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Tissue Engineering in the Twenty-First Century

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Abstract

In the 20th century, free tissue transfers have been successfully introduced using microvascular anastomosis techniques. Transplants not only include whole organs such as the kidney, liver and lung, but also bone, muscle and skin. However, there are a limited number of organs available for transplantation. This leads to the patient not only suffering from the malfunctioning tissue or organ, but also from the psychological trauma of an indefinite waiting period. The rapidly evolving field of tissue engineering is beginning to have an impact on free tissue transfers including organ. Small biopsy specimens can be grown into a large number of cells. These cultured cells can then be seeded onto biodegradable polymers, which serve several purposes. Firstly, the polymers function as a cell delivery system that enables the transplantation of a large numbers of cells into an organism. Secondly, they create a three-dimensional space for cell growth and serve as a template, thereby providing a structure for the extracellular matrix. These approaches have been demonstrated as practical strategies for the reconstruction of many tissues such as the liver, intestines, heart valve leaflets, bone and cartilage.

Key Words: Cell, culture, biology, polymer, biodegradation, liver, intestine, heart, finger, bone, cartilage, capillary

INTRODUCTION

In the 20th century, free tissue transfers have been successfully introduced using microvascular anastomosis techniques. These transfers not only include whole organs such as the kidney, liver and lung, but also bone, muscle and skin. However, the surgical procedures for severely impaired or congenital defects have been challenging, as they require many complicated reconstructive operations. Often a prosthetic or artificial replacement procedure may be compromised by a limited tolerance to external forces or by an intractable infection. Another difficulty with using artificial organs is that these devices do not grow in children. Therefore, they need to be replaced as the child matures. In recent years, free tissue transfers, which include either autologous tissue or allografts, have been developed to replace diseased parts of the body. However, this process is limited by donor site morbidity and whole tissue availability. While the number of transplants has been increasing year by year, large waiting lists remain. Even if a patient is fortunate enough to receive an allograft, lifelong immunosuppression is essential.

New techniques of tissue engineering are rapidly emerging and may make inroads into the care of patients in the 21st century.[1-5] Small biopsy specimens, even those with only a few isolated cells, can be cultured into over a large number of cells. These cultured cells can then be seeded on biodegradable polymers, which serve several purposes. Firstly, the polymers function as a cell delivery system that enables the transplantation of large numbers of cells into an organism. Secondly, they create a three-dimensional space for cell growth and serve as templates, which provide structural cues that direct tissue development. The matrix temporarily provides the necessary biomechanical support in the construct while the cells lay down their own extracellular matrix. The cells' extracellular matrix will ultimately provide the structural integrity and biomechanical profile of the engineered tissue. The long-term foreign body reaction problem that is related to polymers can be eliminated if the polymers are completely absorbed into the body, leaving only the naturally regenerated matrices. With regards to biocompatibility, polyglycolic acid (PGA) with poly-L-lactic acid (PLLA) and poly-lactic-co-glycolic acid (PLGA) polymers have

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been shown to be both biodegradable, and effective as scaffolds for cell delivery in the generation of new tissues.

In this paper, an outline of our research findings regarding tissue engineering during the last decade is provided and a prediction relating to new bridge tissue engineering that spans from biotechnology to clinical medicine during the 21st century is also given.

FABRICATION OF THE LIVER

Each year 26,000 people die of end-stage liver disease in the United States, with an estimated annual cost of 9 billion dollars. Liver transplantation is the only established successful treatment for end-stage liver disease, and currently more than 3,000 liver transplants are performed annually in the United States. It must be pointed out that severe organ donor shortage is a major limitation and it is this problem that has stimulated investigation into tissue engineering.

Since 1986 our approach has been to investigate tissue engineering as a novel means of recovering lost organ function in order to treat the end-stage liver disease.[6] Our hepatocytes transplantation strategy is to seed and culture dispersed parenchymal cells, which are harvested from the liver, onto synthetic biodegradable artificial polymers. These polymer constructs are then implanted into the animal. The biodegradable polymer scaffolds serve as a template to guide cell organization and growth. As the cell-polymer constructs are incorporated into the recipient, the polymer scaffold dissolves, leaving behind only the new natural tissue.

