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Immunolocalization of VEGF-A and orosomucoid-1 in odontogenic myxoma

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Abstract

Objective: The aim of the present study was to determine and establish the immunohistochemical distribution of VEGF-A and ORM-1 protein in odontogenic myxomas to suggest a possible function in the biological behavior of odontogenic myxomas. *Materials and Methods*: A total of 33 odontogenic myxoma cases and three tooth germs were included. Immunohistochemistry was performed to localize VEGF-A and ORM-1 proteins in tumor cells, endothelial cells and extracellular matrix in the odontogenic myxomas. The intratumoral microvessel density (MVD) was determined with CD34 and Factor VIII antibodies. *Results*: Immunopositivity was strong in the endothelial cells, which compose various vessels, and in the randomly oriented stellate, spindle-shaped and round tumoral cells with long cytoplasmic processes. More than half of the extracellular matrix lacked expression of VEGF-A. ORM-1 expression was strong in both endothelial cells and tumor cells, and the myxoid extracellular matrix was positive, with moderate or strong immunoexpression in all cases. An important finding of this study was the statistically significant positive correlation between the expression of ORM-1 and VEGF-A in tumor cells (p=0.02). *Conclusions*: The results of this study suggest that the expression of VEGF-A and ORM-1 may be associated with two mechanisms (angiogenesis and tumor structural viscosity) that may influence tumor growth in odontogenic myxoma.

Keywords: odontogenic myxoma, VEGF-A, ORM-1, immunohistochemistry.

Introduction

Odontogenic myxomas are considered benign odontogenic tumors with locally aggressive behavior and non-metastasizing neoplasia of jawbones. Despite the benign nature of these lesions, odontogenic myxomas frequently display aggressive infiltration of the adjacent tissue as well as tendency of recurrence after surgical removal. There is a high rate of local recurrence after curettage alone, and in some cases, adequate resection is required [1, 2].

Tumor growth requires blood supply, which provides oxygen, nutrients and growth factors required for tumor cell growth and division. The blood supply also removes the tumor bed catabolites produced by these cells [3].

Angiogenesis is the process of growth and development of new capillary blood vessels from pre-existing vessels. When pathological, this process contributes to the development of numerous types of tumors and the formation of metastases. For growth, tumors require new blood vessels to form so that they can feed themselves. Angiogenesis involves complex cellular and molecular interactions between cancerous cells, endothelial cells and the components of the extracellular matrix; namely, the existence of specific proteins secreted by the tumoral cells capable of stimulating the proliferation of capillary endothelial cells. Among them, vascular endothelial growth factor (VEGF) has been found in several types of tumors [4].

VEGF is a sub-family of growth factors. Specifically, it is the platelet-derived growth factor family of cystine-knot growth factors. These are important signaling proteins that are implicated in both vasculogenesis and angiogenesis. The most important member is VEGF-A; this protein has been shown to stimulate endothelial cell mitogenesis and cell migration. VEGF-A is also a vasodilator that increases microvascular permeability and was originally referred to as vascular permeability factor [5, 6].

Intratumoral microvessel density (MVD) determined by staining endothelial antigens on histological sections may be used as a quantitative measure of angiogenesis. Small blood vessels as well as capillaries can be detected on immunohistochemistry with a range of specific antibodies. Many studies published to date have used Factor VIII-related antibody (von Willebrand factor), while others have used markers such as CD31 and CD34 [7]. The assessment of microvessel densities (MVD), detected by antibody to CD34, is frequently used to quantify angiogenesis in archival tissue, and it is correlated with poor outcome in several tumors.

Orosomucoid-1 (ORM-1) belongs to a group of acute-phase proteins found in plasma. Such proteins undergo dramatic changes in concentration as a response to a

disturbance of homeostasis. These plasmatic proteins constitute a group of serum factors related to different immunological regulator functions. They have also been associated with tumor development and growth. However, it is uncertain whether the serum levels of acute-phase proteins, such as ORM-1, increase as a response to tumor growth or because of neoplastic cell production [8].

Human hepatocytes are the primary site of ORM-1 production, but endothelial cells and some tumor cells can also produce this protein [9–11].

In 2012, our group used proteomics and western blotting to show the presence of ORM-1 protein in odontogenic myxomas and dental follicles [8].

It was recently reported that ORM-1 has pro-angiogenic properties and supports the angiogenic effect of VEGF-A. Thus, ORM-1 appears to be involved in the regulation of angiogenesis [12].

The aim of the present study was to establish the immunohistochemical distribution of ORM-1, VEGF-A, CD34 and Factor VIII-related antigen, and determine the possible correlation between the angiogenic activity through immunoexpression of VEGF-A and the presence of protein ORM-1 in odontogenic myxomas.

