NF-κB Signaling Pathway Confers Neuroblastoma Cells Migration and Invasion Ability via the Regulation of CXCR4

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Background: Accumulating evidence implicates the transcription factor NF-κB as a positive mediator of tumor metastasis, but the molecular mechanism(s) involved in this process remains largely unknown. In this study, we investigated the role of NF-κB signaling pathway in the regulation of CXC chemokine receptor-4 (CXCR4) in neuroblastoma metastasis.

Material/Methods: NF-κB, CXCR4 mRNA and protein expression were measured by RT-PCR, and Western blot. Tumor necrosis factor-a (TNF-a) was used to induce the upregulation of NF-κB and CXCR4. The knockdown of NF-κB and CXCR4 was achieved by PDTC. Transwell assay was used to investigate the role of NF-κB (p65) in neuroblastoma cell migration and invasion. An in vitro co-culture system was established to investigate the role of tumor microenvironment in regulation of the NF-κB signaling pathway.

Results: Over-expression of NF-κB (p65) promoted tumor migration and invasion through the upregulation of CXCR4; however, knockdown of NF-κB(p65) inhibited tumor migration and invasion through blocking the expression of CXCR4. Consistently, in the co-culture system, the expression of CXCR4 was partly dependent on the expression of NF-κB (p65).

Conclusions: Our studies reveal critical roles for the NF-κB signaling pathway in neuroblastoma migration and invasion. The mechanism may be through up-regulation of CXCR4, mediated by the NF-κB signaling pathways. Targeting NF-κB signalling pathways and ultimately CXCR4 could be a strategy in neuroblastoma therapy.

MeSH Keywords: Neoplasm Metastasis • Neuroblastoma • Receptors, CXCR4 • Transcription Factor RelA

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Background

Neuroblastoma, the most common extracranial solid tumor in children, is a heterogeneous tumor that arises from the neural crest [1]. Neuroblastomas account for approximately 15% of childhood deaths from cancer. At the time of diagnosis, more than 70% of patients have metastatic disease [2]. The disease displays a remarkable clinical diversity, ranging from spontaneous regression to fatal progression and dissemination to privileged sites, such as bone-marrow and liver [3]. However, the molecular mechanisms and/or intrinsic factors controlling neuroblastoma cancer metastasis are not well understood.

Chemokines and their receptors were originally described as essential mediators of leukocyte directional migration, and have further emerged as crucial players in all stages of tumor development. The binding of chemokines to their cognate receptors elicits typical cellular responses, such as directional migration. CXCR4 is the most frequently expressed chemokine receptor on tumor cells [4–6]. CXCR4’s ligand is the small chemokine SDF-1α, also known as CXCL12. In addition to its critical role in tumor cell growth, survival, and angiogenesis in multiple cancers, the CXCR4/SDF-1α pair has been shown to mediate homing and metastatic secondary growth in SDF-1α-producing organs, such as liver and bone marrow [7,8]. Research suggests that neuroblastoma cells are equipped with a bone marrow homing system that may mediate the establishment of bone marrow metastasis by neuroblastoma cells [9].

Numerous in vitro and in vivo studies have suggested that NF-κB plays an important role in regulating cell proliferation, angiogenesis, adhesion, invasion, and metastasis [10]. The NF-κB pathway can be activated by a large variety of factors, including cytokines and stress stimuli. NF-κB dimerizes function as a transcription factor in the nucleus and are sequestered in an inactive form in the cytoplasm, bound to inhibitors of kappa B proteins (IκB), most often IκBα. Upon stimulation by pro-inflammatory cytokines, such as TNF-α and IL-1, I kappa B kinase (IKK) is activated. IKK phosphorylates IκBα, which is then degraded by the proteasome, allowing translocation of the NF-κB dimers to the nucleus [11]. NF-κB was also shown to induce the expression of CXCR4 [12].

