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Review article

From deceased to bioengineered graft: New frontiers in liver transplantation

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ABSTRACT

In the worldwide context of graft shortage, several strategies have been explored to increase the number of grafts available for liver transplantation (LT). These include the use of marginal and living donors, split livers, and the improvement of marginal donor grafts (machine perfusion). However, recent advances in the understanding of liver organogenesis, stem cells, and matrix biology provide novel insights in tissue engineering. Today, the newest technologies and discoveries open the door to the development of new methods for organ implementation such as the recellularization of natural scaffolds, liver organoids, bio-printing, and tissue or generation of chimeric organs. These approaches might potentially to generate an unlimited source of grafts (allogenic or chimeric) which will be used in the near future for LT or as a temporary bridge toward LT. This qualitative review focuses on all methods of organ implementation and highlights the newest developments in tissue engineering and regenerative medicine.

Contents

1. Introduction

Over the last two decades, major advances have been achieved in liver transplantation (LT) but access to allografts remains the main limitation [1]. This issue has led to the expansion of deceased donor selection criteria and the utilization of extended criteria allografts (grafts with steatosis, malignancies, viral infections, elderly donors, and donors after cardiac death) which have been historically associated with a high

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risk of primary non-function (PNF) or delayed graft function (DGF) and consequent high recipient morbidity and mortality [2]. Despite recent encouraging literature resulting in the utilization of these marginal grafts [3,4], the need remains and developing finding innovative ways to increase the pool of available grafts is mandatory. Tissue engineering and regenerative medicine (TERM) provide novel insights in liver grafts implementation. Organ engineering (recellularization of natural scaffold technologies and three-dimensional [3D] bioprinting) is a promising system with the potential to generate an unlimited source of grafts (allogenic or chimeric) or capable of bridging a patient either to LT or to recovery of the native liver through endogenous regeneration. This review provides an updated insight of current state of the art in the field of liver regenerative medicine. Advantages and limitations of current approaches to liver tissue engineering are explored.

2. Present and future alternatives for LT

Alternatives to LT, such as liver support systems (including bioartificial livers), have been extensively explored in the past but none have been adopted in daily clinical practice [5]. For example, the Molecular Adsorbent Recirculation System (MARS) is the most studied liver support system which is developed on the concept of albumin dialysis. The patient's blood is led through the hollow fibre capillaries of a high flux dialysis filter. Albumin solution, which is circulated in the extracorporeal circuit, passes the membrane counter directionally, allowing albumin-bound toxins in the blood to cross the membrane and bind to the albumin of the MARS circuit. The membrane is, however, impermeable to albumin. When passing the absorber and filter cartridges, the toxins are cleared by the filter and albumin is regenerated and able to accept new toxins when passing the membrane again. However, there is no substantial data demonstrating an overall survival benefit with MARS treatment [6] and more, MARS has not been approved by the FDA as a bridge to LT, as its safety and efficacy have not been demonstrated in controlled randomized trials [7].

TERM is a cross-cutting interdisciplinary field that applies the principle of engineering and life sciences to promote or enhance the intrinsic regeneration capacity of an organism with the aim of restoring damaged tissue function and/or structure or even to replace partial or whole damaged tissues/organs with artificial organs created in vitro (Fig.1).

Scaffolds play an important role in tissue engineering. They mimic the tissues native environment and support the cells inside the construct. They are made from biomaterials that are fully biocompatible [8]. Scaffolds are mainly produced by three approaches: (i) decellularization of live tissues; (ii) synthetic production; and (iii) the 3D-printing/bio-printing of a scaffold or of a seeded tissue/organ from a computer-aided design model. Different approaches are used depending upon the final use of the tissue/organ. However, the scaffold interacts with other cells, supporting cells in a two or three-dimensional environment, by providing anchoring points and by allowing cells to proliferate and/or migrate, and thus requires bioactivity [9]. It would be highly advantageous if the materials could be modified by the cells themselves or by the environment. Biodegradable materials are degraded over time, to be replaced by the cells own extracellular matrix (ECM) [10] as in the case of absorbable sutures or prosthetic materials. Ideally, the scaffold mimics the ECM [11] of the tissue with different characteristics, including biophysical and biochemical properties. The ECM is capable of absorbing and maintaining amounts of water and hydrogels (hydrophilic cross-linked polymer networks). Therefore, they are of interest to different applications in the biomedical field, including soft tissue engineering. Biological hydrogels have been formed from agarose, alginate, chitosan, hyaluronan, fibrin, and collagen, as well as many other materials.