In order to replace the defective tissue and restore liver function, it is essential that there is sufficient engraftment and an adequate mass of transplanted cells that survive. However, hepatocytes have high metabolic needs and require both a steady oxygen and nutrient supply both *in vitro* and *in vivo*. To overcome these conflicting issues, several improvements in the fabrication of biodegradable polymer scaffolds, culture conditions of hepatocytes *in vitro*, and implantation techniques *in vivo* that allow better gas and nutrient exchange have been demonstrated.

There are many important conditions needed for the fabrication of biodegradable polymers. They must have a large enough surface area to allow the attachment of a large number of implanted hepatocytes. In addition, they must be able to provide a steady supply of oxygen and nutrients, and facilitate waste removal by diffusion in culture *in vitro* during the initial period after implantation until the development of neovascularization. These polymers also require the provision of a space for hepatocytes reorganization and neovascularization from the surrounding tissue *in vivo*. For these reasons we have fabricated a biodegradable polymer tube with a highly microporous three-dimensional structure created from sheets of a nonwoven PGA fiber mesh sprayed on the outer surface with a 5% PLGA chloroform solution. Each fiber was 15 μm in diameter with an average pore size of 250 μm.[7]

An additional approach to the fabrication of a porous polymer uses the technique of three-dimensional printing (3DP). This technology provides complex and three-dimensional scaffolds in any shape or size with a high degree of both macro-architectural and micro-architectural complexity. The 3DP technique can create polymer scaffolds with a complex intrinsic network of interconnected vascular channels.[8]

The optimization of hepatocytes seeding onto these three-dimensional polymer devices is critical for maximizing the mass of cells that can be transplanted. This is not only to uniformly distribute the cells throughout the complex polymer scaffold, but also to help ensure the long-term survival of the cells and their function as tissue. We have designed a simple system for dynamic hepatocytes seeding and culturing, and demonstrated the successful attachment, uniform distribution throughout the polymer scaffolds, and survival of a large number of cells after 7 days of flow culture. The hepatocytes demonstrated an increasing rate of albumin synthesis over the culture period.[7,9] Moreover, the hepatocytes seeded onto the 3DP polymer scaffolds reformed the histotypical structures in the channels of the polymer devices. These results suggest a huge potential for providing immediate access of the implanted hepatocytes to the blood supply.[10]

Hepatocytes have tremendous regenerative capacity with hepatotrophic stimulation *in vivo* and most of these factors seem to derive from the portal blood. It has been demonstrated that both the engraftment and proliferation of transplanted hepatocytes in a peripheral site and in small intestine mesentery improve with portacaval shunts and partial hepatecto-

mies. The diversion of these hepatotrophic factors that are naturally circulated in the portal blood to the transplanted cells led to increased vessel proliferation.[11]

For sufficient engraftment and survival of an adequate mass of transplanted cells to replace lost liver function, the gap between *in vitro* and *in vivo* must be bridged. For this reason, prevascularized polymer sponge devices were fabricated in advance, which were injected with a hepatocytes density equivalent to a normal liver. Seven days after implantation it was estimated that between 10.8% and 18.0% of these hepatocytes were engrafted and functioning.[12]

Another approach is the implantation of hepatocytes onto biodegradable polymer scaffolds with direct access to the portal blood. In preliminary studies, small intestinal submucosa (SIS) were grafted between the portal vein and inferior vena cava as a small-caliber venous conduit, which may be used for the implantation of a tissue engineered liver. Hepatocytes demonstrated clusters of viable cells in the portal blood two days after implantation.[13] In addition, we are developing tissue with its own blood supply, which is described at the end of this paper.

