RESEARCH COMMUNICATION

5-Aminoisoquinolinone Reduces the Expression of Vascular Endothelial Growth Factor-C via the Nuclear Factor-kappa B Signaling Pathway in CT26 Cells

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Abstract

Objective: VEGF-C has recently been identified as a key molecule which is involved in tumor lymphangiogenesis. The aim of this research was to investigate the role of PARP-1 inhibition in the regulation of VEGF-C expression in CT26 cells. Methods: CT26 cells were treated with or without the PARP-1 inhibitor 5-aminoisoquinolinone (5-AIQ). The expression of PARP-1, NF-kB, and VEGF-C proteins in CT26 cells was measured by Western blot analysis and the VEGF-C mRNA level was determined by reverse transcription polymerase chain reaction (RT-PCR). CT26-secreted VEGF-C was detected by enzyme-linked immunosorbent assay (ELISA). Results: The results of Western blot analysis showed that the expression levels of PARP-1, NF-kB, and VEGF-C were reduced in 5-AIQ treated CT26 cells and the levels of VEGF-C mRNA in 5-AIQ treated CT26 were significantly lower than t in 5-AIQ-untreated cells (P<0.05). The concentrations of CT26-secreted VEGF-C were also dramatically decreased (P<0.05). Conclusion: Here, we provide evidence for the first time that PARP-1 inhibition dramatically reduces VEGF-C expression via the nuclear factor NF-kB signaling pathway. We therefore propose that PARP-1 inhibition has an anti-lymphangiogenic effect and may contribute to the prevention of metastatic dissemination via the lymphatic system.

Keywords: PARP-1 - NF-kB - VEGF-C - tumor lymphangiogenesis

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Introduction

Cancer metastatic dissemination plays a pivotal role in the progression of malignant tumors. Therefore, the principal goal for the treatment of malignant tumors is to prevent tumor proliferation, invasion and metastasis. Tumor lymphangiogenesis is a key factor for the spread of carcinomatous cells and therefore, usually affects the prognosis of patients. Various evidences from recent advances have demonstrated that tumor-induced lymphangiogenesis is an important mechanism promoting lymphatic metastasis (Karpanen et al., 2001; Mandriota et al., 2001; Skobe et al., 2001).

PARP-1 is a monomeric, chromatin-bound enzyme, which is abundantly present in eukaryocytes (except yeast) and is activated by DNA single-strand breaks (Isabelle et al., 2010). PARP-1 uses ADP-ribose units from its substrate NAD^+ to build a polyanionic poly (ADP-ribose) polymer onto Glu residues on its acceptor proteins including histones, transcription factors and mainly PARP-1 itself and then recruit the repair apparatus to repair the DNA breaks (Diefenbach and Bürkle, 2005; Woo and Threadgill, 2005). 5-AIQ, a highly water soluble inhibitor, is known to inhibit the activation of PARP-1. Inhibition of PARP-1 activation has been found to prevent angiogenesis (Rajesh et al., 2006; Fauzee et al., 2010), decrease colon injury (Cuzzocrea et al., 2006), reduce the evolution of experimental periodontitis (Di Paola et al., 2004), and protect ischemic-reperfusion induced myocardial injury (Pálfi et al., 2005).

NF-kB is a transcription factor that participates in diverse processes such as immunity, inflammation, cell proliferation, apoptosis or embryonal development (Fauzee et al., 2010; Fauzee et al., 2012; Li et al., 2012) Active NF-kB consist of dimeric combination of members of the NF-kB/Rel transcription factor family (Rothwarf and Karin, 1999).

VEGF-C has recently been identified as a critical regulator in tumor induced lymphangiogenesis. VEGF-C has been found to induce lymphangiogenesis by VEGF receptor-3 (VEGFR-3), which is mainly located in lymphatic endothelial cells. Its over expression in breast cancer is known to promote tumor lymphangiogenesis and enhance regional lymph nodes metastasis (Skobe et al., 2001). A series of reports have shown that inhibition of VEGF-C expression suppresses tumor lymphangiogenesis, tumor growth and regional lymph node metastasis (Sun et al., 2008; He et al., 2009; Zhang et al., 2010). Thus, the inhibition of VEGF-C activation in tumor cells may be useful in suppressing tumor growth and preventing lymph

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node metastasis.

