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PRINCIPLES OF TISSUE ENGINEERING AND RECONSTRUCTION USING POLYMER-CELL CONSTRUCTS

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ABSTRACT

The fields of materials science, cell and molecular biology, and surgical reconstruction are merging to create new devices for surgical transplantation and reconstructive applications. The field of artificial devices for implantation has matured over the last 40 years. Likewise, the field of transplantation and tissue reconstruction has undergone enormous change and improvements over the last 30 years. It has been proposed that these fields merge to create new tissue substitutes for functional replacement, therapy or reconstructive applications. Over the last five years, our lab has experimented with the concept of using man-made, biodegradable polymer systems as scaffolding for cell implantation devices. They have been designed to maximize diffusion parameters allowing nutrient exchange, gas exchange, and waste exchange. Vascular ingrowth occurs in the implant with subsequent resorption of the original polymer. This leaves a permanently engrafted new tissue which is a chimera of donor cells for functional replacement and recipient mesenchymal elements including blood vessels and supporting tissue. We have experimented in several model systems including hepatocyte implants, chondrocyte implants for cartilage reconstruction, urethelial implants for urinary reconstruction, and more recently small bowel and bone. Across this broad front of tissue types, much new knowledge has been gained and there continues to be hope that this will achieve clinical application.

INTRODUCTION

The fields of organ transplantation and tissue reconstruction suffer from three major problems 1) a scarcity of donor or reconstructive tissue, 2) a high cost, and 3) problems associated with tissue rejection or side-effects of the immunosuppresion used to reduce tissue rejection. In 1989, for example, 30,000 deaths resulted from liver disease but only 2160 transplants were performed (1). Many of these deaths could have been avoided if a sufficient number of donor organs were available. One possible solution to these problems is to engineer new tissue by transplanting selected cell populations (2). If functional tissue can be reconstructed using cell transplantation it may alleviate the problem of donor tissue scarcity as the cells from one organ could be used for several recipients, and/or the available cells may be multiplied *in vitro* before implantation, potentially creating an endless supply of transplantable tissue. Additionally, the cost of isolating the cells and subsequently introducing them to the recipient may be much less than whole organ transplantation. In applications such as bone or cartilage replacement it may also eventually be possible to isolate cells from a healthy site in the patient and subsequently implant his/her own cells after expansion of the cell population *in vitro*, thus alleviating the need for immunosuppression. The development of cell transplantation technology may also be important to the development of novel approaches for gene therapy (3).

However, for cell transplantation to successfully replace lost and/or deficient tissue several requirements must be met. These requirements include isolation of the cell type of interest from the donor tissue, survival of transplanted cells *in vivo*, and organization of the cells into a functional structure. Isolation techniques for a variety of cell types have been developed and outlined in the available literature, and the direct injection of isolated cell suspensions into a variety of implantation sites has been reported(4). However, these injected cells exhibit limited viability *in vivo*, and their ability to organize into higher order structures is limited. To surmount these challenges it has been proposed that the fields of materials science and cell biology be incorporated into this approach to develop materials which will promote cell adhesion and tissue-specific function while additionally providing a template for tissue organization. If these materials were also capable of degrading as tissue organization proceeded, one would ultimately obtain a completely natural tissue with no foreign body.

We have thus been investigating synthetic, biodegradable polymer systems for use in cell transplantation. The attachment and function of hepatocytes to films of the related polymers poly (D, L-lactic-co-glycolic acid) and poly (L-lactic-acid) has been previously described (5), and in this paper we will describe the *in vitro* interactions of hepatocytes with polymeric meshes formed of a random weave of polyglycolic acid (PGA) fibers. Preliminary *in vivo* results utilizing these matrices for hepatocyte delivery will also be presented.

MATERIALS AND METHODS

Cell isolations and attachment

Hepatocyte isolations were performed utilizing collagenase perfusion of Fischer rats as previously described (6). Cellular debris was subsequently removed utilizing a Percoll gradient (Sigma, St. Louis, MO; 7). After isolation hepatocytes were suspended in Williams E media (Gibco, Grand Island, NY) supplemented with 20 mU/ml insulin and 5 nM dexamethosone (Sigma), 10 ng/ml epidermal growth factor (Collaborative Research, Bedford, MA), 20 mM sodium pyruvate and 50 ug/ml ascorbic acid (Gibco), and a mixture of penicillin and streptomycin (100 mU/ml; Irvine Scientific, Santa Ana, CA).

