Amelioration of Murine Schistosoma mansoni Induced Liver Fibrosis by Mesenchymal Stem Cells

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Schistosomiasis is a common chronic helminthic infection of the liver that causes hepatic fibrosis and portal hypertension, contributing to the death of over half a million people a year. Infusion of autologous bone marrow cells into patients with hepatic cirrhosis has been reported to ameliorate symptoms of portal hypertension and improve liver function, either by conversion of the infused mesenchymal stem cells (MSCs) to hepatocytes or by modulating the hepatic fibrosis. Here, we have investigated the antifibrotic effect of mesenchymal stem cells (MSCs) using S. mansoni-induced liver fibrosis in mice, which causes an intense, stable fibrosis. MSCs derived from bone marrow of male mice were then infused intravenously into female mice that had received intraperitoneal injection of S. mansoni cercariae. Mice were divided into four groups: Untreated control; MSCs infusion only; Schistosomiasis only; and Schistosomiasis plus MSCs infusion. Serum alanine aminotransferase (ALT) and liver histopathology were evaluated. Expression of the collagen gene (type I), transforming growth factor (TGF-β), matrix metalloproteinase (MMP2), tissue inhibitor of metalloproteinase (TIMP-1), stromal cell–derived factor–1 (SDF-1) and its receptor (CXCR4) were analyzed. MSC infusion resulted in significant decrease in liver collagen and TGF-β gene expression in the Schistosomiasis mice. The ratio of MMP-2 to TIMP-1 expression increased. SDF-1 and CXCR4 mRNA expression also increased. There was overall improvement of liver histology and a statistically significant reduction of serum ALT level. MSCs infusion ameliorated S. mansoni-induced liver fibrosis, probably by modulating the relative expression of MMP and TIMP. The findings support the hypothesis that MSCs participate in liver regeneration and functional improvement by reducing liver fibrosis.
that the observed salutary effect of bone marrow-derived cells on cirrhotic livers are more likely related to their collagen-modulating effect. Therefore, we hypothesized that in the presence of stable hepatic fibrosis, such as in hepatic schistosomiasis, the anti-fibrogenic effect of transplanted MSCs should predominate over their fibrogenic effect, resulting in a net reduction of hepatic fibrosis. Much of the information on the effect of stem cell transplantation of liver fibrosis or cirrhosis has been obtained from mice or rats exposed to hepatotoxins, such as carbon tetrachloride. However, in these models, liver fibrosis reverses spontaneously after discontinuation of the toxin. In contrast, in the murine Schistosomiasis model, in which the hepatic collagen turns over slowly, providing a relatively stable level of fibrosis, which should permit unambiguous interpretation of the result of autologous bone marrow MSC transplantation.

Chemokines are thought to play an important role in recruiting bone marrow-derived cells to the liver. This family of small secreted proteins (8–13 kDa) is best known for the activating leukocytes and activating their migration. The homing of MSCs in injured liver.

Materials and methods

Preparation of BM-derived MSC

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old male Balb/C mice with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL). Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). Cells were incubated at 37 °C in 5% humidified CO2 for 12–14 days as primary culture or upon formation of large colonies. When large colonies developed (80–90% confluence), cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% trypsin in 1mM EDTA (GIBCO/BRL) for 5 min at 37 °C. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50 cm² culture flask (Falcon). The resulting cultures were referred to as first-passage cultures [20]. MSCs in culture were characterized by their adhereness and fusiform shape. [21] We also detected CD29 gene expression by RT-PCR as a marker of MSCs. [22,23]

S. mansoni-induced liver fibrosis model and MSC administration

Forty female Balb/C mice, aged 4 weeks, weighing 30-40 g, were included in this study. Animals were treated in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Cairo University. They were divided into the following groups:

Group I: Control group; ten healthy mice.

Group II: Ten mice received MSCs in Phosphate Buffer Saline (PBS) injected into the tail vein. MSCs were derived from the tibia and femur of male Balb/C.

Group III: Ten mice infected with Schistosoma Mansoni cercaria (60/mouse) injected intraperitoneally. They also received intravenous PBS.

