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Review

Management of Liver Failure: From Transplantation to Cell-Based Therapy

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The severe shortage of deceased donor organs has driven a search for alternative methods of treating liver failure. In this context, cell-based regenerative medicine is emerging as a promising interdisciplinary field of tissue repair and restoration, able to contribute to improving health in a minimally invasive fashion. Several cell types have allowed long-term survival in experimental models of liver injury, but their therapeutic potential in humans should be regarded with deep caution, because few clinical trials are currently available and the number of patients enrolled so far is too small to assess benefits versus risks. This review summarizes the current literature on the physiological role of endogenous stem cells in liver regeneration and on the therapeutic benefits of exogenous stem cell administration with specific emphasis on the potential clinical uses of mesenchymal stem cells. Moreover, critical points that still need clarification, such as the exact identity of the stem-like cell population exerting the beneficial effects, as well as the limitations of stem cell-based therapies, are discussed.

Key words: Intrahepatic stem cells; Endothelial progenitor cells; Fetal liver stem cells; Induced pluripotent stem cells; Bone marrow-derived stem cells; Mesenchymal stem cells

INTRODUCTION

Liver failure is a major cause of morbidity and mortality in the Western world (159). Acute liver failure (ALF) is a rare disorder in which the rapid deterioration of liver function results in encephalopathy and coagulopathy in a previously healthy individual (12). The development of ALF represents the final common outcome of a wide variety of potential causes including viral hepatitis, drugs and toxins (12). Chronic liver disease (CLD) is a more common disease, in which a gradual destruction of liver tissue occurs over time, the tissue being replaced by newly formed collagen fibers (113). Attenuation of fibrosis progression to prevent the development of cirrhosis and/or hepatocellular carcinoma constitutes a major therapeutic challenge in the treatment of CLD today (113). Nonetheless, although multiple potential targets have been identified, no antifibrotic medication has yet been approved for clinical use (118). Instead, cell-based therapies are raising the possibility of improving liver repair by either hepatocyte regeneration and the prevention and/or reduction of fibrosis.

This review summarizes current knowledge on the use of stem/progenitor cells in hepatic regenerative medicine, focusing particular attention on multipotent mesenchymal stromal cells, which have been shown to possess a high potential for injured liver tissue regeneration in experimental models, compared to other stem-like cell subpopulations.

LIVER FAILURE AND REGENERATION CAPACITY: THE ROLE OF OVAL CELLS, SMALL HEPATOCYTES, AND HEPATIC STELLATE CELLS

A current explanation for the collapse of normal hepatic architecture is the imbalance between injury mechanism and regenerative repair. The liver is mitotically a quiescent organ in adult humans. However, the two ma-

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jor types of hepatic epithelial cells—hepatocytes and biliary epithelial cells (BECs)—are capable of proliferation and can, at least in a healthy liver, meet the demand for the replacement of damaged cells (28,29). The best example of their ability to restore the liver mass was seen after partial hepatectomy (PH) in animal models (93), creating the basis for living donor transplantation in humans. Due to this well-established trait of hepatocytes and BECs to regenerate the liver, the existence of hepatic stem cells was a matter of considerable controversy until recent years.

In the 1990s Potten and colleagues defined ''actual'' stem cells of a particular tissue as ''(a) undifferentiated cells, (b) capable of proliferation, (c) able to self-maintain the population, (d) able to produce a large number of differentiated, functional progeny, (e) able to regenerate the tissue after injury, and (f) flexible use of this options'' (82).

Similar to stem cells, progenitor cells retain the differentiation potential and high proliferation capability, but they have lost the self-replication property. Moreover, they are considered committed to the cell phenotypes of their tissues of origin. From this perspective, a progenitor cell can be considered as an adult stem cell (ASC), a term typically used to describe postnatal stem cells, which, as opposed to embryonic (ESCs) or fetal stem cells, persist throughout life (152). Although a useful distinction, over recent decades compelling evidence has suggested a greater developmental potential of progenitors/ASCs, according to which they are thought to be committed but not restricted to a single fate, as was proven in the case of bone marrow-derived cells (BMDCs) (71). Both cell fusion and transdifferentiation may account for this plasticity (170).

Oval Cells

The first indication of the presence of a stem cell population in the liver came in 1956 from Farber's studies of hepatocarcinogenesis in rats (44). Two years later, Wilson and Leduc reported "indifferent cholangiole cells" as the only cells responsible for liver regeneration in a mouse model of dietary injury (160). These small periportal cells with scant cytoplasm and ovoid nuclei were termed "oval cells" (OCs) (44). Due to their location within the intrahepatic biliary tree, OCs have been improperly regarded by some authors as proliferating bile duct cells (58), but the finding of a number of differences in protein expression between these two cell types later contradicted this assumption. The expression pattern of OCs appeared to be rather similar to that of hepatoblasts as they expressed markers of immature liver cells such as alpha-fetoprotein (AFP) and the hepatocyte-lineage marker albumin (42,81). Moreover, OCs expressed the biliary epithelial cell marker cytokeratin (CK)-19 (81), being therefore able to differentiate into both hepatocyte-like cells and biliary-type cells. These bipotential precursors are now believed to represent the progeny of a subset of stem cells with a slow-cycling phenotype that retain long-term self-renewal capacity almost throughout life (149). Impairment of hepatocyte proliferation induces these dormant/facultative pluripotential liver stem cells (FLSCs) to proliferate in order to reestablish homeostasis.

FLSCs likely possess the lineage potential of uncommitted gastrointestinal stem cells. This plasticity appears to be passed on to OC progeny, as indicated by the observation that under certain conditions OCs can be induced to differentiate into nonhepatic lineages, including intestinal and pancreatic epithelium (80,143,164).

