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Review Article

Review: Extracellular Vesicles from Human Liver Stem Cells as an Alternative Therapeutic Approach for the Treatment of Urea Cycle Diseases

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Abstract

The intrahepatic administration of Human Liver Stem Cells (HLSCs) was recently evaluated in infants with inherited hyperammonaemia. As HLSCs are easy to isolate and culture *in vitro*, there is a very good opportunity to test their therapeutic potential in several liver related diseases especially urea cycle disorders. The *in vivo* pro-regenerative effect of HLSCs and their secretome was first demonstrated in different models of acute liver injuries including acute liver injury induced by the analgesic drug *N-acetyl-p-aminophen* and fulminant hepatitis induced by D-galactosamine and lipopolysaccharide. In addition, further research highlighted their effectivity in improving chronic liver diseases such as non-alcoholic steatohepatitis. This therapeutic property exhibited by HLSCs and HLSC-EVs was attributed towards their abilities to modulate inflammatory and fibrotic processes. Recently, we identified a new contribution of HLSC-derived EV cargo in an *in vitro* model of type 1 citrullinemia which occurs due to a defective Argininosuccinate Synthase 1 (ASS1). By implementing an assay comprising of ASS1 mutated HLSCs derived from a patient with type 1 citrullinemia, we observed the restoration of ASS1 enzymatic activity following treatment with HLSC-EVs. This article aims to discuss some of the experimental and methodological aspects of our approach and highlights some of the broad spectrum therapeutic effects of HLSCs and their secretome in various different disease models. We will also focus on some of the different parameters that still remain challenging towards the use of HLSC-derived EVs as a tool for the delivery of functional proteins and mRNAs in urea cycle disorders.

Keywords: Argininosuccinate synthase; Extracellular vesicles; Human liver stem cells; Urea cycle

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Introduction

The urea cycle is a process that primarily occurs in the liver and is involved in the elimination of toxic ammonia waste in the form of urea. It is a conglomerate of multiple enzymatic reactions involved in the endogenous production of arginine, ornithine, and citrulline, as well as the metabolism of adenosine monophosphate and removal of nitrogen waste. Some of these essential enzymes required to catalyse the urea cycle such as Argininosuccinate Synthase 1 (ASS1) are also connected with the nitric oxide production pathway through the urea cycle [1].

Citrullinemia type I is a rare autosomal recessive genetic disorder caused by a deficiency of the enzyme ASS1. The role of ASS1 is to catalyse the condensation reaction between citrulline and aspartate to form argininosuccinic acid in the third step of the urea cycle [1]. Mutations in the ASS1 gene results in the disruption of the urea cycle causing excessive accumulation of nitrogen. The clinical phenotype and its onset depend on the ASS1 residual enzyme activity affected by the specific mutation [1,2]. With an incidence rate of approximately 1 in 57,000 live births [2], therapy for citrullinemia type 1 is limited to dietary protein restriction, administration of arginine supplements and nitrogen scavengers [2]. Liver transplantation has been reported to prolong survival in some patients [3], but scarce organ availability and high costs for surgery has stimulated researchers to search for alternative therapies.

In 2006, we reported the isolation and characterization of a stem cell population derived from healthy human livers known as Human Liver Stem Cells (HLSCs) [4]. They exhibit certain characteristics comparable to bone marrow derived Mesenchymal Stem Cells (MSCs) which include: a similar phenotype, multipotent abilities [4], gene expression profile [5], and immunomodulatory properties [6]. Contemporaneously, they also show a specific commitment towards the liver therefore making them different from MSCs. Over the past few years, we have focused our attention on studying HLSC derived Extracellular Vesicles (EVs) and their functional properties primarily in preclinical models of liver injuries *in vivo*.

Proposed for the first time in 2011, the term EVs was defined as a lipid bilayer vesicle secreted by cells enriched with the cytosol [7]. They are classified according to their size, mechanism of biogenesis

and cargo that includes: nucleic acids, proteins, and lipids [7]. Over the years, special attention has been given on their isolation techniques [8] as well as their paracrine effects; in particular stem cell-derived EVs in different preclinical models *in vivo* [7]. In 2017, our group demonstrated that EVs isolated from HLSCs had the ability to carry and transfer the wild-type version of ASS1 to ASS1-mutated HLSCs *in vitro*, thereby restoring enzyme activity and urea production [9]. In this review we provide an overview of the latest advances in the use of HLSC-EVs as an acellular therapeutic option not only for liver injuries but also for urea cycle disorders.

