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Association of vascular endothelial growth factor and mast cells with angiogenesis in laryngeal squamous cell carcinoma

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Abstract We investigated the expression of vascular endothelial growth factor (VEGF) and microvascular density in 54 cases of invasive laryngeal squamous cell carcinoma (SCC) and in ten samples of normal laryngeal tissue using immunohistochemistry methods. The study also focused on the distribution of mast cells in and around the SCCs. The microvascular density in laryngeal carcinoma tissue was higher than that in normal tissue (P=0.02). VEGF was localized in SCCs, stromal cells, endothelial cells, minor salivary glands, and non-cancer epithelium adjacent to the tumor. VEGF expression in the tumor cells was found in 13 of 54 cases (24.1%), whereas mast cells around the carcinomas were VEGF positive in all 54 cases. Staining of VEGF in SCCs was strong in the area of high microvascular density (P=0.0002). Using a multi-labeling subtraction immunostaining method, VEGF-positive stromal cells were classified mostly as mast cells and, in a few instances, as macrophages. VEGF staining in SCCs was associated with the mast cell count (P=0.0001). There was no distinct correlation between VEGF expression and pTNM stage of an SCC. In conclusion, the results suggest that VEGF might be an important angiogenic factor in cancer invasion. Laryngeal cancer cells and mast cells may control the angiogenic response by releasing VEGF.

Key words VEGF · Angiogenesis · Mast cell · Macrophage · Laryngeal squamous cell carcinoma

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Introduction

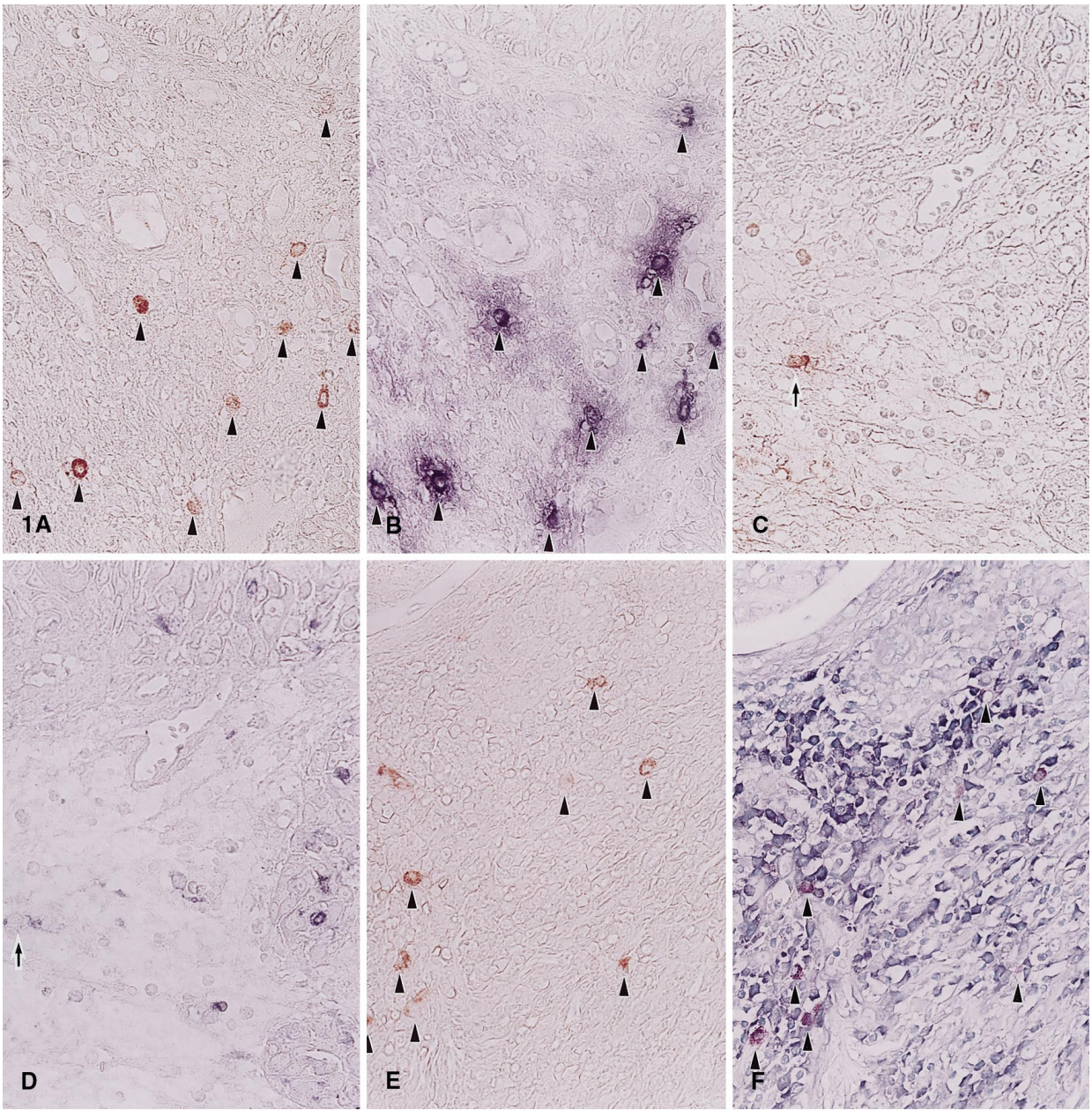
Angiogenesis is essential for tumor growth and invasion. Although it is substantially an endothelial cellular event, there are many types of accessory cells and many kinds of mediators involved in this process. Vascular endothelial growth factor (VEGF), one of the angiogenic and hyperpermeability factors, plays an important role in diverse pathological and physiological conditions including tumor growth [2, 3, 4, 5, 6, 10, 11, 12, 15, 18, 19, 21, 25, 30]. Protein and mRNA of VEGF are overexpressed in some malignant tumors. The source of this overexpression is considered to be tumor cells, epithelial cells, endothelial cells, fibroblastic cells, and macrophages [10, 15].

