Expression of VEGF, VEGF-R2, bFGF and FGF-R in Oral Capillary Hemangioma

Man-Li Chuai1, Kei Sakamoto1, Hachiro Iseki1, Takafumi Makino2, Norihiko Okada3, Hirotsugu Yamamoto4 and Minoru Takagi1

1Molecular Pathology, Graduate School of Tokyo Medical and Dental University, Tokyo, Japan
2Department of Oral Surgery, Nihon University School of Dentistry at Matsudo, Chiba, Japan
3Laboratory Medicine, Graduate School of Tokyo Medical and Dental University, Tokyo, Japan
4Department of Oral Pathology, Nihon University School of Dentistry at Matsudo, Chiba, Japan

Oral capillary hemangioma (OCH) is a benign hamartomatous lesion that exhibits a proliferation of capillary endothelial cells and abnormal capillary formation. Its occurrence is relatively common in the oral cavity, yet the pathogenesis is not well understood. To elucidate the mechanism of OCH development, we examined the expression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), as well as their receptors (VEGF-R2, FGF-R), in OCH of 45 patients using an immunohistochemical staining method. More than 50% of the endothelial cells and the stromal cells of OCH showed significantly elevated expression of VEGF or/and bFGF, accompanied with an increase in the expression of their receptors. The increased number of capillary vessels in OCH was positively correlated with the expression level of these angiogenic factors. Our data indicate that the pathogenesis of OCH is closely associated with overexpression of angiogenic factors, which may result in autocrine or paracrine stimulation of endothelial cells and their precursors.

Key words: oral capillary hemangioma, VEGF, bFGF, angiogenesis

Correspondence: Minoru Takagi, Molecular Pathology, Graduate School of Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Phone: +81-3-5803-5451, Fax: +81-3-5803-0188, E-mail: m-takagi.mpa@tmd.ac.jp

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Introduction

Oral capillary hemangioma (OCH) is a relatively common benign vascular lesion, which arises mainly in adults as a solitary rapid-growing nodular mass (1, 2). Histologically, the lesion is characterized by marked increase of capillary-sized blood vessels accompanied with endothelial and stromal cell proliferation (3). Various factors have been proposed to be involved in its pathogenesis, including traumatic stimulation (4, 5), hormonal influences, viral oncogenes and arteriovenous malformations (6). Trauma is believed to play a major role; however, the cause and underlying mechanism for its development has yet to be elucidated.

During the process of angiogenesis, endothelial cells become stimulated, and some migrate to leave the blood vessel. These cells then divide and multiply to form a sprout on the capillary. This process is regulated by various growth factors that are secreted by macrophages, pericytes, endothelial cells and other stromal cells. Among these angiogenic factors, VEGF and bFGF are shown to be the most potent ones (7). VEGF stimulates proliferation and migration of vascular endothelial cells (8) through association with one of its three transmembrane receptors (VEGF-R1/Flt-1, VEGF-R2/KDR/Flk-1, VEGF-R3/Flt-4) (9-11), of which VEGF-R2 appears to be the dominant signaling receptor in VEGF-induced mitogenesis (12). The association with VEGF is thought to promote multimerization of VEGF-R, which activates its tyrosine kinase domain, triggering downstream signal transduction pathways. bFGF is a member of the FGF family and regulates a diverse range of physiologic and pathological processes including angiogenesis (13), through activation of FGF receptors (FGF-R).

Lines of evidence have demonstrated that these angiogenic molecules play definitive roles in stimulating capillary formation; however, it is not known whether they...
participate in the development of OCH. To gain insight into the pathogenesis of OCH, we collected 45 cases of surgically removed OCH and examined the expression of VEGF, bFGF, VEGF-R2 and FGF-R by immunohistochemical staining method. We evaluated the level of expression and the degree of capillary formation in OCH and analyzed their correlations.

