Rehmannia glutinosa oligosaccharide induces differentiation of bone marrow mesenchymal stem cells into cardiomyocyte-like cells

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ABSTRACT. The aim of this study was to observe the effect of *Rehmannia glutinosa* oligosaccharide (RGO) on differentiation of bone marrow mesenchymal stem cells (MSCs) into cardiomyocyte-like cells . Rat MSCs were isolated, treated, and grouped as follows: RGO treatment group, 5-azacytidine (5-aza) treatment group, RGO + 5-aza treatment group, and control group. Following a four-week induction period, cardiac troponin I (cTnI) levels in MSCs were quantified by chemiluminescence, and the levels of myocardial enzymes creatine kinase (CK) and creatine kinase isoenzyme-MB (CK-MB) were measured using a dry chemistry analyzer. The cTnI- and connexin 43 (Cx43)-positive MSC population was identified by

immunofluorescence, and expression levels of cTnI and Cx43 were analyzed by western blots. Following induction, cTnI, CK, and CK-MB levels were significantly higher in the RGO + 5-aza group as compared with the RGO and 5-aza groups ($P < 0.05$). In addition, fluorescence intensity of cTnI and Cx43 was higher in the RGO + 5-aza group as compared with the RGO and 5-aza groups. No cTnI- or Cx43-positive cells were detected in the control group. Western blot analysis further confirmed that cTnI and Cx43 were not expressed in the control group, while cTnI and Cx43 was higher in the RGO + 5-aza group than in the RGO and 5-aza groups. These results suggest that MSCs can be induced by RGO to differentiate into cardiomyocyte-like cells *in vitro*, and that RGO in combination with 5-aza enhance differentiation of MSCs.

Key words: *Rehmannia glutinosa* oligosaccharide; Mesenchymal stem cells; Cardiomyocyte-like cells; Cell differentiation

INTRODUCTION

In recent years, stem cell transplantation in the heart has become a new research focus (Dey et al., 2010). Bone marrow mesenchymal stem cells (MSCs) have been commonly used as donor stem cells for transplantation as they can differentiate into diverse cell types, including cardiomyocyte-like cells and vascular endothelial cells (Antonitsis et al., 2008; Yue et al., 2008). 5-Azacytidine (5-aza) is often used to induce differentiation of MSCs into myocardial cells; however, 5-aza is associated with various adverse effects as an antitumor drug (Borodovsky et al., 2013). A number of studies focusing on traditional Chinese medicine extracts found that *Rehmannia glutinosa* oligosaccharide (RGO), extracted from *R. glutinosa*, could promote the differentiation of MSCs into skeletal muscle myoblasts *in vitro* (Chen et al., 2015). In addition, RGO has also been shown to regulate immunity, protect the myocardium, as well as exert antidiabetic and antitumor effects (Lai et al., 2012). However, there has been little research comparing the effects of 5-aza and RGO on cell differentiation. More robust MSC differentiation can likely be induced by combining 5-aza and RGO treatments than by either treatment alone. The aim of this study therefore was to compare the effects of 5-aza, RGO, and a combination of both treatments on MSC differentiation.

MATERIAL AND METHODS

Experimental animals

Seven-week-old purebred Wistar rats (clean grade, male, and average body weight: 180 ± 10 g) were provided by the Experimental Animal Center of the General Hospital of PLA (Beijing; certificate No. 20050013). This study was carried out in strict accordance with recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Military General Hospital of Beijing PLA.

Three individual experiments were performed in triplicates using three samples. The rats were divided into four experimental groups consisting of 12 animals in each cohort. Four indexes were observed: morphological characteristics of MSCs, the effect of RGOs on biochemical markers of heart muscle MSCs, identification of MSCs following their induction, and western blot analysis after MSC induction.