HLSCs and their Secretome: Mediators of Liver Repair Activity

HLSCs have proven to be a resident stem cell type exhibiting strong therapeutic potential in various preclinical models of liver damage [5,9-11]. One of the first studies in our lab showed that, HLSCs were able to engraft and contribute to hepatic regeneration in an acute liver injury model induced by *N-acetyl-p-aminophen* in Severe Combined Immunodeficient Mice (SCID) [4]. Furthermore, through a murine model of fulminant hepatitis, we demonstrated that engrafted HLSCs persisted as functioning undifferentiated population in the liver tissue of SCID mice after 21 days [12]. The engraftment of HLSCs was also observed in a murine model of Non-Alcoholic Steatohepatitis (NASH), whereby HLSCs were able to persist in the liver parenchyma of non-immunocompetent mice for at least 3 weeks [5]. This therefore, confirms the ability of HLSCs to integrate with the liver parenchyma regardless of the type of liver damage. Recently, HLSCs were administered in a Phase I clinical study in neonates suffering from inherited neonatal-onset hyperammonaemia [13]. The purpose of the trial was to assess the safety of intrahepatic administration of HLSCs and their effect on the biochemical parameters, and maintenance of patient metabolic stability in view of liver transplantation [13]. Whether HLSCs contribute towards correcting the urea cycle enzymatic deficiency by integrating in the liver parenchyma *in vivo* or through the synergistic paracrine mechanism of their secretome still remains to be defined.

The HLSC secretome which includes biologically active molecules released either in the soluble form or packaged into EVs also plays an important role in various physiological processes [7,12,14] (Figure 1). Interestingly, through different *in vivo* experimental models, we found that the HLSC secretome also contributes towards the broad spectrum pro-regenerative effects comparable to HLSCs, suggesting that also paracrine factors have therapeutic properties. For instance, soluble molecules present in HLSC Conditioned Medium (CM) ameliorated liver function and improved morphology and overall survival in a murine model of fulminant hepatitis [12]. Five of the most concentrated growth factors that were identified in HLSC-CM such as interleukin 6 and 8 (IL6 and IL8), Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF) and Macrophage Stimulating Protein (MSP) have been described to exhibit liver regeneration function [12]. By neutralizing HGF in the HLSC-CM with a blocking antibody, we demonstrated that HGF was a significant mediator of hepatic regeneration induced by HLSC-CM [12].

HLSC-EVs are also considered to be part of the HLSC secretome, and are known to exhibit therapeutic effects. For instance, HLSC-EVs promoted hepatocyte proliferation and reduced apoptosis in a rat model of 70% hepatectomy [15]. The presence of AGO2 human mRNA as well as de novo protein in the rat liver parenchyma demonstrated not only the horizontal transfer of human mRNA from EVs, but also the translation of the mRNA to protein in the target cells [15]. More recently, in a murine model of NASH, we demonstrated that HLSC-EVs exerted antifibrotic and anti-inflammatory properties similar to their cellular counterparts [10]. Protein array analysis of HLSC-EVs in this study revealed the enrichment of 251 proteins that could mediate inflammatory pathways [10]. Furthermore, similar antifibrotic and immunomodulatory effects were also observed by Kholia et al., in their model of aristolochic acid nephropathy [16], as well as by Grange et al., in their model of diabetic nephropathy [17]. Although both the models of nephropathy were diverse, the effects exerted following HLSC-EVs treatment were similar and partially attributed to miRNAs enriched within the EVs [16,17]. Several of these enriched miRNAs including miRNA-29a, miRNA-21, miRNA-30a, miRNA-24, and the let-7 family, were identified to function as regulators of profibrotic proteins such as collagen I, snail, and FAS ligand, therefore contributing towards the antifibrotic effects observed [17]. Apart from liver injury, HLSC-EVs have also been identified to exhibit a potential therapeutic role in acute models of kidney injury [18]. Antitumor effects in various models of cancer through the delivery of anticancer miRNAs as well as by inhibiting cancerogenic angiogenesis has also been reported [19, 20]. These studies therefore highlight the potential therapeutic and regenerative abilities of HLSC-EVs in a wide spectrum of pathologies.