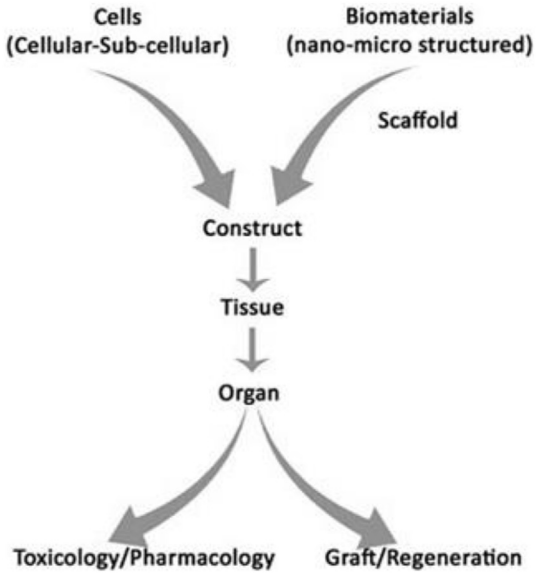

Fig. 1. Schematic view of the tissue engineering and regenerative medicine (TERM) steps and possible finale use of the obtained construct, tissue or organ (Teodori_et_al Journal_of_Biophotonics 2017. Reproduced with permission [51].

As scaffolds, hydrogels are used to provide bulk and mechanical constitution to a tissue construct, whether cells are adhered to or suspended within the 3D gel framework. When cellular adhesion directly to the gel is favored over suspension within the scaffold, the incorporation of various peptide domains into the hydrogel structure can dramatically increase the tendency for cellular attachment. In hydrogels, peptides can be incorporated on the surface or throughout the bulk of the gel, and have shown enhanced cellular migration, proliferation, growth, and organization in tissue regeneration applications [12]. Another biophysical property, which can influence cellular behavior, is scaffold stiffness because cells are capable of 'sensing' the stiffness of their microenvironment and respond subsequently [13]. Mesenchymal stromal cells (MSCs), for example, can differentiate toward different cell lineages purely based on the stiffness or elasticity of the substrate [14]. Cells are also influenced by biochemical factors, such as growth factors and cytokines, which are produced and released by specific trigger responses. These molecules influence cell survival, proliferation and/or differentiation making themimportant for tissue engineering purposes, as they can be used to either differentiate cells, stimulate cells to "recreate" a tissue [15], and/or to mimic the native tissue environment. In fact, the integration of angiogenic growth factors into implantable scaffolds may promote the recruitment of host vessels. For instance, preceding hepatocyte delivery with the implantation of scaffolds that releases angiogenic vascular endothelial growth factor (VEGF) enhanced capillary density and improved engraftment in rat liver lobules [16]. Similarly, fibroblast growth factor 2 (FGF2) coated scaffolds served as a supportive environment for mouse ESC-derived hepatocyte inoculation in vivo, a mouse liver failure model [17].

In addition to vascular integration, an improved understanding of multicellular organization and morphogenesis in the liver may also aid in the formation of functional biliary transport systems. Various invitro models have been developed that exhibit organized bile canaliculi [18] or artificial duct structures, but their incorporation into implantable systems has yet to be fully explored. Although early work demonstrated engrafted bile ducts in ectopic sites [19], the degree to which the biliary tree must be reconstructed has not yet been established; in ectopic cell transplantation experiments,hepatocytes do not appear cholestatic, and biliary products do appear to find their way to the digestive tract. One hypothesis is that the biliary products are redirected or 'leak' into the bloodstream where they circulate and are processed by the remnant liver into bile. This scenario would argue against the removal of the diseased liver in the setting of transplantable tissue engineered constructs and is consistent with the functional outcome achieved in peritoneal transplantation of mature hepatocytes and hepatocyte-like cells that lacked biliary networks [20].

