

# Odontogenic keratocyst expresses vascular endothelial growth factor: an immunohistochemical study

G. K. Mitrou<sup>1</sup>, K. I. Tosios<sup>1</sup>, A. Kyroudi<sup>2</sup>, A. Sklavounou<sup>1</sup>

<sup>1</sup>Department of Oral Pathology and Surgery, Dental School, University of Athens, Athens, Greece; <sup>2</sup>Department of Histology and Embryology, Medical School, National and Kapodestrian University of Athens, Athens, Greece

**BACKGROUND:** Vascular endothelial growth factor (VEGF) expression may act as a sensitive measure of the angiogenic potential of a lesion. Furthermore, VEGF has been implicated in the pathogenesis of cystic tumors and inflammatory odontogenic cysts. Thus, we studied the expression of VEGF in the epithelium of odontogenic keratocyst (OK) in association with cell proliferation and apoptosis.

**METHODS:** Forty-two cases of OK, 26 cases of dentigerous cyst (DC), and 15 cases of residual cyst (RC) were retrospectively examined by immunohistochemistry for VEGF, Ki67/Mib-1 and anti-caspase-3. For VEGF and caspase-3, the intensity of immunostaining was qualitatively assessed, while for the evaluation of Ki67 the average number of positively stained nuclei in 10 high-power microscopic fields (×400) was calculated.

**RESULTS:** The VEGF expression was stronger in OK when compared with DC ( $P < 0.007$ ). The rate of nuclear Ki67 expression in OK was significantly higher than that in DC ( $P < 0.001$ ) and RC ( $P < 0.001$ ). Cytoplasmic caspase-3 expression was statistically more intense in RC than in OK ( $P = 0.001$ ) or DC ( $P < 0.001$ ). A statistically significant correlation was seen in OK for Ki67 ( $P < 0.001$ ) and VEGF ( $P = 0.023$ ), but not for caspase-3. Multiple regression analysis revealed a linear relationship between VEGF and Ki67.

**CONCLUSIONS:** The VEGF was expressed in the epithelium of OK, DC, and RC with a variable intensity, and in OK VEGF expression was related to Ki67. It is suggested that VEGF expression by the odontogenic epithelium is not induced solely by inflammation.

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**Keywords:** caspases; dentigerous cyst; Ki67; odontogenic keratocyst; residual cyst; VEGF

## Introduction

Vascular endothelial growth factors (VEGF) comprise a family of multifunctional proteins mainly involved in normal and pathologic angiogenesis, defined as the formation of new vessels by sprouting of the pre-existing endothelium (1). Angiogenesis is fundamental in embryonic development, the reproductive cycle and wound healing (2, 3). It is, also, vital in pathologic conditions such as cancer, rheumatoid arthritis, retinopathies, and psoriasis.

The VEGF family includes VEGF-A or VEGF, also known as vascular permeability factor (VPF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factors (1, 4, 5). VEGF is a dimeric, heparin-binding glycoprotein, critical for many of the individual steps involved in the complex process of angiogenesis (6) and a potent inducer of vascular permeability (7). It stimulates proliferation and migration of endothelial cells, promotes survival and differentiation of endothelial cells, triggers the production of metalloproteinases that are required for the degradation of basement membranes, contributes to the formation of tumor stroma, and attracts monocytes that produce angiogenic factors (8). It is, also, thought to elicit the formation of blood vessel *de novo* (2).

There are four major isoforms of VEGF (VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, VEGF<sub>206</sub>), derived from alternative splicing of the VEGF gene located on chromosome 6p21.3, that differ in the number of amino acids of the mature secreted protein (2). VEGF<sub>165</sub> is the most frequent and the most mitogenic isoform. VEGF expression is regulated by various factors, such as hypoxia, cytokines [interleukin (IL)-1, cyclooxygenase-2-derived prostaglandins], endotoxins, estrogens, growth factors [platelet-derived growth factor (PDGF), transforming growth factors (TGFs), insulin-like growth factor (IGF) 1, fibroblast growth factor (FGF), and keratinocyte growth factor], and intracellular signal transducers. Other growth factors are also involved in angiogenesis.

