

Original Research Article

Synthetic Polypeptide Derived from Viral Macrophage Inflammatory Protein II Inhibit VEGF Production of Human Glioma U87 Cells through SDF-1 α /CXCR4-Mediated AKT Signaling Pathway

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Received: 18 March 2014

Revised accepted: 3 July 2014

Abstract

Purpose: To evaluate the effect of synthetic polypeptide (N15P) derived from viral macrophage inflammatory protein II (vMIP-II) on the secretion of vascular endothelial growth factor (VEGF) as well as investigate the signaling pathways involved in stromal cell-derived factor-1 α (SDF-1 α)/CXC Chemokine Receptor 4 (CXCR4) axis-induced VEGF in glioblastoma U87 cells.

Methods: Glioblastoma U87 cells were exposed to SDF-1 α , N15P with various concentrations. The expression of CXCR4, SDF-1 α and VEGF mRNA were assessed by RT-PCR, while expression level of VEGF was tested by ELISA and protein kinase B (Akt) phosphorylation detected by Western blot.

Results: The results showed that CXCR4, SDF-1 α , VEGF are expressed in human glioblastoma U87 cell lines. SDF-1 α caused a dose-dependent sensitivity of cell proliferation with a maximum effect at 15 μ mole/ml, while N15P decreased cell viability in U87 cells in a dose-dependent manner. SDF-1 α stimulated the activation of VEGF, and N15P inhibited the activation of VEGF with or without SDF-1 α stimulation. VEGF production in U87 cells was associated with Akt pathway. These changes in intracellular processes were blocked by N15P in a dose-dependent manner.

Conclusion: The results suggest that N15P suppress SDF-1 α /CXCR4 Mediated VEGF production through Akt signaling pathway and this may be a potent therapeutic strategy in glioblastoma.

Keywords: Viral macrophage, Inflammatory protein II, Glioblastoma, CXC chemokine receptor 4, Stromal cell-derived factor-1 α , Protein kinase B

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INTRODUCTION

High-grade gliomas are considered to be the most common and serious primary gliomas in adults [1]. They are highly mobile, invasive, and difficult to completely resect through surgery and the survival rate of patients with malignant glioma is low [2]. So, a better understanding of glioma pathogenic mechanism with a view to finding a

new effective therapeutics for glioblastoma is needed.

CXCR4 is a prognostic marker in many different cancers [3], including leukemia [4], breast [5], lung [6], prostate [7], ovarian [8] and colorectal cancers [9], where the SDF-1/CXCR4 axis plays an important role in cancer progression, demonstrating that CXCR4 is essential for development, hematopoiesis, organogenesis, as

well as vascularization. Since CXCR4 plays critical roles in different steps of tumorigenesis, the CXCL12/CXCR4 axis is a potential target for therapeutics that block CXCL12/CXCR4 interactions or which inhibit activities of downstream signaling.

Viral macrophage inflammatory protein-II (vMIP-II), encoded by human herpesvirus-8, has antagonistic activity on the chemokine receptors CCR1, CCR2, CCR5, CXCR3 and CXCR4, CCR3 and CCR8 *in vitro* [10]. Therefore, N15P was modified by structural matching, downsizing, and reduction of peptide character in attempt to develop new low-molecular weight CXCR4 antagonists and showed remarkable inhibitory activity against HIV entry. Based on our previously published work on vMIP-II [11,12], we synthesized a novel and small molecule selective CXCR4 antagonist N15P (LGASWHRPD KCCLGY) derived from the N-terminal of vMIP-II [13]. In this study, we evaluated the inhibitory effects of N15P on the production of VEGF and the role of AKT signal pathway *in vitro*. These results indicate that N15P has therapeutic potential for angiogenesis in malignant tumor.

EXPERIMENTAL

Reagents and materials

Anti-phospho-Akt (Ser⁴⁷³), and anti-Akt antibodies were purchased from Cell Signaling Technology (USA). LY294002 was purchased from Calbiochem (San Diego, CA). Human-SDF-1 α was purchased from BD Pharmingen (USA). The inhibitor was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) with the final concentration not exceeding 0.1 %. N15P was entrusted to the Shanghai Yao Qiang Bio-Tech Co, Ltd, China (the final product was at least 95 % pure).

Cells and culture conditions

U87 cell line was purchased from the Cell Bank, Chinese Academy of Sciences (Shanghai, China). U87 glioma cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal calf serum (FCS)(GIBCO, USA). Cells were passaged every three or four days and checked routinely for contamination.

