Review Article

Hepatic Tissue Engineering Using Scaffolds: State of the Art

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Abstract

Severe hepatic failure accounts for many deaths and raises medical costs each year worldwide. Currently, liver transplantation is the most common therapeutic option for patients with end-stage chronic liver disease. Due to decrease in the number of organ donors, many in need of transplantation continue to remain on the waiting list. Hepatic Tissue Engineering is a step toward alleviating the need for organ donors. Regenerative medicine and tissue engineering require two complementary key ingredients as follows: 1) biologically compatible scaffolds that can be readily adopted by the body system without harm, and 2) suitable cells including various stem cells or primary cells that effectively replace the damaged tissues without adverse consequences. Yet many challenges must be overcome such as scaffold choice, cell source and immunological barriers. Today, hepatogenic differentiation of stem cells has created trust and promise for use of these cells in hepatic tissue engineering and liver replacement. However, using suitable scaffolds is an important key to achieving the necessary functions required for hepatic replacement. In recent years, different scaffolds have been used for liver tissue engineering. In this review, we have presented different concepts in using cell /scaffold constructs to guide hepatic tissue engineering.

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Introduction

Every year, the number of patients needing a hepatic transplant increases. Many of those in need of a transplant have suffered from full hepatic failure caused by disease, genetic complications or adverse drug reactions. Currently, there are many people waiting to have a liver transplant. However, there are not enough organ donors.

At the moment, there are about 700 patients waiting to have a liver transplant in Iran, but the number of liver donors is less than 200. At present, liver transplantation is the only therapeutic option for patients with end-stage chronic liver disease and severe liver failure.

However, the efficacy of liver transplantation is limited by the shortage of available organ donors, risk of rejection, infections, and other complications caused by the lifelong immunosuppression [(1)].

Tissue engineering proves to be a temporary treatment for patients suffering from hepatic failure [(2)]. For successful tissue regeneration, the cells constituting tissues to be regenerated are necessary. Considering the proliferation activity and differentiation potential of cells, stem cells are practically promising. Self-renewal is a unique property of stem cells that gives multi-potential differentiation ability to them.

Today, there are different studies showing

hepatogenic differentiation capacity of the stem cells [3-5]. However, the challenge remains to develop robust protocols, to generate functional hepatocytes from stem cells suitable for the transplantation.

A complementary key ingredient in regenerative medicine and tissue engineering is to make a use of biologically compatible scaffolds that can be readily adopted by the body system without harm [6]. Advances in polymer chemistry have facilitated the engineering of synthetic matrices that can be precisely manipulated with regard to physical and mechanical characteristics. This review has presented some directions that the field of liver tissue engineering is heading.

Hepatic biology

The liver is a highly metabolic, complex array of vasculature, endothelial cells and parenchymal cells that performs many functions in the body. The bulk of the liver is primarily composed of parenchymal cells such as hepatocytes, hepatocyte precursor cells (oval cells or Ito cells), stellate cells, kuppfer cells, epithelial cells, sinusoidal epithelial cells, biliary epithelial cells and fibroblasts [7]. Hepatocytes constitute approximately 70% of the cellular population of the liver and perform major metabolic functions such as plasma protein synthesis and transport, xenobiotic metabolism, glucose homeostasis, urea synthesis, and ketogenesis [8]. Thus, hepatocytes used for tissue engineering purposes must be able to perform these basic functions.

Cell source

In the field of hepatic tissue engineering, choosing cell type and cell source is important because it is necessary to choose cells that demonstrate the particular phenotype of interest. The various cell types that have been studied include stem cells, hematopoietic cells, oval progenitor cell and mature hepatocytes [9-11]. Deciding which cell type to use is dictated by the need and desire for the cells to perform in a predicted manner, exhibiting certain characteristics.

Hepatic progenitor cells found within the liver have already begun to differentiate, but still have several options before becoming destined to a specific cell line. These cells will not necessary become mature hepatocytes, but may in fact differentiate into other functional cells of the liver, such as bile duct cells [12,13]. Hepatic progenitor cells are often distinguished as primary or small hepatocytes. Mature hepatocytes can be obtained either from the perfusion of an intact or resectioned liver or from an established cell line.

Currently, primary mature hepatocytes, the most common cellular component in current liver tissue engineering, do not replicate sufficiently *in vitro* to meet the requirements of clinical use and do not maintain their differentiated properties *in vitro* [14]. So the search for novel cell resources has prompted investigations into generating hepatocytes from stem cells of extra hepatic origin, based on their availability and unrestricted potential to propagate and differentiate [15-17].

