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Research Article

The hypoxia-inducible factor-responsive proteins semaphorin 4D and vascular endothelial growth factor promote tumor growth and angiogenesis in oral squamous cell carcinoma

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ABSTRACT

Growth and metastasis of solid tumors requires induction of angiogenesis to ensure the delivery of oxygen, nutrients and growth factors to rapidly dividing transformed cells. Through either mutations, hypoxia generated by cytoreductive therapies, or when a malignancy outgrows its blood supply, tumor cells undergo a change from an avascular to a neovascular phenotype, a transition mediated by the hypoxia-inducible factor (HIF) family of transcriptional regulators. Vascular endothelial growth factor (VEGF) is one example of a gene whose transcription is stimulated by HIF. VEGF plays a crucial role in promoting tumor growth and survival by stimulating new blood vessel growth in response to such stresses as chemotherapy or radiotherapy-induced hypoxia, and it therefore has become a tempting target for neutralizing antibodies in the treatment of advanced neoplasms. Emerging evidence has shown that the semaphorins, proteins originally associated with control of axonal growth and immunity, are regulated by changes in oxygen tension as well and may play a role in tumor-induced angiogenesis. Through the use of RNA interference, *in vitro* and *in vivo* angiogenesis assays and tumor xenograft experiments, we demonstrate that expression of semaphorin 4D (SEMA4D), which is under the control of the HIF-family of transcription factors, cooperates with VEGF to promote tumor growth and vascularity in oral squamous cell carcinoma (OSCC). We use blocking antibodies to show that targeting SEMA4D function along with VEGF could represent a novel anti-angiogenic therapeutic strategy for the treatment of OSCC and other solid tumors.

Abbreviations: SEMA4D, semaphorin 4D; sSEMA4D, soluble SEMA4D; AP, alkaline phosphatase; VEGF, vascular endothelial growth factor; HIF, hypoxia inducible factor; OSCC, oral squamous cell carcinoma; HUVEC, human umbilical vein endothelial cell; shRNA, short hairpin RNA; FBS, fetal bovine serum; BSA, bovine serum albumin; CM, conditioned media; ERK, extracellular signal regulated kinase; BME, basement membrane extract

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer among men in the developed world and among the most fatal cancers at any anatomic site [1]. Because even advanced lesions are usually not painful to the patient, OSCC in areas that cannot be easily visualized such as the lateral and ventral tongue and the posterior oropharynx in particular can evade early detection, eventually requiring aggressive surgical intervention and chemo- or radiotherapy to establish local control. Like many other solid tumors, OSCC lesions represent a heterogeneous population of genetically labile malignant cells that can evolve mutations under the strong selective pressures of such cytotoxic therapies, leading to the emergence of resistant cell populations and eventual treatment failure. A promising alternative treatment strategy is anti-angiogenic therapy, which instead targets the endothelial cells that line the vessels that feed the tumor, thus bypassing complications such as resistance to chemotherapy that can arise in a background of genetic instability when targeting cancer cells themselves.

Growth and metastasis of solid tumors requires induction of angiogenesis, the creation and remodeling of new blood vessels from a pre-existing vascular network, to ensure the delivery of oxygen, nutrients and growth factors to rapidly dividing transformed cells. Without the ability to induce angiogenesis, most neoplasms would fail to grow larger than 2 mm in diameter or metastasize [2]. One way tumor cells acquire the ability to induce angiogenesis, and hence to grow and metastasize, is through activity of the hypoxia-inducible factor (HIF) family of transcription regulators, the most important effectors of the adaptive response to hypoxia in multicellular organisms. Initially identified by Semenza and colleagues in the early 1990s, the HIF transcriptional complex is composed of two polypeptides: the α and β subunits [3]. While the β subunit is expressed constitutively, HIF activity is regulated at the posttranscriptional level by the α subunits, which are stabilized or degraded in conditions of low or high oxygen tension, respectively [4]. As an active dimer, HIF binds to hypoxia response elements within the promoters of target genes resulting in the activation of a pro-survival program that opposes apoptosis, inhibits generation of reactive oxygen species, and activates the transcription of pro-angiogenic proteins such as vascular endothelial growth factor (VEGF). In OSCC, high levels of the HIF-1α subunit are correlated with poor prognosis [5], reduced disease-specific survival [6] and tumor progression, including enhanced size of primary lesion and lymph node metastasis [7]. HIF-1α is also over-expressed in the majority of patients with squamous cell carcinoma of the oropharynx and here too is correlated with a lower rate of remission, greater incidence of lymph node metastases, and poorer disease-free and overall survival [8].

