Table 1). In contrast, cells transfected with CAT-2 or CAT-2/Tea were not infectible with amphotropic BAG. Both amphotropic and ecotropic BAG viruses infected NIH3T3 fibroblasts equally well.

**Table 1.** Percentage infected cells after exposure to amphotropic or ecotropic BAG virus.

| cell line | amphotropic BAG | ecotropic BAG | no virus |
|---|---|---|---|
| NIH3T3 | 8.2 | 7.8 | 0 |
| CHO K1/MCAT-1 | 0 | 5.3 | 0 |
| CHO K1/MCAT-2 | 0 | 0 | 0 |
| CHO K1/MCAT-2 /Tea | 0 | 0 | 0 |

## Discussion

We have demonstrated that the susceptibility of rat hepatocytes to infection by ecotropic MuLV is correlated with the expression of the ecotropic virus receptor (rodent CAT-1). CAT-1 expression was observed in primary hepatocytes plated on a collagen matrix that acquired susceptibility to ecotropic BAG infection. In contrast, ecotropic receptor expression was absent in vivo in both normal, and regenerating adult liver, at time points shown by others to be associated with maximal cell proliferation (5,23), and sensitivity to infection by amphotropic MuLV (13), but not by ecotropic MuLV (17).

Primary rat hepatocytes in culture were 100-1000-fold less infectible than fibroblasts, suggesting that either the amount of receptor protein on the surface of these cells is low, or that other factor(s) required for infection are limiting. MuLV infection of primary hepatocytes was achieved during the first two days in culture when $^{3}$H-thymidine incorporation into nuclei was greater than 50% (data not shown), indicating a high rate of DNA synthesis. Therefore, factors related to DNA replication are unlikely to explain the difference in infectivity.

The factors responsible for induction of ecotropic receptor expression in cultured hepatocytes are not known. The use of culture conditions that allow hepatocytes to maintain a well differentiated status, characterized by production of plasma proteins, urea, bile salts, cytochrome P450 (8,9), and the ability to respond to cytokines (3), did not suppress CAT-1 expression. Furthermore, receptor expression was induced within a few hours in culture (data not shown). As has been previously suggested, the absence of CAT-1 expression in liver may be important to prevent access of the plasma arginine pool to the high levels of hepatic arginase, the enzyme that catalyses hydrolysis of arginine into ornithine and urea. This suggests that the regulation of CAT-1 expression might be coupled to the urea cycle (32,33). Indeed, we have previously observed that urea production is decreased in cultured hepatocytes, even under conditions associated with recovery of albumin and fibrinogen secretion (8). Alternatively, CAT-1 expression might also be repressed as a consequence of the close association between hepatocytes and non-parenchymal cells in the intact liver. CAT-1 expression in vitro could, therefore, be induced by separation of the hepatocytes from non-parenchymal cells (e.g. endothelial cells and Kupffer cells). Further experiments using cocultures of hepatocytes with endothelial and/or Kupffer cells are needed to answer these questions.

Based on chromosome mapping studies and similarity to CAT-1, it has been proposed that CAT-2 might be a receptor for amphotropic MuLV (22). We found CAT-2 expression in primary hepatocytes in vitro and in hepatocytes from regenerating liver, cells which are reportedly susceptible to amphtropic MuLV infection (13,27). However, expression of either of the two alternatively spliced forms of CAT-2 in nonpermissive Chinese hamster ovary cells was not sufficient to confer infectivity by amphotropic BAG using a protocol suitable to demonstrate CAT-1-mediated infection by ecotropic MuLV.

The expression of the ecotropic receptor may be sufficient to determine successful infection of primary hepatocytes in culture by ecotropic MuLV. This conclusion is supported by the absence of CAT-1 expression in regenerating liver, a tissue that is not susceptible to ecotropic viruses, but can be infected by amphotropic MuLV (17,21). Receptor expression, therefore,

appears to be an important determinant of retroviral tissue tropism. The finding that ecotropic MuLV infection of hepatocytes can only be achieved in vitro does not preclude the use of these viruses as vectors in gene therapy, since in vitro infected autologous hepatocytes were recently shown to be able to survive and function after re-implantation into the donor animal (26,35). Additional investigation of the function and regulation of retrovirus receptors will improve the ability to use retroviruses to target genes to specific cells or tissues.

**Abbreviations used:**

CAT: cationic amino acid transporter
MCAT: mouse cationic amino acid transporter
IU: infectious unit
MuLV: murine leukemia virus

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## CHAPTER 8

# Evidence of metabolic activity of adult and fetal rat hepatocytes transplanted into solid supports

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**Abstract**

This study was undertaken to assess the metabolic effect of fetal and adult hepatocyte transplantation in the Gunn rat, genetically incapable of bilirubin conjugation. A comparison was made between fetal and adult hepatocytes transplanted into the spleen, and those injected into polytetrafluoroethylene (PTFE) solid supports, that had previously been implanted intraperitoneally. Between 4 and 12 weeks after intrasplenic transplantation of adult liver cells, serum bilirubin was significantly decreased when compared with control animals ($39.6 \pm 5.6$ %; $p<0.01$ vs. controls). Intrasplenic transplantation of fetal hepatocytes resulted in a maximal decrease of $33.2 \pm 9.1$ % at 8 weeks postoperatively ($p<0.02$ vs. controls). Similar declines of serum bilirubin levels were found after transplantation of adult or fetal liver cells into the solid supports. At 12 weeks after transplantation, bilirubin conjugates were detectable in the bile of all animals that underwent intrasplenic hepatocyte transplantation and in 60% of those that underwent the solid support procedure, whereas none could be detected in control animals. Histological evidence of surviving cells was obtained in all but one animal at 12 weeks, and confirmed at 12 months postoperatively. It is concluded that the (PTFE) solid support technique offers an attractive alternative to the intrasplenic route, and that both fetal and adult hepatocytes, transplanted in either way, still exert their conjugating activity after 12 weeks.

## Introduction

Orthotopic as well as heterotopic liver transplantation are now established techniques to treat end-stage liver disease (19,20). Both modalities are also capable of correcting metabolic defects resulting from inborn errors of liver metabolism (11,19). However, they are still associated with considerable morbidity, mortality, and cost.

In patients with an inborn error, the liver's structural integrity is primarily undisturbed as the genetic defect involves only one enzymatic function (18). The long term objective in treating these patients should therefore be to correct the genetic defect in the host's hepatocyte genome, whereafter the hepatocytes should be re-implanted into the patient. Such a strategy can only succeed, if techniques for hepatocyte transplantation (HTX) are available, that lead to sustained metabolic activity of the cells after retrovirus-mediated gene transfer and transplantation. In rat models several techniques for HTX have been explored, of which the intrasplenic route has been most widely used (3,6,9,12,23). More recently, attempts have been described to transplant cells with, or into different so-called solid supports, with varying degrees of success (1,21,22). The concept of solid supports is attractive, because they can provide a threedimensional scaffold to which implanted cells could adhere following in vitro manipulation and transplantation. In addition, they do not pose the threat of portal thrombosis or hemorrhage seen in intraportal or intrasplenic transplantation, and, theoretically, do not limit the number of cells to be inoculated. The recent description of neovascularization of polytetrafluoroethylene (PTFE) solid supports is promising, since this would circumvene problems related to the initial vascular supply and nutrition of the implanted cells (21).

The present study was performed to assess the metabolic activity of rat hepatocytes transplanted into prevascularized PTFE solid supports in Gunn rat recipients. The homozygous Gunn rat suffers from a genetic defect in bilirubin conjugation, resulting in severe unconjugated hyperbilirubinemia and absence of conjugates in the bile. Non-icteric heterozygous littermates, used as donors in this study, are capable of glucuronide formation, but to a lesser extent than normal rats (25). A comparison was made with the 'classic' intrasplenic hepatocyte transplantation technique. Also, the effectiveness of transplantation of adult hepatocytes on bilirubin conjugation was compared with that of fetal liver cells. To our knowledge, this is the first report describing such comparisons. Furthermore, the formation of biliary bilirubin conjugates by hepatocytes transplanted into solid supports has never been demonstrated. Fetal liver cells may also be of benefit in the concept of retrovirus-mediated gene

transfer, since they would seem to be more susceptible to retroviral infection (7, own unpublished results).

## Materials and Methods

*Animals.* Approximately 8 weeks old homozygous Gunn-rats (RHA/N- J/J) were used as recipients. As donors for adult hepatocytes, ca. 8 weeks old heterozygous rats (RHA/N- J/+) were used. Eighteen days old RHA/N- J/+ fetuses were the source of fetal liver cells. Rats were purchased from Harlan Sprague Dawley Inc., Indianapolis, IN, USA. All animals were kept on standard laboratory chow and water ad libitum. Approval of the Committee for Animal Research of the Erasmus University, Rotterdam, The Netherlands, was obtained before the experiments were carried out.

*Hepatocyte isolation.* For the isolation of adult hepatocytes, a modification of the two-step collagenase perfusion technique as described by Seglen was used (16). After cannulation of the portal vein, the liver was perfused for approximately 10 min with $Ca^{++}$-free Berry buffer (2) at a flow rate of 15-20 ml/min. The perfusion was gassed with a mixture of 95% $O_2$ and 5% $CO_2$, and kept constantly at a temperature of 37°C and a pH of 7.4-7.5. When the liver had discoloured and the perfusate become clear, recirculation was started with a 0.05% collagenase (type IV, Sigma Chemicals, St. Louis, MO, USA) and 5mM $Ca^{++}$ containing Hanks' balanced salt solution. After 10 min of recirculation, the liver was excised and the capsule removed to free the cells. The initial cell suspension was filtered through a 200 $\mu$m sieve, washed in Hanks' salt solution, and centrifugated for 3 min at low speed (50 g). This cycle was repeated for 3-4 times, until the supernatant was clear. Next, the cells were counted, resuspended in Williams-E medium (Gibco Life Technologies, Grand Island, NY, USA) at a concentration of $2.10^7$ viable cells per ml, and stored at 4°C until transplantation.

Fetal cells were obtained through a modification of the collagenase digestion method as described by Radford and Bhathal (15). Fetal livers were rinsed free of blood in Hanks' solution. They were then minced with a surgical blade in Hanks' solution containing 10% newborn calf serum, 0.5 mM EGTA, and 80 $\mu$g/ml DNAse (Type I, Sigma). Subsequently, the pieces were placed in 10 ml of Hanks' solution containing 10% newborn calf serum, 80 $\mu$g/ml DNAse, and 3 mg/ml collagenase type IV, and incubated for 5 min at 37°C under constant agitation. The medium containing the dissociated cells was replaced by Hanks' solution containing 10% newborn calf serum and 1 mM EGTA. To the remaining undissociated pieces fresh digestion solution was added and the process repeated. The cells

from the two digestions were then filtered, washed, and allowed to settle down twice for 20 min to allow separation from hemopoietic cells. Cell viability as assessed by trypan blue exclusion ranged from 80% to 95%.

*Solid supports.* Solid supports were obtained through the courtesy of W.L. Gore & Associates Inc. (Flagstaff, AZ, USA), and consisted of multifilament, angel-hair fibers of expanded polytetrafluoroethylene (PTFE). They were prepared by a modification of the method described by Thompson et al. (21). Sterile PTFE fibers were coated with collagen IV for 2 hrs at 37°C, followed by air-drying overnight. Supports of 0.25 g dry weight were incubated for 2 hours with Williams-E medium, and subsequently implanted into the abdominal cavity of the recipient animal.