Materials and Methods

A total of 33 cases of odontogenic myxomas retrieved from the files of the Laboratory PERIBACT (Mexico), and School of Dentistry, Universidad Juárez del Estado de Durango (UJED) (Mexico) and three tooth germs (in bell stage) retrieved from the Department of Histology, Universidad de la República, Montevideo (Uruguay) were included in the study. The following histological criteria for the diagnosis of odontogenic myxoma were established: intraosseous neoplasm characterized by stellate and spindle-shaped cells embedded in an abundant myxoid or mucoid extracellular matrix, with a few collagen fibers [13]. The protocol was approved by the School of Dentistry UJED institutional Committee of Research and Ethics under the registration No. FO1201, written informed consent was obtained from each participant of your study. The specimens were harvested from non-decalcified portions of the tumors, fixed in 10% buffered formalin and embedded in paraffin.

For immunohistochemical analyses, 2 μm sections were treated with 0.1 mol/L sodium citrate (pH 6.2) and Tween 20 to expose the antigenic epitopes. Endogenous peroxidases were blocked with 0.9% hydrogen peroxide, followed by incubation with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 5 minutes to eliminate non-specific binding. Monoclonal antibodies against orosomucoid-1 (ORM-1, clone 2F9-1F10, Abcam, Cambridge, UK; 1:100 dilution), VEGF-A (VEGF-A, clone EP1176Y, GeneTex, USA; 1:50 dilution), CD34 (CD34, clone QBEnd-10, Dako, Denmark; 1:50 dilution) and polyclonal Factor VIII-related antigen (von Willebrand Factor, Dako, Denmark; 1:200 dilution) were used for immunohistochemical detection. The tissue sections were incubated with primary antibodies for 30 minutes and then incubated with a biotinylated anti-mouse/anti-rabbit antibody and a Streptavidin–Peroxidase complex for 15 minutes each (LSAB+ labeled Streptavidin–Biotin; Dako). For control studies of the antibody, serial sections were treated with 2% BSA–PBS in place of the primary antibodies and were absent of staining. Human liver tissue was used for positive control.

The reaction products were visualized using the substrate 3,3'-diaminobenzidine–H_2O_2 (Dako). Sections were counterstained with Mayer's Hematoxylin.

The percentages of tumor cells, endothelial cells and extracellular matrix that were positively stained for ORM-1 and VEGF-A were calculated using a 10× microscope objective, corresponding to an area of 5.3 mm^2. For each case of odontogenic myxoma five areas were randomly selected and photographed using a digital camera (Olympus XC50). The percentage of VEGF-A and ORM-1 positive tumor cells was determined in each area. The results are expressed as the percentage of total cells positive for VEGF-A and ORM-1 for each odontogenic myxoma studied. The immunoreactivity was semiquantitatively scored using a four-grade scoring scale as follows: 0 (no staining); 1 (+, low): 1–10% positive cells; 2 (++, intermediate or moderate): 11–50% positive cells; and 3 (+++, high): > 50% positive cells. The same scoring system was used for the extracellular matrix expression of ORM-1 and VEGF-A.

Microvessel density counting was performed with CD34 and Factor VIII-related antigen antibodies. Initially, the most vascularized tumor areas were selected under low power (×40 and ×100) using a light microscope to identify the areas containing the greatest number of stained vessels (so-called "hot spots"). MVD was assessed under ×200 magnification according to a method described by Weidner *et al.* with the use of "count point" option of the built-in software in five different vascular "hot spots" [14]. Each brown stained cell or cell cluster that was clearly separated from adjacent microvessels, tumor cells and other connective tissue elements were considered as a single countable microvesssel. Vessels characterized by thick muscular walls or with lumen greater then 20 μm in diameter were excluded from the count.

Comparison between groups was conducted using the *chi*-square test. Spearman's correlation test was used to evaluate the correlation between VEGF and ORM-1 immunopositivity. The results were considered significant when $p \leq 0.05$. The results were analyzed using SPSS 16.0 statistical software (SPSS Professional Statistics, SPSS Inc., Chicago, IL, USA).

Results

The age range for odontogenic myxoma cases was 10–74 years (mean: 30.4 years). There was a predominance of female patients (25/33, 75.7%). In our series, the mandible was affected in 63.3% (21/33) of the cases, most of these involved the posterior area (in the molar regions) and the maxilla was affected in 36.3% (12/33) predominantly in the premolar and molar regions. The mean size of the tumors was 3.3 cm. In 56.6% of the cases showed root displacement, and only 16.6% showed root resorption. Only one case showed recurrence.

The histology findings revealed stellate, spindle-shaped and some round tumor cells loosely arranged in an abundant loose myxoid stroma with a few collagen fibers, only four cases (4/33, 12%) showed some epithelial odontogenic cells throughout the myxoid ground substance.

The cases studied were positive for VEGF-A in both tumor cells and endothelial cells, with predominantly high positivity ($p<0.05$) (Figure 1). Tumor cells were highly positive in 30/33 (90.9%) of the cases, 2/33 (6.1%) had intermediate positivity, and 1/33 (3%) exhibited low positivity, the endothelial cells were highly positive in 32/33 (97%) of the cases and only 1/33 (3%) had low positivity (Figure 1). Evaluation of the positivity in the components deposited in the extracellular matrix revealed that more than half of the cases 17/33 (51.5%) were negative for VEGF-A, whereas 48.5% showed immunopositivity (12/33, 36.4% intermediate and 3/33, 9.1% low), and only one case (3% of the total) had high positivity (Table 1).

