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SUCCESSFUL INTEGRATION OF TRANSPLANTED MESENCHYMAL STEM CELLS INTO THE LIVERS OF *SCHISTOSOMA MANSONI*-INFECTED MICE

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ABSTRACT

One of the most serious consequences of *Schistosoma* (*S.*) *mansoni* infection is hepatic schistosomiasis or periportal fibrosis. Treatment with praziquantel (PZQ) remains the mainstay of schistosomiasis control. Stem cells and their possible use in cell therapy have drawn much attention recently, due to their potential for self-renewal and differentiation. The present study aimed to investigate the ability of mesenchymal stem cells (MSCs) to integrate into the livers of *S. mansoni*-infected mice. *S. mansoni*-infected mice (60±10 cercariae/mouse, s.c.) received intra-hepatic injection of MSCs ($1.5x10^6$ cells/mouse), alone or combined with oral PZQ (500 mg/kg/day, for 2 days, seven weeks post infection). At the 10th month post infection, flow cytometry and immunohistochemical analysis for human-specific β2-globulin were performed. Immunohistochemical results showed positive hepatic expression for β2-globulin. Interestingly, the integration of MSCs was found to be enhanced in transplanted groups which received PZQ. This was shown in the increased hepatic expression levels of β2-globulin in the group which received combined treatment. In conclusion, the results of the present study showed that MSCs were capable of integrating into the liver tissue of infected mice and the observed effects were enhanced when PZQ was given in combination.

INTRODUCTION

Liver fibrosis occurs in the setting of chronic injury caused by different etiologies constituting a serious worldwide public health problem. Whereas in acute hepatic injury nonviable cells are replaced by normal tissue, during chronic injuries a persistent repair response may lead to fibrosis and scar formation as a result of an imbalance between proliferation and degradation of the extracellular matrix components (Bataller and Brenner, 2005). Chronic infection by *Schistosoma mansoni* (*S. mansoni*) is one of the experimental models of hepatic fibrosis used to elucidate the mechanisms involved in the fibrogenic processes.

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In schistosomiasis, the main immune-inflammatory response is directed against the parasite eggs, which, when led to portal circulation, may become lodged into hepatic portal venules, eliciting a granulomatous response. In the mouse model of schistosomiasis, the persistence of the stimulus leads to the development of two pathological patterns; isolated granulomas or periportal fibrosis, the latter resembling the pipe-stem fibrosis found in the severe hepatosplenic form of human disease (Andrade and Cheever, 1993). In Egypt, schistosomiasis, caused mainly by *S. mansoni,* is a continuing health problem despite attempts to control this parasitic infection over many years (El-Khoby *et al*., 2000). There is as yet no vaccine available and the current mainstay of control is chemotherapy with praziquantel (PZQ) (Utzinger *et al*., 2001). For so many years, chemotherapy has been the cornerstone of controlling schistosomiasis. The safety, broad spectrum and reasonable price of PZQ have made it a safe agent for treatment (Cioli, 2000). Moreover, PZQ has proven efficacy

against all schistosome species adapted to humans with a high cure rate (Kumar *et al*., 1994). Currently the only curative treatment for advanced liver cirrhosis is liver transplant. Despite of technical advances, significant morbidity and mortality remain. In addition, liver transplantation is severely limited by available liver shortage, resulting in longer waiting time and increased mortality of patients (Fox and Chowdhury, 2004). This limitation made hepatocyte transplantation a better alternative to liver transplantation since hepatocytes are liver parenchymal cells that do a lot of catabolic and anabolic metabolisms in the liver (Strom *et al*., 1997; 1999). But allogenic hepatocytes could be obtained only from transplantation-inconsistent liver because of steatosis, cirrhosis, fibrosis, and other reasons. Therefore, hepatocyte transplantation is also limited by donor liver shortage. Moreover, Fisher and Strom (2006) reported that the successful hepatocyte transplantation in animal studies was not translated to clinical human experiences. The mismatch between the number of patients requiring transplantation for end stage liver disease and the number of available organs is set to grow, highlighting the need to develop new strategies to stimulate liver regeneration and reduce liver scarring. Recent work suggests that these two aims are inextricably linked, and that reducing hepatic fibrosis can result in activation of hepatic progenitor cells resulting in parenchymal regeneration (Kallis *et al*., 2011).