*Transplantation technique.* A cell suspension of ca. 1 ml, containing $2.10^7$ viable cells, was prepared. This volume was then injected into the splenic parenchyma, or into a solid support that had been implanted into the peritoneal cavity 4 weeks earlier. During the intrasplenic injection the hilar vessels were clamped. Leakage of cell suspension and blood was minimized by tamponade of the injection site with a cotton tip. All animals belonging to the six groups defined below received immunosuppression by intramuscular administration of 15 mg/kg cyclosporin A on days 0, 3, and 7, and weekly thereafter. All procedures were performed under ether anaesthesia.

*Experimental design.*

The experimental groups were defined as follows:

Group A: Intrasplenic HTX of $2.10^7$ viable fetal hepatocytes (n=6)

Group B: HTX into a solid support of $2.10^7$ viable fetal hepatocytes (n=6)

Group C: Intrasplenic HTX of $2.10^7$ viable adult hepatocytes (n=9)

Group D: HTX into a solid support of $2.10^7$ viable adult hepatocytes (n=6).

Control groups were as follows:

Group E: Intrasplenic HTX of $2.10^7$ viable, but lethally irradiated adult hepatocytes (i.e., 60 Gy prior to HTX) (n=6)

Group F: HTX into a solid support of $2.10^7$ viable, but lethally irradiated adult hepatocytes (n=6).

*Evaluation.* Blood samples (maximal volume 1 ml) were collected from the retro-orbital space two days before HTX, and weekly thereafter. From these, serum bilirubin levels were estimated. At 12 weeks postoperatively the common bile duct was cannulated for collection of

bile samples in dim light, followed by sacrifice of each animal for histological examination of the spleen or the solid support. Bile samples of approximately 2 ml were kept at -60°C until measurement of bilirubin and its conjugates by means of the high-pressure liquid chromatography (HPLC) method as described by Spivak and Yuey (17). For histological examination tissues were fixed in 10% buffered formaldehyde, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E), as well as by the periodic acid Schiff (PAS) procedure. In addition, each experimental group (A-D) was supplemented with one animal for long term histology. These animals were kept alive for 12 months postoperatively.

*Statistical analysis.* Values are expressed as means ± SEM. To eliminate the effect of inter-animal variation caused by differing pretransplant values of serum bilirubin, subsequent measurements in each rat were related to these values, and expressed as percentages of the preoperative bilirubin level in that rat. A one-way analysis of variance was used to compare differences in related total serum bilirubin levels between the groups. In case of significant differences (i.e. an F-value indicating a probability of $<0.05$) the Newman Keuls' test was applied to find which pair(s) of means were statistically significant (26). For comparison of the results between groups of bile analyses the non-parametric Mann-Whitney U-test was used (13).

## Results

Fig. 1 shows the course of total serum bilirubin levels after intrasplenic HTX. During the first 3 postoperative weeks a mild decrease in serum bilirubin of approximately 15 % was observed in all groups. Between 4 and 12 weeks post-HTX serum bilirubin in group C (adult cells) had dropped to significantly lower values than present in the control group. In the case of group A (fetal hepatocytes) a comparable reduction of bilirubin levels was observed between 6 and 12 weeks after HTX. At 12 weeks postoperatively the difference between the three groups had lost significance.

The comparable effect on serum bilirubin of HTX in solid supports is demonstrated in Fig. 2. In all groups a decline in serum bilirubin levels was noted during the first 2 postoperative weeks, followed by a gradual return to pretransplant values in group F (control), and a further decline to significantly lower values in both experimental groups B (fetal cells) and D (adult cells) during weeks 5 to 10. Again, no significant differences between the three groups were observed at 12 weeks post-HTX.

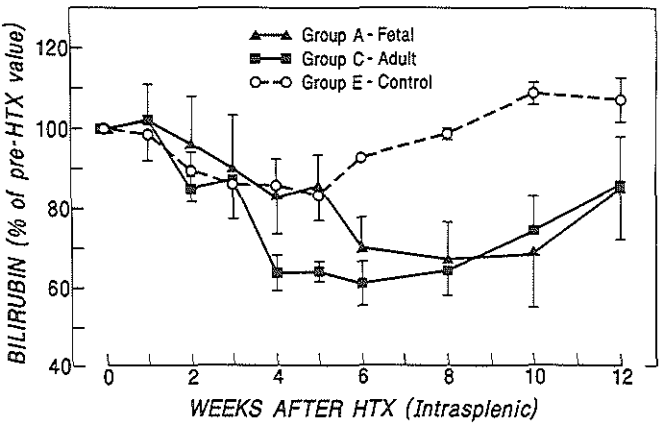


**Figure 1.** Course of total serum bilirubin levels, expressed as percentages of initial, i.e. pretransplant levels (mean±SEM), after intrasplenic transplantation of fetal (A), adult (C), or irradiated donor hepatocytes (E, control). At 4, 5, 6, 8, and 10 weeks post-HTX there was a significant reduction of bilirubin levels in group C when compared with group E ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.001$, and $p<0.02$, respectively). A maximal decrease to 60.4 ± 5.6 % of pretransplant levels was found at 6 weeks post-HTX, representing a decline from 159.8 ± 7.6 $\mu$M to 96.1 ± 5.6 $\mu$M. A similar pattern was observed in group A at weeks 6, 8, and 10 ($p<0.05$, $p<0.02$, and $p<0.05$, respectively) with a maximal decrease to 66.8 ± 9.1 % of pretransplant levels at week 8.

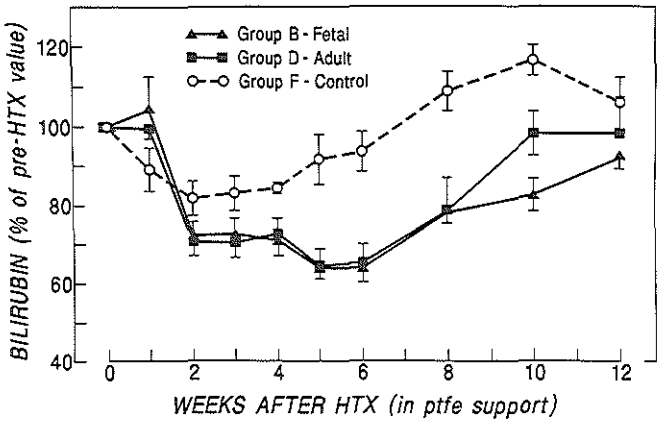


**Figure 2.** Course of total serum bilirubin values, expressed as percentages of pretransplant levels (mean±SEM), after transplantation of fetal (B), adult (D), or irradiated donor hepatocytes (F, control) into collagen coated supports consisting of PTFE 'angel hair' fiber. Between 5 and 10 weeks post-HTX a significantly greater reduction of serum bilirubin levels was observed in groups B and D, than in group F ($p<0.01$, $p<0.01$, $p<0.05$, and $p<0.02$, respectively for group B; $p<0.01$ for group D). Maximal reduction was seen at 5 weeks post-HTX in both groups (63.6 ± 3.0%, and 64.1 ± 3.8% of pretransplant values, respectively).

At 12 weeks after HTX bilirubin-mono-glucuronide was present in all bile specimens of groups A and C (12.6 ± 4.1 , and 15.2 ± 2.3 % of total biliary bilirubin, respectively). Mono-glucuronide was also found in 3/5 of the specimens of group B , and in 4/6 of the ones of group D (respective mean conjugated fractions of 3.2 ± 1.6, and 2.2 ± 1.5 %). Di-glucuronide was only detectable in all bile specimens of group C and in one of group B. In the control groups E and F no biliary bilirubin conjugates were found (Fig. 3).

On opening the abdominal cavity in groups B, D, and F before the actual transplantation, i.e. 4 weeks after insertion of the solid support, macroscopical signs of peritonealization and vascularization of the supports were present without exception (Fig. 4). At 12 weeks post-HTX the white color of the supports on cross section had changed into brownish; those spleens that had served as inoculation sites for HTX invariably revealed macroscopically identifiable areas of a similar light-brown color. On histological examination at 12 weeks postoperatively, morphologically intact hepatocytes could be detected in all spleens of the animals of groups A and C. As a rule, hepatocytes were distributed as clusters throughout the

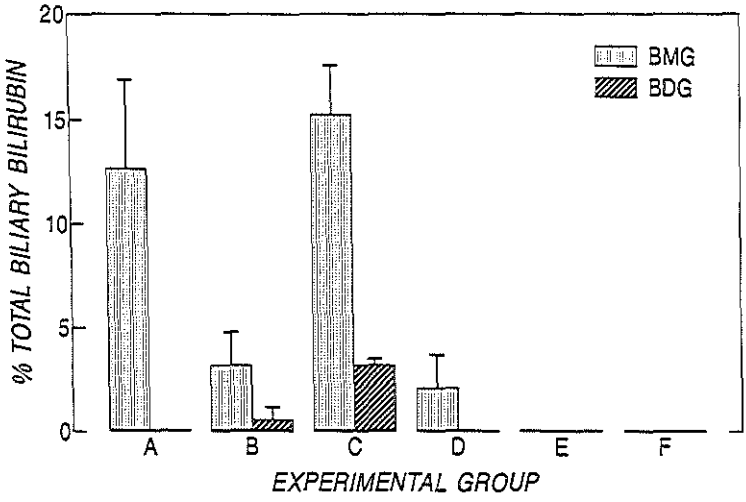


**Figure 3.** Results of bile analyses of samples at 12 weeks after HTX. Conjugated bilirubin was identified as bilirubin-mono-glucuronides (BMG) or bilirubin-di-glucuronides (BDG). Detected fractions are given as mean percentages ± SEM of total biliary bilirubin. In the bile samples of the animals in the control groups (E and F) no conjugates could be demonstrated. Formation of bilirubin glucuronides was more pronounced in groups A and C (intrasplenic HTX of fetal, and adult liver cells, respectively), than in groups B and D (HTX in PTFE-support of fetal, and adult cells, respectively) (group C vs. D: $p<0.01$; group A vs. B: not significant).

splenic parenchyma and hepatic cord formation was present. When fetal cells had been transplanted, bile duct proliferation was more outspoken than in the case of adult cells (Fig. 5). In addition, intact hepatocytes could be identified in histological sections of the solid supports of all animals in group B and all but one of group D. The distribution of hepatocytes inside the supports is illustrated in Fig. 6. They were generally arranged in clusters amidst the PTFE texture and found to be more numerous in the peripheral regions of the supports, when compared to the more central zones. However, central cell localization was not exceptional. Microscopical signs of neovessel formation were frequently present. As in groups A and C, PAS stains always revealed the presence of glycogen granules in the cytoplasm of the surviving hepatocytes. Surviving hepatocytes were also readily detectable in all histological sections of the spleens and supports harvested at 12 months after HTX. Again, these cells stained positively with the PAS stain and revealed cord-like architecture. No hepatocytes were seen in the spleens or supports of the animals in the control groups E and F.