Whether the progenitors of OCs have intra- or extrahepatic origin is still a matter of debate. Although the terminal bile duct system is thought to be the main source of OCs (119,147), they have also been described as deriving from bone marrow (BM) (30,107). Other authors have postulated that OCs may even arise from a dedifferentiation of hepatocytes (19). Light was shed on this controversy between 2003 and 2004 by two separate groups (92,156). Wang et al. observed that OCs induced by chronic administration of 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DCC) neither derived from mature hepatocytes nor originated in significant proportions from progenitors in the BM (156). OCs therefore appear to derive from intrahepatic precursors and are activated when liver damage is so severe that hepatocytes are prevented from entering the cell cycle, as indicated by experimental models in which hepatocytes were rendered unable to proliferate through exposure to mito-inhibitory compounds. In the same study OCs were capable of liver repopulation and rescued liver disease, thus representing a potential cell source for stem cell-based therapy.

Evidence that OCs in the rodent liver can be considered the specific progeny of liver stem cells and not the progeny of hematopoietic stem cells was also provided by Menthena et al. by using three different liver injury protocols for activation and expansion of OCs: D-galactosamine, retrorsine/PH, and 2-acetylaminofluorene (2-AAF)/PH (92). Apart from the above-mentioned protocols, several other models have been developed to induce OC proliferation in rodents, including injury caused by a choline-deficient diet combined with ethionine or AAF, PH combined with dipin, carbon tetrachloride (CCl_4) combined with AAF, as well as allyl alcohol (AA) (31,43,109,131–133).

The stromal-derived factor (SDF)-1/CXCR4 axis is involved in OC activation. Hatch et al. reported that when massive liver injury occurs, hepatocytes located in the proximity of OCs upregulate SDF-1 (57). In turn,

binding of SDF-1 to its unique CXCR4 receptor expressed on the OC surface leads to OC activation and migration along a SDF-1 gradient, thus resulting in liver regeneration. Additional studies revealed that OCs can also synthesize SDF-1 (90). To establish a role of SDF-1 in hepatic regeneration by OCs, these authors injected rats with fucoidan, known to inhibit SDF-1 biological activity. Fucoidan markedly decreased OC accumulation in a majority of treated rats, suggesting that SDF-1 promotes the activation of quiescent hepatic stem cells into OCs and/or stimulates OC growth via an autocrine/paracrine pathway. Impaired OC activation has also been reported after SDF-1 knockdown, further confirming the important role of SDF-1 in the repair process (172).

OCs were detected using histology and immunohistochemistry in liver biopsies from patients with genetic hemochromatosis, alcoholic liver disease, or chronic hepatitis C virus (HCV) infection (83). Importantly, OC numbers increased in direct ratio to the severity of each of the diseases studied. Further investigations revealed that the increase in OC numbers parallels the increase in the mRNA levels of the tumor necrosis factor (TNF) superfamily member lymphotoxin-β (LT-β), the activation of which is presumably responsible for cellular trafficking during chronic HCV infection (84).

Small Hepatocytes

In addition to OCs, there are other potential regenerative cell populations in both rat and human liver that respond to toxic liver injury. Between 1995 and 1996, small hepatocytes with features of committed progenitor cells were fractionated from normal-sized hepatocytes by differential centrifugation (94,144). These so-called small hepatocyte-like progenitor cells (SHPCs) have been found both in the periportal zone of liver lobules (166) and within the hepatic parenchyma (53). SHPCs express albumin but not CK-19 or AFP, thus behaving like unipotential committed hepatocytic lineage progenitor cells.

The activation, emergence, and outgrowth of SHPCs has been observed in response to liver deficit generated via both surgical PH in rats treated with the pyrrolizidine alkaloid retrorsine and exposure to necrotizing agents (14,15).

The origin of SHPCs has long been debated. Both OCs and mature hepatocytes have been indicated as SHPCs precursors (5,153). Strong evidence that SHPCs are not the progeny of OCs was reported by Best and coworkers (16). More recent data indicate that SHPCs are derived from hepatocytes (110). One hypothesis is that SHPCs may arise from a subpopulation of hepatocytes lacking hepatic cytochrome P450 proteins (CYPs), which are required to metabolize retrorsine (53). Nevertheless, despite great efforts in SHPCs research, other studies are needed to ascertain whether or not they represent a distinct population of liver progenitor cells.

Hepatic Stellate Cells

A third group of multipotent progenitors able to support the regeneration of damaged liver could be the hepatic stellate cells (HSCs). During chronic liver injury, massive hepatocyte death and subsequent inflammation are responsible for the conversion of quiescent HSCs into proliferative, fibrogenic, and contractile myofibroblasts (127). Myofibroblasts participate actively in the synthesis of extracellular matrix (ECM) components such as collagens I and III, tenascin, and fibronectin. As a result, a progressive overtaking of functional tissue by scar material occurs, a condition known as fibrosis. Advanced liver fibrosis results in portal hypertension, cirrhosis, and liver failure. Even though HSCs are classically viewed as the primary source of the fibrotic response, other fibrogenic cells and signaling pathways including immune, apoptotic, and angiogenic signaling, as well as responses to oxidative stress, are likely to contribute to the production of ECM (78). Importantly, an increasing body of evidence is indicating that, apart from their role in liver fibrogenesis, HSCs are also key players in liver regeneration (86,112). A close relationship between HSCs, ECM components (laminin and fibronectin), and OCs has been observed in the 2-AAF/PH rat model, highlighting the influence of the hepatic microenvironment on hepatic OC activation and proliferation (171). Of note, Yang et al. suggested that HSCs could be a type of OCs, transiting through a mesenchymal phase before differentiating into mature liver epithelial cells (163). In other words, HSCs could dictate the ultimate outcome of liver injury.