Mesenchymal stem cells (MSCs) are derived from the mesoderm and have self-renewal and multi-differentiation capacity. Under appropriate conditions in vivo and in vitro, they can differentiate into various tissue cells, such as osteoblasts, chondrocytes, adipocytes, muscle cells, neurocytes as well as hepatocytes (Chen *et al*., 2009; Ren *et al*., 2010). For these reasons, MSCs are considered important seed cells for tissue engineering and cell transplantation. To date, there are many studies that have tried to demonstrate a role of MSCs in liver repair after injury, like in animal models of CCl_4 (Sharma *et al*., 2005) as well as Schistosomiasis *mansoni* (Oliveira *et al*., 2008). Based on the previous data, it is expected that MSCs transplantation and PZQ may play a role in the alleviation of liver fibrosis induced by *S. mansoni* infection in mice. The present study aimed at investigating the capability of MSCs to successfully integrate into the liver tissue of *S. mansoni*-infected mice and whether PZQ treatment had an impact on the integration potential.

MATERIALS AND METHODS

Ethics statement

All animal work was conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals.

Animals

Swiss male albino mice CD-1, weighing 18–20 g each, were provided by the Schistosome Biology Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt. They were bred on a standard diet with free accessibility to water. The animals were kept under standard conditions of temperature (25 ± 0.5°C), relative humidity (55 ± 1%) and light cycle (12 h light and 12 h dark).

Infection

An Egyptian strain of *S. mansoni* cercariae was provided by the SBSC of TBRI. Cercariae were shed from laboratory bred infected snails namely, *Biomphalaria alexandrina,* 25-30 days after exposure to miracidia according to the method described by Pellegrino *et al*. (1962). Infection was done by subcutaneous (s.c.) injection of mice with 60 ± 10 *S. mansoni* cercariae suspended in 0.2 ml solution (Holanda *et al*., 1974).

Isolation and culture of MSCs

The MSCs used in this study were originally isolated and expanded from a donated human umbilical cord with the donor's consent after a full-term caesarean delivery, according to the method described by Salehinejad *et al*. (2012).

Detection of MSCs surface markers

The MSCs were analysed for the lack of expression of CD11b by flow cytometry as described previously (Gang *et al*., 2006).

Transplantation of MSCs

Transplantation of MSCs into *S. mansoni*-infected mice was done by single intrahepatic injection using $1.5x10^6$ cells/mouse suspended in Dulbecco's modified Eagle's medium (DMEM) (Elkahfif *et al*., 2011).

Drugs and doses

Praziquantel (E.I.P.I.Co. Pharmaceuticals, Cairo, Egypt) was prepared as suspension in Cremophor-El and given orally seven weeks post infection at a dose of 500 mg/kg/day for two consecutive days (Gonnert and Andrews, 1977).

Experimental design

Infected mice were randomly allocated into the following groups, each consisted of 10 mice:

Group I: This group was given orally Cremophor-El and/or injected with DMEM to serve as the infected control group.
Group II: This group was transplanted with MSCs at the 8^{th} week post infection.
Group III: This group was treated with PZQ at the 7^{th} week post infection.
Group IV: This group received both MSCs transplantation and PZQ treatment, as given in groups II and III.

At the 10^{th} month post infection, all animal groups were sacrificed by decapitation.

Immunohistochemical studies

Immunohistochemistry was performed by using an avidin-biotin complex immunoperoxidase technique (Hsu and Raine, 1981) with anti-human primary antibody against β2-globulin (Santa Cruz Biotechnology Inc., USA) diluted at 1:100, in PBS. We used a streptavidin-biotin-peroxidase preformed complex and peroxidase-DAB (3,3′-diaminobenzidine) (Dako, Denmark), according to the manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin and

mounted with DPX medium. Positive and negative control slides were included in each session. As a negative control, a liver tissue section was processed as described, but with the primary antibody omitted.