The stem cells found in sources such as bone marrow, are the most flexible cells in terms of their undetermined pathway and they express a remarkable ability to differentiate into desired cell types [10,12,13]. The interest in adult stem cells has in particular been triggered by the numerous ethical dilemmas surrounding the use of embryonic stem cells in pre-clinical and clinical research [18]. Among the adult stem cells, human Bone Marrow derived Mesenchymal Stem Cells (hBMSCs) have great potential for liver tissue engineering because autologous BMSCs can be harvested, expanded extensively *ex vivo*, and differentiated into a hepatic phenotype for transplantation back into patient [18,19].

Following Bone Marrow (BM) transplantation, oval cells are derived from the BM donor [20]. Differentiation of hBMSCs into hepatocytes-like cells in standard monolayer or two dimensional (2D) cultures is now well established [21-24].

From stem cells to hepatocytes

Until recently, it was believed that hepatocytes could only be derived from cells of

endodermal origin and their progenitors. However, recent studies suggest that non-endodermal cells may also form hepatocytes *in vivo* and *in vitro*. At present, it is believed that stem cells divide asymmetrically to produce a new stem cell and a progenitor cell that subsequently undergoes differentiation and maturation to form functional tissues [25].

It seems the microenvironment or niche of the stem cell is likely to be one of the factors dictating the type of mature functional cells. The original idea of a stem cell ''niche'' evolved from the concept that stem cells inhabit tissues within an ''inductive microenvironment'' that directs their self-renewal, differentiation, and cell fate in both normal physiology and disease [26-28].

Many developmental regulatory signaling molecules including Wnts, Bone Morphogenic Proteins (BMP), Fibroblast Growth Factors (FGFs), Notch and others may play a role [26,29,30]. In addition to stem cells, the niche microenvironment comprises on stem niche cells (e.g. stromal cells, periductular fibroblasts and stellate cells), parasympathetic nerve endings and specialized extracellular matrix [26, 31-33].

The coordinated signaling between component cells and scaffold, (in) direct cell-cell contacts, and integration of stem cell autonomous properties represent an interactive and dynamic system, organized to facilitate cell fate decisions in a proper spatiotemporal manner [26,30,31].

The microenvironment of developing hepatocytes is a continuously changing process of successively occurring biological events [34]. Each step of cell growth and differentiation is tightly regulated by intra extracellular communication as well as cell autonomous mechanisms [35]. Activin, Fibroblastic Growth Factor (FGF), Bone Morphogenesis Protein (BMP), Hepatocyte Growth Factor (HGF) and Oncostatin M (OSM) are the most essential extracellular signals. HGF is known to mediate growth, proliferation, angiogenesis and cell motility. Growth and differentiation of hepatocytes are known to be controlled by the Epidermal Growth Factor (EGF), FGF, Interleukin-6 (IL-6), Transforming Growth Factor (TGF-a), and Insulin-like Growth Factors (IGF). Corticosteroids, amino acids, OSM, nicotanimide, and Dimethyl sulfoxide (DMSO) stimulate function and differentiation [25, 31, 34, 35-37].

At the intracellular level, the liver-enriched transcription factors including Hepatocyte Nuclear Factor (HNF) 3α,β, HNF4α, HNF1α, β, and HNF6 act consecutively in essence in a cross-regulatory manner at specific development stages to regulate liver-specific gene expression. Interactions between these various compartments accomplish homeostatic regulation of stem/ progenitor cell functioning *in vivo* [25,30,31]. Consequently, identification and simulation of these *in vivo* signaling patterns might comprise an approach to contribute to fate reprogramming of stem/ progenitor cells *in vitro*.

Cell-matrix constructs

Generating cell/ matrix constructs to guide tissue regeneration involves isolating appropriate cell populations and transferring these to polymer scaffolds for *in vivo* implantation. The scaffold functions to (a) provide structural integrity and to define a potential space for the engineered tissue, (b) guide restructuring that occurs through proliferation of cells donor and ingrowths of host tissue, (c) maintain distances between cells that permit diffusion of gas and nutrients and possibly the ingrowths of vasculature from the host bed and (d) to transmit tissue-specific mechanical forces to cue the behavior of cells within it. A biodegradable polymer will degrade and gradually be replaced by regenerated tissue, minimizing the substrate for an inflammatory response [6].

