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TISSUE ENGINEERING

Edited by

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Springer
Preface

This special issue of Advances in Experimental Medicine and Biology includes much of the research presented at the recent Second International Tissue Engineering Conference. Held in Crete, Greece, as part of the Aegean Conference Series, the Second International Tissue Engineering Conference was organized by Dr. Kiki Hellman of the Hellman Group, Dr. John Jansen of the Nijmegen University Medical Center, and Dr. Antonios Mikos of Rice University. The conference brought over 150 researchers from around the world to the Knossos Royal Village Conference Center in Crete from May 22 to 27, 2005.

Following along the lines of the conference program, this volume is divided into seven sections, focusing on stem cells, signals, scaffolds, applied technologies, animal models, regulatory issues, as well as specific tissue engineering strategies. Both original research papers and review papers are presented. The chapters reflect a diverse group of authors, including both clinicians and academicians. Furthermore, the issue contains papers from Asia, Australia, Europe, and North America, demonstrating the international component of the conference.

The intended audience for this issue includes researchers, advanced students, and industrial investigators. This issue should be a useful reference for tissue engineering courses as well as for researchers developing engineered tissues for clinical applications.

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SECTION 1

STEM CELLS
1.1. INTRODUCTION

Regenerative medicine holds promise for the restoration of damaged tissues and organs. The concept of regenerative medicine radically changed when pluripotent human embryonic stem cells were isolated and cultured. However, much more promising perspectives for clinical application are served by recently discovered multipotential adult stem cells, which may give rise to a large family of descendants, depending on the environments they are residing in. Properties and plasticity of stem cells from different sources have been intensely investigated, regarding to their use in therapy. The liver is one target for which development of stem cell-based therapy is of great significance. Even though an injured liver is highly regenerative, many debilitating diseases lead to hepatocyte dysfunction and organ failure.

Liver transplantation is the only effective treatment for severe liver injuries. However, because of organ rejection and the limited number of donors, alternative therapeutic approaches are needed. Stem cell transplantation could offer a potentially unlimited and minimally invasive source of cells for hepatocyte replacement and regeneration and, therefore, might be superior to whole organ transplantation. A first step to establish such therapy is the development of a model where functional hepatocytes can be generated in vitro and the second step is the design of a microenvironment for long-term maintenance of cell and, finally, organ culture.

Many studies have been performed using stem cells to produce functional hepatocytes, but there are still many obstacles like teratoma formation, cell fusion or
endless proliferative ability (potentially carcinogenic), which need to be overcome before clinical use. Nevertheless, investigations to establish a successful liver stem cell-based therapy are on the right track. With humbleness towards this difficult challenge, we will nevertheless attempt to resume achievements and discuss each of the potential stem cell types (ES cells and adult stem cells) (Figure 1.1.) for use in liver therapy.

**1.2. EMBRYONIC STEM CELLS**

Pluripotent embryonic stem (ES) cells, isolated from the inner cell mass (ICM) of blastocysts are capable of giving rise to cells found in all three germ layers of the embryo. They are considered to have the greatest range of differentiation potential and are the cells that the majority of studies have used. In the context of serious ethical concerns, most of experiments are performed on animal ES cells. The pluripotency of ES cells was proven in vivo and in vitro. In vivo, injection of the ES cells generates teratomas harboring derivatives of all three embryonic germ layers. In vitro, after...
removal from the feeder layer or from LIF, ES cells aggregate in suspension to form spheroid clumps of cells called embryoid bodies (EBs).

1.2.1. Mouse ES Cells

Mouse ES cells can be maintained in their undifferentiated state for an indefinite continuous time, on mouse embryonic fibroblasts or by culture with a leukaemia inhibitory factor (LIF), which is a feeder-cell-derived molecule that plays a pivotal role in the maintenance of these cells.

Here we present a few systems of hepatic induction (in vitro and in vivo) from ES cells, utilized by different groups (Figure 1.2.). In vitro strategies include differentiation through EBs formation, co-culture and mono-culture systems, while in vivo strategies utilize animals with liver injury or liver regeneration.

