Original Article

Vascular endothelial growth factor induces multidrug resistance-associated protein 1 overexpression through phosphatidylinositol-3-kinase/protein kinase B signaling pathway and transcription factor specificity protein 1 in BGC823 cell line

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Multidrug resistance (MDR) is one of the most important causes of chemotherapy failure and carcinoma recurrence. But the roles of the MDR-associated protein MRP1 in MDR remain poorly understood. Vascular endothelial growth factor (VEGF), one of the most active and specific vascular growth factors, plays a significant role in proliferation, differentiation, and metastasis of cancers. To explore the effect of VEGF on the expression of MRP1, we used recombinant human VEGF to stimulate K562 and BGC-823 cell lines. Quantitative real-time polymerase chain reaction and western blot analysis showed that the expression of MRP1 at both mRNA and protein levels was increased. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide results also showed that VEGF significantly enhanced the IC50 of the cells treated with adriamycin. To explore the underlying regulatory mechanisms, we constructed MRP1 promoter and the luciferase reporter gene recombinant vector. The luciferase reporter gene assay showed that the activity of the MRP1 promoter was markedly increased by VEGF stimulation, while LY294002, an inhibitor of the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, reduced this effect. Transcription factor specificity protein 1 (SP1) binding site mutation partially blocked the up-regulation of MRP1 promoter activity by VEGF. In summary, our results demonstrated that VEGF enhanced the expression of MRP1, and the PI3K/Akt signaling pathway and SP1 may be involved in this modulation.

Keywords cancer; VEGF; MRP1; PI3K/AKT signaling pathway; SP1

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Introduction

It is well known that the mechanism of multidrug resistance (MDR) in cancer cells is related to the transporters, such as P-glycoprotein (P-gp) and MDR-associated protein 1 (MRP1); the induction of chemotherapeutic drug excretion; the system of cell detoxification; the strengthening of DNA repair; and cell apoptosis. P-gp, which is encoded by MDR1, is a transporter that has received the most attention and has the most clearly characterized mechanisms. In contrast, the mechanism of MRP1 expression remains unclear.

MRP1 is a member of the ATP-binding cassette membrane transporter protein family [1]. Like other ATP-binding transporters, MRP1 is a transmembrane glycoprotein whose transmembrane domain forms the foundation of the drug transport system. The MRP family is composed of seven members, in which MRP1 is the first to be discovered. Its mRNA was detected in 1992 by molecular hybridization technique and confirmed by Southern hybridization [2]. MRP1 is widely expressed in malignant tumor cells. Most importantly, its expression was even detected in some drug-resistant cancer cells that do not express P-gp. Thus, further investigation on MRP1 is urgently required.

Vascular endothelial growth factor (VEGF) is positively correlated with vascularization and malignant potentials of cancer cells [3–5]. It was purified from the medium of bovine pituitary gland folliculostellate cells, and its activity was detected in the mitosis induction of vascular endothelial cells [6]. VEGF is one of the most active and specific vascular growth factors and induces the proliferation, migration, and differentiation of endothelial cells [7]. VEGF can interact with VEGF receptors (VEGFR) to trigger receptor tyrosine kinase activation, and activate the downstream phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway [8], one of the most important signaling pathways in vivo, which is involved in the processes of growth, differentiation, and metabolism [9–11]. In addition, VEGF plays a significant role in the regulation of MRP1. Wortmannin, a specific inhibitor of the PI3K/Akt signaling pathway, has been shown to reduce the expression of MRP1 by inhibiting Akt phosphorylation [12].
Recent studies have shown that VEGF not only is considered the promoter of proliferation and inhibitor of apoptosis in cancer cells, but also functions to strengthen drug resistance. Down-regulation of VEGF reduces the drug susceptibility in some leukemic cells [13]. The expression of VEGF and its receptor Flt-1 is markedly up-regulated in cells that are resistant to adriamycin when compared with those that are sensitive [14]. Therefore, VEGF may be related to the formation of drug resistance. Notably, previous studies have shown that VEGFR-2 inhibitor YM-359445 reduces the proliferation and vascular formation in drug-resistant cancer cells [15]. Moreover, VEGF-165 can induce MDR phenotype in human dermal microvascular endothelial cells to various drugs, such as cisplatin, mitomycin, VP-16, and paclitaxel among others, which is attributed to the overexpression of MRP and lung resistance-related protein [16]. Therefore, VEGF is closely related to MDR and MRP1 expression in malignant cells, and the PI3K/Akt signaling pathway may be involved in this regulation. However, the underlying mechanisms require further investigation.