Isolation and culture of cardiomyocytes from neonatal rats

The cardiac apex of two-day-old male and female Wistar rats was dissected via thoracotomy under sterile conditions. The tissue was washed three times with pre-cooled D-Hank's solution, cut into 1-mm^3 cubes, and digested with 0.0625% trypsin at 37°C. Following precipitation, the supernatant was added to an equal amount of DMEM culture containing 10% FBS. The solution was gently pipetted to yield a single cell suspension, and was followed by centrifugation at 1000 rpm for 10 min. The cell suspension was incubated for 90 min at 37°C with 5% CO_2. Differential wall-adhesion separation method was then used to absorb the cell suspension, and cell concentration was adjusted to 5.0 x 10^5 cells/mL. Cells were seeded in culture plates, and medium was changed after 24 h. Subsequent media replacements were performed every other day.

Isolation and culture of MSCs

Rats were anesthetized by intraperitoneal administration of 20% urethane, and were then dipped in 75% ethanol for 5 min. Under sterile conditions, the femoral were quickly isolated and rinsed with PBS, and the two ends of the bone were cut. Bone marrow was rinsed out with 5 mL low glucose DMEM solution (Thermo Fisher, Waltham, USA) containing 10% FBS (GE Healthcare, Logan, USA). Bone marrow cell suspension was collected, filtered with a 4-gauge needle, and fully dispersed. Prepared cell suspensions were added to the culture bottle, and were incubated at 37°C and 5% CO_2. After 24 h, the medium was changed for the first time, and cell growth was observed under a phase contrast microscope.

Passage and purification of MSCs

As primary cell cultures reached 100% confluency, cells were passaged. Cell digestion was carried out with 0.125% trypsin (Thermo Fisher, Waltham, MA, USA), and was terminated with culture liquid. Adherent cells were detached with repeated pipetting, and were passaged at 1:2 dilution. The newly passaged cells were labeled as P1. Cell growth was observed each day until 100% confluency was reached, at which point the above procedure was repeated up to the eighth generation.

Induction and differentiation of MSCs

Eighth generation MSCs were added to 6-well plates with preset glass bottoms, and were cultured for three days. Cells were treated and grouped as follows: RGO (PLA Pharmacological Experiments Base, General Hospital of Lanzhou Area Command of PLA, Lanzhou, China) treatment group (final concentration = 100 μg/mL), 5-aza (Sigma-Aldrich, St. Louis, MO, USA) treatment group (final concentration = 10 μM), RGO + 5-aza group

(final concentration = 100 µg/mL, 10 µM, respectively), and control group (PBS). For each group, we treated 12 wells for 24 h, after which the medium was replaced. We continued to culture these cells and observed changes in cell morphology for four consecutive weeks; medium was changed every three days.

Determination of myocardial biochemical markers

Following a four-week induction culture period, cells on 6-well plates from each group were washed twice with cold PBS (3 mL), and 0.5 mL cell lysis buffer was added. Cells were scraped off with a cell scraper and by repeated pipetting, collected in 1.5-mL Eppendorf tubes, and centrifuged at 12,000 rpm for 15 min at 4°C. After centrifugation, the supernatant was transferred to another Eppendorf tube. Cardiac troponin I (cTnI) (Santa Cruz, Dallas, TX, USA) expression was detected by chemiluminescence; the myocardial enzymes, creatine kinase (CK) and CK isoenzyme-MB (CK-MB), were detected using a dry chemistry analyzer.

Immunofluorescence

Four weeks following induction and culture, cells were permeabilized with 0.3% Triton X-100, fixed in 4% paraformaldehyde at room temperature for 20 min, and washed three times with PBS for a total of 15 min. Anti-cTnI and anti-connexin 43 (Cx43) antibodies were added; PBS was used as the negative control. Samples labeled with primary antibodies were placed in a wet box and incubated overnight at 4°C. They were washed three times with PBS for a total of 15 min the next day. FITC-labeled goat anti-rabbit secondary antibody (ZSGB-BIO, Beijing, China) was then added, and the samples were incubated for 1 h at 37°C in a wet box. Cells were washed three times with PBS for a total of 15 min, and were labeled with DAPI (25 µg/mL) for 10 min. Lastly, cells were washed (PBS, three times), sealed with 50% buffered glycerol, and imaged using a confocal laser microscope (LEXT-OLS3100).

