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Adipose tissue-derived mesenchymal stem cells and hepatic differentiation: old concepts and future perspectives

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Abstract. – Mesenchymal Stem Cells (MSCs) are multipotent cells, able to differentiate into elements of the mesodermal lineage. Bone marrow and adipose tissue represent the main sources for MSC isolation. In the last decade, several studies have reported the plasticity of MSCs toward a hepatocyte-like phenotype. The use of MSCs to generate hepatocyte-like cells holds great promises to overcome the scarcity of available organs for transplantation. However, little is known about the molecular pathways involved in lineage cross-differentiation and several issues remain to be answered before MSC application in clinical settings.

Aim of this review is to critically analyze the possible sources of MSCs suitable for liver repopulation and the molecular mechanisms underlying MSC hepatic differentiation.

Key Words:

Mesenchymal stem cells, Adipose tissue, Liver regeneration.

Introduction

Mesenchymal Stem Cells (MSCs) were first described by Friedenstein et al[1], more than 30 years ago, as an adherent, fibroblastoid cell population within the bone marrow (BM) that showed inherent osteogenic properties. Numerous studies have demonstrated that these cells are able to differentiate into multiple connective tissue cells, including osteocytes, chondrocytes, adipocytes and stromal cells[2]. Furthermore, in contrast to hematopoietic stem cells, MSCs could be expanded in culture for long periods of time without loss of differentiation capacity.

In addition to BM, MSCs have been isolated from various adult tissues, including muscle[3], adipose tissue[4], connective tissue[5], trabecular bone[6], synovial fluid[7], and from perinatal tissues, such as umbilical cord[8], amniotic fluid[9], and placenta[10]. The presence of MSCs in peripheral blood is still debated as some Authors identified a circulating fibroblast-like population[11], whereas others failed[12].

Adipose tissue has several advantages compared to other adult tissues as source of MSCs, as it is abundant and can be easily removed by simple lipoaspirate. Adipose tissue-derived MSCs (ATMSCs) have been termed processed lipoaspirates[4], adipose tissue-derived stromal cells[13], human multipotent adipose-derived stem cells[14]. ATMSCs can be maintained longer in culture and possess a higher proliferation capacity than BM-derived MSCs. Thus, adipose tissue may be an ideal source of large amounts of autologous stem cells attainable by a less invasive method than BM-derived stem cells.

Despite the numerous efforts that have been made to characterize the immunophenotype of MSCs, no specific surface marker has been identified yet. MSCs are commonly defined as plastic adherent cells that express a panel of surface antigens, including CD90, CD29, CD44, CD73, CD105, CD166 while lacking expression of hematopoietic and endothelial markers, such as CD45, CD14, CD34, and CD31. Moreover, MSCs express human leukocyte antigen (HLA) class I, but not HLA class II. MSCs secrete several extracellular matrix (ECM) molecules, as collagen, fibronectin, laminin and proteoglycans, so that it has been postulated a central role for MSCs in ECM organization. Recently, the low affinity nerve growth factor receptor (CD271)

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has been proposed as a specific marker for MSC isolation. However, the value of CD271-based MSC selection remains unproven[15].

Numerous studies have shown that MSCs have a high degree of plasticity. Indeed, they can not only differentiate into cells of the mesenchymal lineage, but also transdifferentiate into neurons, splenocytes and various epithelial cells, including lung, liver, intestinal, and kidney cells[16-20]. In particular, *in vitro* models using culture medium supplemented with a cocktail of growth factors achieved the transdifferentiation of MSCs into hepatic cells with functional properties such as albumin and urea production and glycogen storage[21]. Moreover, the *in vivo* transdifferentiation of bone marrow stem cells (BMSCs) into hepatic cells has been described in rats[22], mice[23] and humans[24]. Seo et al[25] first reported that human ATMSCs injected to immunosuppressed severely combined immunodeficient (SCID) mice, following toxic liver damage, were able to differentiate into hepatocyte-like cells.

