Journal of Molecular and Genetic Medicine

Xu D, et al., J Mol Genet Med 2016, 10:2
DOI: 10.4172/1747-0862.1000208

Research Article Open Access

High-Expressed CXCR7 Contributes to CXCL12-Mediated Protection from Apoptosis in Lymphoma Cells

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Received date: October 08, 2015; **Accepted date:** April 16, 2016; **Published date:** April 20, 2016

Abstract

Viral genes may act as oncogenes and interact with intracellular proteins, which are related to oncogenesis and tumor growth. Autocrining CXCL12 in growing tumor tissue plays critical roles in modulating cell proliferation and survival through its receptor CXCR4 and CXCR7. Recently, a number of research reports indicated that CXCR7 displays high expression in many kinds of tumor tissues and cell lines and that high -expressed CXCR7 may be involved in cell survival and migration. To date, CXCR7's biological function and signaling in cancer pathogenesis is unclear. In this study, we first found there are higher expression of CXCR7 in both lymphocyte nodes and EBV positive lymphoma cell line IB4. The results further suggested that high-expressed CXCR7 plays a role in CXCL12 mediated anti-apoptosis and inhibition of CXCR7 through CXCR7 inhibitor CCX771 significantly attenuated the function of CXCL12 on anti-apoptosis. Furthermore, we observed that CXCR7 inhibitor directly reduced protection of endogenous CXCL12 from apoptosis in EBV positive lymphoma cell line. Finally, the results revealed that CXCL12 activated CXCR7 trafficking to membrane and interaction of CXCR7 and β-arrestin-2 during anti-apoptosis process. The study suggested that the high-expressed CXCR7 may have effect on anti-apoptosis and inhibitor of CXCR7 may become potential target for cancer therapy.

Keywords: CXCL12; CXCR7; Anti-apoptosis; IB4; Lymphoma

Introduction

Viral genes may act as oncogene and play important roles by interacting with intracellular proteins, which is related to carcinogenesis, growth and the development of tumors [1-5]. Previous studies have reported that Epstein-Barr virus (EBV)-associated latent membrane protein 1 (LMP-1) promotes cell survival and/or migration through the up-regulation of CXCL12 and activation of two CXCL12 (Stromal cell-derived factor-1, SDF-1α) receptors, the chemokine receptor CXCR4 and CXCR7 [6].

In physiologic condition, CXCL12 plays major roles in modulating cell proliferation, survival, and homing through its dominative canonical receptor CXCR4. CXCR7 is highly expressed in the process of biological development, but has reduced expression in cells after birth, and no or low expression in normal blood cells. Former reports suggest that novel receptor CXCR7 activated by CXCL12 can promote cell proliferation and survival under CXCR4 deficit [7]. Interestingly, CXCR7 is higher expressed in many tumor cells and tumor-associated blood cells, and increasing CXCR7 may be involved in cell proliferation, survival, migration and cell adhesion [8-12].

Since CXCR7 has higher expression in many cancer cells, the biological significance of high expression is worth exploring. Past studies have indicated that CXCR7 may be a supportive or necessary factor for CXCR4 to function [13]. In recent years, many studies have reported that CXCR7 is a "decoy" receptor of CXCR4, or "scavenger" of CXCL12 that consumes CXCL12 to weaken the activity of CXCR4 [13]. However, few reports have studied the independent signaling pathway and the effect of CXCR7. For example, our recent study has suggested the function of CXCL12 in anti-apoptosis in human neural progenitor cells [14].

Viral infection and transformation regulate the innate immune system and chemokines for autoimmune diseases or cancers. Viral-encoded proteins possibly act as oncogene proteins in oncogenesis and the development of tumors. But whether virus-encoded protein regulates CXCR7 expression and activation for cancer development is unclear. In this study, we hypothesize that LMP1 activates and up-regulates the expression of new receptor CXCR7 in the EBV-associated lymphomas. We revealed that CXCR7 plays a role in CXCL12 mediated anti-apoptosis and that inhibition of CXCR7 through CXCR7 inhibitor CCX771 partly attenuated the function of CXCL12 on anti-apoptosis in cells.

