DIFFERENTIATION & HOMING OF BONE MARROW-DERIVED MESSCHYMAL STEM CELLS IN THE LIVER SCHISTOSOMA INFECTED MICE

By
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Abstract

Schistosomiasis is an endemic parasitic infective disease that remains widespread in several countries. This work aimed to evaluate the curative influence of bone marrow derived mesenchymal stem cells (BM-MSC) engraftment in liver of schistosomiasis chronic infected mice. 30 Swiss albino mice divided into 3 groups of 10 mice each. G1: normal healthy group, G2: infected with Schistosoma mansoni cercariae subcutaneously and G3: infected and transplanted with BM-MSC on their 8th week post infection by intravenous injection. All mice were sacrificed 4 weeks post-transplantation. Serum was collected for assessment of albumin, ALT & AST levels. Engraftment of BM-MSC was assessed by labeling with PKH26. Histopathological and ultrastructural studies were carried out. Expression of capase-3 & IL-1β was a marker of apoptosis and inflammation respectively were measured by quantitative RT-PCR. Homing of transplanted BM-MSC within the injured liver was confirmed by fluorescent microscopic examination. MSC labeled with PKH26 were recovered in liver. Histopathological and electron microscopic examinations of liver showed differentiation of transplanted BM-MSC into hepatocyte-like cells. Liver function was improved confirmed by elevation of serum albumin and decrease of ALT & AST in G3. Real-time PCR revealed that BM-MSC inhibited capase-3 and suppress interleukin-1β mRNA expression in damaged liver tissues. MSC-treated group had the least apoptosis cells and inflammatory cells. Transplantation of BM-MSCs has therapeutic anti-apoptotic anti-inflammatory mechanisms effect in chronic liver disease.

Key words: Albino mice, S. mansoni, Liver, Mesenchymal stem cells.

Introduction

Schistosomiasis is an endemic parasitic infective disease prevalent in several countries. In spite of the major efforts to control schistosomiasis with considerable reduction in morbidity and mortality, it continue to spread to new areas (Patz et al, 2000). To elucidate mechanisms involved in fibrogenic process of Schistosoma mansoni chronic infection experimental models of hepatic fibrosis are used (Olivera et al, 2008). Along with schistosomiasis, hepatopathies caused by drugs, metabolic & autoimmune diseases, alcohol, viral hepatitis in addition to congenital anomalies considered as leading causes of hepatic fibrosis (Olivera et al, 2008). In Egypt, co-infection of viral hepatitis with S. mansoni considered as one of the leading causes of chronic hepatic disease (Halim et al, 1999). In chronic liver disease new therapeutic strategies are of great interest to minimize damage of hepatic fibrogenesis (Krause et al, 2001).

Mesenchymal stem cells (MSCs) were emerged as an effective advance in treatment of different diseases with minimal invasive procedures and few complications (Salama et al, 2010). MSCs are multipotent, capable of differentiating into functional hepatocytes. They are effective in liver structure regeneration together with recovery of liver functions (Ishii et al, 2008). Apoptosis of hepatocyte, an outcome of chronic hepatic disease, and one of the hall marks of hepatic fibrosis, can lead to a fibrous scar (Friedman, 2008). Caspases are cluster of enzymes participate in regulation of the apoptotic process with the subsequent classical features. Triggering of Caspase-3 in response to variable apoptotic stimuli activate endonuclease and prompt DNA fragmentation (Luo et al, 2010). The damaged
hepatocytes alongside with the involved inflammatory cells release mediators as IL-6, IL-10, IL-13& IL-1β (Nasir et al, 2013). IL-1β, a potent inflammatory cytokine, considered as an important constituent of the innate immune response (Yang et al, 2003), that involved in various cellular functions such as proliferation, activation and differentiation (Dinarello, 1996).

The present study aimed to highlight homing & engraftment abilities of transplanted bone marrow derived MSCs (BM-MSCs) in murine schistosomiasis as a future anti-fibrotic anti-apoptotic therapeutic evolution. Engraftment of transplanted stem cells was assessed by labeling with PKH26 (Paul Karl Horan 26).