Odontogenic keratocyst (OK) is defined in the recent WHO classification as a cystic tumor lined by

Correspondence: Konstantinos Tosios, Department of Oral Pathology and Surgery, Dental School, University of Athens, 2 Thivon Street, 11527 Athens, Greece. Tel: +30 210 7461003, Fax: +30 210 7461220, E-mail: ktosios@dent.uoa.gr  
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parakeratotic squamous epithelium and designated as 'keratocystic odontogenic tumor' (9). Considerable features associated with the OK such as the aggressive, infiltrative behavior and significant recurrence rate (10–13), as well as genotypic findings (14), favor a neoplastic nature. OK shows a high proliferative potential (15–22), balanced by apoptotic cell death (21–23). Cell proliferation and apoptosis in OK has been mainly compared with those in well-established odontogenic cysts, in particular the dentigerous cyst (DC) and the radicular cyst (RC).

Vascular endothelial growth factor has been implicated in the pathogenesis of cystic tumors (24–26) and radicular cysts (27, 28). In addition, extent of neovascularization in tumors is thought to define tumor aggressiveness and invasion, as a dense vascular network ensures the necessary effective metabolic process in tumor cells (3, 29), while acquisition of an angiogenic phenotype appears to be a crucial aspect in the transition from hyperplasia to neoplasia (30). Thus, VEGF may be involved in the pathogenesis and growth of OK, regardless of its neoplastic or dysplastic nature. To the best of our knowledge, VEGF expression in the epithelium of OK has not been characterized.

We report the expression of VEGF in the lining epithelium of OK in association with cell proliferation and apoptosis. Expression is compared with that in DC and RC.

## Materials and methods

Forty-two cases of sporadic OK (26 men and 16 women, average age  $43.9 \pm 18.44$  years), 26 cases of DC (18 men and eight women, average age  $38.04 \pm 15.48$  years), and 15 cases of RC (13 men and two women, average age  $45.73 \pm 10.39$  years), formalin-fixed and paraffin-embedded, were retrospectively studied.

Immunohistochemistry was performed on deparaffinized 4- $\mu$ m sections with a standard streptavidin-biotin-peroxidase technique. Primary antibodies used were monoclonal VEGF that reacts with 189, 165 and 121 amino acid splice variants (sc-7269, dilution 1:100; Santa Cruz Biotechnology, Inc., Heidelberg, Germany), monoclonal Mib-1 (M7240, dilution 1:100; DakoCytomation, Glomstrup, Denmark), and polyclonal anti-caspase-3 (AF835, concentration 0.3  $\mu$ g/ml; R&D systems, Minneapolis, MN, USA). For VEGF and Ki67, the sections were microwaved in 0.01 mol/l citrate buffer, pH 6.0, for  $4 \times 5$  min (800 w, medium power). When cool, sections were immersed for 15 min in 3% hydrogen peroxide, rinsed in distilled water and then treated overnight at 4°C with the primary antibodies against VEGF and Ki67 diluted in 1:20 rabbit serum (X0902; Dako, Glomstrup, Denmark) and tris buffered saline (TBS). The sections were then further treated with the secondary polyclonal rabbit anti-mouse immunoglobulin in 1:200 dilution (E0354; Dako) for 30 min at 37°C. For caspase-3, the sections were boiled in steamer in 0.5 mol/l ethylenediaminetetraacetic acid (EDTA) buffer, pH 8.0, for 45 min, rinsed in distilled water and

then treated for 90 min at 37°C with a mixture of the activated caspase-3 primary antibody diluted in 1:20 swine serum (X0901; Dako) and TBS. This was followed by treatment with the secondary polyclonal swine anti-rabbit immunoglobulin in 1:200 dilution (E0353; Dako) for 30 min at 37°C.

The sections were overlaid with streptABC/HRP complex (K0377; Dako) for 30 min at 37°C. Bound peroxidase was visualized by a 3,3'-diaminobenzidine hydrochloride (DAB; Sigma, St. Louis, MO, USA) and counter-stain was developed with Gill's hematoxylin.

### Evaluation of staining

Immunoreactivity was independently evaluated by two experienced researchers. For VEGF and caspase-3, the intensity of immunostaining was qualitatively assessed as weak, moderate, strong, and very strong, as the full thickness of the epithelium was positive in all lesions. For the evaluation of Ki67 immunostaining, the average number of positively stained nuclei in 10 high-power microscopic fields ( $\times 400$ ) was assessed as follows: weak, < 5 positive epithelial cells per optic field; moderate, 6–10; strong, 11–20; very strong, > 21.