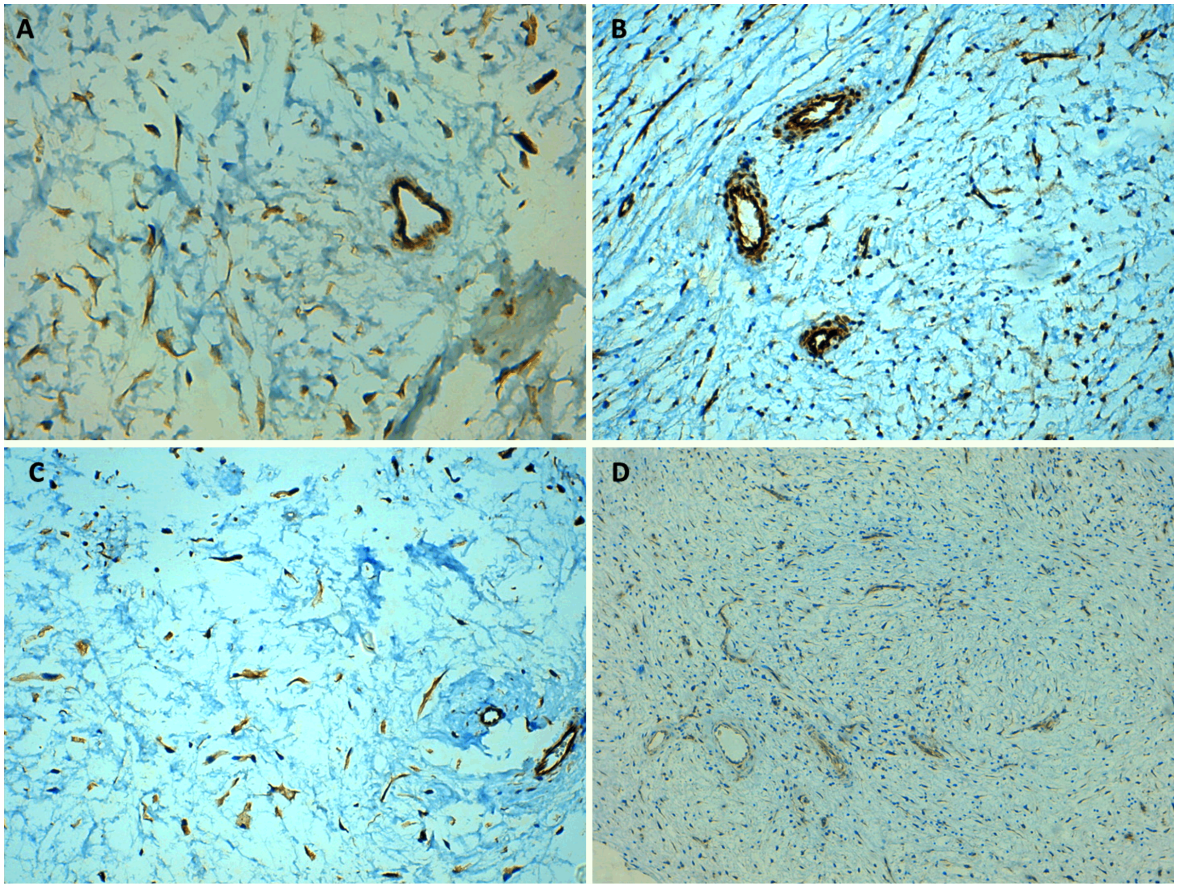

Figure 1 – ***Four different cases (A ×200; B ×200; C ×200; D ×100) with high expression of the VEGF-A protein, where >50% of the tumoral cells and endothelial cells had positive immunostaining.***

Table 1 – ***Differences observed in the expression of VEGF-A and ORM-1 in odontogenic myxoma. Similarity was observed in the expression of these two proteins in both tumor cells and endothelial cells. Differences were found in the extracellular matrix: slightly more than half of the tumors were negative for VEGF-A (p=0.048), while all of the tumors showed expression of ORM-1 in the extracellular matrix (p=0.021). Two tooth germs (dental papilla) showed low immunoexpression for VEGF-A and ORM-1, and one showed high expression for both markers. Some differences between the three tooth germs were found in the extracellular matrix***

