Tenascin-C promotes the migration of bone marrow stem cells via toll-like receptor 4-mediated signaling pathways: MAPK, AKT and Wnt

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Abstract. There are currently limitations in stem cell therapy due to the low rate of homing and proliferation of cells following transplantation. The present study was designed to investigate the effects of Tenascin-C (TN-C) on bone marrow mesenchymal stem cells (BMSCs) and its underlying mechanisms. BMSCs were obtained from C57BL/6 mice. The survival and proliferation of BMSCs was analyzed by Cell Counting Kit-8 assay, migration was evaluated using the Transwell method, and differentiation was assessed by immunocytochemistry and immunofluorescence. In addition, the levels of proteins were detected by western blotting. High concentrations of TN-C promoted the migration of BMSCs. H2O2 at concentrations of 60-90 μmol/ml induced cell death in BMSCs, and thus, it was used to simulate oxidative stress in the microenvironment of acute myocardial infarction (AMI). High concentrations of TN-C were able to protect BMSCs from cell death, and promoted the migration of BMSCs (P<0.05). However, TAK-242 [the inhibitor of Toll-like receptor 4 (TLR4)] reduced the promoting effect of TN-C (P<0.05). By contrast, TN-C had no effect on the proliferation and differentiation of BMSCs. TN-C reduced the phosphorylation levels of p38 mitogen-activated protein kinase (MAPK), and increased the phosphorylation levels of Ser473 protein kinase B (AKT) and β-catenin, all of which were inhibited by TAK-242 (P<0.05). In the simulated AMI microenvironment, TN-C promoted the migration of BMSCs via TLR4-mediated signaling pathways, including MAPK, AKT and Wnt.

Introduction

Many experiments have confirmed that transplantation of bone marrow mesenchymal stem cells (BMSCs) can improve damaged cardiac function after acute myocardial infarction (AMI) (1). However due to local inflammation, ischemia, hypoxia, and other factors, the homing and survival rates of BMSCs after transplantation are still very low (2). Therefore, in order for stem cell therapy to be effective, it is very important to increase the homing and survival rates of BMSCs (3).

Tenascin-C (TN-C) is an extracellular matrix glycoprotein, which is closely associated with inflammation and tissue injury (4). TN-C is detected at low levels in the healthy adult heart, but is more highly expressed under various pathological conditions, such as AMI, myocardial hibernation, myocarditis, and dilated cardiomyopathy. TN-C is secreted by the interstitial cells surrounding the infarcted myocardium in response to adverse conditions, such as ischemia, hypoxia, and other factors, and is involved in injury repair and formation of myocardial fibrosis. TN-C can thus be used as an independent predictor of left ventricular remodeling and long-term prognosis (5-7).

Apoptosis is a form of cell death that is generally triggered by normal, healthy processes in the body. Many factors, such as inflammation and injury, can increase the apoptotic rate of cells, which causes harm to the repair of tissue damage. As a result, excessive apoptosis should be inhibited (8-10).

To date, 11 human and 13 mouse Toll-like receptors (TLRs) have been identified. Recent studies indicate that BMSCs express functional TLRs, including TLR4 (11). When activated, TLRs affect many downstream signaling pathways, such as the mitogen-activated protein kinase (MAPK), AKT, and Wnt signaling pathways. These signaling pathways play important roles in the apoptosis, proliferation, and differentiation of many cells (12-17). However, it is yet unclear whether TN-C binds to TLR4 on the surface of BMSCs and activates some downstream signaling pathways, resulting in its biological effects.

We hypothesized that in the simulated AMI microenvironment, TN-C promotes the migration, proliferation, and
determination of BMSCs via TLR4-mediated signaling pathways, such as MAPK, AKT, and Wnt. This study was designed to investigate the effects of TN-C on BMSCs and elucidate its underlying mechanisms in vitro.

Materials and methods

Animals and preparation of BMSCs. C57BL/6 mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Colonies were subsequently established by in-house breeding at the Laboratory Animal Center of Dalian Medical University. The mice (the experimental mice as well as the breeding pairs) were all housed in a specific pathogen-free animal facility. Adult C57BL/6 male mice (weighing 20-30 g) were obtained from the Laboratory Animal Center of Dalian Medical University. Primary cells from C57BL/6 mice were isolated, cultured, and passaged according to a previously described method, with some modifications (18,19). Cells at passage F3-F4 were harvested at densities of 1 to 2x10^6 cells/ml and used for subsequent experiments.

The study was reviewed and approved by the Institutional Ethics Committee on Animal Resources of Dalian Medical University, and conformed to the guiding principles of the ‘Guide for the Care and Use of Laboratory Animals’ (NIH publication no. 83-23, revised 1996).