Group IV: Ten mice, infected with Schistosoma Mansoni cercaria (60/mouse) injected intraperitoneally, received MSCs at eighth week after infection. Twelve weeks after infection, blood samples were collected from the retro-orbital vein. All mice were sacrificed and livers were harvested for analysis.

Liver histology: Liver samples were collected into PBS and fixed overnight in 40 g/L paraformaldehyde in PBS at 4 °C. Serial 5 μm sections of the right lobes of the livers were stained with hematoxylin and eosin (HE) and were examined histopathologically.

Morphometric analysis: The mean optical density of collagen in liver stained sections was measured. This was accomplished using (The Leica Qwin 500 C) image analyzer computer system (Leica Imaging System Ltd., Cambridge, U.K.), in 10 non-overlapping low power fields/section at 100X magnification in a standard frame of 7099.95μm²

Genomic PCR analysis of male-derived MSCs: Genomic DNA was prepared from liver tissue homogenate of the mice in each group using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA). The presence or absence of the sex determination region on the Y chromosome male (sry) gene in recipient female mice was assessed by PCR. Amplicons (130 bp) were generated using the following primers for the sry gene: forward primer:5'-GGCAGCTACAGCATGATGCAGGAGC-3'; reverse primer:5'-CTGGTCATGGATTGTACTGCAAGG-3' (Unigene Mm.469324). The PCR conditions were as follows: incubation at 94 °C for 4 min; 35 cycles of incubation at 94 °C for 50 s, 60 °C for 30 s, and 72 °C for 1 min; with a final incubation at 72 °C for 10 min. PCR products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide. Positive (male mouse genomic DNA) and negative (female mouse genomic DNA) controls were included in each assay. The presence of RNA in all tissues was assessed by analysis of the “house-keeping” gene β-actin. cDNA was generated from 1 μg of total RNA extracted with AMV reverse transcriptase for 60 min at 37 °C. For PCR, 4 μl cDNA was incubated with 30.5 μl water, 4 μl 25 mM MgCl2, 1 μl dNTPs (10 mM), 5 μl 10× PCR buffer, 0.5 μl (2.5 U) Taq polymerase and 2.5 μl of each primer containing 10 pmol. β-actin primers were: Forward:5'-GCT GAC AAG GAA CAG CAG CTA A-3' and Reverse: 5'-GTT CGA TCA ACC TCC CAT AA AC- 3' (Unigene Mm.402299). The reaction mixture was subjected to 40 cycles of PCR amplification as follows: denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 2 min. The PCR product yielded 218 bp fragment.

RT-PCR for SDF-1 and its receptor CXCR4: cDNA was generated from 5 μg of total RNA extracted with 1 μl (20 pmol) antisense primer and 0.8 μl superscript AMV reverse transcriptase for 60 min at 37 °C. For PCR, 4 μl cDNA was
PCR analysis of CD29 gene expression: Total RNA was extracted from cultured cells using RNeasy Purification Reagent (Qiagen, Valencia, CA), and then a sample (1 µg) was reverse transcribed with AMV reverse transcriptase (RT) for 30 min at 42°C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using specific primers (UniGene Rn.25733) forward: 5'-GTCTAAGCAGCGATGGGTTC-3' and reverse: 5'-ACCATCTACTTCCATCTCCTC-3', and Reverse 5'-CACCATCCACAGGCTATC-3' (UniGene Mm.1401) to give a product of 480 bp. PCR was performed at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute for 35 cycles followed by a 10-minute extension at 72°C. β-actin mRNA in all tissues was assessed as an "invariant" internal control.

Quantitative RT-PCR analyses for collagen (type I), TGF β, MMP-2 and TIMP -1: Total RNA was extracted from liver tissue homogenate using RNeasy purification reagent (Qiagen, Valencia, CA). cDNA was generated from 5 µg of total RNA extracted with 1 µl (20 pmol) antisense primer and 0.8 µl superscript AMV reverse transcriptase for 60 min at 37°C. The relative abundance of mRNA species was assessed using the SYBR® Green method on an ABI prism 7700 sequence detector system (Applied Biosystems, Foster City, CA). Relative expression of collagen, TGFβ, MMP and TIMP mRNA was calculated using the comparative Ct method as previously described. All values were normalized to the 18S rRNA genes and reported as fold change over background levels detected in Schistosomiasis group.