To elaborate the role of CXCR4 in neuroblastoma cell pathology, we examined NF-κB signalling activation as a potential mechanism by which cell metastasis is fostered. We provided evidence that activated NF-κB signaling pathway underlies the molecular basis of the upregulation of CXCR4 in neuroblastoma cells and contributes to enhanced migration and invasion.

Material and Methods

Cell culture and reagents

The human malignant neuroblastoma SK-N-BE(2), SH-SY5Y cell lines, and human mononcytic cell line THP-1 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Science (CBTCCAS), Shanghai, China. The cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, USA). Cells were cultured at 37°C with 5% CO₂ in a humidified atmosphere. Recombinant human TNF-α (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.1% bovine serum albumin (BSA) in PBS and stock solution (10 μg/ml) was stored at −20°C. Recombinant human SDF-1α (Life Technologies, Carlsbad, CA, USA) was prepared in 0.1% BSA in PBS and stock solution (100 μg/ml) was stored at 4°C. PMA (phorbol myristate acetate), a THP-1 cells inducer (Sigma-Aldrich, St. Louis, MO, USA), was prepared in PBS (320 nM) and kept at 4°C until used. PDTC (pyrrolidinedithiocarboxylic acid ammonium salt), a NF-κB inhibitor [13] (Sigma-Aldrich, St. Louis, MO, USA) was prepared in PBS (50μM) and kept at 4°C until used.

In-vitro system of inflammatory microenvironment

THP-1 cells (1×10⁶ per well) were seeded into the upper insert of a 6-well transwell apparatus with 0.4-μm pore size (Corning Costar, Rochester, NY, USA) and treated with PMA at a concentration of 320 nM for 48 h to generate macrophages. After thorough washing to remove all PMA, PMA-treated THP-1 macrophages were co-cultured with SK-N-BE(2) or SH-SY5Y cells (in a 6-well plate, 1×10⁶ cells per well) grown in the bottom compartment of the plate. The THP-1 macrophages were never in direct contact with the co-cultured neuroblastoma cell lines. The cultures were exposed to either vehicle control or PDTC treatments. Cultured SK-N-BE(2) or SH-SY5Y cells without the addition of THP-1 macrophages was used as an experimental control. In the co-culture system, the neuroblastoma cell lines were cultured with THP-1-differentiated macrophages for 24 h and then harvested for use in subsequent experiments.

Semi-quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from cell lines by Trizol (Takara, Dalian, China) according to the protocol supplied by the manufacturers. The purity of the dissolved RNA was assessed by the A260/A280 nm ratio. The RNA integrity was determined by denaturing agarose gel-electrophoresis. Total RNA was extracted following a conventional protocol and was dissolved with 20 μL RNase-free water. The cDNA was synthesized in a 20 μL reaction containing 2 μg total RNA, oligo (dT), and Reverse Transcription
Premix (Takara, Dalian, China). PCR amplification of the cDNA products (3 μL) was performed with PCR premix (Takara, Dalian, China) and the following primer pairs (Sango, Shanghai, China): NF-κB (p65) forward 5’-TCAATGCTACAGGAGCA-3’ and NF-κB (p65) reverse 5’-CAGTGGAAAGGAGACAGA-GAAGA-3’ (308bp); CXCR4 forward 5’-TGGCCTGAAAAGGTGGTCTAG-3’ and CXCR4 reverse 5’-CGATGCTATCCAAATGTAAGT-3’ (333bp); GAPDH forward, 5’-TACGGTGAACCATGAGAATG-GAAGA-3’ and GAPDH reverse 5’-GGATGACTGTGATCCCAATGTAGT-3’ (146 bp). GAPDH was used as the internal control. Amplification consisted of 28 cycles: denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min, followed by a final 5-min extension at 72°C. PCR was performed using a Ge-ne Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide.

