Inhibition of Tumor Angiogenesis and Tumor Growth by the DSL Domain of Human Delta-Like 1 Targeted to Vascular Endothelial Cells[1,2]

Xing-Cheng Zhao[3], Guo-Rui Dou[3], Li Wang[3], Liang Liang, Deng-Mei Tian, Xiu-Li Cao, Hong-Yan Qin, Chun-Mei Wang, Ping Zhang and Hua Han

State Key Laboratory of Cancer Biology, Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Xi'an, China

Abstract

The growth of solid tumors depends on neovascularization. Several therapies targeting tumor angiogenesis have been developed. However, poor response in some tumors and emerging resistance necessitate further investigations of new drug targets. Notch signal pathway plays a pivotal role in vascular development and tumor angiogenesis. Either blockade or forced activation of this pathway can inhibit angiogenesis. As blocking Notch pathway results in the formation of vascular neoplasm, activation of Notch pathway to prevent tumor angiogenesis might be an alternative choice. However, an *in vivo* deliverable reagent with highly efficient Notch-activating capacity has not been developed. Here, we generated a polypeptide, hD1R, which consists of the Delta–Serrate–Lag-2 fragment of the human Notch ligand Delta-like 1 and an arginine-glycine-aspartate (RGD) motif targeting endothelial cells (ECs). We showed that hD1R could bind to ECs specifically through its RGD motif and effectively triggered Notch signaling in ECs. We demonstrated both *in vitro* and *in vivo* that hD1R inhibited angiogenic sprouting and EC proliferation. In tumor-bearing mice, the injection of hD1R effectively repressed tumor growth, most likely through increasing tumor hypoxia and tissue necrosis. The amount and width of vessels reduced remarkably in tumors of mice treated with hD1R. Moreover, vessels in tumors of mice treated with hD1R recruited more $NG2^+$ perivascular cells and were better perfused. Combined application of hD1R and chemotherapy with cisplatin and teniposide revealed that these two treatments had additive antitumor effects. Our study provided a new strategy for antiangiogenic tumor therapy.

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Introduction

It has been recognized for decades that solid tumors require neovascularization essentially through angiogenesis [1,2]. Tumor cells and other microenvironmental cells secrete angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor, which cooperate with other molecules to fulfill angiogenesis in tumors. Therapies targeting tumor angiogenic factors have been developed [3–7], but poor responses in some tumors and emerging resistance have prompted further investigations of new drug targets and strategies [8–10].

The Notch signal pathway plays a pivotal role in physiological vascular development [11–16] and tumor angiogenesis [15,17–22]. In mammals, the canonical Notch pathway is composed of five Notch ligands [Delta-like (Dll) 1, 3, 4, Jagged 1, 2], four Notch receptors (Notch1–4), the transcription factor RBP-J, and the downstream

Abbreviations: Dll1, Delta-like 1; ECs, endothelial cells; HUVECs, human umbilical vein endothelial cells; RGD, arginine-glycine-aspartate; NICD, intracellular domain of Notch receptors

Address all correspondence to: Hua Han, PhD, Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Xi'an 710032, China. E-mail: huahan@fmmu.edu.cn or Ping Zhang, PhD, Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Xi'an 710032, China. E-mail: pingzhang0622@gmail.com

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[3]Equal contributors.

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effectors such as the Hes family molecules [11]. The Notch ligand–receptor interaction mediated by the Delta–Serrate–Lag-2 (DSL) domains of the ligands triggers serial proteolytic cleavages of receptors, which release the intracellular domain of Notch receptors (NICD). NICD further associates with RBP-J and transactivates downstream genes involved in multiple steps of angiogenesis including sprouting, extension, and maturation of neovasculature [11,12,17].

Blocking Notch signal inhibits tumor growth through nonproductive angiogenesis [20–24], but this treatment results in the formation of vascular neoplasms [25]. Activating Notch pathway may be an alternative way to modulate angiogenesis because forced activation of endothelial Notch signaling also inhibits angiogenesis [19,26–29]. However, although a soluble DSL domain of Dll1 is sufficient for triggering Notch signal *in vitro*, it might be inefficient or even inhibitory to Notch signal activation *in vivo*, because of their incompetence in inducing endocytosis of Notch ligands on the signal-sending cells, a necessary step for Notch activation [30,31]. To produce a soluble Notch ligand with a better capability to induce endocytosis *in vivo*, we designed a fusion protein, hD1R, which is composed of the DSL domain of the human (h)Dll1 (amino acids 127–225) and an arginine-glycine-aspartate (RGD)–containing nonapeptide (CRGDCGVRY). RGD targets the integrin αvβ3 expressed on endothelial cells (ECs) in response to angiogenic growth factors and tumors [32–34] and it has been shown that the binding of RGD-containing molecular ligands with cell surface integrins triggers endocytosis [35]. In this study, we demonstrated that hD1R can effectively trigger Notch signaling in ECs and inhibit angiogenesis and tumor growth, thus providing a novel strategy for antiangiogenic tumor therapy.