Odontogenic myxomas	Tumoral cells		Endothelial cells		Extracellular matrix	
	VEGF-A n (%)	***ORM-1 n (%)***	***VEGF-A n (%)***	***ORM-1 n (%)***	***VEGF-A n (%)***	***ORM-1 n (%)***
Negative	0 (0)	0 (0)	0 (0)	2 (6.1)	**17 (51.5)**	0 (0)
+	1 (3)	0 (0)	0 (0)	0 (0)	3 (9.1)	0 (0)
++	2 (6.1)	0 (0)	1 (3)	2 (6.1)	12 (36.4)	12 (36.4)
+++	**30 (90.9)**	**33 (100)**	**32 (97)**	**29 (87.9)**	1 (3)	**21 (63.6)**
Tooth germs	**Mesenchymal cells**		**Endothelial cells**		**Extracellular matrix**	
Negative	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
+	2 (66.6)	2 (66.6)	2 (66.6)	2 (66.6)	1 (33.3)	1 (33.3)
++	0 (0)	0 (0)	0 (0)	0 (0)	1 (33.3)	0 (0)
+++	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	2 (66.6)

Numbers in bold indicate the highest scoring scale for each protein in the tumoral cells, endothelial cells and extracellular matrix. Odontogenic myxomas showed $p<0.05$ in: VEGF-A extracellular matrix *vs.* VEGF endothelial cells, VEGF-A extracellular matrix *vs.* VEGF-A tumoral cells, ORM-1 extracellular matrix *vs.* ORM-1 endothelial cells and ORM extracellular matrix *vs.* ORM-1 tumoral cells.

In contrast, almost all of the cases were positive for ORM-1 and were predominantly in the high category ($p<0.05$). Evaluation of tumor cells showed that 33/33 (100%) cases were classified as high positivity (Figure 2). With regard to endothelial cell positivity, were highly positive in 29/33 (87.9%) of the cases, 2/33 (6%) were

categorized as intermediate, and the remaining 2/33 (6%) were negative (Figure 2C). The extracellular matrix was highly positive in 21/33 (63.6%) cases (Figure 3), and intermediate positivity was observed in 12/33 (36.4%) (Table 1 and Figure 4).

The positive immunoexpression of VEGF-A and ORM-1 in tumor and endothelial cells was similar. Significant differences were observed in VEGF-A (p=0.048) and ORM-1 (p=0.021) immunoexpression between the extracellular matrix localization (Table 1).

Two tooth germs showed low immunoexpression for VEGF-A and ORM-1, and one showed high expression for both markers. Differences between the three tooth germs were found in the extracellular matrix (Table 1 and Figure 5).

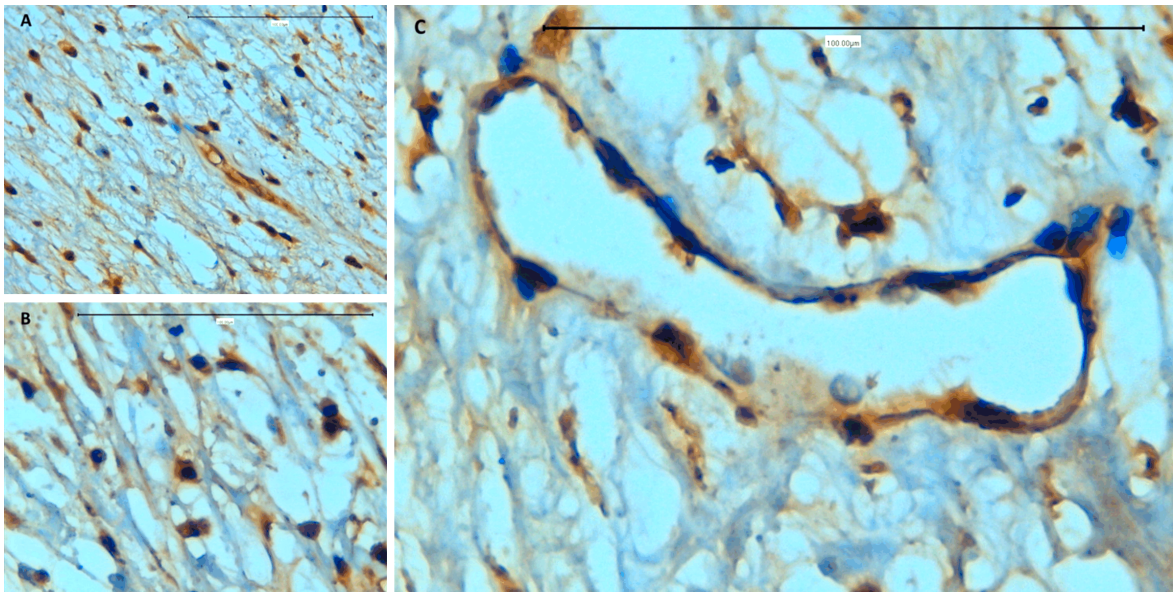

Figure 2 – ***(A) Microphotograph of spindle-shaped tumoral cells and some scattered collagen fibers showed immunopositivity for ORM-1, ×300. (B) The same aspects, at higher magnification, ×400. (C) Endothelial cells of a blood vessel showing positivity for ORM-1, ×500.***