**Materials and Methods**

Experimental animals: Thirty female Swiss Albino mice, 7 weeks old weighing about 25 gm were maintained at appropriate environmental conditions in the Schistosome Biological Material Supply Center (SBSC), Theodor Bilhariz Research Institute (TBRI), Giza. Animal protocols were followed in agreement with the international guidelines for animal research and were permitted by the TBRI’s animal research team.

Animals infection: Each mice was infected by subcutaneous injection of 80±10 cercariae. Mice were classified into 3 main groups of 10 mice each. GI: healthy mice (control group). GII: *S. mansoni* infected mice injected with intravenous phosphate buffered saline (PBS). GIII: infected mice with *S. mansoni* cercariae and received MSCs. After twelve weeks post-infection, blood was obtained from the retro-orbital vein, then all mice groups were sacrificed and livers were processed for paraffin sections and electron microscopic (EM) examination (Philips TEM 208s).

BM-derived MSC Preparation: BM-MSCs were obtained from Department of Biochemistry, Kasr Al-Ainy Medical Faculty.

Donor marrow was obtained by flushing of femur & tibiae of 6-week-old male mice with Dulbecco’s Modified Eagle’s Medium (DMEM) fortified with 10% fetal bovine serum. Nucleated cells were separated according to a density gradient, and then suspended in complete culture medium after adding 1% penicillin-streptomycin to it. Incubation of cells were in 5% humidified CO2 for 12-14 days at 37°C till development of enormous colonies (80-90% confluence). Phosphate-buffered saline (PBS) was used for culture wash then the culture released with trypsin (0.25%) in 1mL ethylene diamine-tetra acetic acid (EDTA for 5 min at 37°C). Finally, centrifugation was done and the cells were suspended with the serum-supplemented medium and incubated in 50cm2 culture flask (Falcon) (Al Hadlaq and Mao, 2004). Characterization of MSCs in the culture was recognized by the fusiform shape and their adhesiveness (Rochefort et al, 2005)

Concerning labeling, MSCs were collected throughout the 2nd passage and marked with PKH-26 dye (Haas et al, 2000). After centrifugation, cells washed two times in a serum free medium, after that they pelleted and resuspended in dye solution.

Cells transplantation: Was done by injection of male non-fractionated BM-MSCs intravenously in infected female mice on 8th week after *Schistosoma* infection in the dose of 1x106 cells/mouse suspended in 0.3ml (PBS).

Histopathological and ultrastructural studies were carried out. Liver function tests were assayed. Expression of caspase-3 and IL-1β as markers of apoptosis and inflammation were measured by quantitative RT-PCR.

Liver function parameters: Level of Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin were determined spectrophotometrically in the serum by an automatic analyzer using conventional laboratory methods.

Liver histopathology: After fixation in buffered formalin (10%), collected tissue were dehydrated then processed for paraffin sections. After that, the samples were deparaffin-
ised then stained by hematoxylin & eosin (H & E). Hepatic inflammation graded (Knodell et al., 1981).

Transmission electron microscope (TEM): Small liver specimens (1mm³) were fixed in 2.5% glutaraldehyde solution followed by 1%. Osmium tetroxide, dried up then placed in epoxy resin. Collected ultrathin sections on copper grids were stained with uranyl acetate & lead citrate. Lastly, they examined and photographed under electron microscope (Philips TEM 208s).

Quantitative RT-PCR analysis for mice caspase-3 & IL-1β: Liver of mice, total RNA was isolated by RNeasy Mini Kit including DNase I digestion according to manufacturer’s guidelines (Qiagen, #74104). Quantiscript reverse transcriptase was used to reverse transcribe RNA. After reverse transcription a Rotor-Gene (Q2plex, Qiagen) was used to achieve quantitative real-time PCR analysis using SYBR green master mix (Roche Diagnostics). Primer 3 web-based tool used to design Primers by (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) depending on the published mice sequence. The subsequent primers were used for the study: mice caspase-3, 5’-GACCATTACATGGGAGCAAGT-3’ and 5’-CCTTCA TCACCATGGCTTAGA3; miceIL-1β, 5’GAGAACACCGACAAAAATA-3’ & 5’-TGGGAACTCTCGACAGCTCAA AC-3’; mice GAP DH, Gen-Bank Accession No. M32599; 5’-TGTGTCGTGCTGGAT CTGA -3’; and 5- CCTGCTTCACCACCTT CTTGA -3’. Mice gene expression was standardized to mice GAPDH. Thermal cycling consist of a primary step at 95℃ for 10min then 40 cycles of amplification (denaturation step at 95℃ for 15s, annealing at 60℃ for 1min) in accordance with the manufacturer’s guide. The critical thresholds (Ct) of the targeted gene were standardized with GAPDH quantities (Ct) by 2ΔΔCt method (Livak and Schmittgen, 2001). Calculation of 2ΔΔCt determined the fold change in gene expression in the treated groups with respect to controls.