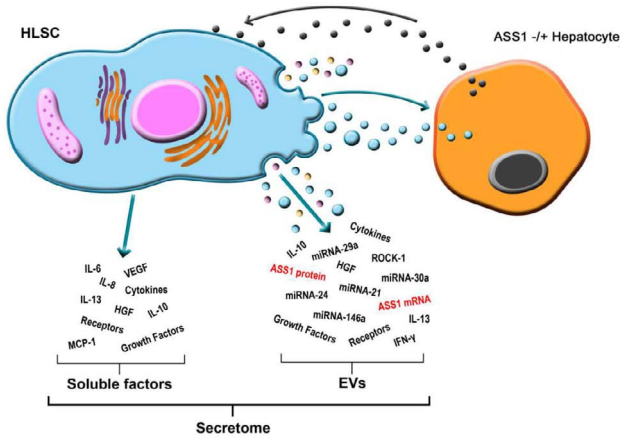

Figure 1: Interaction between HLSC secretome and hepatocytes. The illustration depicts how Human Liver Stem Cells (HLSCs) could influence both wild type (ASS1 +) and ASS1 mutated (ASS1 –) hepatocytes through the transfer of extracellular vesicles. The diagram also shows the components of the HLSC secretome which includes soluble factors released by the cells directly as well as in the form of EVs enriched with various biologically active factors including ASS1 protein and mRNA.

Restoration of ASS1 Activity by HLSC-Derived EVs

Conventional protein-based replacement therapies have several disadvantages mainly due to the fact that cytosolic and mitochondrial enzymes are generally not taken up by cells [3]. EVs on the other hand, have been shown to be taken up by various organs including the liver therefore making them a good therapeutic option to deliver deficient proteins [7,14]. As the urea cycle takes place primarily in the liver, and as HLSCs are of liver origin, we focused our attention in studying the role of HLSC-EVs in the urea cycle disorder citrullinemia type I caused by ASS1 deficiency.

The methodology applied to isolate ASS1 mutated HLSCs was similar to the method described in the past for non-mutated HLSCs [4,9]. Briefly, a fragment of the discarded liver from a citrullinemia type I patient was enzymatically digested and the mixture of hepatocytes plated and cultured for 15 days. The expanded cells were characterized by cytofluorimetric analysis to confirm typical HLSC markers. Mutated HLSCs expressed similar markers as normal HLSCs some of which include surface proteins like CD90, CD73, CD29, CD105, the hepatic marker albumin and cytokeratin 18 [9]. Furthermore, embryonic stem cell markers such as nanog and sox2 were also expressed therefore confirming a phenotypic similarity between the two cell types [9].

SNaPshot sequencing of mutated HLSCs revealed two codon mutations with the substitution of bases C and G with T and A (g.55277 C>T and g.59839 G>A). Furthermore, qRT-PCR analysis showed mutated HLSCs to express the mRNA of both isoforms of ASS1. At the protein level, only isoform 1 was expressed in the mutated HLSCs as was also observed with normal HLSCs. In addition, following differentiation into mature hepatocytes in the three dimensional rotary system, isoform 2 was also expressed in both mutated and normal HLSCs. This therefore confirms that, the mutation probably affects the functional aspect of the ASS1 enzyme as opposed to the protein synthesis that involves transcription and translation [9].

Purified HLSC-EVs were found to be enriched with wild-type ASS1 protein, mRNA, and DNA [9]. We therefore implicated an *in vitro* model to test the transfer of ASS1 from HLSC-EVs whereby ASS1 mutated HLSCs (with diminished ASS1 enzymatic activity) were cultured and co-incubated with HLSC-EVs [9]. Post assay, we observed that the mutated enzymatic defect was corrected, and ASS1 activity as well as urea production restored.