Table 1

| Gene          | Primer Sequence                                      |
|---------------|------------------------------------------------------|
| Collagen-1    | Forward 5'-GGTATGGATCTGTATCTGC-3'                   |
|               | Reverse 5'-AGTCCAGTTCATCGACTTC-3'                   |
| TGF-β         | Forward 5'-CTCCACCTGCAAGACCAT-3'                    |
|               | Reverse 5'-CTTAGGTTCGAGATCGATTCC-3'                 |
| MMP-2         | Forward 5'-TTGAGAAGGATGCGAAGAT-3'                   |
|               | Reverse 5'-ATGGTAAACAGGCTTCATG-3'                   |
| TIMP-1        | Forward 5'-TTCCAGTAAAGGCTCTAGG-3'                   |
|               | Reverse 5'-TTACGACAGTCGACGAG-3'                     |
| 18S rRNA      | Forward 5'-CGGCTACACACATCGAAG-3'                    |
|               | Reverse 5'-GCTGGAATTACCGCCGG-3'                     |

Serum alanine aminotransferase (ALT) assay: ALT levels were determined using conventional laboratory methods.

Statistical analysis: Data are expressed as Mean ± SD. Significant differences were determined using ANOVA and post hoc tests for multiple comparisons using SPSS 9.0 computer Software. P values of < 0.05 were considered significant.

Results

Serum ALT levels: There was a significant improvement in ALT levels in the Schisto/MSC group compared with those in the Schisto group (p<0.01) (Table 2).

Table 2

| Serum ALT (U/L) | Control N=10 | MSCs N=10 | Schisto N=10 | Schisto/MSCs N=10 |
|-----------------|--------------|-----------|--------------|-------------------|
| P1              | 37.6±6.2     | 34.3±5.7  | 70±11.65     | 51.4±8.5          |
| P2              | <0.001       | >0.05     | <0.001       | <0.01             |
| P1, p values for each experimental group versus the control group |
| P2, p values for each experimental group versus the Schisto group |
The collagen, TGF-β, MMP-2 and TIMP-1 gene expression: Quantitative PCR analysis showed that collagen and TGF-β gene expression was reduced by 50-60% after administration of MSCs (Schisto/MSC group) compared with the Schisto group (Fig. 3A and 3B). MMP-2 expression was reduced modestly (~20%) in the Schisto/MSC group compared with the Schisto group (Fig. 3C). In contrast, TIMP-1 expression was reduced by approximately 80% in the Schisto/MSC group. (Fig. 3D)

Liver collagen content: Morphometric study (Table 3) using Sirius red staining (Fig. 1) showed significant decrease in fibrous tissue in Schisto/MSCs group compared with the Schisto group. Histopathological examination of liver tissue showed marked improvement after administration of MSCs (Fig.1)

|                | Control N=10 | MSCs N=10 | S. mansoni N=10 | Schisto/MSCs N=10 |
|----------------|--------------|-----------|----------------|------------------|
| Optical density for red-stained collagen | 12.5±3.8 | 11.9±4.3 | 34.5±7.7 | 23.8±4.1 |
| P1             | <0.001       | >0.05     | <0.001         | <0.001           |
| P2             | <0.001       | <0.001    | <0.001         | <0.001           |

P1 group versus control
P2 group versus Schisto group.

SDF-1 and CXCR4 expression: SDF-1 mRNA was detected in the livers of mice infected with Schistosoma eggs (Schisto) and mice infected with Schistosoma eggs, followed by infusion of mesenchymal stem cells (Schisto/MSC). mRNA levels for collagen-I (Fig.3a.), TGF-β (Fig.3b.), MMP-2 (Fig.3c.) and TIMP-1 (Fig.3d.) were determined by quantitative RT-PCR. The figure shows relative abundance of each mRNA, normalized to the mRNA content in the Schisto mice. The data are means of three determinations.