Western blot

Following induction and a four-week culture, cells were washed with PBS and lysed. The lysate was placed on ice for 30 min and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was transferred to a new Eppendorf tube, and total protein was quantified by the Bradford method. Using myocardial cells of neonatal rats as a positive control, protein from these cells was extracted and quantified according to the Bradford method. SDS protein electrophoresis buffer (6X) was added to sample aliquots, which were then heated for 5 min at 100°C. Samples (40 mg) were loaded onto SDS-PAGE gels and electrophoresed. Proteins were transferred onto polyvinylidene fluoride membranes (GE Healthcare, Pittsburgh, PA, USA) for 60 min (150 mA) and blocked with 15 mL TBST at 4°C overnight. The primary antibodies, anti-cTnI and anti-Cx43 (Boster, Wuhan, China), were added at concentrations of 1:2,000 and 1:1,000, respectively. Blots were washed five times (25 min in total), and the secondary antibody was added at a concentration of 1:2,000. The membrane was gently shaken at room temperature for 1 h. Lastly, the membrane was washed five times (25 min in total), and was imaged via chemiluminescence (KPL, Gaithersburg, MD, USA). Protein levels were quantified using semi-quantitative analysis.

Statistical analysis

Analysis was performed with the SPSS 13.0 statistical software. Data are reported as means ± SD. Differences between groups were compared using single-factor analysis of variance. Statistical significance was defined as $P < 0.05$.

RESULTS

Morphological characteristics of MSCs

Following a 24-h culture period, the majority of primary MSCs had adhered to the plate. Three to four days later, colony growth was observed, and cells exhibited short and fusiform morphology. Cells were 100% confluent after 7-10 days of culture. In addition, cells were completely adherent 24 h following passaging, and were mostly fusiform or fibrous in morphology. An increase in cell volume was observed 72 h following passaging.

Effect of RGO on myocardial biochemical markers in MSCs

After RGO, 5-aza, or RGO + 5-aza treatments, the expressions of cTnI, CK, and CK-MB significantly increased as compared to that of the control ($P < 0.05$). There was no significant difference in the levels of cTnI, CK, and CK-MB between the RGO and the 5-aza group ($P > 0.05$). However, CK and CK-MB levels were significantly higher in the RGO + 5-aza group as compared with the RGO- and 5-aza- only groups ($P < 0.05$). While cTnI level slightly increased in the RGO + 5-aza group as compared to the level in the RGO and 5-aza groups, this difference was not statistically significant ($P > 0.05$, Table 1).

Table 1. Effect of RGOs on the expression of cardiac MSC biochemical markers.

Groups	CtnI (ng/mL)	CK (U/L)	CK-MB (U/L)
RGOs	0.17 ± 0.03*	96.33 ± 8.11*#	74.7 ± 6.40*#
5-aza	0.18 ± 0.02*	104.4 ± 4.07*#	81.93 ± 6.44*#
RGOs + 5-aza	0.24 ± 0.04*	115.7 ± 4.20*	95.3 ± 5.15*
Control	0.013 ± 0.006	0.83 ± 0.15	0

*$P < 0.05$ as compared with control group; #$P < 0.05$ as compared with the 5-aza + RGOs group. All data are reported as means ± SD (N = 3).

Immunofluorescence of induced MSCs

No cTnI- or Cx43-positive cells were observed in the control group, in which MSCs were not induced to differentiate; both cTnI- and Cx43-positive cells were observed in the RGO, 5-aza, and RGO + 5-aza groups. Fluorescent signals were found to be higher in the RGO + 5-aza group than in the RGO and 5-aza groups (Figure 1).