The transcription regulation of MRP1 is complicated. Several regulation sites located at the positive and negative regulatory domains of the MRP1 proximal promoter region, such as TATA and CAT boxes. Sequence analysis shows that these sites contain various transcription factors binding domains, for example, specificity protein 1 (SP1), activator protein 1 (AP1), activator protein 2 (AP2), estrogen response element (ERE), and glucocorticoid response element (GRE) [17]. Thus, it would be expected that some of these transcription factors may contribute to the regulation of MRP1.

Our previous studies have shown that the anti-VEGF antibody dose dependently reduced the expression of MRP1 at both mRNA and protein levels [18]. Here, we used recombinant human VEGF to stimulate K562 and BGC-823 cell lines, and further confirmed the relationship between VEGF and MRP1. The MRP1 promoters with or without SP1 binding site mutation were synthesized and cloned into the luciferase reporter gene vector pGL3-Basic to explore the mechanisms by which VEGF up-regulates MRP1. The results indicated that PI3K/Akt signaling pathway and transcription factor SP1 are involved in this regulation.

**Materials and Methods**

**Reagents**

First Strand cDNA Synthesis kit was bought from Fermentas Corporation (Beijing, China). The luciferase reporter gene vector pGL3-Basic was bought from Shanghai Generay Biological Technology Limited Company (Shanghai, China) and the control vector β-gal was generously provided by Prof. Yongchuang Cheng from Jiangsu University (Zhenjiang, China). Recombinant human VEGF was purchased from Peprotech Inc. (Rocky Hill, USA). All antibodies were bought from Wuhan Boster Biological Technology Limited Company (Wuhan, China). pMD19T-A clone kit and SYBR Green real-time polymerase chain reaction (PCR) kit were obtained from Takara (Shiga, Japan). DNA Gel extraction kit was purchased from Axygen Scientific (Union City, USA). LY294002 was bought from Cell Signaling (Beverly, USA). Lipofectamine 2000, Trizol reagent, Nuclear/Cytosol fractionation kit, and DAB substrate kit were obtained from Invitrogen (Carlsbad, USA). Lumat LB 9507 was bought from Berthold Technologies (Bad Wildbad, Germany). Luciferase reporter gene evaluating reagent kit, β-gal report gene testing reagent kit, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were bought from Biyuntian Biological Technology Laboratory (Shanghai, China). NE-PER nuclear and cytoplasmic extraction reagents and lightshift electrophoretic mobility shift assay (EMSA) kit were bought from Pierce (Rockford, USA).

**Cell lines and cells culture**

Human erythromyeloblastoid leukemia cell line K562 and human gastric carcinoma cell line BGC-823 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). K562 cells and BGC-823 cells were cultured in RPMI 1640 medium (Gibco, San Francisco, USA) and Dulbecco’s modified Eagle’s medium (Gibco) respectively, with 10% newborn calf serum, in a humidified incubator at 37°C with 5% CO2. Cells were passaged every other day, checked routinely, and were free of contamination by mycoplasma or fungi. Both K562 and BGC-823 cells were stimulated with different concentrations of VEGF (0, 0.5, 2, 8, and 32 ng/ml) for 24 h. For quantitative reverse transcriptase (qRT)-PCR and western blot analysis, VEGF was used at the concentration of 32 ng/ml, either alone or in combination with LY294002 (50 μmol/ml).