In order to define hepatic differentiation, liver-specific markers, responsible for hepatocyte endodermal differentiation as well as hepatocyte functions need to be detected. The markers include hepatic transcription factors essential for endodermal development (hepatic nuclear factor (HNF)-3beta/forkhead box A2 (FOXA2) and HNF-4alpha), the carrier proteins (alpha-fetoprotein (AFP), responsible for fatty acids, copper and nickel transport, albumin (ALB), responsible for steroids, fatty acids and thyroid hormone transport, transthyretin (TTR), carrying retinol) as well as enzymes (tryptophan-2,3-dioxygenase (TDO2), catalyzing tryptophan oxidative degradation, glucose-6-phosphatase (G6P), involved in control of glucose homeostasis and cytochrome P450 (CYP) enzymes). The functional assays for hepatocytes are mainly albumin and urea synthesis, glucose production and glycogen storage ability.

1.2.1.1. In Vivo Differentiation

ES cells have a propensity to develop teratomas when implanted into animals. Teratomas form tumors and finally cause the death of the host animal, which introduces a handicap in respect to any naïve utilization of them clinically. However, ES cells have been used by Choi et al. in order to demonstrate their hepatic differentiation potential in vivo\(^5\). They injected ES cells into the spleen of immunosuppressed mice and, as a first, demonstrated that teratomas derived from injected ES cells revealed that some areas contained typical hepatocytes. Chinzei et al. demonstrated that cells isolated from EBs nine or more days after LIF removal have expressed a panel of hepatic markers and were capable of producing albumin and urea\(^6\). After transplantation into partial hepatectomy of female mice pretreated with 2-acetylaminofluorene, ES cell-derived cells survived and expressed ALB, whereas teratomas were found in mice transplanted with ES cells or EBs up to day six. They demonstrated that, while ES cells always developed teratomas in recipient mice, this incidence was decreased in case of implantation of EBs and depended on the culture period of EBs. In vivo differentiation of ES cells carrying green fluorescent protein (GFP) in the AFP locus was performed by Yin et al.\(^7\). They selected a subpopulation of GFP positive and AFP expressing cells from differentiating in vitro ES cells. After transplantation into partially hepatectomized lacZ-positive ROSA26 mice,
GFP positive cells engrafted and differentiated into lacZ-negative and ALB-positive cells. In this case no teratomas were observed. Furthermore, using an animal with injured liver (regenerative condition), Yamamoto et al., reported that efficient differentiation of ES cells into transplantable hepatocytes with therapeutic properties has been successful.8

Figure 1.2. Strategies of hepatic induction from ES cells. In vivo strategies utilize factory on animals with liver injury or liver regeneration. In vitro hepatic differentiation strategies include: embryoid bodies (EBs) formation, co-culture and adherent monoculture systems.

1.2.1.2. In Vitro Differentiation

The EBs mature by the process of spontaneous differentiation and cavitations and the cells acquire markers for differentiated cell types. Dissociation of EBs and plating the differentiated cells as a monolayer reveal many cell lineages. Several growth factors and transcription factors have been shown to be capable of directing differentiation of mouse ES cells. Different matrix proteins may dramatically influence the generation and survival of the developed cells. Usually, collagen is used as the matrix for culturing the cells towards hepatic lineage since the liver bud proliferates and migrate into the septum transversum mesenchyme, which is composed of loose connective tissue containing collagen. Hamazaki et al. demonstrated that mouse EBs can be differentiated into hepatocyte-like cells when cultured on collagen-coated plates with early (fibroblast growth factors (FGFs)), middle (hepatocyte growth factor (HGF)) and late (oncostatin M
Jones et al. confirmed these observations, culturing ES cells carrying a gene trap vector insertion into an ankyrin-repeat-containing gene. This modification induces beta-galactosidase expression when hepatocyte differentiation begins. Kuai et al. reported that beta-nerve growth factor (NGF) also promotes hepatic differentiation, which is increased in the presence of HGF and retinoic acid.