### Statistical methods

Statistical evaluation was performed with, Mann–Whitney, Spearman's rank correlation test, and logistic regression. A  $P \leq 0.05$  was considered to be significant.

## Results

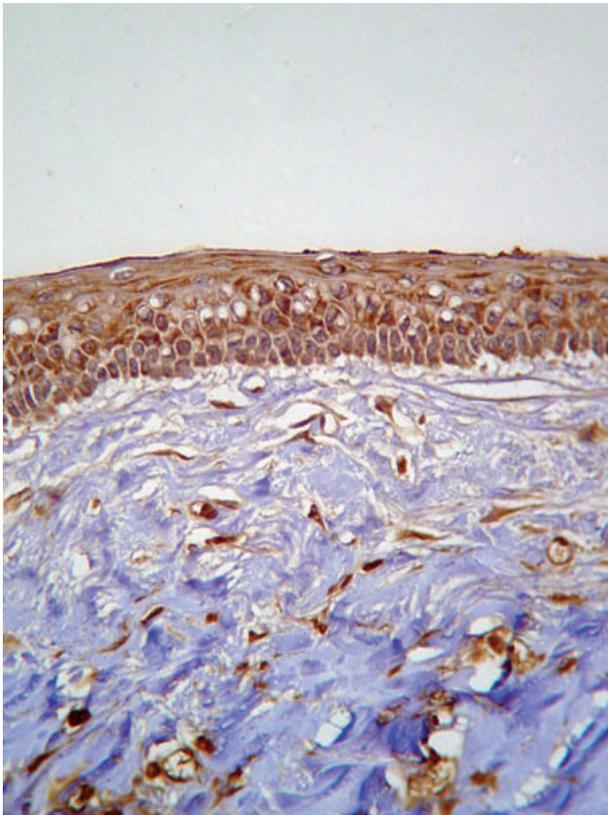
Cytoplasmic immunoreactivity for VEGF was seen in 35 out of 37 OKs and all DCs and RCs, as well as adjacent endothelial cells, fibroblasts, and inflammatory cells. All epithelial cell layers, with the exception of the parakeratin layer in OKs, were stained. VEGF intensity in OKs, DCs, and RCs is summarized in Table 1. Very strong expression was seen in 72.97% of OKs (Fig. 1) and in 46.66% of RCs (Fig. 2), while weak expression was seen in the majority of DCs (38.89%). VEGF expression was stronger in OK when compared with DC ( $P < 0.007$ , Mann–Whitney), but there was no statistically significant difference between OK and RC ( $P = 0.228$ , Mann–Whitney), or DC and RC ( $P = 0.26$ , Mann–Whitney).

Nuclear immunoreactivity for Ki67 was seen in the basal and parabasal layers of the epithelium of OK, while a few positive cells were present in the basal layer

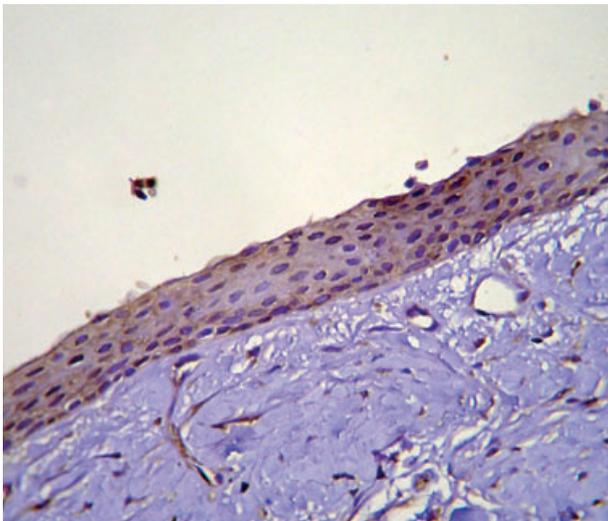
**Table 1** VEGF expression by type of lesion

| VEGF        | OK, n (%)   | DC, n (%)   | RC, n (%)   | Total, n (%) |
|-------------|-------------|-------------|-------------|--------------|
| Negative    | 2 (5.41)    | 0 (0)       | 0 (0)       | 2 (2.86)     |
| Weak        | 5 (13.51)   | 7 (38.89)   | 4 (26.67)   | 16 (22.86)   |
| Moderate    | 3 (8.11)    | 5 (27.78)   | 4 (26.67)   | 12 (17.14)   |
| Strong      | 9 (24.32)   | 4 (22.22)   | 2 (13.33)   | 15 (42.86)   |
| Very strong | 18 (48.65)  | 2 (11.11)   | 5 (33.33)   | 25 (35.71)   |
| Total       | 37 (100.00) | 18 (100.00) | 15 (100.00) | 70 (100.00)  |

VEGF, vascular endothelial growth factor; OK, odontogenic keratocyst; DC, dentigerous cyst; RC, residual cyst.