Furthermore, we found that CXCR7 inhibitor directly reduced protection of endogenous CXCL12 from apoptosis in cancer cell lines. In addition, we found that CXCL12 activated CXCR7 trafficking to membrane during anti-apoptosis process. The study implied that the higher expression CXCR7 provided new targets in developing anti-tumor drugs for their specific role in survival and metastasis of various tumors.

Materials and Methods

Human tissues and cell lines

The human lymphoma lymphocyte nodes and normal tumor-adjacent lymphocyte nodes were used as control, and were collected from the patient diagnosed with B cell or T cell lymphoma in Shanghai Tenth People's Hospital, Shanghai, China. This study was conducted according to the tenets of the Declaration of Helsinki for the use of human lymphocyte nodes in lymphoma and normal tumor-adjacent lymphocyte nodes for Western Blot.

Human EBV-transformed lymphoma cell lines IB4 and EBV negative cell line DG75, originally purchased from American Type Culture Collection, were cultured under 5% CO2 at 37°C in the fresh RPMI-1640 medium, and supplemented with 10% fetal bovine serum and 50 IU/mL penicillin-streptomycin solution (Gibco, Rockville, MD, USA).

Reagents

Primary polycolonal antibodies: PARP (Cell Signaling Technologies, Boston, MA, USA), CXCR4 (R&D Systems, Minneapolis, MN, USA), CXCR7 (Abcam, Cambridge, MA, USA) and β-arrestin-2 (Abcam, Cambridge, MA, USA) and anti-IgG (R&D Systems, Minneapolis, MN, USA). Monoclonal antibodies: CXCR4 (Abcam, Cambridge, MA, USA), CXCR7 (Abcam, Cambridge, MA, USA), GAPDH (Cell Signaling Technologies, Boston, MA, USA) for Western Blot and immunoprecipitation. Second antibody: Horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG or anti-rabbit IgG (Cell Signaling Technologies, Boston, MA, USA).

Western blot analysis

Camptothecin (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as apoptosis inducer. Anti-apoptosis assay of CXCL12, IB4 and DG75 cells were seeded at a density of 5×105 cells/ml in 12-wells plates (Costar, Cambridge, MA, USA), respectively. Cells were pretreated with 100 ng/ml of CXCL12 (R&D Systems, Minneapolis, MN, USA) for 4 hours, then incubated with 10 μM camptothecin for an additional 2 hours, respectively. Cells were harvested for western blot assay.

For dose-dependent anti-apoptosis assay of CXCR7, IB4 cells were incubated with different concentrations of CXCL12 (50, and 100 ng/ml) with or without CCX771 (1 μM) (ChemoCentryx, Mountain View, CA, USA) for 4 hours, and then treated with 10 μM camptothecin for 2 hours.

Protein extracts were detected by BCA protein assay Kit (Pierce Corp., Rockford, IL, USA), and denatured by heating at 100°C for 5 min in RIPA buffer (Pierce Corp., Rockford, IL, USA). The protein mixtures were separated on 10% polyacrylamide gels and transferred to Immuno-Blot polyvinylindene fluoride membrane (Bio-Rad Corp., Hercules, CA, USA). After blocked in Tris-buffered saline (TBS)/ Tween (0.1%) with 5% fat-free milk for 1 h, the membrane was then probed with the appropriate primary antibodies of PARP (Cell Signaling Technologies, Boston, MA, USA), CXCR4, CXCR7, GAPDH and set overnight at 4°C.

The primary antibodies were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG or anti-rabbit IgG, visualized by Pierce ECL system (Pierce Corp., Rockford, IL, USA), and captured by CL-X PosureTM Film (Kodak Corp., Rochester, NY, USA).

Cell extraction assay

In order to investigate the distribution of CXCR7 protein in cells, the Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce Chemical Co., Rockford, IL, USA) was used for the extraction of membrane and cytoplasmic proteins from IB4 cells, according to the manufacturer's instructions. Finally, the cell membrane and cytoplasmic proteins were separated for western blot assay.