Statistical analysis: One-way analysis of variance (ANOVA) was used to assess the influence of mscson serum levels followed by least significant difference (lsd) test as post hoc test. Analysis was performed via statistical package for social sciences version 22.0 (Ibm Corp., Armonk, NY, USA). Results were reported in means ± SD. p value < 0.05 was used to indicate significance

Results

Confirmation of homing of BM-MSCs: Fluorescent microscope was used to distinguish PKH-26 labeled cells appeared as bright dots in liver tissue in MSC treated group (Fig.1A).

Liver histopathology: H & E stained liver sections of GIII showed marked regenerative changes as well as decrease in apoptotic changes in hepatocytes and inflammatory cellular infiltrates compared to the infected control group. Histopathological studies were focused on the homing of transplanted BM-MSCs inside the liver of recipient females that exhibited presence of small dispersed cells resembling hepatocyte, with small rounded central nuclei (Fig.1B), a marked proliferation of primitive and incomplete small groups of bile ducts was observed as well as an increase of recently formed tiny blood vessels in liver parenchyma (Fig.1C). Regarding inflammatory reactions and apoptosis in GIII, there was marked inflammatory cellular infiltrate (grade IV) and mild induced apoptosis within hepatocytes (Fig.1D).

Electron microscopic examination of liver sections in GIII confirmed the regenerative histopathological findings. The peripheral parts of the granuloma revealed the presence of newly formed hepatocytes, in cells opposite the sinusoids as well as the perportal area. These cells were distinguished into: a rather small immature cells with big ovoid nuclei, increased nuclear cytoplasmic ratio with marginal chromatin plus moderate sized cells. Also, there were mature cells with the endoplasmic reticulum and mitochondria upcoming the morphological structures of
liver cells (Fig. 2A). These cells were not detected in the control infected and healthy groups. Mature hepatocytes with features of regeneration were observed as well. Cells with noticeable nucleoli, irregular nuclear membrane and proliferated rough endoplasmic reticulum encircling mitochondria also were detected (Fig. 2B). A newly formed bile ductile and newly formed blood vessels were in infected and transplanted group (Fig. 2C).

Liver function parameters: There was a significant increase in serum albumin in group III in comparison to infected controls (P<0.0001) (Tab. 1). Liver enzymes (ALT &AST) were significantly reduced in GIII comparable to the infected control group (P<0.0001, 0.0004) respectively. A high statistically significant difference in LSD between healthy control versus control infected and the control infected comparable to treated group at all liver functions investigations. But, without significant difference amongst healthy control and treated group at all of those parameters.

Real-time PCR: There was a significant low expression of apoptosis and inflammation related genes, IL-1β as well as caspase-3 in group III when compared to control infected group (Tab. 2).

Table 1: Mean values of ALT, AST and albumin in serum level of groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Albumin (gm/dl)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
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<tbody>
<tr>
<td>Control healthy</td>
<td>3.90±0.29 b</td>
<td>30.2±4.59 b</td>
<td>60.2±12.68 b</td>
</tr>
<tr>
<td>Infected control</td>
<td>2.65±0.71 b</td>
<td>77.2±32.15</td>
<td>156.3±88.76 b</td>
</tr>
<tr>
<td>Infected + MSCs</td>
<td>3.4±0.59 b</td>
<td>36±10.14 b</td>
<td>65.7±11.98 b</td>
</tr>
<tr>
<td>F- value</td>
<td>12.77</td>
<td>17.02</td>
<td>10.68</td>
</tr>
<tr>
<td>P-value</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td>LSD</td>
<td>0.51</td>
<td>18.02</td>
<td>47.93</td>
</tr>
</tbody>
</table>

*a,b=significant difference.*P<0.05, F = Test for ANOVA.