Western blot of induced MSCs

Expressions of cTnI and Cx43 were not observed in control MSCs; the expressions of cTnI and Cx43 were the highest in the positive control group (normal neonatal rat cardiac

myocytes). MSCs in the RGO, 5-aza, and RGO + 5-aza groups expressed both cTnI and Cx43. Similar to previous results, expressions of cTnI and Cx43 were higher in the RGO + 5-aza group than in the RGO- and 5-aza-only groups (Figure 2).
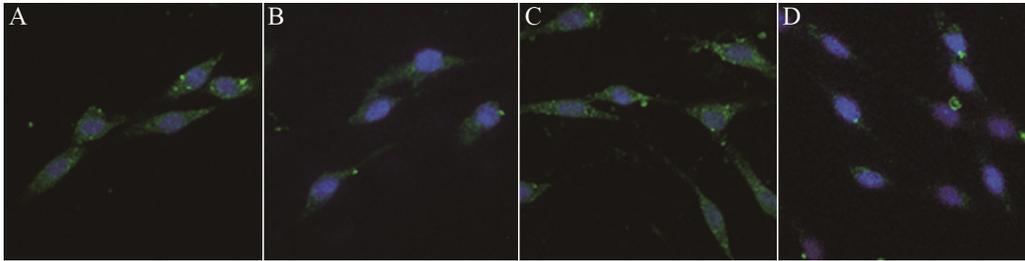

Figure 1. Immunofluorescence identification of induced MSCs (1000X). **A.** RGO group; **B.** 5-aza group; **C.** RGO +5-aza group; **D.** blank control group.
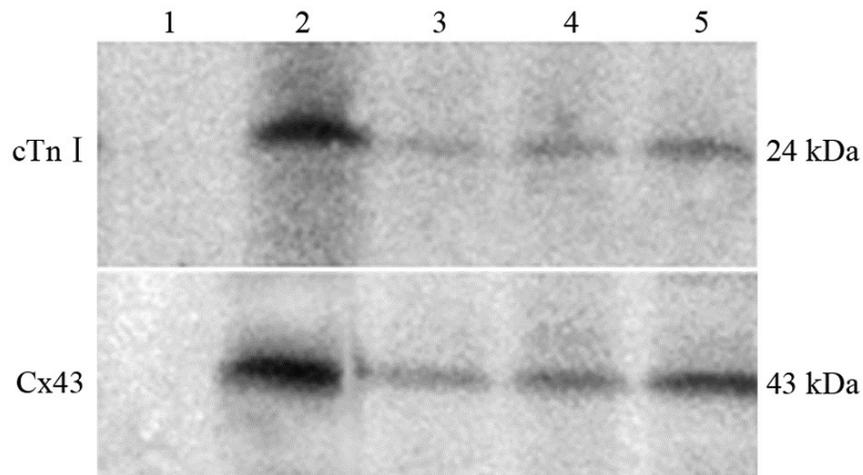

Figure 2. Expression of myocardium-specific proteins detected by western blotting. *Lane 1* = blank control group; *lane 2* = positive control group; *lane 3* = RGO group; *lane 4* = 5-aza group; *lane 5* = RGO + 5-aza group.

DISCUSSION

Injuries to the myocardium can induce myocardial remodeling, which is associated with cellular necrosis that may eventually result in heart failure. Current treatments for heart failure are limited, and cell transplantation has the potential to represent a new form of treatment. MSCs are multipotent cells that have the ability to differentiate into diverse cell types. In addition, they secrete a variety of angiogenic, anti-apoptosis, and mitogenic factors via paracrine effects (Nagaya et al., 2005; Nagaya and Kitamura, 2008). It has been shown that MSCs are able to maintain their multipotency in primary cultures as well as following cell

passaging. For example, Deans and Moseley (2000) reported that MSCs could maintain their multipotency *in vitro* even after 40 generations.

