TISSUE ENGINEERING FOR TISSUE AND ORGAN REGENERATION

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Preface

Over the last decade Tissue Engineering progressed rapidly and first biological substitutes were developed for several tissues in the body. Today, Tissue Engineering is one of the major approaches of Regenerative Medicine and represents a growing and exciting field of research. With the understanding and application of new knowledge of structure, biology, physiology and cell culture techniques, Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence.

The classical principle of Tissue Engineering is to dissociate cells from a tissue biopsy, to expand them in culture, and to seed them onto a scaffold material in vitro in order to generate a viable tissue construct prior to re-implantation into the recipient's organism. In the appropriate biochemical and biomechanical environment these tissues will unfold their full functional potential and serve as native tissue equivalents. Tissue Engineering products may be fully functional at the time of treatment, or have potential to integrate and evolve into the expected functional tissue after implantation. While these steps may seem logical and easy to understand, the underlying biology is far more complicated and more profound questions have to be answered before the engineering of tissue and organs becomes a routine practice.

Even so, the Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material implicate only minimal or no immunogenicity when reimplanted in the patient. This eventually conquers several limitations encountered in tissue transplantation approaches.

This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. With a colorful mix of topics which explain the obstacles and possible solutions, it offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration. As more and more applications move toward clinical application, a reliable preclinical model system to

evaluate the developed techniques becomes crucial. Several animal models and Tissue Engineering approaches for a variety of organ systems are presented in this book.

Finally, I would like to thank all the authors who have supported this book with their contributions.

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Part 1

Cardiac Muscle

1

Myocardial Tissue Engineering

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1. Introduction

Many lives are lost due to heart diseases including myocardial infarction and cardiomyopathy. Recent reports have demonstrated that regenerative medicine has promising potential for recovering severe heart failure. Regenerative therapies for heart failure include cytokine, gene and cell therapy. Because many types of cardiovascular stem cells have been identified and their clinical potentials have been demonstrated for the past decade, cell injection therapy has most attracted both researchers and clinicians (Wollert 2008). On the other hand, significant cell loss due to washing out and cell death has become problematic in cell injection technique. So, as next generation of regenerative therapy for impaired heart, transplantation of myocardial patches fabricated by tissue engineering technology are emerging and are clinically applied. Furthermore, several challenges for fabricating functional myocardial tissues/organs, which are electrically communicated, pulsate synchronously and evoke contraction power, have also started (Zimmermann, Didie et al. 2006). These ambitious challenges may lead to reconstruction of malformed hearts and become alternative therapy for heart transplantation.

Heart tissues are composed of high-dense cylindrical cardiomyocytes and fibroblasts with abundant vascular network and collagen-based extracellular matrix (ECM). Cardiomyocytes pulsate via sodium and calcium ion transient through cell membrane. They are also electrically coupled by gap junctions composed of connexion 43 and rapid electrical propagation realizes simultaneous beating as a whole. Continuous blood flow supplies oxygen and nutrition, and withdraw the waste for high metabolic demand of heart tissues. These structure and function produce mechanical contractions as a blood pump. Therefore the researchers should take into account high density culture of cardiomyocyte and surrounding cells, sufficient micro blood vessel fabrication, cell/ECM orientation and proper cell-to-cell coupling for engineering heart tissues/organs.

Here, previous and current status of cell injection therapy, myocardial patch transplantation and pulsatile myocardial tissue fabrication is described with some future views.

2. Cell injection therapy

Cell injection therapy for damaged heart has been researched since the early 1990's. Many researchers have demonstrated the therapeutic potential of isolated cell transplantation into myocardium using various types of cell sources both in animal models and in some clinical

trials (Puceat 2008). The mechanism of myocardial tissue regeneration has not been completely cleared, but most researchers have agreed that transplanted cells secrete several cytokines which promote neovascularization, prohibit fibrosis, decrease cell death and recruit stem cells, leading to heart function improvement. It has been also asserted that some of injected cells differentiate into functional cardiomyocytes and may directly contribute to heart contraction improvement. Although some differences may exist in according to cell types, multifactorial mechanisms seem to relate with myocardial tissue regeneration.

In addition to cell sourcing, different routes are used for cell administration. Systematic intravenous infusion is performed through a central or peripheral vein. This method is simple and less invasive, however widespread distribution cause low ratio of cell engraftment. Most popular approach is intracoronary cell infusion via a balloon-catheter. Injected cells are reached directly in the target myocardial region, however, cells have to transmigrate across endothelium wall. Intracardiac injection is performed via pericardium during open heart surgery and via endocardium by a catheter with a 3-D electromechanical mapping system (NOGA mapping system). These methods realize relatively targeted delivery, but myocardial damage and arrhythmia induction are problematic. Future clarification will be needed to decide which is the best approach for cell injection.

