Blockade of VEGF-A suppresses tumor growth via inhibition of autocrine signaling through FAK and AKT

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Abstract
Blockade of VEGF signaling using RNA interferences, a neutralizing antibody, an antagonizing soluble VEGF receptor, and a receptor tyrosine kinase inhibitor induced anti-tumor effects in human astrocytoma U251-MG and fibrosarcoma HT-1080 in vitro in a dose-dependent manner. Furthermore, blockade of VEGF-A using the doxycycline-inducible VEGF-A RNA interference system showed a significant anti-tumor effect in a murine HT-1080-xenograft model. Anti-tumor effect through the blockade of VEGF signaling was mediated by FAK and AKT pathway in vitro and in vivo. These results collectively indicate that VEGF-A and its receptors can act as key inducer of tumor growth as well as angiogenesis.

1. Introduction

Even though targeting the VEGF and VEGFRs pathway has been proven effective in the treatment of human cancers [1,2], the multitude of biological actions of VEGF blockade such as vessel disruption and normalization prompts us to exploit the alternative role of VEGF inhibition and its therapeutic potential [3]. Traditional anti-angiogenic strategies attempt to inhibit new vessel formation and/or to destroy existing vessels to starve the tumor from its nutrients [4]; however, it is now becoming increasingly clear that normalizing, and not only pruning, the tumor vessels can be beneficial [3]. Moreover, the heterogeneity of clinical responsiveness and adverse effects has motivated new attraction in re-searching biological meanings of VEGF and its suppression in the complex tumor microenvironment [5,6].

We have previously shown that blockade of VEGF signaling induced a significant anti-tumor effects in various human cancer cell types in vitro, especially human malignant glioblastoma and fibrosarcoma cells [7]. On the contrary, we had also shown that blockade of VEGF improved the perfusion of tumor-associated blood vessels as well as vascular permeability [8]. In the present study, we tried to verify the effects of anti-VEGF treatment on the angiogenesis or tumor-growth, and investigate alternative mechanisms responsible for anti-tumor effects of VEGF blockade other than ablating tumor-associated vessels using in vitro and in vivo models. Our reports are the first to suggest that pertinent inhibition of autocrine and paracrine VEGF signaling function may be effective to inhibit tumor growth in consort with anti-angiogenesis for VEGF-blockade dependent cancers.

2. Materials and methods

2.1. Cell culture and reagents

HT-1080 (a human fibrosarcoma cell line) and U251-MG (a human glioblastoma cell line) were obtained from the American Type Culture Collection (ATCC, Manassa, VA). The cells were grown in DMEM (Gibco BRL, Gaithersburg, MD, USA) medium supplemented with 10% Fetal Bovine Serum (Gibco) and 1 × 10^5 unit/L Penicillin-100 mg/L Streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO2. AKT inhibitor (LY294002) and FAK inhibitor (SC203950) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for FLK-1 and FLT-1 were obtained from Santa Cruz Biotechnology and for phospho-FAK (Y397 and Y576/577), FAK, phospho-AKT (Ser473), AKT,
phospho-ERK (Thy202/204), ERK, NRP-1, caspase-3, cleaved caspase-3, CD31, PCNA, HIF-1α, and GAPDH were obtained from Cell Signaling Technology (Beverly, MA, USA). Recombinant vascular endothelial growth factor-A was obtained from R&D Systems (Minneapolis, MN, USA).

2.2. siRNA transfection

siRNA transfection was performed using an effectene (Qiagen, Hilden, Germany), according to the manufacturer’s protocol as described previously [7]. The siRNA oligonucleotides that encode VEGF-A, FL-1, FLK-1, NRP-1, and scrambled control were obtained from Bioneer (Daejeon, Korea). The sequences of the siRNAs were as follows: VEGF-A, 5′-AAAGUCUAUCCAGACCCCA-3′-dTdT, FL-1: 5′-GACUCUCUCCGCCCAC-3′-dTdT, FLK-1: 5′-CCUCCAUGAGCCCAC-3′-dTdT, NRP-1: 5′-GGCCAAUCCGCUGCCA-3′-dTdT and scrambled control, 5′-CCUACC-GAAUUCGCU-3′-dTdT.