Employing cell/ polymer matrices for tissue regeneration is an approach that permits experimental manipulation at three levels to achieve optimal constructs for individual tissues, i.e. the cells, the polymer scaffolds and the methods used for construct assembly.

Polymer scaffolds

There are multiple approaches to engineering a viable liver, with variables such as cell type, structure, and material. The scaffold is a common feature of many liver tissue engineering projects. Its benefits include providing a place for attachment, increased surface area, support for a larger cell mass, and the capability of shaping specific structures. Importantly the scaffold must be biocompatible and biodegradable allowing the organ to grow "in lieu" of the scaffold and support itself over time. Other aspects that have been studied include surface pattern and structure for optimal attachment, porosity for nutrient and gas exchange, surface factors for increased and supported growth and function, and surface coating of the scaffold [1,6,38]. Scaffolds provide a site of attachment for hepatocytes and are a delivery vehicle for transplantation.

In addition to the basic structural vehicle, several other conditions must be met before a scaffold may be used in tissue engineering applications. The scaffold must be biodegradeable and biocompatible in that they do not leach harmful materials as they degrade. Pore size must be controllable to allow for prevascularization or angiogenesis occurring. Also, the scaffold should have sufficient surface area for cells to attach and be able to provide enough room for the cell colony to expand and proliferate [6,38].

Polymer scaffolds can be constructed from natural or synthetic biomaterials. The hepatogenic differentiation of stem cells in natural matrix such as collagen, fibronectin, gelatin and matrigel has been the subject of different reports [39-42]. Natural polymers are suitable for cell interaction, however, scaffolds fabricated purely from these molecules exhibit poor mechanical strength and are not easy to handle. Large batch to batch variations upon isolation from biological tissues as well as restricted versatility in designing devices with specific biomechanical properties are other limitations assigned to the natural scaffolds [43].

Advances in polymer chemistry have facilitated the engineering of synthetic matrices that can be precisely manipulated with regard to physical and mechanical characteristics. Variables such as polymer porosity and degradation rate can be systematically regulated by altering either the materials employed or polymer-processing methods. A variety of synthetic polymers exist, including polyesters, synthetic polypeptides and hydrogels. The most widely used polymers in tissue engineering have been aliphatic polyesters i.e. Polyglycolic Acid (PGA), Polylactic Acid (PLA), Poly Lactic-co-glycolic Acid (PLGA) and Polycaprolactone (PCL) [44].

These synthetic polymers have advantages in pro-accessibility, good mechanical properties and manipulating degradation rate, but they lack cell recognition signals and hinder successful cell seeding because of their hydrophobic trait. Therefore, to encourage cell ingrowths for better integration between cells and the scaffold, the biologically inert synthetic materials need effective hybridization with bioactive molecules [45,46].

The scaffolds should mimic the structure and biological function of native Extracellular Matrix (ECM). A well known feature of native ECM structures is the nanoscaled dimensions of their physical structure [6,47]. In recent years, with respect to nanofibers for tissue engineering purposes, a wide variety of nanofibrous scaffolds have been produced [48-52].

Design of nanofibers is an important concern in the effective applications of these nanostructured materials. Different techniques have been used for formation of nanofibrous materials [53]. There is increasing interest towards employing electrospinning for scaffold fabrication because the mechanical, biological, and kinetic properties of the scaffold are easily manipulated by altering the polymer solution composition and processing parameters. It has been shown that electrospun 3D nanofibrous structures share morphological similarities to ECM, and are capable of promoting favorable biological responses from seeded cells [54,55].

Kazemnejad et al (2007) designed an artificial nanofibrous matrix that can mimic ECM, to support hepatic tissue engineering. They introduced a scaffold composed of Poly (ε-caprolactone), collagen and polyether sulfone was fabricated by electrospinning technique. It has been reported that the engineered nanofibrous scaffold was a conductive matrix which supports and enhances stem cells development into functional hepatocyte-like cells [56].

Hepatic tissue engineering using scaffolds

Hepatocytes are known to better maintain their differentiated functions in three dimensional (3D) multicellular aggregates or spheroids than in monolayer culture [57,58]. Extensive cell-cell contact between hepatocytes grown in aggregates promotes the formation of gap junctions, tight junctions, and bile canaliculi that are important for stabilizing the hepatocyte phenotype [59,60]. Cells in spheroids also have a morphology and ultra structure similar to those found in a native liver lobule [61-63].