PGA fiber meshes were 6.3 X 12.7 X 0.2 cm laminated sheets of a non-woven random fiber array. Scanning electron microscopy revealed fibers were approximately 14 microns in diameter. The polymer was a generous gift of Davis and Geck Corp. Polymer sheets were coated with a collagen gel by soaking in 10 ml of a 4°C, pH 7.2 solution of 70% Vitrogen™ (Collagen Corp., Palo Alto, CA), and 10% of a 10X solution of phosphate buffered saline (Gibco). Gelation was subsequently initiated by warming the polymer sheet to 37°C. Polymer meshes, either coated or uncoated, were placed flat in a petri dish, and 6 columns of 12 equally spaced 50 µl drops of a 3 X 10^6 cells/ml suspension were added to the top face. The polymer was incubated for 30 minutes at 37°C in a 5% CO_2 incubator to allow the cell suspension to absorb into the hydrophobic polymer mesh, and the polymer was then inverted and the cell suspension application was repeated. After another 30 minute incubation 25 ml of culture media was added to the dish. The polymers were cut into 1 X 3 cm strips 24 hr later, and either cultured in individual 35 mm petri dishes for *in vitro* analysis or implanted. Cultured polymer-cell constructs were refed daily. This cell application technique resulted in 2.1 X 10^6 cells being exposed to each polymer strip.

In Vitro Analysis

Cell attachment was assayed by a combination of a direct Coulter counting and the acid-phosphatase technique (8). In brief, hepatocytes were removed from the PGA mesh utilizing a trypsin-EDTA solution (Sigma) and coulter counted. However, a significant percentage of the cells remained entangled in the polymer. To determine this number the activity of the acid phosphatase cellular enzyme was measured in a known number of the removed cells. The acid-phosphatase activity of the entire polymer with remaining cells was then also measured, and the number of entrapped cells was determined by comparing this enzyme activity to the calibration obtained utilizing a known number of removed cells. The number of removed cells and entangled cells was then added to obtain total attached cells.

To compare the secretory profiles of hepatocytes cultured on PGA meshes with or without collagen gel coating, cells were refed with methionine-free William's E medium (Gibco) containing ^{35}S-methionine (100 µCi/ml, 1156 Ci/mM; ICN, Lesler, IL) as well as the other medium supplements. After two hr secreted proteins were collected, precipitated in ethanol, and separated by molecular weight on 7.5% gels using one dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples containing equal numbers of relative counts were loaded per lane to compare and contrast changes in the pattern of secreted proteins. After protein separation, gels were impregnated with Resolution (Electron Microscopy Co., Chestnut Hill, MA), dried, and exposed to Kodak XOMAT-XAR film at -70°C.

Albumin secretion rates were measured using a quantitative dot-blot assay. Samples of media collected over 24 hr periods were serially diluted, and loaded onto nitrocellulose paper (0.1 µm pore size; Schleicher and Schuell, Keene, NH) using a 96 well minifold apparatus. Serial dilutions of rat albumin (Cappel) were loaded in parallel on each blot. Blotted proteins were exposed to rabbit antibodies against rat albumin (Cappel) for 1 hr at room temperature, followed by exposure to ^{125}I-conjugated donkey anti-rabbit Fab^2 (Amersham; Arlington Heights, IL). The individual dots were then cut out and counted in a gamma counter. The amount of secreted albumin in the medium was determined by comparison with values from the linear portion of the calibration curve calculated using the albumin protein standard. Albumin secretion was normalized for cell number by quantitating the number of cells/polymer in the dish from which the media sample was taken.

In Vivo Implantation and Analysis

Midline incisions were made in anaesthetized Fischer rats, and the small bowel was exposed. Polymer-cell constructs were placed onto the mesentary, and a second leaf of the mesentary was folded over the polymer. The small bowel was subsequently returned to the abdomen, and the abdomen was closed. Rats were sacrificed at various times, biopsies of the mesentary with polymer were fixed in 10% formalin, and paraffin embedded sections (8 µm) were stained for hematoxylin by eosin.