Cell Counting Kit-8 (CCK-8) assay. BMSCs were seeded at 5x10^4 cells/ml (100 µl/well) into 96-well plates and treated with different concentrations of TN-C (0, 0.1, 1, 10, 50, 100, and 150 µg/ml) for 48 h. The cell number was measured using a CCK-8 proliferation assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) following the manufacturer's instructions. The absorbance (optical density, OD) at 450 nm, representing the survival/proliferation of BMSCs, was determined using a microplate reader.

BMSCs were seeded at 5x10^4 cells/ml (100 µl/well) into 96-well plates and treated with different concentrations of H_2O_2 (60 or 90 µmol/ml) and TN-C (0, 1, 10, 50, 100, or 150 µg/ml) for 48 h. Altogether, there were 13 experimental groups that were treated with different concentrations of H_2O_2 and TN-C: H_2O_2 0 µmol/ml, TN-C 0 µg/ml; H_2O_2 60 µmol/ml, TN-C 0 µg/ml; H_2O_2 60 µmol/ml, TN-C 1 µg/ml; H_2O_2 60 µmol/ml, TN-C 10 µg/ml; H_2O_2 60 µmol/ml, TN-C 50 µg/ml; H_2O_2 60 µmol/ml, TN-C 100 µg/ml; H_2O_2 90 µmol/ml, TN-C 0 µg/ml; H_2O_2 90 µmol/ml, TN-C 1 µg/ml; H_2O_2 90 µmol/ml, TN-C 10 µg/ml; H_2O_2 90 µmol/ml, TN-C 50 µg/ml; H_2O_2 90 µmol/ml, TN-C 100 µg/ml; and H_2O_2 90 µmol/ml, TN-C 150 µg/ml. The cell number was analyzed as described above.

BMSCs pretreated with 1 µM TAK-242 (inhibitor of TLR4) were cultured with 60-90 µmol/ml H_2O_2 and 100-150 µg/ml TN-C for 48 h in a cell culture incubator (20). The cell number was analyzed as described above.

Transwell method. To investigate the effects of TN-C alone on BMSC migration, Transwell chambers with 8 µm pores were obtained from Corning Incorporated, (Corning, NY, USA). Pelleted BMSCs were resuspended in Dulbecco's modified Eagle's medium (DMEM) at a concentration of 3x10^5 cells/ml, and then seeded into the upper chambers of the 24-well plate. The lower chambers were filled with 500 µl DMEM at different final concentrations of TN-C (0, 0.1, 1, 10, 50, 100, and 150 µg/ml). Cells were then incubated for 12 h. At the end of the experiment, cells that migrated to the reverse side of the Transwell membrane were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet solution, and then counted under a light microscope at magnification, x100. An average of six visual fields was examined.

To investigate the effects of TN-C and H_2O_2 on BMSC migration, pelleted BMSCs were resuspended in DMEM as described above, and then seeded into the upper chambers of a 24-well plate. The lower chambers were filled with 500 µl DMEM supplemented with different final concentrations of H_2O_2 and TN-C (H_2O_2 0 µmol/ml, TN-C 0 µg/ml; H_2O_2 60 µmol/ml, TN-C 0 µg/ml; H_2O_2 60 µmol/ml, TN-C 10 µg/ml; H_2O_2 60 µmol/ml, TN-C 50 µg/ml; H_2O_2 60 µmol/ml, TN-C 100 µg/ml; H_2O_2 90 µmol/ml, TN-C 0 µg/ml; H_2O_2 90 µmol/ml, TN-C 10 µg/ml; H_2O_2 90 µmol/ml, TN-C 50 µg/ml; and H_2O_2 90 µmol/ml, TN-C 100 µg/ml) and incubated for 12 h. At the end of the experiment, cells that migrated to the reverse side of the Transwell membrane were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet solution, and then counted under a light microscope at magnification, x100. An average of six visual fields was examined. BMSCs pretreated with 1 µM TAK-242 for 2 h were treated with 90 µmol/ml H_2O_2 and 10-100 µg/ml TN-C for 12 h, and then treated as described above. All the above experiments were repeated at least three times independently.

