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Inhibition of cell proliferation and increased-apoptosis of AGS and SNU-5 cancer cells following small interfering RNA (siRNA)-mediated down-regulation of vascular endothelial growth factor receptor 1 (VEGFR1)

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Original Article

Abstract

BACKGROUND: Angiogenesis is vital for development of normal tissue and wound healing; but it play an important role in development of some diseases such as different types of cancer. Vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFR) are two important key factors in this process. Previous studies have shown that down-regulation of VEGFR1 inhibits cell proliferation, migration, and vascular permeability of endothelial cells. So, blocking VEGF and VEGFR1 have been considered as a target to prevent the growth of tumors.

METHODS: In this study, VEGFR1 gene expression was suppressed in AGS and SNU5 cancer cells using RNA interference (RNAi) technology. Down-regulation of VEGFR1 was assessed at mRNA and protein levels using real-time polymerase chain reaction (PCR), and western blot methods. Moreover, the viability and apoptosis of these cells were analyzed using MTT and flow cytometry techniques.

RESULTS: VEGFR1 expression was significantly down-regulated both in mRNA and protein levels. MTT and flow cytometry results revealed that down-regulation of VEGFR1 inhibited cell proliferation, and induced apoptosis of these cancer cells.

CONCLUSION: Our findings suggest that VEGFR1 could play an important role in cell proliferation and tumor growth; and it could be considered as a valuable target for controlling tumor cells, and cancer therapies.

KEYWORDS: Vascular Endothelial Growth Factor, Small Interfering RNA, Down-Regulation, Cellular Proliferation, Apoptosis

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Introduction

After heart diseases, cancer is the second leading cause of morbidity and mortality worldwide,[1] and many efforts and extensive studies have been carried out in the field of cancer treatment and prevention to date.[2] Vascular endothelial growth factor (VEGF) and its receptors (VEGFR) are the key regulators of physiologic angiogenesis, during organ development, embryogenesis, and reproduction, and plays a major role in the pathobiology of cancer and inflammatory diseases.[3-6] They are structurally related members of the receptor tyrosine kinase (RTK) family, and secreted as glycoproteins that mediate critical signaling pathways for survival, proliferation, vascular permeability, and migration of endothelial cells by interaction of VEGF to its receptors, VEGFR1 (or FLT1), VEGFR2 (KDR), and VEGFR3.[7-9] VEGFR2 is dominantly found in endothelial cells, but VEGFR1 is widely expressed in normal and malignant cells such as endothelial, macrophages, hematopoietic stem

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cells, lung cancer, breast cancer, and pancreatic cancer and hematopoietic malignancies.[10,11] Some studies have shown that VEGFR1 plays an important role in malignant growth, and accelerate proliferation of transformed cells.[12,13] Furthermore, in-vivo and in-vitro studies on liver sinusoidal endothelial cells have revealed that VEGFR1 could induce expression of growth factors.[14,15] VEGFR1 is different from other VEGFRs; because it could also be expressed in soluble form.[13] Previous studies in some cancers such as breast cancer revealed that VEGFR1 was significantly upregulated in metastasis stage and was related to relapses, also was associated with shorter survival in resected breast cancer and early-stage non-small cell lung cancer.[13,16]

So, recent studies have considered VEGF and its receptors as crucial targets in inhibition of angiogenesis and growth of tumor cells.[17] Up-regulation of VEGF and its receptors such as VEGFR1 and VEGFR2 stimulates angiogenesis, while inhibition of these proteins using monoclonal antibodies or chemical inhibitors suppresses angiogenesis processes.[18-21] Previous studies such as clinical trials, using an aptamer[22] or an antibody fragment[23] that binds VEGF, have supported this ideal that VEGF pathway could be considered as a crucial therapeutic target for cancers therapy. Other studies used VEGFRs inhibition as an alternative strategy to antagonize VEGF.[5,11,19-21] This is a great strategy because of its potency to inhibit multiple members of VEGF family at once.[24]