In conclusion, in contrast to the intestinal epithelium, the liver does not behave like a classical stem cell-fed lineage renewal system. After ALF parenchymal regeneration occurs through the proliferation of fully differentiated hepatocytes and/or BECs, under conditions of regenerative stress (i.e., during CLD), an unsuccessful or even dangerous emergence and proliferation of stem-like progenitor cells take place. The possibility to expand and differentiate local progenitors or stem cells is an interesting approach for regenerative medicine. Nonetheless, studies investigating whether they could represent a therapeutic option in the clinical perspective to cure liver disease are still lacking.

CELL-BASED THERAPIES FOR LIVER DISEASES: STATE OF THE ART

End-stage liver disease, in particular cirrhosis, represents a worldwide health problem (159). Currently, orthotopic liver transplantation represents the only effective treatment for end-stage liver disease, but its appli-

cability still remains limited by a variety of problems, including the shortage of donor organs. Additional limitations on liver transplantation are the numerous complications that can affect the recipient of a liver transplant. Early failure of the transplant can occur. Some transplants never work, some patients succumb to infection, and some suffer immune rejection. The latter is usually treated with large doses of immunosuppressants, with severe toxic and side effects. Consequently, it has become imperative to develop alternative therapeutic strategies. Some of the important findings in the field of liver cell therapy follow.

Mature Hepatocytes

The transplantation of mature hepatocytes has been widely evaluated in clinical trials in hepatology, resulting in the cure or alleviation of a variety of inherited metabolic disorders of the liver (IMDs) (33,46,60,96). The efficacy of hepatocyte transplantation and bioartificial liver devices has also been evaluated in the setting of ALF (1,2,18,128,139,162), but the long-term efficacy of these treatments remains unclear and the scarcity of donor cells limits these strategies. Although hepatocytes used for cell transplantation are usually isolated from donors with a beating heart, livers retrieved from non-heart-beating donors (NHBDs) could significantly expand the donor pool. Human NHBDs have been considered a valuable source of hepatocytes for cell transplantation only if the livers are harvested within 45 min after death (62). On the contrary, in the mouse, viable hepatocytes have been recently isolated for up to 27 h postmortem, and their engraftment and liver-repopulating capabilities have been confirmed in animals with fumarylacetoacetate hydrolase (FAH) deficiency, a model for the human IMD tyrosinemia type I (40).

Endothelial Progenitor Cells

The shortage of sources of transplantable human hepatocytes has led to the experimental and clinical exploitation of stem/progenitor cells in liver disease. Unfortunately, clinical application has often proceeded without a thorough understanding of the underlying biology of the stem-like cells used, leading to great contradictions concerning the utility of cell-based therapies.

Among the exogenous stem/progenitor cells that have been used for therapeutic purposes are endothelial progenitor cells (EPCs), a minor population of mononuclear cells circulating in peripheral blood (PB) known to play a role in regulating liver regeneration. Treatment with vascular endothelial growth factor (VEGF) has been reported to mobilize EPCs, facilitating liver repair (10). Moreover, human and mouse EPCs have been shown to improve the survival of mice following CCl_4-induced ALF, through the production of growth factors and induction of hepatocyte proliferation (142). Single or repeated EPC transplantation has also been reported to reduce liver fibrosis by the suppression of activated HSCs and an increase in metalloproteinase (MMP) activity (98).

Although from these studies EPC transplantation seems to be a feasible treatment for patients with liver diseases, other studies are needed before clinical trials can begin, because the EPC population actually comprises two subpopulations, known as early EPCs (eEPCs) and outgrowth endothelial cells (OECs) (63), whose molecular fingerprint has only recently been identified. According to Medina et al., eEPCs are hematopoietic cells with a typical monocytic phenotype, and because of their expression of genes involved in inflammation and immune responses they could cause adverse events if injected into a proinflammatory microenvironment (91), such as in the liver during fibrosis development.

Fetal Hepatocytes

Fetal liver is a rich source of stem cells as it has been found to be a major hematopoietic organ during embryo development (38) consisting of up to 60% erythrocytes at certain developmental stages (106). Of the two existing pluripotent hepatic progenitors (hepatic stem cells and hepatoblasts), hepatoblasts are the dominant cell type in fetal and neonatal livers. Their typical features are the expression of high levels of AFP and albumin, low levels of genes characteristic of the stem cell phenotype (CK-19 and c-kit), and of adult liver-specific genes [e.g., connexins, phosphoenol pyruvate carboxykinase (PEPCK), and P450s], as well as a lack of expression of neuronal cell adhesion molecule (NCAM) and claudin-3 (CLDN-3) (129). Evidence shows that hedgehog signaling is conserved in hepatic progenitors from fetal development through adulthood, thus representing a therapeutic target in patients with liver damage (134).

A strong dispute exists about the ethical and legal implications of creating embryos in vitro to derive cells for therapeutic or research purposes. Fetal hepatocytes can be obtained from fetuses after medical abortion. These cells offer a valuable alternative to mature hepatocytes, having several advantages, including greater availability, proliferative capacity, and plasticity, lower immunogenicity, good adaptation, and integration capacity, as well as a greater resistance to cryopreservation and ischemia (111).

Fetal rat liver cells have been reported to proliferate for up to 6 months after transplantation in naive adult rats and to divide as many as 10 times, while adult rat hepatocytes proliferate for only 1 month and divide only three or four times (126). Moreover, they are able to differentiate into both hepatocytes and BECs after transplantation.

Transplantation of human fetal hepatocytes, immortal-

ized by introducing the gene encoding simian virus 40 large T antigen (SV40 Tag), has been reported capable of rescuing mice with ALF induced by 90% hepatectomy (25). Stimulation of the mitotic activity of hepatocytes from fetal hepatocytes has been observed by Kurbatova (73). Of note, human fetal hepatocytes can stimulate the differentiation of human mesenchymal stem cells (MSCs) into hepatocyte-like cells (24).