Miyashita et al. and Chinzei et al. also demonstrated in vitro hepatic differentiation through formation of EBs, without using hepatocyte-specific cytokines. Yamada et al., by using an ES cell line carrying the enhanced green fluorescent protein (EGFP) gene, identified indocyanin-green (ICG) uptake by cells differentiated from mouse EBs and reported the presence of liver-specific markers using RT-PCR and immunocytochemistry. Ishizaka et al. demonstrated that when transfected with HNF-3beta, mouse ES cells were able to differentiate into hepatocytes with liver-specific metabolic functions, after stimulation with FGF-2, dexamethasone, L-ascorbic-2-phosphate, and nicotinamide in a 3-dimensional culture system. The same genetically modified ES cells were differentiated through EBs formation into hepatic-like cells by Kanda et al. on an attached culture system. Importantly, later on they discovered that HNF-3beta transfected ES cell-derived hepatic-like cells have infinite proliferating potential, resulting in tumor formation and finally the death of the animals after transplantation.

EBs offer the advantage of providing a three-dimensional structure, which enhances cell-cell interactions that may be important for hepatocyte development. However, the complexity of the EBs is a problem, because of the cytokines and inducing factors generated within these structures which induce differentiation of other cell lineages as well. Until now, none of the hepatic differentiation systems based on EBs formation revealed an efficient induction of functional hepatocytes sufficient for experimental therapeutic study.

A co-culture strategy was used by Ishii et al. They produced in vitro mature hepatocytes from ES cells (carrying GFP in AFP locus) entirely via isolation AFP-producing cells and subsequent maturation of these cells by co-culture with Thy1-positive (CD90) mouse fetal liver cells. The achieved hepatic-like cells produced and stored glycogen as well as revealed a capacity to clear ammonia. In a co-culture system differentiation takes place in contact with assisting cells (fetal liver cells or stromal cells), because of the presence of differentiation factors; however, undefined factors produced by these supportive cells may influence the differentiation of ES cells into undesired cell types. An additional disadvantage might be the difficulty with separation of the ES cell-derived hepatocytes from assisting cells.

Recently, Teratani et al. showed that ES cells can differentiate into functional hepatocytes without the requirement for EBs formation or in vivo transplantation or a co-culture system. By comparison of genes between CCl4-treated injured and untreated normal mouse liver, their group identified a hepatic induction factor cocktail (HIFC). ES cell-derived hepatocytes, after HIFC induction, expressed multiple liver specific makers: ALB, TDO2, TTR, G6P, cytochrome 450, as well as hepatocyte nuclear factors: HNF-3 beta and HNF-4 alpha. Additionally, AFP expression appeared in early and TDO2 at the late stage of differentiation, which means that ES cell-derived hepatocytes mimic normal liver development. The functionality
shown by glucose-producing ability, the capacity to clear ammonia and urea synthesis ability, display characteristics of mature hepatocytes. Also in this case no teratomas were observed and karyotyping analysis showed a normal chromosome number. Most importantly, transplantation of ES cell-derived hepatocytes in mice with cirrhosis generated by dimethylnitrosoamine (DMN) showed a significant therapeutic effect. This model could be easily adapted into human ES cells, allowing precise control of proliferation and differentiation during production of human hepatocytes. In support of this speculation, differentiation of hepatocytes from *Cynomolgus monkey* ES cells is achievable using the HIFC system (Teratani et al. unpublished data). In regard to utilizing human ES cells in liver therapy, knowledge of molecular mechanisms of hepatic differentiation is needed. Yamamoto et al., based on the HIFC differentiation system, compared the gene expression profile of ES cell-derived hepatocytes with adult mice liver and found significant similarities in gene expression profile. Of 9172 analyzed genes in the HIFC treated ES cells; approximately 200 genes related to liver specific functions were radically altered in comparison with non treated ES cells. By using small interfering RNA (siRNA) technology, HNF-3beta has been found to be essential in in vitro hepatic differentiation, which indicates also that this system progresses via endoderm differentiation, imitating hepatic development in vivo: step 0-pluripotent ES cells, step 1-endoderm specification (HNF-3beta expression), step 2-immature hepatocytes (AFP, ALB), step 3: mature hepatocytes (ALB, TDO2) (Figure 1.3.b.).

