Nitric oxide functions in stromal cell-derived factor-1-induced cytoskeleton changes and the migration of Jurkat cells

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Abstract. Stromal cell-derived factor-1 (SDF-1) regulates multiple cell signal pathways in a variety of cellular functions, including cell migration, proliferation, survival and angiogenesis. SDF-1-induced chemotaxis is an important step of lymphocyte migration. However, the molecular mechanisms that modulate SDF-1-mediated lymphocyte migration are not well identified. Nitric oxide (NO) has been found to function as a signaling molecule in a number of signaling pathways, including migration. In the present study, the potential role of NO in SDF-1-induced migration and the association between NO and the cytoskeletal changes of Jurkat cells was investigated. The present study demonstrated that Jurkat cells induced the production of NO by SDF-1 stimulation, using Griess reaction method and western blot analysis, and that NO was involved in SDF-1-induced rearrangement and polymerization of the cytoskeleton, using NOS inhibitor L-NMMA. Furthermore, NO was required for the migration of Jurkat cells. The research suggested that NO signaling pathways exerted a critical role in SDF-1-induced cytoskeleton changes and the migration of Jurkat cells. This work provides insight into the migration mechanism of acute lymphoblastic leukemia and provides an effective theoretical basis for therapy strategies for acute lymphoblastic leukemia.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a highly invasive blood cancer and is a serious threat to human health. As a common T lymph cell malignancy, T-ALL occurs in adults and children and is characterized by rapid progression and high mortality (1). Despite the development of multi-agent chemotherapy, radiotherapy and hematopoietic stem cell transplantation, ~15% of pediatric and 40% of adult T-ALL patients relapse due to primary resistance to treatment (2-4). The 5-year event-free survival rate increases with age, from 44% for those <12 months to 88% for those aged 1-9 years (5-7). Babies younger than 6 months have a poor outcome (8,9); therefore, the search for novel specific treatment targets to enable a more effective targeted therapy of T-ALL is urgently required.

Cell migration is a crucial process for the deterioration of T-ALL. Stromal cell-derived factor-1 (SDF-1) is a small chemokine that regulates the mobilization, retention, migration, trafficking and homing of hematopoietic stem cells and lymphocytes (10,11). An increasing amount of evidence has indicated that chemotaxis signaling serves a crucial role in the migration of cancer cells (12-15). Chemokines serve roles as signal initiators and are involved in changes of the actin cytoskeleton (16), which are required for cellular morphological changes and motility (17). SDF-1 can induce changes to cell behavior, including changes to cellular morphology and the regulation of cellular motility (18) by modulating cytoskeletal redistribution and assembly (19).

Nitric oxide (NO) is a multifunctional signaling molecule that mediates different signaling pathways and regulates various cellular functions, including vasodilatation, neurotransmission, macrophage-mediated immunity, chemotaxis and cell migration (20-23). NO has been demonstrated to regulate migration in several types of cells, including human neutrophils, endothelial cells, vascular smooth muscle cells, and T cells (24-28). However, the mechanism of NO in T-ALL migration remains poorly understood.

The present study used Jurkat cells, a typical T-ALL cell line to more carefully investigate the role of NO in T-ALL migration. An aim of the present study was to provide further insight into the role of NO in the regulation of chemokine-induced migration in T-ALL.

Materials and methods

Chemicals and reagents. RPMI-1640 (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 100 U/ml penicillin and 100 μ/ml streptomycin (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China), fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), SDF-1 (R&D Systems, Inc., Minneapolis, MN, USA), and nitric oxide synthase inhibitor N^G-monomethyl-l-arginine monoacetate salt

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(L-NMMA, Beyotime Institute of Biotechnology, Haimen, China) were obtained and used without further modification. Total Nitric Oxide assay kit was from the Beyotime Institute of Biotechnology (cat. no. S0021). Anti-NOS2 (Phospho-Tyr151) rabbit polyclonal antibody (NOS2-pY151; cat. no. D151565) and anti-NOS3 (Phospho-Thr494) rabbit polyclonal antibody (NOS3-pY494; cat. no. D151279) were obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

Cell culture. Jurkat cells from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 25 ng/ml SDF-1 and 1 mM L-NMMA in a humidified incubator with 5% CO_2 at 37˚C. A total of 1 mM L-NMMA was dissolved in double distilled water and cells were pre-treated with L-NMMA for 2 h. Jurkat cells were starved with 0.5% serum overnight in all experiments.