The semaphorins and their receptors, the plexins, are a family of proteins characterized by cysteine-rich semaphorin domains originally identified as regulators of axon guidance and lymphocyte activation [9–11]. Our group and others have shown that semaphorin 4D (SEMA4D) is produced and secreted by the transformed cells of many different aggressive carcinomas, including OSCC, and that it acts through its receptor, Plexin-B1, on endothelial cells to promote angiogenesis and enhance tumor growth and survival [12–14]. Why SEMA4D is over-expressed in so many different tumor types remains unknown, but studies demonstrate that like other pro-angiogenic factors, plexins and semaphorins are regulated by changes in oxygen tension [15–18]. Indeed, we have previously shown that Sema4D is induced by hypoxia in a HIF-1-dependent manner and may be another route by which carcinomas promote angiogenesis [18].

The focus of the current study is to investigate the role of HIF-mediated SEMA4D induction in the generation of the pro-angiogenic phenotype in OSCC and determine its biological significance for tumor growth and vascularity when compared to, and in combination with, the better-studied angiogenic factor VEGF. Here we use lentiviral-mediated RNA interference and over-expression techniques, *in vitro* and *in vivo* angiogenesis assays and tumor xenografts to show that both VEGF and SEMA4D transcription is under the control of HIF and cooperate to promote angiogenesis for the purposes of enhancing vascular density and tumor cell proliferation in OSCC. We employ blocking antibodies to demonstrate that targeting SEMA4D along with VEGF might represent a new complementary or parallel mode of treatment for anti-angiogenic therapy of OSCC or other solid neoplasms.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVEC, ATCC, Manassas, VA), 293T cells (ATCC), and the head and neck (HN) squamous cell carcinoma cell lines HN12, HN13, and HN30 [19] were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin/amphotericin B (Sigma).

Immunoblots

Cells infected with lentiviruses expressing the indicated constructs, treated with increasing concentrations of anti-SEMA4D blocking antibody 1.5 h prior to incubation with soluble SEMA4D (sSEMA4D) for 3 min (to determine ERK phosphorylation), or treated with up to 400 ng/ml sSEMA4D under conditions of low serum (to measure caspase 3 activation), were lysed in buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP 40) supplemented with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 μl/ml aprotinin and leupeptin, Sigma) and phosphatase inhibitors (2 mM NaF and 0.5 mM sodium orthovanadate, Sigma) for 15 min at 4 °C. After centrifugation, protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). 100 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Immobilon P, Millipore Corp., Billerica, MA). The membranes were then incubated with the appropriate antibodies. The antibodies used were as follows: SEMA4D (BD Transduction Labs, BD Biosciences, Palo Alto, CA); VEGF (Santa Cruz Biotechnology, Santa Cruz, CA); HIF-1β (BD Transduction Labs); Tubulin (Santa Cruz Biotechnology); Total ERK (Cell Signaling Technology, Danvers, MA); Phospho-ERK (Cell Signaling Technology); Plexin-B1 (Santa Cruz A8); cleaved caspase 3 (Cell Signaling, Danvers, MA); GAPDH (Sigma). Proteins were detected using the ECL chemiluminescence system (Pierce, Rockford, IL).