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Angiopoietin-like 4: A Novel Molecular Hallmark in Oral Kaposi's Sarcoma

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Abstract

Kaposi's sarcoma (KS) remains among the most common causes of oral cancer in HIV-infected individuals. Infection with the KS-associated herpesvirus (KSHV/HHV8) is a necessary event for disease development. Emerging evidence suggests that KSHV infects vascular endothelial (or endothelial progenitor) cells promoting the formation of the KS tumor (or spindle) cell. These cells elaborate angiogenic growth factors and cytokines that promote the dysregulated angiogenesis and profuse edema that characterizes this unusual vascular tumor. Central among these secreted factors is the potent endothelial cell mitogen, vascular endothelial growth factor (VEGF). Indeed, VEGF has proven to be a key player in KSHV pathogenesis and is a molecular hallmark of KS lesions. We have recently shown that a second angiogenic factor, Angiopoietin-like 4 (ANGPTL4), may also play a critical role in KS development. Here we demonstrate that ANGPTL4 is upregulated both directly and indirectly by the KSHV oncogene, vGPCR. We further show that ANGPTL4 is a molecular hallmark of oral KS lesions. Indeed, expression of this protein was observed in more tumor cells and in more biopsies specimens than expression of VEGF (23/25 or 92% vs. 19/25 or 76%, respectively) in oral KS. These surprising results support a key role for ANGPTL4 in Kaposi's sarcomagenesis and further suggest that this angiogenic factor may provide a novel diagnostic and therapeutic marker for oral KS patients.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Keywords

oral cavity; Kaposi's sarcoma; Kaposi's sarcoma associated herpesvirus; KSHV; Human herpesvirus-8; HHV-8; G protein-coupled receptor; vGPCR; Angiopoietin; Angiopoietin-like 4; vascular endothelial growth factor; angiogenesis; vascular permeability

Introduction

KS is a multifocal vascular neoplasm that often affects the oral cavity in immunosuppressed patients[1]. First described as a skin cancer in older men of Jewish or Mediterranean ancestry, a dramatic change in the epidemiology and clinical course of KS occurred with the emergence of the acquired immune deficiency syndrome (AIDS)[2]. Today, KS remains as one of the most common malignancies affecting HIV-infected individuals and is the most frequent cancer among children and adult men in countries of sub-Saharan Africa[2]. Unfortunately, clinical management of KS continues to be a challenge.

A scientific leap in our understanding of the pathogenesis of KS was made possible with the identification of a novel human herpesvirus, HHV8, named Kaposi's sarcoma associated herpesvirus (KSHV), as the etiological agent for this tumor[3]. Subsequent work from several groups suggests that endothelial cell infection with KSHV is indeed a prerequisite for KS development, and results in the formation of the KS tumor (or spindle) cell. These KS spindle cells are the driving force of KS lesion, elaborating angiogenic growth factors and cytokines that promote the formation of this vascular tumor[2].

Emerging evidence supports a key role for a viral protein, the KSHV G protein-coupled receptor (vGPCR), in the initiation and promotion of KS[2]. vGPCR is a member of the family of CXC chemokine GPCRs, with closest homology to CXCR2, but with ligand-independent (constitutive) activity. Endothelial cells expressing vGPCR elaborate angiogenic growth factors and cytokines that have been suggested to promote tumor formation through a unique paracrine mechanism[2]. Indeed, transgenic mice that express vGPCR manifest dermal angioproliferative lesions that closely resemble those seen in KS[4–6]. These observations have prompted intense investigation into identifying the molecular mechanism(s) whereby vGPCR could play a role in Kaposi's sarcomagenesis.

Initial work on the contribution of vGPCR to KS development appropriately centered on the upregulation by this viral receptor of the potent endothelial cell mitogen, VEGF, a key player in KSHV pathogenesis[7,8]. However, we recently identified a novel angiogenic factor, ANGPTL4, which also appears to play an essential role in vGPCR tumorigenesis, promoting angiogenesis and vascular permeability[9]. Here we set out to examine the prevalence of ANGPTL4 upregulation in oral KS lesions.