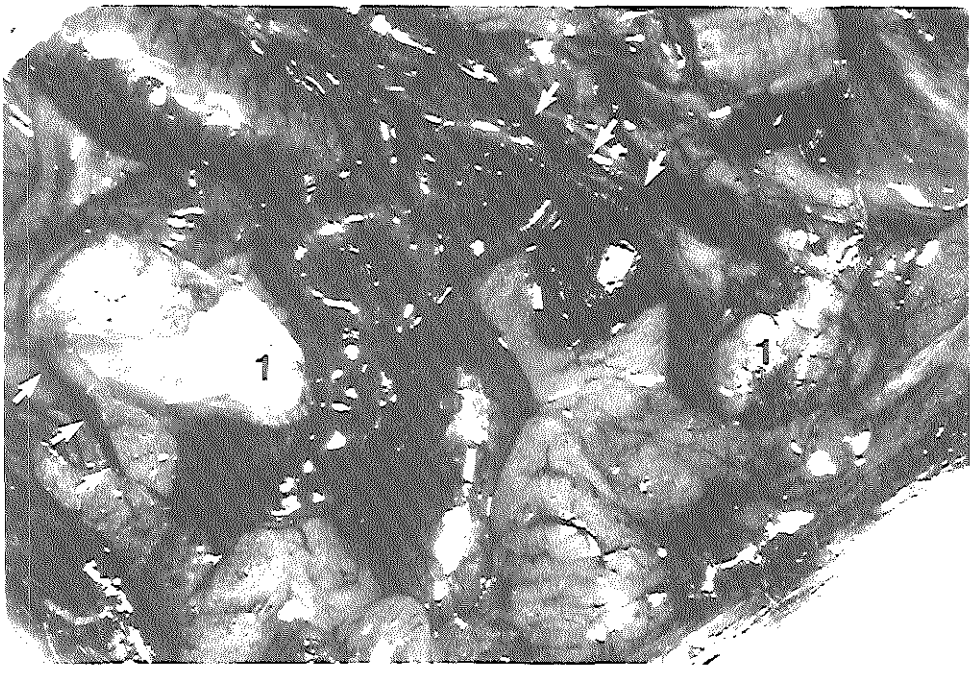


**Figure 4.** Macroscopical appearance of two PTFE solid supports 4 weeks after implantion into the peritoneal cavity (this animal was not included in the experimental groups mentioned, but the demonstrated features are characteristic). The supports are marked (1), and are covered with neo-peritoneum. Large vascular structures can be seen running towards and into the supports (arrows).

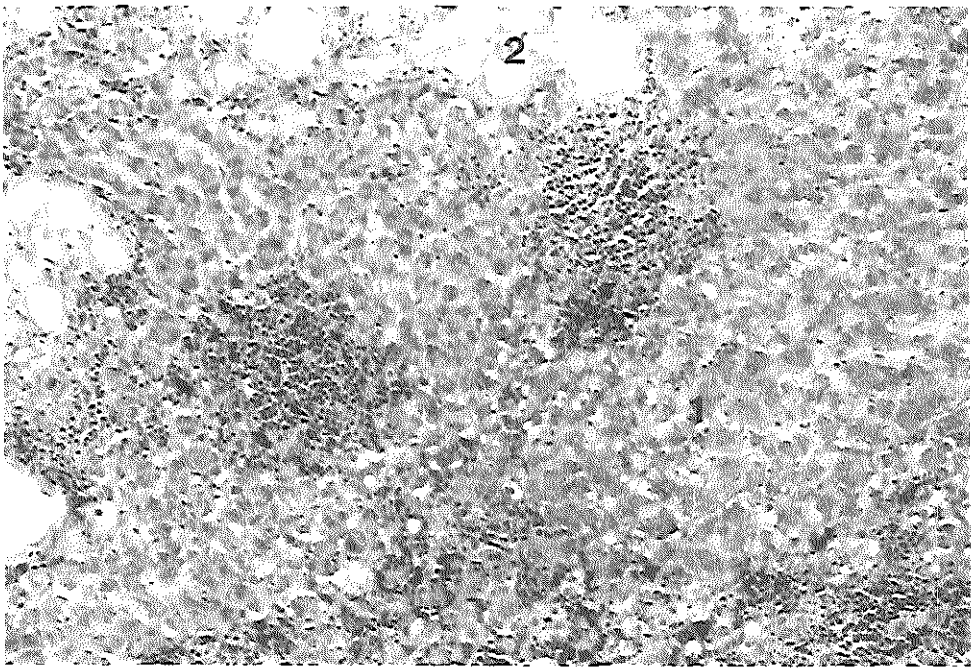


**Figure 5.** Histological section 12 weeks after intrasplenic transplantation of fetal donor hepatocytes. Large clusters of intact hepatocytes (1) are visible. There is marked bile duct proliferation (2). (H&E stain; 40x)

## Discussion

In this study we demonstrated, for the first time, that transplantation of adult and fetal hepatocytes into either PTFE solid supports or the spleen results in comparable, temporary reductions of total serum bilirubin levels in the Gunn rat recipients used. The presence of glucuronides in the bile 3 months after HTX indicates conjugational activity of the cells in all four experimental groups and offers the first description of biliary glucuronide excretion after HTX into solid supports. Histological examination confirmed these findings by demonstrating intact hepatocytes in all but one recipient animal at 12 weeks. Long term survival was confirmed histologically at one year postoperatively.


**Figure 6.** Histological section of PTFE 'angel hair' solid support 12 weeks after transplantation of adult donor hepatocytes. Amidst the PTFE texture (1), clusters of intact hepatocytes (2) can be identified. Cord-like architecture is observed. Note the neovessel formation (3) inside the support (H&E; 40x).

Although several investigators reported decreasing serum bilirubin levels after HTX in Gunn rats, only some presented data on biliary glucuronide formation by the transplanted cells as evidence of functioning hepatocellular grafts (3,23). Our observations support these data.

The discrepancy found between serum bilirubin levels and biliary glucuronide excretion may be explained by the number of successfully engrafted hepatocytes being too small to exert an effect on the total bilirubin level in the serum. It is also possible that the conjugating capacity of the heterozygous donor cells was not sufficient for sustained reduction of total serum bilirubin levels. We chose heterozygous rats to serve as donors in order to reduce the possibility of rejection, since they are bred selectively by backcrossing homozygous Gunn rats with the original Wistar (RHA/N-)rats of which the Gunn is a mutant strain. The strain of Gunn rats

used was the offspring of selective crossing of homozygous mutants. The original experiments were performed under immunosuppression, since a considerable genetic variability of the existing Gunn rat strains has been reported (10). Once the data in immunosuppressed animals were obtained, an additional set of experiments was carried out in order to ensure that the decrease of serum bilirubin values was not due to the administration of cyclosporin A. In these experiments groups C-E were repeated without the use of immunosuppression, resulting in bilirubin patterns similar to the ones presented (data not shown).

The course of serum bilirubin after HTX of irradiated cells (control groups E and F) showed a decrease in the first 3-4 weeks, thereafter returning to pretransplant values. Possibly, the alteration in function of the reticulo endothelial system because of the transplantation as proposed by Woods et al. plays a role in this phenomenon (24). It is also conceivable that the irradiated hepatocytes retained their metabolic activity during this period before degenerating.

Furthermore, it must be noted that total serum bilirubin levels may show considerable inter- and even intra-animal variation. This may in part be due to the interference of bilirubin with sodium and urea transport in the renal medulla, leading to increased susceptibility of Gunn rats to dehydration by polyuria (14). For this reason we related the absolute total serum bilirubin values measured per animal to the pretransplant level in that animal.

HTX of both fetal and adult hepatocytes into PTFE 'angel hair' fiber supports did not lead to the prolonged decline of serum bilirubin as described by Thompson et al. (21). It rather resulted in a course of serum bilirubin similar to that observed after intrasplenic HTX in this study. Moreover, in 60 % of the bile specimens examined in both groups we were able to detect biliary glucuronides indicating conjugational activity, whereas none could be detected in the control animals. Quantitative evaluation showed a significantly lower amount of conjugates in the rats with supports than in those that had undergone intrasplenic HTX. Perhaps, omitting heparin-binding growth factor 1 (HBGF-1) from the coating process did have an adverse effect on hepatocyte survival in the supports, since it has been reported that this factor may not only enhance neovessel formation, but also stimulates hepatocyte proliferation in vitro (8). We excluded HBGF-1 from the coating process, after pilot studies performed by us had failed to show it to be uniformly essential for hepatocyte survival or function (unpublished results). In these studies we also observed neovascular formation without the addition of the growth factor (both macroscopically and histologically). Indeed, on reopening the peritoneal cavity for transplantation 4 weeks after insertion of the supports we encountered remarkable macroscopical signs of vascularization (Fig. 4).

The PTFE supports offer great possibilities for gene therapy experiments with the ultimate goal of treating a patient with an inborn error of liver metabolism by autotransplantation of his/her hepatocytes after correction of the genetic defect by retrovirus-mediated gene transfer (5). To date, only one in vivo study reported genetically modified hepatocytes to be successfully transplanted into a collagen support (1). But an important limitation of collagen supports is their rapid reabsorption in vivo, with subsequent loss of the threedimensional scaffold to which the cells adhere. This disadvantage is circumvened by the nonabsorbable PTFE fiber supports. Furthermore, the latter can be site-specifically implanted into the peritoneal cavity prior to the actual transplantation, with pre-vascularization and its putatively advantageous effects on (auto)graft nutrition and survival as a consequence. This means that their potential application is not limited to hepatocellular manipulation and transplantation alone, but that they could be used as a receptacle for a variety of other cell types.

Histological examination revealed clusters of intact hepatocytes in all but one animal of the experimental groups. The increased tendency to bile duct proliferation after intrasplenic transplantation of fetal hepatocytes is in concordance with the observations of other investigators (4). Glycogen particles were present in all hepatocellular grafts as demonstrated by the PAS staining procedure, again indicating their metabolic activity.

In conclusion, graft function in the Gunn rat model used can be assessed best by estimation of bilirubin conjugates in the bile and not by total serum bilirubin levels. Although the intrasplenic route for the transplantation of adult hepatocytes remains the 'golden standard', we showed that inoculation of fetal hepatocytes in a recipient spleen is equally effective. We demonstrated conjugational activity of hepatocytes, both fetal and adult, engrafted in PTFE solid supports. These supports provide an attractive alternative technique for hepatocyte transplantation, certainly in the light of future developments in gene therapy.

**Acknowledgement**

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## CHAPTER 9

# Proliferative responsiveness of rat hepatocytes transplanted into spleen or solid support

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Submitted for publication
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## Abstract

Understanding the regenerative behavior of transplanted hepatocytes is of great importance for developing and improving such novel therapeutic strategies as hepatocellular transplantation and ex-vivo gene therapy. In this study the proliferative responsiveness of transplanted syngeneic rat hepatocytes was examined in relation to the timing of the administration of a mitogenic stimulus. For this purpose nuclear BrdU incorporation after partial hepatectomy was investigated during the early post-transplant phase. The response of intrasplenically transplanted hepatocytes was compared to that of liver cells engrafted in polytetrafluoroethylene solid supports that had been implanted intraperitoneally 4 weeks prior to transplantation. Non-stimulated, engrafted hepatocytes exhibited a labeling index of ~0-1% independent of the transplantation technique used. This "spontaneous" labeling index did not change with time. Partial hepatectomy, executed simultaneously with hepatocyte transplantation through either technique, did not result in significant alteration of this proliferation index. Delayed kinetics were found not to be responsible for this lack of responsiveness. When the mitogenic stimulus was given between 2 and 6 weeks post-transplantation, a significant increase in labeling index was observed in comparison to sham treated control animals. Maximal labeling indices of ~3-4% were found if the stimulus took place at 4 weeks post-transplantation. Both the pattern, and the extent of the proliferative response seen in liver cells engrafted in solid supports were similar to the ones found in intrasplenic hepatocytes, indicating adequate vascularization of the supports. This data provides the first description of proliferative response in hepatocytes transplanted by the solid support technique, which may offer an attractive alternative to the intrasplenic route. It further suggests that, in analogy with the *in vitro* situation, a lag time exists between hepatocyte isolation/transplantation, and their maximal responsiveness to physiological stimuli. It is concluded that timing of the administration of a proliferative stimulus is important for obtaining a maximal response of transplanted hepatocytes during the early post-transplantation phase.

