Published Online July 19, 2011

Cell-based therapeutics for liver disorders

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Introduction: Due to a lack of adequate liver donors and post-surgical complications, researchers propose that cell therapy should be an alternative treatment for patients with end-stage liver diseases.

Data sources: We performed a literature review on cell-based therapy for liver disorders.

Areas of agreement: Due to growing numbers of patients on waiting lists for liver transplantation, a substitute treatment strategy is needed for our patients. Cell therapy can save patients who are in life-threatening situations, enabling them to have more time and increase their chances of survival. Pluripotent stem cells can be a good resource for cell-based therapy after the establishment of efficient differentiation protocols in addition to the settlement of ethical and immunological issues. Cell-based therapy will be applicable after the approval of its efficiency via animal model studies.

Areas of controversy: Transplanted cells cannot integrate into the recipient liver and lose their functionality after a limited time. The rate of homing and transdifferentiation of transplanted cells into hepatocytes is scant.

Growing points: Application of autologous bone marrow mononuclear cells (MNCs), hematopoietic and mesenchymal stem cells (HSCs and MSCs) has improved the general conditions of certain patients. Although this improvement is temporary, new studies have focused on increasing their performance.

Timely areas for developing research: The safety, feasibility and efficacy of applying MNCs, HSCs and MSCs in liver disorders have been proven in clinical trials. Patient-specific cell therapy after the production of induced pluripotent stem cells and new discoveries in somatic cell conversion during transdifferentiation are promising insights.

Keywords: cell therapy/liver disorders/regenerative medicine/tissue engineering

Accepted: June 14, 2011

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DOI:10.1093/bmb/ldr031

Introduction

Regenerative medicine is a new approach toward the treatment of complicated diseases such as liver disorders. Because of the high mortality rate in end-stage liver diseases and lack of whole organ donors for liver transplantation (LT), cell-based therapies have been considered an alternative strategy for hepatic injuries. Therefore, different types of stem cells have been used in various experiments to evaluate their regenerative potential. The results of these studies are promising; however, we need additional evidence before their broad application in the clinic setting. Here, we will review the basic considerations of the liver, followed by a discussion of different types of stem cells for cell-based therapies along with tissue engineering and bio-artificial liver devices.

Basic considerations of the liver

The liver is the largest internal organ that performs a vast variety of biochemical reactions (Supplementary data, Fig. S1). Approximately 80% of the liver consists of parenchymal cells in lobules (Supplementary data, Fig. S2), which are the functional units of the liver. The liver's extracellular matrix (ECM) is composed of a reticular network of glycoproteins[1] with dynamic sophisticated components, which have an important role in maintaining the functionality as well as the structure of this organ. The liver originates from the ventral foregut endoderm (Fig. 1). Provoking signals such as bone morphogenic protein-2 (BMP2) and fibroblast growth factor-4 (FGF4) induce endodermal cells to migrate from the ventral foregut epithelium to a cellular plate called the septum transversum to form a hepatic bud. The hepatic bud consists of hepatoblasts that are bi-potential cells that can differentiate into hepatocytes and cholangiocytes. Additionally, hematopoietic cells migrate and colonize in the hepatic bud and perform hematopoiesis. In this regard, liver formation is a consequence of expansion and proliferation of hepatoblasts.[2]

In vertebrates, the liver is the only internal organ that can regenerate itself and restore its primary mass.[3] Only 25% of a liver is needed to regenerate an entire organ via hepatocyte replication. Labeling studies have shown that after partial hepatectomy, all hepatocytes in the remaining portion of a liver undergo mitosis to restore the liver mass. In severe damage, however, the regenerative capacity is insufficient and leads to end-stage liver disease.[4] Transplantation of functional cells can

