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Elevated VEGF-D Modulates Tumor Inflammation and Reduces the Growth of Carcinogen-Induced Skin Tumors[1]

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Abstract

Vascular endothelial growth factor D (VEGF-D) promotes the lymph node metastasis of cancer by inducing the growth of lymphatic vasculature, but its specific roles in tumorigenesis have not been elucidated. We monitored the effects of VEGF-D in cutaneous squamous cell carcinoma (cSCC) by subjecting transgenic mice overexpressing VEGF-D in the skin (K14-mVEGF-D) and VEGF-D knockout mice to a chemical skin carcinogenesis protocol involving 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate treatments. In K14-mVEGF-D mice, tumor lymphangiogenesis was significantly increased and the frequency of lymph node metastasis was elevated in comparison with controls. Most notably, the papillomas regressed more often in K14-mVEGF-D mice than in littermate controls, resulting in a delay in tumor incidence and a remarkable reduction in the total tumor number. Skin tumor growth and metastasis were not obviously affected in the absence of VEGF-D; however, the knockout mice showed a trend for reduced lymphangiogenesis in skin tumors and in the untreated skin. Interestingly, K14-mVEGF-D mice showed an altered immune response in skin tumors. This consisted of the reduced accumulation of macrophages, mast cells, and $CD4^+$ T-cells and an increase of cytotoxic $CD8^+$ T-cells. Cytokine profiling by flow cytometry and quantitative real time PCR revealed that elevated VEGF-D expression results in an attenuated Th2 response and promotes M1/Th1 and Th17 polarization in the early stage of skin carcinogenesis, leading to an anti-tumoral immune environment and the regression of primary tumors. Our data suggest that VEGF-D may be beneficial in early-stage tumors since it suppresses the pro-tumorigenic inflammation, while at later stages VEGF-D-induced tumor lymphatics provide a route for metastasis.

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Introduction

Non-melanoma skin cancer (NMSC) is the most frequent human cancer worldwide and its incidence is increasing among Caucasian people due to population aging. Approximately 20% of NMSCs are cutaneous squamous cell carcinomas (cSCC) and it is the second most common subtype after basal cell carcinoma. cSCC is mainly caused by repeated exposure to solar ultraviolet radiation throughout life, but, for example, chronic ulcers, ionizing radiation, chemicals, papillomavirus infection, and immunosuppressive medication are also risk factors. Approximately 20% of all the skin cancer related deaths are caused by metastatic cSCC. The average rate of the nodal metastasis of cSCC is around 5%, and the five-year survival rate of metastatic cSCC is less than 35% [1–4]. Metastatic cSCC most often spreads via tumor-associated lymphatic vessels to sentinel and distant lymph

Abbreviations: cSCC, cutaneous squamous cell carcinoma; DMBA, 7,12-dimethylbenz[a]anthracene; IFN, interferon; IL, interleukin; K14, keratin 14; KO, knockout; LN, lymph node; NMSC, non-melanoma skin cancer; TG, transgenic; TGF, transforming growth factor; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WT, wild type

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nodes (LNs), and sometimes also to distant organs, preferentially to other sites of the skin as well as to the lungs, liver, brain, and bone [5].

Increasing experimental and clinical evidence suggests that the growth of tumor-associated lymphatic vessels, in other words, tumor lymphangiogenesis, is actively induced in many tumor types [6], including cSCC [7], and that it promotes early LN metastasis. The extent of tumor lymphangiogenesis has also been suggested to serve as an independent predictor of LN metastasis and patient survival [8,9].

Vascular endothelial growth factors (VEGFs) C and D (the c-fos-induced growth factor [FIGF]) are key stimulators of lymphangiogenesis. They activate the VEGF receptor 3 (VEGFR-3) that is constitutively expressed in lymphatic endothelium [10,11]. At least in part, the signals from VEGFR-3 are transduced via the protein kinase C-dependent activation of the MEK/ERK1/2 mitogen-activated protein kinase signaling cascade and induction of Akt1 phosphorylation [12]. VEGFR-3 is expressed also in angiogenic blood vessel endothelia in tumors and thus signaling through VEGFR-3 can activate tumor angiogenesis [13]. While VEGF-C is essential for the formation of lymphatic vasculature during embryonic development [14], VEGF-D is not [15,16]. Nevertheless, a detailed analysis of dermal lymphatic network in the *Vegfd*$^{-/-}$ mice showed that this factor is needed for the maintenance of caliber and functional capacity of initial lymphatics [17]. In humans, both ligands can also bind to VEGFR-2 on blood vascular endothelial cells to activate angiogenesis and to support primary tumor growth and metastasis [18]. In contrast to human VEGF-D, mouse VEGF-D only binds to VEGFR-3 [19].