Talens-Visconti et al[26] have recently confirmed the possibility of generating hepatocyte-like cells from ATMSCs. Aurich et al[27] demostrated long-term engraftment of human ATMSCs derived hepatocyte-like cells in a xenogeneic transplantation model of liver regeneration. In particular, the Authors reported that engraftment was significantly improved using ATMSCs pre-differentiated to hepatocyte like cells *in vitro* as compared with undifferentiated MSCs. Differentiated ATMSCs were capable of extensive proliferation within the host liver similar to hepatocytes during liver regeneration. After 10 weeks, more than 10% of all hepatocytes in the host liver were replaced by hepatocyte-like cells derived from ATMSCs. The Authors concluded that engraftment was significantly more efficient using ATMSCs pre-differentiated to hepatocyte like cells *in vitro* as compared with undifferentiated ATMSCs. In a previous study, the same Authors had observed that using human BM-derived MSCs in the same mouse model, a repopulation of only 1% of the liver mass was obtained by the transplanted cell[28]. Overall, repopulation rates of more than 10% make ATMSCs better candidates than BM-derived MSCs for the stem cell based liver therapies[27].

ATMSC and Liver Regeneration

The liver is a remarkable organ, given its inherent capacity to fully restore itself after significant hepatic tissue loss. In most instances, mature hepatocytes can undergo several cell divisions and restore the hepatic mass. Furthermore, numerous studies have reported the presence of a stem cell compartment within the liver that can participate to the process of repair in certain circumstances[29]. However, several debilitating diseases tend to compromise the regenerative ability of both hepatocytes and the local reservoir of hepatic stem cells. In such conditions, the liver is unable to maintain a functional mass, and clinically, this phenomenon is mirrored by liver failure[29]. Orthotopic liver transplantation (OLT) represents the gold standard for the treatment of end-stage liver disease. Nevertheless, only a minority of candidates undergo OLT, given the scarcity of donor organs. Indeed, it was estimated that about 35% of patients on the waiting list in Europe do not receive the required organ (http://www.eurotransplant.nl/) and in the United States, the percentage appears to be even higher (http://www.unos.org/)[30]. Other adverse factors such as rejection, problems associated with the long-term use of immunosuppressants, and perioperative morbidity and mortality contribute to additional complications. Hepathocyte-based therapy has been proposed as a potential alternative to OLT. In particular, transplantation of hepatocytes seems to be a versatile alternative to whole organ transplantation in various rodent animal models such as mice, rats and rabbits[31,32] but, to date, hepatocyte transplantation trials in humans have shown poor results[33]. Probably, this is due to the fact that the experimental methods used in animal models to enhance liver repopulation are not applicable in clinical settings. In addition, human hepatocytes are currently available only from marginal donor livers that are not allocated for transplantation, thus yielding cells of low quantity and quality[34].

Hence to need to find alternative cell sources that led to the development of protocols to generate hepatocytes from stem cells. In the past, it was believed that hepatocytes could only be derived from cells of endodermal origin. However, subsequent studies have suggested that nonendodermal cells may also form hepatocytes *in vivo* and *in vitro*[35-39]. Recently, BM-derived stem cells have been extensively investigated as potential sources for liver regeneration. In 1999, Petersen et al., first showed that liver stem cells might be derived from BM, in a rat model of liver injury[40].

However, initial reports of the hepatic potential of hematopoietic stem cells were later shown to have resulted from fusion between transplanted donor cells and the resident recipient hepatocytes[41].

In the last eleven years, many researchers have reported that MSCs might be more suitable for clinical transplantation than hematopoietic stem cells[42]. Both BMSCs and ATMSCs can be made to differentiate *in vitro* into functional hepatocytes using various experimental protocols, based on either a direct induction[25], or a sequential addition of growth factors[43]. These experiments were conducted on unsorted cell cultures[25-26] or following immunomagnetic selection of particular cell subpopulations[44]. The results of different protocols change the *in vivo* functional capacity of the transplanted cells profoundly.

