Acta Medica et Biologica
Vol. 47, No.3, 97-102, 1999

In Vitro Assessment of Microencapsulation in Providing Immunoprotection Against Antibody-Mediated Cell Lysis

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Received December 4 1998; accepted March 8 1999

Summary. **Xenotransplantation of porcine organs to untreated primates results in hyperacute rejection involving natural antibodies and complement. These antibodies rapidly interact with the vascular endothelium of discordant xenograft, activate the complement, and cause destruction of the vascular endothelium.**

The present study investigated the efficacy of two modalities to overcome the above barrier. The study was carried out on non-treated porcine hepatocytes, UV-B treated hepatocytes, and encapsulated hepatocytes which were incubated with normal human AB serum and complement-inactivated serum. Antibody-mediated cytotoxicity to porcine hepatocytes was assessed by MTT (tetrazolium) assay. Complement inactivation of normal human AB serum resulted in significantly less cell lysis in comparison with normal human serum. Non-irradiated and UV-B irradiated hepatocytes did not show any difference, indicating that surface-antigenic epitopes are not altered by UV-B irradiation. Microencapsulation of hepatocytes in alginate poly-L-lysine capsules, however, provided protection against antibody-mediated cell lysis, suggesting that this immunoisolation technique may be useful in xenotransplantation.

Key words—porcine hepatocytes, antibody-mediated cytotoxicity, MTT assay, microencapsulation.

INTRODUCTION

Hepatocyte transplantation is emerging as a simpler and cost effective approach for the treatment of acute liver failure and hepatic dysfunctions associated with enzymatic deficiencies or metabolic abnormalities[1-10].

Studies carried out by several investigators have shown that isolated hepatocyte transplantation has a promising future for the treatment of fulminant hepatic failure. However, the present shortage of human cadaver organs is a potent barrier for the pooling of liver cells. The use of xenogenic tissue has been suggested as a way of overcoming the shortage of human organs for transplantation. Use of non-human primate organs is not practical since sufficient number of organs are not available and also because of ethical considerations. Use of porcine organs is a viable alternative for transplantation. However, when xenogenic tissues are implanted directly into the host animal they are rejected even when the animal is immunosuppressed.

Xenotransplantation of swine tissue into untreated primates results in a hyperacute rejection (HAR) involving natural antibodies and complement[11]. These antibodies rapidly interact with the vascular endothelium of discordant xenograft, activate complement, and cause destruction of the graft vascular system[12,13].

These xenoreactive natural antibodies occur against and interact specifically with the mammalian carbohydrate Gal α-1-3 Gal B 1-4 GlcNac-R, termed the α-galactosyl (α-gal) epitope[14,15]. This epitope presents a major obstacle in xenotransplantation[16].

The α-gal epitope is produced in large amounts (1×10^6-35×10^6 epitopes per cell) in marsupials, in placental nonprimate mammals, in prosimians, and in New World monkeys, but not in Old World monkeys, apes, or humans[17-20]. Anti-gal antibodies, on the other hand, are produced abundantly in humans, apes, and Old World monkeys[17]. The interaction of α-gal antibodies with cells carrying the α-gal epitope leads

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to cell lysis.

The α-gal epitope is also reported to be present in microglial cells present in porcine fetal brain[21], against which there is a complement activating, naturally occurring IgM and IgG in the human serum[22].

Two approaches for preventing immunization include treatment of donor cells/tissues by different types of irradiation (γ-irradiation, x-ray irradiation, UV-irradiation)[23-27] and immunoisolating transplanted cells by microencapsulation, which provides a physical barrier between the host immune system and the transplanted cells[10,28,29].

In the present study we have attempted to determine whether UV-B irradiation immunomodulates the antibody response to porcine hepatocytes carrying the α-gal epitope and whether immunoisolation of these cells by microencapsulation prevents cell lysis by means of a simple antibody-mediated cytotoxicity/MTT (tetrazolium) assay.