**qRT-PCR**

RNA samples from the K562 and BGC-823 cells were extracted by Trizol reagent. cDNAs were prepared by first strand cDNA synthesis kit according to the protocol. The MRP1 primers were synthesized by Shanghai Shenggong (Shanghai, China). The sequence of MRP1 primers were as follows: MRP1_F, 5'-GTACATTAAATGATCTGGTC-3', MRP1_R, 5'-CGTCTCATCAGCTTGATCCGAT-3'. qRT-PCR was performed on MX3000p (Stratagene, La Jolla, USA). The reaction conditions were 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 20 s at 54°C. A standard curve and a melting curve were created automatically when the reactions were completed.
Western blot analysis

The protein samples (30 μg per lane) from the stimulated BGC-823 and K562 cells were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. After electrophoresis, the proteins on the gel were transferred onto nitrocellulose (NC) membrane. The NC membrane was blocked with 50 g/L milk in Tris-buffered saline with Tween for 1 h at room temperature, followed by incubating with primary antibody for 2 h and secondary antibody for 50 min at room temperature. The membrane was washed three times after each incubation. 3,3′-Diaminobenzidine substrate kit was used to visualize the positive strips on the membrane.

MTT assays

A total of 1 × 10⁴ BGC-823 or K562 cells in 0.1 ml culture medium were plated into each well of 96-well plate, and cultured with different concentrations of VEGF (0, 0.5, 2, 8, and 32 ng/ml) for 24 h. The cells were then incubated with medium plus adriamycin (0, 0.5, 1.0, 1.5, 2.0, and 2.5 μg/ml). After 48 h incubation, 10 μl of MTT (6 g/L) was added into each well and the incubation was continued for further 4 h at 37°C. Finally, the culture medium was removed and 200 μl of dimethylsulfoxide was added to each well. The absorbance was determined at 570 nm. The percentage change in the absorbency value (A) of the cells treated with both VEGF and adriamycin compared with the A value of the control cells was calculated as: Change (%) = A value (adriamycin group − control)/A value of control group × 100%. The IC₅₀ was calculated by a software according to the percentage change shown above.

Construction of the MRP1 promoter luciferase plasmids and luciferase reporter assays

The MRP1 promoters, with and without SP1 mutations, were designed and cloned into the luciferase reporter gene vector, named pGL3-Basic-MRP1w (wild-type MRP1) and pGL3-Basic-MRP1m (MRP1 with SP1 combining site mutations). A total of 2.5 × 10⁵ untreated BGC-823 cells were plated into each well of six-well plate for 24 h. Cells at 80% confluence were transiently co-transfected with the luciferase promoter luciferase plasmids (with or without SP1 mutations) and control vector β-gal, using Liopfectamine 2000 reagent according to the instructions of the manufacturer. The medium was changed to complete medium at 4–6 h after transfection. The transfected cells were then treated with various concentrations of VEGF for 6, 12, or 24 h, either alone, or after pre-treatment with different concentrations of LY294002 (0, 10, 20, 30, 40, and 50 μmol/ml) for 1 h. The luciferase reporter assays were performed with Lumat LB 9507.

Electrophoretic mobility shift assays

Nuclear protein extracts of the control BGC-823, VEGF (32 ng/ml)-treated cells, and LY294002 (50 μmol/ml) pre-treated cells were prepared with NE-PER nuclear and cytoplasmic extraction reagents. EMSA were performed with LightShift EMSA kit following the protocol of the manufacturer. Biotin-labeled SP-1 oligonucleotides (5′-ATT CGA TCG GGG CGG GAG C-3′, 5′-G CTC GCC CCG CCC CGA TCG AAT-3′) of 50 ng were mixed with 10 ng nuclear protein in binding buffer and incubated for 20 min at room temperature. The protein complexes were resolved in 6% DNA retardation gels.

Statistical analysis

Results were shown as mean ± standard error. Statistical analysis of the data was performed using the unpaired two-tailed Student’s t-test, one-way analysis of variance, and linear correlation analysis by SPSS17.0 software (Bizinsight Beijing Information Technology Co., Ltd., Beijing, China). Differences were considered statistically significant when P < 0.05.

