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Superoxide Stimulation Enhances CXCR4 Expression in Heart Muscle-Derived Stem Cells via ASK1 Activation

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Abstract **Background:** Stem cell therapy for cardiac regeneration is hindered by poor homing to infarcted legion because of poor expression of homing factor, CXCR4, on the stem cell surface. A strategy for enhancing CXCR4 is promising to accelerate stem cell homing to infarcted lesion. **Methods and Results:** Heart muscle-derived Sca-1(+) cells (HDSCs) were isolated from normal mouse heart using surface antigen Sca-1. The expression of CXCR4 was determined by the fluorescence-activated cell sorting by labeling the cells with CXCR4 antibody with fluorescence. The expression of CXCR4 was enhanced by stimulation of hydrogen peroxide (0.1, 0.33, 1μM) for 24h. The Western blotting showed no significant increase in the protein level of CXCR4. The enhancement of CXCR4 can be attributed to translocation of CXCR4 from the cytosolic fraction to cell surface. H_2O_2 stimulation enhanced ASK1 p966 phosphorylation. **Conclusions:** The expression of CXCR4 on HDSCs was augmented by H_2O_2 through the translocation from cytosolic to membrane surface through ASK1 phosphorylation.

Key words: stem cells, CXCR4, superoxide, heart muscle

Introduction

Cell transplantation therapy has been a promising therapeutic strategy for the treatment of heart failure caused by either ischemic or non-ischemic etiology. The potential cell sources for heart regeneration are fetal cardiomyocytes,[1,2] and embryonic stem cell-derived cardiomyocytes,[3] but these cell sources are problematic because of the ethical view point. Skeletal myoblasts,[4] bone marrow-derived stem cells,[5,6] and heart-derived stem cells,[7-9] can be other candidates and has been applied for the regeneration therapy in ischemic and non-ischemic heart failure patients. Although the results are promising, the low efficiency of the stem cell differentiation into cardiomyocytes is a problem to be solved. The observed improvement of the ejection fraction in these studies can be attributed to the paracrine effect of the growth factors or cytokines secreted from the transplanted stem cells. In the case of the injection of skeletal myoblasts which does not differentiate into cardiomyocytes,[10] the lack of gap junction without expression of Cx43 may cause lethal arrhythmias after cell transplantation.[11,12] Bone marrow-derived stem cells improved cardiac function in animal models[5,6], but most of the transplanted cells did not regenerate cardiomyocytes.[13,14] Heart-derived stem cells identified using stem cell markers such as Sca-1, c-kit and Isl-1 can differentiate into cardiomyocytes.[7-9] These cells injected into

infarcted mice may home to the impaired cardiomyocytes.[7] This is caused by the function of CXC chemokine receptor 4(CXCR4)-CXC chemokine stromal cell- derived factor 1(SDF1) interaction.[15] However, the expression of CXCR4 on the heart -derived stem cells are scarce, and this may hinder the homing and repopulation of the implanted stem cells in the infarcted area. Tang et al. showed that the ischemic preconditioning enhanced the expression of CXCR4 in the heart-derived progenitor cells.[16] Here, we tested a new strategy to improve CXCR4 expression on the cell surface and the underlying signal transduction has been explored.

Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised at 1996).

Animals The C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan) and bred in the Animal Center of Yamaguchi University.

Isolation and Culture of Heart-Derived Sca-1(+) Cells

Heart-derived Sca-1(+) cells (HDSC) were isolated using a magnetic cell sorting system (MACS, Miltenyi Biotec) as described previously[7]. Briefly, C57BL/6 mice (8 weeks old) were anesthetized with sodium pentobarbital (50mg/kg, intraperitoneal) and euthanized by neck translocation. Heart was dissected, and minced with scissors on ice-cold dish with saline solution. The minced heart tissue was digested in the solution of 0.4% type II collagenase (Worthington Biochemical, USA) for 30 min at 37℃ with agitation. After the filtration with a 40 μm mesh filter (Falcon, USA), anti-Sca-1 antibody conjugated with biotin (1:100) was added to the cell mixture for 15 minutes on ice. Then, anti-biotin microbeads (1:4) were added for 10 minutes at 4℃ and the cell mixture was sorted with magnetic cell separation columns twice. The sorted cells were cultured in DMEM culture medium with 10% FBS, antibiotics, and growth factors such as PDGF-bb (10 ng/ml, R & D, USA), EGF (10 ng/ml, Sigma, USA), and LIF (2.5 ng/ml, Sigma, USA) at 37℃ with 5% CO_2.