MTT assay

Cells were seeded in 96 - well plate at 1×10^4 cells/ml. After adherence, the cells were treated with DMEM containing 10 % fetal calf serum

alone or DMEM containing 0 - 20 μ mol /ml SDF-1 α or SDF-1 α with 0 - 5 nmol/ml N15P. MTT (5 mg/l) was added to each well at intervals. After incubation for 4 h, culture medium was removed and 150 μ l DMSO was added. The absorbance at 490 nm was measured with a BIO-RAD Model 550 and the results were expressed as the percent viability, which is directly proportional to number of metabolically active cells: % Viability = OD in sample well/OD in 0 nmol /ml SDF-1 α well \times 100, considering the cell viability of the control group was 100 %.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from human glioblastoma cells U87 using TRIzol Reagent (Invitrogen, USA). For RT-PCR to detect SDF-1, VEGF and CXCR4 mRNA expression, 1 μ g of total RNA from U87 cells treated with different factors was subjected to RT-PCR using two-step RT-PCR kit according to the protocol described by the supplier. Each PCR reaction and the PCR products analyses were performed as described in [14]. The primer pairs were as follows: β -actin (600 bp), 5' - GTACCACTGGCATCGTGAT GGACT - 3' (sense) and 5' - ATCCACACGGAGTACTTGGCGCTCA - 3' (antisense); CXCR4 (274 bp), 5' - CTGAGAAGCATGACGGACAA - 3' (sense) and 5' - CGCCAACATAGACCACCTTT - 3' (antisense); SDF-1 (276 bp), 5' - ATG AAC GCC AAG GTC GTG GTC - 3' (sense) and 5' - GGT CTG TTG TGC TTA CTT GTT T - 3' (antisense); VEGF (VEGF121: 515 bp; VEGF165: 646 bp), 5' - ATG AAC TTT CTG CTG TCT TGG G - 3' (sense) and 5' - CTG TAT CAG TCT TTC CTG GTG AG - 3' (antisense).

Western blot

To analyze the phosphorylation of AKT induced by CXCR4 activation, U87 cells were pretreated with 0 - 5 nmol/ml N15P or 10 μ mol/ml LY294002, followed by 0 - 20 μ mol/ml SDF-1 α stimulation for 24 h. Then the cells were solubilized, total protein concentrations of whole cell lysates were collected, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described [15].

VEGF Enzyme-linked immunosorbent assay

U87 cells were pretreated with 0 - 5 nmol/ml N15P, 10 μ mol/ml LY294002 for 4 h, followed by 0 - 20 μ mol/ml SDF-1 α stimulation for 24 h. After incubation for 24 h, supernatant was collected and VEGF concentration was measured using

ELISA kits (R&D Systems, USA). Triplicate cultures of cells were tested for each experimental condition, and the average value was recorded as pg/ml.

Statistical analysis

Data are expressed as mean ± standard deviation (SD), and the statistical significance between test and control groups was analyzed with GraphPad Prism 5.0. When two groups were compared, unpaired Student's t-test was used. When multiple groups were evaluated, one-way ANOVA was used. $P < 0.05$ was considered to be statistically significant.

RESULTS

Expression of CXCR4 and VEGF mRNA in human glioblastoma U87 cells

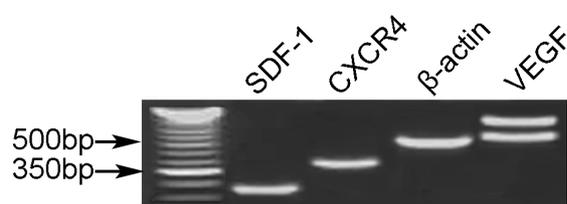


Figure 1: The mRNA expression of CXCR4, VEGF, SDF-1 in U87-MG glioblastoma cell line. β -actin amplification was performed as internal control for the PCR reaction

The expression of the chemokine receptor CXCR4, SDF-1 α and VEGF in U87 cells were first studied by RT-PCR. As shown in Figure 1,

U87 tumor cells expressed CXCR4, SDF-1 and VEGF mRNA (Figure 1).

Inhibitory test on U87 cell growth *in vitro*

To understand the effect of N15P on tumor viability, we examined the viability of U87 cells treated with N15P by MTT assay. Cells were serum starved for 24 h and treated with increasing concentrations of SDF-1 α (0 - 20 μ mol/ml) for 24 h. The results indicate that SDF-1 α caused a dose-dependent sensitivity of cell proliferation with a maximum effect at 15 μ mol/ml in U87 cell lines (Figure 2A). When treated with increasing concentrations of 0 - 5 nmol/ml N15P for 24 h, N15P caused a dose-dependent decrease of cell viability in U87 cell line (Figure 2B).

VEGF production

SDF-1 α stimulates activation of VEGF (Figure 3). N15P inhibited the activation of VEGF with or without SDF-1 α stimulation. Moreover, VEGF activation was blocked by the PI3K inhibitor LY294002.