Mast cells also have been implicated as having an important role in neovascularization [8, 20, 22, 23, 29]. Higher than normal numbers of mast cells have been reported in various diseases associated with angiogenesis, such as rheumatoid arthritis [23, 32], wound repair [29], varicose veins of the lower limbs [31], and some tumors [8, 20, 22]. Mast cells can produce, store, and release many kinds of chemical mediators, including histamine, tryptase, chymase, heparin proteoglycan, basic fibroblast growth factor, and cytokines [8, 20, 22, 23, 29]. However, the role of mast cells in angiogenesis is not fully understood.

The study focused on the localization of VEGF and mast cells in invasive laryngeal squamous cell carcinoma (SCC).

Materials and methods

For this study, we collected carcinoma tissue samples from 54 SCC patients who had undergone total laryngectomy or laryngo-micro-laser surgery at our hospital between 1992 and 1996. All patients had invasive SCC. Patients with carcinoma in situ, verrucous carcinoma, and other types of carcinoma were excluded. None of the patients had received radiotherapy or chemotherapy before the surgical intervention. SCCs were classified according to the *Union Internationale Contra la Cancrum* (UICC) TNM classification of 1987 [14]. The average age of patients at surgery was 67 years (range 41–89 years). The tumor locations were as follows: supraglottic (6 cases), glottic (42 cases), subglottic (3 cases),