Figure 3. Relative mRNA abundance in liver: RNA was extracted from livers of mice infected with Schistosoma eggs (Schisto) and mice infected with Schistosoma eggs, followed by infusion of mesenchymal stem cells (Schisto/MSC). mRNA levels for collagen-I (Fig.3a.), TGF-β (Fig.3b.), MMP-2 (Fig.3c.) and TIMP-1 (Fig.3d.) were determined by quantitative RT-PCR. The figure shows relative abundance of each mRNA, normalized to the mRNA content in the Schisto mice. The data are means of three determinations.

CD29, a marker for MSCs, was expressed in MSCs culture. The sry gene that was used as the Y chromosome marker was detected only in liver tissue from the Schisto/MSCs group, but not in the in control, Schisto or MSCs groups (Fig.2).

Figure 1. Photomicrograph of liver tissue from mice infected with Schistosoma mansoni cercariae. Panels a, b and c: No infusion of MSCs. Panel a, hematoxylin/eosin-stained liver sections, showing characteristic granuloma around the S. mansoni ova and sinusoidal dilatation. Panels b and c: Sirius red-stained liver sections (original magnification 100x and 400X, respectively) showing granulomas containing dense collagen fibers. Panels d, e and f: Livers from mice infected with S. mansoni cercariae followed by infusion of bone marrow-derived MSCs. Panel d: hematoxylin/eosin-stained liver sections, showing smaller Schistosoma granulomas compared with those shown in Panel a, and improved liver histology. Panels e and f: Sirius red-stained liver sections (original magnification 100x and 400X, respectively) showing markedly reduced intensity of Sirius red staining, compared with the sections shown in panels b and c, indicating reduction of collagen content in the granulomas.

Figure 2. UV transilluminated agarose gel of PCR products of sry gene (A) and β-actin (B) in the studied groups. M: PCR marker, lane 1: control group, lane 2: MSCs group, lane 3: Schisto group, and lane 4: Schisto/MSCs group.
that both collagen production and collagenase activity and TIMP gene expression. It has been shown previously expression and (b) a change in the balance between MMP the resorption of collagen: (a) decreased collagen I gene which revealed two major factors that are known to influence reduction of hepatocellular injury.

Interestingly, after MSC administration, MMP-2 gene expression was reduced modestly, suggesting that with the attenuation of the inflammatory signal(s) and in the face of decreased collagen production and deposition, MMP gene expression is also decreased.

TIMPs regulate the action of MMP2 by blocking collagenase activity. Therefore, it is noteworthy that within one month of MSCs administration, TIMP-1 expression was markedly reduced compared with livers of untreated mice. This decrease is highly relevant as TIMP-1 is the predominant gene expressed in the granulomatous livers and its reduced expression is expected to enhance collagenase activity and fibrous tissue degradation. Thus, the balance in MMP/TIMP expression is shifted towards MMP, even in the face of lowered MMP gene expression. This shift constituted the basis of scar tissue resorption. These observations are in agreement with those on carbon tetrachloride-induced reversible rat liver fibrosis, where resolution of the fibrosis is also associated with decreased MMP expression.

In contrast to the reported studies in TIMP-1- and TIMP-2-deficient C57BL/6 mice, which found no role for these inhibitors in schistosome egg-induced fibrogenesis, here we showed an association between greatly diminished TIMP-1 expression and enhanced resorption of liver fibrous tissue.

The cellular source(s) of collagenases need further clarification. Previously, it has been shown that both macrophages and eosinophils isolated from the vigorous granulomas secrete collagenases. Additionally, MMP-10 expression has been shown in myofibroblasts present in the granulomas after acute S. mansoni infection. With the involution of the granulomas in drug-treated mice, the cellular content of the lesions diminished gradually, but MMP gene expression remained elevated at 6 and 12 months after infection, despite marked reduction in the number and cellular content of the granulomas, suggesting that, in addition to hepatic myofibroblasts, collagenases are secreted by other liver cells, such as Kupffer cells and hepatocytes.