Co-Immunoprecipitation analysis

To detect the interaction of CXCR7 and CXCR4, IB4 cells were treated with 100 ng/ml of CXCL12 for 4 hours, and lysed with cold RIPA buffer (Pierce Corp., Rockford, IL, USA). Lysates were further incubated with monocloning anti-CXCR4 antibody or monocloning anti-CXCR7 antibody or anti-IgG (R&D Systems, Minneapolis, MN, USA) as negative control, and rotated at 4°C overnight, after which protein G sepharose (Thermo Scientific Inc., Waltham, MA, USA) was added with an additional hour at 4°C.

Afterwards, immunoprecipitates were washed twice with SDS-PAGE sample buffer (50 mm Tris-HCl, pH7.4, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue), and immunoblotted with rabbit anti-CXCR7 or rabbit anti-β-arrestin-2 to detect CXCR7/CXCR4 or CXCR7/β-arrestin-2 interaction using western blot.

Statistics

All results are reported as mean ± SD. Values of $P<0.05$ were considered statistically significant. Statistical analysis was performed using prism software.

Results

CXCL12 employed for anti-apoptosis in EBV transformed lymphoma cells with higher expressed CXCR7

The study first detected the protein expression of CXCR7 in lymphoma lymphocyte nodes and IB4 cell line. Furthermore, LMP-1 was detected in EBV transformed lymphoma cell line IB4 and EBV negative lymphoma cell line DG75. The results showed higher expression of CXCR7 in lymphoma lymphocyte node and IB4 cell lines (Figures 1A and B), and LMP-1 in IB4 cells (Figure 1B). To understand the function of CXCL12 in EBV transformed cancer cells, the cells were pre-treated with 100 ng/ml of CXCL12 for 4 hours and then treated with 10 μM camptothecin (chemical apoptosis inducer) for 2 hours.

We detected cleaved PARP level (apoptosis indicator) using Western Blot, and cell lyses were collected to detect cleaved PARP and total PARP. The cleaved PARP decreased with CXCL12 (Fig. 1C and D), which indicated that CXCL12 rescue camptothecin induced apoptosis in IB4 cell lines. The result suggested that CXCL12 plays a crucial role as an anti-apoptosis cytokine on IB4 cells. We also double-checked the expression of CXCR7 and CXCR4 upon CXCL12 stimulation in IB4 cells (Fig. 1D). The results indicated that the expression of both receptors did not show significant changes with three independent tests.