AKT signaling pathway

SDF-1 α significantly increased AKT phosphorylation in U87 tumor cells. Pretreatment of the U87 tumor cells with N15P degraded SDF-1 α -induced AKT phosphorylation in a dose dependent manner (Figure 4).

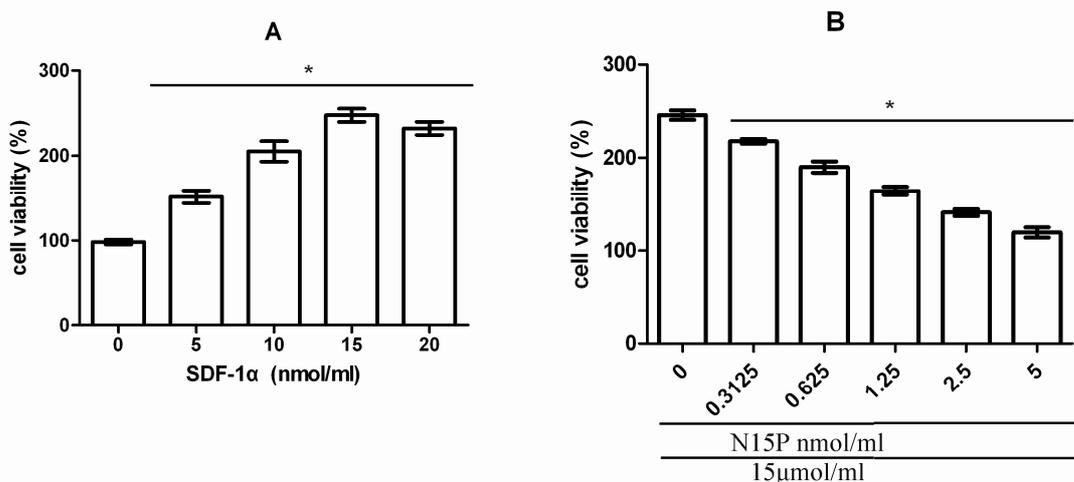


Figure 2: Cell viability of U87 cells measured by MTT assay; **A** = SDF-1 α caused a dose-dependent induction of proliferation with maximum effect at 15 mol; **B** = treatment with N15P decreased proliferation of U87 cells dose-dependently; * $p < 0.05$, compared with control group

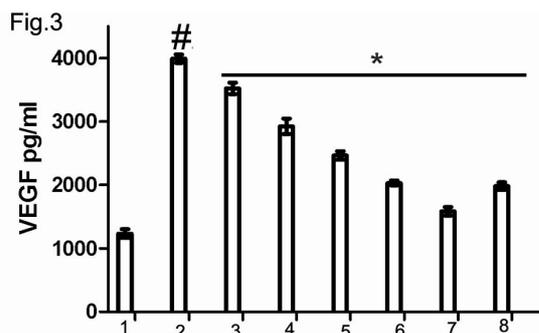
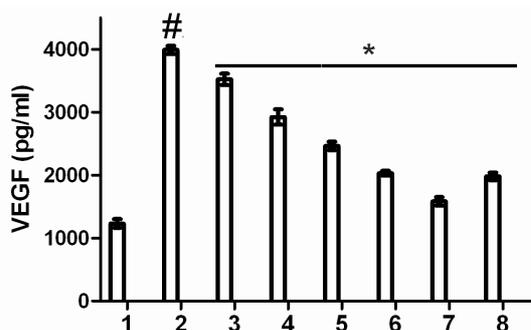


Figure 3: Reduction of SDF-1 α -induced VEGF production in U87 tumor cells by N15P. 1 = 0 nmol/ml SDF-1 α ; 2 = 15 nmol SDF-1 α ; 3 = 15 nmol/ml SDF-1 α + 0.3125 nmol/ml N15P; 4 = 15 nmol/ml SDF-1 α + 0.625 nmol/ml N15P; 5 = 15 nmol/ml SDF-1 α + 1.25 nmol/ml N15P; 6 = 15 nmol/ml SDF-1 α + 2.5 nmol/ml N15P; 7 = 15 nmol/ml SDF-1 α + 5 nmol/ml N15P; 8 = 15 nmol/ml SDF-1 α + 10 μ mol/ml LY294002; # p < 0.05, compared with lane 1; * p < 0.05, compared with lane 2

DISCUSSION

VEGF, one of the major angiogenic factors, is a critical mediator of angiogenesis and tumor proliferation [16] and there is strong evidence

that VEGF plays a key role in tumor angiogenesis process [17]. Recently, the role of CXC-chemokine modulation of VEGF signaling played in tumorigenesis has been elucidated in various cancers, including gliomas [18]. Therefore, understanding the regulatory mechanisms of VEGF expression in glioblastoma multiforme cancer cells may be vital for novel therapies to fight various cancers. So, in this study, we first analysed the mRNA expression of CXCR4, SDF-1 α and VEGF in human glioblastoma cells by RT-PCR, the result of CXCR4, SDF-1 α and VEGF expression in U87 glioblastoma tumor cells, their occurrence in U87 glioblastoma tumor cells suggest that they may play an important role in glioblastoma.