Total nitric oxide assay. Total nitric oxide production in the culture medium was determined by measuring the concentration of nitrate and nitrite, a stable metabolite of NO, by the modified Griess reaction method (29,30). The procedure was performed according to the manufacturer's protocol of the Total Nitric Oxide assay kit (Beyotime Institute of Biotechnology). RPMI-1640 medium was used as the control for SDF-1.

Western blot assay. Cells were stimulated at 37˚C for 0, 1, 2, 3 or 4 h and lysed in ice-cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM each NaF, Na3VO4, and β-glycerolphosphate, 10 μg/ml aprotinin and leupeptin). After 30 min on ice, lysates were centrifuged at 13,000 x g for 30 min. Protein concentration was determined by the BCA kit (cat. no. P0010) from Beyotime Institute of Biotechnology. A total of 20 μg lysate from each sample were subjected to 10% SDS-PAGE. Proteins were transferred to the nitrocellulose membranes using chilled transfer buffer (25 mM Tris, 192 mM glycerin, and 20% methanol) at 100 V for 1 h. Following protein transfer, nitrocellulose membranes were washed with TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for ≥3 times, and were immediately incubated with 3% bovine serum albumin (BSA, cat. no. SW3015) from Beijing Solarbio Science and Technology Co., Ltd., Beijing, China. After the incubation for 1 h at room temperature, the membrane was incubated with primary antibodies NOS2-pY151 and NOS3-pY494 respectively diluted at a ratio of 1:1,000 in TBST at 4˚C overnight, washed with TBST 3 times at room temperature, and incubated with HRP-conjugated goat anti-rabbit secondary antibody (cat. no. D110058; Sangon Biotech Co., Ltd., Shanghai, China) diluted at a ratio of 1:1,000 in TBST at 37˚C for 1 h. For the detection of the reference gene, mouse monoclonal antibody against actin (cat. no. D190606; Sangon Biotech Co., Ltd., Shanghai, China) and HRP-conjugated goat anti-mouse IgG (cat. no. D110087; Sangon Biotech Co., Ltd., Shanghai, China) were used. Chemiluminescent detection was performed by using ECL plus Western blot reagents (BeyoECL Plus; cat. no. P0018; Beyotime Institute of Biotechnology). Western blot assay was repeated three times, analyzed by image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA), and expressed as fold increase relative to the basal level in unstimulated cells.

Cell viability assay. The MTT assay was employed to quantify cell viability. Jurkat cells were washed once with RPMI-1640, and ~$3x10^5$ cells were seeded to each sample. The cells were then cultured at 37˚C for 6 h with different concentrations of L-NMMA (0, 0.25, 0.5, 1, 2 and 4 mM). At the end of the treatment, MTT reagent (5 mg/ml, 20 μl) was added to each sample and cells were incubated at 37˚C for 3 h, DMSO (200 μl) was then added to each sample, in order to dissolve the cell precipitation. A total of 150 μl liquid was transferred to the well of a96-well plate and the optical density value of each well was determined using a plate reader at 570 nm.