**Western blot analysis**

Cells were washed twice with cold phosphate-buffered saline (PBS, pH=7.0), and lysed in RIPA buffer (150 mM NaCl, 1%Nonidet P-40, 1% deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 8.0) supplemented with protease inhibitors. The protein concentration of each sample was assayed using the bicinchoninic acid method (BCA kit) (Pierce, Rockford, IL, USA). Equal amounts of protein (50 μg) were subjected to SDS-PAGE on 10% gel. Then the protein was blotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% non-fat milk in 20 mM of TBS with 0.1% Tween for 1 h at room temperature with shaking, they were incubated with the primary antibodies at 4°C overnight followed by incubation in mouse anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:6000; Santa Cruz Biotechnology) for 1 h. Proteins were detected using Pierce ECL Western Blotting Substrate (Santa Cruz Biotechnology, Inc.) with LAS-3000 (Life Science-Fujifilm Global). Primary antibodies employed in this study included anti-NF-κB (1:2000, Santa Cruz, CA, USA), anti-CXCR4 (p65) (1:1000, Abcam, Cambridge, MA), anti-CXCR4 (1:2000, Abcam, Cambridge, MA).

**Transwell migration assay**

Assays were performed using a transwell (Corning Costar, Rochester, NY, USA) containing a polycarbonate membrane filter (8-μm pore size) for 24-well plates according to the manufacturer’s instructions. SK-N-BE(2) or SH-SY5Y cells (5×10⁴) either untreated or pretreated for 24 h with TNF-α (20 ng/ml) were seeded onto the upper surface of a filter that had been coated with 50 μl of Matrigel with a volume of 200 μl DMEM medium containing 2% FBS in the presence or absence of PDTC (50 μM) and placed into the lower wells containing 500 μl of complete medium with SDF-1α (100 ng/ml) to induce cell migration. This transwell migration chambers were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. Following incubation, cells on the upper side of the membrane were removed by cotton swabs, and cells on the bottom surface of the membrane were fixed in 95% ethanol for 10 min at room temperature, stained with 0.1% crystal violet for 30 min, then washed 3 times with PBS. The number of migration cells in 10 randomly selected microscopic fields (>200 magnifications) per membrane was counted.

**Statistical analysis**

Statistical analysis was done using SPSS software 17.0. The data are expressed as the mean ±SD. The Student’s t-test and one-way analysis of variance test were used to compare data between the different groups. P-values<0.05 were considered statistically significant.

**Results**

**NF-κB contributes to CXCR4 upregulation in neuroblastoma cells**

The CXCR4 promoter region has been shown to contain NF-κB response elements [14]; therefore we wanted to determine whether the NF-κB pathway plays a role in the induction of CXCR4 in response to TNF-α in neuroblastoma cells. SK-N-BE(2) or SH-SY5Y neuroblastoma cells were treated with TNF-α for 24 h. Reverse transcription-PCR and Western blotting detection revealed that the expression of NF-κB and CXCR4 was upregulated significantly. However, when pretreating SK-N-BE(2) or SH-SY5Y cells with pyrrolidine dithiocarbamate (PDTC), which is a potent anti-oxidant inhibitor of NF-κB, the expression CXCR4 in either SK-N-BE(2) or SH-SY5Y cells were inhibited (P<0.05; Figures 1 and 2).

**Upregulation of CXCR4 in neuroblastoma cells were mediated by NF-κB signaling pathway in co-culture system**

In the present study, PDTC, a specific inhibitor of the NF-κB pathway, was added to macrophages/ SK-N-BE(2) or SH-SY5Y co-culture system for 24 h at a concentration of 50 μM. This treatment resulted in significant reduction in CXCR4 mRNA and protein levels in co-cultured SK-N-BE(2) or SH-SY5Y cells. Nuclear factor-kB P65 protein was also significantly decreased in SK-N-BE(2) or SH-SY5Y cells in the presence of PDTC (P<0.05; Figure 3).

