Regenerative Capacity of Transplanted Mesenchymal Versus Mononuclear Stem Cells in Carbon Tetrachloride Induced Liver Injury in Rats

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Abstract

Aim of the Work: Our study aimed at investigation of the capacity of allogenic bone marrow derived Mesenchymal Stem Cells (MSCs) versus Mononuclear Stem Cells (MNSCs) to regenerate liver in carbon tetrachloride (CCl4) animal model of liver fibrosis. The role of regulatory T cells (CD4+ & IL2α/CD25+) in controlling homing of the transplanted stem cells was also evaluated.

Animal: After induction of liver fibrosis for 8 weeks, the survived female rats were divided into positive control group (CO+) and two groups treated with MSCs and MNSCs isolated from Sprague Dawley male rats. To provide independent evidence of homing of transplanted male donor cells, real time PCR was done to detect sex determining region on the Y chromosome (Sry) gene. N-terminal Procollagen III (PII-INP), liver function test (alanine aminotransferase ALT, aspartate aminotransferase AST, Gamma Glutamyl Transferase GGT and Albumin Alb) were assessed. Histopathological examination of livers was done to evaluate the regeneration of hepatocytes and improvement of fibrosis. Expression of CD4 and IL2α/CD25 was also evaluated.

Results: In group received MSCs, Sry gene was detected in 100% of rats and in 84.6% of MNSCs treated group. Serum concentration of PIIINP showed good improvement in group treated with MSCs (160.938 ± 33.741), compared to MNSCs (190.582 ± 27.842) after 8 weeks of transplantation. Compared to CO+, liver functions (ALT, AST and GGT) were improved in treated groups in time dependent fashion. The group received MSCs showed good improvement compared to MNSCs treated group. After 8 weeks of MSCs transplantation 42.7% of rats showed resolution of fibrosis with variable improvement of hepatitis activity. 57.1% of rats treated with MNSC for 8 weeks showed improvement of the hepatitis to milder degree of activity and resolutions of fibrosis. Cytoplasmic expression of CD4 and IL2α/CD25 was positive in most of animals which received MSCs and MNSCs. There was significant correlation between CD4 and IL2α/CD25 in both groups.

Conclusion: MSCs are capable of regeneration of hepatocytes and restoration of hepatic architecture compared to MNSCs. Expansion of regulatory T cells played an important role in homing of MSCs and improving the immunomodulatory properties of MSCs.

Key Words: Bone marrow stem cells – Mesenchymal stem cells – Mononuclear stem cells – Regulatory T cells – Tregs – Liver fibrosis.

Introduction

MOST of the disease burden associated with liver fibrosis and cirrhosis is the development of liver failure, portal hypertension, hepatocellular carcinoma and represent a major cause of morbidity and mortality worldwide. Approximately 20% of HCV infected patients develop complications [1].

Since liver transplantation is the only available current therapy for end stage liver failure and there is an ever increasing shortage of donor livers, stem cell based therapy has received attention as a possible alternative to liver transplantation [2].

In recent years, liver related stem cells have become a hot spot of research. Well known endogenous liver stem cell markers can be expressed by bone marrow derived stem cells [3]. Mesenchymal Stem Cells (MSCs) are reported to have the capacity to differentiate into hepatocytes in vitro and in vivo [4]. Transplantation of MNSCs has the ability to regenerate liver and reduce fibrosis in regenerating liver [5].

One of the obstacles against the application of allogenic stem cell therapy is graft rejection. Regulatory T cells have a critical role in maintaining immune tolerance and prevent transplant rejection [6]. It was reported that MSCs increase the percentage of CD4+CD25+ regulatory T cells (Tregs) on coculture with T lymphocytes, suggesting that these regulatory cells may amplify MSCs mediated immunosuppressive effects [7,8]. To the best of our
knowledge the role of Tregs in homing of MNSCs was not clear.