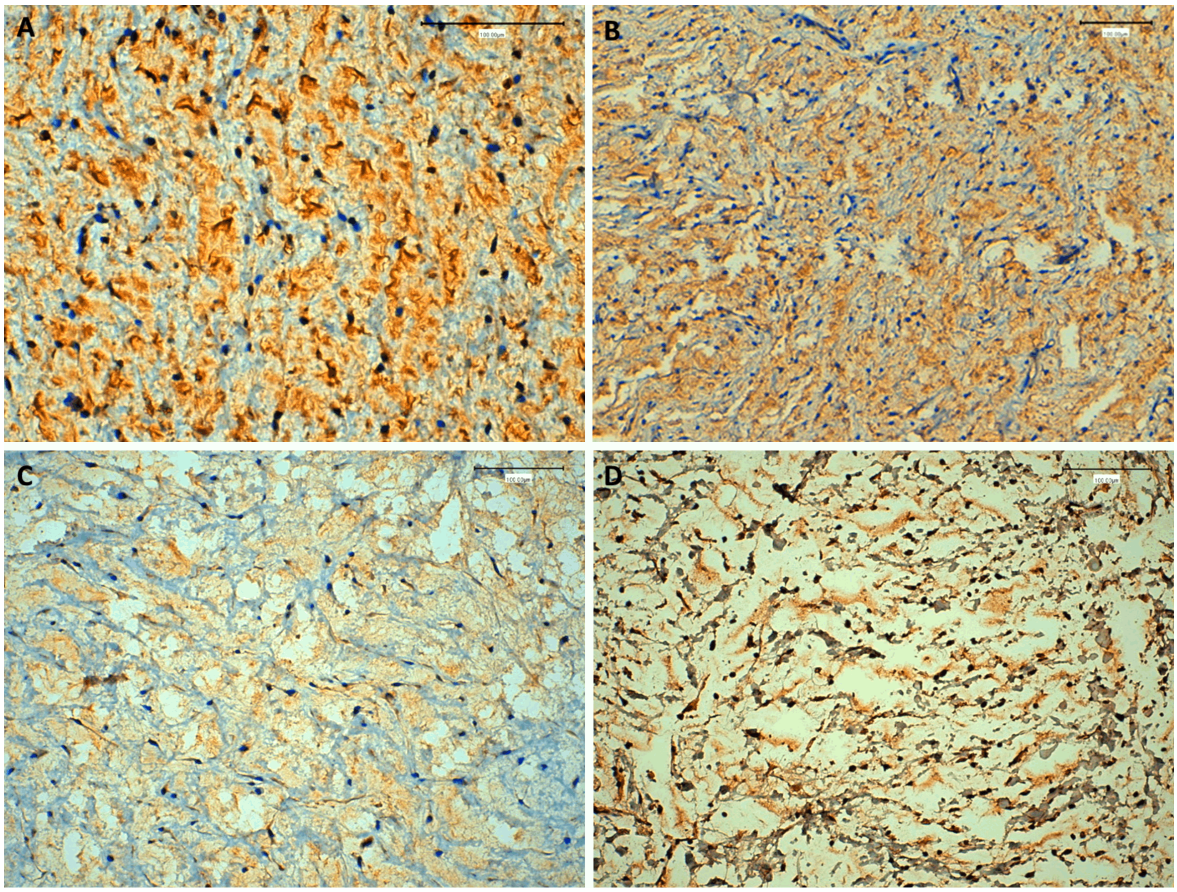

Figure 3 – ***The four different cases (A, ×200; B–D, ×100) with highest expression of the protein ORM-1, where >50% of the extracellular matrix showed positive immunostaining.***