From MSCs to Hepatocytes

Hepatic differentiation of MSCs is a multistep process tightly regulated by intra- and extracellular communications. Little is known about the complexity of the molecular pathways involved in lineage cross-differentiation. The most important extracellular signals involved in hepatogenesis are activin A, fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), hepatocyte growth factor (HGF), and oncostatin M (OSM)[45-49]. A wide variety of experimental conditions have been applied thus far to trigger the differentiation of cultured MSCs into functional hepatocytes. Among these, the most promising approaches are based on reconstructing the *in vivo* microenvironment *via* addition of soluble medium factors. Significantly optimized differentiation was obtained *via* exposure of MSCs to hepatogenic factors in a time-specific sequential manner, reflecting their secretion pattern during *in vivo* hepatogenesis. A mixture of FGF and HGF followed by OSM have been applied to obtain functional hepatic conversion of MSCs[26,50]. Basically, soluble medium factors such as dexamethasone, insulin-transferrin-selenium (ITS), and nicotinamide synergistically affect the hepatic driving pathways[25]. Serum-free conditions have been applied on a routine basis for hepatic differentiation of MSCs[25,26,51,52].

Another critical factor affecting cellular differentiation status is the cell spatial distribution. Differentiation to a hepatocyte phenotype was seen only when cells were seeded at high confluence (upon 60%-100% confluence), which may cause a post-mitotic state required for differentiation and promote maximal cell-cell contact[28,53-57]. Minor roles are ascribed to the type of coatings used (the natural scaffold collagen turned out to be most effective)[54,57].

Hepatic differentiation has been assessed by means of immunohistological and molecular approaches. MSC-derived hepatocyte-like cells may be characterized *in vitro* at four levels: morphology, RNA, protein, and activity. However, each of these methods has potential pitfalls that complicate interpretation of the results. Many of the genes usually used to test the differentiation of MSCs toward the hepatic lineage are not expressed exclusively by hepatic cells and thus cannot be considered as ''true'' hepatocyte markers[58-61]. Hence, exclusive analysis of one marker cannot count as proof for a genuine hepatic phenotype. On the other hand, some genes, like alpha-fetoprotein (AFP), are expressed very early in embryonic development and during the fetal stages. Their expression gradually levels off with increasing development and disappears entirely in adult life[62]. AFP thus represents a reliable marker to discriminate between distinct developmental stages. Alternatively, most, but not all, metabolic and detoxifying enzymes become functional during the terminal step of liver organogenesis. Therefore, to state the differentiation stage of the resultant hepatocyte-like cells, functional assays need to be carried out. At present, functional analysis is particularly focused on glycogen uptake, urea metabolism, and albumin (ALB) secretion.

To date, studies on animal models reported the beneficial effect of ATSMCs in promoting hepatic tissue regeneration. Recently, Banas et al[63], evaluated the therapeutic potential of ATMSCs for the treatment of liver failure. In this study, the Authors transplanted human ATMSCs into immunodeficient mice with acute liver failure caused by carbon tetrachloride (CCl_4) injection and revealed the ability of ATMSCs to incorporate into the liver and improve its function. The Authors postulated that benefic effects of human ATMSC transplantation may be due to MSC ability to produce a large number and volume of bioactive factors. Interestingly, the Authors compared *in vitro* production of cytokines/growth factors by undifferentiated ATMSCs with BM-derived MSC and observed a higher production of bioactive factors in ATMSCs than in BM-derived MSCs.

These results have been also confirmed by Kuo et al[64], who demonstrated that undifferentiated MSCs were able to rescue rats from D-galactosamine-induced fulminant hepatic failure by soluble factors, indicating that they might act by paracrine mechanisms.

Overall, these findings confirmed the ATMSCs efficacy in animal models of liver diseases and in the clinical settings for liver disease treatment.

Molecular Mechanisms Underlying ATMSCs Differentiation in Hepatocyte-Like Cells

To date, the molecular mechanisms underlying the differentiation of ATMSCs are largely unknown. Recently, Yamamoto et al[65] examined the gene expression profiles of AT-MSC-derived hepatocytes in order to identify the genes responsible for hepatic differentiation, using several microarray methods. The resulting sets of differentially expressed genes (1639 clones) were comprehensively analyzed to identify the pathways expressed in AT-MSC-derived hepatocytes. Microarray analysis revealed that the gene expression pattern of AT-MSC-derived hepatocytes was similar to that of adult human hepatocytes and liver. Further analysis showed that enriched categories of genes and signaling pathways such as complementary activation and the blood clotting cascade in the AT-MSC-derived hepatocytes were relevant to liver-specific functions. Interestingly, decreases in Twist and Snail expression indicated that mesenchymal- to-epithelial transition (MET) occurred in the differentiation of AT-MSCs into hepatocytes.