2.3. Measurement of cell death

To assess cell death, LDH (lactate dehydrogenase) assay (Promega, Madison, WI, USA) was carried out according to the manufacturer’s protocol. After 30 min incubation at room temperature, the absorbance was measured at 490 nm by using a microplate reader (Bio-Rad, Richmond, CA, USA). To evaluate cell viability, WST-1 reagent (Nalgene, Rochester, NY) was used as previously [7].

2.4. Western blot analysis and ELISA

Nuclear/cytosol Fractionation Kit (Bio Vision, Mountain View, CA, USA) was performed according to the manufacturer’s instruction using the tumor samples extracted from mice. Extracted proteins were separated by SDS–PAGE as previously described [9]. The cytoplasmic fractions were probed with GAPDH, and nuclear fractions were probed with PARP (poly ADP-ribose polymerase). Human VEGF Duoset™ ELISA Development System (R&D system, Minneapolis, USA) was performed according to the manufacturer’s instruction using the blood samples extracted from mice.

2.5. Generation of VEGF-specific shRNA stable cell lines

For stable suppression of VEGF-A expression by short hairpin (sh)-activated gene silencing vector system, plasmids expressing shRNAs were constructed by synthesizing cDNA oligonucleotides bearing the target sequence, XhoI and HindIII linker, and then ligated into XhoI and HindIII sites of pSIN5-Ts-shRNA vector (pSIN5-shRNA, Clontech). The target sequences for VEGF, corresponding to region at nucleotides 379–397 of human VEGF mRNA (GeneBank accession No. GI6631028), were 5′-AAATGTGAATGCAGACCAA-3′ and 5′-CUCCUAUGAGCCCAC-3′-dTdT, NRP-1: 5′-GGCCAAUCCGCUGCCA-3′-dTdT and scrambled control, 5′-CCUACC-GAAUUCGCU-3′-dTdT.

2.6. Xenograft tumor model

Balb/c nude mice were obtained from Orient (Seongnam, Korea) at 5–6 weeks of age. All mice were housed and handled in accordance with the Animal Research Committee’s Guidelines at KAIST (Daejeon Science Town, Daejeon, Republic of Korea). When tumors reached an average size approximately 3 cm³, water containing 200 mg/L doxycycline was fed to mice. Tumor volume was calculated using the following formula: V = 0.523 LW² (L = length, W = width). Animals were anesthetized by ketamine and xylazine, perfused with PBS, and followed by fixation with formalin (Sigma–Aldrich, St. Louis, MO, USA). Specimens were excised, immersed in formalin, and transferred to 30% sucrose (Sigma) solution. Immunohistochemistry was performed using the VECTASTAIN® ABC-kit (Vector Laboratories, Inc., Burlingame, CA, USA) as manufacturer’s recommendation.

2.7. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Levels of significance for comparisons between two independent samples were determined using the Student’s t-test. Groups were compared by one-way analysis of variance (ANOVA) with Tukey’s post hoc test applied to significant main effects (SPSS 12.0 R for Windows; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Blockade of VEGF-A induced apoptotic cell death via inhibition of AKT and FAK

Previously, we have demonstrated that VEGF blockade induced a significant cell death in human glioblastoma and fibrosarcoma cell lines [7]. We further confirmed the functional dependency of HT-1080 and U251-MG cells on VEGF-A by showing the dose-dependent induction of cell death by inhibition of VEGF signaling using anti-VEGF-A monoclonal antibody, soluble VEGF receptor and receptor tyrosine kinase inhibitor (Fig. 1).

To elucidate the anti-cancer mechanism of VEGF-A blockade, we examined the signal transduction pathways that are specifically activated by VEGF stimulation (Fig. 2A). Treatment with VEGF-A induced a time-dependent increase in FAK and AKT phosphorylation. Inhibition of VEGF-A-induced activation of AKT and FAK by pharmacological inhibitors induced a significant cell death in a dose-dependent manner, which was significantly suppressed by pretreatment of a pan-caspase inhibitor, z-VA (Fig. 2B). The involvement of caspases was confirmed by immunoblot analysis specific for active caspase-3 (Fig. 2C). Treatment with pharmacological inhibitors of AKT and FAK induced a significant cell death even in the absence of exogenous VEGF-A (data not shown). The involvement of autocrine FAK and AKT activation in HT-1080 cells was confirmed by immunoblot analysis (Fig. 2D). Similar results were obtained using U251-MG cells (data not shown). Interestingly, inhibition of FAK also suppressed phosphorylation of AKT; while specific inhibition of AKT had little effect on FAK phosphorylation, suggesting that FAK might be an upstream signaling event in this setting. To confirm that, we further inhibited FAK expression using siRNAs to demonstrate that knockdown of FAK suppressed both FAK and AKT phosphorylation in HT-1080 cells (Fig. 2E). Similar results were also obtained in U251-MG cells (data not shown).