Results

VEGF-induced drug resistance in cancer cells

Our previous studies have suggested that the expression of MRP1 at both protein and mRNA levels is dose dependently reduced by anti-VEGF antibody [18]. In this study, to further explore the relationship between VEGF and MRP1, we used recombinant human VEGF to simulate the K562 and BGC-823 cells. The results of qRT-PCR showed that VEGF markedly increased the mRNA expression of MRP1 in a dose-dependent manner in both BGC-823 and K562 cells [P < 0.01; Fig. 1(A), Supplementary Fig. S1(A)]. Compared with the control group, the cells treated with VEGF at 32 ng/ml showed the greatest enhancement in the expression of MRP1 mRNA, with 3.5-fold and 2.0-fold increases in BGC-823 and K562 cells, respectively (P < 0.01). Consistent with the results of qRT-PCR, western blot results showed that VEGF also significantly enhanced the expression of MRP1 [Fig. 1(B), Supplementary Fig. S1(B)]. With the concentration of VEGF being increased, the expression of MRP1 protein was significantly enhanced. MTT assay results indicated that VEGF dose dependently enhanced the IC₅₀ of BGC-823 and K562 to adriamycin [P < 0.01; Fig. 1(C), Supplementary Fig. S1(C)]. Compared with the control group, the IC₅₀ were 2.4-fold and 1.8-fold higher in the groups of BGC-823 and K562 cells stimulated by 32 ng/ml of VEGF, respectively (P < 0.01).

VEGF enhanced the activity of the MRP1 promoter

Promoters, one of the most important transcriptional elements, play a crucial role in the regulation process of gene expression. In order to explore the functions of VEGF affecting the MRP1 promoter, we synthesized the promoter of MRP1 and cloned it into the luciferase reporter gene vector...
pGL3-Basic, named pGL3-Basic-MRP1w. The construct or the empty pGL3-Basic vector was then transiently transfected into BGC-823 cells, along with the control vector β-gal. We treated the transfected BGC-823 cells with different concentrations of VEGF (0, 0.5, 2, 8, and 32 ng/ml) for 6, 12, or 24 h. The results showed that the activity of the MRP1 promoter was stable after VEGF treatment for 6 h ($P < 0.05$). When the cells were treated with VEGF for 12 h, the activity of the MRP1 promoter increased in a dose-dependent manner ($P < 0.01$), with the highest level at VEGF concentration of 32 ng/ml, 4.4-fold greater than the control group ($P < 0.01$). When the cells were treated with VEGF for 24 h, the activity of MRP1 promoter also increased ($P < 0.01$), but the dose-dependent effect was not significant (Fig. 2).

The PI3K/Akt signaling pathway affected the functions of VEGF by increasing MRP1 promoter activity and Akt phosphorylation

Previous studies have suggested that inhibitors of the PI3K/Akt signaling pathway, such as wortmannin, can significantly increase the susceptibility of cancer cells to chemotherapeutic drugs [19]. Here, LY294002, another inhibitor of the PI3K/Akt signaling pathway, was used to explore the effects of this pathway on the regulation of MRP1 promoter activity by VEGF. The BGC-823 cells transfected with pGL3-Basic-MRP1w and β-gal were pre-treated with LY294002 at different concentrations for 1 h, followed by stimulating with 32 ng/ml of VEGF for 12 h. The results indicated that the activity of the MRP1 promoter was not obviously altered, when the cells were pre-treated with 10 or 20 μmol/ml LY294002 compared with the control group ($P > 0.05$; Fig. 3(A)). However, when the cells were pre-treated with LY294002 at 30–50 μmol/ml, the activity of the MRP1 promoter was markedly reduced ($P < 0.01$). The cells pre-treated with 50 μmol/ml of LY294002 showed the greatest effect, with the activity of the MRP1 promoter reduced by 60.8% compared with the control group.