**Figure 1** Intense vascular endothelial growth factor (VEGF) expression in the lining epithelium of an odontogenic keratocyst (OK) (original magnification  $\times 400$ ).



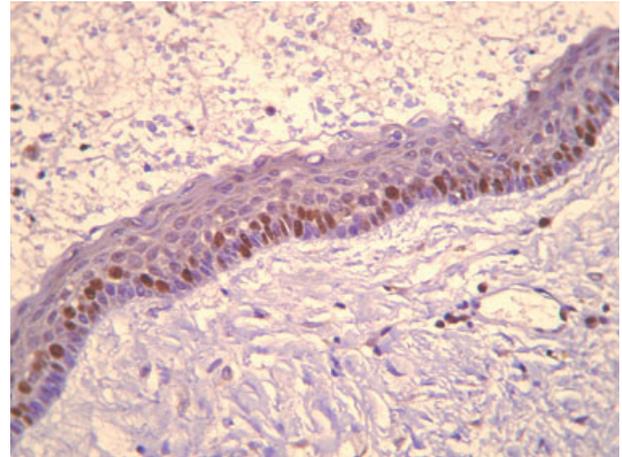
**Figure 2** VEGF expression in a residual cyst (original magnification  $\times 400$ ).

of DC and RC. Results are summarized in Table 2. Most OKs expressed Ki67 strongly or very strongly (47.22% and 19.44%, respectively; total 66.66%) (Fig. 3). In contrast, the majority of DCs (89.47%) and RCs (92.31%) showed weak expression of Ki67.

**Table 2** Ki67 expression by type of lesion

| Ki67        | OK, n (%)   | DC, n (%)   | RC, n (%)   | Total, n (%) |
|-------------|-------------|-------------|-------------|--------------|
| Weak        | 4 (11.11)   | 17 (89.47)  | 12 (92.31)  | 33 (48.53)   |
| Moderate    | 8 (22.22)   | 1 (5.26)    | 1 (7.69)    | 10 (14.71)   |
| Strong      | 17 (47.22)  | 1 (5.26)    | 0 (0)       | 18 (26.47)   |
| Very strong | 7 (19.44)   | 0 (0)       | 0 (0)       | 7 (10.29)    |
| Total       | 36 (100.00) | 19 (100.00) | 13 (100.00) | 68 (100.00)  |

OK, odontogenic keratocyst; DC, dentigerous cyst; RC, residual cyst.



**Figure 3** Intense Ki67/Mib-1 expression the basal and parabasal layers of an OK (original magnification  $\times 400$ ).

The rate of Ki67 expression in OK was significantly higher than that in DC ( $P < 0.001$ , Mann–Whitney) or RC ( $P < 0.001$ , Mann–Whitney), and there was no statistically significant difference between DC and RC ( $P = 0.761$ , Mann–Whitney).

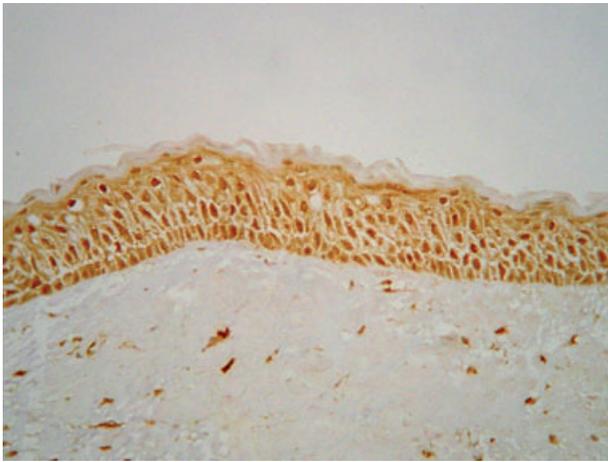
Cytoplasmic and nuclear caspase-3 expression was seen in the full thickness of the OKs, DCs, and RCs lining epithelium (Table 3, Fig. 4). There was no statistical difference between OK and DC ( $P = 0.757$ , Mann–Whitney), but RCs showed statistically more intense expression than OK ( $P = 0.001$ , Mann–Whitney) or DC ( $P < 0.001$ , Mann–Whitney).