## Introduction

Hepatocyte transplantation (HTX) would offer an attractive alternative to orthotopic liver transplantation in the treatment of inborn errors of liver metabolism, in which the host liver remains structurally intact. One approach would involve the removal of hepatocytes from the affected individual, introduction of the defective gene *in vitro*, and reimplantation of the cells into a receptive locus. Such a strategy, commonly referred to as ex vivo gene therapy (21), requires both successful engraftment of the hepatocytes, and stable integration and expression of the introduced gene after HTX.

Several methods for HTX have been reported to be successful in partially, or temporarily restoring the defective liver function in animal models of inborn errors of metabolism, of which the intrasplenic injection has been most extensively studied (3,4,9,17,25-27). Recently, the intrasplenic technique has received renewed interest since it has been reported to enable hepatocyte delivery to and engraftment in the recipient's liver (10,20). Furthermore, it has been demonstrated that transient, and prolonged expression of an introduced gene after ex vivo gene therapy and intrasplenic transplantation can be achieved with allogeneic (29,30), and autologous hepatocytes (21), respectively. However, the metabolic correction of the inborn error involved was only partial.

One recently proposed alternative to intrasplenic injection involves the use of so-called solid supports consisting of polytetrafluoroethylene (PTFE) 'angel hair' fibers (24). These supports can be implanted intraperitoneally several weeks before the actual transplantation, thereby allowing time for the initial foreign body response to subside, and vascularization to occur. In addition, they are simple to use, and do not pose the threat of portal thrombosis and hemorrhage as do the intraportal, or intrasplenic injections. Moreover, since more than one support may be used per recipient, the number of cells that can be transplanted might be less restricted. Recently, our group has provided evidence of long-term engraftment and metabolic function of hepatocytes transplanted into such supports in Gunn rats (2).

A strong stimulus for hepatocellular proliferation may result in an increase in the proliferative activity of transplanted hepatocytes, if delivered in a post-transplant phase when the cells are (optimally) responsive to such a stimulus. Knowledge of the regenerative potential of transplanted hepatocytes would be useful for augmenting the mass of transplanted hepatocytes, e.g. by mitogens. This issue has been addressed by few investigators, and their observations were based on regenerative stimuli that were either given at a very late postoperative stage

(11,28), or prior to transplantation (13,19), when transplanted hepatocytes may not be fully responsive (15,27). The effects of a stimulus, e.g. partial hepatectomy, administered during the early postoperative phase has never been studied. Furthermore, it is not known whether liver cells engrafted in PTFE solid supports have the ability to respond to such a stimulus.

This study was undertaken to analyze the responsiveness of transplanted, syngeneic rat hepatocytes to a 70% partial hepatectomy delivered during the first 6 weeks post-HTX, in an attempt to define an early optimum time zone for the delivery of the stimulus. In addition, the proliferative responsiveness of hepatocytes after transplantation into PTFE supports was investigated. For these purposes a non-autoradiographic method for visualization of cells in the S-phase of the cell cycle was used.

## Materials and Methods

*Animals.* Adult male rats from an inbred Wistar strain (WAG-Rij), weighing 250-350 g, were used as hepatocyte donors and recipients. All animals were purchased from Harlan CPB, Zeist, The Netherlands. They were kept under standard laboratory conditions with 12/12h dark/light rhythm and standard laboratory diet. All procedures were performed under ether anesthesia. Approval of the Animal Welfare Committee of the Erasmus University was obtained prior to initiating experiments.

*Hepatocyte isolation.* The technique used for hepatocyte isolation was based on the two-step collagenase perfusion technique originally documented by Seglen (23), using the modifications described elsewhere in detail (2). Cell viability ranged from 80-95% as judged by trypan blue exclusion. The cells were suspended in Williams-E medium (Gibco BRL Life Technologies BV, Breda, The Netherlands) at a concentration of $1x10^7$ viable hepatocytes per ml, and kept on melting ice until transplantation.

*Hepatocyte transplantation.* Hepatocytes were transplanted either intrasplenically, or into intraperitoneal supports. For intrasplenic HTX, 1 ml of the cell suspension was injected into the splenic parenchyma, while the hilar vessels were being clamped. Leakage of blood and cell suspension was minimized by tamponade of the injection site. Solid supports consisted of multifilament, angel-hair fibers of expanded polytetrafluoroethylene (PTFE), which was generously provided by W.L. Gore & Associates, Inc. (Flagstaff, AZ). The supports were prepared as previously described in detail (2). Four weeks prior to HTX, supports (0.25g dry weight) were implanted intraperitoneally. HTX into a PTFE support was performed by

relaparotomy, followed by injection of 1ml of cell suspension into the PTFE texture, which by then was covered by vascularized (neo)peritoneum. No mortality was associated with either transplantation procedure.

*Proliferative stimulus.* Animals were divided into 2 groups. In the first group (PH), HTX into spleen or PTFE support was followed by a 70% partial hepatectomy according to the technique originally described by Higgins and Anderson (12). This technique involves resection of the left lateral and median lobes of the liver. In the second group of animals (sham) either HTX-technique was followed, instead, by a sham procedure characterized by laparotomy and gentle manipulation of the liver. Partial hepatectomy or sham procedure were performed at the time of transplantation, or at 2, 4, or 6 weeks after intrasplenic HTX. Those animals that had undergone HTX through the solid support technique were subjected to partial hepatectomy or sham procedure at only 2 time points, i.e. simultaneously with, or 4 weeks after HTX. Partial hepatectomy was well tolerated by the animals, and no associated mortality was encountered. Unless stated otherwise, animals were sacrificed at 48 h following partial hepatectomy for histological processing and determination of the proliferative activity of the transplanted hepatocytes.

*Determination of the proliferative activity of transplanted hepatocytes.* The bromodeoxyuridine (BrdU) pulse labeling technique was used to detect cells in the S-phase of the cell cycle. This is a non-radiographic technique based on the incorporation of BrdU, a thymidine analogue, in the DNA of replicating cells. A monoclonal antibody specific for BrdU allows for immunocytochemical staining of BrdU-labeled cells (8,22). Comparison of this technique with [$^3$H]thymidine-incorporation has shown that both will label proliferating liver cells to a similar extent (16).

Two hours prior to sacrifice the animals received an intraperitoneal injection of 1 ml BrdU (50 mg/kg). After sacrifice the liver, spleen/solid support, and a segment of small intestine were removed for histological processing. Tissues were then fixed in 10% neutral buffered formaldehyde, and paraffin embedded. Immunocytochemical staining was performed using an indirect, two-step labeling technique with peroxidase conjugated IgG according to the method described by Schutte et al. for intestinal sections, and by Frederiks et al. for liver (8,22). Monoclonal anti-BrdU antibodies were purchased through Eurodiagnostics, Apeldoorn, The Netherlands. For optimal interpretation, counterstains with H&E, or PAS were performed. Staining of small intestine was used as a positive control for the procedure by staining BrdU-labeled proliferative intestinal mucosa cells in the crypts of Lieberkühn.

The proliferative activity of engrafted syngeneic hepatocytes was expressed as the labeling index (LI). This LI was defined as the ratio (%) of the number of BrdU-labeled hepatocytes to the total number of hepatocytes counted. In each recipient spleen a minimum of 500 hepatocytes was counted. Since the numbers of hepatocytes found per histological section of PTFE supports were lower than those found in spleen, a minimum of 100 cells was counted per support to determine the LI.

Values are expressed as mean ± SEM. A one-way analysis of variance was used to determine which groups were statistically significantly different. For comparison of two groups, the non-parametric Mann Whitney U-test was used.

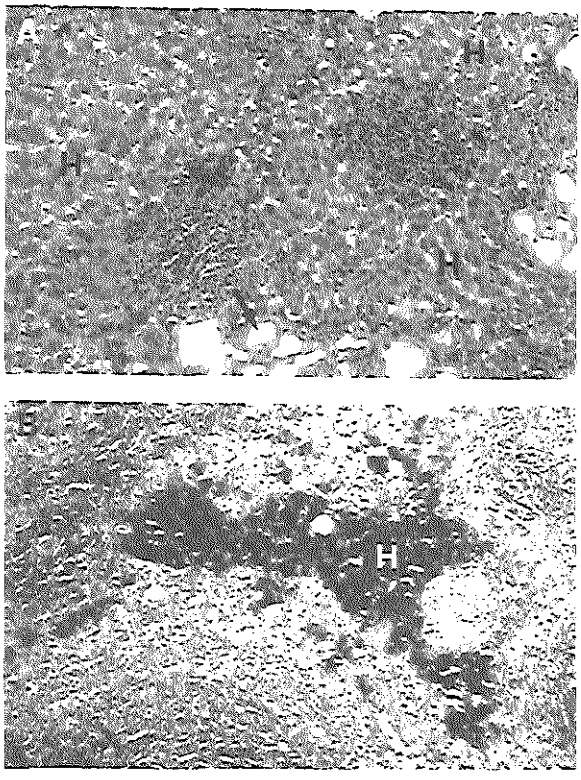


**Figure 1.** Histological section of spleen after intrasplenic transplantation of syngeneic hepatocytes. A) H&E-stain, showing extensive clusters of engrafted hepatocytes (H). Bile duct formation is present (arrow). B) PAS-stain of a cluster of intrasplenically engrafted hepatocytes (H), showing staining of glycogen containing cytoplasm (40x).

## Results

A typical example of the morphological appearance of syngeneic hepatocytes after intrasplenic transplantation is shown in Fig. 1. Surviving hepatocytes were arranged in clusters, which were distributed throughout the recipient spleen (Fig 1A). The majority of intrasplenically engrafted hepatocytes retained their typical histological features, including positive staining for glycogen by PAS (Fig. 1B), and formation of hepatic chords. In addition, bile duct formation was found in the vast majority of sections. In some cells degenerative features were observed, including cytoplasmic vacuolization and accumulation of fatty deposits. Hepatocytes, engrafted inside a PTFE support, were predominantly found in its peripheral regions, although localization in the central portions was by no means exceptional (Fig. 2). Evidence of surviving hepatocytes was obtained in virtually all histological sections of PTFE supports. Typically, the

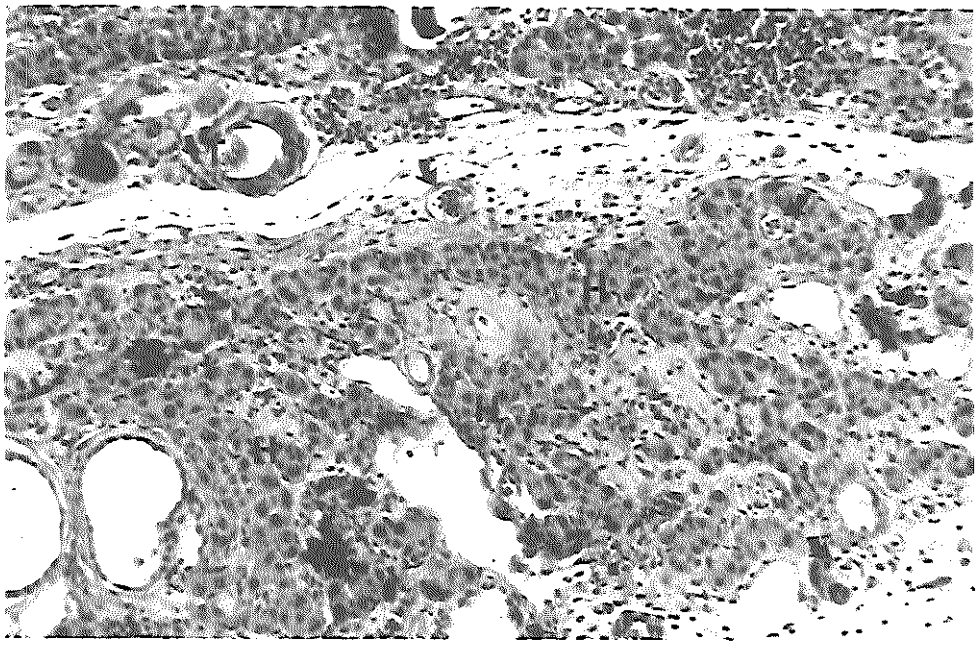

**Figure 2.** Histological section of PTFE ('angel hair') solid support 6 weeks after transplantation of syngeneic hepatocytes. HTX was performed 4 weeks after implantation of the support into the peritoneal cavity. PTFE-texture can be seen on cross-section (T). Engrafted hepatocytes (H) at the periphery of the support are shown. Signs of (neo)vascularization are indicated (arrows). (H&E, 40x).

cells were polygonal in shape, arranged in clusters with chord-like architecture, and also stained positively with PAS. Macroscopical, as well as histological evidence of (neo)vascularization of the supports could invariably be detected (Fig. 2). These observations confirm recent histological findings by our group after HTX in Gunn rats (2).