RNA interference (RNAi) is a fundamental biological process of sequence-specific, post-transcriptional gene silencing, by which animal and plant cells regulate gene expression. This process is initiated by complementary base-pairing of RNAi with target RNA, which recruits cellular RNases that mediate RNA degradation.[25,26] These molecules are 19-23 double strand nucleotide, which is homologous in sequence to the silenced gene.[27] The strategy of RNAi to inhibit gene expression and function has developed rapidly as a research tool to a promising therapeutic approach.[28] This technology is now routinely used in scientific researches, and some biotechnology companies have reported the use of RNAi as therapeutic agents because of its potential to targeted gene silencing.[29] Small interfering RNAs (siRNAs) molecules are 19-23 double strand nucleotide sequences, which are homologous in sequence to the silenced genes, provide a useful means to selectively down-regulate mRNA transcripts and studying the function of gene products.[30]

In this study, the mRNA expression of VEGFR1 was down-regulated using synthetic siRNA in AGS and SNU-5 cells. We showed that suppression of VEGFR1 inhibited cell proliferation and growth, and stimulated apoptosis of these cells.

Materials and Methods

AGS and SNU-5 cells (from Pasteur Institute, Tehran, Iran) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Inoclon, Iran) containing 10% fetal bovine serum (FBS) (Gibco), streptomycin (100 µg/ml), and penicillin (100 U/ml) (Life Technologies). Cells were incubated in a humidified 5% CO_2 incubator at 37 °C for 48 hours, and passaged using trypsinization. Up-regulate the expression of target gene was induced by hypoxic situation for 24 hours. Anti-VEGFR1 siRNA was designed with oligowalk online software. The siRNA sequenced designed to target VEGFR1 was GTGGCTACTCGTTAATTATCA. Working solution of siRNA was prepared according to manufacturer's protocol and stored at -20 °C.

AGS and SNU-5 cells were grown to ~80% confluency in RPMI-1640 medium for 24 hours before transfection. Lipofectamine 2000 (Invitrogen, USA) was used for transfection of anti-VEGFR1 siRNA according to manufacturer's instructions.

To assess the mRNA expression of target

genes, total RNA was extracted from the cells using TRIzol® Reagent (thermo fisher, US) according to the manufacturer's instructions. Agarose gel electrophoresis and spectrophotometry (NanoDrop™ 2000, thermo scientific) were used to measure RNA quality and quantity, respectively. 2 µg of total RNA was used for cDNA synthesis using 2-steps real-time polymerase chain reaction (RT-PCR) kit (Vivantis Technologies, Selangor, Malaysia), and OligodT and Random Hexamer primers, according to the manufacturer's instructions.

mRNA expression levels of genes were evaluated using quantitative real-time (qRT) PCR method. Appropriate primers were designed using Snap Gene and Oligo 7 software. The mRNA expression of VEGFR1 (forward: 5'-GGCTTCTGACCTGTGAAGCAAC-3', reverse: 5'- GAACTCTCGTGTTCAAGGGAGTG-3') was assessed and normalized to mRNA expression level of GAPDH (forward: 5'-GTGAACCATGAGAAGTATGACAA-3', and reverse: 5'-CATGAGTCCTTCCACGATAC -3') as an internal control gene.

qRT-PCR was performed using StepOne ABI system (Applied Biosystems, CA, USA). The final volume of reactions was 20 µl, which contained 20 ng of cDNA, 5X HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), and 200 nM of forward and reverse primers. The thermal reaction condition was as follows: denaturation of templates at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 15 seconds, and annealing/extension at 60 °C for 30 seconds. Dissociation curve analysis and 2% agarose gel electrophoresis were used to verify the specificity of PCR products.

To examine the protein expression level of VEGFR1, total protein was extracted from cells by radio-immunoprecipitation assay (RIPA) lysis buffer (150 mmol/l NaCl, 50 mmol/l Tris-HCl, pH of 7.5, 1% Nonidet P-40, and 0.25% Na deoxycholate) containing protease inhibitors, and stored in -80 °C. Protein samples were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then transferred to polyvinylidene fluoride (PVDF) membrane using Bio-Rad Trans-Blot® SD semi-dry system. Membranes were blocked with 5% (w/v) bovine serum albumin (BSA) in phosphate buffer saline (PBS) for 1 hour at room temperature, washed with PBS + 1% Tween20 (PBST), and then incubated with the following primary antibodies overnight at 4 °C; washed with PBST again, and incubated at room temperature with anti-rabbit secondary antibodies (Santa Cruz Biotechnology, CA, USA), goat polyclonal antibody actin (Cyto-Matin Gene, Iran) for 1 hour. Proteins were visualized with ECL Western Blotting kit (Cyto-Matin Gene, Iran).

