·Basic Research·

# Effect of soluble vascular endothelial growth factor receptor-1 on proliferation of human multiple myeloma cells

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**[Abstract] Background and Objective:** Vascular endothelial growth factor (VEGF) is the most important angiogenic factor of multiple myeloma (MM). This study was to investigate the effect of transfection of human soluble vascular endothelial growth factor receptor-1 (sFlt-1) gene on the proliferation of human MM cell line RPMI8226. **Methods:** The recombinant plasmid pcDNA3-sFlt-1 was constructed and transfected into RPMI8226 cells. The expression of sFlt-1 was identified by reverse transcription-polymerase chain reaction (RT-PCR) and ELISA. The effects of sFlt-1 protein on the proliferation and VEGF expression of RPMI8226 cells were investigated by MTT assay and ELISA, respectively. **Results:** The recombinant plasmid pcDNA3-sFlt-1 was successfully transfected into RPMI8226 cells. sFlt-1 protein expression was identified by ELISA, which inhibited the proliferation of RPMI8226 cells and reduced VEGF concentration in the culture supernatant. **Conclusion:** RPMI8226 cells can express sFlt-1 protein with high biological activity when transfected with the sFlt-1 gene, which inhibits the proliferation of RPMI8226 cells.

**Key words:** Vascular endothelial growth factor receptor, transfection, multiple myeloma

The growth and metastasis of tumor is a process which depend on angiogenesis. Tumor angiogenesis is mediated by pro-angiogenic factors which produced by tumor cells and/or tumor infiltrating inflammatory cells. Vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors. VEGF plays the pro-angiogenic role through binding with the specific VEGF high-affinity tyrosine kinase receptors VEGFR-1/FLT-1 and VEGFR-2/KDR/FLK on endothelial cells.[1] Natural soluble vascular endothelial growth factor receptor-1 (sFlt-1) in human body is a good antagonist of VEGF and can competitively inhibit the pro-angiogenic role of VEGF.[2] In this study, we transfected the constructed eukaryotic expression vector sFlt-1 into multiple myeloma (MM) cell line RPMI8226 to measure the expression of sFlt-1 in cells after transfection and explore the possibility of VEGF as a target to treat MM.

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## Materials and Methods

### Materials

Eukaryotic expression plasmid pcDNA3-sFlt-1 and MM cell line RPMI8226 were preserved by our laboratory; G418, Trizol, Lipofectamine 2000 were purchased from Invitrogen Company; reverse transcription-polymerase chain reaction (RT-PCR) kit was from Fermantas MBI Company; restriction endonuclease *Eco*R I and *Bam*H I dNTP, *Taq* enzyme, plasmid extraction kit, gel extraction kit were from TaKaRa Company; fetal bovine serum (FBS), DMEM medium were from Gibco Company; RPMI-1640 culture medium, DNA marker, MTT, DMSO were from Beijing Dingguo Biotechnology; competent DH5α were from Guangzhou Ruizhen Biotechnology; sVEGFR-1 ELISA Kit and VEGF ELISA Kit were from Wuhan Boster Biotechnology. The remaining reagents were all domestically produced analytical reagents purchased from Guangzhou Chemical Factory.

### Methods

***Construction and identification of the recombinant plasmid pcDNA3-sFlt-1*** Previously constructed recombinant plasmid pcDNA3-sFlt-1 was identified.[3]

***Transfection of plasmid pcDNA3-sFlt-1 into RPMI8226 cells*** RPMI8226 cells were seeded in 24-well culture plates ($2\times10^5$ cells/well), cultured for 24 h with serum-free and antibiotics-free DMEM medium for cell adherence. The cells were divided into

empty vector pcDNA3 group and pcDNA3-sFlt-1 group. The plasmids were extracted by Ultra-pure plasmid DNA extraction and purification kit. The plasmid pcDNA3 and pcDNA3-sFlt-1 were transfected by Lipofectamine 2000 into RPMI8226 cells. After 48-hour transfection, the medium was replaced with RPMI-1640 containing G418 (600 mg/L), cells continued to be cultured. The supernatants at 24 h, 48 h and 3 days, 7 days, 14 days, 21 days after transfection were collected and cryopreserved for further use. Transfection efficiency was detected by flow cytometry.

