The role and mechanism of CXCR4 and its ligand SDF-1 in the development of cervical cancer metastasis

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Background and Objective: The chemokine receptor CXCR4 and its sole ligand stromal cell-derived factor-1 (SDF-1) not only actively participate in inflammation, hematopoiesis, infection of HIV, but also play a pivotal role in migration, invasion and metastasis of some malignant tumors. This study was to investigate the role of CXCR4/SDF-1 axis in mediating metastasis in cervical cancer cells through activating the mitogen-activated protein kinase (MAPK) pathway and the possible mechanism. Methods: Intracellular calcium mobilization was observed under laser scanning confocal fluorescence microscopy. The phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in HeLa cells after the binding of SDF-1α to CXCR4 was measured by Western blot. Adhesion of CXCR4/SDF-1 to cervical cancer cells and secretion of matrilysin (MMP) were detected by adhesion assay and gelatin zymography, respectively. Results: After SDF-1α was bound to CXCR4, a rapid and robust mobilization of intracellular calcium in Hela cells was initiated. The difference between the average baseline fluorescence intensity (FI) and the peak FI was significant (p < 0.01). ERK-1/2 was rapidly phosphorylated in Hela cells after its exposure to SDF-1α, and the strongest phosphorylation occurred at 30 min. The adhesion ability of Hela cells to fibronectin (FN) and laminin (LN) was increased after SDF-1α treatment (p < 0.05 and p < 0.01, respectively), while pretreatment with the fibronectin (FN), collagen type VI, laminin (LN), Fluo-3AM were from Sigma. The laser scanning confocal microscope was produced by Olympus, Japan.

Materials and Methods

Materials. The cervical cancer cell line HeLa was purchased from the Cell Bank of Wuhan University. Rabbit anti-ERK1 polyclonal antibody, mouse anti-pERK monoclonal antibody, and rabbit anti-α-actin polyclonal antibody were bought from Santa Cruz Company, USA. Recombinant SDF-1α was the product of Peprotech Company. The MEK11 inhibitor, PD98059, was from the Promege Company, USA. Recombinant SDF-1α was the product of Peprotech Company.

Methods. Cell culture. HeLa cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS), and incubated in a saturated humidity atmosphere containing 5% CO₂ at 37°C.

Calcium mobilization. HeLa cells were cultured in a special dish. The fluorescence intensity (FI) before and after the treatment of 50–200 ng/mL SDF-1α was measured by continuous dynamic scanning under confocal microscopy at 37°C with 10 μmol/L Fluo-3AM in dark. The excitation and emission waves of Fluo-3AM combined with calcium were 488 nm and 530 nm, respectively. Under X100
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magnification, the average FI of 8–10 fields, in total 50 cells, was taken as the index of the relative level of $[Ca^{2+}]_{i}$. The relative change of the free calcium concentration before and after SDF-1α treatment was indicated by the change of intracellular IF and amplitude. The peak value of the relative fluorescent unit was recorded as the fluctuation of intracellular calcium concentrations.

**Western blot.** HeLa cells in the logarithmic phase of growth were starved in non-serum medium overnight, followed by addition of SDF-1α (100 ng/mL) to incubate for 0–30 min. Then cells were harvested, and the total protein was extracted. The protein was separated on a 12% SDS-PAGE gel, and then transferred onto a nitrocellulose membrane. After being blocked in 5% skim milk for 2 h, the membrane was incubated with rabbit anti-ERK1 polyclonal antibody (1:400), mouse anti-pERK monoclonal antibody (1:400), rabbit anti-β-actin polyclonal antibody (1:400) at 4°C overnight, and washed in TBS (Tris-Buffered Saline Tween-20). The membrane was then incubated with secondary antibodies (1:1000) for another 2 h. Subsequently, the membrane was immersed in diaminobenzidine substrate buffer (DAB). Images of protein bands were developed and photos were taken.

**Adhesion assay.** A 96-well plate was coated with COL, FN and LN, respectively. HeLa cells in the logarithmic phase of growth were detached from the flask using trypsin and resuspended in non-serum medium. After being treated with a MEK inhibitor, PD98059 (30 μmol/L), at 37°C for 15 min, cells were seeded and incubated with different concentrations of SDF-1α (0–200 ng/mL) at 37°C for 2 h. After removal of non-attaching cells with PBS, 200 μL fresh medium containing 20% FBS and 20 μL MTT (5 mg/ml) was added into each well, and incubated at 37°C for 4 h. After addition of 200 μL DMSO, the absorbance of each well was measured using an enzyme-labeling instrument at 490 nm.