Materials and Methods

Cell lines and reagents

pCEFL AU5 vGPCR, pCEFL AU5 GFP, pBIG AU5 vGPCR and pCEFL Tet REV TA have been previously described[5,9]. HMEC1s were obtained from the CDCs (Atlanta, GA) and grown as described elsewhere[9]. Cells were transfected with Polyfect (Qiagen). Conditioned media was prepared as previously described[9]. Recombinant proteins were purchased from Pepro Tech. Concentrations used are: IL-8 (50 ng/ml), GROα (50 ng/ml), PDGF (25 ng/ml), IL-1β (10 ng/ml), IL-10 (25 ng/ml), IL-6 (2 ng/ml), TNFα (25 ng/ml), IP-10 (50 ng/ml), SDF1α (80 ng/ml), VEGF (50 ng/ml), and ANGPTL4 (5 μg/ml).

Additional information can be found in the Supplemental Materials and Methods.

Results

KS is a vascular tumor promoted by KSHV infection and the resultant expression of different viral genes and microRNAs[2]. Work from several labs has supported a key role for dysregulated expression of the KSHV-encoded GPCR (vGPCR) in the promotion of KS[2]. Transgenic mice expressing this viral receptor in endothelial cells manifest vascular tumors (vGPCR tumors) histologically similar to human KS, with expression of vGPCR limited to a few cells, suggestive of a paracrine mechanism for vGPCR tumorigenesis[5]. Indeed, expression of vGPCR in cultured endothelial cells stimulates the release of angiogenic growth factors and pro-inflammatory chemokines and cytokines[7,8,10–13]. Of interest, expression of vGPCR in immortalized human dermal microvascular endothelial cells (HMEC1s) led to an increase in the mRNA levels of a novel hypoxia-regulated factor, *angiopoietin like-4*, a member of the family of Angiopoietin-like proteins (ANGPTLs), which has been shown to play an important role in the control of angiogenesis[9,14] (Fig. 1A). Indeed, induction of vGPCR expression in HMEC1s using a tetracycline-inducible expression system led to a robust upregulation of ANGPTL4 translation and secretion (Fig. 1B–C).

Immunohistochemical staining of murine vGPCR tumors also demonstrated high levels of expression of ANGPTL4 in most tumor cells (Fig. 1D). These lesions similarly showed elevated levels of another vGPCR upregulated factor, VEGF[7,8]. However, expression of vGPCR was limited to only a few tumor cells, consistent with a paracrine role for vGPCR in the upregulation of these growth factors (Fig. 1D). Indeed, when we treated HMEC1 with media conditioned by endothelial cells expressing vGPCR, we observed an induction of ANGPTL4 in treated cells (Fig 1E). An increase in ANGPTL4 was also found when HMEC1s were exposed to individual chemokines, cytokines and growth factors found in vGPCR conditioned media[10] (Fig. 1E). Collectively, these results suggest that vGPCR upregulates ANGPTL4 by both direct and paracrine mechanisms.

To study the relevance of ANGPTL4 as a potential diagnostic marker in oral KS, we obtained 25 biopsy samples from patients with oral KS tumors. Demographic data of the patients and clinical information of the lesions are included in Table 1. KSHV infection in all the cases was confirmed by the presence of the KSHV Latency-Associated Nuclear Antigen 1 (LANA1) in the tissue. We then performed immunohistochemical analysis on all the biopsies with specific antibodies against ANGPTL4 or VEGF (Fig. 2). Table 2 includes the grading of immunohistochemical reactivity to these antibodies, according to the percentage of positive tumor cells. 23/25 (92%) of the KS lesions tested showed upregulation of ANGPTL4 in tumor cells. This compares to 19/25 (76%) of KS lesions that demonstrated upregulation of VEGF expression. As shown in Table 3, 10/25 (40%) of the KS lesions had high levels of expression of ANGPTL4 in the majority of tumor cells compared to 7/25 (28%) of KS lesions with high levels of VEGF. Collectively, these results support a fundamental role for ANGPTL4 in Kaposi's sarcomagenesis.

Discussion

KS is a multifocal vascular tumor with lesions predominately affecting the skin and oral mucosa[1]. KS tumorigenesis develops in response to infection by KSHV; indeed, expression of KSHV-encoded latent genes (e.g. LANA1) can be detected in most tumor cells within oral KS lesions. Interestingly, the KSHV lytic protein, vGPCR, expressed only in a few tumor cells, appears to be important in KS development through a unique paracrine mechanism[2]. The role of vGPCR in KS initiation and maintenance suggest that this viral

receptor may provide a new perspective in the search for therapeutic alternatives for KS patients.

vGPCR expression leads to the elaboration of numerous inflammatory and angiogenic factors which are postulated to help promote the proliferation and survival of neighboring endothelial cells[15]. Among the proteins upregulated by vGPCR, VEGF has been shown to be a key player in KSHV pathogenesis and a molecular hallmark of KS lesions. Interestingly, we have recently shown that vGPCR upregulation of a novel Angiopoietin-like factor, ANGPTL4, may also play an essential role in KS development by the promotion of angiogenesis and vascular permeability[9].