![Figure 1.3. Hepatic induction system in adherent monoculture](image)

(a) Schematic representation of the differentiation protocol for the induction of the hepatocytes from ES cells by HIFC in monolayer culture. (b) Graph representation of AFP (alpha-fetoprotein), ALB (albumin) and TDO2 (tryptophan 2,3-dioxygenase) expression during HIFC treatment.
Experiments on human ES cells are limited because of obvious serious ethical concerns; however, there are strong speculations that after some modifications, experiments performed on animal models could be adapted into humans.

1.2.2. Human ES Cells

Schuldiner et al. showed the potential of human ES cells to differentiate into three embryonic germ layers after stimulation with different growth factors. EBs were dissociated and plated onto fibronectin-coated dishes and treated with growth factors, none of which induced the differentiation into any one specific cell type. Rambhatla et al. used sodium butyrate to induce hepatocyte differentiation in human ES cells, through EBs formation. Characteristics of hepatocyte morphology as well as ALB, alpha-1-antitripsin (AAT), cytokeratin (CK)-8 and CK-18, inducible cytochrome P450 expression and glycogen accumulation have been observed; however, sodium butyrate induced significant cell death. Levenberg et al. used biodegradable scaffolds of PLGA-poly(lactic-co-glycolic acid) and PLLA-poly(L-lactic acid) to induce tissue-like structures, after seeding ES cells or EBs and they found hepatocyte differentiation after stimulation with activin-A and insulin-like growth factor (IGF). 14 days after implantation of 2-week-old constructs into SCID mice; immunostaining analysis of cytokeratin and AFP indicated that the implanted constructs continued to express these human proteins.

ES cells have great potential, although they face limitations inherent in procurement from fetal tissues, problems related to histocompatibility and ethical concerns. Such handicaps might be sidestepped in the future by somatic cell nuclear transfer of a patient’s own skin cells into donated oocytes. Further investigations concerning genomic stability, differentiation fidelity and cellular “reprogramming” need to be performed. Many controversies have emerged, but there are speculations that human ES cells might be investigated and used in regenerative medicine in the future without the need for embryos or oocytes.

1.3. ADULT STEM CELLS

Many adult tissues contain populations of multipotent stem cells, which have the capacity for renewal after trauma, disease, or ageing and indefinite proliferative potential, which makes them a very attractive and ethically non-controversial tool in stem cell therapy. In adults, there is a spectrum of stem cells with a different scale of potentiality (multipotent, unipotent) and quantity. They “are ready” to receive signals from circulating blood (also containing multipotential adult stem cells) to control homeostasis.

The liver, besides liver-derived bipotential cells, which can give rise to hepatocytes and biliary epithelial cells, is supported by stem cells deriving from bone marrow and blood; however, the mechanism by which this balance is achieved is still enigmatic and controversial.
1.3.1. Bone Marrow

Bone marrow (BM) as a source of heterogeneous populations of stem cells (hematopoietic stem cells (HSC), mesenchymal stem cells (MSCs)), has been shown to contribute in liver regeneration.

1.3.1.1. Non-Fractioned BM

A huge focus on BM as a source of stem cells for regenerative medicine started when its contribution to liver regeneration in vivo was described. Petersen et al. showed that transplantation of unfractioned male BM into the livers of lethally irradiated female rats, whose livers were injured by 2-acetylaminofluorene and CCl₄, rescued the animals from radiation-induced BM ablation and simultaneously produced small numbers of BM-derived hepatic stem cells. They demonstrated that host liver contained hepatocytes carrying genetic markers derived from implanted BM cells. Additional evidence of BM-derived hepatic stem cells was demonstrated by Theise et al., who also used the gender mismatch BM transplantation strategy and showed that over a six month period 1-2% of hepatocytes in mice liver may be derived from BM in the absence of any liver damage. Further studies made by the same group demonstrated that also in humans, hepatocytes can derive from BM. They examined the livers of female patients, who had received a BM transplant from male donors, and female livers transplanted into male recipients, which had to be removed for recurrent disease. In both cases Y-chromosome-positive hepatocytes were identified, but the degree of hepatic engraftment of HSCs into the human liver was highly variable. The most impressive generation of hepatocytes from BM cells has occurred after transplantation of BM into mice with lethal hepatic failure resulting from homozygous deletion of the fumaryl acetoacetate hydrolase (Fah) gene, corresponding to human tyrosinemia type 1. Subsequent studies in the Fah-deficient model suggest that differentiation of HSCs to hepatocytes results from the fusion of HSCs descendant cells with Fah-negative hepatocytes, giving heterokaryotic cells. The fusion might be observed as a result of the genetic alterations in the Fah-deficient mice; however, it remains unclear.