Materials and Methods

Expression of Recombinant Proteins in Escherichia coli

The cDNA fragment encoding the DSL domain of hDll1 (NM_005618) was amplified by using polymerase chain reaction (PCR) from a human cDNA library. The product was fused with an RGD motif (hD1R) or a DGR motif (hD1D) by PCR. The PCR primers are listed in Table W1. The resultant gene fragments were cloned into pET32a(+) between the *Nco*I and *Xho*I sites to construct pET32a-hD1S (stop), pET32a-hD1R (RGD fusion), and pET32a-hD1D (DGR fusion), respectively (Figure 1*A*). For the production of the recombinant proteins, *E. coli* BL21 was transformed with the plasmids. Positive clones were expanded in Luria-Bertani medium, and cells at the exponential stage were induced with 0.5 mM isopropyl β-D-thiogalactoside. The Trx-hD1S, Trx-hD1R, and Trx-hD1D proteins were purified by using Ni^{2+}-NTA columns (Invitrogen, Carlsbad, CA) according to the manufacturer's manuals. To obtain the S-tagged hD1S, hD1R, and hD1D proteins, Trx-hD1S, Trx-hD1R, and Trx-hD1D were cleaved by using thrombin (Novagen, Darmstadt, Germany) and further purified by using Ni^{2+}-NTA columns following the supplier's instructions. For Western blot, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membrane. Membranes were probed by using anti-His (Sigma, St Louis, MO) or anti–S-Tag antibody (Novagen) at appropriate dilutions, followed by incubation with an HRP-conjugated secondary anti-mouse IgG antibody (Millipore, Billerica, MA). Blots were developed by using the enhanced chemiluminescence system (Clinx Science Instruments, Shanghai, China).

Cell Culture

The tumor cell lines U87, LLC, and MCF-7 (ATCC, Manassas, VA) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate. Human umbilical vein ECs (HUVECs) were cultured in the endothelial cells media (ECM; ScienCell, San Diego, CA) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and endothelial cell growth supplement (ECGS; ScienCell). The recombinant hD1S, hD1D, or hD1R was added at the concentration of 1 μg/ml. γ-Secretase inhibitor (GSI; DAPT; Alexis Biochemicals, San Diego, CA) and Dynasore (Sigma) were used at the concentration of 75 and 40 μM, respectively. For the cell adhesion assay, wells of 96-well dishes were coated with 100 μl of different recombinant proteins (50 μg/ml) overnight, and then 1×10^5 HUVECs were seeded in each well. In some cases, an RGD peptide (CRGDCGVRY) or a DGR peptide (CDGRCGVRY; CL Bio-Scientific, Xi'an, China) was included at the concentration of 1 μg/ml. Cells were cultured for 1.5 hours, and nonadhesive cells were discarded by rinsing with phosphate-buffered saline (PBS). Adherent cells were stained with crystal violet, photographed, and quantified by light absorbance at A620 using a spectrometer.

Flow Cytometry

For flow cytometry, single-cell suspensions were prepared from cultured cells or mouse lymphoid tissues and stained with appropriate antibodies. Then, cells were analyzed by using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) and the CellQuest software. Dead cells were excluded using propidium iodide staining. S-Tag was stained with a rabbit anti–S-Tag antibody followed by fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Other antibodies used for staining were given as follows: anti-CD19 (1D3; BD Pharmingen, San Jose, CA), anti-CD21 (7G6; Biolegend, San Diego, CA), anti-CD23 (B3B4; Biolegend), anti-CD3 (145-2C11; Biolegend), anti-CD4 (RM4-5; BD Pharmingen), and anti-CD8 (53-6.7; BD Pharmingen).

Quantitative Reverse Transcription–PCR

Total RNA was extracted by using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was prepared by using a reverse transcription (RT) system (Takara Dalian, Dalian, China). Quantitative real-time PCR was performed in triplicates by using a kit (SYBR Premix EX Taq; Takara) and the ABI PRISM 7500 Real-Time PCR System, with β-actin as an internal control. The PCR primers are listed in Table W1.

Fibrin Bead Assay

HUVECs were tested for angiogenic sprouting with the fibrin bead assay using a kit (Amersham Pharmacia Biotech, Piscataway, NJ) according the supplier's instructions [36]. Briefly, HUVECs were cultured in EGM-2 medium (Clonetics, Walkersville, MD) the day before beading. Cytodex 3 microcarrier beads (Amersham) were prepared according to the supplier's instructions, and the beads were incubated with HUVECs (400 cells per bead) at 37°C for 4 hours. The coated beads were transferred to a tissue culture flask (T25; Falcon, Bedford, MA) and left overnight in 5 ml of EGM-2 at 37°C and 5% CO_2. The beads were washed three times and were suspended at a density of 500 beads/ml in the fibrinogen solution with 0.625 U/ml thrombin, and the fibrinogen/bead suspension (0.5 ml) was distributed in each well of a 24-well plate. After clotting at room temperature for 5 minutes and then at 37°C and 5% CO_2/95% air for 15 minutes,

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