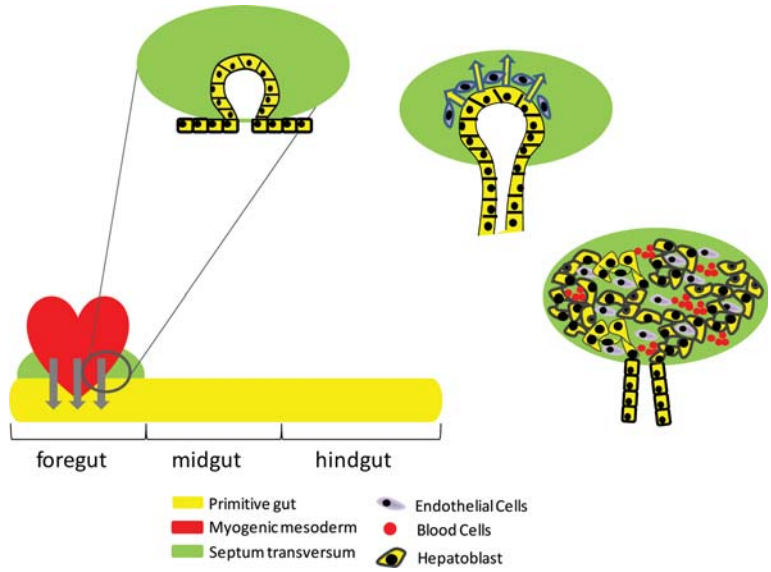

Fig. 1 Liver development. Adjacent endoderm to myogenic mesoderm receive provoking signals (BMP2, FGF4) to form hepatic diverticulum. The hepatoblast migrate to STM by EMT and form the liver bud which expands and proliferates upon triggering by secreted factors from resident HSCs to form the liver. BMP2, bone morphogenic protein, FGF4, fibroblast growth factor 4; STM, septum transversum mesenchyme; EMT, epithelial mesenchym transition.

promote liver regeneration through compensating the functionality of disrupted resident hepatocytes and relieve stress.[5]

However, despite the remarkable capability of the liver for self-repair, the vast verities of pathogenic factors have caused liver-related disorders to remain as a major health challenge. Table 1 presents the basic etiologies of liver injuries.

Table 1 Basic etiologies of liver injury.

Infectious
Viral hepatitis (hepatotropic and opportunistic), bacterial, fungal, parasitic
Immune mediated
Autoimmune hepatitis, GVHD[a], primary biliary cirrhosis
Drug and toxin induced
Mushroom ingestion, idiosyncratic drug reaction, acetaminophen, suicide attempt
Genetic and metabolic
Inherited metabolic diseases, fatty liver disease
Miscellaneous
Obstructive cholestasis, vascular disorders, heat stroke, life style and habits (alcoholism, fast foods, insufficient physical activity, stress), neoplasms (primary and secondary)

[a]GVHD, Graft versus host disease.

Cellular and molecular mechanism in chronic liver injury

Activation of hepatic stellate cells is the main event for cell-mediated mechanisms of liver injury.[6] Activated stellate cells proliferate and undergo a remarkable change in their phenotype and function. These cells secrete extraordinary amounts of ECM components, including collagen and laminin. Accumulation and condensation of this fibril structure surrounds the regenerative hepatic nodules. Due to chronic irritation of hepatic tissue and in consequence, release of inflammatory mediators, circulating white blood cells migrate to injured sites and produce more chemokines and cytokines which activate the hepatic stellate cells.[7]

Animal models in liver disorders

Animal modeling of specific diseases enables researchers to understand the pathophysiology of disorders and discover possible pathophysiological mechanisms. A good model needs to prepare a selective advantage of transplanted cells over resident hepatocytes. Besides, it should provide an appropriate niche for transplanted cells in order to have efficient homing.[8] Administration of hepatocyte inactivators such as retrorsine in addition to irradiation leads to selective advantages for transplanted cells. Traditionally, liver animal models have been divided into the following groups:[9] (i) toxin-induced models that involve the administration of hepatotoxins such as acetaminophen, CCL4, ethanol and D-galactosamine. Single dose or long-term treatment of animals with hepatotoxins leads to acute or chronic hepatic failure. (ii) Surgical models made by total or partial hepatectomy, ligation of the portal vein or hepatic artery as well as bile duct and portocaval shunts. (iii) Models of hereditary liver defects: FAH^{-} (fumarylacetoacetate hydrolase)[10] and uPA^{-} (urokinase-type plasminogen activator)[11] are two examples of this group, which are suitable experimental models for cell-based therapies because of selective advantage and a proper niche. However, none of these liver models can exactly imitate human pathophysiologic conditions. Therefore, additional investigations are necessary.