A trial recently concluded in a cohort of 25 patients with end-stage liver cirrhosis of different etiologies confirmed the ability of human fetal liver-derived stem cells to improve clinical and biochemical parameters of liver function (68). However, caution should be taken with regard to this cell therapy due to the risk of immune rejection and therefore it appears to be mainly applicable in patients with depressed immune function or human fetuses (45).

Induced Pluripotent Stem Cells

Ethical concerns and the risk of rejection related to embryonic and fetal liver stem cells can be overcome by reprogramming somatic cells through the introduction of specific transcription factors, such as octamer binding transcription factor 3/4 (Oct3/4) and sex-determining region Y box 2 (Sox2) with either Krueppel-like factor 4 (Klf4) and c-Myc (141) or Nanog and Lin28 (168). The induced pluripotent stem cells (iPSCs) generated in this way have been demonstrated to possess a hepatocyte-lineage differentiation potential comparable to that of ESCs and to integrate into the hepatic parenchyma in vivo (135,136,140).

iPSC technology enables the generation of an almost inexhaustible source of immune-compatible cells for tissue regeneration. However, the viral transfection systems used to insert the genes at random locations in the host's genome have aroused important concerns about the safety of iPSCs for use in human patients. An important turning point came with the development of a protocol for generating iPSCs without using viruses to introduce genetic material into the host cells. This protocol involves the repeated transfection of two expression plasmids, one containing the complementary DNAs (cDNAs) of Oct3/4, Sox2, and Klf4, and the other containing the c-Myc cDNA, in mouse embryo fibroblasts (101). In an attempt to determine the regenerative capabilities of iPSC-derived hepatocytes, Espejel et al. first created iPSCs by using this virus-free technique, and subsequently generated chimeric mice by injecting iPSCs into blastocysts obtained from FAH-deficient mice (41). To prevent damage to FAH-deficient hepatocytes, foster mothers carrying the iPSC-injected blastocysts were treated with the drug 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). Eight of the total offspring of 24 pups presented chimerism in digits and survived even when maternal milk lacked NTBC. In vivo analyses showed that iPSCs were intrinsically able to differentiate into fully-mature hepatocytes leading to the complete restoration of liver function. Moreover, iPSC-derived hepatocytes were able to repopulate the livers of adult FAH-deficient mice off NTBC and responded to 2/3 PH with the rapid and coordinated cell cycle entry and progression characteristic of normal liver regeneration. Importantly, FAH-expressing hepatocytes in iPSC chimeras resulted from direct iPSC differentiation and not from the fusion of iPSC progeny with blastocyst-derived hepatocytes. In the same issue of *The Journal of Clinical Investigation*, the generation of a library of human iPSC lines from individuals with a range of IMDs was also reported (116). Dermal fibroblasts obtained from skin biopsies from individuals with IMDs were reprogrammed to pluripotent stem cells using the four-factor approach developed by Takahashi and colleagues (141) and subsequently differentiated into hepatocytes using a three-step protocol. The human iPSC-derived hepatocytes showed a strong morphological resemblance to human hepatocytes and shared with them in vitro functional characteristics, including albumin secretion and cytochrome P450 metabolism. Importantly, iPSC-derived hepatocytes replicated key features of the diseases from which they were derived, thus demonstrating the ability to derive large numbers of patient-specific hepatocytes to use as instruments in the study of the pathogenesis, disease mechanism(s), and possible cures of IMDs.

In addition to the above-mentioned protocol for the generation of iPSCs without using viruses, a nonintegrating strategy using synthetic mRNA has also been developed to prevent cancer risk (158). Other studies will be necessary to confirm the safety and efficiency of this cell population.

Bone Marrow Hematopoietic Stem Cells

In the last few decades, evidence has been provided to support spontaneous progenitor cell mobilization from the BM to the periphery during various diseases. The mobilization of $CD133^+$ hematopoietic progenitor cells into the PB has been described in both patients undergoing PH and cirrhotic patients (49,50). In the latter, c-kit^+ and Bcrp-1^+ populations have also recently been found to be recruited (50). Due to their well-known contribution to the repair of solid organs, BM stem cells have been considered the most promising candidates for regenerative medicine, when compared with other stem/progenitor cell populations.

Petersen et al. were the first to examine the possibility that BM contains a population of pluripotent stem cells with epithelial cell lineage capability, able to regenerate the liver following injury (108). In 2000, La-

gasse et al. demonstrated that intravenous injection of purified hematopoietic stem cells in mice with FAH deficiency rescues the mouse and restores the biochemical function of its liver (74). Further studies highlighted that it was not a case of transdifferentiation, but of cell fusion of hematopoietic stem cells (157). Nevertheless, others showed that hematopoietic stem cells become liver cells when cocultured with injured liver separated by a barrier (i.e., without fusion) (64). Indeed, when transplanted into liver-injured mice, hematopoietic stem cells converted into viable hepatocytes, restoring liver function.

The fate of ECM following BM transplantation in mice was investigated by Sakaida et al. (124). In their study, the subpopulation of Liv8-negative BM cells degraded collagen fibers, reducing CCl_4-induced liver fibrosis. Fibrinolysis was likely dependent on: 1) the increase in MMPs levels in transplanted BM cells, especially of MMP-9, whose production may be related to both the migration of BM cells to the inflamed liver and ECM degradation; 2) a reduction in the number of activated HSCs, possibly through apoptosis induction by BM cells. Importantly, transplanted cells differentiated into albumin-producing hepatocytes, thus providing evidence of the transdifferentiation ability of a nonhematopoietic subpopulation of BM. Following on from the reports of BM cells functioning as a potential source of hepatocytes to replace or restore hepatic tissues in rodents, Theise et al. investigated whether extrahepatic stem cells could also contribute to liver regeneration in humans (146). Biopsy and autopsy liver specimens from human recipients of therapeutic BM or liver transplants, in which there was gender discordance between the donor and recipient, were analyzed for marrow-derived hepatocytes and BECs. According to the results of mouse studies, human BMDCs could differentiate into both of the above-mentioned hepatic epithelial cells (146).