Then we studied the direct interaction of SDF-1 α -CXCR4 in glioblastoma viability *in vitro* using U87 human glioblastoma cell lines. The results showed that in U87 cell lines, stimulation with exogenous SDF-1 α induces a significant dose-dependent proliferation with the maximal effect at 15 nmol/ml. N15P, the CXCR4 receptor antagonist, was used to study its function in U87 cell tumor progress. The results showed that N15P inhibited the proliferation cell proliferation, thereby inhibiting tumor progress.

It has been shown that SDF-1 α /CXCR4 axis in tumor cells can promote VEGF production and glioblastoma is one of the most vascularized tumors [19]. PI3K/AKT signaling is critical to the evolution of aggressive tumourigenesis, and activation of the AKT pathway is known to be involved in CXCR4-mediated angiogenesis [20].

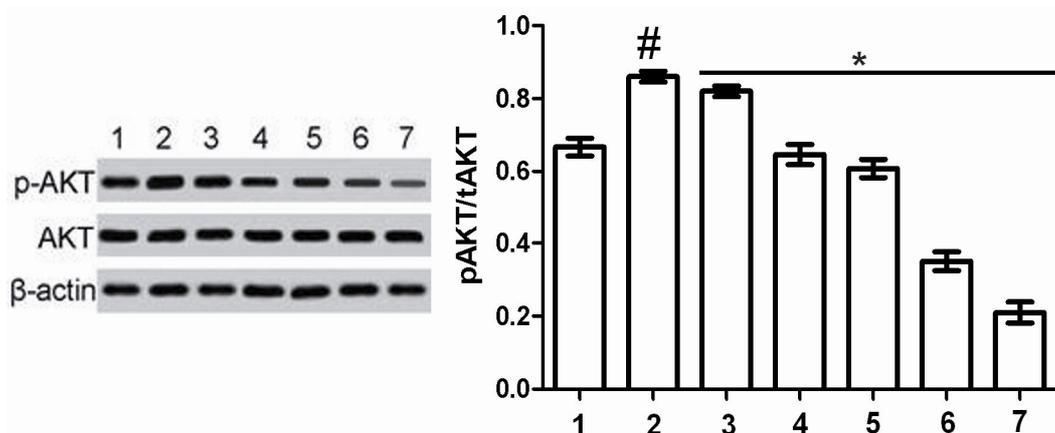


Figure 4: AKT Signaling Pathway. 1: control; 2:15 μ mol/ml SDF-1 α ; 3:0.3125 nmol/ml N15P + 15 μ mol/ml SDF-1 α ; 4: 0.625 nmol/ml N15P + 15 μ mol/ml SDF-1 α ; 5:1.25 nmol/ml N15P + 15 μ mol/ml SDF-1 α ; 6:2.5 nmol/ml N15P + 15 μ mol/ml SDF-1 α ; 7:5 nmol/ml N15P + 15 μ mol/ml SDF-1 α . # p < 0.05, compared with lane1. * p < 0.05, compared with lane 2

Thus we further set out to investigate the critical determinants of the SDF-1 α /CXCR4-induced activation of intracellular signaling pathways of U87 cells. In this paper, we found that SDF-1 α induces a significant effect on VEGF production and VEGF production in U87 cells after CXCR4 stimulation was dependent on PI3K/AKT pathway. In addition, these changes in intracellular processes were blocked by N15P in a dose-dependent manner. This phenomenon indicates that activation of PI3K/Akt is an important step in the signal that leads to secretion of VEGF in U87 cells.

Our preliminary study demonstrated that N15P may be a potent antagonist of SDF-1 α -CXCR4 signaling and can block the physiological action mediated by SDF-1 α -CXCR4 axis, resulting in inhibition of the AKT phosphorylation and blockade of SDF-1 α -induced cell proliferation. Collectively, these results may represent a new method for development of new therapeutic strategies for glioma. Thus our further studies will set out to elucidate the critical determinants and the mechanism of the SDF-1 α -induced cellular response and the inhibition of N15P on SDF-1 α -mediated tumor process of glioblastoma *in vitro* and *vivo*. Consequently, the studies discussed above are ongoing.

CONCLUSION

These results suggest that N15P suppress the SDF-1 α /CXCR4-Mediated VEGF production through Akt signaling pathway and this may be a potent therapeutic strategy in glioblastoma.

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