3.2. Inhibition of tumor-derived VEGF-A suppressed tumor growth independently from anti-angiogenic activities

We constructed a doxycycline-inducible sh-VEGF HT1080 cell line (supplementary figure), then investigated the effect of specific targeting of tumor-derived VEGF-A in vitro and in vivo. First, we checked the knock-down efficacy of VEGF-A in these cells in vitro (Fig. 3A). Treatment with tetracycline induced a significantly reduction of VEGF-A accompanied by inhibition of AKT and FAK phosphorylation, confirming that AKT and FAK are downstream signals responsible for VEGF-dependency. Secretion of VEGF-A was also significantly suppressed by inducible expression of shRNA specific for VEGF-A (data not shown). Cell viability also decreased significantly upon induction of VEGF knockdown in vitro (Fig. 3B). Such an anti-proliferative effect of VEGF knockdown was also observed in HT-1080 xenograft model transplanted with the doxycycline-inducible sh-VEGF HT1080 cells (Fig. 3C). The tumor-laden mice were fed with doxycycline when the tumors reached to an average size approximately 3 cm³. Control tumors grew to an average size of 6.24 ± 1.9 cm³ by 46 days after transplantation; while the shRNA-inducible tumors remained to an average size of 2.94 ± 0.9 cm³ by 46 days after 6 days of doxycycline treatment. The serum level of human-specific VEGF-A was significantly lower in shRNA-inducible tumors compared to the control (Fig. 3D).

We further investigated the possible therapeutic mechanisms of inhibition of tumor-derived VEGF-A by histochemical analysis. As expected, the vessel areas and number of CD31-positive endothelial cells were significantly reduced in VEGF-suppressed tumors (Fig. 4A and B), which were consistent with previous results of ours and others [3,8]. To verify the effect of tumor vessel reduction, we next examined the level of HIF-1α protein, a hypoxia-sensitive protein, in the cytosol and nuclear fractions extracted from the tumors (Fig. 4C). In VEGF-suppressed tumors, the level of HIF-1α protein was substantially lower compared to the control tumors, indicating that the tumor vessel reduction was not associated with vessel disruption leading to tissue hypoxia but rather tumor vessel normalization. To confirm the hypothesis, we analyzed the involvement of tissue hypoxia with tumor cell apoptosis in the core and peripheral regions of the tumors (Fig. 4D). In the central necrotic regions, both
VEGF-suppressed and control tumors exhibited high levels of HIF-1α and active caspase-3. The number of HIF-1α positive cells was lower in VEGF-suppressed tumors even though the difference is not statistically significant. On the contrary, in the peripheral regions where the blood perfusion is higher than the core regions [8], the level of HIF-1α protein was quite lower in both VEGF-suppressed and control tumors; however, the level of active caspase-3 was significantly higher in VEGF-suppressed tumors. These results suggest that the reduction of tumor size in VEGF-suppressed tumors was not associated with tissue hypoxia. The in vivo involvement of downstream kinases for VEGF dependency was also confirmed by immunoblot analysis using tumor extracts (Fig. 4E).