We focused on the antifibrotic effect of MSCs on liver of S. mansoni-infected mice. In this model, once the granulomas form around the S. mansoni ova, the rate of spontaneous change of the collagen content of the granulomas is low, thereby providing a relatively stable in vivo model for analyzing collagen turnover. In addition to collagen content, we analyzed the expression of MMP2 and TIMP-1, which exert opposing effects on the net hepatic collagen content. Histopathological examination of the liver of the S. mansoni-infected mice showed characteristic granulomas, which were cellular and contained thick collagen bands. After MSC infusion, the granulomas decreased in size and number and became less cellular, although the concentric fibrous bands were still discernible. Sirius red staining showed reduction of liver collagen content significantly compared with post-infection control values. Furthermore, MSC administration resulted in significant improvement in ALT levels, indicating reduction of hepatocellular injury.

Interestingly, the transplanted MSCs were detectable in the liver by sry gene analysis only after induction of S. mansoni granulomas, indicating that liver injury is required for recruitment of the MSCs to the liver. Analysis of expression of the chemokine (SDF-1) and its receptor (CXCR4) was undertaken to determine their potential role in mobilizing and recruiting MSCs to the liver. SDF-1 mRNA was not detectable in normal livers harvested from the control mice, but was found after induction S. mansoni-induced liver infection control values. Furthermore, MSC administration resulted in significant improvement in ALT levels, indicating reduction of hepatocellular injury.

Figure 4. RT-PCR for mRNAs SDF-1 gene (panel A), CXCR-4 (panel B) and β-actin (panel C). mRNA was extracted from livers of mice from various experimental groups and amplified by RT-PCR. Amplicons were subjected to agarose gel electrophoresis and stained with ethidium bromide. M, PCR marker; lane 1, control group; lane 2, MSCs group; lane 3: Schisto group; lane 4, Schisto/MSCs group.

Discussion

We show that infusion of bone marrow MSCs in a murine Schistosomiasis model of relatively stable hepatic fibrosis results in significant reduction of hepatic collagen content, probably by altering the relative expression of MMP and TIMP genes. This was detected at one time point; 4 weeks post-administration of MSCs (not MSC-conditioned media). The findings are relevant in understanding the mechanism of improvement of liver function in patients with cirrhosis of the liver after infusion of autologous bone marrow cells with or without fractionation.
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fibrosis, with or without MSC infusion. In contrast, mRNA for the chemokine receptor, CXCR4 was detected only in S. mansoni-infected mice only after MSC infusion. Son et al. [36] suggested that SDF-1, CXCR4 and HGF-c-met axes, along with MMPs, may be involved in the recruitment of expanded MSCs to damaged tissues. Also, Xu et al. [37] suggest that the SDF-1/CXCR4 axis is important in the complex sequence of events triggered by bleomycin exposure which eventually leads to lung repair. SDF-1 participates in mobilizing bone marrow-derived stem cells, via its receptor CXCR4. The signal transduction pathways initiated by the binding of SDF-1 to CXCR4 are not fully understood. In human T and immature CD34+ cells, SDF-1 activates PI3K, the phospholipase C/PKC (PLC/PKC) cascade, and MAPK

In the present study, resorption of fibrous tissue was observed at one month post MSCs administration. This is more rapid than fibrous tissue resorption observed with anti-schistosoma drug treatment, where resorption starts 3 months after treatment. [24] Consistent with this, after anti-schistosoma drug treatment, liver fibrosis undergoes slow resorption. [41-46] In mice, the slow resorption of fibrous tissue following treatment has been linked to the timing of the treatment. Early treatment, started at 8 weeks post-infection, has yielded better resorption of the fibrous tissue compared to CXCR4 are not fully understood. In human T and immature CD34+ cells, SDF-1 activates PI3K, the phospholipase C/PKC (PLC/PKC) cascade, and MAPK

In conclusion, MSC infusion ameliorates S. mansoni-induced liver fibrosis in mice. The antifibrotic effect may be mediated by changes in the relative levels of the MMP and TIMP gene expression. The findings are consistent with the notion that the infused MSCs may mediate the improvement of liver function in hepatic cirrhosis by modulating the liver architecture through reducing liver fibrosis.

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