In Table 4, statistically significant correlation is shown in OK between Ki67 and caspase-3 ( $P = 0.02$ ), and in DC between caspase-3 and VEGF ( $P = 0.02$ ). Multivariate regression analysis (Table 5) revealed a linear relationship between VEGF and Ki67 in OK.

**Table 3** Caspase-3 expression by type of lesion

| Caspase-3 | OK, n (%)   | DC, n (%)   | RC, n (%)   | Total, n (%) |
|-----------|-------------|-------------|-------------|--------------|
| Weak      | 5 (13.51)   | 3 (12.50)   | 0 (0)       | 8 (10.53)    |
| Moderate  | 13 (35.14)  | 10 (41.67)  | 0 (0)       | 23 (30.26)   |
| Strong    | 19 (51.35)  | 11 (45.83)  | 15 (100)    | 45 (59.21)   |
| Total     | 37 (100.00) | 24 (100.00) | 15 (100.00) | 76 (100.00)  |

OK, odontogenic keratocyst; DC, dentigerous cyst; RC, residual cyst.



**Figure 4** Intense caspase-3 expression in the lining epithelium of an OK (original magnification  $\times 400$ ).

**Table 4** Spearman's rank correlation for VEGF, Ki67 and caspase-3

|    | <i>Ki67-VEGF</i> |                | <i>Caspase 3-VEGF</i> |                | <i>Ki67-caspase 3</i> |                |
|----|------------------|----------------|-----------------------|----------------|-----------------------|----------------|
|    | <i>Rho</i>       | <i>P-value</i> | <i>Rho</i>            | <i>P-value</i> | <i>Rho</i>            | <i>P-value</i> |
| OK | 0.21             | 0.24           | 0.20                  | 0.26           | 0.41                  | 0.02           |
| DC | -0.23            | 0.4            | 0.58                  | 0.02           | -0.01                 | 0.95           |
| RC | 0.36             | 0.23           | N/C                   | N/C            | N/C                   | N/C            |

OK, odontogenic keratocyst; DC, dentigerous cyst; RC, residual cyst; N/C, not calculated.

**Table 5** Multivariate logistic regression of Ki67 and VEGF in OK, when compared with DC/RC

|      | <i>Odds ratio</i> | <i>95% CI</i> | <i>P-value</i> |
|------|-------------------|---------------|----------------|
| Ki67 | 13.281            | 3.749-47.04   | <0.001         |
| VEGF | 0.987             | 0.049-1.976   | 0.971          |

VEGF, vascular endothelial growth factor; OK, odontogenic keratocyst; CI, confidence interval.

## Discussion

Vascular endothelial growth factor has been studied in inflammatory periapical lesions (27, 28). Leonardi et al. (27) found that VEGF was expressed in the epithelial component in seven radicular cysts, 17 periapical granulomas with epithelial proliferation, and Malassez's rests, while reaction of fibroblasts and inflammatory cells was heterogeneous. Graziani et al. (28) found heterogeneous, weak-to-moderate expression of VEGF in the lining epithelium in 24 radicular cysts, and strong in the connective tissue. Both VEGF expression and CD34 microvascular density (MVD) were higher in cysts showing more intense inflammation. Both studies emphasized the association of VEGF expression with inflammation and pointed to the up-regulation of cytokines that induced VEGF expression, such as IL-1 $\alpha$ , IL-6, TGF- $\beta$ , IGF-1, in periapical lesions as a possible mechanism (28). In our study, VEGF expres-

sion in the lining epithelium of OK, DC, and RC was not associated with the presence of inflammation. Furthermore, IL-1 $\alpha$  and IL-6 have been found to be produced by the epithelial cells of OK, regardless of inflammation (31, 32). Although it can be assumed that pre-existing inflammation may upregulate VEGF expression, particularly in RCs, our findings are suggestive of a role for VEGF regardless of inflammation.