In our recently published study, we performed a high throughput molecular analysis of ATMSCs before and after hepatogenic conversion in order to clarify the molecular events involved in controlling the plasticity of AT-MSCs that give rise to hepatocytes[66]. We achieved the hepatogenic conversion of ATMSCs, using a two-step protocol with sequential addition of growth factors. Under this regimen, spindle-shaped fibroblastoid cells differentiated to a layer of compact polygonal cells, characteristic of an epithelium. Interestingly, these cells acquired specific liver functions, as shown by their ability to store glycogen and to express hepatic-associated genes and proteins. Overall, we identified several targets that depict the numerous biological functions exerted by the liver, including protein metabolism, innate immune response regulation, and biodegradation of toxic compounds. Moreover, modulation of molecules involved in adhesion and migration capacity confirmed that a mesenchymal-epithelial transition occurred. This process and the reverse epithelial-mesenchymal transition (EMT) are key developmental programs playing a fundamental role in the differentiation of multiple tissues and organs during embryogenesis[67]. In particular, we observed decreased expression of N-cadherin-2 (CDH2) and vimentin, along with downregulation of gremlin, a key mediator of EMT in ATMSC-derived hepatocyte-like cells. Finally, we identified several members of the transforming growth factor-beta (TGF-β) [(small mother against decapentaplegic (SMAD7), latent-transforming growth factor beta-binding protein 2 (LTBP2)] and WNT [Frizzled-4 (FZD4), Frizzled-6 (FZD6), dickkopf-related protein 3 (DKK3)] signaling pathways, that have been extensively described for their involvement in EMT process[68]. Altogether, these data suggest that cellular plasticity observed in ATMSCs is dependent of mesenchymal-epithelial transition and that subtle regulations of the canonical pathways BMP7, WNT and TGF-β may be important to allow MSCs to transdifferentiate into another lineage.

Clinical Applications

Given their multipotential differentiation potential and their extensive self-renewal, MSCs have been considered a promising candidate for cell-based therapy and tissue engineering. These cells have the ability to proliferate to an extensive but finite degree, an important characteristic that should reduce concerns about potential tumorigenicity of these cells upon transplantation *in vivo*. Moreover, their unique immuno-privileged status may allow them to be used for autologous and allogeneic transplantation. At present, various clinical trials have been conducted with MSC concerning three main applications:

1. *MSCs and tissue engineering.* Tissue engineering may represent an alternative for obtaining tissues and organs needed for transplantation, circumventing the lack of a sufficient number of donors. Basically, tissue engineering consists of donor's cells isolation and expansion, followed by a reimplantation procedure in combination with a scaffolding material. The

recent advent of new bio-scaffolds allowing the three-dimensional culture of MSCs in bioreactors might lead to the formation of whole tissues and organs. A few studies in animals have shown the feasibility of MSCs transplantation in repairing and regenerating damaged tissues of mesodermal origin, such as bone defects[69,70]. ATMSCs and a reasorbable macroporous sheet has been successfully applied in a seven-year old girl suffering from widespread calvarial defect[71].