Clinical experience in using BMDCs for the treatment of liver damage is still limited. Most stem cell-based therapies in hepatology have been based on the collection of mononuclear fractions of the BM or PB samples, following mobilization by granulocyte colony-stimulating factor (G-CSF) administration and subsequent reinfusion. Infusion therapy of either unfractionated autologous BM cells (145) and in vitro-expanded autologous BM $CD34^+$ cells (67,104) has been shown to improve liver function in patients with liver insufficiency. Among the PB-derived $CD34^+$ cells, a rare cell population with a small lymphocyte-like morphology and potential to generate multiple tissue types was fractionated by adherence to plastic (54).

Overall, in these studies, BM stem cell transplantation improved residual liver function in patients with chronic liver failure. However, the observations were found in small groups of patients, trials were not randomized and also lacked a control group of stem cell-untreated patients. A larger cohort of patients with chronic HCV liver disease has only recently been studied. In the work of Salama et al. (125), 140 patients were randomized into two groups. Group 1, comprising 90 patients, received G-CSF for 5 days, followed by autologous $CD34^+$ and $CD133^+$ stem cell infusion in the portal vein. Group 2, comprising 50 patients, received regular liver treatment only and served as a control group. A significant improvement in mean serum albumin and bilirubin levels was observed in the transplanted patients compared to the control group (125). Thus, autologous $CD34^+$ and $CD133^+$ stem cell transplantation may be safely administered and appears to offer some therapeutic benefit to patients with viral hepatic end-stage liver disease.

Although G-CSF treatment has often been able to collect sufficient numbers of $CD34^+$ cells for autologous transplant, a great body of evidence is indicating that G-CSF mobilization alone—independently of the reinjection of ex vivo expanded autologous hematopoietic cells—can induce hepatoprotective and regenerative effects through multiple mechanisms (35,48,148,165). The beneficial effects of G-CSF have been recently attributed to the downregulation of SDF-1 expression in BM and upregulation in a liver with massive injury, with the formation of a SDF-1 gradient between the two compartments and chemo-attraction of BM $CD34^+$ cells to the site of injury (79).

Mesenchymal Stem Cells

Several investigations have shown that the nonhematopoietic adherent cell population of BM could be useful for liver regeneration. The hypothesis that these cells take part in normal tissue repair is long-standing. Cohnheim was the first to suggest in 1867 that BM may be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair (27), but it was not until the early 1970s that Friedenstein and his colleagues provided direct evidence of the existence of "clonogenic fibroblast precursor cells" (CFU-F) in the BM, spleen, and thymus of adult mice (47). CFU-F were selected by means of their adherence to tissue culture plastic and acquisition of a spindle-cell morphology. Cells were also found to differentiate into colonies that resembled small deposits of bone or cartilage. These observations were extended by other groups throughout the 1980s (3,6,21). At the beginning, these fibroblast-like plastic-adherent cells were called "bone marrow stromal cells" (BMSCs). In 1991, Caplan proposed the term "mesenchymal stem cell" on the basis of the ability of this cell population to differentiate towards tissues of mesenchymal origin (20), but the currently recom-

mended designation proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) is "multipotent mesenchymal stromal cells," because convincing data in support of a "stemness" phenotype for this population are still lacking (61). Nonetheless, the acronym MSC can be applied to both cell populations.

Over the years, it has become progressively clear that MSCs are not an exclusive feature of BM, but can also be isolated from other adult organs and tissues, including adipose tissue (174), umbilical cord blood (UCB) (17,39,52,70), PB (22,175), connective tissues of the skeletal muscle and dermis (167), the heart (11,59), liver (11), and spleen (59) (Fig. 1A). A great deal of effort has been directed toward defining a general marker signature of MSCs. Unfortunately, MSC populations have emerged as heterogeneous cell populations whose composition likely depends on isolation methods and expansion conditions, which vary considerably among researchers. As a consequence, despite the large number of markers studied to date, none of them can be regarded as specific to MSCs.

The ISCT has recently proposed minimal criteria to define human MSCs (37). First, MSCs must be plastic adherent when maintained in standard culture conditions. Second, MSCs must express CD105, CD73, and CD90, but not CD45, CD34, CD14 or CD11b, CD79α or CD19, and human lymphocyte antigen-DR (HLA-DR) surface molecules. Third, MSCs must differentiate into osteoblasts, adipocytes, and chondroblasts in vitro.

In 2004, Lee et al. demonstrated that MSCs derived from both human BM and UCB could differentiate into functional hepatocytes-like cells in vitro (75). Enrichment of nonhematopoietic BM and UCB cells was achieved by negative immunoselection. Single cell-derived populations were then obtained via limiting dilution, and hepatic differentiation was finally induced with a two-step protocol using hepatocyte growth factor