In the UK, over 40% of the grafts proposed for LT are declined because of criteria or co-morbidities judged beyond marginal criteria [21]. This provides a major opportunity to explore alternative uses of human livers found to be unsuitable for LT following organ procurement. In particular, while cellular viability is easily compromised, ECM is better maintained in discarded livers and it may be used as scaffold for normal human liver cells and to recreate functional human liver tissue in vitro. A major advantage of using the liver ECM as a scaffold for tissue engineering purposes is that all structural and functional components of the ECM, which make liver micro-environments tissue-specific, are present within the scaffold [22]. The liver ECM can be obtained by decellularization (Fig. 2). During this process, all cellular components are removed from the ECM, without damaging ECM. Decellularization of the left liver can be completed within 14 days of perfusion while 6 weeks are necessary for a whole human liver. Decellularization can be achieved using various methods. It has been done for murine [23], porcine [24], and human liver [25] and different decellularization protocols have been described but all use a combination of chemical and enzymatic methods. The decellularization chemical protocol, based on a retrograde perfusion through the hepatic venous system, is characterized by the combination of different cell-damaging factors (CDFs): i) mechanical cell-damaging (freezing/thawing) to favor cell destruction; ii) isotonic stress enabling cell lysis; iii) enzymes to allow cell detachment; iv) action of detergents to remove debris; and v) flow shear stress to facilitate penetration into the hepatic sinusoid leading to the detachment of cells and debris. Once decellularization is complete, human liver scaffolds can be dissected by scalpel cleavage to obtain liver cubes utilized for 3D-platform for biocompatibility and bioengineering studies. Tissues are considered decellularized when no DNA fragment larger than 200 base pairs remain within the matrix. The resulting decellularized liver matrix is biocompatible and biodegradable but the most important advantage of utilizing the whole organ scaffold is that the architectural layout and ECM of other tissue types, such as the biliary tree and the vasculature network, are present avoiding the need to add them to the scaffold. Because of all these reasons, the liver ECM is the best scaffold for liver tissue engineering. A liver scaffold decellularized can be repopulated [26] (partially or completely) with functional cells using different methods and with different goals (2D coating, 3D hydrogel, or liver organoid proliferation). The latter could be used for an auxiliary partial LT [27]. Moreover, human hepatocytes could be added to a swine ECM resulting in the development of a chimeric liver (CL). In 2013, Hata et al. [28] investigated feasibility of this process, developing a rodent model of CL by repopulation of rat hepatocytes in a mouse and successfully transplanted the auxiliary CL into a rat recipient with vessel reconstruction. However, utilization of xenogeneic (porcine) scaffolds raises concerns about surgical technique and xenozoonosis such as porcine endogenous retrovirus [29]. Nevertheless, cautious and longer investigations to secure human patient safety are indispensable.

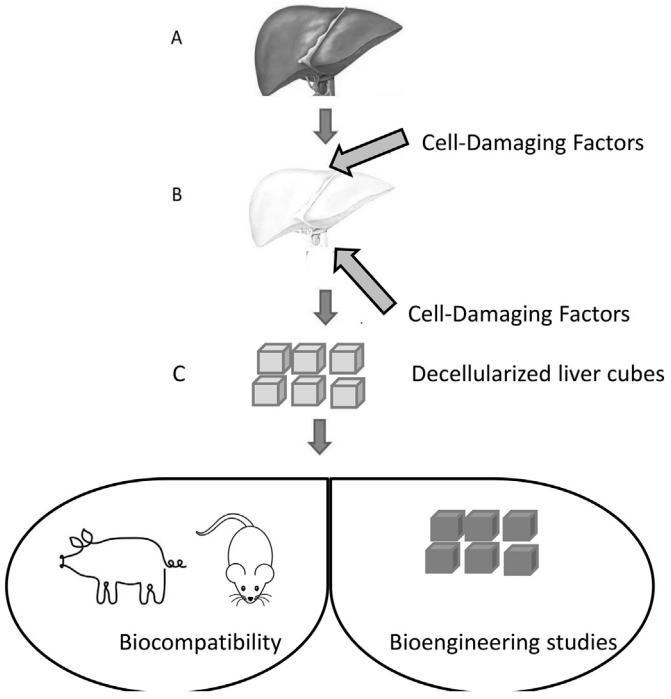

Fig. 2. Decellularization of a whole liver (A). The decellularization chemical protocol, based on a retrograde perfusion through the hepatic venous system, was characterized by the combination of the different Cell-Damaging Factors (B). Decellularized whole or split liver (B) is translucent because of the dissolution of cells. Once decellularization is completed, human liver scaffolds are dissected by scalpel cleavage to obtain liver cubes (C) utilized for 3D-platform for biocompatibility and bioengineering studies (D, E).