ANGPTL4 is known as a gene with upregulated expression in endothelial cells exposed to hypoxia, ischemic tissues, and in hypoxic areas of solid tumors[16]. Although the precise function of this factor in cancer biology is still unclear, emerging evidence implicates ANGPTL4 in the promotion of tumor progression, angiogenesis, and tumor dissemination. In breast cancer, ANGPTL4 has been recently shown to prime tumor cells for lung metastasis and trigger the disruption of vascular endothelial cell-cell junctions[17]. Similarly, expression of ANGPTL4 in gastric cancer and in esophageal and oral tongue squamous cell carcinoma correlates with lymphatic and venous invasion, degree of tumor differentiation, and poor survival[18–20]. ANGPTL4 has also been shown to be a diagnostic marker for primary and metastatic clear cell renal-cell carcinoma[21]. Conversely, this protein has also been suggested as an anti-angiogenic and anti-metastatic factor[14]. Collectively, these incongruent data suggest that the contribution of ANGPTL4 to cancer may be dependent on the tumor type and tissue environment.

Here we provide evidence that ANGPTL4 is upregulated both directly and indirectly (through a paracrine mechanism) by the KSHV oncogene, vGPCR. We further demonstrate that ANGPTL4 is a molecular hallmark of oral KS lesions. Indeed, expression of ANGPTL4 was observed in more tumor cells and in more biopsies specimens than expression of VEGF in oral KS. Almost half (12/25) of the KS biopsy specimens had a higher percentage of cells expressing ANGPTL4 compared to VEGF; only 7/25 (28%) of the KS biopsies demonstrated a higher percentage of cells expressing VEGF compared to ANGPTL4. Moreover, only 1/25 (4%) of the KS biopsies tested expressed VEGF but not ANGPTL4. Conversely, 4/25 (16%) of the KS biopsies tested expressed ANGPTL4 but not VEGF. As demonstrated in Table 3, 10/25 (40%) of the KS lesions had high levels of expression of ANGPTL4 in the majority of tumor cells compared to 7/25 (28%) of KS lesions with high levels of VEGF in the majority of tumor cells. Collectively, these results support a fundamental role for vGPCR paracrine upregulation of ANGPTL4 in Kaposi's sarcomagenesis and further suggest that this angiogenic growth factor may prove to be an essential diagnostic as well as therapeutic target in KS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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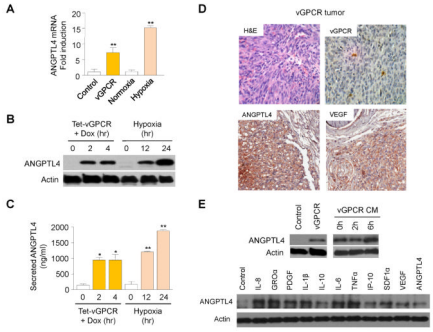

Figure 1. Direct and Paracrine upregulation of ANGPTL4 by vGPCR

(A) *angptl4* mRNA levels (qRT-PCR), upon transfection of pCEFL AU5 vGPCR (vGPCR) or pCEFL AU5 GFP (Control) in HMEC1. Induction of *angptl4* mRNA by hypoxia (1% O_2; 24 hr) was used as a control. (B–C) Cellular ANGPTL4 (WB) (B) and secreted ANGPTL4 (ELISA) (C) of HMEC1 transfected with pCEFL Tet REV TA and pBIG AU5 vGPCR (Tet-vGPCR). Cells were left untreated or treated with (1 μg/ml) Dox for 2h or 4h. Induction of ANGPTL4 expression by hypoxia (1% O_2; 12hr or 24hr) was used as a control. (D) Representative H&E staining and immunohistochemical detection of (AU5) vGPCR expressing cells as well as ANGPTL4 and VEGF expression in murine vGPCR tumors. (E) Upregulation in HMEC1 of ANGPTL4 upon transfection of pCEFL AU5 vGPCR (vGPCR) or pCEFL AU5 GFP (Control), treatment with conditioned media of vGPCR-expressing cells (vGPCR CM), or exposure to individual recombinant factors present in vGPCR conditioned media.