However, neither the effect of PARP-1 inhibition on regulating VEGF-C expression nor the effect of PARP-1 inhibition on tumor associated lymphangiogenesis has been determined yet. Hence, we investigated the effect of PARP inhibition on regulating the VEGF-C expression by NF-kB signal pathways in CT26 tumor cells.

Materials and Methods

Materials and Main Reagents

CT26, a murine colon carcinoma cell line, was kindly provided by Prof. YQ. Wei, Hua xi Hospital, Sichuan University. 5-AIQ was kindly supplied by Prof. Michael D. Threadgill. PARP-1 polyclonal antibody, NF-kB monoclonal antibody and β-actin polyclonal antibody were from Santa Cruz Biotechnology. VEGF-C antibody was from Abcam Inc. RNAiso Plus Reagent and PrimeScript II 1st Strand cDNA Synthesis Kit were from Takara Biotechnology Co., Ltd.

Cell Lines and Culture Conditions

CT26 cells were grown in RPMI 1640 medium, supplemented with 10% FBS, Penicillin (100 unit/ml) and Streptomycin (100 μg/ml). The CT26 cells were cultured in a humidified 37 °C incubator with 5% CO_2, supplemented every 2 days with complete medium. Once confluence was reached, the CT26 cells were used in respective experiments.

Western Blot Analysis

The CT26 cells were grown at 3×10^4 cells ml/L in a 100ml culture flask and pretreated with or without 5-AIQ for 16 h, then washed with ice-cold PBS and lysed with M-PER® Mammalian Protein Extraction Reagent, following manufacturer's instructions. The nuclear proteins were extracted with NE-PER® nuclear and cytoplasmic extraction reagents according to the manufacturer's instructions. Western blot analyses were carried out using procedures described by Lee et al., 2006. Concentrations of protein were determined by the Bradford protein assay using BSA as standard. Equal amounts of protein (25 ug) prepared from the cells were separated on SDS-polyacrylamide gels (8%) and then transferred onto a PVDF membrane. After blocking non-specific binding sites, membranes were incubated with rabbit anti-mouse PARP-1 polyclonal antibody (1:300), with mouse anti-mouse NF-kB monoclonal antibody (1:500), with rabbit anti-mouse VEGF-C (1:200) or with rabbit anti-mouse β-actin polyclonal antibody (1:1000) over-night at 4 °C. The immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence.

RT-PCR

For RT-PCR, total CT26 cellular RNA was extracted from controls and treated samples with the RNAiso Plus method and cDNA was synthesized with PrimeScript II 1st Strand cDNA Synthesis Kit according to the manufacturer's protocol. The first-strand cDNA synthesis was implemented with Reverse Transcription System. 10 μl of cNDA products was amplified in a 50 μl PCR system. PCR primer sequences were as follows: VEGF-C (sense) 5'-TTCTCGGAAGCGGAGCCCGA-3' and (antisense) 5'-GCCTTTCCGCAGCTGGCACT-3', β-Actin:(sense) 5'-GACCCAGATCATGTTTGAGACC-3' and (antisense) 5'-GCCAGGATAGAGCCACCATA-3', respectively. PCR analysis was performed under the following conditions: 33 cycles of denaturation for 10 seconds at 940 C, annealing for 30 seconds at 600 C, and extension for 45 seconds at 720 C. All samples, including the control group, were run in triplicate. PCR products were then analyzed by 1.5% agarose gel electrophoresis and PCR fragments were visualized by ethidium bromide staining.

ELISA

Tested supernatants were obtained on the 16th hour of 5-AIQ-treated or untreated CT26 cells. Enzyme-Linked Immunosorbent assay was performed on 100 μl aliquots of the cultures' supernatants from both 5-AIQ-treated and untreated CT26 cells; in order to estimate the concentration of VEGF-C. The mouse ELISA kit was used according to manufacturer's instructions and the OD450 was measured using a Microplate reader model 3550.