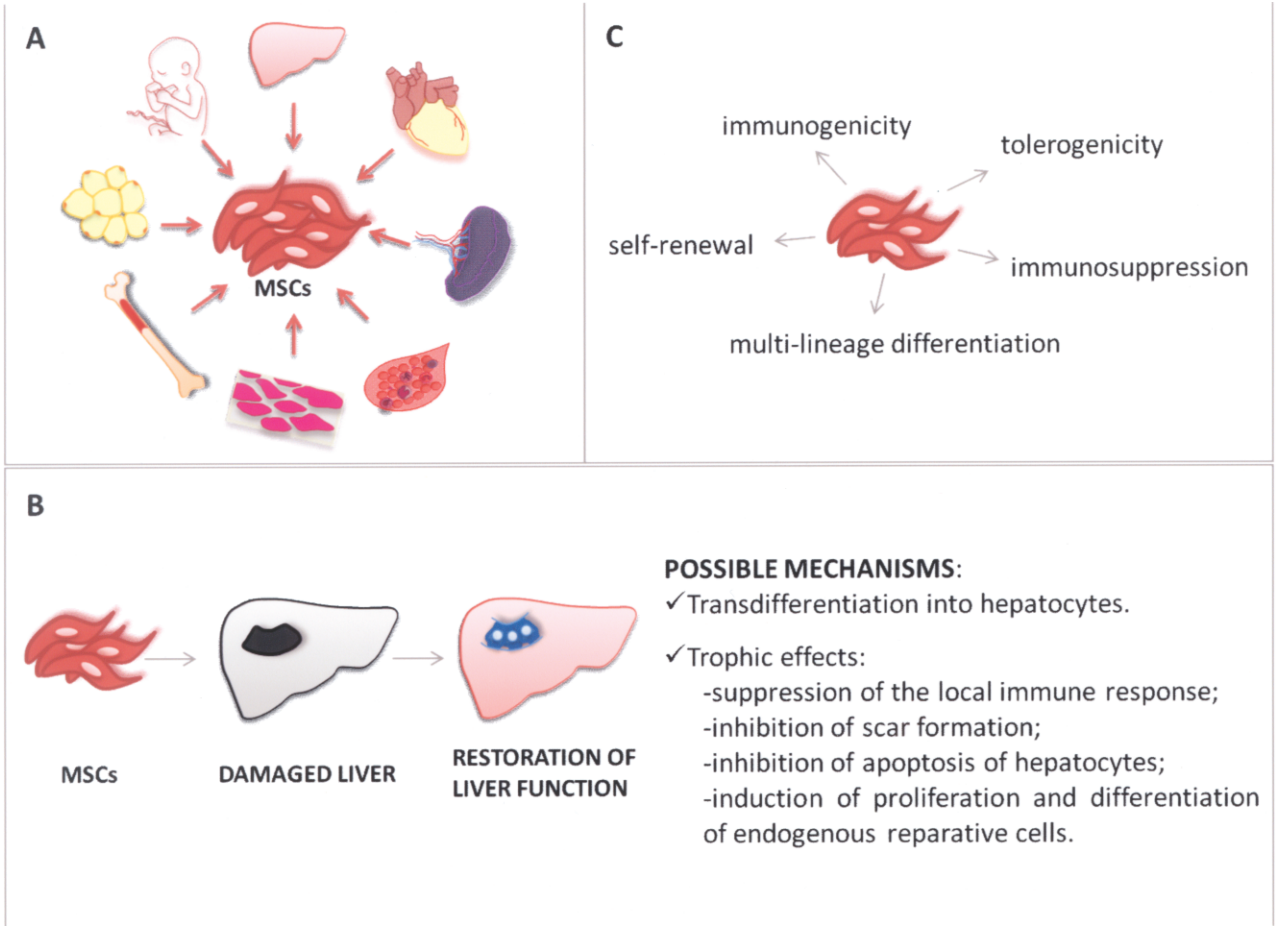

Figure 1. (A) Mesenchymal stem cells (MSCs) can be isolated from bone marrow, adipose tissue, cord blood, liver, heart, spleen, peripheral blood, and connective tissue. (B) Representative restoration of liver function following transplantation of MSCs and possible mechanisms involved. (C) Simplified scheme of the most common properties of MSCs.

(HGF) and oncostatin M. Differentiated cells were found to be capable of albumin production, glycogen storage, urea secretion, uptake of low-density lipoprotein, and phenobarbital-inducible cytochrome P450 activity, thus confirming MSC potential to generate liver cells.

A growth medium containing HGF and epidermal growth factor (EGF) was also used to differentiate human BM MSCs, generating a cell population able to integrate and repopulate the murine host liver injured by PH (4). Human BM MSCs facilitated recovery and decreased fibrosis in rat models of CCl_4-induced liver damage (23,55) and were capable of enhancing the repopulation of endogenous cells following necrotizing injury, suggesting an MSC paracrine effect (72). Indeed, MSC-conditioned medium (MSC-CM) contained high levels of chemokines and significantly improved the survival of rats with fulminant hepatic failure (FHF), via the alteration of leukocyte trafficking (105). MSC-CM had an effect on resident liver cells as suggested by the 90% reduction in apoptotic hepatocytes and a threefold increase in the number of proliferating hepatocytes in vivo (151). Downregulation of proinflammatory cytokines [interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, IL-2, IL-1ra] and upregulation of anti-inflammatory cytokines (IL-10) were observed following treatment. Figure 1B shows a scheme summarizing possible mechanisms of MSC-mediated liver regeneration.

Wharton's jelly, the main component of umbilical cord ECM, is thought to be a rich source of MSCs. Like BM MSCs, under appropriate culture conditions UCB MSCs can also differentiate into hepatocytes (75,77). Despite this, until recently there has been little investigation into the effect of UCB MSCs on liver injury. Two studies on CCl_4-induced liver fibrosis in rats have suggested an antifibrotic activity of transplanted UCB MSCs (65,150). However, a more recent study has indicated only a partial improvement in liver function in mice subjected to the same fibrosis induction, due to their inability to accelerate the capillarization and venularization of hepatic sinusoids (117).

The ability of MSCs to express genes of therapeutic potential has been reported in numerous studies in various regenerating organ systems, including the liver (69, 76,85,88,100,114,161,169). Indeed, following HGF exogenous expression, MSCs were shown to improve regeneration of the liver grafts in the rat model of 30% small-for-size liver transplantation (SFSLT) (169).