RESULTS

In vitro Cell Attachment and Function

To determine the suitability of the PGA meshes to support hepatocyte adhesion and survival, suspensions of hepatocytes were placed on uncoated

Figure 1. Hepatocytes attached for three days to PGA fibers either left uncoated (A) or coated with a type I collagen gel (B).

PGA meshes, and cell attachment and function was measured. Large numbers of hepatocytes attached to the PGA (Fig. 1A), and quantitation of cell adherence 24 hr. after cell exposure revealed a 36 ± 6 % efficiency of attachment. Furthermore, the number of cells within the polymer mesh remained constant for the subsequent 13 days of the experiments (data not shown).

The secretion of albumin, a liver-specific protein secreted by hepatocytes, was then quantitated to assess the function of the cells. A significant level of albumin was secreted by these cells over the first few days in culture, and measurable quantities were found even out to 13 days (Fig. 2). Thus, these polymer devices were capable of supporting hepatocyte adherence and liver-specific function, although function decreased at longer times.

To increase the attachment efficiency of the PGA meshes they were subsequently coated with a type I collagen gel before exposure to cells. Type I collagen is a naturally occurring extracellular matrix molecule, and is well known to mediate hepatocyte adhesion *in vitro*. We hypothesized that coating the PGA with a gel of this protein would promote specific binding

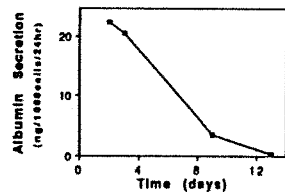

Figure 2. Albumin secretion from hepatocytes cultured on uncoated PGA fibers.

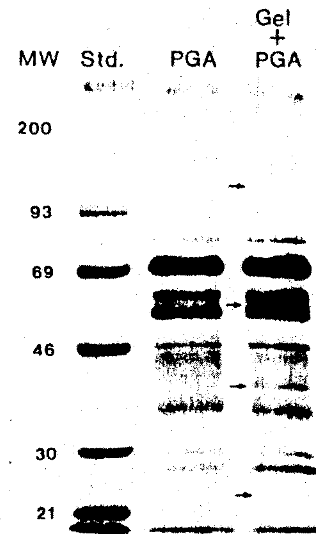

Figure 3. Autoradiograph of SDS-PAGE gel showing the pattern of protein secretion from hepatocytes cultured for 11 days on uncoated PGA (PGA) or PGA coated with a type I collagen gel (Gel + PGA). Molecular weight standards were loaded in the far left lane (Std.) for reference, and the appropriate molecular weights, in kDa, of these standards is labelled on the left. Arrows highlight differences in the protein bands under the two experimental conditions.

interactions between hepatocytes and the PGA mesh, and thus increase cell attachment. Collagen gel coated PGA devices did indeed promote hepatocyte attachment as 49 ± 9 % of the cells attached under these conditions (3.3 ± 0.7 X 10^5 cells/cm^2). Interestingly, the morphology of hepatocytes attached to the collagen gel coated PGA differed markedly from hepatocytes attached to uncoated PGA (Fig. 1 A & B). Hepatocytes cultured on collagen coated PGA clumped together into multicellular aggregates, while hepatocytes on uncoated PGA initially remained mainly as single cells, and only began to assemble into multi-cellular structures after several days in culture.

The function of hepatocytes cultured on PGA with or without the collagen gel coating was then investigated by examining the pattern of protein secretion from hepatocytes under both conditions. One-dimensional

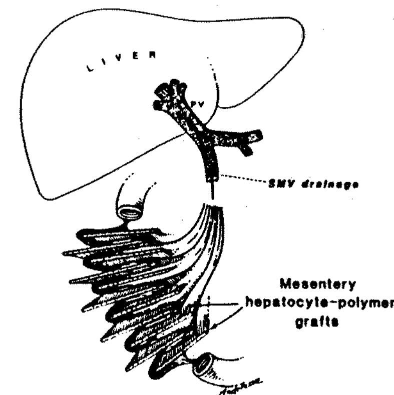

Figure 4. Schematic of the implantation procedure. A midline incision is made, the small bowel is exposed, and the hepatocyte-polymer grafts are placed between mesenteric folds. As shown here, multiple grafts can be placed in the mesentary. It is also illustrated how the blood supply for the implants is the superior mesenteric vein (SMV), which drains the entire small bowel. The SMV itself drains into the portal vein (PV), and the PV subsequently perfuses the liver. The implants are thus exposed to the same blood supply as the native liver.