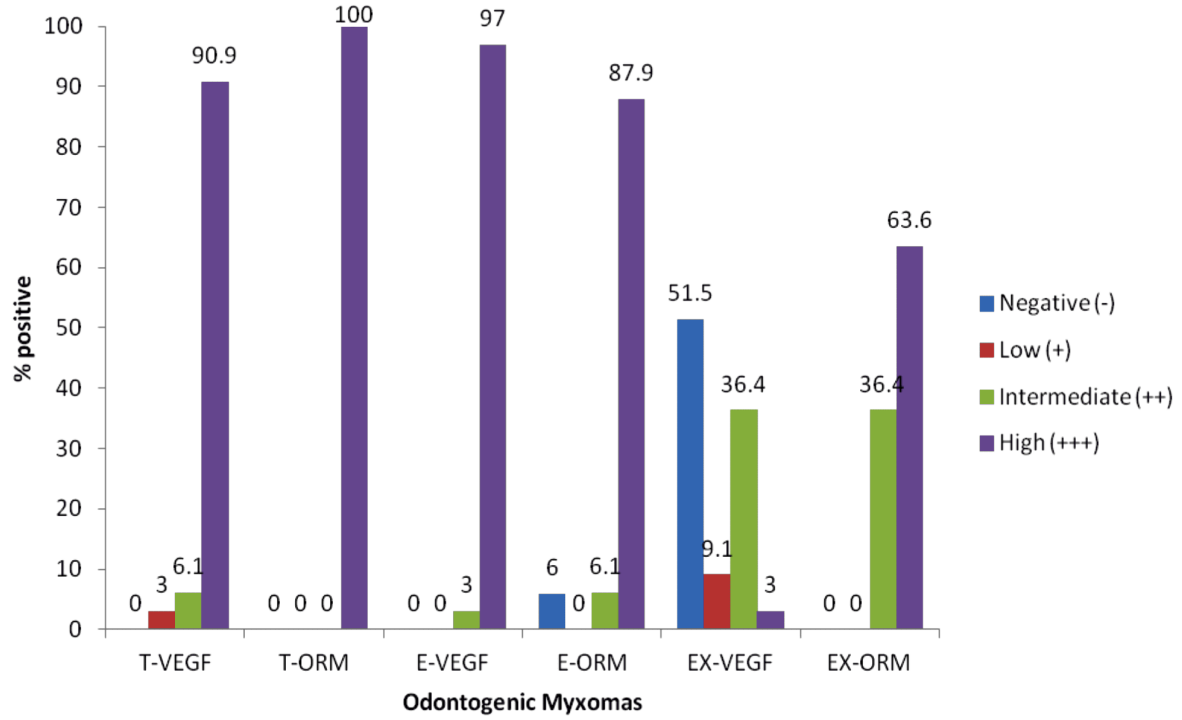

Figure 4 – ***Odontogenic myxomas. This chart shows how the expression of VEGF-A and ORM-1 was similar in tumor and endothelial cells, however in the extracellular matrix can be appreciated clear differences. T: Tumoral cells; E: Endothelial cells; EX: Extracellular matrix.***

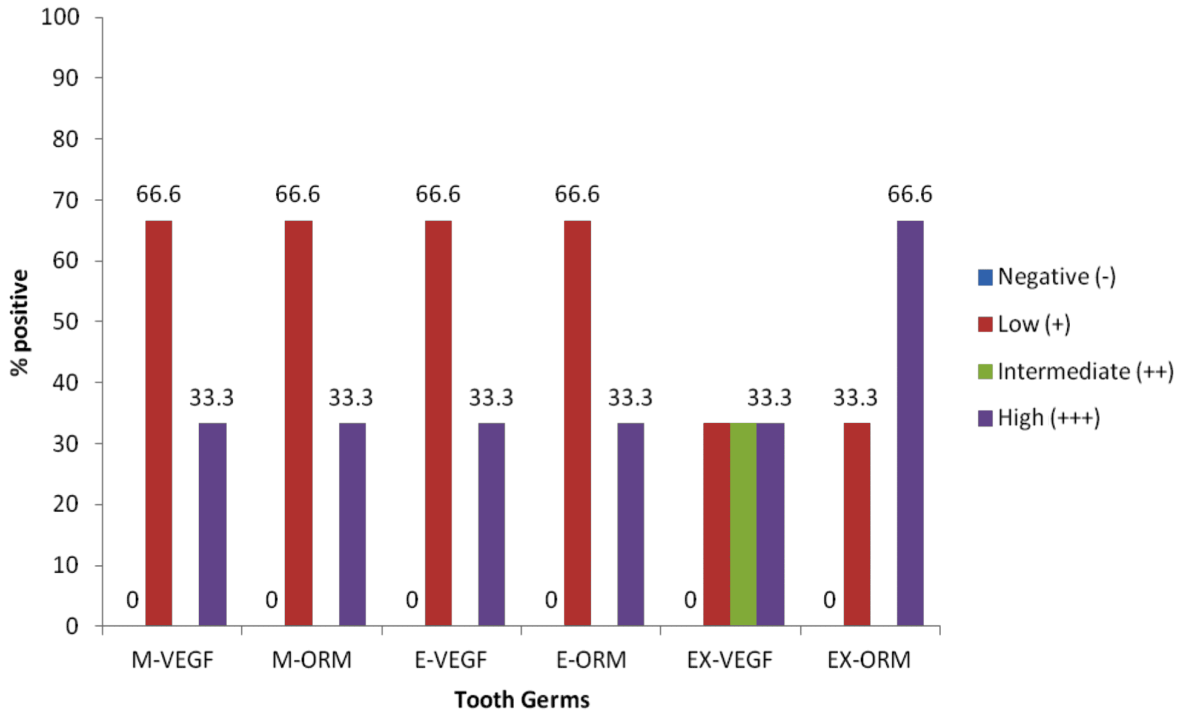

Figure 5 – ***The expression of VEGF-A and FORM-1 was similar in mesenchymal cells and endothelial cells of the three tooth germs, however in the extracellular matrix can be appreciated some differences. M: Mesenchymal cells; E: Endothelial cells; EX: Extracellular matrix.***

The most relevant result found was the statistically significant correlation between the expression of VEGF-A and ORM-1 in the odontogenic myxoma cases (p<0.002) (Table 2).