1.3.1.2. Purified BM-Derived Hematopoietic Stem Cells

Wang et al. did not observe cell fusion when transplanting purified human HSCs CD34⁺ or CD34⁺ CD38⁻ CD7⁻ from BM (and umbilical cord blood (UCB)) into non-obese diabetic immunodeficiency (NOD/SCID) mice and (NOD/SCID) beta-2-microglobulin–null mice. They demonstrated after CCl₄ administration the presence of ALB in mice serum, and human ALB and CK19 mRNA in mice livers; however, they did not detect AFP. A recent report by Jang et al. showed as well that HSCs can differentiate without fusion into hepatocytes and that an injured liver’s function was restored after transplantation. They suggested that microenvironmental cues rather than fusion might
be responsible for hepatocyte differentiation. Transdifferentiation is a heavily debated topic and the mechanisms are not understood. It might be a rare and unphysiological event, occurring under special conditions only. Nevertheless, fusion of donor cells derived from BM or elsewhere with resident hepatocytes does not preclude stem cell-based therapies. It can bring as well a new opportunity for delivering new genetic material to cells for gene therapy.

There have also been reports that BM-derived HSCs can differentiate into mature hepatic phenotypes in vitro. Miyazaki et al. showed that BM-derived HSCs (CD34⁺, Thy-1⁺ (CD90) and c-kit⁺ (CD117) express hepatic markers such as HGF receptor (c-Met) and AFP. After culturing with growth factors (HGF, epidermal growth factor (EGF), BM stem cell-derived hepatic-like cells expressed ALB (protein) and TDO2 (mRNA). Fiegel et al. selected CD34⁺ cells and cultured them on a collagen matrix with growth factors. Unlike CD34⁻ cells, CD34⁺ derived cells expressed ALB and CK-19 mRNA. Similarly, Okumoto et al. used selected HSCs from rat BM and cultured them with mature hepatocytes or only with HGF. The cells co-cultured with hepatocytes expressed HNF-1alpha, CK8, AFP and ALB mRNA, when cultured with HGF expressed HNF-1alpha and CK8.

In a co-culture system the cell fusion problem might be avoided; however, a proper separation protocol has to be developed.

1.3.1.3.  Mesenchymal Stem Cells

Previously, a well-characterized BM stromal cell population emerged as a focus for regenerative therapy. There is a little confusion in terminology with some authors suggesting that subpopulations named colony forming units of fibroblasts (CFU-F), multipotent adult progenitor cells (MAPCs), MSCs or stromal cells are quite similar or highly related. Kucia et al. showed that human BM is composed of a heterogeneous nonhematopoietic (CXCR4+, CD34+, AC133+, lin-, CD45-) tissue-committed stem cell subpopulation. They postulate that in BM there are stem cells with different levels of differentiation beginning from primitive pluripotent stem cells to tissue committed stem cells and so they suggest that the subpopulation of stem cells (e.g. HSCs, MSCs) taken into investigations should be carefully considered. However, this subpopulation is different from MSCs (CXCR4-, CD34-). The predominant source for MSCs is the adult BM, but they can also be obtained from various tissues of the human body, including compact bone, peripheral blood, adipose tissue, cord blood, amniotic fluid and other fetal tissues. MSCs are capable of self renewal and multilineage differentiation (adipogenic, osteogenic, chondrogenic, myogenic) and can be expanded in vitro, making it possible to engineer transplantable tissue in association with appropriate scaffolds. Schwartz et al. showed that rat, mouse and human BM-derived MAPCs, cultured with FGF-4 and HGF on Matrigel, can differentiate into cells expressing several liver-specific markers. Sato et al. showed that human BM-MSCs xenografted into liver of the rat differentiate into human hepatocytes, which express liver-specific markers, without fusion. Zhao et al. have demonstrated a protective effect of MSCs isolated from rat BM on fibrosis caused by CCL4 and DMN. The HIFC system has been adapted by Teratani et al. to contribute to hepatic differentiation of human BM-derived MSCs.
Hepatocytes derived from human MSCs reveal morphological, biological, functional and therapeutic evidences.