2.1 Skeletal myoblasts

Skeletal myoblasts were the first cell source to enter the clinical application for heart tissue repair. They lie in a quiescent state on the basal membrane of myofibers and have the potential to start to proliferate and differentiate into functional skeletal muscle in response to muscle damage. They can be isolated autologously and be expanded from a single biopsy. In addition, skeketal myoblasts are relatively resistant to ischemia. Menasche and colleagues first applied skeletal myoblast injection via epicardium for patients undergoing coronary artery bypass grafting (CABG) (Menasche, Hagege et al. 2001). The phase I clinical study (MAGIC I) have shown the feasibility of skeletal myoblast implantation, however, increased risk of ventricular arrhythmias after the operation. Then, MAGIC II trial was performed to clarify the safety and efficacy, in which all patients received preventive medication and an implantable cardioverter-defibrillator for rescuing critical ventricular arrhythmias. In result, skeletal myoblast injection failed to significantly improve heart function, leading to sample size reduction (Menasche, Alfieri et al. 2008). On the other hand, the trial indicated the possibility that high dose cell injection might recover left ventricular dilatation. In addition, the other clinical trials of catheter-based myoblast implantation via endocardium have revealed functional efficacy (Opie and Dib 2006). According to these results, not the regenerative potential of myoblasts themselves but the amount of injected cells and delivery system may affect the efficacy. Therefore, it seems that skeletal myoblasts should not be excluded as a cell source for heart tissue repair. More optimization of cell delivery and comparison of cell sources can address these critical issues.

2.2 Bone marrow-derived cells

Bone marrow-derived cells are the most used cells in clinical trials for myocardial tissue repair (Wollert 2008). The discovery of circulating progenitor cells originated from human bone marrow has stimulated research and clinical use of bone marrow-derived cells

(Asahara, Murohara et al. 1997). Bone marrow cells contain different stem and progenitor cells which will differentiate into various types of cells including endothelial cells, smooth muscle cells and cardiomyocytes. Bone marrow mononuclear cells (BMNCs), which can be isolated simply by gradient sedimentation after bone marrow aspiration without culture expansion, have been clinically injected via coronary artery from the first. BMNCs include heterogeneous cell population of monocytes, hematopoietic stem cells and endothelial progenitor cells (EPCs). Therefore, some groups have used BMNCs selected by surface markers ($CD34^+$, $CD133^+$) and demonstrated more efficacy of their injection. As another cell population, mesenchymal stem cells (MSCs) have been researched and clinically used. Although, MSCs represent between 0.01 and 0.001% of all nucleated cells in bone marrow, they can be readily expanded in culture. MSCs have the potential to differentiate into various types of cells and injected MSCs in heart seem to differentiate into myocardial composing cells. Recent studies have revealed rare happening of cardiomyocyte differentiation, therefore MSCs seem to recover heart function via their cytokine secretion and partial differentiation into vascular cells. As a unique feature, MSCs have the potential to escape from immune detection due to the direct inflammatory inhibition and the lack of cell-surface molecules. This property has realized allogenic mesenchymal stem cell transplantation in clinic and has given high impact on cell therapy research field.

Recent randomized controlled trials of bone marrow-derived cell injection revealed overall feasibility and safety. However the data has revealed only marginal increases of ejection fraction (EF) even in positive studies (0-5%) (Martin-Rendon, Brunskill et al. 2008). For establishing more effective bone marrow-derived cell therapy, optimization of cell source, cell dose, delivery method and deliver timing will be needed.

2.3 Adipose-derived stem cells

In addition to bone marrow-derived MSCs, stem cells isolated from the stroma of adipose tissues have represented regenerative potential for heart tissues (Psaltis, Zannettino et al. 2008). Adipose tissue-derived stem cells (ASCs) display features similar to that of bone marrow-derived MSCs and their angiogenic potential have been reported. Some studies have also revealed cardiomyocyte differentiation from ASCs. It has not been clarified which mesenchymal stem cells are superior to other cell types, however, reatively easy isolation of adipose tissue may push the clinical application of ASCs.

2.4 Cardiac stem cells

Cardiac stem cells (CSCs) are also possible cell source for myocardial tissue regeneration. Two groups first reported CSC existence in 2003 (Beltrami, Barlucchi et al. 2003; Oh, Bradfute et al. 2003). Until then, it was common knowledge that heart was a post mitotic organ, but those reports accelerated the researches for identifying surface marker of CSCs and culturing them. Islet-1, Sca-1 and c-kit have been known as CSC markers. Recently, it has been also confirmed that heart has renewal ability at normal state and the annual rate of turning over is 1% at the age of 25 (Bergmann, Bhardwaj et al. 2009). Although the ability of CSCs may increase after heart injury, newly formed cardyomyocytes are not sufficient for replacing damaged muscle tissues. Therefore isolation and expansion of CSCs have been extensively examined. Some groups have used a different approach to make cardiospheres from biopsied myocardium, which lead to efficient CSC expansion (Lee, White et al. 2011).