REGENERATION OF SMALL INTESTINE

Short bowel syndrome is a clinical condition that afflicts both adults and children, which is characterized by malabsorption and malnutrition after a massive small bowel resection. With the development of total parenteral nutrition, many patients may survive for extended periods. However, total parenteral nutrition is accompanied by various complications, such as hepatic dysfunction, progressive nephric insufficiency, bone demineralization, and catheter sepsis. The annual mortality of these patients with this syndrome is estimated to be between 2% and 5%.

Various surgical therapies aimed at increasing the intestinal surface area and slowing intestinal transit so as to increase the absorptive rate have been attempted, but with limited clinical success. Recently, small bowel transplants have been performed on patients with short bowel syndrome. However, this procedure has limitations as there are difficulties in controlling rejection, immunosuppression related complications, and donor supply.

As a promising alternative approach to the treatment of short bowel syndrome, our laboratory has investigated the transplantation of intestinal cells using synthetic biodegradable polymer scaffolds for generating new intestinal tissue.[6] Since an epithelial-mesenchymal cell-cell interaction is thought to be critical during embryonal organogenesis for crypt cells to survive, proliferate, and differentiate, we have been using crypt cells as an epithelial mesenchymal unit called the intestinal epithelial organoid unit. This unit consists of a villus structure with an overlining epithelium and a core of stromal cells, which preserves the epithelial-mesenchymal interaction. Epithelial organoid units were isolated from neonatal rat intestine by enzyme digestion and differential sedimentation. Organoid units were subsequently seeded onto a tubular scaffold created of nonwoven PGA fiber and implanted into the omentum of adult rats. The organoid units survived, became vascularized, proliferated, and formed cyst like structures after implantation. The inner lumen was lined with a well-developed neomucosal layer characterized by crypt-villus structures, and it was surrounded by a smooth muscle layer.[14] Immunofluorescent microscopy of the neomucosa showed apical staining of the brush border enzymes, sucrase and lactase, and basolateral staining for the basement membrane protein, and laminin. Electrophysiology of neomucosa exhibited a similar transepithelial resistance to adult ileal mucosa.[14]

Intestinal epithelial organoid units and polymer constructs were implanted into the omentum of adult rats that had received a 75% resection of their small bowel to investigate the methods of stimulation for optimizing neointestinal regeneration. The neointestinal cyst lengths and diameters were significantly larger, and histological analysis demonstrated a better differentiation of the tissue engineered small intestinal mucosa than in the control group. A small bowel resection provided significant regenerative stimuli for the morphogenesis and differentiation of the tissue-engineered small intestine.[15]

The successful anastomosis between tissue-engineered intestine and native small bowel was also demonstrated where the anastomosis had a positive effect on the development of the neointestine. Three weeks after implantation, the tissue-engineered intestine was anastomosed, either side-to-side or in an end-to-end fashion, to the native jejunum. Histological examination performed 10 weeks after implanta-

tion showed that after anastomosis the tissue-engineered intestines were lined by a neomucosa with continuity to native small bowel across the anastomosis site. The tissue engineered intestines with anastomosis had greater villus number, height, and surface length than the controls without anastomosis.[16,17] The anastomoses maintained a high patency rate and the tissue engineered intestine increased in size for up to 36 weeks.[18] We are now focusing on the functions of the tissue-engineered neointestine, such as absorption, wall mobility, and neural innervation.

RECONSTRUCTION OF HEART VALVE LEAFLETS

Valvular heart disorder is a major cause of morbidity and mortality. In 1997 78,000 valve replacements were performed in the United States alone. Although ordinary valve replacement surgery can effectively improve patients' quality of life, the implants are different from living valves and have some important restrictions. Major problems include thromboembolism, which limits durability, donor organ scarcity, rejection and infection.

The reconstruction of heart valves using tissue engineering techniques has many potential benefits. They include the avoidance of anticoagulant therapy, a potential for growth, greater durability and longevity, and the opportunity to use viable, autologous tissue that can utilize the body's mechanisms for repair and remodeling.