Cells were seeded onto 96-well plates (10^3 cells/well), and were subsequently incubated for 24 hours. Cell viability was assessed by the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, methyl-thiazol-tetrazolium (MTT) assay (Roche, Germany)[31] at 24, 48, and 72 hours post transfection with anti-VEGFR1 siRNA. Quant Universal Microplate Spectrophotometer (BioTek, Winooski, VT) was used to measure the absorbance at 570 nm.

Annexin-V-PI detection kit (Roche) was used to assess the number of apoptotic cells, according to manufacturer's instruction. AGS and SNU-5 cells were seeded in a 24-well flat-bottomed plate, and incubated for 24 hours at 37 °C, then transfected with anti-VEGFR1 siRNA. 48 hours post transfection, cells were collected and washed with PBS, then resuspended with PI and Annexin V in the binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, and pH of 7.4). After 15 minutes of incubation at 37 °C, cells were analyzed by flow-cytometer (FACScan™ system, Becton Dickinson, NJ, USA).

T-test and one way ANOVA methods were

performed for analyzing data using Graphpad Prism 6 Demo and SPSS (version 22, IBM Corporation, Armonk, NY, USA). A P-value ≤ 0.05 was considered significant, and data were shown as mean ± standard deviation (SD).

Results

mRNA expression level of VEGFR1: mRNA expression level of VEGFR1 was analyzed using qRT-PCR technique 48 hours post transfection of anti-VEGFR1 siRNA in AGS and SNU-5 cells. mRNA expression levels of *VEGFR1* and *GAPDH* (as a reference gene) were assessed both in treated and untreated cells. T-test analysis of qRT-PCR results revealed that the mRNA expression level of *VEGFR1* gene decreased significantly in both cells transfected with siRNA compared to non-treat cells (Figure 1). This findings indicated that anti-VEGFR1 siRNA application successfully down-regulated the mRNA level of VEGFR1.

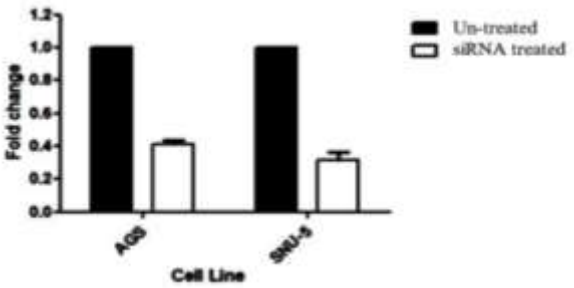

Figure 1. The quantitative analysis of expression level of VEGFR1 gene down-regulated in AGS and SNU-5 cells after treatment with anti-VEGFR1 siRNA. Each real-time PCR examination was carried out at least in triplicate. Data are shown as fold change in relative expression compared with GAPDH on the basis of Comparative Ct (2-ΔΔCt) method. Values are shown as mean ± SD.
siRNA: Small interfering RNA; VEGFR1: Vascular endothelial growth factor receptor 1; SD: Standard deviation

Western blot analysis: After analyzed on SDS-PAGE, protein were transferred to PVDF membrane using western blotting technique to analysis VEGFR1 protein level in AGS and SNU-5 cells. As shown in figure 2, cells treated with anti-VEGFR1 siRNA showed significant reduction of VEGFR1 protein level compared to untreated cells that expressed a 17kDa VEGFR1 band while. This shows that the use of anti-VEGFR1 siRNA targeted VEGFR1 mRNA specifically, and influenced its protein production. β-actin was used and expressed as a positive control in this experiment.

Figure 2. Analysis of siRNA effect on VEGFR1 protein expression in AGS and SNU-5 cells using western blot. β-actin was used as positive control. Negative control is without protein. As shown, a 19 KDa protein was expressed in cells transfected without siRNA, but transfection with anti-VEGFR1 siRNA has significantly reduced the level of VEGFR1 protein.
siRNA: small interfering RNA; VEGFR1: Vascular endothelial growth factor receptor 1

Cell viability assay: MTT assay was performed to evaluate the viability of AGS and SNU-5 cancer cells transfected with anti-VEGFR1 siRNA after 24, 48, and 72 hours. The results of MTT assay revealed that viability of AGS and SNU-5 cells treated with anti-VEGFR1 siRNA significantly reduced in a time-dependent manner compared to untreated cells (Figure 3). This results indicated the cytotoxicity of VEGFR1 suppression to these cancer cells.