Immunofluorescence assay. To detect the distribution of F-actin, Jurkat cells were pre-treated with L-NMMA (1 mM) at 37˚C for 2 h, and then stimulated with SDF-1 at 37˚C for 4 h. At 4 h time, chilled 500 μl PBS was added to cells to rapidly stop the reaction. Jurkat cells were then fixed with 4% paraformaldehyde for 20 min at room temperature. After washing twice with PBS, the Jurkat cells were permeabilized with 0.2% Triton X-100 in PBS (containing 5mM EDTA and 2% FBS) for 10 min. Following washing twice with PBS, the cells were stained with $2x10^{-7}$ M Fluorescein isothiocyanate (FITC)-conjugated phalloidin at room temperature for 20 min, washed three times with PBS, and then observed under a confocal microscope at x600 magnification.

Flow cytometry. The amount of F-actin in cells was examined by flow cytometry. Following stimulation with SDF-1, Jurkat cells were fixed with 4% paraformaldehyde at room temperature for 20 min, permeabilized with 0.2% Triton X-100 in PBS (containing 5 mM EDTA and 2% FBS) for 10 min, blocked in 1% BSA (cat. no. SW3015) from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China) in 0.05% Tween 20 and PBS for 30 min at room temperature, stained with $2x10^{-7}$ M FITC-conjugated phalloidin (cat. no. P5282-.1MG; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 20 min, and then washed with PBS. Cells were examined by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA), analyzed by FlowJo 7.6 software (TreeStar, Inc., Ashland, OR, USA), and the results were expressed as the relative fluorescence index (RFI) by dividing the fluorescence value of the experimental groups by that of the control group. RPMI-1640 medium was used as the control for SDF-1.

Transwell migration assay. The migration assay was performed using 3.0 μm Transwell inserts in 24-well culture plates. The Jurkat cells were resuspended in serum-free RPMI-1640 at a density of $3x10^6$ cell/ml and 100 μl cell suspensions were pre-treated with L-NMMA (1 mM) at 37˚C for 2 h and seeded onto the upper chambers. Next, 500 μl of RPMI-1640 supplemented with SDF-1 was added to the lower chamber. After 4 h incubation at 37˚C, the migrated cells were imaged at x100 magnification under a light microscope, and the relative migration rates were analyzed. RPMI-1640 medium was used as the control for SDF-1.

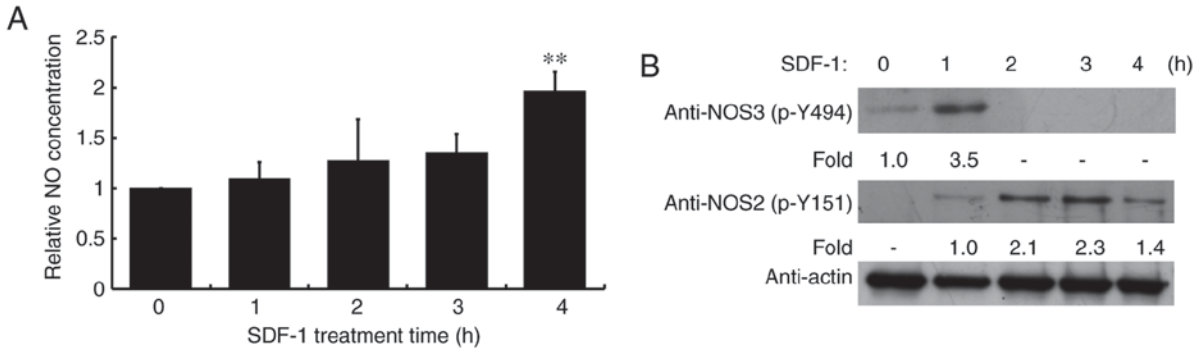

Figure 1. SDF-1 stimulation induced NO production in Jurkat cells. (A) Cells were treated with SDF-1 (25 ng/ml) for indicated time or RPMI-1640 medium as a control, and then NO concentration was analyzed. Data of three independent experiments were analyzed. **P<0.01. (B) Cells were treated with SDF-1 (25 ng/ml) for indicated time and the activities of NOS2 and NOS3 were analyzed by western blotting with antibody to NOS2 (p-Y151) and NOS3 (p-Y494), respectively. Fold changes relative to actin were shown. SDF-1, Stromal cell-derived factor-1; NO, nitric oxide.