Western blot results also suggested that VEGF up-regulated the protein expression of MRP1, but LY294002 could partially block this up-regulation, which was consistent with our previous experiments (Fig. 3(B)).

A previous study has proved that PI3K/Akt signaling pathway is activated in drug-resistant cells, with increased level of p-Akt [19]. To evaluate whether VEGF was able to enhance the expression of Akt and p-Akt in BGC-823 cells, we tested the expression of Akt and p-Akt under different conditions by western blot. The level of Akt was not changed at the three conditions, but the level of p-Akt was significantly increased in BGC-823 cells treated with VEGF compared with the control group. However, LY294002 pre-treatment diminished this increase, which indicated that VEGF increased the phosphorylation of Akt, while the inhibitor of the PI3K/Akt signaling pathway reduced this effect (Fig. 3(C)).

VEGF increased the expression of SP1 and its DNA combining activity

A GC-rich region which is the binding site of SP1 locates on the promoter of MRP1 [18,20]. Therefore, we assumed that VEGF might regulate MRPl expression partially through SP1. Next, we detected the expression of SP1 protein in BGC-823 cells by western blot. The results showed that the
expression of SP1 was increased by VEGF, and LY294002 pre-treatment reduced this increment [Fig. 4(A)]. Furthermore, we detected the DNA binding activity of SP1 by EMSA. Figure 4(B) showed that the DNA binding activity of SP1 was also enhanced by VEGF, and abolished by LY294002.

SP1 mutations partially blocked the up-regulation of MRP1 promoter activity by VEGF

In order to determine the contribution of SP1 to the up-regulation of MRP1 promoter activity by VEGF, a luciferase reporter gene vector contained SP1 mutations on the promoter of MRP1 was constructed, named pGL3-Basic-MRP1m. The recombinant vectors, pGL3-Basic-MRP1m, pGL3-Basic-MRP1w, or the empty vector pGL3-Basic, were transiently transfected into BGC-823 cells, along with the control vector β-gal, respectively. The activity of MRP1 promoter in the cell group transfected with pGL3-Basic-MRP1m was reduced by 23.6% compared with the activity in the pGL3-Basic-MRP1w group (P < 0.05), although it was still 1.8-fold higher than the control group (P < 0.05). These results suggested that SP1 binding sites mutation in the MRP1 promoter partially decreased MRP1 promoter activity [Fig. 5(A)].

Next, we detected the role of VEGF in activating SP1-mutated MRP1 promoter. BGC-823 cells transfected with pGL3-Basic-MRP1m were treated with different concentrations of VEGF for 6, 12, or 24 h. The MRP1 promoter
activity was stable when the cells were stimulated with different concentrations for 6 h \( P > 0.05; \text{Fig. 5(B)} \). However, when the VEGF treatment time was extended to 24 h, MRP1 promoter activity was enhanced dose dependently \( P < 0.01 \) and VEGF at concentration of 32 ng/ml induced a 2.2-fold increase of MRP1 promoter activity compared with the control group \( P < 0.01 \). A 0.7-fold increase of MRP1 promoter activity was also observed at 12 h when the cells were treated with 32 ng/ml of VEGF \( P < 0.01; \text{Fig. 5(B)} \).

### Discussion

MRP1 is widespread in many types of malignant tumor cells, such as gastric carcinoma, leukemia, multiple myeloma, lymphoma, non-small-cell lung carcinoma, and breast cancer. The study on 44 acute myeloblastic leukemia patients suggested that the patients with overexpressed MRP1 had a lower survival rate than the patients without MRP1 overexpression [21]. Therefore, investigating the regulatory mechanisms of MRP1 might provide a new potential approach to enhance cancer cell susceptibility to chemotherapeutic agents and to diminish patient mortality.

VEGF is an important growth factor which is involved in all the processes of tumor growth [22]. Its expression in serum is highly related to the prognosis of cancer patients, and it may reflect prognosis more precisely than the tumor node metastasis stages [23]. Recently, more and more studies have shown that VEGF is highly related to MDR in cancer cells. Therefore, MDR and MRP1 expression may be strongly related to VEGF in cancer cells, and VEGF inhibition may enhance drug susceptibility of cancer cells.