Apoptosis assay: To evaluate the number of apoptosis AGS and SNU-5 cells, Annexin-V-PI kit was used. With regards to MTT assay results, 72 hours post transfection with anti-VEGFR1 siRNA, cells were collected and treated with Annexin V-FITC and PI, and analyzed by flow-cytometer, according to manufacturer's instruction.

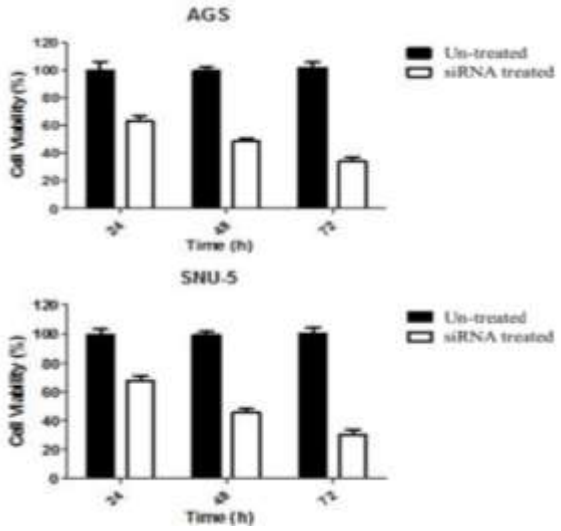

Figure 3. Survival ratios of AGS and SNU-5 cells treated with anti-VEGFR1 siRNA. The 80% confluence cell cultures were treated with siRNA. Cell viability was significantly reduced in a time-dependent manner. At 48h after treatment more than 50% of cells die. Results represented as means of three independent experiments by MTT assay (P ≤ 0.050)

siRNA: Small interfering RNA; VEGFR1: Vascular endothelial growth factor receptor 1; MTT: Methyl-thiazol-tetrazolium

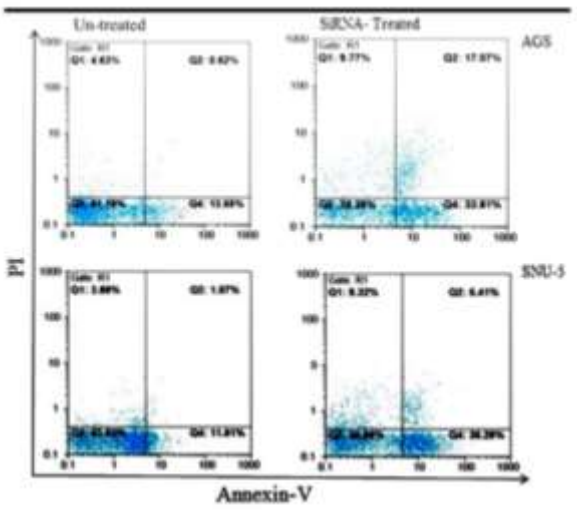

Figure 4. Flow Cytometry analysis of AGS and SNU-5 cells stained with Annexin V-FITC and PI. (A) Cells without treatment were used as controls. (B) Cells underwent apoptosis induced by anti-VEGFR1 siRNA. Diagram Q1 to Q4 represents necrotic, early apoptotic, late apoptotic and live cells, respectively. Treated cells showed significantly increased in the rate of early and late apoptosis compared with control.

siRNA: Small interfering RNA; VEGFR1: Vascular endothelial growth factor receptor 1

Results of apoptosis assay revealed that the number of apoptotic cells had notably increased following transfection with anti-VEGFR1 siRNA in comparison to non-transfected cells. Total apoptosis had elevated 3.7 and 3.2 times in treated group compared to control group in AGS and SNU-5 cells, respectively (Figure 4).