Table 2: Effect of BM-MSCs transplantation on apoptosis and inflammation related genes expression.

<table>
<thead>
<tr>
<th>GENE</th>
<th>Caspase-3</th>
<th>IL1β</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1±0.18 b</td>
<td>1.1±0.16 b</td>
</tr>
<tr>
<td>Infected</td>
<td>4.23±0.23 b</td>
<td>4.97±0.34 b</td>
</tr>
<tr>
<td>Infected + MSCs</td>
<td>1.61±0.11 b</td>
<td>1.94±0.27 b</td>
</tr>
</tbody>
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Discussion

Liver fibrosis complicates chronic liver diseases of different etiologies. In Egypt, co-infection of viral hepatitis and S. mansoni considered as the most important cause of chronic hepatic illness (Halim et al, 1999). S. mansoni infection reflects a good experimental model of chronic hepatic disease and fibrosis. Regression of fibrosis occurs spontaneously as soon as the stimulus for liver damage is removed (Olivera et al, 2008). It was crucial to find novel effective therapeutic methods as a challenge aimed to improvement of the damage produced by fibrogenesis in chronic hepatic diseases. Stem cell therapy had supported tissue regeneration with minimal invasive procedures and few complications (Lorenzini et al, 2008). BM-MSCs are clever in homing to specific tissues with the ability to differentiate and stimulate local repair response (Fu and Li, 2009).

The current research determined the effect of murine BM-MSCs transplantation on diseased liver as an experimental in vivo trial of hepatocyte differentiation from BM-MSCs. After transplantation, the fluorescent microscope detected PKH-26 labeled MSCs incorporated in the liver tissues. Some authors reported the homing of inoculated MSCs in damaged liver (Anan et al, 2016; Fikry et al, 2016).

The MSC can protect against S. mansoni-induced liver fibrosis through alteration of liver microenvironment at engraftment site. Numerous cytokines and growth factors as hepatic growth factor could be secreted by MSC (Matsuda-Haashii et al, 2004). It exhibited anti-apoptotic effect on hepatocytes as well as having a crucial role in hepatic.
regeneration (Ueki, 1999) as well as nerve growth factor (NGF) (Li et al., 2002). In the present study, there were newly formed hepatocytes at the edge of the granuloma in hepatocytes facing the sinusoids and similarly around periportal areas. The newly formed blood vessels and the newly formed bile ductules were seen in infected transplanted group. The oval cells count raised by 3-4 folds in liver of infected mice following MSCs transplantation denoting new hepatocytes formation (Oliveira et al., 2008). Similar findings were recorded by other authors (Elkhaff et al., 2010a, Shizhu et al., 2012; Yu et al., 2012). The origin from the injected MSCs or from the liver itself regenerated hepatocytes. The noted improvement after the MSCs inoculation; could attributed their soluble growth factors secretion more than their transdifferentiation to liver cells (Alison et al., 2009).