The effects of timing of the proliferative stimulus on the cytokinetic response of engrafted intrasplenic hepatocytes is shown in Table 1. When compared to the sham-treated animals, partial hepatectomy performed immediately following HTX (day 0) did not significantly influence the LI. In contrast, when partial hepatectomy was performed at a later stage, i.e. 2 to 6 weeks after HTX, it consistently resulted in a significant increase of the LI in the intrasplenic hepatocytes. A maximal response of approximately 4% BrdU-labeled hepatocytes was seen when partial hepatectomy took place 4 weeks after HTX. A slightly, but not significantly lower LI response was found for cells that were stimulated 6 weeks post-transplantation. The spontaneous, i.e. non-stimulated, LI in the early postoperative period was found to lie roughly between 0 and 1%. There were no significant differences between any of the sham groups, as judged by one-way analysis of variance.

**Table 1.** Labeling index of intrasplenically transplanted hepatocytes in response to 70% partial hepatectomy as a function of the time of hepatectomy. This time is expressed in days after HTX.

| Group | Days after HTX | n | Labeling Index (%) | PH vs. Sham |
|---|---|---|---|---|
| PH | 0 | 7 | 0.58 ± 0.16 | n.s. |
| Sham | 0 | 5 | 0.30 ± 0.20 | |
| PH | 14 | 5 | 2.21 ± 0.14 | $p < 0.005$ |
| Sham | 14 | 5 | 0.89 ± 0.26 | |
| PH | 28 | 5 | 3.96 ± 0.66 | $p < 0.005$ |
| Sham | 28 | 5 | 0.96 ± 0.09 | |
| PH | 42 | 5 | 3.15 ± 0.43 | $p < 0.002$ |
| Sham | 42 | 4 | 0.54 ± 0.09 | |

Values are given as mean±SEM. No statistically significant differences exist between groups PH 28 and PH 42.

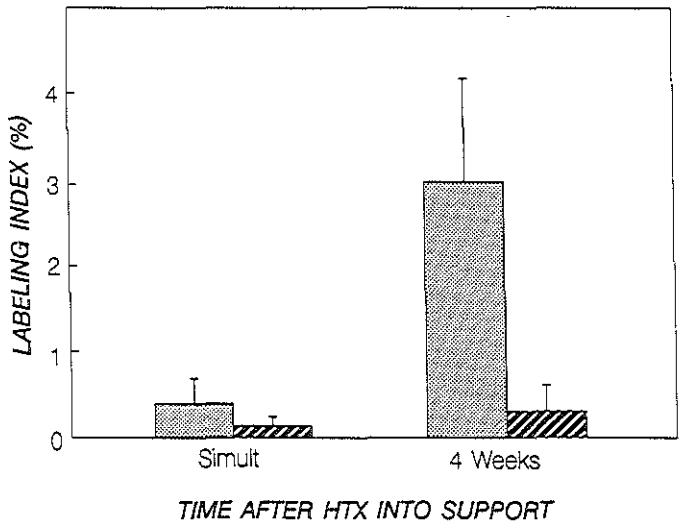


**Figure 3.** Labeling index of syngeneic hepatocytes transplanted into PTFE solid supports. Recipient animals were subjected to either 70% partial hepatectomy (dotted bars), or sham procedure (dashed bars) immediately (simult), or 4 weeks after HTX. The labeling index is expressed as the ratio of BrdU-labeled hepatocytes to the total number of counted hepatocytes per support. Data are given in mean±SEM (n=5 for each group).

Similar responses were seen in hepatocytes that had been transplanted into the pre-vascularized PTFE solid supports. Fig. 3 shows that simultaneous (i.e. d.0) HTX and partial hepatectomy did not lead to a significant increase in LI of the engrafted hepatocytes. In contrast, when partial hepatectomy was executed at 4 weeks post-HTX, the LI increased significantly to ~3% ($p<0.05$ when compared to controls).

The above experimental results were based on BrdU pulse labeling at 48h after partial hepatectomy. However, the lack of detectable increase in LI as a result of simultaneous partial hepatectomy might be explained by altered (i.e., slower) hepatocellular response kinetics (19), which could be attributed to the recent isolation and transplantation procedures. We addressed this possibility by varying the time between a combined intrasplenic HTX-partial hepatectomy, and the administration of the BrdU-pulse. No significant differences between sham-treated and hepatectomized animals were found at any of the time points ranging from 2 to 14 days postoperatively (Table 2).

**Table 2.** Labeling index of hepatocytes after combined HTX-partial hepatectomy as a function of the time between stimulus and BrdU pulse.

| Group | Days after PH | n | Labeling Index (%) | PH vs. Sham |
|---|---|---|---|---|
| PH | 2 | 7 | 0.73 ± 0.17 | n.s. |
| Sham | 2 | 5 | 0.61 ± 0.25 | |
| PH | 7 | 4 | 0.87 ± 0.24 | n.s. |
| Sham | 7 | 4 | 0.70 ± 0.14 | |
| PH | 14 | 4 | 0.85 ± 0.23 | n.s. |
| Sham | 14 | 4 | 0.82 ± 0.20 | |

Fig. 4 shows the visualization of intrasplenic hepatocytes in the S-phase of the cell cycle by nuclear BrdU incorporation. Both micrographs represent labeled intrasplenic hepatocytes, counterstained with either H&E (4A), or PAS (4B). Positive control BrdU-stains without exception revealed proliferating, DNA synthesizing cells at the base of the crypts of Lieberkühn (data not shown).

## Discussion

From this data it is clear that the responsiveness to a potent proliferative stimulus of transplanted hepatocytes changes over time. While simultaneous partial hepatectomy and HTX does not lead to increased BrdU incorporation, a significant response is seen when partial hepatectomy takes place between 2 and 6 weeks post-HTX. A maximal response of ~3-4% BrdU labeled hepatocytes is reached around 4 weeks, whereafter it appears to stabilize. This pattern of responsiveness was not only observed in intrasplenically engrafted hepatocytes, but also in liver cells that had been transplanted into prevascularized PTFE 'angel hair' solid supports. The "spontaneous" LI of non-stimulated hepatocytes, transplanted in either fashion, does not undergo significant changes in this early post-HTX time frame.

These findings are in excellent agreement with those of Gupta et al. (11), who induced proliferative activity of intrasplenic hepatocytes at 10 months post-HTX, and observed a spontaneous LI of 0.9% to increase to 2% after partial hepatectomy. In another report on the late post-transplant phase, Vroemen et al. (28) noted a 3% spontaneous LI at 12 and 20 weeks after HTX, which was elevated to 10% after partial hepatectomy at 12 weeks. Although the

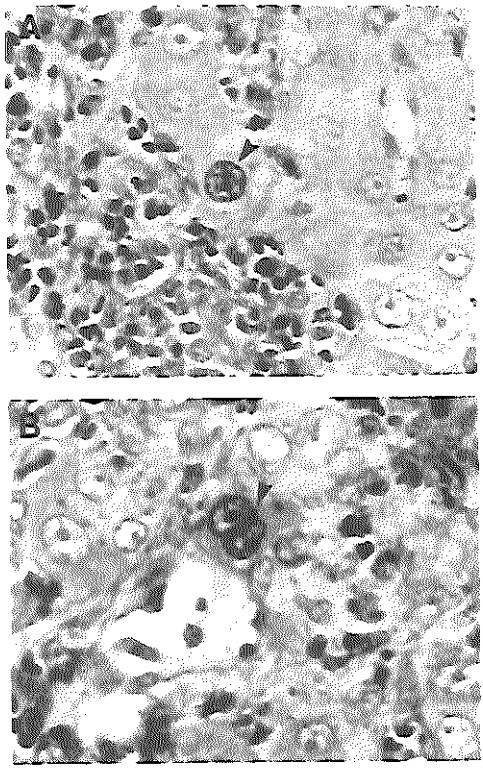


**Figure 4.** BrdU staining of intrasplenically engrafted hepatocytes following 70% partial hepatectomy. Partial hepatectomy was performed at 4 weeks post-HTX. DNA synthesizing hepatocytes are clearly visible (arrow) amidst a cluster of engrafted hepatocytes. A and B are 2 representative examples, counterstained with H&E, and PAS, respectively (40x).

absolute values in that study are higher, the relative increase of LI after stimulation is very similar to our findings, suggesting that inter-observer variation may account for this difference.

This study offers the first description of proliferative responsivity of hepatocytes transplanted into intraperitoneal PTFE solid supports. It is of interest to note that not only the time course, but also the extent of the response to partial hepatectomy were similar to the observations in intrasplenically engrafted hepatocytes. Together with the histological and macroscopical data

presented, this provides ample evidence for adequate vascularization of the PTFE support in its function as graft-receptacle. This notion is further supported by our recent findings concerning long-term metabolic activity and histological survival (up to 12 months postoperatively) after transplantation of congeneic hepatocytes with this technique into Gunn rat recipients, which are deficient in bilirubin conjugation (2). The solid support technique, therefore, offers an attractive alternative to the intrasplenic route, in particular with regard to the concept of (ex-vivo) gene therapy. It is a simple, rapid, two-stage procedure, bearing minimal complication hazards, and, potentially, poses less restrictions to the cell number that can be transplanted. The supports become adequately vascularized, and provide a non-biodegradable, three-dimensional scaffold necessary for cell engraftment after in vitro manipulation and transplantation.

The lack of effect of partial hepatectomy, executed simultaneously with HTX, on the LI of transplanted hepatocytes is contradictory to the findings of Nordlinger et al. (19), who observed that partial hepatectomy prior to HTX caused a rise in the LI from 0 on days 1 and 2, to maxima around 3% between days 3 and 7 postoperatively. Apart from the fact that comparisons in that study did not include sham-treated control animals, such a delay of the proliferative response would seem rather extensive considering the rapidity with which events take place in vivo during liver regeneration (18). We did not find a similar delay in response kinetics to play a role in our experiments. Accordingly, Jirtle et al. (14) reported that pre-transplant hepatectomy induced maximal proliferative indices between 1 and 3 days after the stimulus. They also described the stimulatory signal to rapidly diminish beyond 2 days postoperatively. It must be noted, however, that comparison with the latter study is hampered by the different techniques used by the investigators, including HTX in the interscapular fat pad, clonogenic substance in the suspending medium, and quadruple pulse labeling.

Notwithstanding the above comments, an altered state of responsiveness of hepatocytes immediately following isolation and transplantation may account for the observed lack of response to a simultaneous regenerative impulse. This hypothesis would be consistent with previous reports describing a lag time between HTX and maximal expression of hepatospecific function (2,3,27), and morphology (15). Interestingly, a similar lag time has been observed in long-term culture before hepatocytes reach a stable phenotype (6), and express maximal response to physiologic stimuli (1). This lag time has been attributed to reassembly of intracellular components, e.g. polyribosomes, following the isolation procedure (5).