Although MSCs have been reported to possess a high liver regeneration ability (26), a study comparing the ability of syngeneic MSCs and hepatocytes to contribute to liver regeneration in different models of artificial liver injury indicated that only hepatocytes may be able to engraft and proliferate, while MSCs may not be able to enter the liver parenchyma because of the lack of important adhesion molecules, being therefore cleared during the first 2 days after injection (115).

The first human study on MSC transplantation for liver cirrhosis was reported by Mohamadnejad et al. (95). In their study, infusion of autologous cultured MSCs through the peripheral vein was reported to be safe and feasible and was associated with a slight improvement in liver function tests and model for end stage liver disease [MELD] scores in two out of four patients examined. This group is currently studying the efficacy of this new treatment strategy in a phase II multicenter randomized placebo-controlled trial, enrolling 50 patients with decompensated cirrhosis. Moreover, other clinical trials are currently ongoing to evaluate the safety and efficacy of both autologous BM- and allogenic UCB-MSCs transplantation for the treatment of liver failure and cirrhosis.

Table 1 shows some of the already concluded clinical trials for liver cirrhosis.

LIMITS OF MESENCHYMAL STROMAL CELL THERAPY FOR LIVER REGENERATION

Heterogeneity of BM is a well-accepted feature. In recent years, great effort has been devoted to determining which BM cell types hold clinical promise. From these studies MSCs have emerged as among the most promising candidates for tissue regeneration/repair, due to their intrinsic capacity for self-renewal, potential to differentiate into multiple cell lineages, as well as immunogenic and immunosuppressive properties (51,123) (Fig. 1C). The recent finding of MSCs in other tissues has fostered the growth of knowledge about how these cells can replace damaged tissues.

Despite the hopes and enthusiasms of researchers, the therapeutic potential of MSCs for supporting liver regeneration in clinical practice has been somewhat unclear. Existing contradictory data on MSC therapeutic utility might be due to a variety of factors, including the use of separate sources of MSCs, caveats, models of liver injury, as well as routes of cell delivery. The differences in behavior of MSCs derived from the same source observed by separate research groups could also reflect the use of different methodologies of cell preparation as well as the extraction site.

Conventionally, MSCs are generated by plating cells from different sources into culture flasks and selecting plastic-adherent cells with a spindle-shape fibroblastic morphology. This isolation procedure has several limitations, including the influence of undesired cocultured cells on the growth and differentiation of MSCs during the first culture period (56). Moreover, early removal of nonadherent cells may result in the elimination of a late-

Table 1. Clinical Trials of Stem/Progenitor Cell Therapy on Human Patients With Liver Cirrhosis

Authors (Year)	No. of Patients	Indication	Etiology	Treated Group (*n*)	Control Group (*n*)	Cell Source	Type of Stem/Progenitor Cells	Delivery of Stem/Progenitor Cells	Improvement Shown?
Gordon et al. (54) (2006)	5	liver cirrhosis	1 AL/HBV; 2 AL/HCV; 1 AL; 1 N.D.	5	0	G-CSF–mobilized peripheral blood	$CD34^+$ cells	portal vein/hepatic artery	yes
Terai et al. (145) (2006)	9	liver cirrhosis	3 HBV; 5 HCV; 1 N.D.	9	0	bone marrow	monuclear cells	peripheral vein	yes
Mohamadnejad et al. (95) (2007)	4	liver cirrhosis	4 N.D.	4	0	bone marrow	mesenchymal stem cells	cubital vein of the arm	yes
Khan et al. (67) (2008)	4	liver cirrhosis	1 HBV; 3 HCV	4	0	G-CSF–mobilized bone marrow	$CD34^+$ cells	hepatic artery	yes
Pai et al. (104) (2008)	9	liver cirrhosis	7 AL; 2 AL/HBV	9	0	G-CSF–mobilized peripheral blood	$CD34^+$ cells	hepatic artery	yes
Khan et al. (68) (2010)	25	liver cirrhosis	23 AL; 1 AL/HBV; 1 AL/HCV	25	0	human fetal liver	$EpCAM^{+ve}$ cells	hepatic artery	yes
Salama et al. (125) (2010)	140	liver cirrhosis	140 HCV	90	50	G-CSF–mobilized bone marrow	$CD34^+$ $CD133^+$ cells	portal vein	yes

AL, alcoholic liver disease; HBV, hepatitis B virus infection; HCV, hepatitis C virus infection; N. D., not defined; G-CSF, granulocyte colony-stimulating factor.

adhering MSC subset (56). In fact, the existence of late-adhering MSCs has been proposed, at least with respect to BM (8,155). Baksh et al. observed that not all MSCs directly isolated from BM were readily adherent capable when exposed to tissue culture plastic (8). Specifically, if nonadherent cells were removed 24 h postseeding, there were no detectable colony-forming unit-fibroblasts (CFU-Fs) and CFU-osteoblast (CFU-Os) development from the adherent cells that remained, thus suggesting that accessory cells present in the suspension cell fraction provide growth factor and cytokine signals necessary for MSC attachment and subsequent proliferation. The suspension culture had a $CD45^{-}$ $CD49^{elow}$ phenotype in accordance with previous indications (32) and maintained the capacity to differentiate into fibroblastic and osteogenic cells. Importantly, MSC suspensions were smaller in size than cultured expanded MSCs, making them ideal candidates for cellular therapies requiring systemic infusion. In fact, the majority of systemically infused plastic-adherent MSC cultures are prevented from homing to damaged tissues due to their large size and repertoire of cell surface adhesion receptors, which cause them to be trapped especially within the lungs, leading to pulmonary embolism (130).