Statistical analysis. All experiments were performed in triplicate. The data were expressed as the mean ± standard deviation and were analyzed by one-way analysis of variance with Bonferroni's post-hoc test using the statistical software package SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

NO was produced by SDF-1 stimulation. NO serves a critical role in signal transduction. Therefore, cellular NO levels were analyzed following SDF-1 treatment for 1, 2, 3 or 4 h. NO levels were significantly increased following SDF-1 stimulation for 4 h compared with the control group (Fig. 1A). Additionally, NO synthases 2 and 3 were phosphorylated following SDF-1 treatment (Fig. 1B), corresponding to activities, which catalyze the generation of NO.

NO was required for cell migration. To investigate the role of NO in SDF-1-induced cell migration, the NOS inhibitor, L-NMMA was used. The results demonstrated that pretreatment of Jurkat cells with L-NMMA led to the inhibition to cell migration towards SDF-1 (Fig. 2A), which suggested that NO was required for cell migration. Jurkat cells were then treated with different concentrations of L-NMMA and found that the different concentrations of L-NMMA had no significant effect on cell viability (Fig. 2B), suggesting that the inhibitory effect of L-NMMA on cell migration was not due to a reduction in cell viability.

NO is involved in SDF-1-induced cytoskeletal changes. The mechanism by which NO affects SDF-1-induced Jurkat cell migration was further tested by observing and measuring the distribution of the F-actin cytoskeleton, in the presence or absence of L-NMMA when stimulated by SDF-1. The results showed that L-NMMA significantly inhibited the F-actin rearrangement induced by SDF-1 (Fig. 3A) and the F-actin polymerization-induced by SDF-1 (Fig. 3B). NO was required for F-actin rearrangement and polymerization in response to SDF-1. This suggests that SDF-1-induced migration was regulated by NO production and the subsequent F-actin rearrangement and polymerization.

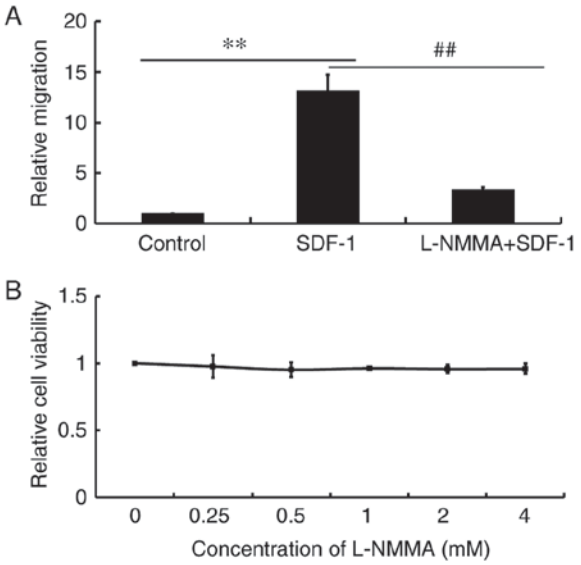

Figure 2. NO was involved in cell migration. (A) Cells were pre-treated with L-NMMA (1 mM) or an equal volume of RPMI-1640 medium for 2 h. The cell migration was detected by Transwell assay in the presence of SDF-1 (25 ng/ml) or equal volume of RPMI-1640 medium. The relative migration was the relative migrated number of cells. (B) Effects of different concentrations of L-NMMA on cell variability were detected by using MTT assay. The relative cell viability was the relative number of cells. Data of three independent experiments were analyzed. **P<0.01 compared with control group; ##P<0.01 compared with SDF-1 treatment group. SDF-1, Stromal cell-derived factor-1; L-NMMA, N^G-monomethyl-l-arginine monoacetate salt.