**NF-κB mediates migration towards SDF-1α in neuroblastoma cells**

To evaluate the role of NF-κB in regulating the migration of neuroblastoma cells towards SDF-1α, the transwell migration assay was performed. As shown in Figure 4, TNF-α pre-treated cells showed a significant increase in migration towards...
SDF-1α as compared to cells exposed to SDF-1α alone (P<0.05; Figure 4). Knock-down of NF-κB expression with PDTC and migration of the SK-N-BE(2) or SH-SY5Y cells towards SDF-1α was significantly decreased (P<0.05; Figure 4).

Discussion

Neuroblastoma is the most common tumor in children less than 1 year of age worldwide. Metastasis is present in 70% of neuroblastoma patients and is responsible for many neuroblastoma deaths [15]. Although aggressive and intensive multimodality therapies (e.g., surgery, cytotoxic chemotherapy, and radio-metabolic treatment) have produced some improvements in the overall cure rate of neuroblastoma patients, the prognosis of patients with metastatic neuroblastoma remains poor.

The expression of CXCR4 on malignant epithelial cells and on cells from several hematopoietic malignancies implies that the SDF-1α/CXCR4 pathway may influence the biology of cancer and play a pivotal role in directing the metastasis of CXCR4+ tumor cells to organs that express SDF-1α [16,17]. Although a great deal of work has contributed to our understanding of the basic pathophysiology of SDF-1α/CXCR4, the signaling properties of CXCR4 and SDF-1α in the proposed regulation of organ-specific metastasis have not been well characterized, but studies point to genetic and microenvironmental factors [18]. The role of the microenvironment in the establishment

![Image of Figure 1](image-url)
**Figure 2.** NF-κB activation contributes to the regulation of CXCR4 in neuroblastoma cells (A1, A2, A3), (B1, B2, B3). Cells were treated with TNF-α (20 ng/ml) or NF-κB inhibitor PDTC (50 μM) for 24 h. NF-κB(p65) and CXCR4 expression were analyzed by Western blot analysis. NF-κB (p65) and CXCR4 protein expression were upregulated in SK-N-BE(2) or SH-SY5Y cells after being pre-treated with TNF-α and were downregulated when pre-treated with PDTC. β-actin was used as the loading control.

* P<0.05 vs. untreated group. Data are expressed as mean ±SD.

**Figure 3.** The macrophages-increased upregulation of CXCR4 expression was dependent on the NF-κB signaling pathway in the co-culture system (A1, A2), (B1, B2). The SK-N-BE(2) or SH-SY5Y cells expressed more CXCR4 after co-culture with THP-1-derived macrophage cells. The upregulation of CXCR4 expression was inhibited after treatment with PDTC. NF-κB(p65) and CXCR4 expression was measured by Western Blot. β-actin was used as the loading control. CO: co-culture. *P<0.05 vs. co-culture group. Data are expressed as mean ±SD.
of a metastatic lesion has become increasingly important because we now know that several factors secreted by stromal cells regulate metastatic pattern in a variety of tumor types. Hypoxia in the tumor microenvironment and NF-κB have both been implicated in CXCR4 overexpression.

The role of the NF-κB signaling system in bridging inflammation and cancer is currently well established. Furthermore, NF-κB is increasingly recognized as a crucial player in many steps of cancer initiation and progression. Elevated NF-κB activity is observed in various cancers, including neuroblastoma. The activation of NF-κB induces the expression of various molecules, including cyclooxygenase-2, matrix metallopeptidase-9, and adhesion molecules such as intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and endothelial-leukocyte adhesion molecule 1, all of which have been linked with cancer cell invasion and metastasis [19,20]. We speculate that inhibition of NF-κB activity may suppress neuroblastoma cell migration and invasion.