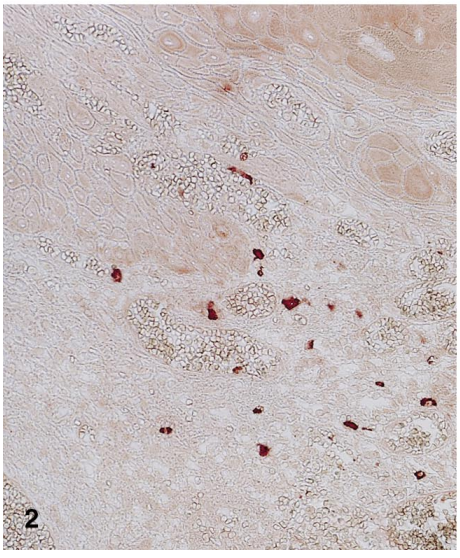


Fig. 1 Multi-labeling subtraction immunostaining in exactly the same areas on the same slide (**A** and **B**, **C** and **D**, **E** and **F**). Note that most mast cells are labeled by vascular endothelial growth factor (VEGF) (**A** and **B**, **E** and **F**, *arrowheads*). In contrast, only a few macrophages express VEGF (**C** and **D**, *arrow*). **A**, **C**, **E** VEGF expression (red). **B** Tryptase expression (blue). **D** CD68 expression (blue). **F** Toluidine blue stain (×400)

Fig. 2 Expression of vascular endothelial growth factor (VEGF) in laryngeal carcinoma. VEGF is immunolocalized in both cancer cells and stromal cells around cancer nest (×200)

and transglottic (3 cases). Forty-five cases were T1 or T2, and nine were T3 or T4. Autopsy tissue samples from ten subjects without a history of laryngeal disease and 30 tumor-free biopsy specimens from laryngeal carcinoma patients were prepared as controls.

Of the 54 SCCs, 36 were well differentiated (G1), 14 were moderately differentiated (G2), and the remaining 4 were poorly differentiated (G3). Six patients showed lymph-node metastases

and 25 showed local recurrence. Histologic typing was based on the World Health Organization system [28]. Formalin-fixed, paraffin-embedded sections were cut and stained with hematoxylin and eosin (H&E) and toluidine blue (pH 7.2).

Immunostaining was carried out using primary antibodies (Abs) against VEGF (MAB293, R&D System, Minneapolis, Minn.) and CD34 (QB-END/10; Novocastra Laboratory, Tokyo, Japan) with a streptavidin–biotin complex (SAB) kit (Nichirei Co., Tokyo, Japan). For each experiment, negative control samples were processed using non-immune mouse immunoglobulin G (IgG) of the same IgG subtype at the same concentration of anti-VEGF Ab. Sections were stained in batches of ten slides each for VEGF and CD34, and staining was detected using an alkaline-phosphatase complex (SAB-AP) kit and a peroxidase complex (SAB-PO) kit (Nichirei). New fuchsin (Nichirei) and 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, Mo.) were used as the chromogen.

Multi-labeling subtraction immunostaining was performed in a stepwise fashion as previously described [32]. The primary Abs were antitryptase Ab (mast-cell-specific marker; Dako, Glöstrup, Denmark), antimacrophage Ab (CD68, PG-M1, Dako, Tokyo, Japan), and anti-VEGF Ab (R&D system). As usual, the first immunostaining for VEGF was performed on sections using a SAB-PO kit (Nichirei) and 3-amino-9 ethylcarbazole (AEC, Sigma; positive, red color). After immunostaining, the slide was immediately mounted on a cover glass using an aqueous mounting medium (Biomeda, Foster City, Calif.). Within 20 min, microscopic photographs were taken (Fig. 1A, C). Then the slide was immersed in phosphate-buffered saline (PBS) without calcium and magnesium (pH 7.2) and gently shaken manually until the coverslip came off. Then the slide was meticulously washed with PBS and immersed in 99.8% methyl alcohol for 10 s to extinguish the positive deposits of the first immunostain. After treatment with citrate buffer (pH 6.0) for 10 min at 90°C, the second immunostaining was performed on the same slide glass using an antitryptase SAB-AP kit. For the second staining, fast blue (Nichirei; positive, blue color) was used to detect tryptase (Fig. 1B) and CD68 (Fig. 1D). Finally, the exact area on the same slide was compared with the results of the first immunostaining. To check the specificity of immunostain, control stainings were performed with non-immune mouse IgG.

Double immunostaining was also performed using primary antibodies against VEGF, tryptase, and CD68 with SAB-PO and SAB-AP kits. For double staining, peroxidase/DAB (brown color), alkaline phosphatase/new fuchsin (red color), and alkaline phosphatase/fast blue (blue color) were used for CD68, VEGF, and tryptase, respectively. We compared the results of multi-subtraction immunostaining and double immunostaining.