Recellularizing of a liver scaffold is a complex process. Cells have to migrate in the vascular tree and bile ducts without damaging the scaffold. While attempts using murine and porcine scaffold are ongoing, upscaling whole human organ scaffolds remains a challenge [23]. Moreover, ensuring that cells end up in the right location is a further challenge. Hepatocytes, for example, may be injected in vessels into the empty ECM or as cholangiocytes via bile ducts. In the first case, clogging of the blood vessels with hepatocytes and consequent thrombosis after transplantation of the organoid may occur. The vascular system and biliary tree also need to be fully repopulated with a layer of endothelium and cholangiocytes, respectively. The integrity of the vessels barrier and biliary ducts is fundamental to distinguish structures and to ensure functionality of the recellularized scaffold. Furthermore, different types of cells in specific ratios are required. For example, hepatocytes are required in large quantities. Cells from organoids can either be differentiated toward hepatocytes or cholangiocytes in vitro before injection into the liver graft. However, cells can be injected as undifferentiated organoids or as a mixture of both differentiated and undifferentiated cells with a certain ratio.

Hepatocyte transplantation involves the injection of cells obtained from healthy donors into the diseased liver of the recipient and the donor cells are maintained and expanded in vitro. However, since it is estimated that a human liver contains approximately 300 billion cells, the first challenge is to obtain a sufficient number of hepatocytes from healthy donors [30,31]. Moreover, when hepatocytes are cultured on relatively hard plastic in vitro, they quickly dedifferentiate and lose functionality [32]. Potential alternatives include pluripotent and adult stem cells with the potency to differentiate into functional hepatocytes. In 2008 Pai et al. [33] proposed the administration of autologous expanded mobilized adult progenitor CD34+ cells into the hepatic artery of patients with alcoholic liver cirrhosis. Significant decrease of bilirubin and transaminases were reported. Induced pluripotent stem cells, which can potentially be differentiated into cells with hepatocyte-like morphology and function, are promising. However, these so-called iHEPs do not fully mimic all the characteristics of hepatocytes [34]. MSCs are another source of adult stem cells that may be used in an alternative transplantation approach and are known to prevent or reduce ischemia/reperfusion injury in donor livers [32]. MSCs show enough plasticity to be differentiated into hepatocyte-like cells [35] but, at present, they cannot be differentiated into fully functional hepatocytes [36].

Cells are paramount in creating functional tissue construct in vitro. Since the liver is involved in several complex functions such as homeostasis, glucose and lipid metabolisms, detoxification, production of serum proteins, and secretion of bile, different cell types are present in the liver of an adult human. The majority of these cells are hepatocytes [37] (one-third of the liver in cell number and 70–85% of the liver volume). Non-parenchymal cells exist as cholangiocytes, endothelial cells (which create a barrier between the parenchyma and blood), Kupffer cells, and stellate cells [37] (important for liver immunity and its response to damage). In order to create a functional tissue construct to replace the damaged liver, all cell types have to be obtained, expanded,

and seeded within the scaffold material. To obtain a wide number of cells, different culture platforms have been developed [38]. These might increase the primary hepatocyte yield, however, these approaches are laborious and are still not able to supply sufficient numbers of functional cells. Therefore, other potential cell sources should be considered. Hepatic cell lines, (adult) stem cells and/or progenitor cells, are interesting alternatives that could be expanded in vitro and differentiate into all liver cell lines in order to replace primary hepatocytes, cholangiocytes, and other cell types of the human liver [39]. Although fewer cells are required to repopulate the graft, extensive proliferation and differentiation are still needed before becoming functional hepatocytes. Recently, a new 3D culture method has been established for the long-term expansion of liver-derived stem cells. These stem cells self-organize into so-called liver organoids, which are transplantable structures because they retain many characteristics of the original epithelial architecture [40].

The first organoid culture system was developed almost ten years ago when a 3D long-term culture was established from murine small-intestinal stem cells, which closely resembled crypt-villus units. These intestinal stem cells were marked by the expression of the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5). The Lgr5-positive cells were shown to be multipotent stem cells able to form all cell types of the intestinal epithelium by lineage tracing [41]. These adult stem cells were cultured in a specific mouse-derived hydrogel in vitro, which allowed cells to organize into 3D crypt-villus units containing both self-renewing stem cells and differentiated cells of all intestinal epithelial lineages. Adaptations to this culture method have allowed organoid cultures from many stem cell sources and, to date, several 3D cell culture systems are currently available to create liver organoids [41]. In general, these systems display better physiologic and metabolic aspects of intact liver tissue than 2D culture systems. However, none of these systems reliably mimic human liver development, including the parallel formation of hepatocyte and cholangiocyte anatomical structures. However, such models of tissue development have important applications in the discovery and treatment of human diseases. For example, the Gunn rat model of inherited bilirubin-UGT deficiency, such as the Crigler–Najjar syndrome [42], and the inv-mouse (partial deletion of the inversin gene) model of biliary atresia [43] have been particularly helpful in the study of hepatic and biliary diseases, respectively. However, these models are not optimal for the study of human-specific congenital diseases and corresponding new therapeutic targets, due to differences in liver fetal development between species. Hepatocyte maturation is a dynamic process highlighted by changes in levels of various cytokines and transcription factors associated with differentiation and maturation of hepatoblasts into hepatocytes. These disease models can be used to study disease mechanisms and discover new diagnostic, therapeutic and prognostic approaches. Besides providing a better model for human liver development, liver organoids may be used for drug development and toxicity screening applications [44].