Cell-based therapeutic approach for liver disorders

Since 1983, the gold standard for treatment of end-stage cirrhotic patients has been LT. Development in post-surgical management

strategies and HLA matching in addition to wonderful surgical techniques and efficient immunosuppression have increased survival rates of patients after LT. The lack of sufficient donors and a continual increase in the number of patients on waiting lists has led researchers and physicians to consider alternative therapies. Prevention of fibrosis progression and acceleration in healing mechanisms toward normal liver architecture are crucial goals in these substitute treatments.[12] Possible cell resource candidates for cell-based therapy of liver diseases are listed below are listed below and also in Fig. 2.

Hepatocytes and intrahepatic stem cells

The feasibility of allogeneic primary hepatocyte transplantation and strong evidence for its therapeutic efficiency have been demonstrated.[13,14] These functional cells can support the basic metabolic activity of liver for certain period of time and can bridge the patients to life from lethal condition in liver failure. Despite promising results in these studies, the major limiting factor in their application is a lack of availability from healthy donors as well as difficulties in long-term maintenance.[15]

Liver progenitor cells located in small biliary canals (Hering) are another choice for liver regeneration. These cells (first described as oval cells in rodents) activate when liver injury occurs. Liver progenitor cells are bi-potential and can differentiate into hepatocytes and cholangiocytes. Signaling molecules that lead to activation of these cells are secreted by non-parenchymal cells in the liver.[16,17]

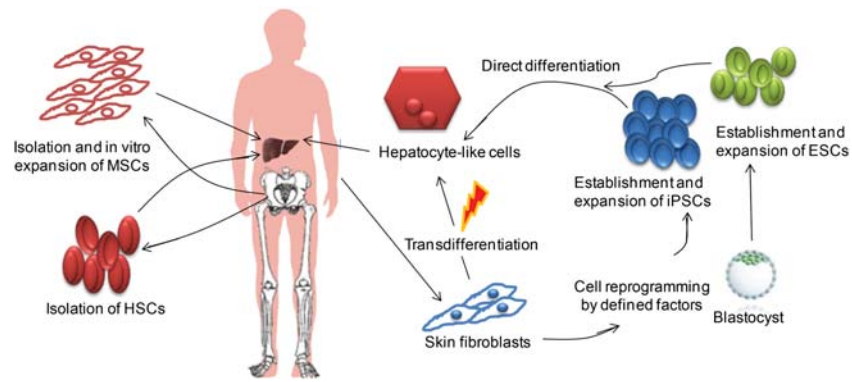

Fig. 2 Different cell sources for liver regeneration.

Table 2 Recent autologous bone marrow-derived stem cell transplantation in liver disorders.