Immunostaining was classified into three groups according to intensity: non-immunoreactive (–), weakly stained (±), and strongly stained (+). A tumor sample was considered positive if the tumor cells were +. The mast cells and VEGF-positive stromal cells were counted. Within one high-power field, five regions were counted, each of which was 0.16 mm^2 in size. The counts were expressed as mean±SEM per region within a ×400 high-power field. Microvascular density was determined using immunostaining for CD34. Microvascular counts were calculated in the area of highest microvascular density (i.e., vascular "hot spots") in five regions of a ×200 microscopic field (0.723 mm^2 per field), as described previously [15].

Statistical comparisons were made using the two-tailed Student' *t*-test or the chi-square test with StatView 4.5 and DA stat for Macintosh. *P* values of <0.05 were regarded as significant.

Results

Localization of VEGF expression

Thirteen of the 54 SCCs (24.1%) showed positive immunostaining for VEGF (Fig. 2). Among these, samples from nine patients also showed positive staining for VEGF in the non-cancerous epithelium, minor salivary glands, and the ducts near the SCCs. VEGF-positive staining was observed in vascular endothelial cells adjacent to SCCs in three samples. In all 54 cases, stroma had VEGF-positive cells concentrated around vascular channels, in fibrosis, near necrotic foci, and in the inflamed lesions. The numbers of VEGF-positive stromal cells were higher in VEGF-positive SCCs (3.54±0.35) than in VEGF-negative SCCs (1.05±0.19) (*P*<0.0001, Fig. 3). The counts of VEGF-positive stromal cells (1.65±0.22) were higher in SCCs than in controls (1.11±0.11).

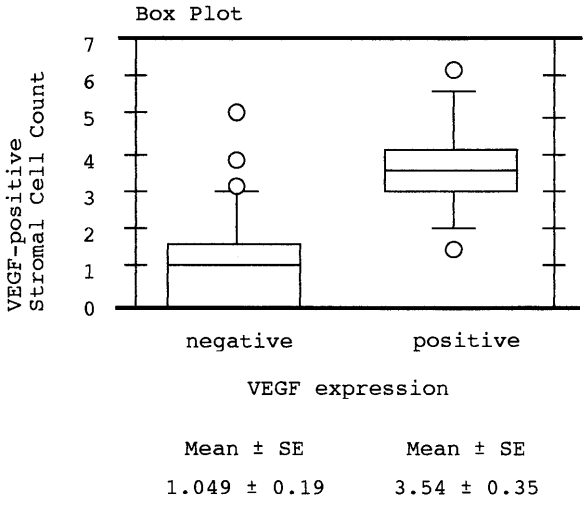


Fig. 3 Correlation between vascular endothelial growth factor (VEGF) expression of laryngeal squamous cell carcinomas (SCCs) and stromal cell count. The VEGF-positive stromal cell count is higher in VEGF-positive SCCs than in VEGF-negative SCCs (*P*<0.0001)

Normal laryngeal epithelium from controls showed no VEGF expression. No significant correlation was seen between VEGF expression and clinicopathologic parameters (histologic type, T category, lymph-node metastasis, distant metastasis, and stage).

Mast cells

Mast cells were identified best by toluidine blue stain and immunostain for tryptase. They were usually large and oval, short spindled, or stellate. The distribution of all mast cells was similar to that of VEGF-positive stromal cells. In SCCs, mast cells were frequently accumulated as groups of several cells in fibrosis and in the perivascular region in groups. In controls, they were scattered singly in the submucosa. The number of mast cells was higher in SCC patients (2.37±0.30) than in controls (1.56±0.24). A significant correlation was seen between mast cell count and VEGF staining of SCCs (*P*<0.0001, Fig. 4).