Inflammatory cytokines produced by tumor cells or inflammatory cells in the tumor microenvironment can promote tumor progression through the induction of genes dependent on the NF-κB signaling pathway [21]. The NF-κB complex is normally confined to cytosol through its interaction with the IκB protein. Upon stimulation, IκB is degraded and NF-κB is activated. In this study, we presented evidence that TNF-α can induce CXCR4 expression in neuroblastoma cells. Furthermore, blocking the NF-κB pathway with PDTC suppressed CXCR4 expression. There was another obvious upregulation of CXCR4 expression in SK-N-BE(2) or SH-SY5Y cells after they were cocultured with macrophage, an alternative source of TNF-α in the neuroblastoma microenvironment [22]. Importantly, this upregulation could be inhibited by NF-κB inhibitor PDTC.

Overexpression of CXCR4, whose involvement in various human tumors is well known, has frequently been observed in neuroblastoma tissues to increase metastasis. In our study, we observed a marked increase in migration towards SDF-1α in TNF-α–pretreated SK-N-BE(2) or SH-SY5Y cells, and the treatment with a NF-κB inhibitor PDTC resulted in a significant suppression of SK-N-BE(2) or SH-SY5Y cells migration towards SDF-1α. The expression of CXCR4 on malignant epithelial cells and on cells from several hematopoietic malignancies implies that the SDF-1α/CXCR4 pathway may influence the biology of cancer and play a pivotal role in directing the metastasis of CXCR4+ tumor cells to organs that express SDF-1α [23]. A previous study indicated that an SDF-1α/CXCR4 axis may be involved in attracting neuroblastoma cells to bone marrow, which was one of the favorable site of metastasis formation by neuroblastoma [9].

It is now recognized that metastasis of solid tumors requires collaborative interactions between malignant cells and a diverse assortment of “activated” stromal cells at both primary and secondary tumor locations. Macrophage involvement in carcinogenesis, tumor invasion, and metastasis [24,25] is generally blamed for stimulating TAMs (Tumor-associated macrophages), a major source of TNF-α in the tumor microenvironment [26–28], to release a variety of growth factors, cytokines, and inflammatory mediators. Inflammatory factors in the tumor microenvironment activated NF-κB, and constitutive NF-κB

**Figure 4.** NF-κB mediates migration and invasion towards SDF-1α in neuroblastoma cells (A, B). SK-N-BE(2) or SH-SY5Y cells pretreated or untreated with TNF-α (20 ng/ml) for 24 h were seeded in the migration chambers in the presence or absence of PDTC (50 μM) and placed into wells in the presence of SDF-1α (100 ng/ml). They were allowed to migrate for 24 h. Representative figures indicate the average number of migrated cells per field. The results are shown as the mean ±SD from triplicate cultures. *P<0.05 vs. untreated group.
activation further upregulates CXCR4 and major inflammatory factors such as TNF-α, IL-6, IL-1, and IL-8, which are potent activators of NF-κB. Thus, it is believed that NF-κB and inflammation constitute a positive feedback loop that promotes neuroblastoma tumor cell survival and progression. However, the possibility of other transcription factors besides NF-κB contributing to the upregulation of CXCR4 should not be ignored. For instance, Ets-1, another HGF and hypoxia-inducible transcription factor, is able to activate the transcription of CXCR4 [29].

In summary, we found that the inflammatory factor TNF-α promoted human SK-N-BE(2) or SH-SY5Y cells migration and invasion through activation of NF-κB and up-regulation of CXCR4 expression. Inhibition of the NF-κB pathway suppressed cell migration in SK-N-BE(2) or SH-SY5Y cells [30].

Conclusions
The overall findings of this study lead to our conclusion that neuroblastoma cells functions in part, through the NF-κB signaling pathway to upregulate CXCR4 expression to foster migration and invasion. Our data suggest that the NF-κB/CXCR4/SDF-1α pathway may be a potential regulator of neuroblastoma cell metastasis. Targeting NF-κB signalling pathways and ultimately CXCR4 could be a therapeutic strategy in neuroblastoma.

Conflicts of interest
None declared.

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