The effect of MSCs based on primary hepatic illness. It either substitutes the injured tissue or enhances hepatic regeneration via antiapoptotic, anti-inflammatory as well as their proliferative action (Christ and Stock, 2012). Histopathological data of newly formed blood vessels was confirmed by TEM (Elkhaff et al., 2010b). In the present study, liver functions were improved by significant elevation of serum albumin level and suppression of transaminase activity ALT, AST in mice transplanted with MSCs as compared with control infected group. Consistent with current results, infused BM-MSCs were confined to liver with subsequent improvement of liver functions (Abdel Aziz et al., 2012). BM-MSCs transplantation in mice with fibrosed liver induced by administration of the chemokine ligand 4 (CCL4), exhibited significant decrease in hepatic fibrosis evaluated by enhancement of liver functions, such as levels of albumin, bilirubin, as well as prothrombin time (Sakaida et al., 2004). Oyagi et al. (2006), Wu et al. (2005) and Luk et al. (2005) reported that autologous transplanted BM-MSCs could distinguish to liver cells, reestablish albumin level and inhibit transaminase action and hepatic fibrosis in experimentally injured liver. S. mansoni infection resulted in hepatocellular injury; enzymes were released from the affected liver cells into the blood stream (Oyagi et al., 2006). AST, ALT and albumin levels proved to be indicators to the schistosomiasis pathological changes. Aminotransferases are indicators for toxicity of the cells. Their elevation provided an evidence for S. mansoni cell toxicity. So, the current study revealed that the induced inflammatory reactions in the liver of infected mice were confirmed by hepatic dysfunctions shown by increased AST and ALT levels. This was attributed to egg deposition with liver cell injury. Therefore, levels of transaminase revealed a close association with cellular necrosis beside increased permeability to release the enzymes to the circulation (El-Rigal and Hetta, 2006). Reduced serum albumin level reflects an impairment of protein synthesis caused by destruction of parenchymal liver cells (Abdel-Rahium et al., 1990). There was a noted significant decrease in serum albumin caused by S. mansoni infection (Mahmoud et al., 2002). It was reported that the elevation of serum albumin and reduction in ALT & AST levels express a stabilization of the hepatic function induced by treatment with MSCs. So, the reversal of liver functions in fibrotic liver either caused by inhibition of parenchymal cells apoptosis or inhibition of proliferation and infiltration of inflammatory cells (Pulavendran et al., 2010).

The quantitative RT-PCR showed that there was a significant the low expression of caspase-3 and IL-1β (apoptosis & inflammation-related genes) in the BM-MSCs-treated group comparable with controls. These findings evidently demonstrate that BM-MSCs can improve hepatic dysfunction in vivo by reducing inflammation; apoptosis also, promoting proliferation as well as recovery (reinforced by ALT & AST normal levels). The results showed that MSCs were innovative cells that could repair liver damage as well
as dysfunction. However; the mechanisms of hepatocytes differentiation as well as hepatic function restoration are indistinct. It was found that BM-MSCs secrete hepatocyte growth factor (HGF) alongside with suppression of inflammation following transplantation to CCL4-injured mice (Oyagi et al, 2006). HGF has the capability to stimulate hepatic differentiation in addition to decrease the hepatocytes death (Matsuda et al, 1995).

**Conclusion**

The outcome results showed that the BM-MSCs have a potential therapeutic effect on *Schistosoma mansoni*-induced fibrotic liver model by capacity to differentiate into the hepatocytes, and, via anti-apoptotic and anti-inflammatory mechanisms. So, it could be used as a new therapeutic line in chronic liver disease.

**Conflict of interest:** The authors declared that they neither have conflict of interests nor received fund.

**References**

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Explanation of figures

Fig.1: Liver section from infected transplanted group. A- PKH26 marked cells seem like bright dots in hepatic tissue (Fluorescent Microscope, X 200). B-new hepatocytes like (black arrow; H&E, x400) C-proliferating bile ducts and congested blood vessels (black arrow; H&E,x400) D-mild inflammatory infiltrate (yellow arrow) and mild apoptosis (black arrow; H&E X100).

Fig.2: Electronmicrograph of infected transplanted group. A- Newly formed small sized hepatocyte (h) with oval shaped nucleus (n) surrounded by developing cytoplasm. An adjacent regenerating hepatocyte with larger nucleus (N), well developed cytoplasm (C) with abundant mitochondria (M). Nucleus chromatin formed of euchromatin with prominent nucleolus. B- Signs of regeneration, increase in rough endoplasmic reticulum (rER) encircling proliferated mitochondria (M). Nuclear chromatin formed mainly of euchromatin with prominent paracentral nucleolus (n) and segregation of its constituents (arrow). C- Newly formed blood vessel (blue arrow) with luminal RBC, an adjacent progenitor cell (P) surrounded by few cytoplasmic organelles (C),macrophage (m) with cytoplasmic Try lysosomes (red arrow). D- Area of inflammatory cellular infiltrates represented by newly formed blood vessels (red arrows), progenitor cells (P), macrophage (m) with cytoplasmic lysosomes (blue arrow).