Accumulation in the cystic fluid of OK, DC, and radicular cysts of serum proteins from the vasculature have been thought to elevate the hydrostatic pressure and maintain their expansion (33). Smith et al. (33) considered histamine release from mast cells as a major initiating event in this process, but VEGF is a 50 000 times more potent inducer of vascular permeability than histamine (7). On the other hand, mechanical forces have been shown to up-regulate VEGF and VEGF-inducing growth factors (TGF, PDGF, and FGF) in endothelial cells (34). Thus, it would be reasonable to suggest that there is a positive feedback mechanism, where VEGF induces an increase in cystic pressure and is induced by the pressure exerted on the lining epithelium from the cystic fluid.

Vascular endothelial growth factor expression was more intense in OK, which is currently considered as a cystic tumor rather than a developmental cyst (9). Cystic tumors of the brain (24, 26) and the thyroid (25) show increased concentration of VEGF in the cystic fluid that is produced by parenchymal cells. In cerebral hemangioblastomas and gliomas, VEGF is thought to increase the osmotic pressure of luminal fluid through a mechanism similar to that in inflammatory odontogenic cysts, and thus promote the expansion of the lesion (24, 26). In enlarging or recurrent cysts of thyroid adenomas and thyroid adenomatous goiters with cystic degeneration, a paracrine mechanism that stimulates fenestration of endothelial cells in blood vessels, increasing microvascular permeability and accumulation of cyst fluid, is suggested by the production of VEGF by thyrocytes (25). VEGF has also been found to be produced by cancer cells in malignant tumors, such as primary head and neck carcinomas (35-37). In the latter lesions, high expression by tumor cells is strongly correlated with high expression of VEGFR-2 and VEGFR-3 by the same cells, suggesting the presence of an autocrine loop that induces epithelial proliferation. An autocrine proliferative effect has also been shown in kidney cysts developing in acquired dominant polycystic kidney disease (38, 39). VEGFRs are expressed in the cyst-lining epithelium and normal and cystic biliary epithelial cells have increased rates of proliferation when treated with VEGF in a dose-dependent manner, similar to that seen on endothelial cells.

In our material VEGF was expressed by OK epithelium and a linear relationship between VEGF and Ki67 was found, although no statistically significant correlation was established. Further studies that would also evaluate VEGFR expression by epithelial cells in OK would be of interest.

Vascular endothelial growth factor is a sensitive measure of the angiogenic potential of a lesion (2) and

it is well known that tumors need a rich vascular supply in order for the cells to survive and grow, but also to reach the circulation and metastasize (40, 41). Tumor cells secrete angiogenesis-stimulating proteins, such as VEGF, thymidine phosphorylase, basic bFGF, hepatocyte binding growth factor, and others, whose specific receptors are found on the endothelial cells. In addition, tumor-associated macrophages, lymphocytes, and fibroblasts of the tumor stroma can also produce angiogenic factors, promoting tumor growth and spread (42). Other markers of angiogenesis, such as the MVD would be of interest to be evaluated in OK.

*In vitro*, VEGF has been shown to be involved in chemotaxis, formation and survival of osteoclasts; osteoclast-induced bone resorption; and induction of RANK expression in osteoclasts. As the RANK/RANKL/OPG system has been implicated in bone resorption in OK (43), the role of VEGF as an inducer of osteolysis would be interesting to be evaluated.

Ki67/Mib-1 showed intense expression in the paracanal layers of OKs and weak basal expression in DCs and RCs, as has been previously reported (17, 19, 23, 44). Nuclear and cytoplasmic expression of caspase-3 in all epithelial layers of OKs, RCs, and DCs has been described by Kimi et al. (21), although in another study with RCs and radicular cysts it was limited to the superficial layers (44). The statistically significant correlation between Ki67/Mib-1 and caspase-3 found concurs with the suggestion of Kichi et al. (23) that a balance between proliferation and apoptosis may maintain the thickness of the epithelium and prevent formation of tumor masses, in spite of the prominent proliferative activity. The linear correlation between VEGF and caspase-3 expression in RC may be explained by up-regulation of both VEGF from pre-existing inflammation and caspase-3 resulting from the removal of the inciting factor, i.e. the tooth.

We conclude that VEGF expression by epithelial cells in OK may promote growth via an autocrine proliferative effect on the cystic epithelium, while paracrine stimulation of the vascular network may maintain survival and growth, and promote enlargement and expansion through protein accumulation in the cystic cavity and bone resorption. The role of VEGF in the pathogenesis of OK and odontogenic cysts should be further evaluated, as angiogenesis could be a potent target for the therapeutic management of those lesions.

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