From the data presented, one could postulate that the optimal time for the delivery of a strong proliferative stimulus to intrasplenically transplanted hepatocytes lies around the 4th week post-

HTX. Whether this would lead to the desired augmentation of transplanted liver cell mass remains to be determined. Vroemen et al. (28) found no detectable increase in splenic liver mass after late partial hepatectomy. However, this may have been caused by the fact that most, if not all, of the regenerative stimulus from partial hepatectomy is directed to the liver itself. Indeed, inhibition of the proliferative capacity of the liver induces significant growth of (preneoplastic) intrasplenic hepatocytes following hepatectomy (7). This is also consistent with the observation that porto-caval shunting prior to HTX causes an increase in LI (19), and splenic colonization (13) of engrafted hepatocytes. Although the presented behavior of intrasplenic hepatocytes, and liver cells in supports, may be indicative for the responsiveness of all ectopic hepatocytes, intrahepatically translocated hepatocytes may respond in a different fashion to mitogenic stimuli due to their location inside the target organ. Transplantation of hepatocytes from transgenic animals could provide insight into related questions (10,20).

In conclusion, timing of the exposure of transplanted hepatocytes to a strong mitogenic stimulus is likely to be important for obtaining a maximal proliferative response by the engrafted cells, due to changes in their responsiveness over time in the early post-HTX period. HTX by means of the novel PTFE 'angel hair' solid support technique seems an attractive alternative to the classic intrasplenic injection, enabling hepatocytes to engraft, function, and respond physiologically to a proliferative stimulus. This knowledge may be helpful for establishing improved protocols for HTX and ex-vivo gene therapy in the treatment of inborn errors of liver metabolism.

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# CHAPTER 10

Discussion and Conclusions

## Discussion

Currently, the only definite treatment available for both acute fulminant hepatic failure, and inborn errors of liver metabolism consists of liver transplantation. Although this procedure clearly has improved the survival of afflicted patients, it is associated with considerable morbidity, mortality, and cost, and the problem of donor scarcity has not yet been resolved (38). Furthermore, timing of transplantation in cases of fulminant hepatic failure is difficult: early transplantation may replace a host liver that, given time, would have resumed regeneration, while OLT in later stages of the disease may be too late to prevent death (37,38). In patients with an inborn error of metabolism, which has not (yet) led to secondary liver damage, OLT would seem overtreatment for the correction of one single metabolic defect. Consequently, most transplantations in these patients have thusfar been delayed until after the development of secondary liver damage (37,38). Such disadvantages have prompted research into alternative therapeutic strategies that would provide some form of liver support. Most of the alternatives now focus on the use of hepatocytes, since hepatocyte-based systems appear to generate the most promising experimental results. Arguments for the use of hepatocyte-based support systems include i) reduction of the mortality, morbidity and costs associated with OLT, ii) preclusion of unnecessary removal of the patient's own liver, iii) allowance of the possibility of spontaneous recovery of the host liver, and iv) possibilities for storage and gene-therapy (20,39).

In **Chapter 1**, an overview of the literature is presented in an effort to place the basis of this thesis in its proper context. Hepatocyte-based liver support systems can be divided into extracorporeal devices, and implantable systems. From this chapter it becomes clear that acute fulminant hepatic failure may be best treated by extracorporeal systems, that would ideally be available on demand, and serve as a bridge to either liver transplantation, or spontaneous functional recovery and regeneration of the affected liver. On the other hand, inborn errors of liver metabolism may benefit most from the more permanent concept of implantable systems. The ultimate goal of the latter approach should be to genetically modify part of the patient's own hepatocytes, e.g. by ex-vivo retroviral infection, and subsequently reimplant these cells by autologous transplantation.

The rationale and objectives of the studies presented in this thesis are explained in the second section of Chapter 1, where the experimental work is introduced. The objectives of the studies were as follows:

**PART I**: To obtain insight into hepatocellular behavior under conditions that might be encountered during extracorporeal support, e.g. exposure to mediators of the acute phase response, and plasma perfusion in an extracorporeal circuit.
**PART II**: To define optimal freezing conditions required for long-term hepatospecific function of hepatocytes in stable double gel culture by means of a systematic analysis of the effects of each of the major steps involved in a freeze-thaw cycle on hepatocellular morphology, function, and cytoskeletal organization.
**PART III**: To obtain further insight into the mechanisms involved in retroviral infection of hepatocytes, in particular the role of putative virus receptors, and to evaluate a novel technique for hepatocyte transplantation using prevascularized PTFE solid supports.

**PART I** focuses on the in vitro behavior of hepatocytes to conditions that they could encounter while functioning as part of an extracorporeal device. These in vivo conditions include elevated levels of cytokines, since connection of an indiviual to an extracorporeal circuit has been shown to evoke a typical acute phase response by exposing his circulation to artificial surfaces. Hepatocytes were cultured according to the double gel, or sandwich technique, in which configuration they have been shown to remain phenotypically stable for at least 6-8 weeks (14,15). The experiments described in **Chapter 2** demonstrate that hepatocytes cultured in this double gel configuration are capable of responding to cytokines in a physiological way, while retaining normal, stable long-term phenotype. Cytokine administration was carried out after 16 days of undisturbed culture. Individual IL-6 was shown to induce a complete acute phase protein response similar to the in vivo situation, IL-1ß resulted in a partial response when administered in high doses, while TNF-α did not affect the secretion rates of the proteins examined. Furthermore, all responses were followed by a return towards homeostasis upon withdrawal of the stimulus. Both the kinetics, and the extent of the response closely resemble the in vivo patterns seen in rat and mouse (9,27,31,36). Comparison with prior in vitro studies is difficult, since those studies have either used transformed cells (4,7) or short term conventional cultures of unstable primary hepatocytes (5,18). Nevertheless, the extent of the observed response to IL-6 and the lack of effect of TNF-α are comparable to the findings of others (5,8,18). The partial response we found elicited by IL-1ß is similar to in vivo observations after administration of IL-1 (27), but contradicts previous findings in hepatoma cells (4,7). The study confirms the importance of IL-6 as principal mediator of the acute phase response, and supports the notion that the response is primarily regulated at the pre-translational level (3,7). This study represents the first demonstration of such long-term physiologic behavior in cultures of primary, i.e. non-transformed, hepatocytes and emphasizes the unique properties of the double gel culture configuration. In a somewhat broader sense, the double gel culture system provides a means

by which more insight can also be obtained into the various mechanisms involved in the hepatic acute phase response, and may be valuable in subsequent attempts to control, or rather modulate, these mechanisms, for instance aimed at circumventing the hypo-albuminemic part of the response.

The feasibility of using the double gel culture technique in designing a hepatocyte-based extracorporeal liver support system has been more directly addressed in **Chapter 3**. Before shifting attention towards the effects of such a system on the animal model under investigation, we reasoned it essential to be informed about the effects such a system, and its connection to a laboratory animal, might have on hepatocyte morphology and function. Until now, such data has not been reported. For this purpose, a microscopy perfusion chamber was designed and built, which could house hepatocytes cultured according to the double gel technique. This chamber allowed continuous monitoring of cellular behavior under different circumstances, particularly during extracorporeal perfusion. Having ascertained that the thermal, chemical, and flow characteristics of this chamber were within acceptable limits, we then attached it to normal rats through an extracorporeal circuit containing a plasmaseparator. Six hours of continuous extracorporeal perfusion of sandwiched hepatocytes resulted in reversible cytoplasmic changes, e.g. increasing fatty deposits, and unaltered cell shape indices. It further led to a temporary increase in albumin secretion during the first post-perfusion day, followed by a return towards stable protein secretion levels, which were maintained for at least 6 days after perfusion. These results are in contrast to the short-term observations of Arnaout et al. (6), who found that microcarrier-attached hepatocytes, inoculated in the extracapillary chamber of a hollow fiber device, only remained viable for a maximum of 4h, whereafter they rapidly degenerated and died. The more favourable outcome in our study may reflect the fact that our system combines a stable culture configuration with the required thermal and perfusion characteristics.

From the results presented in Part I it would seem likely that hepatocytes cultured in the double gel configuration could well be used in the design of more practical extracorporeal liver support systems. They are phenotypically stable, express a variety of hepatospecific functions, and are capable of responding physiologically without subsequent destabilization. Furthermore, they undergo only slight cytoplasmic alterations when exposed to normal animal plasma during extracorporeal perfusion. In the near future, many other hepatocellular properties, e.g. detoxifying capacity, could be relatively easily measured 'on line' during extracorporeal perfusion using the microscopy chamber described. Furthermore, one could continually monitor the effects of partial hepatectomy, or chemically induced liver failure, on the cultured hepatocytes in the system. However, for a clinically useful device to be realized, technologies to expand the cell mass will have to be developed. Issues dealing with device scale-up, such as

the reduction of dead volume, distribution of oxygen, and miniaturization of the supporting structure, need to be thoroughly addressed.

A second aspect that needs to be considered in the development of liver support systems involves hepatocyte storage. In effect, the problem of donor organ scarcity will not be resolved by using hepatocyte-based support systems alone, but will largely depend on adequate storage techniques. This is particularly true for extracorporeal devices, which will most likely be needed at very short notice for acutely ill individuals. **PART II** of this thesis focuses on the in vitro effects of cryopreservation on cultured hepatocytes. Although several investigators have reported attempts to cryopreserve isolated hepatocytes, the results of these studies are confusing, and are often based on viability assays in selected cell populations, rather than on long-term hepatospecific function (10,11,17,22,24). The sandwich culture configuration provides an attractive alternative for the study and evaluation of hepatocyte cryopreservation, since it contains a fixed number of stabilized hepatocytes expressing differentiated long-term function and, thus, would eliminate cell selection in the post-freeze evaluation. Furthermore, one could speculate that increased cell stability may lead to increased capability to withstand stresses associated with a freeze-thaw cycle.

Typically, cryopreservation protocols contain multiple steps, each of which needs to be carefully evaluated in order to minimize cellular damage (32). The first (and last) step of every protocol involves the addition (and removal) of cryoprotecting agents to the cellular environment. Although meant to reduce cell damage under sub-zero temperature conditions, the addition of such agents has been associated with considerable osmotic stresses, for which reason tedious stepwise addition and removal procedures have become common practice (30). In **Chapter 4**, it is shown that hepatocytes, cultured in sandwich collagen configuration can withstand one-step addition/removal of substantial concentrations of the most widely used cryoprotectant dimethyl sulfoxide ($Me_2SO$). Long-term morphology, cytoskeletal organization, and protein secretion capacity remained undisturbed when the cells were exposed at room temperature to initial $Me_2SO$ concentrations of up to 5.0 M, provided the pre-freeze exposure time was 30 min or less. When 2.0 M was used, the exposure time could be extended to 120 min prior to freezing without causing deleterious effects. These results compare favorably to those of other groups, (11,17), who reported a rapid decline in hepatocellular viability and protein secretion after brief exposure to $Me_2SO$ concentrations of more than 1.0 M. The beneficial effect of the double gel configuration on survival and function of hepatocytes following $Me_2SO$ exposure may be explained by two mechanisms. First, the collagen top layer covering the cells may be reponsible for a more gradual exposure of the hepatocytes to the cryoprotectant, thus reducing the osmotic stresses induced by one-step addition/removal.

Second, the sandwiched hepatocytes may be less vulnerable to the toxic effects of $Me_2SO$, which may explain the retention of function and cytoskeletal organization even after very long exposure times of 1-2 hours.