It has also been demonstrated that an increased level of Ecadherin mediated cell adhesion between cultured hepatocytes, induces higher levels of liver-specific functions [64]. Many studies have also highlighted the benefit of matrigel, a basement membrane extract from the Engelbreth-Holm-Swarm mouse sarcoma that serves as a complex ECM, in prolonging the maintenance of adult hepatocyte functions and in promoting the maturation of hepatic progenitor cells.

Differentiation of stem cells and hepatic progenitor cells is the most complete on 3D matrigel [40,65,66], and liver-specific functions of adult hepatocytes are better maintained when they are plated on a combination of ECM molecules [67,68]. To date, various coatings like fibronectin, collagen, and matrigel have been used to support the differentiation of stem cells to hepatocytes [40,69-71]. Therefore, *in vitro* selective growth and differentiation of MSCs in 3D biocompatible polymer scaffolds could be very efficient to develop a liver tissue having a clinically significant mass and maintain liver-specific functions.

However, the use of such natural scaffolds has been associated with some limitations. The problem with the control of pore size and porosity, large batch to batch variations upon isolation from biological tissues and poor biomechanical strength are the major concerns. As such artificial microenvironments including nanofibers designed to produce differentiated cells from stem cells and progenitor cells need to support both adult progenitor cell proliferation and differentiation [72-79].

Although there is a significant interest in using nanofibers in tissue engineering from stem cells, reports on the transdifferentiation of stem cells into the hepatic lineage in a nanofibrous configuration is scanty. More recently, differentiation of Human cord blood-derived unrestricted somatic stem cells into hepatocyte-like cells on poly (epsilon-Caprolactone) nanofiber scaffolds has been reported [80].

Thereafter, Kazemnejad et al (2009) differentiated hBMSCs into hepatocyte-like cells on an artificial nanofibrous matrix composed of PCL, collagen and PES. Based on the experimental evidences the expression of liver specific genes such as albumin, alpha fetoprotein, cytochrome P450 3A4, cytokeratin-18 and cytokeratin-19 detected by RT-PCR, showed progressive expression during 3 weeks of differentiation on 3D scaffold. Moreover, the hepatocyte like cells displayed several characteristics of metabolic functions as judged by production of albumin, urea, transferrin, Serum Glutamic Pyruvic Transaminase (SGPT) and Serum Oxaloacetate Amino Transferase (SGOT) [81].

In an another study, Kazemnejad et al demonstrated that the PCL/ collagen/ PES nanofibers not only allow the hBMSCs to differentiate into hepatocyte, but also enhance MSCs development into functional hepatocyte-like cells when compared to the conventional culture system. They reported that the levels of the mentioned markers (except SGOT) in differentiated cells on scaffold were

significantly greater than that in 2D culture system ($p<0.05$). So it seems that the high porous PCL/ collagen/ PES architecture provides an ECM-like nano-environment that is conducive to normal hepatic differentiation [82].

Detailed knowledge about the part played by the scaffold architecture for enhancing the stem cell differentiation especially into hepatocytes needs further studies. It is assumed that the presence of biological signals from the biomimetic nanofibers provides a nano-environment resembling a 3D natural ECM which would enhance the biological activity of growth factors and cytokines for inducing differentiation.

In vivo studies of hepatic tissue engineering

Hepatic tissue engineering is focused on creating a whole, implantable and functional liver. Many approaches have been used such as direct cellular injection onto present vascular beds, micro-carrier attachment and scaffold implants seeded with cells. One obstacle that must be overcome in complex, vascular organs such as the liver is the feeding of nutrients and removing waste from the cells in the interior. Two approaches to solve this problem are to allow the process of angiogenesis to occur into the cell aggregate or preestablish a vasculature bed and seed the cells around the network.

The success of tissue engineering over other organs such as bone, cartilage and skin has been achieved, because they are not as highly metabolic and do not require an extensive vasculature. In addition, these vascularized organs do not need to achieve the large dimensions that many vital organs do. Direct cellular injection into a vascularized area of the body has been the focus for the cells to sustain the metabolic activity of the organ. Cells have been injected with or without a hydrogel into various vascular beds, cavities, and organs within the body [83]. Several areas that have been injected with cells are the spleen, pancreas and peritoneal cavities.