MATERIALS AND METHODS

Hepatocyte preparation

Porcine hepatocytes were isolated by the collagenase digestion method by Berry and Friend (1969)[30] as modified by Habibullah et al. (1990)[31]. Briefly, porcine liver, which was obtained from a local slaughter-house, was perfused with Hank's buffer (flow rate 30-50 ml/min) until the perfusate became clear. The perfusion was stopped and the liver was gently pressed with forceps to remove the buffer. The liver was then incubated with 0.05% Collagenase (Sigma) for 10 min. The liver capsule was removed with forceps and the liver was cut into fine pieces and incubated at room temperature with constant stirring to disperse the hepatocytes completely. The digested tissue specimen was filtered through a nylon mesh (pore size 37 μ) to remove vascular and connective tissue. Hepatocytes were separated by gravity-sedimentation method. Viability of isolated hepatocytes was determined by trypan blue bio-exclusion method.

Encapsulation

Encapsulation of hepatocytes was done by the method of Cai et al. (1989) with slight modifications at 4℃[32]. Three million freshly prepared hepatocytes were suspended in 2 ml 0.9% sodium chloride containing 3% alginic acid (Sigma Chemical Co. St. Louis, MO), 0.045% collagen (Sigma), supplemented with 2% dextrose and loaded in a 10 ml syringe with a 23 G needle. Spherical droplets of the suspension were formed by dropping the suspension on a 1.3% calcium chloride solution. Following washing with 50 ml of 0.1% 2N-cyclohexylethane sulphonic acid (CHES) and 0.9% sodium chloride, the gel spheres were suspended in 25 ml of 0.05% (w/v) poly-L-lysine (MW 51,000; Sigma) for 10 min. Finally the capsules were washed with 0.1% CHES and 0.9% sodium chloride.

UV-B irradiation

Three million freshly isolated porcine hepatocytes were subjected to UV-B (302 nm) irradiation at 800 and 1500 Jm^{-2} using a standard UV lamp (UVM-57, Ultraviolet Products Limited, USA) and radiometer assembly (UVX-31, Ultraviolet Products Limited, USA). A non-irradiated group served as the control.

Antibody-mediated cell cytotoxicity/MTT (tetrazolium) assay

MTT assay was carried out as per the procedure by Mossmann (1983)[33]. Free, treated and encapsulated hepatocytes at a concentration of 3 million cells were incubated in presence of 1 ml Dulbecco modified Eagle's medium (DMEM, Sigma) (Control) or medium+80% complement inactivated or normal human AB serum at 37°C/2 hrs.

The cells were centrifuged and incubated with 1 ml of MTT (C,N-diphenyl-N'-4,5-dimethyl thiazol 2 yl tetrazolium bromide) (0.1%) at 37° C/1 hr. Cells were pelleted by centrifugation (700 xg/10 min) and then incubated at 37℃ with 2 ml iso-propanol until the blue formazon dissolved. Absorbance of the supernatant was read against appropriate blanks at 570 nm using a Beckman spectrophotometer.

Six experiments comprising 10 replicates per experiment were performed using free, treated, and encapsulated hepatocytes. Each experiment was performed using hepatocytes from a different donor. Data obtained for sets of porcine hepatocytes incubated with complement-inactivated and normal human serum were analyzed by Student's t-test and values were deemed significantly different when $p<0.05$. Results were expressed as mean±S. D.

RESULTS

In experiments designed to investigate the effect of complement-mediated lysis induced by xenoreactive antibodies, formazon produced by free hepatocytes exposed to normal human serum was significantly less than that produced by free hepatocytes exposed

Table 1. Formazon production by free, UV-B irradiated, and encapsulated porcine hepatocytes after incubation with normal human AB serum and complement inactivated human AB serum

	Normal human serum				Complement inactivated human serum		
Exp. No.	Control	Free hepatocytes	UV-B irradiated hepatocytes	Encapsulated hepatocytes	Free hepatocytes	UV-B irradiated hepatocytes	Encapsulated hepatocytes
1.	100.0	52.2	49.2	98.9	96.4	95.4	90.8
2.	100.0	58.8	56.4	93.1	102.8	93.1	99.6
3.	100.0	46.4	47.2	103.4	98.8	105.6	100.0
4.	100.0	61.2	59.8	95.6	96.8	89.8	102.4
5.	100.0	49.8	44.2	89.8	88.4	98.6	105.3
6.	100.0	50.1	43.6	97.6	90.6	88.8	98.6
A.M.	100.0	53.1	50.1	96.4	95.6	95.2	99.5
± S.D.	-	5.7	6.6	4.7	5.3	6.2	4.8

Values are derived from A570 values and represent formazon production. Control values have been standardized as 100, against which values for human serum and complement inactivated human serum have been corrected. Each experiment was performed on porcine hepatocytyes from a different donor and comprised 10 replicates.