**Gelatin zymography.** After HeLa cells in the logarithmic phase of growth were maintained in non-serum DMEM with different concentration of SDF-1α (0–1000 ng/mL) for 24 h, the supernatant was collected. Proteins were electrophoresed in a 10% SDS-PAGE gel containing 1% gelatin at 4°C. After electrophoresis, the gel was eluted and washed in elution and washing buffer twice, incubated with incubation buffer at 37°C for 42 h. Then the gel was stained for 3 h, destained with solution A, B, C for 0.5, 1, and 2 h to display the MMP-2 bands against the blue background. The gel was scanned with BIO-RAD Quantity One software to semi-quantitatively analyze gelatinase activity by calculating the product of the areas and the intensity of the band.

**Statistical analysis.** Data were expressed as mean ± SD. SPSS 12.0 was applied to analyze the data. ANONA was applied to compare the mean value between multiple groups; and t test was applied to compare the mean value of two groups. p < 0.05 was considered as significantly different.

**Results**

SDF-1α induced transient changes of intracellular calcium concentrations. The time course of the dynamic curve indicates that the FI was proportional to the calcium concentration. After addition of SDF-1α, the intracellular calcium concentration increased rapidly in a short time, reached the peak, and maintain at the peak (Fig. 1). Differences between the average basic value and the peak value were statistically significant (p < 0.01). Observation under

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**Figure 1.** Intracellular calcium mobilization in HeLa cells after exposure to 200 ng/mL SDF-1α.

**Figure 2.** Images of laser scanning confocal fluorescence microscopy in HeLa cells after exposure to SDF-1α (200 ng/mL). Well-distributed green fluorescence is observed in cells when intracellular calcium is rapidly increased. (A) at 1 s; (B) at 59 s.
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were not significantly different in the 50 ng/mL and 200 ng/mL SDF-1α groups, with or without the pretreatment of PD98059 (p > 0.05). The adherent cell number significantly differed in the rest of the groups treated with SDF-1α (p < 0.05) (Table 1).

Differences in the adhesion ability of HeLa cells to COL between SDF-1α treatment groups at different concentrations and control groups were not statistically significant (p > 0.05). SDF-1α treatment did not enhance the adhesion ability of HeLa cells to COL, and PD98059 did not prevent cells from adhering.

CXCR4/SDF-1 regulated the activity of MMP-2. HeLa cells secreted a certain amount of active MMP-2 (62 ku), which was dramatically elevated after SDF-1α treatment at different concentrations (50–1000 ng/mL) for 24 h. The level of MMP-2 reached the peak when the concentration of SDF-1α was 800 ng/mL, and started to decline afterwards (Fig. 4).

Discussion

CXCR4/SDF-1 not only takes part in various normal biological functions, but is also active in tumor growth and malignancy. Similar to the homing of hematopoietic stem cells and lymphocytes, chemokines play important roles in the organ specific metastasis of tumors. It is reported that metastasis in many tumors are related to CXCR4/SDF-1. The invasion and metastasis of cervical cancer are resulted from, to some extent, the interaction of CXCR4/SDF-1. However, the underlying mechanism remains unknown.

Tumor metastasis is a successive and selective process, which is promoted under various stimulation. Chemokines expressed at the metastatic areas lead the tumor cells to migrate to specific sites. Enhanced adhesion ability enhances the invasive ability of tumor cells. Matrix metalloproteinases degrade the basement membrane of the metastatic site.

Our study was based on the hypothesis that HeLa cells could adhere to the metastatic sites by expressing the CXCR4 receptor, and thereafter secrete matrix metalloproteinases to degrade the

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**Table 1** The adhesion ability of Hela cells to FN and LN after different treatments

<table>
<thead>
<tr>
<th>Group</th>
<th>SDF-1α concentration (ng/mL)</th>
<th>FN</th>
<th>LN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A490nm</td>
<td>Increased adhesion to FN(%)</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>0.80±0.357</td>
<td>–</td>
</tr>
<tr>
<td>+FN</td>
<td>–</td>
<td>1.33±0.216</td>
<td>–</td>
</tr>
<tr>
<td>+LN</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FN+SDF-1α</td>
<td>50</td>
<td>2.10±0.212</td>
<td>57.74</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2.25±0.182</td>
<td>69.20</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>2.16±0.539</td>
<td>62.40</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.59±0.400</td>
<td>19.86</td>
</tr>
<tr>
<td>FN+SDF-1α+PD98059</td>
<td>50</td>
<td>1.40±0.369</td>
<td>5.10</td>
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<tr>
<td></td>
<td>100</td>
<td>1.45±0.393</td>
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<tr>
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<tr>
<td></td>
<td>200</td>
<td>1.33±0.356</td>
<td>0.43</td>
</tr>
</tbody>
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FN: fibronectin, LN: laminin, *p < 0.05, †p < 0.01, vs. control. Values are presented as mean ± SD.