***Identification of transfected cells*** Transcription level of sFlt-1 gene was detected by RT-PCR. The cells of pcDNA3 transfection group, pcDNA3-sFlt-1 transfection group and non-transfection group were collected separately. Total RNA was extracted by Trizol, amplified by RT-PCR using the same primers and reaction system as stated in reference 3.

The expression of sFlt-1 protein in supernatants collected at different time after transfection was detected by ELISA kit according to the manufacturer's instructions.

***Detection of the recombinant sFlt-1 protein function*** RPMI8226 cells were cultured with DMEM medium containing the supernatants collected at 24 h, 48 h, 3 days, 7 days, 14 days, 21 days after transfection. Cell proliferation was determined by MTT assay. The concentration of VEGF in RPMI8226 cell supernatant was detected by ELISA kit according to the manufacturer's instructions.

### Statistical analysis

Statistical analysis was performed in software package SPSS 13.0. One-way ANOVA was used to do multiple comparisons of the means among the groups; *t* test was used to compare between the two groups. Significant difference was reported when $P < 0.05$.

## Results

### Transfection efficiency

The transfection rate of RPMI8226 cells detected by flow cytometry was (10.9 ± 1.7)%, with a maximum of 13.9%.

### The expression of sFlt-1 in transfected cells

RT-PCR showed a distinct DNA band (940 bp) of sFlt-1 gene in pcDNA3-sFlt-1 transfection group, but no band of target gene in pcDNA3 transfection group and non-transfection group (Figure 1), suggesting that pcDNA3-sFlt-1-transfected RPMI8226 cells expressed sFlt-1 gene at transcriptional level.

The expression of sFlt-1 protein was detected at 24 h after transfection by ELISA, which increased at 48 h, reached the bottom at the third day, and then gradually recovered. There was still expression of sFlt-1 protein at 21 days after transfection (Figure 2).

### The functions of recombinant sFlt-1 protein

MTT results showed that the supernatants collected at different time after transfection (24 h, 48 h and 3 days, 7 days, 14 days, 21 days) inhibited the proliferation of RPMI8226 cells. Its inhibitory effect at 48 h [(16.07 ± 9.31)%] was higher than that at 24 h [(10.35 ± 6.97)%], and was fall down at the 3rd day [(10.74 ± 7.36)%] then increased gradually [(15.07 ± 9.02)% at the 7th day] with obvious effect shown at the 14th day [(20.25 ± 12.13)%] and 21st day [(29.37 ± 14.37)%]. One-way ANOVA showed that the supernatants collected at 48 h, 7 days, 14 days, 21 days after transfection inhibited the proliferation of RPMI8226 cells significantly as compared with that in non-transfection group (*P* values were 0.017, 0.025, 0.004, and < 0.001, respectively).

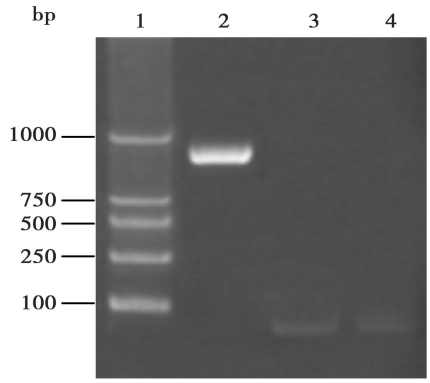


Figure 1 Identification of sFlt-1 gene in transfected RPMI8226 cells by RT-PCR

Lane 1, marker; lane 2, pcDNA3-sFlt-1-transfected cells; lane 3, pcDNA3 transfected cells; lane 4, untransfected cells.