Organoid cultures were also initiated from human adult liver tissue. They have an extensive proliferative capacity, having the potential to grow approximately one million cells from one single stem cell within two months. Secondly, they have proven great genetic stability as demonstrated by the karyotypic analysis of chromosome numbers and detailed sequencing of the whole genome which confirmed stability over time [45]. This is in contrast to other hepatocyte-like culture systems, such as induced pluripotent stem cells, which are prone to acquire genetic variations in vitro [46]. Thirdly, organoids are bipotent (hepatocyte and cholangiocyte cell lines). Differentiation in vitro requires a change in composition of the medium, after which cells differentiate toward either the hepatocyte or cholangiocyte fate. Upon hepatocyte differentiation, besides acquiring hepatocyte morphology and upregulation of classic hepatocyte markers, organoids gain some hepatocyte function as well. They were shown to take up glycogen and LDL and produce albumin and bile acid salts, although to a lesser extent than primary hepatocytes. Organoid differentiation toward cholangiocytes is less established. However, differentiation toward a cholangiocyte-like cell with corresponding phenotype and function has been obtained with induced pluripotent stem cells. During differentiation, these cells are switched to organoid culture conditions to facilitate the final maturation toward cholangiocyte-like cells, suggesting that organoid-forming cells are quite capable of differentiating toward the cholangiocyte fate [47]. Finally, adult tissue-derived organoids retain more commitment to their tissues of origin. This is in contrast to embryonic-type and induced pluripotent stem cells which are omnipotent and not committed to a particular tissue or organ type.

Another effective method of bioengineering for solid tissues (such as the liver) is bio-printing. In the past 10 years, 3D printers have been actively adapted to be compatible with manipulation of living mammalian cells developing patterned 2D cultures and 3D tissue structures in which multiple distinct cell types can be organized in a space relative to each other per user specifications. The bio-ink material is crucial because it provides a spectrum of biochemical (i.e., chemokines, growth factors, adhesion factors, or signaling proteins) and physical (i.e., interstitial flow, mechanical and structural properties of extracellular matrix) cues which promote a favorable environment for cell survival, motility, and differentiation [48]. In TERM, scaffolds could be fabricated by biomaterials and serve as ECM. In an earlier work, a 3D hepatocyte/gelatin construct was printed from a 38-layer assembly [49]. The laminated hepatocytes remained viable and performed biological functions in the construct for more than two months. Recently, metabolically active, anatomical, 3D hepatic tissues have also been developed successfully [50]. However, organs such as the liver have highly complex architectures and properties and may require a combination of several bio-printing techniques along with specifically designed bio-inks to reproduce structural heterogeneity and functionality. Although attractive, bio-printing remains nowadays an arduous challenge but this technology has already demonstrated a remarkable potential for future development and the 3D scale-up of functional organs.

In conclusion, a worldwide shortage of liver grafts available for LT has led scientists to develop other promising therapies. ECDs and regenerative medicine are future solutions. In TERM, decellularized livers constitute a good option for obtaining a scaffold as the vascular and biliary architectures are well preserved. Moreover, in decellularized livers, ECM maintains both viable and functional hepatocytes and cholangiocytes. This approach has the potential to generate an unlimited source of grafts (allogenic or chimeric), to provide a better timing for the procedure and to improve patient quality of life after surgery. Moreover, they could be used in the near future as a temporary bridge in LT (e.g., auxiliary partial orthotopic or heterotopic transplantation of the engineered liver graft) until an allograft becomes available. However, difficulties in engraftment and scaffold repopulation need to be resolved and problems with xenozoonosis and rejection still persist. Combined efforts in research from different specialists (surgeons, hepatologists, pathologists, and bioengineers) should achieve future clinical success.

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