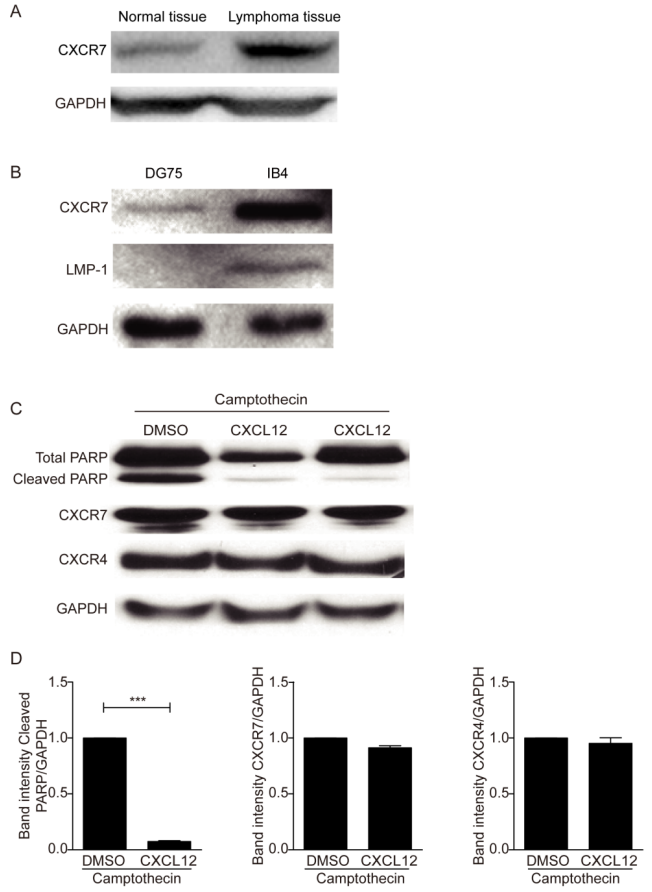

Figure 1: CXCL12 protects cancer cells from apoptosis induced by camptothecin. The expression of CXCR7 in lymphoma lymphocyte nodes and adjacent tissues (A). The expression of CXCR7 and LMP-1 in DG75 and IB4 cells was performed (B). To understand the function of CXCL12 in IB4 cells, cells were pretreated with 100 ng/ml of CXCL12 for 4 hours, and then treated with camptothecin (10 μM) for 2 hours. The results showed that CXCL12 significantly protected cells from apoptosis. The levels of Total PARP, cleaved PARP, CXCR4, or CXCR7 were determined by Western blot. GAPDH was used for loading control. Expression of CXCR4 and CXCR7 both displayed no significant changes (C and D).

CXCR7 is associated with CXCL12-mediated anti-apoptosis of lymphoma cells

Recently, CXCR7 has been found to have higher binding affinity to CXCL12 than CXCR4 [15]. Therefore, we explored which receptor was involved in CXCL12-mediated anti-apoptotic function in cancer cells. We first detected the effects on anti-apoptosis of CXCL12 with dose-dependent manner in the cells. The gradient data further confirmed the function of CXCL12 on anti-apoptosis (data not shown). Moreover, we utilized commercial CXCR7 inhibitor CCX771 to inhibit the activation of CXCL12 to CXCR7. The cells were pre-treated with CXCL12 and CCX771 for 4 hours and then treated with camptothecin for 2 hours. Afterwards, Western Blot for PARP was used to exam the anti-apoptosis reaction. The results showed that CXCL12 could totally abolish camptothecin induced PARP cleavage but only diminished in part upon blocking of CXCR7 using CCX771 (Figure 2A). These repeated experiments demonstrated that CXCL12 might be involved in anti-apoptotic effect through CXCR7 in lymphoma cells in addition to CXCR4 (Figure 2B).

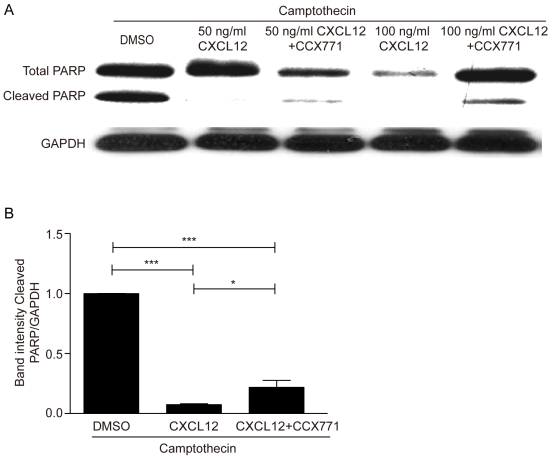

Figure 2: CCX771 inhibits CXCL12 mediated anti-apoptosis. IB4 cells were pretreated with different concentrations of CXCL12 (50 and 100 ng/ml) with or without CCX771 (1 μM) for 4 hours, and then incubated with 10 μM camptothecin for 2 hours. Total PARP and cleaved PARP protein levels were determined by Western blot. GAPDH was used for loading control. CCX771 significantly abolished the effect of CXCL12 on anti-apoptosis (A and B).