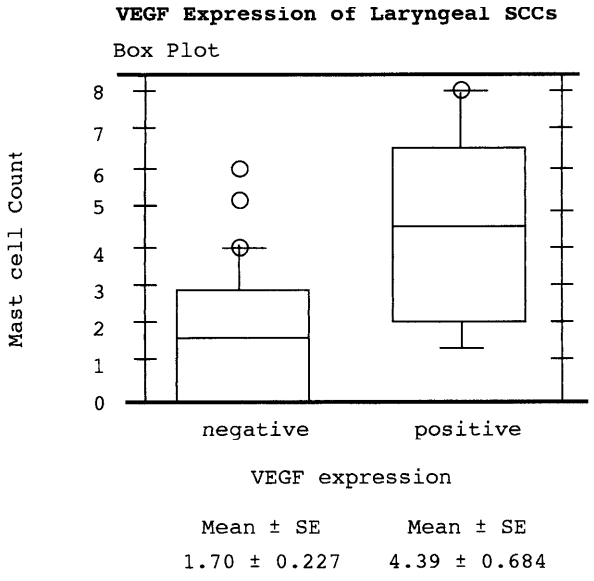


Fig. 4 Correlation between vascular endothelial growth factor (VEGF) expression of laryngeal squamous cell carcinoma (SCC) and mast cell count. The mast cell count is higher in VEGF-positive SCCs than in VEGF-negative SCCs ($P<0.0001$)

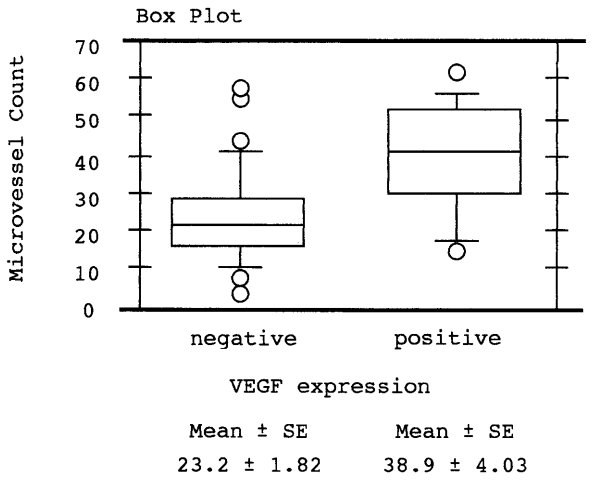


Fig. 5 Correlation between vascular endothelial growth factor (VEGF) expression of laryngeal squamous cell carcinomas (SCC) and microvascular count. The microvascular count is higher in VEGF-positive SCCs than in VEGF-negative SCCs ($P<0.0002$)

Microvascular density

CD34 was a useful marker for detecting endothelial cells of small blood vessels. Microvascular counts varied from 5 to 60 per ×200 field (average 25.8 per ×200 field). The microvascular count was higher in SCCs (27.00±1.91) than in controls (15.67±2.17, $P=0.021$). The microvascular count also was higher in VEGF-positive SCCs (38.92±4.03) than in VEGF-negative SCCs (23.22±1.82, $P=0.0002$, Fig. 5).

Multi-labeling subtraction immunostaining

To clarify the cell type of VEGF-positive stromal cells, multi-labeling subtraction immunostaining was performed, and it clearly demonstrated that most VEGF-positive stromal cells were also labeled by antitryptase Ab in both SCC specimens and in controls (Fig. 1A, B). These were regarded as mast cells. In contrast, only a few VEGF-positive cells were labeled by CD 68, and these were classified as macrophages (Fig. 1C, D). The proportions of mast cells and CD68 macrophages in the total VEGF stromal cells were 89.8% and 10.2%, respectively. No cross-reaction occurred between the two antibodies.

Double immunostaining

The results of double immunostaining were similar to those of multi-labeling subtraction immunostaining (data not shown).