Cell source and quantity ($\times 10^6$)	Patient (no.)	Route of administration	Follow-up (weeks)	Outcome	Ref.
HSCs					
$CD133^+$ (2.4–12.3)	Liver malignancy. Cell transplantation before partial hepatectomy to induce healthy lobe hypertrophy (3)	Portal vein	3	2.5-fold increased healthy lobes hypertrophy compared with the control group	21
CD34+ (1–100)	Liver insufficiency (5)	Portal vein or portal artery	8	Improved level of albumin and bilirubin	25
$CD34^+$ (3–10)	Decompensated cirrhosis (4)	Hepatic artery	24	Serum albumin improvement but results were not similar in all patients. Some side effects noted	26
$CD34^+$ (2300)	Alcoholic liver cirrhosis (9)	Hepatic artery	12	Significantly decreased bilirubin and improved ALT, AST and Child-Pugh score	77
$CD34^+$ partially differentiated into hepatocytes (1000)	Hepatitis C (36), end-stage autoimmune liver disease (12)	Portal vein and hepatic artery	48	Improved serum albumin, bilirubin, INR and ALT, 100% ascites removal.	22
MSCs					
MSCs (31.73)	Liver cirrhosis (4)	Peripheral vein	48	Improved end-stage liver score, increased physical and mental scales, increased liver volume in 6 months	20
MSCs differentiated into hepatocytes (30–50)	Hepatitis B (4), hepatitis C (2), alcoholic liver cirrhosis (1), cryptogenic (2)	Portal vein	24	Improved end-stage liver disease score, albumin, bilirubin and creatinine	19
MNCs					
MNCs (5.20)	Liver cirrhosis (9)	Peripheral vein	24	Improved serum albumin, total protein and Child-Pugh score, decreased α-fetoprotein	23
MNCs (200–1500)	Liver cirrhosis (8)	Hepatic artery	56	Improved level of albumin and decrease in bilirubin	27
MNCs (160–1310)	Chronic liver diseases (10)	Hepatic artery	16	Improved serum albumin, bilirubin and INR	24
MNCs (1340–1500)	End-stage liver disease (6)	Portal vein	96	No significant difference in serum parameters and liver volume	78
$CD133^+$ (6–14)					

ALT, alanine transaminase; AST, aspartate transaminase.

Mesenchymal stem cells

MSCs have been isolated from different sources such as bone marrow, adipose tissue, umbilical cord and amniotic fluid. Their high capability for self-renewal and differentiation make them an important cell source in regenerative medicine. In comparison with other pluripotent cells, MSCs are accessible, safe (do not form tumors) and without ethical problems.[18]

Autologous bone marrow MSCs have been used for both chronic liver disorders and compensated cirrhotic patients (Table 2). Many researchers have noted the presence of bone marrow stem cells in the liver following hepatic injury.

Kharaziha *et al*.[19] have shown remarkable improvements in liver functions after cell therapy during 24 weeks of follow-up. No side effects, mortality or morbidity were noted. As a result, they have recommended infusion of MSCs for improvement of liver functions. Application of MSCs in decompensated liver diseases as investigated by Mohammadnejad *et al*.[20] also showed improved MELD scores in patients. Infusion of MSCs increased the liver volumes in cirrhotic patients.

Mononuclear cells and hematopoietic stem cells

HSCs are commonly used in clinical trials for treatment of hepatic disorders. Their application has improved the functionality of primary hepatocytes (Table 2). BM-derived CD133+ cells have the ability to improve liver cell repopulation and accelerate liver regeneration.[21] In a recent study, it was demonstrated that transplantation of $CD34^+$ cells in patients with end-stage liver cirrhosis significantly decreased ascites in all patients and improved clinical and biochemical indices in most patients. However, no significant alterations in liver function parameters, liver enzymes, serum albumin, creatinine, serum bilirubin and/or liver volume after transplantation of both types of cells was found.[22] Additionally, serum albumin, total protein as well as the Child-Pugh score for cirrhotic patients improved significantly 24 weeks following transplantation of autologous BM-derived mononuclear cells (MNCs) into the peripheral veins of patients.[23] Transplantation of BM-derived MNCs via the portal artery led to enhancement of serum albumin, and a decrease in bilirubin and international normalized ratio (INR).[24] It was also demonstrated that the levels of serum albumin and bilirubin improved after transplantation of an adherent subpopulation of CD34+ cells via the portal vein or artery.[25] These subpopulations were isolated from mobilized stem cells from BM by G-CSF administration.

Mohamadnejad *et al*.[26] in 2007 performed a similar study which was terminated due to the possible side effects and risks of CD34+ cell infusion through the hepatic artery, although they observed some improvements in a number of patients.