Inhibition of CXCR7 naturally attenuated autocrining CXCL12 mediated anti-apoptosis in lymphoma cell lines

To further clarify the involvement of CXCR7 in anti-apoptosis of cancer lines in natural pattern, we used CXCR7 inhibitor CCR771 to directly stimulate IB4 cells. The data showed that CCR771 attenuated the activation of secreted endogenous CXCL12 to CXCR7 and decreased protection from apoptosis in IB4 (Figure 3A). The repeated results indicated that CXCR7 did play a critical role in anti-apoptosis through CXCL12 activation to CXCR7 in cancer cells (Figure 3B).

CXCR7 mediated anti-apoptosis requests CXCR7 trafficking

Comparing with CXCR4, CXCR7 has higher expression in cytoplasm and lower expression in cell membrane [7]. To detect cellular CXCR7 behavior upon CXCL12 simulation, Cellular extraction assays were used to detect CXCR7 expression in cell membrane (CXCR7 in membrane, M-CXCR7) and cytoplasm (CXCR7 in cytoplasm, C-CXCR7), respectively. Western Blot analysis showed that CXCL12 enhanced CXCR7 expression in membrane (M-CXCR7, Figure 4A, and line 3). Furthermore, the results demonstrated anti-apoptosis function upon CXCR7 trafficking. The expression in membrane was reduced with CCX771 to endogenous CXCL12 (Figure 4B). The results showed trafficking of CXCR7 in part from cytoplasm to membrane upon CXCL12 treatment (Figure 4C).

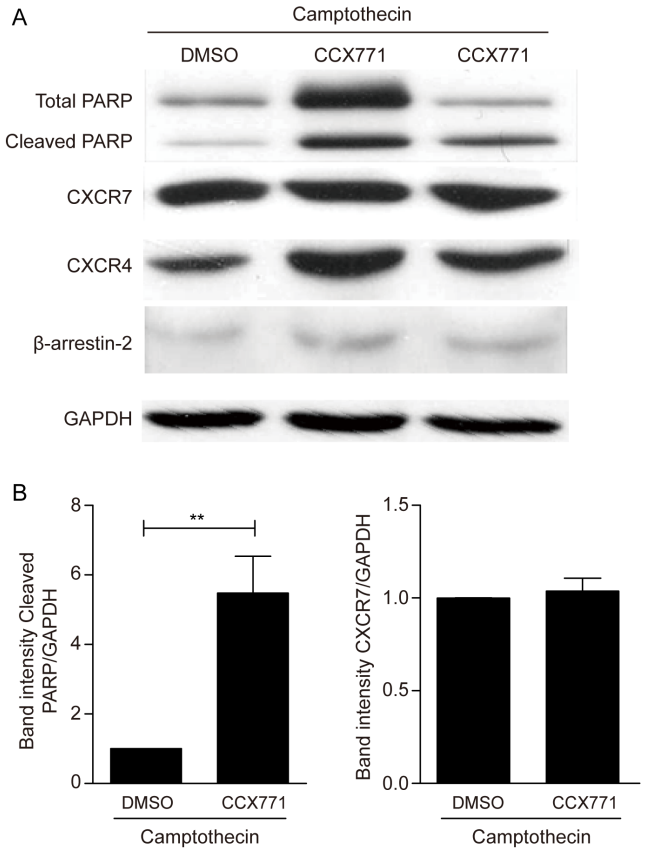

Figure 3: CCX771 inhibits endogenous CXCL12 mediated anti-apoptosis. IB4 cells were incubated with 1 μM CCX771 alone for 4 hours, and then treated with 10μM camptothecin for 2 hours. Cleaved PARP, CXCR7, CXCR4, β-arrestin-2 proteins levels were determined by Western blot. GAPDH was used for loading control (A). The rate of cleaved PARP was shown upon CCX771 treatment. The expression of CXCR7 indicated no significant change (B).

CXCL12 enhanced CXCR7 and CXCR4 interaction and recruit β-arrestin-2 during CXCR7 trafficking

To further study the mechanism of CXCL12 mediated anti-apoptosis, IB4 cell line was treated with CXCL12 and camptothecin. The cells were collected next day for immunoprecipitation of CXCR7, with pull-down of CXCR4. The immunoprecipitation results showed that protein-protein interaction between CXCR7 and CXCR4 was enhanced by CXCL12 treatment at 4 hours. The results implied CXCL12 enhanced CXCR7 and CXCR4 physical interaction (Figure 4B).