Materials and methods

Specimens
Surgical specimens of OCH from 45 patients registered at the Dental Hospital of Tokyo Medical and Dental University were collected for the study. The specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Sectioning and staining for hematoxyline and eosin were performed following the standard laboratory protocol. Diagnoses of OCH were confirmed by two pathologists.

Immunohistochemistry and evaluation of immunostaining
Immunohistochemical staining was performed by streptavidin-biotin (SAB) immunoperoxidase staining method. Antibodies used were; Anti-VEGF monoclonal antibody (Santa Cruz Biotechnology), anti-VEGF-R2 polyclonal antibody (TaKaRa), anti-bFGF monoclonal antibody (Wako), anti-FGF-R monoclonal antibody (Wako) and anti-Factor VIII monoclonal antibody (Dako) (Table 1). Paraffin-embedded tissues were sectioned at 4-micrometer thickness, attached to polyllysine-coated glass slides, deparaffinized and hydrated. To inactivate endogenous peroxidase activity, the sections were soaked in 0.3% hydrogen peroxide in absolute methanol for 30 min, followed by washing in phosphate-buffered saline (PBS). For detection of VEGF, VEGF-R2 and bFGF, sections were pretreated with microwave oven heating for 5 min in citrate buffer (pH 6.0). Non-specific binding was blocked by incubating the sections in 10% rabbit serum for 30 min. Incubation with the primary antibody was performed overnight at 4°C. After washing 3 times in PBS, the secondary antibody coupled to biotin was applied and incubated for 30 min. After washing, the streptavidin-conjugated peroxidase solution was applied. Coloration was performed in 3,4-diaminobenzidine tetrahydrochloride substrate and counterstained with methyl green. As negative controls, normal mouse serum or PBS was substituted for the primary antibodies.

The level of expression was graded as follows: expression in less than 50% of endothelial cells and stromal cells as negative or slightly positive (±); in 50-75% cells as moderately positive (+); and in more than 75% cells as strongly positive (++)

Microvessel counting
Microvessel counting was performed after immunostaining for Factor VIII that is expressed exclusively in capillary endothelial cells. The lumina encircled by factor VIII-positive cells were identified as a capillary. Capillary density was measured within a 0.45 square millimeter field that contain the highest number of capillaries, using the counting software WINROOF (Mitani) (14). The data was represented as the numbers of capillaries per area, termed as microvessel count (MVC), and percentage of areas occupied by capillaries, termed as microvessel square (MVS). Statistical analysis was performed according to the Student t-test. Correlational coefficients were calculated after giving a value of one to (±), two to (+) and three to (++). Correlational coefficients larger than 0.2 were regarded as significant.

Results
Clinical and histopathological findings
The patients were 19 men and 26 women, with ages ranged from 2 to 78 years (mean 44.8 years). Two patients were pregnant and six were noticed to have experienced trauma at the same site. The numbers of tumor sites were 16 of lip, 19 of tongue, 5 of cheeks, 1 of palate, 1 of palate...

<table>
<thead>
<tr>
<th>Table 1: Antibodies used in this study</th>
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<tr>
<td><strong>Clone</strong></td>
</tr>
<tr>
<td>VEGF</td>
</tr>
<tr>
<td>VEGF-R2</td>
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<tr>
<td>bFGF</td>
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<td>FGF-R</td>
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<td>Factor VIII</td>
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3 of gingiva and 1 of pharynx. There was no site-dependent difference in sex and age distribution. All the OCH lesions were located in the submucosal connective tissues and had a lobular architecture. The lobules were composed of ovoid to spindle cells with moderate amounts of amphophilic cytoplasm, and the nuclei were ovoid to round with delicate chromatin and small nucleoli. The cells in these lobules were arranged in clusters and nests with a considerable number of small capillaries lined with swollen endothelial cells and the stroma rich in spindle cells with or without slight inflammatory cell infiltration. Some capillaries were present in the interlobular fibrous stroma, interconnecting the lobules in a random fashion.