In the current study we compared the efficacy of allogenic transplanted bone marrow derived stem cells to regenerate liver function and structure in animal model of liver fibrosis. The role of regulatory T cells in homing of the transplanted stem cells was also evaluated. We extended our work to cover different types of bone marrow derived stem cells (mesenchymal, hematopoietic and mononuclear) to clarify the impact of Tregs transplanted cell homing.

Material and Methods

Study design: It is an experimental multifaceted pre-post interventional study. The study was carried out in the Pathology Department in collaboration with the Stem Cell Research Unit in the Physiology Department during 2014, Faculty of Medicine, Suez Canal University.

Animals: Female Sprague Dawley rats aged 7-8 weeks, weighing 150-250gm, were injected by CCl4 at a dose of 0.2mL/100g body weight of 40 mL/L CCl4 dissolved in corn oil [4]. After 8 week of induction of liver fibrosis, the survived rats were randomly divided into CO+, MSCs and MNSCs treated groups, each group contained 13 rats.

MSCs preparation (plastic adherence): Bone marrow was flushed from the tibia and the femur of male Sprague Dawley rats. The harvested cells were seeded at the culture plate in Modified Eagle’s Medium (MEM) medium supplemented with 100 ml/l FBS, 100 µ/ml Penicillin, 100 µ/ml Streptomycin, 50U/ml Gentamycin and 2mM/l Glutamine (Lonza, Belgium). The plate was incubated in 37ºC with 5% CO2 environment. MSCs were preferentially attached to the polysytrene surface.

MNSCs preparation: The harvested bone marrow cells were layered carefully over Ficoll Paque (GE Healthcare Amersham Biosciences corp, USA). Cells at the interphase layer were carefully collected. Cell suspension was prepared for transplantation.

The prepared cell suspension contained MSCs and MNSCs were injected into the tail vein of female rats in a dose of (1 x 10^6 cells/rat).

Marker of liver fibrosis and assessment of liver function: Pre and post therapeutic assessment of liver fibrotic marker and biochemical changes of liver functions were done (Enzym (Glory Science co., Ltd, USA). Automated biochemical assay of ALT, AST and GGT was done (Chema Diagnostica, UK), in addition to serum albumin (Human Gesellschaften für biochemica und diagnostic mbH, Germany).

Detection of male donor stem cells: After 6 and 8 weeks of stem cell transplantation, rats were sacrificed; liver samples were immediately frozen in liquid nitrogen and preserved at 80. Genomic DNA was purified from the liver tissue (DNeasy Blood & Tissue Kit, Qiagen, USA). Sry gene was detected by Real Time Polymerase Chain Reaction (RT-PCR). Sry gene amplification sequence was (forward 5’-catacgaggttaagtcc-3’, reverse 5’-atacaggtagttgtgcc-3’) (Qiagen, USA).

Histopathological evaluation of liver samples: Liver biopsies were preserved in 10% neutral buffered formaldehyde solution. Serial 5 µm sections of livers were stained with H & E and Masson trichrom. The grade of necroinflammatory changes and the stage of liver fibrosis were assessed according to the metavir scoring system [9].

Immunohistochemistry; expression of CD4 and IL2α/CD25: Frozen sections were tested with the antibody aganist CD4 and IL2α/CD25 (Abcam, USA). The slides were evaluated according to Allred Scoring Guideline [10]. Computerized image analysis of cells was done using ZEN 2012, SP2, blue edition software (Carl Ziess, Germany®).

Statistical analysis:

Values are presented in mean and standard deviation. Significant comparisons were determined using one way ANOVA test. The significance of differences among proportions was evaluated by Pearson’s Chi square test. All analyses were performed using the statistical package for social science, SPSS, version 18, 2009. Results were considered significance at p-value <0.05.