To understand the mechanism of CXCR7 mediated anti-apoptosis, and in addition to the interaction with CXCR4, another key protein arrestin-2 was tested in IB4 cells. The data showed the interaction of CXCR7 and β-arrestin-2 was enhanced by CXCL12 treatment. The results indicated that CXCR7 activation recruited β-arrestin-2 to form complex with/without CXCR4 upon CXCL12 stimulation, which may contribute to anti-apoptosis function in cancer cells (Figure 4C). Further molecular events need to be clarified (Figure 5).

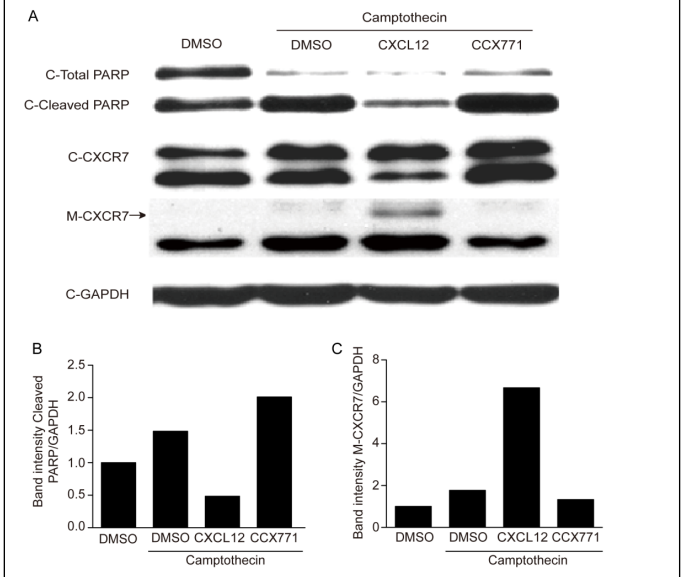

Figure 4: CXCL12 mediated anti-apoptosis requests CXCR7 trafficking. The membrane and cytoplasmic proteins of IB4 were extracted to detect the level of CXCR7 (A) and cleaved PARP (B) by western blot assay, respectively. The cleaved PARP displayed increased expression after incubation with 1 μM CCX771 (line 4). GAPDH was used for loading control. Immunoblotting analysis illustrated CXCR7 trafficking from cytoplasm (C-CXCR7) to membrane (M-CXCR7) upon CXCL12 stimulation (C).

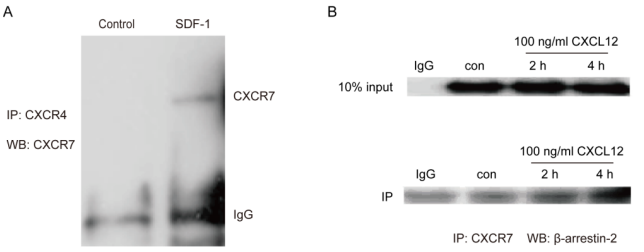

Figure 5: CXCR7 interacted with CXCR4 and β-arrestin-2. IB4 cells were incubated with 100 ng/ml of CXCL12 for 4 hours, and immunepreciptation (IP) was performed with monoclonal antibody against CXCR4 (A) or CXCR7 protein (B); CXCR7 (A) or β-Arrestin-2 (B) protein levels in the lysates were determined by immunoblotting, respectively. Mouse IgG was used as control for immunoprecipitation. Immunoblotting of cell extracts before immunoprecipitation was used as control (Input).

Discussion

In physical status, CXCR7 expresses with low level to balance CXCL12/ CXCR4 signaling. This study focused on CXCR7's function with higher expression in cancer tissues and cancer cell lines. The

study of the function of high-expressed CXCR7 has important significance.