Median MVD's assessed with CD34 and Factor VIII were 7.5 (range: 3.4–21.4) and 5.2 (range: 2.1–12.3), respectively for odontogenic myxomas, and 7.3 (range: 3.8–9.4) and 6.2 (range: 2.8–8.7) respectively for tooth germs, these results were very similar, with no significant differences (Table 3). Some areas that had higher MVD showed tendency to high ORM-1 and VEGF-A but without statistically significance.

There were no statistically significant differences in ORM-1 and VEGF-A expression among morphoclinical factors like age, gender, location, size, and recurrence (Table 4).

Table 2 – ***Spearman's correlation test indicated significant correlation between VEGF-A and ORM-1 expression in the tumoral cells in the odontogenic myxoma***

	Tumoral cells		Endothelial cells		Extracellular matrix	
	VEGF-A	***ORM-1***	***VEGF-A***	***ORM-1***	***VEGF-A***	***ORM-1***
Spearman correlation	***r*=0.537**		*r*=-0.048		*r*=-0.050	
	***p*=0.003**		*p*= 0.798		*p*=0.789	

Numbers in bold indicate $p<0.05$.

Table 3 – ***Median MVD's assessed with CD34 and Factor VIII***

	Odontogenic myxoma	Tooth germ
	Mean (range)	***Mean (range)***
CD34	7.5 (2.1–12.3)	7.3 (3.8–9.4)
Factor VIII	5.2 (3.4–21.4)	6.2 (2.8–8.7)

Table 4 – ***Clinico-pathological features and expression of ORM-1 and VEGF-A in odontogenic myxoma. Association between proteins expression and clinico-pathological factors using chi-square test***

Factors	Total	VEGF-	VEGF+	VEGF++	VEGF+++	ORM-	ORM+	ORM++	ORM+++	*P*-value
Age [years]										
0–19	6									
T		0	0	0	6	0	0	0	6	
E		0	0	0	6	0	0	0	6	
Ex		2	0	4	0	0	0	3	3	
20–39	17									
T		0	0	2	15	0	0	0	17	
E		0	0	1	16	1	0	2	14	
Ex		14	0	3	0	0	0	5	12	
40–59	7									
T		0	1	0	6	0	0	0	7	0.524
E		0	0	0	7	1	0	0	6	
Ex		0	1	5	1	0	0	1	6	
30–39	0									
T		0	0	0	0	0	0	0	0	
E		0	0	0	0	0	0	0	0	
Ex		0	0	0	0	0	0	0	0	
60–79	3									
T		0	0	0	3	0	0	0	3	
E		0	0	0	3	0	0	0	3	
Ex		1	2	0	0	0	0	3	0	
Gender										
Male	8									
T		0	0	1	7	0	0	0	8	
E		0	0	0	8	1	0	0	7	
Ex		5	0	3	0	0	0	7	1	0.295
Female	25									
T		0	1	1	23	0	0	0	25	
E		0	0	0	25	3	0	0	22	
Ex		12	3	9	1	0	0	5	20	
Location										
Mandibula	21									
T		0	1	0	20	0	0	0	21	
E		0	0	0	21	0	0	0	21	
Ex		9	3	8	1	0	0	6	15	0.424
Maxilla	12									
T		0	0	2	10	0	0	0	12	
E		0	0	1	11	2	0	2	8	
Ex		8	0	4	0	0	0	6	6	
Size [cm]										
No data	12									0.380
0–3	9									
T		0	1	1	7	0	0	0	9	
E		0	0	0	9	0	0	0	9	
Ex		2	2	4	1	0	0	5	4	
3.1–6	9									
T		0	0	0	9	0	0	0	9	
E		0	0	1	8	0	0	0	9	
Ex		6	0	3	0	0	0	3	6	

Factors	Total	VEGF-	VEGF+	VEGF++	VEGF+++	ORM-	ORM+	ORM++	ORM+++	*P*-value
6.1–9	3									
T		0	0	1	2	0	0	0	3	
E		0	0	0	3	0	0	0	3	
Ex		1	0	2	0	0	0	2	1	
>9	0									
T		0	0	0	0	0	0	0	0	
E		0	0	0	0	0	0	0	0	
Ex		0	0	0	0	0	0	0	0	
Recurrence										
No	32									
T		0	0	2	30	0	0	0	32	
E		0	0	1	31	2	0	2	28	
Ex		17	3	12	0	0	0	11	21	0.116
Yes	1									
T		0	1	0	0	0	0	0	1	
E		0	0	0	1	0	0	0	1	
Ex		0	0	0	1	0	0	1	0	

T: Tumoral cells; E: Endothelial cells; Ex: Extracellular matrix.

Discussion

Similarly to tumors, odontogenic myxomas require a blood supply that delivers oxygen, nutrients and growth factors to support tumor growth.

The process of vessel formation is based on a complex interplay of various signal transduction pathways. Among those pathways, special interest has been devoted to VEGF, a growth factor that acts to initiate proliferation, vessel sprouting processes, and can promote the formation of new vessel-like structures [15].