The polymeric scaffold used for reconstructing the heart valve leaflet was composed of PGA and PLGA polymers.[19-21] The bioprosthetic leaflet was a composite, laminated structure. The outer layer of the implantation device was constructed from a nonwoven mesh made from pure PGA fibers. The inner portion of the leaflet was constructed from a woven mesh made of PLGA. The inner layer of woven material was sandwiched by an outer layer of randomly arrayed fibers, which were thermally fused together at multiple points along their interface. The implantation devices were square sheets with surface dimensions measuring 3×3 cm and a thickness of 3.2 mm. The maximum tensile strength of polymers was greater than native leaflets. The scaffolds were biodegradable via hydrolysis over a 6−8 week period.

The cells were isolated from either the carotid or femoral arteries of a lamb. Small diced pieces of artery were placed in Dulbeco's modified Eagles's medium with 10% fetal bovine serum and a 1% antibiotic solution for 8−10 weeks. Mixed populations of smooth muscle cells and endothelial cells were detached. After reaching confluence, they were labeled with an acetylated low-density lipoprotein (LDL) probe, which selectively tagged the endothelial cells with a fluorescent dye and allowed the division of the cells into two populations using a fluorescent activated cell sorter. Immunohistochemical analysis showed that the LDL-negative cells were mostly stained with actin and resembled smooth muscle cells or myofibroblasts. The LDL-positive cells were stained with factor VIII for the endothelial cells. Those two cells were passed separately to obtain sufficient cell numbers. One million LDL-negative cells were seeded onto the polymeric scaffold every day over 12-day period. Subsequently, three million LDL-positive cells were seeded covering the scaffold with a monolayer of cells. After 2 weeks, the constructs were implanted into the sheep whose arteries were originally harvested. Under sterile conditions, a cardiopulmonary bypass was prepared via left thoracotomy. After this, a pulmonary arteriotomy was performed and the right posterior pulmonary leaflet was excised. The tissue engineered leaflet was then tailored and implanted.

Post-operative echocardiograms showed no evidence of stenosis or regurgitation. However, the implanted leaflet was thicker and did not move as freely as the native leaflets. Histologically, the tissue engineered leaflet demonstrated an appropriate cellular architecture and an extracellular matrix similar to the native leaflets.

STRUCTURAL GENERATION OF BONE AND CARTILAGE

Reconstruction of a human digit is a major challenge in tissue engineering since the joint is a complicated structure consisting of various types of cells and their specific extracellular matrices. The three sources of structural cells used in the current study were osteoblastic cells, chondrocytes and tenocytes. These three types of cells were implanted *in vivo* in order to create a whole tissue engineered unit of a joint.[22] Firstly, the periosteum was applied to induce new bone formation since it exhibits an osteo-

genic potential through the release of periosteum-derived osteoblastic cells. When the periosteum was placed directly onto the PGA polymer, the periosteal cells migrated from the periosteum, attached and then spread readily on the polymer, generating bone. Secondly, chondrocytes and tenocytes were seeded directly onto the polymer scaffold and shown to regenerate cartilage and tendon tissue on and in the amorphous fibrous mesh of the PGA and PLLA.

Two types of polymer scaffolds were created for the fabrication of the phalanges and joints. One scaffold was only a mesh of PGA fibers for cartilage and tendon support. The other scaffold was produced by cutting a PGA mesh into three-dimensional cubes. The PGA meshes were subsequently immersed in a 2% chloroform solution of PLLA, which resulted in cross-linking between the PLLA chains and PGA fibers. This technique aids the polymer scaffolds in retaining specific shapes. Prior to fixation, each wet PGA mesh was reshaped by placing it into negative molds of the phalanges made from a dental impression material. After evaporation of the solvent, the polymers were removed from the negative molds.