Results

The mortality rate was 29% after CCl4 induction of liver fibrosis. The survived rats divided into CO+, MSCS and MNSCs treated groups, each included 13 rats. Liver fibrosis and hepatitis activity were noted in sampled rats with more predominance after 8 weeks of induction as evident by marked elevation of PIIINP (217.56±56.543). Compared to the baseline data, liver enzymes (ALT, AST and GGT) showed marked elevation. Albumin level remained within normal ranges. Liver biopsies showed marked hepatitis activity (A3) and bridging fibrosis with pseudo lobules (F3) Fig. (1).

Sry gene has been detected in the male cells transplanted into female rats in 100% of the group.
received MSCs and in 84.6% of the group treated by MNSCs.

Serum concentration of PIIINP showed marked elevation in CO+ group (370.89±70.540) compared to treated groups. MSCs treated group showed good improvement of PIIINP (160.938 ±33.741) compared to the group received MNSCs (190.582 ±27.842). Comparing the mean concentration of PIIINP among the studied groups and the CO+ group after 6 and 8 weeks of therapy was statistically insignificance (Table 1).

The mean levels of liver enzymes (AST and ALT) showed continuous elevation in CO+ group. After 8 weeks of transplantation, the group received MSCs showed improvement of ALT (74±41.46) and AST (117.85±52.52) compared to ALT (86.8 ± 16.6) and AST (179±69.5) in MNSCs treated group. There was significant difference between the mean levels of ALT and AST of group treated with MSCs for 6 and 8 week. The mean level of GGT showed good improvement in group received MSCs versus the CO+ and MNSCs group. Albumin level remained within the normal ranges (Table 1).

Necroinflammatory changes and liver fibrosis were persistent in CO+ group. Groups received bone marrow derived stem cells showed regression of fibrosis with improvement of hepatitis activity Figs. (2,3). After 8 weeks of stem cell transplantation; liver fibrosis showed complete resolution in 42.9% of the group received MSCs and in 57.1% in MNSCs treated group. The correlation between liver fibrosis in both groups did not reach the significance. There was statistically insignificant correlation between the studied groups regarding histopathological examination (Table 2).

Cytoplasmic expression of T regulatory cells (CD4 & IL2α/CD25) was evaluated after rat bone marrow derived stem cell transplantation. Positive control group showed negative expression of T regulatory cells (CD4 & IL2α/CD25). After 6 weeks of MSCs transplantation, most of the studied cases were negative for regulatory T cells expression. CD4 positive cells were expressed in 42.8% of MSCs after 8 weeks of treatment, proportional scores was 1 (PS=1). Most of rats received MSCs showed positive expression of IL2α/CD25 with moderate intensity. IL2α/CD25 was expressed in 42.8% of cases (PS=3) Fig. (4). There was statistically significant correlation (p=0.025) between the expression of CD4 and IL2α/CD25 after 6 weeks of MSC transplantation.

Most of rats which received MNSCs showed positive expression of regulatory T cells with variable proportional scoring. Study of the correlation between CD4 and IL2α/CD25 expression was statistically significant after 6 weeks of MNSCs transplantation. Positive cytoplasmic expression of CD4 in group received MNSCs was noted Fig. (5). 42.8% of cases showed positive expression of CD4 cells with PS=3 (Table 3).

Table (1): Mean serum level of PIIINP and liver function tests after 6 and 8 weeks of bone marrow derived stem cell transplantation.