This knowledge was used in the subsequent freezing experiments described in Chapter 6. Prior to actual cryopreservation experiments, however, a freezing device had to be constructed, which had to control cooling and warming rates over several orders of magnitude, achieve uniform heater layer temperatures down to -80°C without significant thermal gradients, and have multi-step protocol ability. We designed such a device, and a detailed characterization of its features has been given in **Chapter 5**. Since it met the prerequisites for reliable and reproducible freezing protocols, the freezing device was used throughout the experiments described in **Chapter 6**. This chapter comprises a stepwise analysis of the damaging effects of several parameters on long-term protein secretion and morphology of a population of hepatocytes in sandwich configuration. The results indicate that cooling rate, warming rate, and final temperature are critical factors determining post-freeze survival. Optimized conditions included a dehydration period at -12°C, a cooling rate of 5°C/min, and a warming rate of $\geq$ 400°C/min. Under these circumstances, sandwiched hepatocytes were able to fully recover from the damage inflicted by freezing to -40°C, while freezing to -80°C led to ~30% loss of long-term protein secretion. This study represents the first description of long-term functional recovery of cultured hepatocytes following a freeze-thaw cycle.

The optimal protocol used in this study is comparable to procedures described for isolated hepatocytes, but our results regarding post-freeze viability and function are significantly better (10,11,17,34). This difference may be explained by the presence of an extracellular matrix and a physiologically polarized cytoskeleton, which may stabilize the plasmamembrane and cell organelles against the freeze-thaw stresses. This hypothesis is supported by the fact that the estimated water permeability and nucleation parameters of cultured hepatocytes approach values found for other mammalian cells, and are in contrast with those of freshly isolated hepatocytes (21,45). In addition, the presence of a collagen gel in the intact matrix may alter the structure of the external ice and, subsequently, the interaction of extracellular ice with the plasma membrane (2). Most intriguing are the findings concerning the restoration of full protein secretory capacity during the first several days following a freeze-thaw cycle to -40°C. The repair processes involved have yet to be understood, and experiments to evaluate these processes are currently being designed. Although these results are a very promising first step towards application of cryopreservation of cultured hepatocytes, many problems still need to be resolved. These include definition of the desired subzero temperature for adequate storage, extension of the preservation time at that temperature, and methods to overcome mechanical stresses to the

collagen lattices resulting from lower temperatures. Furthermore, this area, too, needs serious engineering efforts to design devices that can not only cool and warm with the same degree of reliability as the one described here, but can also serve as storage containers for cultured hepatocytes.

**PART III** of this thesis addresses the concept of ex-vivo gene therapy. This novel therapeutic approach relies on an effective combination of two technologies, i.e. retrovirus-mediated gene transfer, and hepatocellular transplantation. Although several investigators have established the feasibility of gene transfer into hepatocytes in vitro (29,44), reproducibility and infection efficiency need to be improved. In an attempt to further characterize the mechanisms involved in successful retroviral infection of hepatocytes, the experiments described in **Chapter 7** have concentrated on the expression of a recently identified cationic amino acid transporter (CAT-1), which also serves as ecotropic virus binding site (1,26). The results of these experiments indicate that expression of CAT-1 is correlated with susceptibility of hepatocytes to infection by ecotropic retrovirus in vitro and in vivo. CAT-1 was found not to be expressed in either resting, or regenerating rat liver in vivo, in which situations infection with ecotropic virus could not be achieved (23). In contrast, receptor expression was shown to be induced as soon as 5 hours after hepatocyte isolation and seeding in culture, and it remained expressed through several weeks of (double gel) culture. Ecotropic infection could be confirmed in early stages of hepatocyte culture. A cationic amino acid transporter closely related to CAT-1, and termed CAT-2, was previously suggested to be likely to encode for the amphotropic receptor (40). Experiments to this nature were also performed, but failed to support this hypothesis. The factors responsible for induction of ecotropic receptor expression in cultured hepatocytes are unknown. However, the absence of CAT-1 expression in normal liver in vivo may be important to prevent depletion of the plasma pool of arginine by preventing access to intracellular hepatic arginase, which catalyses hydrolysis of arginine into ornithine and urea. Therefore, the regulation of CAT-1 expression may be related to the urea cycle (43). Alternatively, CAT-1 expression in cultured hepatocytes could be induced by disruption of the close association between hepatocytes and non-parenchymal cells. Further experiments using cocultures of hepatocytes with sinusoidal endothelial, and/or Kupffer cells are necessary to answer these questions.

In **Chapters 8 and 9**, an alternative technique for hepatocyte transplantation has been characterized. This technique involves the use of prevascularized solid supports, consisting of collagen coated PTFE 'angel hair' fibers. Prevascularization was allowed by implantation of these supports into the peritoneal cavity of recipient rats ~4 weeks prior to the transplantation procedure, which consisted of injection of suspended hepatocytes into the supports. The

experiments described in **Chapter 8** revealed evidence of metabolic activity of hepatocytes from heterozygous donors, transplanted into homozygous Gunn rats, suffering from severe hyperbilirubinemia due to a genetic defect in the conjugation of bilirubin. At 3 months after transplantation into either splenic parenchyma, or PTFE solid support, the appearance of significant levels of bilirubin conjugates could be detected in the recipients' bile. The biliary glucuronide levels in the animals from the solid support group were lower than in those that had undergone intrasplenic injection. The use of fetal hepatocytes did not lead to results that differed significantly from those seen with adult hepatocytes. Surviving engrafted hepatocytes could easily be identified histologically in all experimental groups for at least one year post-transplantation. The results emphasize the importance of evaluating biliary conjugates as the ultimate proof of a functioning hepatocellular graft in the Gunn rat, and are supported by previous reports on hepatocyte transplantation by means of intrasplenic injection (42), and intraperitoneal insertion on microcarriers (12).

The in vivo proliferative responsiveness of hepatocytes transplanted in either way was the subject of the investigations presented in **Chapter 9**. The aim of these experiments was to define a time frame during which optimal proliferative responsiveness of transplanted hepatocytes could be identified in the early posttransplant phase. Ultimately, such knowledge could lead to augmentation of the transplanted cell mass by means of strong proliferative stimuli. As shown in the chapter, the responsivity of transplanted hepatocytes changes over time: while no increase of the proliferative index could be observed after intrasplenic transplantation and simultaneous partial hepatectomy, significant increases were found when the hepatectomy was performed at 2, 4, or 6 weeks after hepatocyte transplantation. Maximal responsiveness was seen around the 4th post-transplant week. An identical pattern was found for hepatocytes transplanted into prevascularized PTFE solid supports. Since it is known that at least part of the regenerative stimulus is conveyed through humoral signals (33), these latter observations demonstrate the adequate vascularization of hepatocytes inside the supports. The lack of effect of partial hepatectomy performed immediately after the transplantation, may be due to an altered state of responsiveness of hepatocytes directly following isolation. This hypothesis is supported by previous reports which describe a lag time between hepatocyte transplantation, and maximal expression of hepatospecific function (13,41), and morphological appearance (28). Interestingly, a similar lag time can be seen in the in vitro situation (15, this thesis Chapter 2), and may reflect the reassembly of intracellular components, such as polyribosomes, following the isolation procedure (16).

The results from these final three chapters represent yet another step towards the realization of clinical protocols for ex vivo gene therapy for inborn errors of liver metabolism. The

mechanisms that regulate the expression of CAT-1, including its induction in vitro, have not yet been clarified. Once further insight into these mechanisms has been obtained, modulation of the expression might improve the efficiency of retroviral infection. The solid support transplantation technique clearly needs optimization with regard to the maximum amount of cells that can be transplanted per support, and the maximal number of supports that may safely be implanted intraperitoneally. Once optimized, this technique may offer distinct advantages over the conventional methods, in particular by circumventing such problems as hemorrhage, and portal hypertension or thrombosis seen after intrasplenic and intraportal injection (19,25). Furthermore, since more than one support could be implanted into the abdominal cavity, the number of hepatocytes that can be transplanted may be less restricted than in intrasplenic inoculation. In addition, the solid support technique provides investigators with a means to further characterize functional and response properties of hepatocytes after implantation into an in vivo milieu. Of the three parts that constitute this thesis, the concept of ex vivo gene therapy addressed in the third part seems to be closest to clinical application. The recent description of long-term partial correction of hypercholesterolemia in rabbits after autotransplantation of genetically modified hepatocytes is encouraging, but awaits confirmation from other investigators (35). Several hurdles remain to be taken, the most prominent of which involve the development of adequate, reliable, and safe transplantation techniques in large animal models, and more efficient methods for gene transfer.

## Conclusions

The major conclusions that can be drawn from the work presented in this dissertation can be summarized as follows:

1. In restoring the characteristic polarity of hepatocytes, the sandwich, or double gel, collagen culture configuration renders hepatocytes capable to respond to physiological stresses, e.g. the acute phase response, without losing their phenotypic stability.
2. Connection of hepatocytes in double gel culture to a rat through an extracorporeal circuit for 6 hours induces minor morphological changes, while their long-term functional capacity remains intact.
3. One-step addition and removal of 2.0 M dimethyl sulfoxide to sandwiched hepatocytes followed by 60-120 min exposure at 22°C prior to freezing does not adversely affect hepatocellular morphology, cytoskeletal organization, or long-term functional capacity.
4. Under optimal freezing conditions, using the described freezing unit, ~70% functional recovery of hepatocytes in sandwich configuration can be achieved for at least 2 weeks following freezing at -80°C.
5. In addition to proliferative activity, receptor expression in hepatocytes seems to be an important determinant in their susceptibility to ecotropic retroviral infection. The mechanisms regulating receptor expression in vitro and in vivo need to be determined.
6. The novel technique of hepatocyte transplantation into PTFE solid supports, that were prevascularized by intraperitoneal implantation, is a valuable asset in the concept of ex-vivo gene therapy for treatment of inborn errors of liver metabolism, since it allows transplanted hepatocytes to engraft, function over the long term, and respond to proliferative stimuli.
7. Timing of the administration of a strong proliferative stimulus to transplanted hepatocytes is important for maximal cytokinetic response.

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# SAMENVATTING

Dit proefschrift bestaat uit drie delen welke alle betrekking hebben op leverondersteunende behandeling met behulp van hepatocyten. De ontwikkeling van dergelijke alternatieve behandelingsstrategieën is van belang voor afwijkingen waarbij de aangedane lever slechts in één (enzymatische) functie tekort schiet (bijv. aangeboren stofwisselingsziekten), of waarbij de lever potentieel nog kan herstellen door regeneratie (zoals bij acuut fulminant leverfalen). Op dit moment staat ons, als enige doeltreffende therapie van deze afwijkingen, alleen orthotope levertransplantatie (OLT) ten dienste met de daaraan verbonden nadelen van morbiditeit, mortaliteit, kosten, en donorschaarste. Op hepatocyten gebaseerde leverondersteunende systemen (HLS) zouden in dergelijke gevallen kunnen dienen ter vervanging van de defecte enzymfunctie, respectievelijk ter overbrugging van de periode tot hetzij spontaan herstel van de aangedane lever, hetzij OLT. De volgende argumenten pleiten vóór het gebruik van HLS als alternatief voor OLT: i) vermindering van de aan OLT verbonden mortaliteit, morbiditeit en kosten, ii) verlaging van het donor-orgaan tekort, iii) vermijding van onnodige acceptor hepatectomie, iv) behoud van de mogelijkheid op spontaan herstel van de aangedane lever, en v) mogelijkheid tot opslag en gentherapie.