**Immunohistochemical findings**

VEGF and bFGF were expressed in both capillary endothelial cells and stromal cells (Fig. 1-a, Fig. 1-b and Fig. 2-a). VEGF was expressed more in the endothelial cells than in the stromal cells, whereas bFGF expression was equally strong in both cell types. The control connective tissues adjacent to the lesions usually showed

![Image of immunohistochemical staining for VEGF and VEGF-R2 in OCH](image1)

![Image of immunohistochemical staining for bFGF and FGF-R in OCH](image2)

**Table 2: Result of immunohistochemical staining in OCH and control tissue**

<table>
<thead>
<tr>
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<th>OCH (n=45)</th>
<th>control tissue (n=25)</th>
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<tbody>
<tr>
<td></td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>VEGF</td>
<td>4 (9%)</td>
<td>14 (31%)</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>2 (4%)</td>
<td>4 (9%)</td>
</tr>
<tr>
<td>bFGF</td>
<td>4 (9%)</td>
<td>15 (33%)</td>
</tr>
<tr>
<td>FGF-R</td>
<td>21 (47%)</td>
<td>9 (20%)</td>
</tr>
</tbody>
</table>

+++: strong positive, +: moderately positive, ±: weak positive.
only slight positivity (<50% of the cells) for these angiogenic factors. In contrast, all the OCH lesions showed a moderate or strong positivity for at least either one of bFGF or VEGF, which means that more than 50% of the cells forming OCH lesion are overexpressing bFGF or VEGF. Strong positivity (>75% of the cells) of VEGF was observed in 60% OCH lesions (4% in control tissue) and bFGF in 58% OCH lesions (8% in control tissue) (Table 2). 70% of the cases were strongly positive for both bFGF and VEGF (Table 3). Moreover, the expression level of VEGF and bFGF was positively correlated, where the correlational coefficient was 0.83, indicating their synergistic upregulation (Table 3). The receptors were also overexpressed by a large population of the OCH cells. Strong positivity was noted for VEGF-R2 in 87% (8% in control tissue) and FGF-R in 36% cases (20% in control tissue) (Table 2). Expression of FGF-R was observed mainly in the endothelial cells (Fig. 2-b), whereas VEGF-R2 was expressed both in the endothelial cells and the stromal cells.

**Microvessel counting**

MVC in OCH (mean ± SD, 50.9 ± 21.7) was five times higher than that in adjacent connective tissue (8.2 ± 2.2). High MVC in OCH was positively correlated with the high level expression of the angiogenic factors when compared to the control tissue (Fig. 3). We examined the correlation between the expression level of the angiogenic factors and MVC within the OCH group. Again, statistical analysis revealed that high MVC is correlated with the expression level of VEGF, VEGFR and bFGF. The correlational coefficients were 0.21, 0.31 and 0.35 respectively. The correlation between the FGFR expression level and MVC was not significant. There was no significant correlation between MVS and any of the four angiogenic molecules (Fig. 4).

**Discussion**

Angiogenesis is controlled by a variety of angiogenic factors and angiogenesis inhibitors (15). Normal tissue maintains a subtle balance of these angiogenesis mediators. When stimulators are present in excess of inhibitors in such circumstances as wound healing or tumor progression, the balance is tipped in favor of blood vessel growth (16, 17). The role of VEGF and bFGF in blood vessel growth has been studied mainly in light of tumor angiogenesis (18). VEGF is highly expressed in tumor tissue in contrast to relatively low expression in normal adult tissue (19), which led to identification of VEGF as a key factor in angiogenesis. VEGF expression is stimulated

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**Table 3: Comparative distribution of expression levels of VEGF and bFGF in OCH**

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<thead>
<tr>
<th></th>
<th>VEGF</th>
<th>bFGF</th>
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<tr>
<td>±</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>3</td>
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<tr>
<td>++</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>16</td>
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</tbody>
</table>