gel electrophoresis of the proteins secreted by the cells showed that the pattern of protein secretion was overall very similar for both conditions over the 11 days of the experiment, although small differences were noted (Fig. 3). Thus, the enhancement of hepatocyte adhesion obtained by utilizing a collagen gel coating did not appear to detract from the subsequent function of the cells. Although the function of hepatocytes is clearly impaired at later times in culture (Fig. 2), Figure 3 clearly shows that even at these later times the cells still continue to exhibit a wide range of liver-specific proteins. We have identified several of the bands on Figure 3 by Western blot analysis, including albumin, transferrin, fibrinogen, and fibronectin (data not shown). All of these are liver-specific proteins secreted by differentiated hepatocytes.

In vivo Implantation

These promising *in vitro* results next led us to implant hepatocyte-PGA devices in the mesentary of Wistar rats to determine 1) if hepatocytes could be successfully delivered *in vivo* with these polymer devices, and 2) whether the *in vivo* environment was capable of promoting cell survival. The highly vascularized mesentary was chosen as the implant site because it was thought that this location could support the metabolic needs of the implanted cells, and the mesentary could be accessed non-traumatically to introduce the cells without causing a surgical wound at the implant site.

Hepatocytes were allowed to adhere to the PGA meshes for 24 hr. *in vitro*, the PGA-cell constructs were then implanted in the mesentary (Fig. 4), and subsequently biopsied for histological evaluation at serial time points. Importantly, tissue ingrowth with accompanying vascularity consistently occurs, and this new tissue remains stable over the entire time-course of the experiment even though the polymer fibers are completely degraded after 3 months. Histological sections of implants 3 weeks after transplantation exhibited numerous hepatocyte nodules in the mesentary (Fig. 5), and surviving cells have been detected out to 6 months after implantation. Although preliminary morphometric analysis suggests a large cell loss in the first few hours after implantation, studies with the Gunn rat suggest transplanted cell retain the ability to conjugate bilirubin, an important liver function seriously impaired in this animal model. Furthermore, staining of histological sections with antibodies directed against albumin demonstrated that the implanted cells continued to produce this liver-specific plasma protein (9).

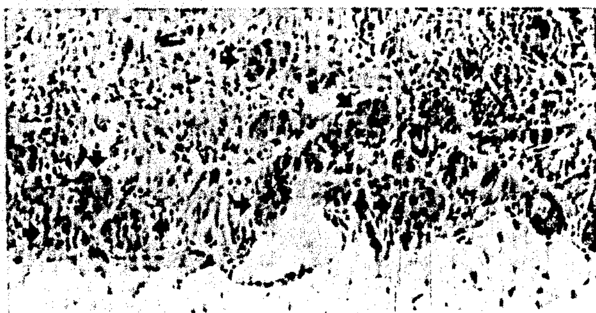

Figure 5. Histological section of mesentary 3 weeks post-implantation with hepatocyte-polymer graft. Numerous nodules of viable hepatocytes are appreciated (arrows) in the newly created tissue, and the native mesenteric tissue is visible at the bottom of the section.

CONCLUSIONS

Polymeric devices consisting of a random weave of PGA fibers have been shown to promote hepatocyte adhesion and function *in vitro*. The decrease in hepatocyte function *in vitro* was significant, but not surprising as cultured hepatocytes typically exhibit a rapid loss of differentiated in culture (10). Indeed, the ability of these devices to promote significant hepatocyte function for shorter times is promising, as these polymer devices will typically be transplanted one or two days after cell introduction.