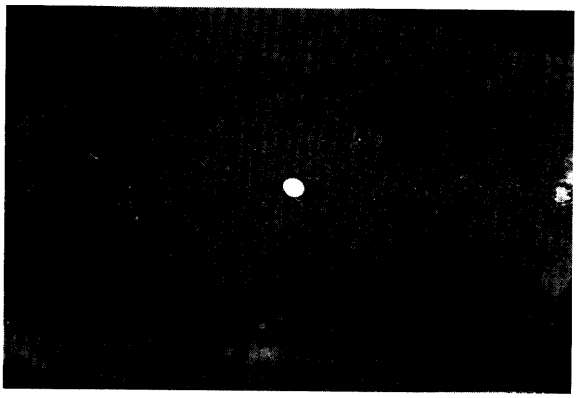

Fig. 1. Photograph showing a typical alginate poly-L-lysine microcapsule containing porcine hepatocytes.

to complement-inactivated human serum: formazon production was reduced by 42±5%. This difference was significant in each experiment ($p<0.05$).

The production of formazon by UV-B irradiated hepatocytes exposed to normal human serum was significantly lower ($p<0.05$) as compared with UV-B irradiated hepatocytes exposed to complement-inactivated human serum. Formazon production was reduced by 45±6%.

Standardized data from individual experiments showing the effect of complement-mediated lysis on free, UV-B irradiated, and encapsulated hepatocytes is expressed in Table 1. Encapsulation of hepatocytes in alginate poly-L-lysine microcapsules negates the effect of complement-mediated lysis -- i. e. there was no significant difference between the production of formazon by encapsulated hepatocytes exposed to normal human AB serum and complement inactivated human serum. Fig. 1 shows a typical microcapsule.

DISCUSSION

Fulminant hepatic failure (FHF) is the most complicated of all liver diseases and causes high morbidity and mortality (>80%) in the most advanced medical centers. FHF is associated with extensive necrosis rather than a loss of proliferative ability of surviving hepatocytes[34,35]. The treatment of different acute and subacute liver diseases is restricted due to the functionally complicated status of the liver.

Studies carried out by several investigators have clearly shown that isolated hepatocyte transplantation has a promising future for the treatment of FHF[1-4]. However, human donor organs are in short supply. Pigs represent a viable alternative for donor organs since they breed easily and have a short gestation time. Unfortunately, xenotransplantation between distantly related 'discordant' species such as pig to primate or pig to human results in hyper acute rejection (HAR) due to the presence of circulating preformed natural antibodies. Binding of these antibodies results in the activation of endothelial cells

and host complement, leading to graft vascular endothelial injury and vessel thrombosis.

These xenoantibodies recognize the α-galactosyl or Gal epitope (Gal α 1-3 β 1-4 GlcNac-R), which is a terminal dissacharide on glycoproteins and glycolipids[36,37]. The Gal epitope is formed due to the activity of α(1-3)-galactosyl transferase (Gal T) enzyme. Absence of this enzyme leads to non-expression of the Gal epitope in humans, apes, and Old World monkeys, which instead have high titer antibodies (both IgG and IgM) against the Gal epitope[38].

The importance of anti-Gal xenoantibodies was demonstrated initially by competitive studies wherein α-galactosyl sugars inhibited binding of human xenoantibodies to porcine endothelial cells[18,38-40]. Other studies using COS cells derived from Old World monkeys and, therefore, lacking the Gal epitope showed cell lysis by human serum only after transfection with a construct containing the cDNA for the Gal T enzyme[40]. Adsorption of human serum with COS cells expressing the GalT gene did not react with porcine endothelial cells.