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**Fig. 3: Comparison of microvessel count (MVC) and microvessel square (MVS) between OCH (n=45) and control tissue (n=25).**
following tissue hypoxia through regulation by other cytokines and growth factors (20). When the tissue runs short of oxygen, cells are signaled to enhance expression of VEGF and VEGF-R, resulting in increased angiogenesis for restoring blood flow to tissues (21). Although VEGF is the predominant stimulatory factor of angiogenesis, other protein factors have also been implicated to participate in the process (22). Most prominent of those is bFGF, which stimulates fibrosis and angiogenesis following tissue damage associated with chronic inflammation. It triggers the migration of fibroblasts to the site of injury and their subsequent proliferation and differentiation (23).

We demonstrated that VEGF and bFGF, as well as their receptors, were upregulated in OCH cells compared with the adjacent connective tissue. We also found a correlation between the expression level of these angiogenic factors and MVC; with higher expression of VEGF or bFGF, there was an enhancement of angiogenic activity and a corresponding increase in the vessel density. These observations suggest that overexpression of VEGF and bFGF plays a substantial role in development of OCH. OCH often develops at the site of injury. In our file, 6 cases showed a previous traumatic event and were thought to be associated with trauma. Although there is no conclusive evidence that injury causes OCH development, it is certain that the post-traumatic inflammatory tissue is rich in bFGF, VEGF and other cytokines to stimulate angiogenesis at its initial stage. However, inflammatory cell infiltration of most OCH cases was of only limited numbers, and the endothelial cells appear to be proliferating as if by an autonomous mechanism without aid from inflammatory cells. Studies have shown that bFGF and VEGF have a synergistic effect in angiogenic stimulation. bFGF can increase VEGF and VEGF-R expression in endothelial cells, marking bFGF as an important downstream mediator of bFGF-induced angiogenesis in tumor development (24-27). In addition, VEGF can release the stored bFGF from heparan sulfate proteoglycan in the extracellular matrix, thus potentiating its activity. VEGF, bFGF and their receptors were co-expressed in the capillary endothelial cells and stromal cells in OCH, implying that they may act by autocrine or paracrine mechanism among OCH cells. The co-overexpression of both factors and receptors in OCH cells suggests that they may have a synergistic role, stimulating endothelial cells and their precursors beyond the control of angiogenesis inhibitors supplied from the surrounding tissues. It should be noted that bFGF is relatively abundant in stromal cells, whereas VEGF was more expressed in endothelial cells rather than

![Graphs showing correlation between expression levels and microvessel count (MVC) and microvessel square (MVS) in OCH.](image)

Fig. 4: Correlation between the expression levels and microvessel count (MVC) and microvessel square (MVS) in OCH.
stromal cells. This biased co-localization suggests that abundant production of bFGF by the stromal cells might play an important role in inducing and maintaining VEGF expression in the endothelial cells.

Estrogen has been demonstrated to upregulate VEGF. In our file, 2 cases were considered as pregnancy tumors, which implies that abundant estrogen in blood flow may have somehow stimulated the local VEGF production and resulted in OCH development. Indeed, some OCH cases have been shown to be associated with exogenous estrogen and progesterone administration. In addition, there was one case of a young woman that may be associated with hormonal effects. The other 37 cases were associated neither with injury nor with pregnancy, and the exact etiology was unclear. This category may include genuine neoplasms that erroneously produce excessive angiogenic factors, but further study will be required to clarify these points.

In cancer therapy, clinical applications of various angiogenesis inhibitors have been used in an attempt to reduce tumor development. For example, the monoclonal antibody to VEGF has been used for cancer treatment, in which the density of vessel was obviously lower than one without antibody treatment. Although the first-choice treatment for OCH is a surgical excision, those investigations suggest a new possibility for treatment in which we might inhibit VEGF or bFGF signaling by pharmacological or even genetical methods.

References
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