2. *MSCs and immune-mediated disorders.* One of the most exciting aspects of MSCs biology is the so-called *immunoprivilege*. MSCs express few HLA class I and no HLA class II molecules, suggesting that they can evade allogeneic immune response. These unique immunologic properties were first demonstrated in sheep, in which human MSCs were shown to evade rejection[72]. *In vitro* experiments showed that MSCs exert immunoregulatory functions by suppressing T cell[73], B cells[74] and natural killer cells[75] proliferation, and by affecting dendritic cell maturation[76]. The mechanisms underlying immunoregulation remain unclear: some investigators suggested a cell-to-cell contact mediated suppression, while others hypothesized a soluble factor mediated mechanism[77]. MSCs have been applied in hematology to facilitate hematopoietic stem cells (HSCs) engraftment and lessen the risk of graft versus host disease (GVHD). GVHD constitutes the most frequent form of rejection associated with the transplantation of allogeneic hematopoietic grafts, characterized by the destruction of host tissues and organs by the transplanted cells. Leblanc et al[78] reported the safety and efficiency of BMSC infusion for the control of GVHD in a few patients subjected to allogeneic HSC transplantation. Furthermore, Lazarus et al[79] reported that co-infusion of HSCs and MSCs in patients suffering from haematological malignancies reduced transplant side effects and enhance marrow recovery after myeloablative treatment.
3. *MSCs and solid organ transplantation.* MSCs have recently emerged as promising candidates for cell-based immunotherapy to induce tolerance after allogenic solid organ transplantations[80,81]. Furthermore, MSCs have a great regenerative potential and can, therefore, contribute to the marginal organ regeneration after transplantation, thus improving overall clinical outcome[82] Animal models featuring MSCs in solid organ transplantation emerged for the first time in 2006[83,84]. Unfortunately, the great part of results from *in vitro* studies could not be transferred in models of transplantation. In fact, many *in vitro* effects attributed to MSCs depend on cell-cell contact or at least the close vicinity of MSCs and effector cells. Moreover, organ recipients are heavily immunosuppressed during the induction phase in order to protect the grafts from rejection. Drugs that affect the immune system add extra complexity to the MSC-induced tolerance. Pop et al[83] reported no effects on graft survival when MSCs were applied concurrently with cyclosporine after heart transplantation in rats. In contrast, other groups[84] have demonstrated prolonged graft survival when MSCs were applied before transplantation with or without additional injections in corresponding models. Recently, it has been shown that MSC injection together with low doses of mycophenolate promotes long-term graft survival[85].

Clinical use of MSCs for repair/regeneration of solid organ is in its infancy. However, several concerns remain before their general application in clinical settings. A main limit is the lack of a standardized protocol for isolating a specific cell population. Each group of investigators using ATMSC defines these cells in different ways, ranging from specific-antigen profile to simple plastic adherence property. The heterogeneity in the methods of isolation generates variable results and makes the interpretation of data very difficult.

In vivo studies reported that transplanted MSCs may contribute to scar-forming myofibroblast in the liver[86]. This result suggests that MSCs could enhance the fibrotic process instead of regenerating the parenchymal tissue.

Another issue in using MSCs for liver regeneration is the possible differentiation into mesodermal cells within the liver. I.e., one cannot exclude the possible spontaneous differentiation of ATMSCs into adipose cells. In our laboratory, we performed some experiments with intrasplenic injection of ATMSCs in a murine model of liver damage induced by monocrotaline. Our results showed the presence of adipose cells in the spleen of transplanted animals while differentiation of ATMSC into hepatocyte-like cells was not established (data not published). Other studies reported unintended differentiation of MSCs following *in vivo* transplantation. Yoon et al[87] re-

ported the generation of calcification/ossification into the infarcted myocardium of rats transplanted with bone marrow cells. The mechanism by which locally transplanted BM cells induced calcification was not clearly defined, but MSCs as bone and cartilage precursors were highly suspected.

The safety of using MSCs could be also questioned as MSCs can also undergo malignant transformation, thus giving rise to tumors. MSCs, and in particular ATMSCs, have a high proliferation rate *in vitro*. ATMSCs have been proved to undergo spontaneous transformation in long term culture (4-5 months)[88]. Another study suggested that cultured BMSCs bear karyotype mutations and may develop osteosarcoma in the lung upon *in vivo* injection[89].

Overall, these data highlight that numerous unanswered issues have to be elucidated to ensure safety and efficiency of MSC-based therapies in hepatology. To date, 2 clinical trials using autologous ATMSCs for liver regeneration have been deposited (http://clinicaltrials.gov/), but the recruitment's process has been suspended.

Conclusions

The use of MSCs to generate hepatocyte-like cells hold great promises to overcome the scarcity of available organs for transplantation. In particular, human adipose tissue may represent a novel source for therapeutically applicable MSCs in liver disease.

However, many concerns remain before MSCs application in Clinics. A deeper understanding of the signals emanating from the stem cell niche, a more accurate analysis of cell-intrinsic mechanisms underlying differentiation-inducing signals, together with the standardization of the processes of isolation, expansion, differentiation and reinfusion of MSCs are mandatory prior to consider these cells for clinical purposes.

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