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Mobilization of endogenous bone marrow-derived stem cells in a thioacetamide-induced mouse model of liver fibrosis

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ABSTRACT

The clinical significance of enhancing endogenous circulating haematopoietic stem cells is becoming increasingly recognized, and the augmentation of circulating stem cells using granulocyte-colony stimulating factor (G-CSF) has led to promising preclinical and clinical results for several liver fibrotic conditions. However, this approach is largely limited by cost and the infeasibility of maintaining long-term administration. Preclinical studies have reported that StemEnhance, a mild haematopoietic stem cell mobilizer, promotes cardiac muscle regeneration and remedies the manifestation of diabetes. However, the effectiveness of StemEnhance in ameliorating liver cirrhosis has not been studied. This study is the first to evaluate the beneficial effect of StemEnhance administration in a thioacetamide-induced mouse model of liver fibrosis. StemEnhance augmented the number of peripheral CD34-positive cells, reduced hepatic fibrosis, improved histopathological changes, and induced endogenous liver proliferation. In addition, VEGF expression was up-regulated, while TNF-α expression was down-regulated in thioacetamide-induced fibrotic livers after StemEnhance intake. These data suggest that StemEnhance may be useful as a potential therapeutic candidate for liver fibrosis by inducing reparative effects via mobilization of haematopoietic stem cells.

1. Introduction

Liver fibrosis occurs as a result of chronic injury leading to the excessive accumulation of extracellular matrix and scar tissue formation. If not efficiently treated, liver fibrosis may lead to cirrhosis, inducing permanent and irreversible damage to liver structure and function with fatal consequences (Friedman et al., 2013). The most common causes of liver fibrosis are infection with hepatitis B or C virus, which represents a major public health problem that affects millions of people worldwide. Studies on the epidemiology of hepatitis C virus (HCV) infections have suggested that Egypt has one of the highest prevalence rates of HCV in the world, with seroprevalence rates of 30–40% in villagers over the age of 30 (Lehman and Wilson, 2009). With the development of new antiviral strategies, viral eradication and treatment of hepatitis can be anticipated in some patients, even in those with chronic viral hepatitis. However, liver transplantation is still the only current radical treatment in patients in which decompensated liver cirrhosis has already occurred (Dhingra et al., 2014). However, several problems, such as organ shortage, surgical complications, and expensive costs, underlie the need to develop new therapeutic strategies to attenuate liver scarring and enhance liver regeneration (Saito et al., 2013).

Stem cell therapeutic strategies are being evaluated as an attractive promising approach for liver repair. Several studies have reported the ability of various types of stem cells to improve the pathological outcome of liver cirrhosis and to attenuate the clinical symptoms of the disease (El-Ansary et al., 2012; Takami et al., 2012; Ali and Masoud, 2012; Zhang et al., 2012; Wang et al., 2012; Agaev et al., 2014). However, exogenous stem cell therapeutic strategies carry several potential risks that may limit their wider clinical application. For instance, stem cell therapy is an invasive technique that requires repeated injections often in the portal vein or hepatic artery (Kharaziha et al., 2009; Salama et al., 2010; Wang et al., 2012). Another limitation is that stem cells are exposed to several manipulations during their expansion in vitro before being transplanted, these manipulations lead to their contamination and/or cause deleterious changes in their intrinsic characteristics due to several intracellular and extracellular influences, adding additional burden on the diseased liver (Herberts et al., 2011). Based on the fact that bone marrow-derived stem cells have the ability to migrate to sites of tissue damage and participate in tissue regeneration, stimulating the mobilization of endogenous bone marrow-derived stem cells may provide a promising non-invasive alterative to exogenous stem cell transplantation.

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Many different soluble factors have the ability to mobilize bone marrow-derived haematopoietic stem cells (BM-HSCs) from the bone marrow to the peripheral circulation and hence increase their total number (Weissman et al., 2001). Granulocyte-colony stimulating factor (G-CSF) was the first factor described to have this feature. However, investigating the therapeutic potential of G-CSF has been largely limited due to the significant risks accompanied with its use for long periods of time (Bensinger et al., 1996; D'Souza et al., 2008; Barnes et al., 2014). StemEnhance (SE) is a natural stem cell mobilizer that can trigger a much milder mobilization of BM-HSCs, and its considerable safety allows for a sustained oral daily intake over long periods of time. SE is a natural water-soluble extract of the cyanophyta *Aphanizomenon flos-aquae* (AFA), which was recently shown to increase the number of circulating BM-HSCs by approximately 25% within 60 min after oral consumption (Jensen et al., 2007). Previous experimental studies reported that mobilization of BM-HSCs with SE promoted muscle regeneration in cardiotoxin-induced muscle injury (Drapeau et al., 2010) and ameliorated manifestations of diabetes in rats (Ismail et al., 2013). However, the potential effectiveness of SE in ameliorating liver cirrhosis has not been investigated.

In the current study, we sought to evaluate the effect of SE administration in thioacetamide-induced liver fibrosis in mice. In all experimental groups, the percentage of CD34-positive cells in the peripheral circulation was assessed using flow cytometry 7 days after starting SE administration in SE-treated groups. At the end of the experiment, liver function and histological assessments were conducted to investigate the potential reparative effect of the endogenously increased haematopoietic CD34-positive cells and the possible mechanisms underlying this effect.