Statistical Analysis

Data are given as the mean ± standard deviation (SD) in quantitative experiments. The significance of the data was calculated by Student's t-test and statistical calculations were performed using SPSS version 13.0. Values of P of less than 0.05 were considered to be statistically significant.

Results

The effects of 5-AIQ on the expressions of PARP-1, NF-kB and VEGF-C

To determine whether 5-AIQ affects the expressions of PARP-1, NF-kB and VEGF-C, CT26 cells were incubated with or without the 5-AIQ. Expressions of PARP-1, NF-kB and VEGF-C were determined by Western blotting, which showed that compared to the control group, there were

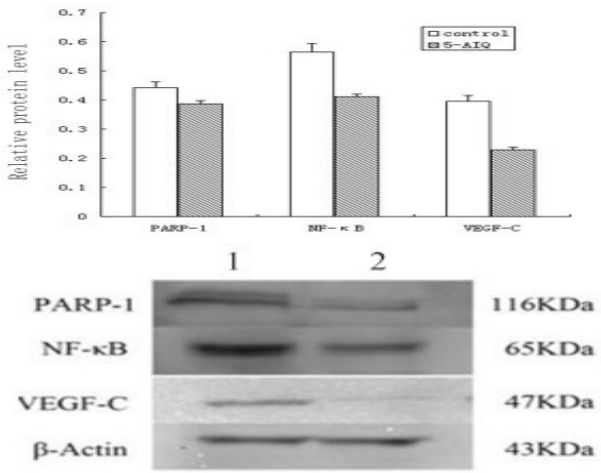

Figure 1. (A, B) PARP-1 Inhibition Reduced Expression of PARP-1, NF-kB and VEGF-C by Western Blot Analysis. The β-actin protein was used as an internal loading control. Lane 1: 5-AIQ-untreated group; Lane 2: 5-AIQ-treated group

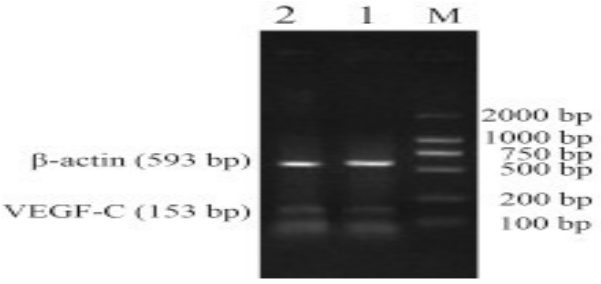

Figure 2. PARP-1 Inhibition Decreases The Expression of VEGF-C mRNA by RT-PCR Analysis. β-actin mRNA was used as an internal loading control. Compared with control cells, VEGF-C mRNA expression was significantly decreased in 5-AIQ-treated cells. Lane M: DNA standard (DL2000); Lane 1: 5-AIQ-treated group; Lane 2: 5-AIQ-untreated group

Table 1. Effects of 5-AIQ on Concentrations of the VEGF-C in 5-AIQ-treated CT26 Cells Supernatants

Group	VEGF-C(pg/ml)
Control	896.72±53.92
5-AIQ-treated	90.44±10.32*

a significant decrease in the expressions of the PARP-1, NF-kB and VEGF-C protein in the experimental group (5-AIQ treated tumor cells) (Figure 1 A, B).

The Effects of 5-AIQ on VEGF-C mRNA level

The mRNA level of VEGF-C was high in the control group while this same expression was significantly lower in the 5-AIQ-treated group ($P<0.05$, Figure 2). The result demonstrates that PARP-1 inhibition down-regulates VEGF-C at mRNA level.