Clinical trials for injection therapy of autologous CSCs isolated from biopsy sample are now on going.

2.5 Embryonic stem cells

Although abundant studies demonstrated that MSCs, ASCs and CSCs have the potential of cardiomyocyte differentiation regarding gene and protein expression, there are no studies clearly showing beating cardiomyocytes differentiated from those stem cells. On the other hand, many researchers have confirmed that embryonic stem cells (ESCs) can differentiate into beating cardiomyocytes in vitro and implantation of ESC-derived cardiomyocytes improves damaged heart function. Several signal pathways for cardiac differentiation have been already clarified and various molecules have been reported as its promoters. For example, noggin increased cardiac differentiation efficacy via regulation of Bone morphogenetic protein (BMP) signalling pathway (Yuasa, Itabashi et al. 2005) and insulin-like growth-factor-binding protein 4 (IGFBP4) promotes cardiogenesis by inhibitor of canonical Wnt signalling (Zhu, Shiojima et al. 2008). In addition, fibloblast growth factor (FGF), retinoic acid, ascorbic acid and cyclosporine A have been reported to have the potential to enhance cardiac differentiation from ESCs. The important issue as well as cardiac differentiation is purification of cardiomyocytes from heterogeneous cell mixture, because contamination of immature cells leads to teratoma formation. Although gene-modified ESCs harboring neomycin resistance gene or green fluorescent protein (GFP) gene in the cardiac-specific gene locus are very useful in non-clinical experiments, safe and efficient isolation technologies will be needed for clinical application. Culture media control focusing on the differences of cell metabolism may be useful for safe cell selection. Moreover immune response of the host is another critical issue. Nucler transfer or cell banking is possible approach avoiding immunoreaction.

Electrical communication and simultaneous beating of implanted ESC-derived cardiomyocytes should be also requested for improving damaged heart function without arrhythmia. In vivo electrophisiological analyses and the transplantation technology for synchronization will be essential for clinical application of these cells.

2.6 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) also hold great promise for myocardial tissue engineering (Vunjak-Novakovic, Tandon et al. 2010). Terminally differentiated cells can be reprogrammed to have the same potential as ESCs by introducing 3 or 4 transcriptional factor genes. Furthemore none-gene transfer technologies have been developed in the world. The superiority of iPSCs to ESCs is autologous cells, which do not cause immune response. Cardiac differentiation of human iPSCs has been reported in the same manner with ESCs.

Several critical issues must be clarified for clinical use, but ESCs/iPSCs-derived cardiomyocytes should contribute to myocardial tissue engineering in the view point of their pulsatile function and scaling-up.

2.7 Problems of cell injection therapy

Cell injection therapies for heart failure are now world-widely performed. While moderate success of direct cell injection has been observed, the efficacies seem not to reach the level that general clinicians think cell therapy a reliable treatment for heart failure. More

optimization of cell source, cell preparation process, injection route, injection timing and patient population may increase the effectiveness; however one of the essential issues is cell delivery methodology. Cell injection therapy has significant difficulties about cell retention in the target tissue. The shape, size, and position of the grafted cells are often uncontrollable and large amount of the cells are washed-out. Moreover, once retaining cells die due to necrosis and apoptosis. Time course quantification with TUNEL assay demonstrated that a large number of the grafted cells die within a few days after injection in rat models (Zhang, Methot et al. 2001). In the clinical trial using bone marrow-derived cells, it has been also demonstrated that only 1-3% of the cells infused via coronary arteries could be detected by 3D positron emission tomography (PET) imaging of the patient heart. In this study, a large percentage of cells were found in the liver and spleen immediately after the procedures (Hofmann, Wollert et al. 2005). To clear the problem of cell loss, hydrogel-cell mixture injection has been pursued. Fibrin, collagen and alginate hydrogels are now used. Hydrogels with cells are injected as a liquid phase through syringe or catheter, then, they are polymerized and fixed in the target tissues (Kofidis, de Bruin et al. 2004). In hydrogel-cell mixture injection therapy, local tissue damage due to space occupation of hydrogel itself and inflammatory reaction due to hydrogel biodegradation are problematic.

Therefore, more advanced cell delivery systems have been requested to spread the regenerative therapy as one of the reliable treatments for heart failure.