In a recent study, Couto *et al*.[27] showed early improvements in the level of bilirubin and serum albumin after transplantation of autologous BM-derived MNCs. They also monitored the kinetics of transplanted cells to evaluate their distribution via cell-labeling with Tcm^{99}. Whole-body scans performed 3 and 24 h after infusion showed a trend of labeled-cell reduction in the liver.

Fusion of cells with hepatocytes, their transformation into hepatocytes as well as paracrine secretion of cytokines and growth factors by transplanted cells are possible mechanisms for improvement.[28] However, the enhancement in functional performance of the treated liver in many patients is undeniable. Additionally, the definitive differentiation of these stem cells into hepatocytes is questionable.[29]

Embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of blastocysts and can differentiate into definitive endoderm (DE), the first step in hepatic formation. Their exposure to hepatogenic factors leads to the formation of hepatic cells. Based on lessons from *in vivo* developments, in the first step of direct differentiation, ESCs that have been exposed to activin A and Wnt for 3 days formed DE.[30] Then, FGFs and BMPs were added to cells for 5 days. Early hepatocytes were exposed to hepatocyte growth factor, dexamethasone and oncostatin M for 10–15 days for additional maturation. ESC-derived hepatocyte-like cells have been transplanted in animal models with improvement in hepatic function.[31] Here, the term *in vitro* hepatocyte-like cells (HLCs) indicates some of the properties of mature hepatocytes (for review see ref. 32). Although these cells are promising, their clinical application is a challenging issue. Ethical problems and immunologic rejection are main limiting factors as well as their potential to be teratogenic.

Induced pluripotent stem cells

In a groundbreaking 2006 report, Yamanaka and Takahashi[33] surprised the scientific community when they discovered that the skin fibroblasts of an adult can be directly reprogrammed to a pluripotent state through the ectopic expression of certain transcription factors,

thus producing induced pluripotent stem cells (iPSCs). This was performed with the retroviral transduction of *Oct4* (also known as *Pou5f1*), *Sox2*, *Klf4*, and *c-Myc* genes. Many studies have demonstrated that mouse and human iPSCs are highly similar to their respective embryo-derived ESCs counterparts in morphology, molecular and phenotype aspects.[34,35] Numerous studies have since reproduced these results from almost any somatic tissue and mammalian species[36] with the use of different approaches.[37] Additionally, in human iPSCs the evidence of functional differentiation into specialized cell lineages of all three embryonic germ layers has been demonstrated. This wide differentiation potential provides fascinating possibilities and tools for developmental studies, genetic diseases, in addition to their application in drug discovery and regenerative medicine (reviewed in ref. 38).

It has been shown that HLCs could also be generated from human iPSCs (for review see ref. 32). The differentiated HLCs showed several similarities in morphology, the expression of a set of proteins, such as α-fetoprotein and albumin, and functionality such as glycogen synthesis, detoxification and engraftment, after transplantation into a suitable animal model.[9,39] However, it has been shown that HLCs derived from human ESCs and human iPSc exhibited broad similarity as well as meaningful differences.[40]

Moreover, since animal models do not always faithfully mimic human diseases, many groups have successfully reported a wide range of iPSCs from patients with different diseases (for review see refs 41 and 42). Therefore, it is possible to generate iPSCs from patients who have inherited liver diseases. Recently, several liver-specific disease iPSCs, such as familial hypercholesterolemia, glycogen storage diseases, Crigler Najjar syndrome, alpha 1-antitrypsin deficiency and familial hypercholesterolemia have been launched.[43,44] These cells can be used as suitable specific models to study the pathogenesis, mechanism(s) and possible treatment for inherited liver disorders.