The periosteum, chondrocytes and tenocytes were isolated from newborn calves. The periosteum was wrapped around the center section of the PGA-PLLA copolymer phalanges molds. The chondrocytes and tenocytes (each numbering 30×10^6) were both seeded on each sheet of polymer mesh. Every construct was incubated in M199 and F12 medium with a 10% fetal bovine serum, respectively, for one week before implantation. The chondrocyte-polymer sheets were sutured to the periosteum-polymer constructs of the distal and middle phalanges to create opposing articular surfaces. A joint was then formed by wrapping this composite with additional PGA sheets seeded with tenocytes. This created a distal interphalangeal joint. A 0.5 mm thick silicone sheet was inserted between the phalanges to prevent contact between the two adjacent equivalent articular surfaces. All the constructs were implanted into the dorsal subcutaneous space of athymic mice. After 5 months the implants were dissected in half and examined histologically.

After 5 months of implantation, the whole joint constructs maintained their original shape and formed phalangeal joints resembling their normal counterparts. A longitudinal cross-section showed that the periosteum-polymer composite had developed into bone tissue, whereas the articular chondrocyte-polymer construct had developed into cartilage. Moreover, the tenocyte-containing polymer had formed the tenocapsule of the joint, which was histologically distinguishable from surrounding connective tissue. The structural arrangement of the engineered tissues was quite similar to that of a normal joint. Subcutaneous implantation of the sutured composite polymers with periosteum cells, chondrocytes and tenocytes resulted in new tissue formation with the shape and dimensions of human phalanges and joints.

CAPILLARY NETWORK INNOVATION

Vascularization is essential for allowing the implanted tissue-engineered organs to survive. All past approaches in tissue engineering aimed at achieving permanent vascularization have relied on the ingrowth of capillaries into the neo-organs, which are about $5-10\ \mu m$ in diameter. This strategy has worked well for many thin tissues with low metabolic needs. However, it is unsuitable for thick and complex structures such as large vital organs, including the liver, kidney, and heart.

The Micro Electro Mechanical System (MEMS) has been contributing a broad variety of applications, such as motor vehicles, navigation systems, chemical sensing and, most recently, biomedical engineering. The use of the MEMS micro-structural technique in biomedicine demonstrates the expansion of the semiconductor wafer process technology originally developed for the computer industry. Since quality control down to the submicron level is routinely achieved in an integrated circuit (IC), MEMS technology translates this level of power into mechanical structures with scales stretching from 1 cm to $1\ \mu m$. Standard micromachining enables patterns of an arbitrary geometry to be imprinted into wafers using a series of subtractive etching methods. Three-dimensional structures can be realized by the superposition of these processes using precise alignment techniques. We have reported microfabrication strategies on silicon and Pyrex that generate hepatic and vascular tissue that may be combined to form thick three-dimensional vascularized organs.[23]

A schematic design of a vascular branching network was used as a template for micromachining. This patterning was transferred and etched to silicon

and Pyrex wafers. Typical trench depths were achieved 20 μm on silicon and 10 μm on glass. Endothelial cells were then cultured on the Pyrex and silicon wafers and proliferated to confluence within 4 days. On a silicon surface coated with a collagen solution, the endothelial cell sheets were lifted maintaining intact monolayer sheets. Hepatocytes also were attached to Pyrex and they spread as well as they had on silicon wafers. Moreover, they could also be lifted as intact monolayers. Histological assessment showed that both the endothelial cells in a single cell array and the hepatocytes in a more round multilayered array were viable. Hepatocytes secreted 154 μg/day albumin during the culture period of 4 days and stained positive for albumin in immunohistochemical staining.

This preliminary study demonstrated that silicon microfabrication technology can be used to form large sheets of liver tissue, which establishes the feasibility of etching ordered branching arrays of channels, whose luminal surface can be lined with liver cells. In addition, organized sheets of hepatocytes and endothelial tissue can be lifted from the surface of silicon or Pyrex wafers and then folded into compact three-dimensional configurations.

CONCLUSION

Tissue engineering has rapidly developed, becoming a promising technology for health care. The critical problem remaining is to engineer new functional vascular networks for large, complex vital neo-organs that can function continuously in the human body. A combination of various cell types and biodegradable polymer scaffolds with vascular networks may solve this problem, and may lead to the beginning of neo-organ transplantation in the 21st century.

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