Flow Cytometry

Antibodies raised for Sca-1 (Becton Dickinson, USA) were added to the freshly isolated HDSC (20,000 cells) suspended in 200 μl of PBS (1:100). Isotype-specific negative IgG was used as a negative control. Surface antigens were detected with fluorescence-activated cell sorting (FACS, FC500, Coulter, USA). FACS analysis showed that the Sca-1 positivity of freshly-isolated HDSCs was 92 ± 2%.

Western blotting

Cultured HDSCs were lysed with RIPA buffer containing a protease inhibitor cocktail (Roche, IN, USA) in a glass homogenizer on ice. Homogenates were mixed with an equal volume of lysis buffer and centrifuged at 1,000 g for 20 min. The supernatant was used to measure protein concentration by the Bradford method. An equal amount of protein from each group (50 μg) was resolved by electrophoresis on a 10% polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane. Non-specific binding was blocked by immersing the membrane in the solution with 5% non-fat milk diluted in Tris-buffered saline for 1h. The membrane was then incubated with primary antibodies against CXCR4, ASK1 at 4℃ overnight. Blots were then incubated with secondary antibody at room temperature for 2hs. Bands were visualized by enhanced chemiluminescence (ECL) and quantified using a Lumino image analyzer (LAS-1000; Fuji Film, Japan). Band density was expressed as a percentage of control.

CXCR4 expression on cell surface

HDSCs cultured for 2 weeks were trypsinized and washed by centrifugation and resuspended in 6-well culture plate (Corning 3516, USA) at a density of 2x 10^5 cells per well in DMEM supplemented with 0.5% FBS. In the control group cells were cultured without H_2O_2 for 24hs; in the H_2O_2 group cells were cultured for 24hs with three doses of H_2O_2 (0.1, 0.3, 1 μM). The cells were isolated by trypsinization and washed by centrifugation and used for

FACS analysis. The number of experiment was 4 for each group.

ASK1 phosphorylation

7x 10^5 cultured HDSCs were used for each group. The effect of 24h H_2O_2 exposure on protein level of ASK1 and the phosphorylation of ASK1 at serine 83 and serine 966 were analyzed by Western blotting. (N=4)

Statistics

Data are expressed as means ± SD. The difference between the groups was analyzed by ANOVA and when the significant difference was detected, Scheffe's multiple comparison was used. P value less than 0.05 was considered significant.

Results

Cell surface expression of CXCR4

In the control group, the cell surface expression of CXCR4 was 11.9 % ± 4.7% (Fig. 1a). In the H_2O_2 (0.33 μM) exposed group, the expression of CXCR4 was increased to 29.2 % ± 5.8% ($P<0.05$ vs. control, Fig. 1b). The expression of CXCR4 was not dose dependent between 0.1 to 1.0 μM (data not shown).

Changes in CXCR4 protein level

CXCR4 protein level was not changed after 24h H_2O_2 exposure. The expression of ASK1 protein level was not significantly changed after 24h H_2O_2 exposure. However, the phosphorylation of ASK1 p966 was significantly increased without change in ASK1 p86. (Figs. 2 and 3).

Discussion

Cardiac-derived stem cells can be collected by using surface marker, Sca-1[17] and the cells were shown to differentiate into cardiomyocytes.[18-19] But the cell-therapy using these cells are not so potent because of the low expression of CXCR4 which accelerates the cells home to the injured lesion.[20-21] Thus, the methods to enhance the expression of CXCR4 on these cells are required. In this study we found that 24h H_2O_2 exposure can increase the cell surface expression of CXCR4 on HDSCs. Furthermore, we clarified that the increase in cell surface expression of CXCR4 were not accompanied by the increase in CXCR4 protein, but the ASK1 phosphorylation at serine 966 was augmented. To the best of our knowledge, this is the first evidence