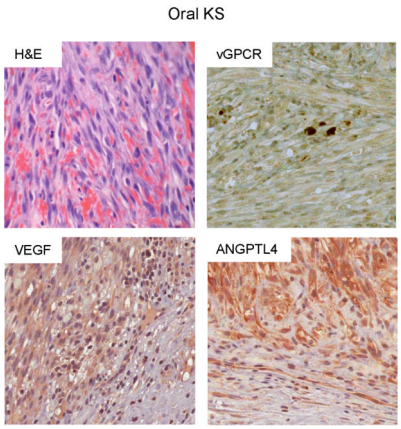

Figure 2. Overexpression of ANGPTL4 in oral KS

Representative H&E and immunohistochemical staining of human oral KS tissue with specific antibodies against vGPCR, ANGPTL4 or VEGF.

Table 1

Demographic data of the patients (age, gender, race and HIV status) and clinical information of the oral lesions (location, size, color, clinical presentation) included in our studies. HHV8 infection in all the cases was confirmed by the presence of the latency-associated nuclear antigen 1 (LANA1).

Case	Age	Sex	Race	Location	Size	Color	Clinical Presentation	HIV status	LANA1
1	34	M	W	Palate	1.5cm	red	N/A	Positive	+
2	29	M	W	Gingiva	N/A	red/purple	N/A	N/A	+
3	44	M	W	Palate	3.5×2.5cm	Blue	Swelling	N/A	+
4	40	M	W	Palate	N/A	Blue	N/A	Positive	+
5	60	M	W	Palate	1 cm	red	Exophytic granulation tissue	N/A	+
6	36	M	W	Palate	4×3 mm	Purple	Pedunculated mass	N/A	+
7	31	M	W	Gingiva	N/A	Purple	Multiple, spongy lesions	Positive	+
8	39	M	W	Gingiva	N/A	Purple	Soft, multiple	N/A	+
9	57	M	W	Mucobuccal fold	N/A	Dark	Firm, pedunculated	Positive	+
10	39	M	W	Gingiva	N/A	N/A	N/A	Positive	+
11	42	M	W	Gingiva	1 cm	Purple	Nodular, multiple	Positive	+
12	27	M	W	Tongue	N/A	Blue	Multiple	Positive	+
13	32	M	W	Maxillary tuberosity	N/A	Purple	Exophytic	Positive	+
14	38	M	W	Gingiva	1×1×2 cm	Blue	Swelling	Positive	+
15	33	M	W	Tongue	N/A	N/A	Verrucous cast	Positive	+
16	25	M	W	Palate	2.5 cm	N/A	Pedunculated mass	Positive	+
17	48	M	W	N/A	2×2×.5cm	Red/Purple	N/A	Positive	+
18	31	M	W	Gingiva	1 cm	N/A	Enlarged operculum	N/A	+
19	47	M	W	Hard palate	N/A	Purple	N/A	Positive	+
20	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+
21	22	M	W	Lip vestibule	0.5 × 0.8	Blue	Swelling	Positive	+
22	32	M	W	Hard palate	3 × 4	Red	Exophytic mass	Positive	+
23	45	M	W	Hard palate	1.5 × 1.2	Red	Macule	Positive	+
24	31	M	W	Hard palate	2 cm	Blue	Pedunculated mass	Positive	+
25	56	M	W	Hard palate	Unknown	Red/Blue	Swelling	Positive	+

N/A: Information not available in chart

Table 2

Levels of expression of ANGPTL4 or VEGF in oral human KS lesions. Immunohistochemical reactivity was graded in a semiquantitative manner according to the percentage of positive tumor cells (− = 0%; + = <20%; + + = 20%–50%; +++ = >50%).

Case	ANGPTL4	VEGF
1	++	+
2	+	+++
3	++	−
4	+++	−
5	+	+++
6	+++	++
7	+++	++
8	−	+++
9	+++	++
10	++	+
11	−	−
12	+	++
13	++	+
14	+	+
15	+++	−
16	++	++
17	++	−
18	+++	+++
19	+	++
20	++	+++
21	+++	+++
22	+++	+++
23	+	++
24	+++	−
25	+++	+

Table 3

Stratification of results of immunohistochemical reactivity according to the percentage of positive tumor cells (− = 0%; + = <20%; ++ = 20%–50%; +++ = >50%).

	ANGPTL4 n (%)	VEGF n (%)
Negative	2 (8)	6 (24)
<20%	6 (24)	5 (20)
20–50%	7 (28)	7 (28)
>50%	10 (40)	7 (28)
Total	25 (100)	25 (100)