Anti-gal antibodies are the primary mediators of hyper acute rejection (HAR), a rapid process mediated by the binding of xenoreactive antibodies and subsequent complement activation. New strategies to eliminate the contribution of anti-Gal antibodies to HAR have been proposed. These include depletion of the antibody either nonspecifically by plasmapheresis or specifically by immuno-adsorption. Other strategies depend on depleting or eliminating the Gal epitope from the donor[41,42]. Inactivation of the GalT gene has the potential advantage of eliminating anti-Gal antibody involvement in HAR permanently and completely. However, at present it is not possible to generate pigs lacking the Gal epitope because porcine pleuripotent cells, which are essential for gene inactivation, are not yet available. In addition, it has been suggested that deletion of the Gal epitope may be lethal. Studies with isolated porcine cells have shown that the α-gal epitope is also present on microglial cells present in the porcine brain as well as porcine pancreatic islet cells[21,43]

The application of immunoisolation technologies to prevent host sensitization to implanted cells is a feasible approach to avoid xenograft rejection. It has been reported that the alginate poly-L-lysine microcapsule membrane acts as an immune barrier during *in vivo* transplantation of microencapsulated cells[44]. It has also been demonstrated *in vitro* that the microcapsule membrane blocks the passage of antibodies and provides cell protection against the cytotoxic effect of anti-islet antibodies[43,45].

Heald et al. (1994) have shown that the capsule membrane can efficiently prevent the passage of xenoreactive antibodies leading to complement mediated damage by using an MTT (tetrazolium) assay. MTT, a pale, yellow substrate, is cleaved to form formazon, a dark, blue product, by the oxidative processes performed by living cell mitochondria[43]. The MTT assay has been used by us to investigate the effect of xenoreactive antibodies upon hepatocyte metabolism *in vitro*, and in addition, to test the efficacy of hepatocyte encapsulation as well as effect of UV-B irradiation on the functional efficacy of hepatocytes. UV-B irradiation has been shown to alter the cell surface properties[23-25] as well as immunogenicity of hepatocytes to a certain extent[46].

Microencapsulation of hepatocytes affords protection against anti-gal antibodies. The alginate poly-L-lysine membrane is not permeable to antibodies as our results suggest. No significant hepatocyte lysis occurred and values were close to control values when free hepatocytes were incubated in the absence of the antibody. This suggests a lack of penetration by anti-gal antibodies and non-binding since the presence or absence of the complement did not show any significant decrease in the amount of formazon formed.

UV-B irradiation did not cause any significant increase in formazon formation as compared with free hepatocytes (Table 1). This suggests that the galactosyl epitope remains unaltered after UV-B irradiation and that these epitopes are available to react with anti-gal antibodies and undergo lysis in the presence of the complement. This is further supported by the result that complement inactivation of human serum results in significantly higher formazon values in case of UV-B irradiated cells, indicating that while anti-gal antibodies may have become bound, cell lysis does not occur in the absence of the complement.

In all experiments, free hepatocytes incubated with normal human serum produced significantly less formazon than those incubated in the absence of serum. Formazon production by encapsulated hepatocytes exposed to normal human AB serum and complement inactivated human serum was similar to that by free hepatocytes incubated in the presence of medium or complement inactivated human serum (Table 1). In the above experiments, complement-mediated lysis and resultant reduction in hepatocyte metabolism was prevented by encapsulating hepatocytes within alginate poly-L-lysine microcapsules. The method used to prepare the microcapsules was one adapted from the method by Cai et al. (1989) with slight modifications[32]. Encapsulated hepatocytes fun-

ctioned satisfactorily as indicated by their ability to reduce MTT to formazon.

Our experiments confirm the ability of alginate poly-L-lysine encapsulation to prevent the passage of xenoreactive antibodies and subsequent complement-mediated lysis. In accordance with the results earlier obtained with islet cells by Heald et al (1994)[43]., our results suggest that the MTT (tetrazolium) assay may be used to assess the metabolic status of encapsulated or irradiated hepatocytes, and combined with a complement-mediated cytotoxicity assay, constitutes a simple and affordable procedure. In conclusion, immunoisolation of hepatocytes by microencapsulation appears to be a promising tool for the xenotransplantation of hepatocytes.

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