ICC/IF staining. Pelleted BMSCs were resuspended in DMEM at a concentration of 1x10^5 cells/ml. Ten pieces of 10 mm cell sheets were disinfected with 75% alcohol, air-dried, and placed into a sterile 12-well plate. Then, 100 µl of the above BMSC suspension was directly seeded onto each piece of cell sheet, and cultured for 2 h under standard cell culture conditions. Following that, 1 ml DMEM was added to each well, and the cells were cultured for another 24 h. DMEM with different final concentrations of TN-C (0, 0.1, 1, 10, 50, 100, and 150 µg/ml) was then added to the cell sheets and they were returned to culture, with the culture medium replaced every 3 days. After 3 weeks, the cell sheet slices were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100 at room temperature, blocked with goat serum at 37°C for 1 h, incubated for 2 h with 50 µl 1:100 primary antibody against mouse α-actin (1 mg/ml) at 37°C, incubated for 1 h with 50 µl 1:200 fluorogenic secondary antibody (FITC-labeled, 1 mg/ml) at 37°C, and counter-stained for 1 min with 10 µl DAPI at room temperature. Cell differentiation was observed under
a fluorescence microscope. The nuclei were stained blue, whereas the α-actin was green.

As described above, the BMSC suspension was directly seeded onto each piece of cell scaffold, and then returned to the incubator for 2 h. Following that, 1 ml DMEM was added to each well and they were cultured for 24 h. Next day, DMEM with different final concentrations of H₂O₂ and TN-C, as described in section 2.4.2, was added to the cell scaffolds, and they were cultured and processed as described above. Finally, cell differentiation was observed under a fluorescence microscope.

Western blotting. BMSCs were treated with different factors (60 μmol/ml H₂O₂; H₂O₂ 60 μmol/l, TN-C 100 μg/ml; H₂O₂ 60 μmol/ml, TN-C 100 μg/ml, TAK-242) for 48 h; untreated normal cells were used as control. At the end of the treatment, the cytoplasmic proteins were extracted using a cell lysis solution (RIPA; PMSF: Phosphatase inhibitor=100: 1: 20), and the total protein and phosphorylated protein levels of p38 MAPK, AKT (Ser473), and β-catenin were then analyzed by western blot analysis as described previously (21,22).

Statistical analysis. All statistical analyses were performed using GraphPad Prism v5.0 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS v13.0 (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). All OD values, cell numbers, and protein levels were compared between two groups using one-way analysis of variance (ANOVA) with LSD analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of BMSCs. The cells obtained from the mouse bone marrow were CD29⁺ (96.9%), CD34⁺ (96.3%), CD44⁺ (40.9%), and CD45⁻ (0.7%), which confirmed that these cells were BMSCs (Fig. 1).

Effect of TN-C on the survival/proliferation of BMSCs. TN-C did not promote the proliferation of BMSCs: OD values of BMSCs treated with different concentrations of TN-C (0.1, 1, 10, 50, 100, or 150 μg/ml) were no higher than those of control BMSCs (P>0.05); however, OD values of BMSCs treated with high concentrations of TN-C (100 or 150 μg/ml) were lower than those of controls (P<0.05; Fig. 2A).

H₂O₂ reduced the survival rate of BMSCs: OD values of BMSCs treated with either 60 or 90 μmol/ml H₂O₂ were lower than those of control BMSCs (P<0.05), which showed that 60-90 μmol/ml H₂O₂ could cause BMSC death (Fig. 2B).

TN-C protected BMSCs from cell death caused by H₂O₂: OD values of BMSCs treated with 60 μmol/ml H₂O₂ together with high concentrations of TN-C (100 or 150 μg/ml) were higher than those of BMSCs treated with 60 μmol/ml H₂O₂ alone (P<0.05). OD values of BMSCs treated with 90 μmol/ml H₂O₂ together with high concentrations of TN-C (100 or 150 μg/ml) were also higher than those of BMSCs treated with 90 μmol/ml H₂O₂ alone (P<0.05; Fig. 2C and D).

TAK-242 reduced the protective effect of TN-C: OD values of BMSCs treated with TAK-242 were less than those of normal cells treated with the same concentrations of H₂O₂ and TN-C (P<0.05; Fig. 2E).

Effect of TN-C on the migration of BMSCs. Migrated BMSCs were observed under an inverted microscope at high magnification, x200. Long spindle-shaped or irregularly shaped cells stained purple with crystal violet were the migrated BMSCs. After treatment with different factors, including TN-C, H₂O₂, and TAK-242, the migrated BMSCs were observed and counted.

High concentrations of TN-C promoted the migration of BMSCs: High concentrations of TN-C (10-150 μg/ml) promoted the migration of BMSCs, whereas low concentrations of TN-C (0.1-1 μg/ml) showed no significant effect (Fig. 3A).

H₂O₂ promoted the migration of BMSCs: The number of migrated BMSCs in cultures treated with 60 or 90 μmol/ml H₂O₂ was greater than that in control untreated cultures (P<0.05), demonstrating that H₂O₂ promotes the migration of BMSCs. However, we found no significant difference between the groups treated with 60 or 90 μmol/ml H₂O₂ (P>0.05; Fig. 3B).