Evidence that proliferative and differentiation potentials depend on BM MSCs in vivo location was given for the first time by Matsubara et al. They showed that unlike iliac BM MSCs, alveolar BM MSCs had a poor adipogenic or chondrogenic potential, suggesting that the site of aspiration influences cell behavior (89). Importantly, alveolar BM MSCs were not obtained from all alveolar aspirates of patients >50 years of age, suggesting an age-related marked decline in the proliferative capacity of this cell type. At that time, the effects of aging on MSCs had already been investigated, with some groups finding an age-related decline and others seeing no change (9,36,66,87,97,99,102,103,137). A more recent study has shed new light on this controversy by using a wider range of donor ages and by measuring a variety of markers of cellular aging, oxidative damage, and senescence. Briefly, an age-related decline in overall BM MSC "fitness" was found, thus posing important concerns over use of autologous aged MSC for cell-based therapies (138).

In MSCs, the expression of telomerase is also disputed. Baxter et al. found that even a limited expansion in vitro induces severe dramatic telomerase shortening (9). However, according to other investigators, MSCs do not exhibit telomere maintenance mechanisms (13). Studying adult and pediatric MSCs, Baertschiger et al. found that although an inverse relation between human donor age and average life span of the cells in culture exists, expansion ability is not dependent on telomerase activity (7).

One of the characteristics of aging is the increase in the likelihood of neoplastic transformation (120). Indeed, Rubio et al. reported the spontaneous transformation of MSCs from human adipose tissue, but while in their model transformation occurred following long-term in vitro culture (4–5 months) (121), other investigators revealed that mouse BM MSCs undergo chromosomal aneuploidy within three passages (173). Therefore, cell expansion ex vivo may increase the risk of spontaneous transformation, posing several questions: Is MSC therapy safe? Does MSC therapy provide a real benefit in the treatment of liver and other diseases?

Discrepancies between studies on MSC engraftment and function in liver after delivery may then be related to the transplantation of undefined populations of MSCs. Although clinical trials have been conducted for the treatment of a variety of diseases, including multiple sclerosis, diabetes, and skeletal and heart diseases, MSCs are still in fact not well defined by physical, phenotypic, and functional properties. Even though their heterogeneity is a well-accepted feature, to date no studies reporting the successful separation of distinct cell subpopulations have been published. Thus, the largely conflicting results existing in the literature about liver disease are likely dependent on the heterogeneity of the initial population. It would be very useful to separate different phenotypic subpopulations on the base of their migration, antifibrogenic, self-renewal, and death resistance in vitro properties. Correlation of the molecular signature and/or pathways activation of the so-selected subpopulations with different experimental outcomes could provide important lessons and guidance for the treatment of liver failure and cirrhosis, allowing MSC therapy to develop toward an efficient biomedical application. Wagner et al. reported that microarray analysis might provide a better tool for the characterization of MSCs rather than surface antigen phenotyping. In their study, MSCs from three different sources and fibroblasts were compared under two growth conditions. Although the MSC populations were quite different in genetic profile, they shared a panel of 25 genes, which were upregulated in comparison with fibroblasts (154). Thus, this study offers the potential to enhance the quality of clinical trials contributing to the establishment of standard guidelines for the molecular identification of MSCs.

A fundamental limitation in MSC therapy is its undervalued profibrogenic potential. A contribution to liver fibrosis of both whole BM cells (122) and MSCs (34) through differentiation into myofibroblast-like cells has been observed. Baertschiger et al. observed that when injected into the spleen, few MSCs migrated into the liver after PH. These cells integrated randomly into the tissue and were not able to differentiate into hepatocytes. The MSCs instead retained a mesenchymal morphology,

expressing vimentin and α-smooth muscle actin (αSMA). Further, their localization merged with collagen deposition in transplanted liver, indicating a potential harmful effect on the liver parenchyma (7).

In conclusion, more research is needed to optimize the techniques by which MSCs are isolated, expanded in vitro, and infused to improve long-term functional integration in vivo.

CONCLUDING REMARKS

The diagnosis and treatment of end-stage liver disease represent an important challenge for clinicians. The recent investigations on MSCs have offered a new perspective for liver regeneration: these cells can be easily isolated, expanded and infused in the recipient, leading to improved liver function. Moreover, MSCs are an attractive vehicle for gene therapy, allowing the cure of IMDs in an autologous fashion.

Although the MSC potential to transdifferentiate in vitro into functional hepatocyte-like cells has been described, the evidence from in vivo animal studies is contradictory. In addition, to our knowledge there has been no research to date to show that MSCs can integrate into damaged human liver and differentiate into hepatocytes and BECs. This concern remains to be investigated. In our opinion, the functional changes seen in experimental models of liver disease and in the few concluded and ongoing clinical trials of MSC therapy are most likely dependent on the immunomodulatory properties of MSC-secreted paracrine-soluble factors. Although from this perspective MSC-CM administration appears to be a valuable alternative to cell transfer, we believe that MSC infusion could ensure the sustained release of a cocktail of factors exerting several actions, including the inhibition of apoptosis and promotion of proliferation of endogenous hepatocytes, as well as attenuation of inflammation and liver fibrosis regression.

MSCs have the features to provide effective low-cost treatment. However, autologous MSC therapy could be difficult to achieve due to the need to obtain a biopsy from a patient's sick body. Also, it is not clear whether each donor can produce sufficient MSCs of required potency when needed. An alternative approach is to use allogenic MSCs. The advantages of allogenic MSCs are many: the donor can be chosen in advance, qualified and tested for the presence of various diseases. Moreover, a ready-to-use MSC culture can be immediately available when needed.

A problem to be faced when using allograft materials is the risk of rejection. Importantly, apart from an immunomodulatory function (51), MSCs also show an immunogenic function, mainly due to the absence of costimulatory molecules (123). Therefore, allogenic MSCs are not associated with graft rejection in treated patients.

Briefly, the key advantages of using MSC therapy would be the reduced waiting time before transplantation, the opportunity to utilize small samples of adult tissues to obtain an initial MSC culture, a reduced rejection risk, and higher success rates of liver regeneration.

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