Polymer-cell chimeras have furthermore been successfully utilized as a delivery vehicle for hepatocyte transplantation, and to promote the formation of a stable tissue encompassing the transplanted cells over extended periods of time. A similar approach has also been taken in the transplantation of chondrocytes and uroepithelial cells to successfully construct new cartilage (11) and urothelial tissue (12). This approach thus appears to be very promising for replacing lost or deficient function in a variety of tissues. It may even one day be possible to obtain a small cell sample from an afflicted individual, allow the disease-free cells to multiply *in vitro*, and after a sufficient cellular mass is obtained the cells could be transplanted back into the original donor. By utilizing a patient's own cells the problems of tissue rejection would be avoided. If desirable, it may also be possible to genetically correct cellular deficiencies, such as single enzyme defects, before implanting the cells.

We hypothesize that the chemical (i.e., cell adhesion) and physical (i.e., porosity, fiber diameter) properties of these devices is critical to the interaction of the polymer-cell constructs with the native tissue, and thus to its ability to support the metabolic needs of the transplanted cells while also promoting the vascularization of the new tissue. These properties are currently being optimized to allow reproducible and large-scale cell transplantation, and we are continuing to investigate methods of stimulating the proliferation of the cells after transplantation. This approach to creating new, functional tissue is promising, and the materials and techniques developed in these studies may have applications for a wide variety of diseases and therapies.

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BIODEGRADABLE CELL TRANSPLANTATION DEVICES FOR TISSUE REGENERATION

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ABSTRACT

Biodegradable polymers can be utilized as templates for cell transplantation and regeneration of metabolic organs and structural tissues. Candidate materials must be adhesive substrates for cells, promote cell growth and allow for retention of cell function. However, the processing requirements of such materials into highly porous three-dimensional structures with large surface per volume and an interconnecting pore network limits their potential application for tissue regeneration. A new processing technique was developed to produce uniform, three-dimensional cell transplantation devices of poly(lactic-co-glycolic acid). The process involved the preparation of highly porous membranes by a solvent-casting and particulate-leaching technique followed by their lamination. The device structural and mechanical properties depended on those of their constituent membranes, as evaluated by mercury porosimetry, scanning electron microscopy, and thermomechanical analysis. Cells to be seeded into the devices were injected from catheters incorporated within their structure. In vitro studies with model suspensions of dyed microspheres allowed for visual evaluation of the internal pore structure of various layered devices. From these studies, numerous parameters of device design for cell seeding were determined including pore size and injection rate. The membrane lamination technique produced devices without interfaces between layers as determined by microsphere injection and scanning electron microscopy.

Introduction

The use of biodegradable polymers to regenerate metabolic organs, such as liver and pancreas, and repair structural tissues like cartilage and bone by cell transplantation was recently explored [1-4]. To create organ function, individual cells are harvested from donor tissue, the cells are attached to a suitable scaffold, and the construct is implanted at a site where the immobilized cells grow and function. The three-dimensional scaffold serves as both a physical support and an adhesive substrate for isolated parenchymal cells during *in vitro* culture and subsequent implantation [3,5]. These scaffolds are designed to mimic their counterparts, the natural connective tissues of the body. Therefore, a successful cell transplantation support must exhibit a large number of diverse characteristics including high surface area to volume ratio, high percentage porosity, ease of processability into desired three-dimensional forms, and mechanical strength. In addition these polymers must be biocompatible, biodegradable, and allow expression of normal cell function.

The polymer provides a sturdy scaffold to the transplanted cells and the means of organization to the ingrowing tissue. High porosity values are required in order to accommodate a large number of cells. As most cells are anchorage-dependent, large values of the total pore area are necessary for high cell growth rates. Also, the pore diameter (or the interstitial distance) must be much larger than the particular cell diameter and an interconnecting pore network structure is essential for tissue ingrowth, vascularization, and diffusion of nutrients [6].

Natural tissues such as collagen [7], and synthetic materials such as tassels of poly(lactic-co-glycolic acid) (PLGA) fibers [6,8] have been used as transplantation devices for hepatocytes and chondrocytes to regenerate liver and cartilage function, respectively. Although these materials were useful in demonstrating the feasibility of organ regeneration, they lacked the necessary structural stability, and three-dimensional configuration.

In this work, a novel processing technique is reported to laminate highly porous, thin polymer membranes, and, thus, prepare devices with suitable pore morphology, strength, and shape. The devices are physically joined without any modification of the internal pore structure, and can be configured into shapes approproate for organ implants.

Materials and Methods

1. *Processing Technique*