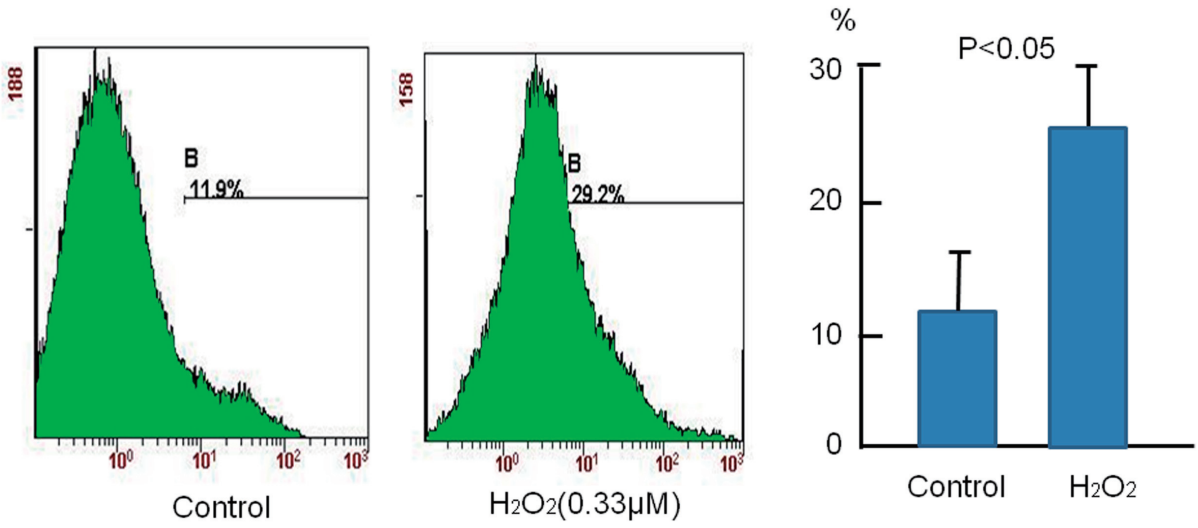

Fig. 1a

Typical example of CXCR4 expression on the cell surface detected by fluorescence-activated cell sorting (FACS). 0.33 μM H_2O_2 increased the expression of CXCR4. b, Results of the FACS analysis about CXCR4 positivity. H_2O_2 significantly increased CXCR4 expression ($P<0.05$, N=4). Data represent the mean ± SD.

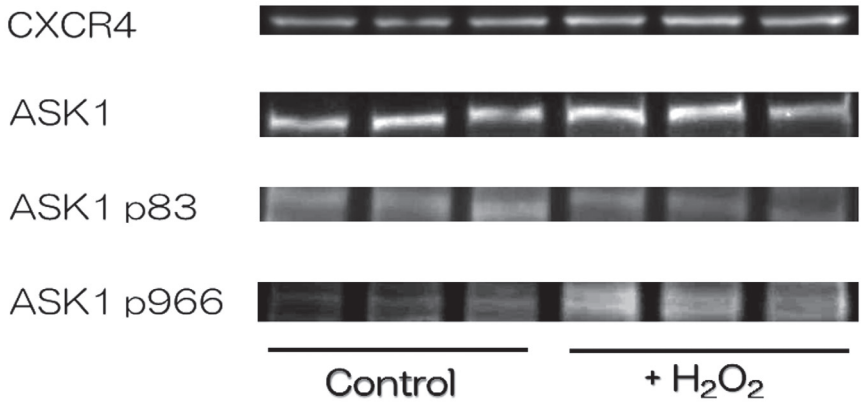

Fig. 2
Western blotting of CXCR4, ASK1, ASK1 p83, and ASK1 p966 before and after H_2O_2 exposure. ASK1 p966 increased after H_2O_2 exposure.