High VEGF-C or VEGF-D expression has been shown to boost VEGFR-3-mediated signaling to promote tumor lymphangiogenesis and to enhance LN and distant organ metastasis in various experimental mouse tumor models [20–24]. Conversely, the blocking of VEGF-C and VEGF-D from binding to VEGFR-3 inhibits these processes [22,25]. Reduced peritumoral lymphangiogenesis and LN metastasis have been demonstrated in *Vegfd*$^{-/-}$ mice in an orthotopic pancreatic tumor model [16], while the effect of VEGF-C deficiency on tumor growth is not known so far, due to embryonic lethality of the constitutive *Vegfc* deletion. Depending on the experimental model, human VEGF-D has been observed to either promote [22,24] or inhibit [23] angiogenesis and tumor growth. In a range of human cancers, the expression of VEGF-C or VEGF-D positively correlates with LN metastasis and with poor patient outcome [8,26,27]. Collectively these data indicate that both VEGF-C and VEGF-D may play major roles in tumor lymphangiogenesis and in metastatic spread to sentinel and distal LNs and beyond. Targeting these factors and their downstream signaling pathways could thus be a promising therapeutic strategy to prevent tumor progression and metastasis.

Although much is known about VEGFs in tumor growth and metastasis, the specific roles of VEGF-D in cutaneous cancers have not been studied previously. Here we investigated the effects of VEGF-D on the development and progression of skin tumors by subjecting both *Vegfd*$^{-/-}$ mice [15] and transgenic (TG) mice that overexpress mouse VEGF-D in the skin (keratin 14 [K14]-mVEGF-D mice) [28] to a two-stage chemical skin carcinogenesis model [29]. We show that while *Vegfd* deletion has rather marginal effects on skin tumor growth and lymphangiogenesis, TG overexpression of VEGF-D promotes tumor lymphangiogenesis and metastasis. Interestingly, VEGF-D also induces significant changes in immune cell and cytokine profiles in the developing skin tumors, leading to a shift from a protumoral Th2 to antitumoral Th1/Th17 response, and substantial regression of primary tumors in the K14-mVEGF-D mice.

Materials and Methods

Mice

Age- and sex-matched transgenic K14-mVEGF-D mice overexpressing mouse VEGF-D in the skin under the K14 promoter [Tg(KRT14-Figf)1Ali] [28] in the FVB/N background (TG) and VEGF-D knockout (KO) (Figf^{tm1Mach}) [15] mice, backcrossed into a FVB/N (Harlan, the Netherlands) background for at least five generations, were used for the skin carcinogenesis studies.

Chemical Skin Carcinogenesis

Each experimental group consisted of 15–30 TG, KO or wild type (WT) littermate control mice of 9–12 weeks of age. Mice were subjected to the chemical skin carcinogenesis involving 7,12-dimethylbenz[a]anthracene (DMBA; Sigma-Aldrich) and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; Sigma-Aldrich) treatments, and tumor development, size and, macroscopic appearance were monitored once a week for up to 30 weeks, as described earlier [30]. Mice were sacrificed at weeks 10, 15, 25, and 30, or at predetermined humane end points (engraved or ulcerative cSCCs, tumors with a diameter over 10 mm, or excessive tumor load per mouse). Tumor incidence (the percentage of mice with a tumor) and cumulative tumor multiplicity (the number of papillomas divided by the total number of mice alive at the time when the first mouse was removed from the experiment due to humane end points, as described by Abel et al. [29]) were recorded. To induce epidermal hyperplasia and chronic inflammation, the shaved dorsal skin of nine-week old mice was treated four times, at two-day intervals, with 5 μg of TPA in acetone and skin samples were collected on the ninth day from the first application. Mice treated with acetone were used as controls. Untreated dorsal skin was also collected from nine-week old mice. All the animal experiments were approved by the Finnish National Animal Experiment Board.

Tumor and Tissue Sample Harvesting

Mice were sacrificed by CO_2 inhalation and cervical dislocation; skin or skin tumors, and inguinal, axillary, and brachial lymph nodes were dissected. Tissue samples were either fixed in fresh phosphate-buffered 4% paraformaldehyde for 24 hours at 4 °C and embedded in paraffin or snap-frozen in liquid nitrogen. Hematoxylin-eosin-stained 5 μm sections from the subcapsular region of LNs were evaluated for the presence of metastases.

Histochemical and Immunohistochemical Analyses

Fixed tissue sections were: treated with trypsin for 30 min at 37 °C and stained with rat anti-mouse CD-31 antibody (BD Biosciences PharMingen) or rat anti-mouse VEGF-D antibody (ReliaTech); or heated in boiling EDTA-Tris buffer (pH 9.0) for 15 minutes and stained with rat anti-mouse monoclonal F4/80 antibody against macrophages (Serotec), rat anti-mouse CD45R antibody against B-lymphocytes (BD Biosciences) or rabbit anti-mouse VEGF-C antibody (Santa Cruz Biotechnology Inc.); or boiled in 10 mmol/L citrate buffer (pH 6.0) for 15 minutes and stained with goat anti-mouse VEGFR-3 antibody (R&D Systems) or rabbit anti-mouse LYVE-1 antibody [14]. The tyramide signal amplification kit (Perkin-Elmer) was used according to the manufacturer's instructions to intensify color reactions. A biotinylated anti-rat (Vector Laboratories), anti-rabbit (Jackson Immunoresearch), or anti-goat (R&D Systems) antibody was used as secondary antibody. A Histomouse-SP Broad Spectrum (AEC) kit was used to stain helper