Discussion

Chemokines mediate cell migration in a key step in the inflammatory response and the metastasis of cancer (31). SDF-1 is a chemokine that exerts its effects in a variety of types of cells (31,32). SDF-1 serves a crucial role in lymphocyte trafficking and homing, and is particularly important in the migration of lymphocytes (33). In Jurkat cells, MAPK/Erk, Akt, in addition to NO are involved in the process of SDF-1 induced migration (19). However, the mechanism by which NO functions in SDF-1 signaling remains unclear. Therefore, an aim of the present study was to identify the specific mechanism of NO that affects SDF-1-induced migration. The present study demonstrated a crucial role of NO in SDF-1-induced

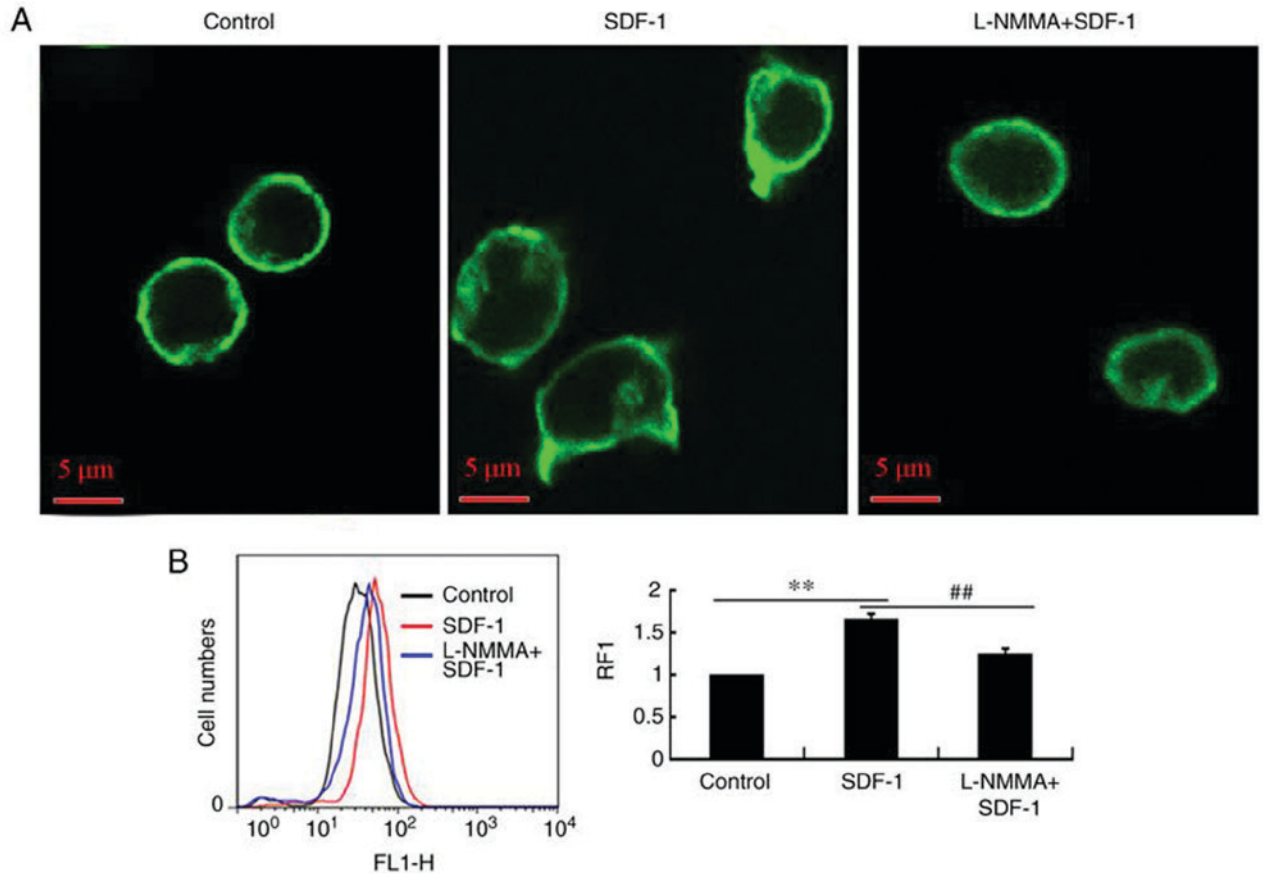

Figure 3. NO was essential to SDF-1-induced cytoskeleton changes. (A) Cells were pre-incubated with L-NMMA (1 mM) or equal volume of RPMI-1640 medium for 2 h, induced by SDF-1 (25 ng/ml) for 4 h, labeled with FITC-phalloidin, observed under a confocal microscope. (B) The amount of F-actin was detected by flow cytometry, and the results were expressed as relative fluorescence index which divides the fluorescence value of the experimental groups by that of the control group. Data of three independent experiments were analyzed. **P<0.01 compared with control group; $^{\#\#}$P<0.01 compared with SDF-1 treatment group. SDF-1, Stromal cell-derived factor-1; L-NMMA, N^G-monomethyl-l-arginine monoacetate salt; FITC, Fluorescein isothiocyanate.

cytoskeleton changes and cell migration. SDF-1 induced the generation of NO in Jurkat cells. NO signaling is involved in migration by promoting the rearrangement and polymerization of the cytoskeleton (Fig. 4). These important results indicate effective therapeutic targets to prevent unwanted cell migration.