The effect of 5-AIQ on the concentrations of CT26-secreted VEGF-C

We evaluated VEGF-C protein expression and secretion of 5-AIQ-treated CT26 cells conditioned medium using ELISA; 5-AIQ-untreated CT26 cells served as control (Table 1). PARP-1 inhibition dramatically reduced concentrations of the secreted VEGF-C in CT26 cells through ELISA method. Compared with the control group assessed, concentrations of the VEGF-C in 5-AIQ-treated CT26 cells supernatants ($P<0.05$) were significantly lower. Only minimal expression was seen in 5-AIQ-treated CT26 cell lines. Concordance between VEGF-C mRNA and protein levels in 5-AIQ-treated CT26 cells was observed clearly.

Discussion

PARP-1 is involved in many cellular processes, including the control of repair of damaged DNA (especially single-strand breaks), the regulation of gene expression and transcription. However, till now very little is known about the involvement of PARP-1 inhibition in tumor induced lymphangiogenesis. Therefore, in this study, we have shown that PARP-1 inhibition by 5-AIQ decreases the expression of VEGF-C secreted by CT26 mouse colon adenocarcinoma cells.

Our previous studies had also showed that PARP-1 inhibition can dramatically decrease the activation of nuclear factor-kB and its DNA-binding activity (Li et al., 2009, Li et al., 2012). PARP-1 has been reported to be a co-activator of NF-kB in many inflammatory disorders (Pétrilli et al., 2004; Zheng et al., 2004). Indeed, it has been proposed that acetylation of PARP by p300/CREB-binding protein plays an essential regulatory role in NF-kB-dependent transcription gene expression (Hassa et al., 2005). A series of evidences have shown that NF-kB binds to an NF-kB sequence in the VEGF-C promoter (Chilov et al., 1997; Zhang et al., 1997). NF-kB also regulates heregulin-beta-1-induced VEGF-C expression in human breast cancer cells (Tsai et al., 2003). Over expression of Her-2/neu causes VEGF-C production via NF-kB activation cascade in epithelial ovarian carcinoma (Hsieh et al., 2004). The pro-inflammatory cytokine, interleukin-1β (IL-1β), was found to induce inflammatory lymphangiogenesis via enhanced production of VEGF-C expression through NF-kB activation (Watari et al., 2008).

Cumulative data show that VEGF-C mediates the increased formation of lymphatic vessels and lymphatic spread (He et al., 2005; Renyi-Vamos et al., 2005). Moreover, studies using experimental animal models have demonstrated that inhibition of VEGF-C/VEGFR-3 signal transduction would reduce the spread and metastasis of tumor cells via lymphatic lumens (Chen et al., 2005; Lin et al., 2005; Roberts et al., 2006). Inhibition of tumor secreted VEGF-C is known to be a therapeutic target as well as VEGF-C specific inhibition using small interfering RNA (SiRNA) vectors has been indicated to suppress lymphatic metastasis (Chen et al., 2005). There is a substantial evidence that tumor derived VEGF-C plays a crucial role in lymphangiogenesis (Skobe et al., 2001; Chen et al., 2005; Lin et al., 2005; Roberts et al., 2006; Sun et al., 2008; He et al., 2009). Hence, these results directly hint the important role of the VEGF-C/VEGFR-3 signal transduction pathway in tumor associated lymphangiogenesis together with lymphatic metastasis.

Based on our results and findings, we speculate that one mechanism by which PARP-1 inhibition reduces the expression of VEGF-C is via the suppression of NF-kB signaling pathways. Besides, several conserved putative binding sites such as NF-kB, Sp-1 and AP-1 transcription factors are located in the upstream sequence of the VEGF-C promoter region (Chilov et al., 1997). Therefore, it is hypothesized that diminished VEGF-C production can be partly due to the inhibitory effect of PARP-1 on NF-kB signalling activation. However, we cannot remove the possibility of participation of other signal pathways and molecules in the inhibitory effect of PARP-1 on lymphangiogenesis.

In summary, PARP-1 inhibition appears to act as a suppressor of VEGF-C expression in CT26 cells and our results provide the first concrete data that PARP-1 inhibition has the potential to suppress tumor-related lymphangiogenesis.

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