There are approximately more than 50 different chemokines in higher vertebrate animals. To date, all the cytokines achieve function through the transduction of G protein receptors (GPCRs). Chemokine CXCL12 belongs to small molecular weight cytokines, and can be divided into two categories of CXCL12a and CXCL12b. Past studies have shown that CXCL12 binds CXCR4 to activate GPCR downstream signaling pathways, thereby promoting cell survival and migration. In recent years, research on CXCR7, another receptor of CXCL12, has become a hot topic. Recent studies have demonstrated the important effect of CXCR7 on tumor growth and metastasis. However, in regards to how CXCR7 affects the classical pathway of CXCL12/CXCR4 and whether CXCR7 has an independent function are still unclear.

Different from CXCR4, new receptor CXCR7 does not activate G protein pathway (due to the lack of DRYLAIV sequence). CXCR7 is mainly expressed in cytoplasm, and CXCR4 and CXCR7 can form a heterodimer under physiological conditions. This phenomenon that prompts the interaction between the receptors may adjust certain biological function. Our research shows that CXCL12 promotes this interaction, and this phenomenon indicates the interaction between CXCR4 and CXCR7 is affected by the inflammatory environment or activated by CXCL12 synchronously to perform the next step in biological function.

In this process, there are several possible mechanisms of CXCR7 function: 1. Collaborating with CXCR4 to activate G protein pathway to promote cell survival and migration; 2. CXCR7, as a scavenger, balance or impair the inherent function of CXCL12/CXCR4; 3. In a certain state (i.e., high expression of CXCR7), CXCL12/CXCR7 forms separate pathways (Endocytosis) to compensate or replace the effect from activation of CXCL12/CXCR4 on G proteins. Study results from tumor cell migration have confirmed that CXCR7 can activate the intracellular transport of β-arrestin-2 through endocytic pathway to participate in anti-apoptosis and migration of cells.

The study of CXCR7 function can be concentrated on two paths: 1. How does CXCR7 affect CXCL12/CXCR4 pathway (G protein)? To date, most studies believe CXCR7 serve as a “scavenger" receptor to modulate CXCL12/CXCR4 passage; 2. In high expression of CXCR7 condition, such as cancer and inflammation, can CXCL12/CXCR7 alone exert biological function through endocytic pathway?

The study of the function of high expressed CXCR7 has important significance: 1. CXCR7 knockout mice exhibit congenital heart defects and short-term mortality after birth, indicating the importance of CXCR7 in development; 2. There is physiological significance in the comparison of G protein pathway and endocytic pathway; 3. Compared to CXCR4, CXCR7 has greater than ten folds affinity to CXCL12, and this biochemical characteristic determines the important role of CXCR7; 4. Based on the powerful small molecule binding force, CXCR7 inhibitor (inhibition of CXCL12/CXCR7 binding, such as CCX771) and/or activators may play an important regulatory role, which is involved in cancer cell survival and migration.

Based on higher expression of CXCL12 receptor CXCR7 in EBV associated tumor cells, this study indicated that potential CXCR7 blocker CCX771 significantly affects the survival of tumor cells. On this basis, we will study the mechanism of high-expressed CXCR7 adjusted by EBV and its pathways on survival to reveal the important biological functions and independent role of CXCR7. In addition, high expressed CXCR7 may be a potential target for gene therapy or cell therapy. Camptothecin is well used as apoptosis inducer for neuron apoptosis study [16]. Our former results suggested CXCL12 could significantly rescue camptothecin-induced apoptosis in hNPCs [14].

In conclusion, our study confirmed apoptosis induced by camptothecin might be protected from further cell damage using CXCL12. We also explored the mechanism of how CXCR7 and CXCR4 are involved in this process. After all, our findings give us the outlook that CXCL12 and its two receptors are potential targets in promoting cancer cell survival.

Acknowledgment

This research was supported by funds provided through the Major State Basic Research Development Program of China (973 program) (2014CB965000 and 2012CB518900) and the National Natural Science Foundation of China (81471936, 81271420, and 31230005).

Conflict of Interest

All authors declared no competing financial interest.

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