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Inhibition of VEGF-dependent angiogenesis by the anti-CD82 monoclonal antibody 4F9 through regulation of lipid raft microdomains

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ABSTRACT

CD82 (also known as KAI1) belongs to the tetraspanin superfamily of type III transmembrane proteins, and is involved in regulating cell adhesion, migration and proliferation. In contrast to these well-established roles of CD82 in tumor biology, its function in endothelial cell (EC) activity and tumor angiogenesis is yet to be determined. In this study, we show that suppression of CD82 negatively regulates vascular endothelial growth factor (VEGF)-induced angiogenesis. Moreover, we demonstrate that the anti-CD82 mAb 4F9 effectively inhibits phosphorylation of VEGF receptor 2 (VEGFR2), which is the principal mediator of the VEGF-induced angiogenic signaling process in tumor angiogenesis, by regulating the organization of the lipid raft microdomain signaling platform in human EC. Our present work therefore suggests that CD82 on EC is a potential target for anti-angiogenic therapy in VEGFR2-dependent tumor angiogenesis.

1. Introduction

CD82 (also known as KAI1) belongs to the tetraspanin superfamily of type III transmembrane proteins, and is involved in the regulation of cell adhesion, migration and proliferation through the activation of downstream signaling processes [1]. We previously showed that CD82 was preferentially expressed on $CD4^+CD45RO^+$ memory T cells and that treatment with the anti-CD82 monoclonal antibody (mAb) 4F9 co-immobilized with a submitogenic dose of anti-CD3 mAb led to marked T cell proliferation [2]. Moreover, we dissected key aspects of the CD82-and β1 integrin-mediated signaling pathways through studies with Jurkat T cells with marginal expression of Cas-L/NEDD9 as well as those involving CD82-mediated tyrosine phosphorylation of Cas-L in peripheral T cells and H9 cells [3]. Meanwhile, CD82 expression on cancer cells suppresses tumor progression [1]. CD82 was originally identified as a suppressor of metastasis in a genetic screening assay for rat AT6.1 prostate cancer cells [4]. Subsequent studies showed that CD82 functions as a wide-spectrum suppressor of invasion and metastasis during cancer progression in many solid tumors [1,5]. Mechanistically, CD82 inhibits receptor tyrosine kinases (RTKs) (*e.g.*, epidermal growth factor receptor and hepatocyte growth factor

Abbreviations: BSA, bovine serum albumin; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; mAb, monoclonal antibody; RTK, receptor tyrosine kinase; shCD82, shRNA against CD82; shRNA, short hairpin RNA; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; [^{3}H]-TdR, tritiated thymidine.

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receptor) and integrin signaling by promoting their internalization [6,7]. Recent studies demonstrated that CD82 expressed on melanoma cells suppresses environmental angiogenesis by inhibiting the production of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) in melanoma cells [8].

Cancer angiogenesis is another fundamental process involved in tumor growth as it ensures adequate supply of oxygen and nutrients to promote cell growth and motility through the development of new blood vessels, potentially causing cancer progression and metastasis [9]. Angiogenesis involves coordinated endothelial cell (EC) proliferation, migration, branching and tube formation [10]. The VEGF family is a group of key proteins involved in the angiogenic pathway and is highly expressed in many tumor types [11]. Following increased production of VEGF induced by hypoxia, inflammatory cytokines, activation of oncogenes or silencing of oncosuppressor genes, the VEGF receptors (VEGFRs), which are expressed in tumor environmental vascular ECs, are activated to mediate cancer angiogenesis, promoting tumor growth and metastasis [12–14]. Therefore, suppression of cancer angiogenesis pathway by such agents as the anti-VEGF mAb bevacizumab, anti-VEGFR2 mAb ramucirumab, or the RTK inhibitors sorafenib and sunitinib represents novel treatment approaches for anticancer therapy [9,12,15].

Although CD82 is also expressed on EC as well as many neoplasms [1], a role for CD82 in EC activity and angiogenesis has not yet been clearly established. Recent study using *Cd82*-null mice showed that perturbation of CD82-ganglioside-CD44 signaling attenuates pathological angiogenesis by inhibiting EC movement [16], while *Cd82*-null mice display normal vessel development without obvious vascular defects [17]. Although VEGF-dependent EC activity plays an essential role in angiogenesis [18], a functional role for CD82 in association with VEGF/VEGFRs in EC activity has not yet been elucidated.

In this study, we show that suppression of CD82 negatively regulates VEGF-induced angiogenesis. Moreover, we demonstrate that the anti-CD82 mAb 4F9 effectively inhibits phosphorylation of VEGFR2, which is the principal mediator of the VEGF-induced angiogenic signaling process, by regulating the organization of the signaling platform, *i.e.*, lipid raft microdomains, in human EC. As a result, our present study suggests that CD82 on EC is a potential target for anti-angiogenic therapy in tumor angiogenesis.