Combined treatment with TN-C and H₂O₂ further promoted the migration of BMSCs: When BMSCs were treated with either concentration of H₂O₂ (60 or 90 μmol/ml) together with different concentrations of TN-C (10, 50, or 100 μg/ml), increased numbers of migrated cells were observed compared to those in cultures treated with different concentrations of H₂O₂ alone (60 or 90 μmol/ml) (P<0.05). However, there was no significant difference among the groups treated with different concentrations of TN-C (10, 50, or 100 μg/ml) (P>0.05; Fig. 3C).

TAK-242 reduced the migration-promoting effect of TN-C: In cultures of BMSCs treated with 90 μmol/ml H₂O₂, different concentrations of TN-C (10 or 50 μg/ml), and 1 µM TAK-242, there were fewer number of migrated cells than in cultures treated with 90 μmol/ml H₂O₂ and different concentrations of TN-C (10 or 50 μg/ml) (P<0.05). However, in cultures of BMSCs treated with 90 μmol/ml H₂O₂, 100 μg/ml TN-C, and 1 µM TAK-242, the number of migrated cells was higher than that in cultures treated only with 90 μmol/ml H₂O₂ and 100 μg/ml TN-C (P<0.05; Fig. 3D).

TN-C was unable to promote differentiation of BMSCs. Staining for α-actin was negative in all culture groups, indicating that none of the conditions tested induced the BMSCs to differentiate into cardiomyocytes (Fig. 4A).

When the same experiment was performed in the presence of H₂O₂ (60 or 90 μmol/ml) to simulate oxidative stress in the microenvironment of AMI, TN-C was still unable to induce the differentiation of BMSCs into cardiomyocytes (Fig. 4B).

The effect of TN-C on MAPK, AKT, and Wnt signaling pathways. TN-C decreased the phosphorylation levels of p38 MAPK, which were inhibited by TAK-242: The phosphorylation level of p38 MAPK in the 60 μmol/ml H₂O₂ group was higher than that in the control group (P<0.05), whereas the phosphorylation level of p38 MAPK in the 100 μg/ml TN-C group was lower than that in the 60 μmol/ml H₂O₂ group (P<0.05). In contrast, the phosphorylation level of p38 MAPK in the TAK-242 group was higher than that in the 100 μg/ml TN-C group (P<0.05; Fig. 5A and B).

TN-C increased the phosphorylation levels of Ser473 AKT, which were inhibited by TAK-242: The phosphorylation level of Ser473 AKT in the 60 μmol/ml H₂O₂ group was lower than that in the control group (P<0.05), whereas the phosphorylation level of Ser473 AKT in the 100 μg/ml TN-C group was...
higher than that in the 60 μmol/ml H₂O₂ group (P<0.05). Furthermore, the phosphorylation level of Ser473 AKT in the TAK-242 group was lower than that in the 100 μg/ml TN-C group (P<0.05; Fig. 5C and D).

TN-C increased the phosphorylation levels of β-catenin, which were inhibited by TAK-242: The phosphorylation level of β-catenin in the 60 μmol/ml H₂O₂ group was lower than that in the control group (P<0.05), whereas the phosphorylation level of β-catenin in the 100 μg/ml TN-C group was higher than that in the 60 μmol/ml H₂O₂ group (P<0.05). Furthermore, the phosphorylation level of β-catenin in the TAK-242 group was lower than that in the 100 μg/ml TN-C group (P<0.05; Fig. 5E and F).

**Discussion**

Our results showed that TN-C acts in a dose-dependent manner to promote the migration of BMSCs. When H₂O₂ was added to the culture to simulate oxidative stress in the cardiac microenvironment after AMI, TN-C promoted the migration of BMSCs and protected them from cell death. However, TN-C had no effect on promoting the proliferation or differentiation of BMSCs. Investigation of possible signaling mechanisms indicated that TN-C bound to TLR4 expressed on the surface of BMSCs, and then activated the downstream signaling pathways, including MAPK, AKT, and Wnt.
Many signaling molecules and their ligands are involved in the migration of BMSCs to areas of damage. Among them, stromal cell-derived factor-1 (SDF-1) is, so far, the only known natural chemokine that can bind to and activate the CXC chemokine receptor type 4 (CXCR4) receptor (23-25). In rats, this interaction between SDF-1 and CXCR4 has been shown to play a key role in the homing of BMSCs to the infarct area (23). Here, we demonstrated that TN-C promotes the migration of BMSCs.
BMSCs in vitro, but it is unclear whether TN-C still exerts the same effect in vivo.