Statistical analysis

All values are presented as means ± standard error (S.E.). Statistical analysis was performed by one-way analysis of variance (One-way ANOVA) followed by Bonferroni post hoc test for multiple comparisons using Graph Pad Prism (v5). Values of $P<0.05$ were considered to be statistically significant.

RESULTS

Flow cytometry

Flow cytometric analysis showed that MSCs were negative for CD11b.

Immunohistochemical studies

Human-specific antibody with no cross-reactivity to mouse antigens were used to label the liver-associated marker, namely, β2-globulin. As shown in Figure (1), there was no positive expression observed in infected control and PZQ-treated groups.

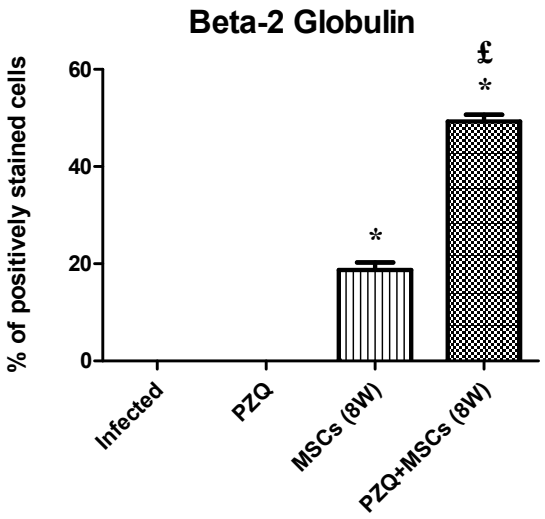

Figure 1. Effects of treatment with MSCs, given either alone or combined with PZQ, on the immunohistochemical expression of human β2-globulin in the liver sections of mice infected with *S. mansoni*

MSCs ($1.5x10^6$ cells/mouse) were injected at the 8th week (W) post infection. PZQ (500 mg/kg/day) was orally given at the 7th W post infection for 2 consecutive days. Animals were sacrificed at 10th month post infection. Values are presented as means ± S.E. (n=10). Significantly different ($P<0.05$) * versus Infected control and PZQ-treated groups and £ versus MSCs (8W).

However, the percentage of positively-stained cells of β2-globulin in the group which received MSCs was found to be 18.70±1.58 (Table 1, Figure 2). Combining PZQ to MSCs given at the 8th week post infection caused a 2.64-fold increase in the hepatic expression of β-2 globulin, as compared to the group treated with MSCs alone.

Table 1. Effects of treatment with mesenchymal stem cells (MSCs), given either alone or combined with praziquantel (PZQ), on the immunohistochemical expression of β2-globulin in hepatic sections of mice infected with *S. mansoni.*

	Infected control	PZQ	MSCs (8W)	PZQ+MSCs (8W)
% of positively-stained cels	--	--	18.70±1.58*	49.30±1.35*£

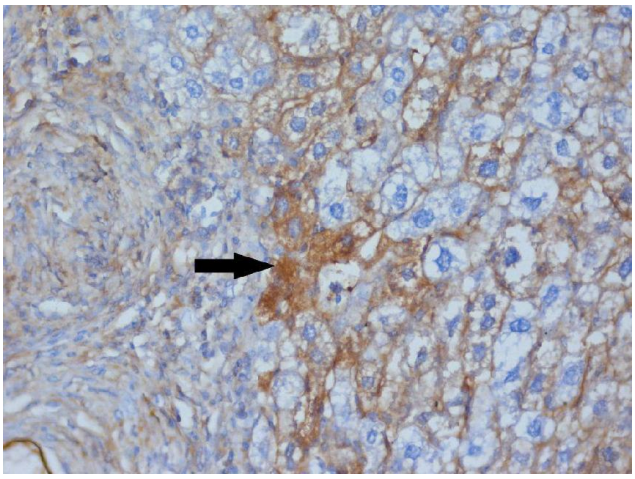

Figure 2. Photomicrograph of immunohistochemical staining (arrow) of human β2-globulin in liver section of MSC-transplanted (8W) mouse showing positive expression. No expression was observed in control and PZQ-treated groups