<table>
<thead>
<tr>
<th>Post therapy</th>
<th>Groups</th>
<th>PIIINP ng/dl</th>
<th>ALT U/l</th>
<th>AST U/l</th>
<th>GGT U/l</th>
<th>Alb mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 weeks</td>
<td>CO+</td>
<td>308.30±61.66</td>
<td>99.14±41.62</td>
<td>223.29±65.03</td>
<td>92.14±43.01</td>
<td>3.21±1.43</td>
</tr>
<tr>
<td></td>
<td>MSCs</td>
<td>167.96±39.73</td>
<td>97.85±30.85*</td>
<td>196.28±27.76*</td>
<td>56.71±15.87</td>
<td>3.57±0.11</td>
</tr>
<tr>
<td></td>
<td>MNSCs</td>
<td>223.89±29.729</td>
<td>97.71±13.31</td>
<td>194.42±23.16</td>
<td>72.7±22.89</td>
<td>3.50±0.33</td>
</tr>
<tr>
<td>8 weeks</td>
<td>CO+</td>
<td>370.89±70.54</td>
<td>99.57±41.62</td>
<td>258.29±24.4</td>
<td>92.14±43.19</td>
<td>4.26±0.76</td>
</tr>
<tr>
<td></td>
<td>MSCs</td>
<td>160.94±33.74</td>
<td>74±41.46*</td>
<td>117.8±52.52*</td>
<td>50.85±32.02</td>
<td>3.38±1.5</td>
</tr>
<tr>
<td></td>
<td>MNSCs</td>
<td>190.582±27.842</td>
<td>86.8±16.6</td>
<td>179±69.5</td>
<td>132±60.42</td>
<td>4.60±3.25</td>
</tr>
</tbody>
</table>

*: p<0.05.

Table (2): Post therapeutic evaluation of hepatitis activity and stages of fibrosis.

<table>
<thead>
<tr>
<th>Post therapy</th>
<th>Groups</th>
<th>Grade of activity (A)</th>
<th>Stage of fibrosis (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A1</td>
<td>A2</td>
</tr>
<tr>
<td>6 weeks</td>
<td>CO+</td>
<td>66.6%</td>
<td>16.7%</td>
</tr>
<tr>
<td></td>
<td>MSCs</td>
<td>85.7%</td>
<td>14.3%</td>
</tr>
<tr>
<td></td>
<td>MNSCs</td>
<td>83.3%</td>
<td>16.7%</td>
</tr>
<tr>
<td>8 weeks</td>
<td>CO+</td>
<td>57.1%</td>
<td>42.9%</td>
</tr>
<tr>
<td></td>
<td>MSCs</td>
<td>28.6%</td>
<td>71.4%</td>
</tr>
<tr>
<td></td>
<td>MNSCs</td>
<td>57.1%</td>
<td>42.9%</td>
</tr>
</tbody>
</table>
Table (3): Cytoplasmic expression of CD4 and IL2a/CD25 in MSCs and MNSCs treated groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>PS=0</th>
<th>PS=1</th>
<th>PS=2</th>
<th>PS=3</th>
<th>PS=0</th>
<th>PS=1</th>
<th>PS=2</th>
<th>PS=3</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSCs 6 wk</td>
<td>50%</td>
<td>33.3%</td>
<td>16.7%</td>
<td>0%</td>
<td>33.3%</td>
<td>50%</td>
<td>16.7%</td>
<td>0%</td>
<td>0.025*</td>
</tr>
<tr>
<td>MSCs 8 wk</td>
<td>14.3%</td>
<td>42.8%</td>
<td>14.3%</td>
<td>28.6%</td>
<td>14.3%</td>
<td>28.6%</td>
<td>14.3%</td>
<td>42.8%</td>
<td>0.333</td>
</tr>
<tr>
<td>MNSCs 6 wk</td>
<td>16.7%</td>
<td>33.3%</td>
<td>33.3%</td>
<td>16.7%</td>
<td>16.7%</td>
<td>33.3%</td>
<td>33.3%</td>
<td>16.7%</td>
<td>0.047*</td>
</tr>
<tr>
<td>MNSCs 8 wk</td>
<td>14.3%</td>
<td>14.3%</td>
<td>28.6%</td>
<td>42.8%</td>
<td>14.3%</td>
<td>14.3%</td>
<td>24.8%</td>
<td>28.6%</td>
<td>0.243</td>
</tr>
</tbody>
</table>

Discussion

The efficacy of allogenic transplanted bone marrow derived MSCs and MNSCs to regenerate liver function and structure in animal model of liver fibrosis was studied. The role of Tregs in homing of the transplanted stem cells was also evaluated.