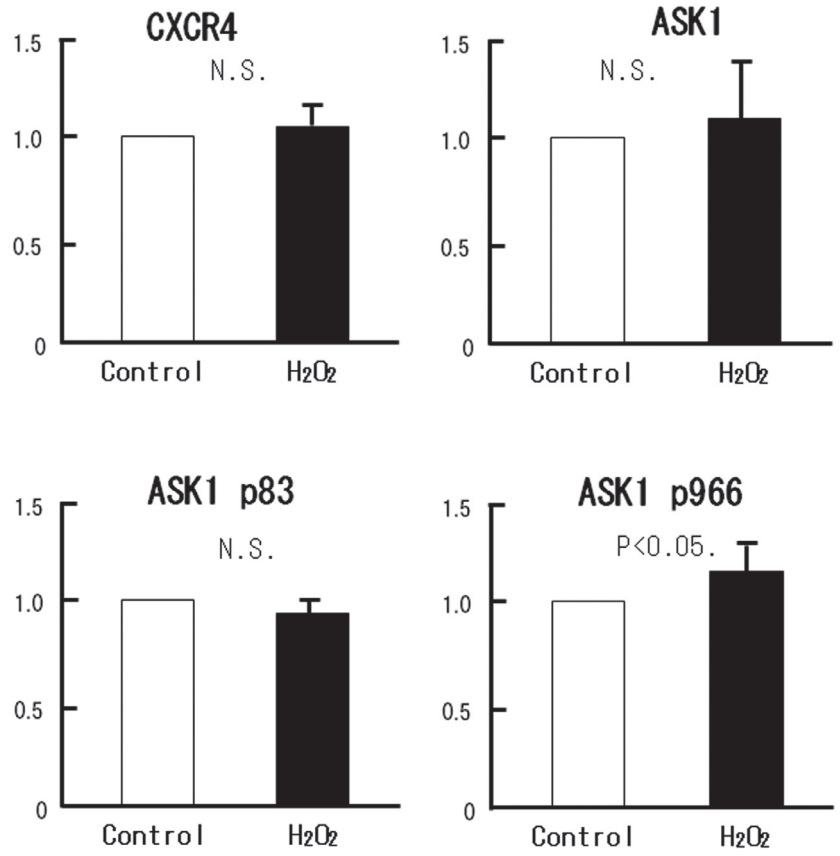

Fig. 3
Results of Western blotting of CXCR4, ASK1, ASK1 p83 and ASK1 p966. Data are expressed as the ratio to control. ASK1 p966 significantly increased after H_2O_2 exposure. Data represent the mean ± SD.

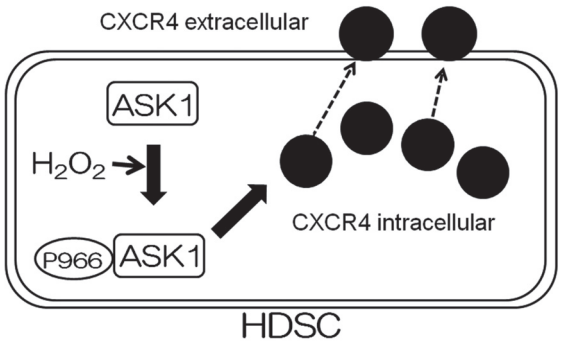

Fig. 4
Schematic explanation of translocation of CXCR4 molecules from intracellular to extracellular membrane caused by H_2O_2 via ASK1 phosphorylation.

that the increase in cell surface CXCR4 induced by H_2O_2 is attributed to the translocation of CXCR4 from inside the cell to the cell surface membrane mediated by the phosphorylation of ASK1 p966. To strengthen our results, the surface biotinylation assay should be performed in the future studies. The H_2O_2-ASK1 pathway is known as the pro-apoptosis pathway connected to the downstream signaling pathway including JNK and P38 MAPK.[22-25] However our study showed that the JNK and P38MAPK was independent of the CXCR4 translocation (data not shown), and suggested other pathway such as PKD1 may be involved in the phosphorylation of ASK1 p966.[21] This pathway remains to be elucidated in the further study. Previous studies have suggested that the increase in CXCR4 expression accelerated the homing of the stem cells to the injured lesion and promoted the regeneration.[26-32] We need to examine the in vivo effect of the increased expression of the CXCR4 caused by H_2O_2 on the extent of regeneration of infarcted mice model.

Limitation

Although the low concentration of H_2O_2 can increase CXCR4 expression on the cell surface, the high dose of H_2O_2 can cause cell death either by apoptosis or necrosis.[22-25] In our study, the extent of apoptosis was not checked, but 0.33 μM H_2O_2 may not be so pro-apoptotic, and suitable for the induction of CXCR4 on cell surface. The addition of H_2O_2 inhibitor may further strengthen our study, and we will do this in the future studies. The downstream pathway related to ASK1 p966 has not been clarified. This should be further clarified . In conclusion, low dose H_2O_2 exposure can improve expression of CXCR4 on the cell surface of HDSC through the phosphorylation of ASK1 p 966.

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