A major discovery in the EV field was the demonstration of the presence of mRNA and miRNA in the EV cargo [21,22]. In addition, several other studies have reported that these mRNAs could be translated into proteins when taken up by target cells [22-25]. To study the contribution of HLSC-EV enriched ASS1 mRNA in our experimental settings, we transiently transfected HLSCs with ASS-shRNA. After confirming the silencing of ASS1 mRNA following transfection in HLSCs, EVs were isolated and purified from these cells and applied in the *in vitro* ASS1 enzymatic assay. Unlike normal HLSC-EVs, the EVs from ASS1 silenced HLSCs were unable to restore urea production in hepatocytes differentiated from mutated ASS1-HLSCs. This therefore suggests that the restoration mechanism may depend on the horizontal transfer of intact functional ASS1 mRNA and protein [9].

The direct delivery of mRNA has also been shown to be an effective mode of treatment for urea cycle deficiencies. For instance, a group studying Ornithine Transcarbamylase (OTC) deficiency (another enzyme involved in the urea cycle) successfully normalised blood ammonia and improved the survival of OTC-deficient mice following direct administration of human OTC mRNA [26]. Although this direct delivery of mRNA has some advantages over viral vectors which tend to elicit host immune reactions [3,7,27], one major limitation is that they are unstable and have a relatively short half-life *in vivo* [3,27]. On the other hand, as EVs are membrane bound particles, the cargo enriched within them including mRNAs and proteins are not only very well protected from degradation, but also display a prolonged biological activity *in vivo*, therefore making them suitable candidates for therapy [7, 28].

Apart from mRNA and protein, SNaPshot sequencing analysis revealed the enrichment of HLSC-EVs with fragments of non-mutated ASS1 DNA [9]. Furthermore, sequencing mutated HLSCs treated with wild type HLSC-EVs, we discovered that the amplitude of the peaks of the bases mutated was reduced. However, this horizontal transfer of DNA was not relevant for the correction of ASS1 enzyme activity in the mutated HLSCs [9]. Additional studies are therefore required to evaluate whether DNA transfer by EVs could possibly correct enzymatic defect at a genetic level.

EV Isolation Methodology Plays an Integral Part in EV Research

As mentioned above, EVs is a collective name for vesicles that are released by various different processes influenced mainly by the cargo, and the state at which the cell is *in situ* [29]. Before working with EVs, a set of requirements for the obtained EVs has to be defined, as this will determine the method of isolation. For instance, if EVs are to be used as therapeutic vehicles, it is imperative to use an isolation method that will preserve their structure and integrity of the EVs. If diagnostics is the main objective, then a suitable isolation method that will provide maximum yield with purity has to be selected. In addition, the heterogeneity nature of EVs will also influence the method of choice with modifications to isolate a specific EV sub-type such as exosomes, or microvesicles.

Over the years various methods have been developed to isolate EVs [8]. The more recent methods developed over the last decade include: affinity interaction based isolation involving the use of antibodies that bind to specific EV receptors [30]; precipitation with various dense agents (polyethylene glycol, sodium acetate, and protamine) that are based on the solubility and or aggregation properties of EVs [31-33] and more recently microfluidics [34,35]. The more traditional methods such as differential ultracentrifugation [36], gel filtration chromatography [37], and microfiltration [30], are based on various characteristics of EVs such as size and buoyancy and are still considered to be the more popular methods for EV isolation [8]. Each of these methods however has their advantages and limitations, which need to be taken into consideration. For the purpose of this review, we will be discussing on the method of differential ultracentrifugation as it was the preferred method of EV isolation when the urea study was performed. This protocol was initially developed to isolate exosomes from reticulocyte cultured medium and then eventually modified and adapted for the isolation of EVs from various cell types and biological fluids [38]. Majority of the studies that have been performed since the late 90s used differential ultracentrifugation for the isolation of EVs as it was considered to be the classical gold standard and most widely used method by researchers in the EV field and therefore was adopted for our study [29].