Our experiments showed that 60-90 μmol/ml H₂O₂ causes apoptosis of BMSCs. Different concentrations of TN-C were able to promote migration of BMSCs in the simulated oxidative stress environment of AMI, modeled in vitro by H₂O₂. However, it is unclear whether this effect would be reproduced in vivo, where many other factors are involved. Hence, further experiments are needed.

TLR4 is the best-studied immune sensor, which detects invading microbes. It is broadly distributed on cells throughout the immune system. It has been revealed that BMSCs also express functional TLR4 (26-33). Activation of TLR4 signaling in BMSCs has diverse effects and is likely to influence their survival, differentiation, proliferation, migration, and pro-inflammatory cytokine secretion ability (26,30,31). After AMI, ischemia leads to the activation of TLR4/MyD88-dependent and independent downstream pathways. Among these, activation of the MyD88-dependent signaling pathway is thought to mediate the response of the innate immune system, promote the rapid release of cytokines and inflammatory mediators in the heart tissue, and recruit inflammatory cells to the lesion sites for repair (34-36). Lipopolysaccharide serves as a specific ligand for TLR4, activating the TLR-4/MyD88 pathway to promote BMSC proliferation and reduce BMSC apoptosis, which suggests that the TLR4 pathway is involved in promoting the survival and proliferation of BMSCs (34-36).

In this study, we showed that TN-C (10 or 50 μg/ml) promotes BMSC migration, and this effect can be reduced by treatment with the TLR4 inhibitor, TAK-242. These results suggest that TN-C binds to TLR4 through which it exerts its effects. However, when the concentration of TN-C was 100 μg/ml, the migration of BMSCs was not reduced, but was promoted. The possible mechanism was that TN-C (100 μg/ml) could activate other receptors on the surface of BMSCs when TLR4 was inhibited by TAK-242. Further analysis of the downstream signaling pathways showed that TN-C reduced the phosphorylation levels of p38 MAPK, but increased the phosphorylation of both Ser473 AKT and β-catenin, and all of these effects could be inhibited by TAK-242. Taken together, these results demonstrate the possible mechanism of action of TN-C, wherein TN-C binds to TLR4 expressed on the surface of BMSCs, and activates the MAPK, AKT, and Wnt signaling pathways to exert its biological effects. This result is consistent with that reported in a previous study (28-33). There are many signaling pathways and proteins involved in the action of TN-C, but in this study, only the major signaling pathways and proteins were investigated. To further elucidate whether TN-C exerts its effects through the TLR4-mediated signal transduction pathways, more research is required.
There is a bottleneck in stem cell therapy due to the low homing and survival rates of stem cells after transplantation. Our results showed that TN-C promoted the migration of BMSCs as well as protected them from cell death. This study provides a new theoretical basis for improving the homing and survival rates of transplanted cells, which is very important for effective stem cell therapy.

When AMI occurs, a series of complex changes take place in the microenvironment of the infarct area, and hence the effect of $\text{O}_2$ alone cannot reflect the real situation in the body following AMI. As a result, in vivo experiments are crucial.

When AMI occurs, a series of complex changes take place in the microenvironment of the infarct area. Consequently, the effect of $\text{O}_2$ alone cannot reflect the real situation in the body, and as a result, in vivo experiments are necessary. However, addition of extracorporeal $\text{H}_2\text{O}_2$ to simulate oxidative stress in the microenvironment following AMI showed that TN-C reduces BMSC apoptosis and promotes the migration of BMSCs under these conditions. This finding provides a new theoretical basis for animal experiments.

In summary, TN-C acts in a dose-dependent manner to promote the migration of BMSCs in vitro. In the simulated AMI microenvironment, TN-C promotes the migration of BMSCs and protected them from cell death, but did not promote BMSC proliferation or differentiation. The possible mechanism suggested was that TN-C binds to TLR4 expressed on the surface of BMSCs, and then activates the downstream signaling pathways, such as MAPK, AKT, and Wnt. This study provides a new theoretical basis for improving the homing and survival rates of transplanted cells, which is very important for effective stem cell therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conception: HD, MJ and RH. Data curation: HD, MJ, DL, SW, JZ and RH. Experiments: HD, MJ, DL and RH. Analysis: HD, DL, XS and RH. Validation: HD, MJ, SW, XS and RH. Funding: RH. Project administration: HD, MJ, DL and RH. Original draft of manuscript: HD and MJ. Reviewing and editing of manuscript: HD and RH.

Ethics approval and consent to participate

The present study was reviewed and approved by the Institutional Ethics Committee on Animal Resources of Dalian Medical University (Liaoning, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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