NO is a potent signaling molecule that contributes to the migration of cells in human cancers (34). The results of the present study demonstrated that SDF-1 induced NO production, this is due to chemokine-induced changes of the cytoskeleton and cell migration by triggering multiple signaling pathways (35). The change of the actin cytoskeleton is critical for migrating cells (36). Here, pre-treatment of Jurkat cells with an NOS inhibitor was demonstrated to markedly inhibit SDF-1 induced cytoskeletal rearrangement and polymerization (Fig. 3A and B). Additionally, NO was involved in SDF-1-induced cell migration (Fig. 2A). These results suggest that SDF-1-induced chemotaxis can produce NO and NO-mediated cytoskeleton changes that are involved in subsequent cell migration. Similarly, it has been reported that NO modulates cell migration by the regulation of cytoskeletal proteins (37,38).

Based on the results of the present study, a mechanism for chemokine-mediated signaling in T-ALL was described, in which NO signaling regulated SDF-1-induced changes of actin cytoskeleton and cell migration. Importantly, an inhibitory role of L-NMMA in the migration of T-ALL was demonstrated, and these results will contribute to the design of more effective treatment strategies for T-ALL.

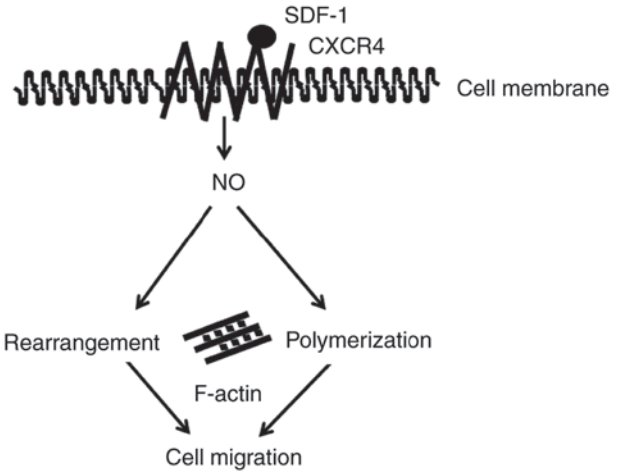

Figure 4. A diagram of the signal transduction pathway induced by SDF-1 involves NO and downstream F-actin rearrangement and polymerization, which leads to cell migration. SDF-1, Stromal cell-derived factor-1; NO, nitric oxide; CXCR4, C-X-C chemokine receptor type 4.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Author's contributions

JXL designed some of the experiments and wrote this manuscript. DW and DYL conducted the experiments. LW designed some of the experiments and was involved in revising the manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflict of interest

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