Multipotential MSCs comprise a promising tool for cell therapy (Figure 1.4.). They can be obtained from the patient’s own bone marrow (or other sources), expanded in vitro and implanted back into the liver as a native source for liver regeneration (this theory however needs to be confirmed) or after differentiating ex vivo (possibly with genetic modifications), maintained on a proper scaffold and implanted back into a diseased liver (without risk of rejection). There is high hope that in the future stem cell-derived hepatocytes might be used together with the advanced tissue engineering technology in entire liver system development.

1.3.2. Adipose Tissue

Adipose tissue derived stem cells (ADSCs) are a heterogeneous population of cells similar to BM-derived MSCs and are similarly able to differentiate into multiple lineages (adipocytes, osteoblasts, myoblasts, chondroblasts, neuroblasts). Their
potentiality to differentiate into hepatocytes has been observed by Seo et al.\textsuperscript{51}. Banas et al. has recently confirmed that the HIFC system allows hepatic induction from ADSCs (unpublished observations). ADSCs present an attractive tool for cell therapy, because they are easy to obtain, in large quantities and with minimal invasiveness.

1.4. STEM CELLS IN FETAL TISSUES

Lazaro et al. established a primary culture of human fetal liver hepatocytes, which retained hepatocyte morphology and gene expression patterns for several months\textsuperscript{52}. After treatment with OsM, these fetal hepatocytes matured. Moreover, during culturing they formed a 3-dimensional structure, which after section revealed liver-like morphology. Importantly, the primary culture of mature hepatocytes either does not replicate sufficiently in vitro to produce the number of cells necessary for transplantation or does not maintain differentiated properties in vitro, and so fetal liver might be a better source of hepatocytes; however there are ethic concerns regarding utilization of fetal organs.

Human umbilical cord blood (UCB), placenta and amnion are normally discarded at birth and so they provide an easily accessible alternative source of stem cells. Human UCB, commonly used as part of clinical application for several hematopoietic diseases, besides HSCs, contains stem cells with properties similar to BM-derived MSCs. Lee et al. demonstrated a subpopulation of UCB-derived MSCs able to differentiate into hepatic-like cells, which were positive for immunofluorescence albumin staining and exhibited an ability to uptake low-density lipoprotein (LDL)\textsuperscript{53}. Hong et al. reported differentiation of human UCB-derived stem cells into cells expressing hepatic markers (e.g. ALB, AFP, CK18)\textsuperscript{54}.

1.5. LONG-TERM MAINTENANCE OF HEPATIC FUNCTIONS BY BIOMATERIALS

Biomaterials with distinct properties are necessary to accommodate the growth and interactions of multiple cell lineages in composite tissue constructs\textsuperscript{55}. The microenvironment for long-term culture with a possibility to maintain stem cell-derived hepatocyte functions is important in the context of stem cell-based liver therapy and tissue engineering\textsuperscript{56}. The ideal scaffold is non-immunogenic, non-toxic, biocompatible, biodegradable and easy to manufacture. Scaffolds should permit easy diffusion of nutrients and cellular waste products and provide good mechanical support for cells during the repair process. Scaffolds currently used are natural or synthetic polymers. Liver tissue engineering started with maintaining a long-term culture of hepatocytes. Highly porous biocompatible polymers have been utilized, mainly poly (lactide-co-glycolide)\textsuperscript{21}, polyurethane, collagen, chitosan, alginate, hydrogel. In the three-dimensional (3D) culture system, using poly-N-para-vinylbenzyl-lactonamide (PVLA)-coated reticulated polyurethane Sato et al. maintained specific hepatocyte functions for up to 40 days in an in vitro culture. When transplanted into the peritoneal cavity of rats, the hepatocytes were able to survive and retain liver specific functions for more than one month\textsuperscript{57}. Ohashi et al. successfully engrafted and maintained hepatocytes at extrahepatic