4. Discussion

In the present study, we demonstrated that selective inhibition of tumor-derived VEGF-A can suppress tumor growth in vivo by inhibition of autocrine tumor growth signals. We further showed that FAK and AKT are the downstream signal transduction pathways responsible for VEGF-A dependency in human glioblastoma and fibrosarcoma cells; while the FAK signaling seems to be upstream to the AKT pathway. This is the first report to our knowledge in that anti-VEGF therapy might exert its anti-cancer effects in some VEGF-dependent malignancies independently from its anti-angiogenic effect whether it is disruptive or normalizing.
**Fig. 3.** In vivo effect of VEGF silencing in tumor xenograft model. (A) and (B) HT-1080 cells stably expressing control or shVEGF vectors were incubated in the absence or presence of 2 mg/L doxycycline, and the cell lysates were examined for expression of VEGF-A, FAK and AKT. Cell viability was measured using WST-1 assay (a representative of more than three independent experiments. n = 5, P < 0.05). (C) The effect of VEGF-A silencing on tumor growth in vivo (P < 0.05). (D) Human-specific VEGF expression was determined by ELISA in the plasma of the mice. VEGF level is expressed as the quantity of relatively secreted human VEGF (n = 3; Tukey’s post hoc test was applied to significant group effects in ANOVA, P < 0.05; y scale 1.00 indicates 13500 ng/L of VEGF-A in the plasma).

**Fig. 4.** Anti-cancer effect of VEGF silencing is not associated with anti-angiogenic action. (A) Blood vessel areas (pixels) and the number of CD31 positive vessels (B) were assessed by immunohistochemical staining in shVEGF expressing HT-1080 tumors. Magnification, ×200. (C) Fractionated lysates (C, cytosol fraction; N, nuclear fraction) from tumor samples was subjected to the immunoblot analysis for HIF-1α expression. GAPDH and PARP were measured to test the efficacy of cell fractionation. (D) The number of HIF-1α and cleaved caspase-3 positive cells was analyzed in tumors. Peripheral region means the outside of three quarters in the section of tumor samples. Magnification, ×200. (E) The lysates from tumor samples were tested for VEGF-A, p-FLK-1, FLK-1, p-FAK, FAK, p-AKT, AKT, PCNA and GAPDH by immunoblot analysis.
and that AKT and FAK signaling pathways can be potential therapeutic targets in VEGF-sensitive tumors.

Multiple pro-angiogenic functions are attributed to VEGFs, including the activation of endothelial cell invasion, migration, and proliferation [10]. Tight balance between pro-angiogenic and anti-angiogenic signals in the tumor microenvironment that governs the response of endothelial cells is critical. Accordingly, VEGF, one of the most effective pro-angiogenic factor, can critically shift the response of endothelial cells is critical. Accordingly, VEGF, one of the most effective pro-angiogenic factor, can critically shift the balance to angiogenesis process [11]. In agreement with this, vessel areas and CD31-positive vessels were significantly decreased when tumor-derived VEGF-A was specifically targeted. The reduction of tumor vessels seems to be the result of tumor vessel normalization rather than disruptive changes of tumor-associated vessels since there were less hypoxic markers in VEGF-suppressed tumors.

Given that inhibition of VEGF decreases tumor growth in vivo, our results are consistent with previous studies [12,13]. However, the reduction of tumor sizes seems to be independent from both tumor vessel normalization and disruption, which are usually regarded the leading mechanisms responsible for anti-cancer effect of VEGF blockade [2,3,14]. In concordance with our initial hypothesis, we could conclude that the reduction of tumor growth was closely related to the autocrine or paracrine VEGF signaling in the cancer cells based on several findings. Firstly, the immune-reactivity to active caspase-3, a marker for apoptotic cell death, was even higher in properly perfused tumor periphery of VEGF-suppressed tumors, indicating that increased cell death was not related to vessel disruption. Secondly, the tissue level of HIF-1α, a specific marker of hypoxia, was significantly lower in VEGF-suppressed tumors, where the tumor growth was markedly inhibited. Lastly, the downstream signals responsible for tumor growth in vitro were also remarkably reduced in vivo in VEGF-suppressed tumors. Even though these results suggest that the reduction of tumor size in VEGF-suppressed tumors might not be associated with tissue hypoxia, the reduction of tissue oxygen demand by substantial cell death might also result in relative non-hypoxic condition in VEGF-suppressed tumors. In summary, blockade of VEGF evoked prominent anti-tumor effect via inhibition of autocrine and/or paracrine VEGF/VEGFRs signaling independently from angiogenesis through the FAK and AKT pathway. This mechanism may contribute to the adoption of patient-specific therapeutic strategy targeting VEGF/VEGFRs signaling.

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