2. Methodology

2.1. Animals

Male C57Bl/6 mice aged 7–8 weeks old were purchased from the Theodor Bilharz Research Institute, Imbaba, Egypt, and maintained in the animal house of Research Institute of Ophthalmology, Giza, Egypt. The mice were subjected to a 12: 12-h daylight/darkness and allowed unlimited access to chow and water. All of the ethical protocols for animal treatment were followed and supervised by the animal facilities at Research Institute of Ophthalmology, Giza, Egypt. All procedures involving the use of the mice were approved by The Animal Care and Use Committee.

2.2. Experimental design

The mice were randomly divided into four groups; control, SE-treated (SE group), thioacetamide-treated (TAA group), and thioacetamide plus SE-treated (TAA + SE group) ($n = 10$ per group). Liver fibrosis was induced in the TAA and TAA + SE groups by intraperitoneal injection (i.p.) of thioacetamide (TAA, Sigma, St. Louis, MO, USA) at a dose of 200 mg/kg b.w. twice a week. After 8 weeks of TAA treatment, the TAA + SE mice were orally administered StemEnhance (SE; StemTech Health Sciences, Inc., UK) at a dose of 300 mg/kg b.w. daily for an additional 4 weeks. In this group, TAA was continuously administered during the additional 4 weeks. The mice in TAA group were given TAA (200 mg/kg b.w. twice a week) for 12 weeks and SE-treated mice were given SE (300 mg/kg b.w. daily) for 4 weeks. With the exception of the flow cytometry data, there was no statistically significant difference between the liver function or histological or immunohistological outcomes of the control and SE groups; therefore, for these measured outcomes, the SE and control groups were pooled into one group (control).

2.3. Biochemical analysis

At the end of the experiment, blood samples were collected from the orbital sinus and incubated for 1 h at room temperature (RT) to allow clotting. Then, the sera were collected by centrifugation at 2400 rounds per min (rpm) for 5 min and stored at −20 °C until use. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed using the Advia Chemical System (Siemens, Germany).

2.4. Flow cytometry

To measure the percentage of CD34-positive cells in peripheral blood, blood samples were collected from mice of all experimental groups in heparin tubes 7 days after SE administration in SE-treated group (SE and TAA + SE). 100 μl of blood was incubated with PE-conjugated rat anti-mouse CD34 (clone RAM34; BD Pharmingen, USA) for 30 min at 4 °C. After red-cell lysis (Versalyse Lysing Solution, Beckman Coulter), the samples were centrifuged, washed twice with phosphate buffer saline (PBS), and fixed with 1% paraformaldehyde (Sigma). Isotype-identical antibody served as controls. The cells were analyzed using a Beckman Coulter EPICS XL flow cytometer.

2.5. Histological and immunohistological (IHC) assessments

At the end of the experiment, each mouse was deeply anaesthetized using ketamine (90 mg/kg) and xylazine (15 mg/kg) (i.p.) and decapitated. Livers were dissected and fixed in 10% neutral-buffered formalin and embedded in paraffin wax. For histological examination, 5-μm sections were deparaffinised and rehydrated using a graded ethanol (100%, 90%, and 70%) series and stained with haematoxylin & eosin (H&E) or with Mallory Trichrome (MT) stain, for collagen fibres.

For immunohistological staining, deparaffinised and rehydrated 5-μm sections were rinsed with PBS and blocked for 30 min in 0.1% H_2O_2, as an inhibitor of endogenous peroxidase activity. After rinsing in PBS, the sections were incubated for 60 min in blocking solution (10% normal goat serum) at RT. The sections were then incubated with the primary antibody (Transforming growth factor beta (TGF-β), 1:100, ThermoScientific; Vascular endothelial growth factor (VEGF), 1:100, Cell Mark; Tumour necrosis factor alpha (TNF-α), 1:500, ThermoScientific; Ki67, 1:500, Dako) at RT for an hour. The sections were rinsed with PBS, followed by 20 min of incubation at RT with the secondary biotinylated antibody. After rinsing the sections in PBS, the enzyme conjugate "Streptavidin-Horseradish peroxidase" solution was applied to the sections for 10 min. The secondary antibody binding was visualized using 3,3-diaminobenzoic acid (DAB) dissolved in PBS with the addition of H_2O_2 to a concentration of 0.03% immediately before use. Finally, the sections were rinsed with PBS and the slides were counterstained of using two drops or 100 μl of haematoxylin. The slides were then washed in distilled water until the sections turned blue. Finally, the slides were dehydrated in ascending grades of ethanol (70%, 95%, and 100%) for 5 min each, cleared in xylene, followed by mounting with Histomount and a coverslip.

For immunohistological quantitative assessment, five non-overlapping fields per section were randomly taken using a Leica DML B2/11888111 microscope equipped with a Leica DFC450 camera. The number of immunopositive cells in fields taken from at least three sections/animal was counted using ImageJ software and averaged per field for each animal. The numbers calculated for at least five animals/experimental group were considered for comparison and statistical analyses.