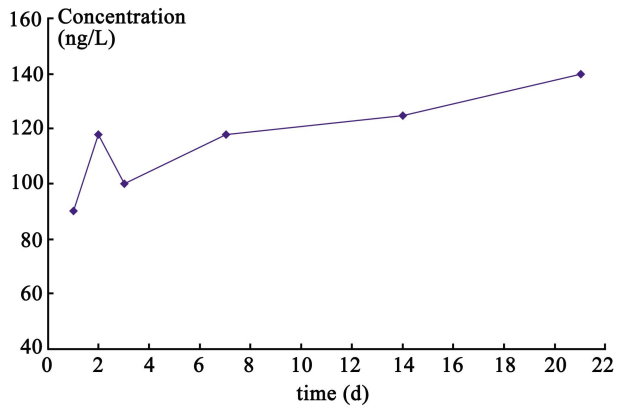


Figure 2 Time-concentration curve of sFlt-1 protein expression in RPMI8226 cells after pcDNA3-sFlt-1 transfection

RPMI8226 cells were cultured with DMEM media containing the supernatant which collected at 24 h. The VEGF protein concentrations in supernatant were (4089.34 ± 792.45) ng/L in pcDNA3-sFlt-1 transfection group at 24 h, (275.12 ± 732.61) ng/L at 48 h, (3929.53 ± 801.17) ng/L at the 3rd day, (3587.13 ± 767.19) ng/L at the 7th day, (3012.41 ± 624.33) ng/L at the 14th day and (2873.57 ± 609.35) ng/L at the 21st day, respectively, while was (4327.92 ± 813.21) ng/L in non-transfection group. It was reduced significantly at 48 h, 7 days, 14 days, and 21 days after transfection as compared with that in non-transfection group (*P* values were 0.002, 0.041, < 0.001 and < 0.001, respectively).

## Discussion

Some in vitro experiments showed that some tumor cells express FLT-1 or FLK, VEGF binding with FLT-1 or FLK on tumor cells can promote the growth of tumor cells. It has been found that VEGF is the most important pro-angiogenic factor in hematological malignancies, including MM and lymphoma. Kumar *et al.*[4] found that 90% of MM patients had the expression of VEGF in plasma cells; all the seven detected MM cell lines expressed FLT-1 and three of them expressed FLK. Studies have shown that serum VEGF levels in MM patients are closely related to severity and prognosis of the disease.[5] VEGF can not only promote bone marrow angiogenesis through the paracrine pathway but also trigger the proliferation and migration of MM cells through the autocrine pathway.[6] Some scholars have therefore suggested to target VEGF and its receptor to inhibit VEGF binding with corresponding receptors on vascular endothelial cells, inhibit angiogenesis, growth and migration of tumors, hence, make the tumors dormant.[7]

sFlt-1 exists as a free molecular state (non-membrane-bound state) in human body and retain the VEGF-binding sites. It has a high affinity with VEGF, can form dimers with the extracellular ligand-binding domain of transmembrane receptor Flt-1 and KDR/FLK. Because sFlt-1 has no activity of tyrosine kinase, it can inhibit the phosphorylation of tyrosine kinase receptors and block the activation of following transduction signals, inhibit the biological function of VEGF promoting angiogenesis.[8]

Our previous study has constructed pcDNA3-sFlt-1 eukaryotic expression vector successfully. In this experiment, we used liposome-mediated method to transfect recombinant plasmid pcDNA3-sFlt-1 into RPMI8226 cells, detected sFlt-1 24 h after transfection which showed stable expression at 21 days after transfection, proving that the sFlt-1 gene transfected into tumor cells and sustained high expression of sFlt-1 protein in tumor locally. In this study, the concentration of sFlt-1 protein in supernatant increased at 48 h after transfection while decreased at the 3rd day, then gradually recovered to stable expression. The reason of the change was unknown. We speculate that the fluctuant sFlt-1 transcription is due to the instable expression of sFlt-1 gene in cells early after transfection.

Our results further showed that the recombinant sFlt-1 protein secreted by transfected tumor cells inhibited RPMI8226 cell growth obviously. The inhibitory effect corresponds to the concentration of sFlt-1 protein in supernatant. VEGF protein content in RPMI8226 cells cultured with transfected supernatant was decreased with time. Our experiments confirmed that the sFlt-1 secreted by transfected RPMI8226 cells has a significant biological activity, and can counteract VEGF secreted by tumor cells. In addition, sFlt-1 could also block autocrine function of VEGF on tumor cells to inhibit tumor cell proliferation.

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