In our study, we utilized a combination of differential ultracentrifugation with density gradient centrifugation to obtain a pure, intact sample of EVs. Differential ultracentrifugation is the classical method of EV isolation that involves separation of particles based on their buoyancy using centrifugation [36]. The method comprises of various steps of centrifugation at varying speeds and time, and floating to remove contaminants and sediment EVs. The speed and duration of the final ultracentrifugation step depends on the sub-type of EVs required for the study. For larger vesicles such as microvesicles (>200 nm) a lower speed of 10,000 g is applied, and for smaller vesicles such as exosomes (<150 nm) a higher speed of 100,000 g is required [8,36].

The heterogeneity of EVs has been very well described in the literature and has been attributed to different release mechanisms and the cargo they carry [14,29]. For instance exosomes are released through the fusion of multivesicular bodies with the cell membrane, whereas ectosomes are released by the outward budding of the cell membrane [29,39]. In our study, we were interested to identify which population of HLSC-EVs was enriched with the ASS1 gene or protein. Therefore, HLSC cell culture supernatants were first ultracentrifuged at 10,000 g (1 hr) to isolate larger vesicles, followed by 100,000 g (1 hr) to pellet the smaller ones [9].

One disadvantage of differential ultracentrifugation is the presence of contaminants such as protein aggregates, non-exosomal particles, nucleic acids, and subcellular components in the EV fraction obtained [8]. In order to remove these contaminants a purifying process is imperative. Density gradient separation allows the efficient removal of various contaminants by separating them according to their buoyant density [40]. This method utilizes two different types of gradient solutions for purification. The first being a sucrose based gradient solution ranging from 1.25 g/ml to 1.1 g/ml from bottom to top (with 0.5 g/ml decrements) [41]. EVs are usually concentrated in the 1.1-1.2 g/ml sucrose density layer (could vary depending on the EV cargo), whereas other impurities would sink towards the higher sucrose density gradient layers [8]. The second gradient solution is iodixanol, and is more preferable over sucrose as the former has the ability to form isosmotic solutions at different densities that preserve EV integrity and therefore biological activity [42]. As our objective was to analyse HLSC-EVs for enrichment of ASS gene and protein and to test their biological activity, we applied a modified version of the iodixanol floating protocol set by Kowal et al., [43]. The pellets of HLSC-EVs obtained were directly resuspended in 60% iodixanol in an ultracentrifuge tube with subsequent gradients of 30%, 15%, and 5% being layered over and ultracentrifuged at 350,000 g (1 h, 4°C). Overlaying the EV pellet with the various gradients of iodixanol allows the EVs to float upwards towards the lower gradients whereas; protein aggregates and other contaminants remain below [43]. On comparing the EVs obtained from the various different gradients with the non-purified 100K EV pellet, we observed that the 15% gradient had the most number of EVs and were positive for: the typical EV marker CD63, the markers for HLSCs, as well as the ASS1 enzyme protein [9]. Furthermore, these populations of EVs were found to be biologically active as observed through the restoration of ASS1 function in ASS1 mutated HLSCs following treatment.

Conclusion

Through these studies we can conclude that HLSC derived EVs are enriched with a complex of biologically active molecules ranging from nucleic acids, to proteins and growth factors and have the ability to influence various pathways both at a molecular and protein level in the target cells. It is due to these properties that they exhibit a broad spectrum therapeutic effect that can be applied in multiple disease pathologies [28,44]. Although various studies have shown the potential therapeutic application of stem cell-derived EVs in a wide spectrum of disease models, our study with HLSC-EVs and ASS1 remains the only one to exist till date in the field of urea cycle disorders. We demonstrated for the first time the ability of HLSC-EVs to restore ASS1 function in ASS1 mutated hepatocytes through the transfer of ASS1 mRNA and protein. Before HLSC-EVs can be introduced to a clinical setting for urea cycle disorders, several aspects have to be addressed such as GMP upscale production, characterization, pharmacokinetics, pharmacodynamics, toxicity and host immune reaction to HLSC-EVs.

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Competing Interest

The authors declare that they have no competing interests. GC is member of the Scientific Advisory Board of Unicyte A.G. MBHS and GC are named as inventor in patents related to the regenerative effects of human liver stem cells.

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