Additionally, it has been demonstrated that iPSC-derived HLCs have both the functional and proliferative potential for liver regeneration after transplantation in an acute liver failure model or after partial hepatectomy in mice with fumarylacetoacetate hydrolase deficiency.[45]

Despite the promising outcomes in animal models, we are far away from their broad clinical applications (for reviews, see refs 32, 36, 37). For example, teratogenicity and their attitude toward malignancy restrict their clinical application in human disorders. Additionally, it has reported recently that transplantation of undifferentiated iPSCs demonstrated T-cell-dependent immune response in recipient syngeneic mice due to the abnormal expression of antigens following genetic manipulation.[46,47] Improvements in the production of safe and

non-immunogenic hiPSCs will enhance the biomedical applications of iPSC derivatives.

Transdifferentiation: a new strategy to reach hepatocyte-like cells

Transdifferentiation is a type of cellular reprogramming that could bring about a direct fate switch from one somatic cell type into another functional somatic cell without intermediate reprogramming into a pluripotent state.[48] Since the evidence for transdifferentiation of somatic cells, such as bone marrow cells into cells of other lineages merely by culturing the cells under specific conditions has not been convincing so far,[49] we do not consider them as transdifferentiation.

Desired induced transdifferentiation is often achieved through ectopic expression of transcription factors as the major players for cell fate conversion.[50–54] Recently, this process has been reported in the conversion of fibroblasts to functional neurons,[55] blood cells,[56] cardiomyocytes,[57,58] chondrocytes[59] and neural progenitor/stem cells.[58] These cells closely resemble the desired cells in terms of morphology, gene expression and functionality. In this process, mature and functional cells can be converted into other developed cells without backward reprogramming into pluripotent cells. During this phenomenon, cells lose their epigenetic marks, and change their morphology and function. Several studies have demonstrated direct conversion of various cells into hepatocytes by ectopic expression of different transcription factors. For example, myeloid cells were converted by ectopic expression of *Hnf4α*,[60] pancreatic cells by *C/ebpβ*[61] and fibroblasts by *Gata4*, *Hnf1α* and *Foxa3*.[44] Another good example is the *in vitro* transdifferentiation of primary pancreatic exocrine cells or pancreatic exocrine AR42J-B13 cells into hepatocytes and ductal cells in the presence of dexamethasone.[61] Transdifferentiated HLCs showed an expression profile and hepatic function close to those of mature hepatocytes.[44] However, some *Cyp* genes were not induced in transdifferentiated cells, and *CK19* and *Afp* were upregulated in transdifferentiation of fibroblasts by *Gata4*, *Hnf1α* and *Foxa3*.[44] Moreover, transplantation of these HLCs in an animal model of fumarylacetoacetate hydrolase deficiency demonstrated partial rescue,[44] which suggested that transdifferentiated HLCs have the future potential for regenerative medicine.

The simplicity of this approach has provided tremendous opportunities for generating 'self' surrogate cells suitable for disease modeling, drug discovery and cell replacement therapies, as well as experiments for basic research in developmental biology. Additionally, this type of reprogramming strategy has also opened up the possibility of direct

conversion of cells *in vivo* for *in situ* regeneration and repair.[62] However, there are some main limitations to the use of this approach in the clinic due to presence of exogenous transcription factors delivered with virus-based vectors. The integrated genes may even change a genome at the single nucleotide level during the reprogramming procedure.[46]

Some key advances aimed at overcoming these safety concerns have been achieved during the iPS cell reprogramming process, which may be applicable to induce transdifferentiation. Such advances include the use of non-integrating viruses like adenoviruses or episomal plasmid transfection,[7,36,63] treatment of cells with cell penetrating recombinant proteins of reprogramming factors,[64] transposon-based systems[65] and a conventional method of plasmid delivery.[66–68] Although these strategies eliminate the threat of random viral integration into the host cell genome, they are generally more technically demanding and less efficient than viral transduction, which is not yet widely adopted.[69] Recently, repeated transfection of modified mRNA encoding reprogramming factors[70] or application of inducible mir-302 expression[71,72] has been shown to be efficient for generating iPS cells, which seem applicable as a safe and efficient way for induced transdifferentiation.