3. Tissue engineering

Recent advance of tissue engineering technologies have realized the transplantation of tissue-engineered construct "myocardial patch" covering over damaged heart surface instead of simple cell injection into myocardium. Grafted cells within myocardial patches can survive more and secrete more cytokines, resulting in more heart function improvement. Furthermore pulsatile myocardial tissues have been successfully engineered by using cardiomyocytes as a seeding cell source. These tissues may directly help heart contraction and total heart wall replacement may be possible in future. There are several contexts of tissue engineering.

3.1 Scaffold-based tissue engineering

Most popular technology of tissue engineering is to seed cells into 3-D pre-fabricated biodegradable scaffolds which are made from synthetic polymer and biological material. Hydrogel formation after mixing cells and scaffold solution is another approach. Decellularized tissues have been also used as scaffolds. These scaffolds play as alternatives for extra cellular matrix (ECM), therefore, their cell-adhesiveness and porosity affect survived cell amount and engineered tissue quality. Scaffold modification can control its biodegradation and tissue formation. Growth factor linkage leads to accelerating tissue formation. Now these scaffold-based tissue engineering has been widely applied to cardiovascular tissue regeneration as well as other tissue repair (Vunjak-Novakovic, Tandon et al. 2010).

3.2 Cell sheet-based tissue engineering

In contrast to scaffold-based tissue engineering, our group have developed unique technique involving cell sheet stacking to fabricate 3-D tissues (Shimizu, Yamato et al. 2003).

Cell sheets are 2-D connecting pure cells without any scaffolds, therefore cell-dense 3-D tissues can be fabricated by stacking cell sheets. Cell sheets are harvested from intelligent culture surface "temperature-responsive culture surface", which are covalently grafted with temperature-responsive polymer, poly (*N*-isopropylacrylamide) (PIPAAm) (Okano, Yamada et al. 1993). The surfaces are slightly hydrophobic and cell-adhesive at 37˚C, on the other hand, the surface changes to hydrophilic and not cell adhesive below 32˚C. Confluently cultured cells on the surface can detach as a contiguous cell sheet simply by reducing temperature. Furthermore, biological molecules underneath cell sheets are also preserved and play a critical role as an adhesive agent during cell sheet stacking. Cell sheet-based tissue engineering has been applied for a wide range of regenerative medicine including corneal epithelial replacement, heart tissue repair, pneumothorax repair, liver tissue repair and so on.

According to the spread of the concept fabricating 3-D tissues from 2-D confluent cells, several other technologies using this concept have emerged. Cell sheet fabrication techniques using fibrin coated dishes or nanofibrous polycaprolactone meshes have been reported (Shin, Ishii et al. 2004; Itabashi, Miyoshi et al. 2005). Cell sheet-like constructs have been also engineered using magnetite nanoparticles (Ito, Hibino et al. 2005). Magnetically labelled cells are attached on culture materials by magnetic force and confluent cells are harvested as a cell sheet by magnetic force release. Thus, cell sheet-based tissue engineering has now spread in the world as scaffold-free tissue engineering.

4. Myocardial patch transplantation

Both scaffold-based and cell sheet-based tissue engineering have been used for myocardial patch fabrication. Not only cardiomyocytes but also other types of cells have been used for creating myocardial patches and some myocardial patches using non-cardiomyocytes have been already clinically transplanted over damaged hearts. (Fig. 1.)

4.1 Scaffold-based mayocardial patch

In myocardial patch fabrication, synthetic polymer, biological material and decellularized tissue have been used as prefabricated scaffolds. Li and colleagues, who were one of the pioneer groups of myocardial tissue engineering, first demonstrated that gelatine sponges seeded with cardiac cells have therapeutic potentials for cryoinjured rat hearts (Li, Jia et al. 1999). Leor and colleagues reported that bioengineered heart grafts using porous alginate scaffolds attenuated left ventricular dilatation and heart function deterioration in infarction model (Leor, Aboulafia-Etzion et al. 2000). Eschenhagen and Zimmermann's group have developed innovative myocardial tissue engineering approach (Zimmermann, Schneiderbanger et al. 2002). They have fabricated 3-D tissues by gelling mixture of cardiac cells and collagen solution. The constructs induced systolic wall thickening of the left ventricle infracted area and improved fractional shortening of damaged hearts in rat myocardial infarction model (Zimmermann, Melnychenko et al. 2006). Small intestinal submucosa (SIS) has also been used as a scaffold for myocardial patch. MSC-seeded SIS improved heart contraction in rabbit infarction model (Tan, Zhi et al. 2009). There have been various types of myocardial patches using different scaffolds and different cell sources. Although implantable human myocardial patches using beating cardiomyocytes have not been established now, clinical trials of collagen-based myocardial patch with bone marrow