DISCUSSION

In schistosomiasis, fibrosis is associated with the granulomatous response to parasite eggs trapped in the liver (Cheever *et al*., 2000). Treatment and control of schistosomiasis rely mainly on PZQ. The search for adjuvant therapies is therefore urgent especially in light of the emerging resistance to the drug (Cioli, 2000). Stem cells are considered an alternative cell source for functional hepatocytes. Several studies have demonstrated that the differentiation of stem cells into hepatocytes is achieved in the appropriate microenvironment following stimulation with hepatic growth factors (Lee *et al*., 2004; Subramanian *et al*., 2011). Mesenchymal stem cells (MSCs) are a type of adult stem cells and, compared to hepatocytes, are better candidates for cell therapy because of their adequate availability, easy accessibility, rapid proliferation, multipotent differentiation, successful integration, and immunological tolerance in the host tissue (Parekkadan and Milwid, 2010). Moreover, several studies have demonstrated that transplantation of MSCs or MSC-derived hepatocyte-like cells improves liver function in rodents (Zhao *et al*., 2005; Piryaei *et al*., 2011) as well as in patients (Peng *et al*., 2011) suffering from liver damage. Based upon the previously mentioned findings, the present study was performed to monitor the integration of transplanted stem cells (MSCs; $1.5x10^6$ cells/mouse) into the livers of *S. mansoni*-infected mice and to test whether PZQ treatment had an influence on the integration process. In this study, we provided data suggesting the successful integration of MSCs in the livers of *S. mansoni*-infected mice. Moreover, it was obvious from the results that PZQ, when given in combination with MSCs, had positively influenced the integration of the

transplanted cells as indicated by an increase in the positive expression of β2- globulin. In this study, after isolation of MSCs, the flow cytometric phenotype analysis revealed that MSCs had negatively expressed CD11b. These results are in agreement with previous studies (Dominici *et al*., 2006; Zhang *et al*., 2009). The hepatic expression of the human-specific marker, β2- globulin, in liver samples from MSCs recipient mice was investigated by immunohistochemical staining techniques. Immunostaining analysis indicated that MSCs positively expressed the aforementioned marker. The expression of the human hepatocyte-specific markers was reported in previous studies (Tsai *et al*., 2009; Yu *et al*., 2012). Moreover, an increased hepatic expression of β2- globulin in the groups which were treated with a combination of MSCs and PZQ was observed, as compared to the MSCs-treated group. These results suggest that the surrounding environment may be essential for transplanted cells to integrate into liver tissue of infected mice, which was at least partially enhanced in this study by PZQ treatment.

MSCs may also protect against *S. mansoni*-induced liver fibrosis by altering the microenvironment of the liver at sites of engraftment. This microenvironment is supposed to be improved in presence of PZQ, since it was reported that after eradication of worms and subsequent reduction of egg deposition, no further stimulation of collagen biosynthesis occurred (Hutadilok *et al*., 1983). Consequently, it has been suggested that once the injurious agent is removed, active collagen synthesis and deposition will return to normal (Rojkind and Kershenobich, 1981). In conclusion, our results suggested that transplanted MSCs were successfully integrated in the livers of mice infected with *S. mansoni* infection. Additionally, combining PZQ to MSCs improved the ability of the latter to integerate into the liver tissue possibly by enhancing the microenvironment at transplantation sites. Further studies are recommended to test the ability of such cells, either alone or combined with PZQ, in differentiating into functioning hepatocyte-like cells and hence repairing *S. mansoni*-induced hepatic fibrosis.

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