Tissue engineering in liver regeneration

Tissue engineering is another approach for patients suffering from end-stage liver diseases. The well-known triads in this field are scaffolds, growth factors and habitant cells within the tissue. Using scaffolds in tissue engineering provides a proper niche for cells. The main characteristics of ECM that should be considered in designing a proper scaffold include: structural and mechanical properties as well as maintenance of cell activity by providing suitable interactions with cells. Cell delivery via scaffolds increases the survival rate and functionality of the transplanted cells.[73] Applicable scaffolds used in liver tissue engineering can be classified as collagen or galactose based as well as hydrogels, which have been applied in some studies for cell delivery to an injured liver (for review see ref. 74). Biomaterials composed of polyglycolic acid, polylactic acid and chitosan are popular biodegradable materials in tissue engineering. Immuno-isolating materials, such as hydrogels that protect transplanted cells from the immune system, can eliminate the use of immunosuppressive drugs.[75] Although some studies have shown the applicability of scaffolds in recovering liver structure, additional research is necessary before it becomes practical in clinical research.

Bio-artificial liver and artificial liver support device

Due to the lack of whole organ donors and current issues in the broad application of stem cells, several studies have attempted to develop non-biological artificial liver support devices (ALSDs) and biological bio-artificial liver (BAL) to save patients who have acute liver failure or rapid deterioration of their hepatic function in chronic liver disorders. These supporting devices have been applied to perform detoxification and synthesis as well as regulatory functions of the liver. ALSDs are based on biochemical and biophysical reactions for detoxification and cannot provide the liver's productive and regulatory functions. BALs are extracorporeal bioreactors charged with functional liver cells. Theoretically, these cell-based devices can provide all crucial activities of a normal liver. The main question in clinical application of these instruments is how to support them with enough functional hepatocytes. It should be mentioned that some unanswered questions exist regarding the application of BALs, such as the best cell candidate for BALs, duration of their functionality and support of bile drainage.[76]

Conclusion, present status and future outlook

Cell-based therapy is an alternative strategy for liver disorders due to the lack of sufficient numbers of organs for transplantation. Hepatocytes and intra-hepatic stem cells are not broadly accessible, therefore extra-hepatic stem cell sources such as ESCs, iPSCs and BM-derived stem cells are alternative options. ESCs and iPSCs have been differentiated efficiently to hepatocyte-like cells. However, concerns with immunological rejection and ethical issues, in addition to their tumorogenecity potential and heterogeneity of their population after directed differentiation remain the main problems for clinical application. Direct transdifferentiation of somatic cells to hepatocyte-like cells without conversion into the pluripotent state can be a new strategy for supplying cell resources, thus allowing them to bypass the tumorigenic state during the pluripotent phase.

Additionally, clinical trials have shown both the safety and feasibility of stem cells in liver cell therapy by bridging patients to LT. However, most were designed for phase I clinical trials. Control group and randomized double-blind trials are necessary to evaluate their efficacy in liver diseases. Currently, according to registered experiments at http ://clinicaltrials.gov several studies are underway. Meanwhile, researchers have considered control groups for ongoing practices. These experiments can be classified in different ways. In some studies, the

BM-derived stem cells are mobilized and/or expanded with G-CSF prior to intervention, and then stem cells are extracted from aspirates of BM or peripheral blood. These cells are introduced through different routes of administration (i.e. intra-portal vein or hepatic artery and/or peripheral veins). Different types of cells have also been applied in the interventions such as CD34, CD133, MNC and MSCs.

A better understanding of the possible mechanisms by which transplanted cells improve physiologic functions of the liver in addition to current advances in practical protocols for differentiation of pluripotent cells, and recent developments in the procedures of cell maintenance bring us to a promising future with cell-based therapies for liver disorders.

Supplementary material

Supplementary material is available at Brimed Journal online.

Funding

This study was funded by grants provided from the Royan Institute.

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