Discussion

VEGF and its receptors play a key role in the process of angiogenesis, which is an important factor in tumor growth and metastasis. Due to the increasing use of therapeutic inhibitors of VEGF signaling pathway in recent years, understanding the mechanisms underlying this pathway has become more important.[32,33] Previous studies had shown that of VEGFR-2 activation can activate downstream signaling pathways including ERK, JNK, PI3K, AKT, P70S6K, and p38MAPK.[32,34]

In other hand, the function of VEGFR1 is not well established.[35,36] Research on different kind of cancers including breast cancer, lung cancer, and gastric cancer has revealed that up-regulation of VEGFR1 is associated with tumor growth, tumor cell activation, and metastasis,[37-41] and its soluble form has been up-regulated in serum of patients with gastric cancer.[42] Blocking the function of VEGF signaling pathway is an important therapeutic approach to control angiogenesis, tumor growth, and metastasis. Previous studies have reported co-expression of VEGF and VEGFR1 in gastric cancer cell lines such as MGC803, AGS-1, RF-1, and RF-48, as well as specimens of gastric cancer.[41]

Many different agents have been reported including chemical compounds and antibodies to block or suppress VEGF signaling pathways in goal of control angiogenesis, tumor growth, and metastasis.[9] Another strategy is to

suppress VEGF receptors to antagonize VEGF. In this strategy, several VEGF family members could be blocked at once. So, VEGF receptors have been considered as therapeutic targets for cancer therapy.[43,44] Currently, about 44 VEGFR inhibitors such as novartis, axitinib, motesanib, sunitinib, and sorafenib are in clinical phase development, and some of them have been used for cancer therapy.[45,46] Hwang et al. have reported that concomitant inhibition of VEGFR1 and VEGFR-2 with paclitaxel increase the TUBB3 expressing cancer cell line cytotoxicity, and their inhibition with paclitaxel, anti-VEGFR1, and anti-VEGFR-2 in AGS cell was more cytotoxic.[42] Moreover, Zhang et al. reported that VEGFR1 was the dominant receptor in tumor microenvironment.[6] Due to these findings, we have postulated that AGS and SNU-5 gastric cancer cell lines express VEGFR1.

Here, we used anti-VEGFR1 siRNA to block VEGFR1 expression in AGS and SNU-5 cell lines. Our results revealed that mRNA and protein expression levels of VEGFR1 significantly decreased in AGS and SNU-5 cells 72 hours post transfection with siRNA compared to control group. These results demonstrated the efficiency of anti-VEGFR1 siRNA to block VEGFR1 function. These findings are consistent with previous studies which demonstrated blockade of VEGFR1 using anti-VEGFR1 antibodies or synthetic compounds, suppressed tumor growth and metastasis in vivo and in vitro.[47,48] In addition, the results of MTT and apoptosis assays demonstrated down-regulation of VEGFR1 could significantly inhibit cell growth and increase apoptosis of AGS and SNU-5 cells. Although, some studies have reported that blockade of VEGFR1 is not adequate without combined suppression of VEGFR-2 to inhibit tumor growth and cell proliferation,[49,50] but other studies demonstrated that suppression of VEGFR1 signaling could inhibit the growth and survival of several mouse tumor models, and increase apoptosis of many cell lines.[41,51,52] A study by Szabo et al. on glioblastoma have revealed that shRNA down-regulation or blocking the phosphorylation of VEGFR1 in vitro and in nude mice could reduce tumor cells growth.[53] Moreover, another research on tumor cell lines such as pancreatic and colorectal cancer cells have demonstrated a possible role for VEGFR1 in cell growth and survival.[54] Furthermore, the inhibition of VEGFR1 signaling in CAKI1 and SKUT1b cells using siRNA oligonucleotides inhibited tumor growth, and decreased survival of cancer cells.[41] These previous works support our findings, and indicate that blocking VEGFR1 using RNAi mechanism suppresses cell proliferation and tumor growth, as well as induction of cancer cells apoptosis.

Conclusion

Our results showed that using specific siRNAs, to down-regulate the expression of VEGFR1 and suppression of its function, can inhibit cell proliferation, and drive apoptosis of tumor cells. As previously proposed, and according to our findings, blocking of VEGFR1 can be considered as a therapeutic target to inhibit tumor growth.

Conflict of Interests

Authors have no conflict of interests.

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