In the case of transplantation into the liver through the portal vein, the number of transplanted cells is limited because intraportal injection of too many cells might cause lethal portal embolism and liver necrosis. The spleen is considered to be a suitable site for implantation because hepatocytes can be stably viable and maintain their functions [84, 85]. The only disadvantage is that the number of transplanted cells might be limited. The peritoneal cavity seems to be the most likely candidate site for implantation, because invasive treatments are not necessary and a large number of cells can be transplanted. However, the disadvantage is the difficulty in maintenance of hepatocyte viability in the peritoneal cavity.

Cells have also been implanted in prevascularized polymer sponges to improve cell survival *in vivo*. Although success has been achieved, the cells are limited by the size and the aggregate they can achieve due to lack of vasculature in the construct [1]. Three dimensional printing of biodegradable polymers allows the ability to create complex shapes and exact replicas of existing structures from Computed Tomography (CT) scans. Isolated hepatocytes on PGA meshes were first transplanted into the mesentery and omentum of syngeneic rats. Cells in these constructs expressed liver specific functions prior to transplantation [86] and survived for extended periods of time following transplantation, organizing into liver-like structures [87]. However, a significant loss of cells was noted post-transplant. This was felt to be due to (a) a failure to meet the oxygen/nutrient requirements of the cell mass and (b) insufficient stimulation of the transplanted cells or to both of these factors.

To augment the supply of these essentials, Stein et al performed prevascularization on a polymer scaffold composed of polyvinyl alcohol and transplanted into recipient animals, i.e. partial hepatectomy and a portal-caval shunt [88]. These procedures resulted in an increased delivery of hepatotrophic factors to the systemic circulation and diminished their clearance by the native liver and led to significant improvement in cell survival.

Higashiyama and his colleague transplanted rat hepatocytes seeded in porous Hydroxyapatite (HA) disks into the peritoneal cavity of Nagase Analbuminemia rats (NARs) [89]. Angiogenesis was observed inside the pores in HA disks, and hepatocyte viability was shown to be maintained for at least 3 weeks, as evidenced by the increase in the serum albumin level. Moreover, these researchers have been attempting to maintain the viability and functions of hepatocytes by co-culturing them with various cells, such as Nonparenchymal liver cells (NPLCs) [90, 91]. They then, co-cultured hepatocytes with BMSCs in HA disks and transplanted the disks into NARs and liver-damaged mice. The increase of serum albumin level in the liver-damaged mice was reported by the transplantation of hepatocytes and BMSCs. The serum level of IL-6 in the liver-damaged mice was also increased by the cotransplantation of BMSCs and hepatocytes [92].

Polymer implantation, surgical stimulation and cell transplantation technologies have been extended to large animal models. Dalmation dogs have a genetic deficiency in uric acid uptake in the liver which results in elevated serum and urine uric acid levels. Implantation of hepatocytes on PGA sheets resulted in partial correction of the enzyme deficiency for up to six weeks [93]. Successful hepatocyte engraftment has also been achieved in swine [94].

These evidences clearly show that more *in vivo* work needs to be accomplished before a viable organ will be available for transplantation.

Conclusion

With the recent advances in the field of hepatic tissue engineering, there is much promise of working towards an implantable whole organ. Many new polymers are being developed that respond to thermal changes, release imbedded or attached growth factors and other mediators, and have degradation characteristics and properties that is ideal for growth, viability, and attachment. An optimal polymer is being developed based on desired characteristics. Recently, electrospun nanofibrous scaffolds showed great promise and potential for liver tissue engineering.

Many other factors are being studied that contribute to cell growth and differentiation. However, further studies need to be performed for the development of a bioartificial liver system.

With the growth of the tissue-engineering field, many ethical considerations must be recognized. Determining which cell source is safest for patients, which cells should be used, whether they are embryonic stem cells, oval progenitors or adult stem cells, and how the cells should be stored and cultured are important issues to take into consideration.

Hepatic tissue engineering is an ever expanding field encompassing and including new areas of study. Because of its multidisciplinary nature, it is important for clinicians, basic scientists and engineers to collaborate and explore all areas of possibilities. With each new advance in the field of tissue engineering, a step towards an implantable liver is realized. Even though the goal of creating an entire implantable organ has not yet been reached, the progress towards this goal is proving to be fruitful to all those involved, mainly the patients who will benefit from the advancements being made.

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