Discussion

Morphologically, this study suggests that VEGF is an important angiogenic factor in stromal invasion by SCC and that cancer cells and mast cells may control the angiogenic response by VEGF. Tumor angiogenesis is essential for tumor growth [13, 27]. It is a complex process that involves the interplay between a variety of inducers and inhibitors of angiogenesis, which are produced by different types of cells such as tumor cells, stromal cells, and endothelial cells [13]. Numerous angiogenic factors have been described in various conditions [13, 20, 26, 27], but in laryngeal carcinoma, the factors remain unknown. VEGF is suspected to play an important role in both tumor and non-tumor angiogenesis [2, 3, 4, 5, 6, 10, 11, 12, 18, 19, 21, 30]. Its overexpression has been reported in esophageal [15] and head and neck carcinomas [6, 25]. High microvascular density is associated with poor prognosis [1, 16], and a close correlation between microvascular density and VEGF expression has been reported in many cancers including some SCCs [10, 12, 15, 18]. To our knowledge, most previous studies, however, focused only on VEGF expression of cancer cells and, thus, did not examine stromal cells. There have been few investigations concerning the relationship between VEGF expression and angiogenesis in many invasive laryngeal SCCs [6, 25]. Our current results indicate that the microvascular density in laryngeal carcinoma is higher than that in normal laryngeal tissue. In patients with VEGF-positive tumors, extensive microvascular formation correlated significantly with VEGF expression. This result suggests that SCCs that express VEGF strongly are potent stimulators of angiogenesis. It also indicates that VEGF serves as a direct mitogenic growth factor for vascular endothelial cells in laryngeal SCCs.

VEGF is thought to induce a vascular stroma by at least two mechanisms [5]. First, VEGF has potent angio-

genic properties due to its role as a direct mitogen of endothelial cells via its binding to two specific tyrosine kinase receptor proteins (flt-1 and kdr) [5]. Second, VEGF acts as a potent mediator of microvascular hyperpermeability. Increased permeability of microvessels results in the extravasation of plasma proteins into the surrounding stroma, leading to proangiogenic alterations of the extracellular matrix [5].

The mechanisms of upregulation of VEGF expression are still unclear. Hypoxia has been reported as a factor in increasing VEGF expression by some tumors [11, 12, 13]. VEGF expression was observed in macrophages around necrotic areas in laryngeal SCCs. However, hypoxia is unlikely to be the only factor in the overexpression of VEGF in laryngeal SCCs, because most VEGF expression is seen in areas distinct from necrotic areas. From the findings of previous studies, we think that various factors produced by stromal cells may stimulate VEGF expression in stromal cells, and influence angiogenesis [26, 27]. The findings of other recent studies demonstrating that tumor cells and other stromal cells secreted certain cytokines, such as transforming growth factor (TGF)-α and platelet-derived growth factor (PDGF) [1, 7, 9, 16, 17, 24], and that, in fact, some of these cytokines upregulate VEGF expression are consistent with this hypothesis [5].

Previous studies have suggested that VEGF-positive stromal cells are macrophages [10, 15] or macrophage-like cells [6], fibroblasts [2], and plasma cells [25]. These studies focused on morphologic features. In the present study, using multi-labeling subtraction immunostaining, we determined that, in addition to CD68 macrophages, mast cells may be a major source of VEGF in SCCs. In support of this hypothesis, the results of our previous investigation suggest that VEGF-positive cells are identical to mast cells in rheumatoid arthritis [32]. In addition, Grutzkau et al. [9], using reverse-transcription polymerase chain reaction (RT-PCR) and Western-blot analysis, detected VEGF in mast cell line HMC-1 and in human skin mast cells. Some previous studies have suggested that VEGF protein-positive cells are also positive for VEGF mRNA [2, 11]. Weninger et al. [30] used MAB293 on human tumor samples and found that the VEGF polypeptide detected by immunohistochemical means was accompanied by VEGF mRNA localization as determined by in situ hybridization and Northern-blot analysis.

Mast cells have been implicated in neovascularization [20]. Some mast-cell products, such as heparin, histamine, tumor necrosis factor-α, and basic fibroblast growth factor, have been reported to stimulate endothelial-cell proliferation. It is possible that one or more of these factors act in concert with VEGF to induce angiogenesis in SCCs. Our present results suggest a possible mechanism to explain the role of mast cells in angiogenesis.

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