Recent data show that MSCs also have immunomodulatory functions (Uccelli et al., 2006; Ramasamy et al., 2008), mainly through the release of soluble cytokines such as prostaglandin E2 (Manferdini et al., 2013), transforming growth factor β, human leukocyte antigen G5, and matrix metalloproteinase (De Miguel et al., 2012). Following transplantation, upregulation of $CD4^+/CD25^+$ regulatory T cells play a significant role in inducing immune tolerance of the graft (Ge et al., 2013). This can be achieved by modulating T cell proliferation as well as by regulating the type and quantity of T cell secretions. At present, MSCs provide a promising option (Selem et al., 2013) in the treatment of dilated cardiomyopathy. In rats with myocardial infarctions, it has been shown that the earlier the MSC transplantation was performed, the better the recovery of heart function was. Analysis of reduction in ventricular remodeling demonstrated that the effect of MSC on heart transplantation was dose dependent (Richardson et al., 2013). Due to their therapeutic potentials, MSCs are one of the most widely studied types of adult stem cells at present.

RGO is a popular traditional Chinese medicine extracted from *R. glutinosa* (Park et al., 2005). RGO can inhibit the progression of experimental thrombosis and atherosclerosis, and can act as an antioxidant (Park et al., 2006; Yu et al., 2006a,b). Previous studies have shown that RGO extract improves glucose metabolism, and can prevent and ameliorate pathologies arising from diabetes (Yokozawa et al., 2004). Other studies have also demonstrated that it improves insulin resistance of HepG2 cells. A more recent study found that it promotes adipose-derived MSC proliferation through VEGF and HGF paracrine signaling, which may reduce H_2O_2-induced MSC apoptosis (Zhang et al., 2012). Preliminary results from our lab suggested that RGO promotes the proliferation of bone marrow MSCs *in vitro* at an optimal concentration of 100 μg/mL. In this study, we found that the levels of cTnI, CK, and CK-MB were elevated in MSC lysates treated with RGO. Moreover, MSCs in the RGO + 5-aza group exhibited higher levels of cTnI, CK, and CK-MB as compared with the RGO and 5-aza groups. Immunofluorescence results indicated that following induction, MSCs expressed differential levels of cTnI and Cx43; the expression was highest in the RGO + 5-aza group. Western blot results confirmed the elevated cTnI and Cx43 expressions following induction, and showed that cTnI and Cx43 levels were the highest in the RGO + 5-aza group. Our study suggested that MSCs are able to adopt the characteristics of cardiac muscle cells following induction. The ability of RGO and 5-aza to induce cardiomyogenic transformation was confirmed at the protein level. MSCs induced by a combination of RGO and 5-aza exhibited the highest expression of cardiac-specific proteins, suggesting that RGO and 5-aza act synergistically to induce more robust differentiation of MSCs into cardiomyocyte-like cells. 5-aza is a DNA methylation inhibitor; it has been widely used to differentiate MSCs into cardiomyocyte-like cells, and the optimal induction dose was determined to be 10 μM (Makino et al., 1999; Tomita et al., 1999). The mechanisms underlying 5-aza induction may be associated with reduction of DNA methylation in cells. However, 5-aza is potentially toxic and may induce cell apoptosis. Our studies demonstrated that RGO could induce differentiation of MSCs into cardiomyocyte-like cells, however, the mechanisms underlying this process are still unclear. A previous study showed that RGO could improve myocardial function by scavenging oxygen free radicals, and can enhance the proliferation of hematopoietic progenitor cells by promoting the production of colony-stimulating factor. It remains to be confirmed whether differentiation of MSCs into cardiomyocyte-like cells by RGO is due to its ability to induce secretion of certain regulatory factors by MSCs.

This study demonstrated that RGO could induce MSCs to differentiate into cardiomyocytes, suggesting that it may be used as a supplemental therapy to MSC transplantation in the treatment of cardiovascular diseases. To conclude, we have provided a novel paradigm for the treatment of diseases that combines traditional Chinese medicine with cell engineering.

Conflicts of interest

The authors declare no conflict of interest.

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