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**Figure 3.** ERK-1/2 phosphorylation induced by CXCR4/SDF-1 (100 ng/mL) in HeLa cells at different time courses.

**Figure 4.** Effects of 24 h-treatment of SDF-1α on the secretion of active MMP-2 in HeLa cells observed by gelatin zymograph.

confocal microscopy showed that Hela cells grew well, presented with intact profiles and evenly distributed green fluorescence (Fig. 2).

CXCR4/SDF-1 induced ERK1/2 phosphorylation in HeLa cells. ERK1/2 was phosphorylated after SDF-1α treatments for 5, 15, and 30 min in HeLa in a time-dependent manner (Fig. 3).

Influence of SDF-1α and PD98059 on the adhesion ability of HeLa cells to ECM. The number of adherent HeLa cells to FN and LN of SDF-1α treatment groups and control groups were statistically significant (p < 0.05) except for the 200 ng/mL SDF-1α group (p > 0.05). After the pretreatment of cells with PD98059, the number of adherent cells to FN and LN decreased. The number of cells adhering to FN was not significantly different in 200 ng/mL SDF-1α treatment group, and the number of cells adhering to LN
basement membrane at these sites. Calcium is the pivot of various signaling pathways. In this study, SDF-1α treatment resulted in a rapid increase of intracellular [Ca^{2+}]_i in HeLa cells. Moreover, the mobilization of intracellular calcium pool and the release of [Ca^{2+}]_i occurred shortly after the binding of SDF-1α to CXCR4 in HeLa cells, which also reflected CXCR4 function on the cell surface. It is reported that the combination of SDF-1 and CXCR4 on the T cell surface could induce cell migration through activating ERK1/2. Brand et al. reported that CXCR4 activation promoted the migration of epithelial cells in intestine, but the effect was inhibited by a MEK-1 inhibitor, PD98059. We observed ERK1/2 phosphorylation induced by SDF-1α in HeLa cells, indicating that CXCR4/SDF-1 could activate the MAPK signaling pathway and activate the downstream molecules to participate in the invasion and metastasis of cervical cancer. The process of tumor metastasis is alike the infiltration of inflammatory cells, including cell rolling, adhesion, migration through endothelium, and adhesion of lymphocytes to CD34⁺ progenitor cells induced by SDF-1 in blood circulation. Adhesion to ECM is a critical step in tumor metastasis. COL, FL, and LN are three important components of ECM. We found that SDF-1α promoted HeLa cells to adhere to FN and LN, and this effect was inhibited by a MEK inhibitor, PD98059, indicating the involvement of the MAPK pathway in the process of SDF-1α promoted metastasis. However, 200 ng/mL SDF-1α treatment did not enhance the adhesion ability of HeLa cell to the matrix (FN and LN), which might be due to saturation of the binding sites on CXCR4. This suggests that adhesion of HeLa cells to FN and LN is partially mediated by the CXCR4/SDF-1 axis. Further research needs to be conducted to investigate whether cell adhesion would be decreased once the binding of saturation is reached. Increased SDF-1α concentration did not reverse the inhibitory effect of PD98059, implying that the MAPK pathway was blocked by PD98059 before SDF-1α treatment. This further confirms that CXCR4/SDF-1 promotes cell adhesion by activating ERK. Matrix metalloproteinase mediated ECM degradation plays an important role in tumor invasion and metastasis. MMP-2 can decompose FN and LN in the basement membrane. This study found elevated secretion of MMP-2 after SDF-1α treatment in Hela cells, indicating that CXCR4/SDF-1 regulates the expression of MMP-2 in, thus to facilitate abscission of tumor cells from the original site, invasion to the extracellular matrix and vessels, and metastasizing to remote sites. In conclusion, CXCR4/SDF-1 participates in tumor invasion and metastasis in Hela cells through regulating the adhesion ability by activating the MAPK signaling transduction pathway and promoting the secretion of MMP-2.

Acknowledgements
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References