Several studies have investigated the molecules involved in the biological behavior of odontogenic myxoma [16–21] but we did not find any that addressed mechanisms associated with angiogenesis and VEGF-A in this particular tumor.

The present study included 33 cases of odontogenic myxoma. It should be noted that we found some level of VEGF-A expression in the tumor cells, vessels and in the extracellular matrix. This immunopositivity was strong in the endothelial cells, which compose the various vessels, as well as in the randomly oriented stellate, spindle-shaped and round tumoral cells with long cytoplasmic processes. The VEGF-A expression indicates a higher ability to increase vascular permeability, possibly resulting in increased tumor growth [22].

More than half of the extracellular matrix was found to have no expression of VEGF-A; however, some cases (only 36.4%) exhibited moderate expression of VEGF-A in the abundant myxoid or mucoid matrix. This evidence suggests that in some cases VEGF-A is released into the surrounding stromal matrix and may contribute to tumor growth in a paracrine manner through angiogenesis processes.

Furthermore, tumor cells may produce VEGF-A not only for vessel sprouting but also for use as an autocrine growth factor. Several studies have demonstrated the existence of VEGF receptors in oral carcinoma cells, suggestive of an autocrine role of VEGF [23, 24].

Recently, our laboratory used proteomics and western blotting to show the presence of ORM-1 protein in odontogenic myxomas and dental follicles [8]. Although its role in reactive and pathologic processes is not fully understood, ORM-1 has been shown to have a number of regulatory functions, including modulation of the immune response and, more recently, angiogenesis [25].

We found that ORM-1 expression was strong in both endothelial cells and tumor cells and that the myxoid extracellular matrix had moderate and strong immuno-expression in some cases. An important finding of this study was the statistically significant positive correlation between the expression of ORM-1 and VEGF-A in the tumor cells (p=0.02).

It has been reported that ORM-1 alone enhances migration but not proliferation of human dermal micro-vascular endothelial cells. However, in the presence of ORM-1 and VEGF-A, endothelial cells are capable of inducing the development of endothelial tubes, suggesting an involvement of ORM-1 in the regulation of angio-genesis [12]. Irmak *et al.* [9] proposed that the highest increase of ORM-1 levels in advanced stages of urinary bladder cancer, which correspond to vascularized tumors, could be due in part to the production of this protein by the augmented number of endothelial cells of angiogenically active blood vessels.

Recently, Ligresti *et al.* [25] found that ORM-1 can potentiate the angiogenic effect of VEGF in endothelial cells in culture and that cultures treated with both ORM-1 and VEGF produced an even higher number of vessels than cultures treated with VEGF alone [25].

With our results we cannot assert that there is really a collaborative-angiogenic effect between VEGF-A and ORM-1 in odontogenic myxomas, we can only state that immune expression of both proteins was found in tumor tissue, but the presence of both proteins in tumor tissue leaves open the possibility to perform other experimental approaches and functional assays focused on elucidate how these proteins may cooperate directly in the growth of this tumor.

When the MDV was calculated with CD34 and Factor VIII related-antigen, the results suggest that myxomas are tumors with moderate to discrete vascularity when compared with more aggressive or malignant lesions. These results prove that addition of angiogenic mecha-

nisms, there are other important factors involved in tumor behavior of odontogenic myxomas.

Another factor that influences the biological behavior of odontogenic myxoma is the classical mucoid consistency. The results from a previous proteomic study by our group suggested that the overexpression of ORM-1 in odontogenic myxomas may be responsible for the classical mucoid appearance of this tumor. ORM-1 is a glycoprotein with very high carbohydrate content (45%), making it a highly viscous mucoprotein [26].

Odontogenic myxoma is a slow-growing tumor consisting of an accumulation of mucoid ground substance, and in some instances, this mucoid mass can be infiltrative and destructive [27]. In the present study, we assessed the presence of the ORM-1 protein using immunohistochemical-staining techniques. We found that all of the cases showed some expression of ORM-1 in the myxoid-mucoid extracellular matrix, with some cases showing strong expression throughout the entire extracellular matrix. This finding supports our theory that this protein is involved in the structural viscosity characteristics of this odontogenic tumor.

A major limitation in this study was a lack of ability to compare the expression in odontogenic myxomas to a normal counterpart (the human tooth germs) in various embryological stages of formation and another odontogenic tumor. Due to the difficulty in obtaining tooth germs, we were able to include only three tooth germs.

Conclusions

The results of this study suggest that the expression of VEGF-A and ORM-1 may be associated with two mechanisms (angiogenesis and tumor structural viscosity) that may influence tumor growth in odontogenic myxoma. Additional studies are needed for better clarification and understanding the angiogenic process and the mechanism by which VEGF-A and ORM-1 cooperate in the biological behavior of odontogenic myxomas. Further evaluation of the association of angiogenic markers and ORM-1 in odontogenic myxomas and comparison of such results with more extensive assessments of human tooth germs and other odontogenic tumors are needed.

Conflict of interests

The authors declare that they have no conflict of interests.

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