In **hoofdstuk 1** wordt een literatuuroverzicht gegeven van de tamelijk prille stand van zaken met betrekking tot alternatieve leverondersteunende behandeling. HLS kunnen worden onderverdeeld in extracorporele en implanteerbare systemen. Extracorporele HLS lijken het meest geschikt voor de behandeling van acuut fulminant leverfalen, terwijl aangeboren stofwisselingsziekten van de lever waarschijnlijk meer gebaat zijn bij implanteerbare systemen (leverceltransplantatie). Deze laatste zouden dan kunnen worden toegepast in combinatie met gentherapie, bijvoorbeeld middels ex-vivo retrovirale infectie van een deel van de eigen levercellen van de patient.

Het eerste gedeelte van het proefschrift bevat twee studies met als doel inzicht te krijgen in het gedrag van hepatocyten onder condities die in een *extracorporeel* HLS verwacht kunnen worden. **Hoofdstuk 2** beschrijft de double gel, of sandwich, kweekmethode voor hepatocyten. Volgens deze techniek worden hepatocyten gekweekt tussen twee lagen collageen-gel. Uit de studie blijkt dat rattehepatocyten in sandwich configuratie een stabiel phenotype behouden gedurende tenminste enkele weken, en bovendien op physiologische wijze reageren met een acute phase respons na blootgesteld te zijn aan individuele cytokines. Deze respons wordt gevolgd door een terugkeer naar homeostase binnen enkele dagen na verwijdering van de stimulus. **Hoofdstuk 3** bevat de beschrijving van een perfusie-kamer waarin het gedrag van hepatocyten in sandwich configuratie kan worden bestudeerd met behulp van time-lapse video-microscopie. Hepatocyten werden geobserveerd onder plasmaperfusie tijdens connectie van de kamer met de circulatie van een normale rat. Uit deze experimenten

blijkt dat 6 uur extracorporele plasmaperfusie leidt tot reversibele cytoplasmaveranderingen in het merendeel van de hepatocyten, terwijl de cellen blijkens albumineproductie normaal blijven functioneren gedurende tenminste de eerste week na perfusie.

Het tweede gedeelte van dit proefschrift besteedt aandacht aan *cryopreservatie* als methode voor langdurige opslag van hepatocyten. De noodzaak hiervan is evident, in het bijzonder voor de extracorporele HLS die op afroep beschikbaar zouden moeten zijn voor de patient met acuut leverfalen. Ook de donorschaarste roept evenwel op tot het ontwikkelen van lange termijn bewaarmethodieken. Echter, de literatuurgegevens omtrent cryopreservatie van geïsoleerde hepatocyten zijn weinig bemoedigend, verwarrend, en veelal gebaseerd op observaties in geselecteerde celpopulaties. Derhalve werd in dit tweede gedeelte de mogelijkheid tot cryopreservatie van gekweekte rattehepatocyten bestudeerd. Het doel van deze studies was het ontwerpen van een bevriezingsprotocol voor in sandwich configuratie gekweekte hepatocyten aan de hand van stapsgewijze analyse van de belangrijkste onderdelen uit het cryopreservatie-proces. **Hoofdstuk 4** gaat in op de effecten van het meest gebruikte cryoprotectant dimethyl sulfoxide ($Me_2SO$) op stabiele hepatocyten in sandwich kweek. In tegenstelling tot geïsoleerde hepatocyten kunnen de cellen in stabiele kweekconfiguratie inéén aan veel hogere concentraties $Me_2SO$ worden blootgesteld zonder nadelige gevolgen. Lange termijn morfologie, actine distributie, en eiwitsecretie blijven onveranderd na toevoeging bij kamertemperatuur van maximaal 5.0 M $Me_2SO$ aan 1 week oude sandwich kweken, mits de blootstellingsduur beperkt wordt tot 30 min. Wanneer 2.0 M $Me_2SO$ wordt gebruikt, kan de tijdsduur veilig worden verlengd tot minstens 2 uur. Derhalve lijkt de bovenste collageenlaag van de double gel configuratie een goede bescherming te bieden aan zowel osmotische, als toxische effecten van $Me_2SO$. **Hoofdstuk 5** geeft een gedetailleerde beschrijving van het in de verdere cryopreservatie-experimenten gebruikte bevriezingsapparaat. Dit speciaal ontworpen, computergestuurde bevriezingsapparaat blijkt aan de volgende vooropgestelde eisen te voldoen: i) gecontroleerde bevriezings- en verwarmings-snelheden over enkele ordes van grootte, ii) uniforme temperatuur tot minimaal -80°C zonder significante temperatuursgradiënten, en iii) mogelijkheid voor het programmeren van zgn. multi-step protocols. Vervolgens worden in **hoofdstuk 6** stapsgewijs de schadelijke effecten van enkele belangrijke bevriezings-parameters op lange termijn eiwitsecretie en morfologie van hepatocyten in double gel onderzocht. Bevriezings-, en verwarmingssnelheid, alsmede eindtemperatuur zijn bepalend voor celoverleving. Tot de geoptimaliseerde condities behoren een dehydratieperiode bij -12°C, een bevriezingssnelheid van 5°C/min, en een opwarmingssnelheid van ≥ 400 °C/min. Onder deze omstandigheden kunnen gekweekte hepatocyten volledig herstellen van de schade door bevriezen tot -40°C, terwijl ~30% van de lange termijn functie verloren gaat wanneer een eindtemperatuur van -80°C wordt gekozen.

Het derde deel van het proefschrift omvat het concept van *ex-vivo gentherapie* van hepatocyten met het oogmerk een enzym(functie)defect te overbruggen. Deze nieuwe strategie rust op de ontwikkeling van twee technologieën: adequate transfectie, bijvoorbeeld middels retrovirale infectie, en hepatocellulaire transplantatie. Retrovirale infectie van hepatocyten is weliswaar eerder beschreven, maar de reproduceerbaarheid en efficiëntie van infectie behoeven nog verbetering. Voorts zijn de mechanismen die ten grondslag liggen aan succesvolle infectie grotendeels onopgehelderd, alhoewel algemeen wordt aangenomen dat celproliferatie een belangrijke rol speelt. **Hoofdstuk 7** gaat nader in op deze problematiek, en onderzoekt de expressie in (ratte)hepatocyten van CAT-1, een recent geïdentificeerde transporter van kationische aminozuren, die eveneens dienst doet als bindingsplaats voor ecotroop retrovirus. Het blijkt dat expressie van CAT-1 door hepatocyten wordt geïnduceerd binnen enkele uren na isolatie en kweek, en dat de receptorexpressie is gecorreleerd aan ecotrope infectiviteit in vitro en in vivo. De door anderen gesuggereerde hypothese dat een tweede aminozuurtransporter (CAT-2), die veel gelijkenis vertoont met CAT-1, de amphotrope receptor zou zijn, wordt niet gesteund door de resultaten uit dit hoofdstuk.

Hepatocellulaire transplantatie geniet een toenemende belangstelling en de intrasplenische injectie is de tot dusverre meest bestudeerde toedieningsweg. Desalniettemin moet rekening worden gehouden met problemen en complicaties die deze techniek bij het grotere proefdier en de mens zou kunnen veroorzaken, en dient naar alternatieve toedieningsvormen gezocht te worden. In **hoofdstuk 8 en 9** treft men de karakterisering aan van een alternatief in de vorm van zgn. geprevasculariseerde, met collageen bedekte 'solid supports' bestaande uit draden van polytetrafluoroethyleen. Deze supports worden 4 weken voor de leverceltransplantatie in de buikholte van een rat geïmplanteerd, zodat voldoende vascularisatie kan optreden. De experimenten beschreven in **hoofdstuk 8** zijn de eerste die daadwerkelijk bewijs leveren voor metabole activiteit van hepatocyten 3 maanden na transplantatie in solid supports: significante hoeveelheden bilirubine-conjugaten konden in de gal van conjugatie-deficiënte Gunn ratten worden aangetoond. De biliaire glucuronide gehalten waren evenwel lager dan die na intrasplenische hepatocyten transplantatie. In beide groepen is lange termijn overleving van getransplanteerde hepatocyten mogelijk tot minstens 1 jaar na transplantatie met behoud van de typische histologische en morphologische kenmerken van normale levercellen. In **hoofdstuk 9** is de reactie van getransplanteerde hepatocyten op een krachtige proliferatieve stimulus onderzocht. Deze blijkt afhankelijk van het tijdstip waarop de stimulus (70% partiële hepatectomie) wordt toegediend: hepatectomie onmiddellijk na transplantatie geeft geen stijging van de proliferatie index te zien, terwijl een maximale stijging wordt geobserveerd wanneer de hepatectomie ~4 weken na transplantatie wordt uitgevoerd. Dit effect op getransplanteerde

hepatocyten is onafhankelijk van de wijze van transplantatie, m.a.w. de hepatocyten getransplanteerd in solid supports reageren op dezelfde wijze als de intrasplenisch getransplanteerde levercellen.

In **hoofdstuk 10** worden de voornaamste onderzoekstresultaten uit dit proefschrift bediscussieerd. De volgende conclusies kunnen aan de hand hiervan worden getrokken als antwoord op de in de introductie gestelde vragen:

1. Door de karakteristieke polariteit van hepatocyten na te bootsen maakt de sandwich, of double gel kweekconfiguratie het mogelijk dat hepatocyten physiologisch kunnen reageren op externe stimuli zonder hun stabiele phenotype te verliezen.
2. Zes uur durende connectie van hepatocyten in double gel met de bloedsomloop van een normale rat middels een extracorporeel circuit leidt tot geringe, reversibele morfologische veranderingen, terwijl de lange termijn functie niet wordt geschaad.
3. Toevoeging (in één stap) van 2.0 M $Me_2SO$, gevolgd door 120 min blootstelling bij kamertemperatuur aan dit cryoprotectant van hepatocyten in double gel heeft geen nadelige gevolgen voor morfologie, actine cytoskelet, of lange termijn functie.
4. Onder geoptimaliseerde condities kan ~70% herstel van de lange-termijn functionele capaciteit van hepatocyten in double gel worden bereikt na bevriezing tot -80°C.
5. Naast proliferatieve activiteit is expressie van de ecotrope receptor een belangrijke determinant voor retrovirale infectiviteit van hepatocyten.
6. De 'solid support' techniek van leverceltransplantatie is een waardevol alternatief voor de intrasplenische route, daar zij eveneens leidt tot lange termijn functie en overleving met behoud van morfologische kenmerken en proliferatievermogen.
7. Het tijdstip waarop getransplanteerde hepatocyten aan een krachtige proliferatieve stimulus worden blootgesteld is van groot belang voor maximaal cytokinetisch effect.

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# CURRICULUM VITAE

The author of this thesis was born in The Hague on January 16, 1958. After finishing high school (atheneum-B) in 1976, he attended medical school at the University of Leiden. Throughout medical school, he was a student assistant at the Eurotransplant Foundation in Leiden (Chairman: prof. dr J.J. van Rood). He graduated from medical school (cum laude) in 1984, whereafter he functioned as a house officer for surgery until 1986 in the Rode Kruis Ziekenhuis, The Hague (Dr J.J. Hamming). His formal surgical training commenced in 1986 in the Zuiderziekenhuis, Rotterdam (Dr G.A. Olthuis), and continued in the University Hospital Dijkzigt in Rotterdam (Prof. dr H.A. Bruining and Prof. dr J. Jeekel) since 1989. His surgical training was interrupted from January 1991 until May 1992 when he fulfilled a fellowship in surgical research at the Massachusetts General Hospital, Boston, USA (R.G. Tompkins, M.D., Sc.D., and M.L. Yarmush, M.D., Ph.D.). Registration as a general surgeon is due in January 1993.