2. Materials and methods

2.1. Antibodies, reagents and cells

Anti-human CD82 mAb 4F9 (mouse IgG1) was established in our laboratory as reported elsewhere [2]. Anti-VEGFR2 rabbit mAb (55B11), anti-phospho-VEGFR2 rabbit mAb (19A10) and anti-CD31 mouse mAb (89C2) were purchased from Cell Signaling Technology (USA). Suramin and recombinant human VEGF were purchased from Sigma–Aldrich (USA) and R&D Systems (USA), respectively. Human umbilical vein endothelial cells (HUVECs) were purchased from KURABO (Japan) and cultured according to the manufacturer's instruction. 293 FT cells for production of lentivirus particles were obtained from the ATCC (USA).

2.2. Generation of CD82-knockdown HUVECs using short hairpin (sh) RNA

Four clones of lentiviral shRNA against human CD82 (shCD82-RNA) and one control shRNA were purchased from Sigma–Aldrich (MISSION™ TRC shRNA Target Set), and each sequence of shCD82-RNA is shown in Supplemental Table. Each vector contained GFP tag as a selection marker. Lentivirus particles of shRNA were produced by 293 FT cells utilizing ViraPower™ Lentiviral Packaging Mix (Invitrogen, USA). After transfection of each shRNA lentivirus particle, GFP-positive HUVECs were purified using BD FACSAria cell sorter (BD Biosciences, USA), and expression of CD82 was confirmed by flow cytometry by the same method as described previously [3].

2.3. Cell migration and proliferation assays

HUVEC migration was assessed using 96-well microchamber plates (BD BioCoat Angiogenesis System, BD Biosciences). Cells were starved for 20 h in HuMedia-EB2 (KURABO) containing 0.1% bovine serum albumin (BSA). Cells were then harvested, resuspended in HuMedia-EB2 containing 0.1% BSA with indicated concentrations of anti-CD82 mAb 4F9 or suramin, and placed in the upper chamber of fibronectin-coated FluoroBlock Cell Culture Inserts (BD Biosciences) at a cell density of 5×10^4 cells per well. Cell migration was initiated by placing medium containing 10 ng/ml VEGF and 0.1% fetal bovine serum to the bottom chamber. After 18 h of incubation, cells were stained with 4 ng/ml calcein AM. Fluorescence in the cells that had migrated through the pores of the fluorescence blocking membrane was directly measured through the bottom of the chambers in a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm (SpectraMax Gemini EM, Molecular Devices, USA) and analyzed using SoftMax Pro 5.2 software (Molecular Devices).

Cell proliferation was measured using [^{3}H]-thymidine ([^{3}H]-TdR) incorporation assay. HUVECs were seeded in HuMedia-EG2 (KURABO) containing indicated concentrations of mAbs or VEGF in collagen-coated 96-well plates (BD Biosciences) at a density of 2.5×10^4 cells per well. [^{3}H]-TdR (1 μCi/mL) was added and the cells were cultured for a further 18 h. Cells were then harvested and their radioactivity level was measured with a Liquid Scintillation Counter (Wallac 1205 Beta Plate; Perkin–Elmer Life Sciences, USA).

2.4. 2-D angiogenesis assay

After treatment with indicated concentrations of mAbs, VEGF or suramin, HUVECs were cultured utilizing the Angiogenesis Kit with the manufacturer's instruction (KURABO). The media containing each concentration of mAbs, VEGF or suramin were changed at 4, 7, 9 days, and cells were immunostained with anti-CD31 mAb at 11 days. Tube formation of HUVECSs was examined by photography under a microscope (Nikon DIAPHOT 300, Nikon, Japan) and quantified in 5 random fields utilizing Angiogenesis Image Analyzer v.2.0.0 (KURABO).

2.5. Measurement of phosphorylation of VEGFR2

HUVECSs were cultured in HuMedia-EG2 with 4F9 or control mouse IgG (each 10 μg/ml) for 12 h, followed by addition of VEGF (10 ng/ml). Cells were harvested at the indicated incubation period with VEGF, and lysates were then subjected to Western blot analysis according to the method described previously [19].

2.6. Lipid raft fractionation

HUVECs were cultured in HuMedia-EG2 with 4F9 or control mouse IgG (each 10 μg/ml) for 12 h, followed by a 5 min incubation period with VEGF (10 ng/ml). Cells were then harvested, and cell lysates were prepared for fractionation by sucrose density gradient ultracentrifugation by the same method as described previously [19].

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