To provide independent evidence of homing of the transplanted male donor cells in livers of female recipient, Sry gene was detected in 100% of animals treated with MSCs. Two out of 13 cases treated with MNSCs were negative for Sry gene. This may reflect rejection of the transplanted cells.
Abdel Aziz et al., [11] stated that groups treated with MSCs showed marked regression of fibrotic process indicated by lowest level of PIIINP. This agreed to our data as the group received MSCs showed good improvement of mean serum level of PIIINP compared to CO+ and MNSCs groups.

The mean levels of ALT and AST were continuously elevated in the positive control group during the experimental period. After bone marrow stem cells transplantation the mean levels of ALT and of AST gradually decreased in a time dependent fashion. The obtained results revealed proliferation of hepatocytes with restoration of hepatic function after stem cell transplantation. The group that received MSCs showed improvement of the mean level of ALT and AST compared to the groups received MNSCs. These findings come in accordance with that revealed the potential of MSCs transplantation to regenerate liver rather than hematopoietic and mononuclear stem cells [12].

We assessed GGT level to get an overview about the efficacy of the transplanted stem cells in regeneration of the biliary epithelium. There was apparent improvement of GGT in group received MSCs compared to other groups after 6 weeks (56.71 ± 15.87) and 8 weeks (50.85±32.02) of transplantation. This may indicate the capacity of MSCs to proliferate and regenerate the biliary epithelium. Our results come in accordance with the study that reported decreased serum GGT after mesenchymal stem cell transplantation, concluded the recovery of liver inflammation after induction of biliary cirrhosis in animal model [13,14].

Our study revealed normal albumin levels in all groups with insignificant difference of the mean albumin levels between the studied groups. This meant that we did not reach the state of chronicity and impairment of albumin level.

In positive control group the histopathological examination results came in accordance with PIIINP and biochemical results, pointing out to persistence of necroinflammatory changes and fibrosis after stop of CCl4 administration. 50% of rats in CO+ group showed regression of fibrosis to milder degree may be explained by spontaneous regeneration of fibrotic liver as previously reported [15].

Homed transplanted MSCs can differentiate and generate hepatocytes as confirmed by histological and biochemical improvement. On the other hand, MSCs have the capacity to reverse fibrosis as revealed by improvement of PIIINP concentration. Our data matched to that reported the capacity of mesenchymal stem cells to differentiate into hepatocytes and have a potential therapeutic effect against fibrotic process through reduction of collagen deposition and through an anti-inflammatory effect [11,13].

Histopathological improvement of hepatitis activity grade and the fibrosis after 6 and 8 weeks of MNSCs transplantation indicated the differentiation capacity to hepatocytes and regression of fibrosis. It was reported significant regression of liver fibrosis after MNSCs transplantation concluding that MNSCs have the ability to regenerate liver and reduce fibrosis in regenerating liver [16].

We achieved our hypothesis regarding the ability of MSCs to expand Tregs cells and augment their immunosuppressive effect. Augmentation of immunomodulatory properties of MSCs, explored their ability to regenerate hepatocytes and improve fibrosis as evident by PIIINP, biochemical and histopathological parameters. Researcher stated that MSCs transplantation prevent acute allogenic graft rejection through expansion of Tregs, as Tregs can amplify MSCs immunomodulation [17,18].

To best of our knowledge, little is known about the role of Tregs in homing of MNSCs. We extend our work to cover this point of interest. We found positive expression of (CD4 & IL2 α/CD25) in 85.7%. The positive expression indicated the role of Tregs cells in homing of MNSCs. However 2 out of 13 animals showed rejection of the transplanted cells evident by negative detection of Sry gene. The heterogeneous mixture of MNSCs may explain the difference of expression between cases.

We concluded that the group that received MSCs showed improvement of liver functions with reduction of liver fibrosis indicated by improvement of PIIINP compared to other groups received MNSCs. Tregs cells played an important role in homing and augmentation of the function of MSCs. Further studies are needed to explain the impact of Tregs cells in homing of MNSCs.

References