Here, we also revealed that VEGF could dose dependently increase the activity of the MRP1 promoter. Previous studies have confirmed that the PI3K/Akt signaling pathway is highly activated in drug-resistant cells, and may induce drug resistance in some leukemic cells. Moreover, LY294002 and Wortmannin, inhibitors of the PI3K/Akt signaling pathway, enhanced the drug susceptibility in such drug-resistant cells [12,19]. In addition, the PI3K/Akt signaling pathway has been shown to up-regulate MRP1 and reduce apoptosis, whereas, LY294002 markedly enhanced apoptosis and antagonized MRP1 [26]. In addition, VEGF activated the PI3K/Akt signaling pathway to induce the transcription of its downstream genes by combining with its receptors [8,27]. Together, these evidences suggest that PI3K/Akt signaling pathway may play a crucial role in the processes involved in MRP1 regulation by VEGF.

Consistent with previous investigation, in this study we found that LY294002 was capable of diminishing the activity of ZK7. Therefore, we considered that VEGF might be an effective predictor for curative effects of chemotherapy. Moreover, exploring the regulatory mechanisms of MDR-related genes induced by VEGF may not only explain the relationship between VEGF and MDR in cancer cells, but also provide a new target to reduce or prevent MDR in cancer cells.

Our previous study have shown that down-regulation of VEGF reduced the expression of MRP1 dose dependently [18]. In this study, we treated BGC-823 and K562 cells with recombinant human VEGF and found that VEGF enhanced the expression of MRP1 at both mRNA and protein levels. MTT assays also showed that VEGF increased the Adriamycin IC50 of K562 and BGC-823 cells, which further demonstrated that VEGF had the ability to increase drug resistance in cancer cells. Therefore, MDR and MRP1 expression may be strongly related to VEGF in cancer cells, and VEGF inhibition may enhance drug susceptibility of cancer cells.
the MRP1 promoter dose dependently in pGL3-Basic-MRP1w transfected cells. Nevertheless, the promoter region of MRP1 contains various positive and negative regulatory elements binding sites, such as SP1 combining sites, a GC-rich region [18]. Moreover, many transcription factors and repressor proteins participate in the regulation of MRP1 expression, and thereby determine the complexity of MRP1 transcription modulation. Furthermore, the PI3K/Akt signaling pathway regulates phosphorylation of SP1, enhances its DNA binding activity, and increases its transcription of downstream genes [27]. As one of the most important transcription factors located in the MRP1 promoter, SP1 has been previously demonstrated to be capable of activating the transcription of the MRP1 promoter. Consistent with this data, our experiments indicated that the DNA binding activity of SP1 was enhanced by VEGF, but LY294002 reduced this effect. In addition, the activity of the MRP1 promoter that contained SP1 mutations was markedly lowered than the activity of that without mutations, although the dose-dependent up-regulation by VEGF remained significant. These results suggested that transcription factor SP1 was at least partially involved in up-regulation of the MRP1 promoter by VEGF. However, considering the complexity of MRP1 transcriptional regulation, other signaling pathways and transcription factors, such as mitogen-activated protein kinases/extracellular signal-regulated kinases, AP1, and AP2 may also participate in this modulation. Moreover, the regulation of MRP1 by VEGF may differ between cell lines, so further evaluation is required.

In summary, in this study we found that VEGF enhanced the expression of MRP1 at both mRNA level and protein level, which increased the MDR in K562 and BGC823 cancer cells. PI3K/Akt signaling pathway and transcription factor SP1 were involved in the regulation. Understanding the mechanisms of MRP1 regulated by VEGF may help to produce a new therapeutic approach for the reversal and prevention of carcinoma MDR. Therefore, more comprehensive exploration of VEGF modulating MRP1 is urgently required.

Supplementary Data

Supplementary data are available at ABBS online.

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