Immunohistochemical coexpression of VEGF and CD34 in ameloblastoma

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Introduction

Angiogenesis (neovascularization) is a multi step process, essential for growth and metastasis of most tumors. It is under the central regulation of a highly specific growth factor (The vascular endothelial growth factor VEGF) that control the proliferation and survival of the endothelial cells (1,2,3). Owing to the amount of VEGF that a tumor produce, a positive feedback loop is created, where in VEGF-induced promotion of angiogenesis allows for enhance tumor growth , which in turn result in increased VEGF secretion (4). And the quantitative estimation of vascular bed of human tumor by studying the mean vascular density (MvD) index (5,6,7,8) as well is important in predicting their relapse and metastasis (9).

Published literatures indicated that VEGF expression has been detected in majority of cancers including head and neck and oral SCC (10) and believed that this expression is an independent prognostic factor in patients with salivary gland tumors and oral SCC (11,12,13). However, its expression in benign lesions and normal tissue adjacent to a tumor has been found to be similar or higher than in tumor (10). Concerning odontogenic tumors, few studies are available. Nevertheless, Kumamoto et al (14) reported an association between VEGF expression and tumor angiogenesis in ameloblastoma.

Materials and methods

Fifty paraffin blocks (with their files) of previously diagnosed ameloblastoma (Am) were collected from Oral Pathol. Dept. Coll. of Dentistry, University of Baghdad and six tooth germs at bell stage were used as control. Routine H&E slides were prepared for histopathological typing according to WHO classification 1992. Cytological patterns of ameloblastic cells were classified into columnar, cuboidal, basal, stellate reticulum, granular and acanthomatous cell types.

IHC staining for VEGF (monoclonal antibody antihuman, Chemicon, Germany) and CD34 (monoclonal mouse anti CD34 antigen; ImmunoTech, France) expression were performed on 4 µm tissue sections mounted on silanised positive charged microscopic slides, following the manufacture instructions.

For each case 5 representative high power fields (40X) were studied to asses VEGF expression and density, each field should contain more than 200 cells (100 outer cells and 100 inner cells, i:e 1000 cell/ case) and another 100 cell of each cellular pattern whenever present were randomly selected. Data presented as percentage of positive cells and score of intensity (tumor cells stained to similar intensity of endothelial cells were categorized as grade 2 (Lime et al 2003).

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Also the number of CD34 positive vessels for each case was calculated in 5 representative high power fields (40X) (that show highest vascular density hotspots in tumoral stroma tissue). The average count of new blood vessels was recorded (follow the criteria described by Weidner et al (15) as MvD (16,17).

In addition, a quantitative description of the shape (as round, elongated and irregular) and size of these vessels was recorded. The size was assessed subjectively as small one when contain up to 3 RBCs (including endothelial cell cluster and single cell sprout not luminated) and medium vessel contains more than 4 RBCs within dilated lumen, whereas large size with muscular wall blood vessels were not included.

Results

Generally VEGF was significantly highly expressed with strong intensity in outer cell layer than inner cell layer of tumor islands and enamel organ, Table (1). The newly formed blood vessels were significantly predominantly rounded and small in size in comparison to dental follicle and papilla of the tooth germ.

Regarding sex variation, females showed significant higher expression of VEGF only in lining tumor cells of UAB (82.7± 14) with stronger intensity (62%) but less MvD around tumor islands (16.68 ± 6.7) than males . On the other hand, old age patients (>41 yrs) showed the lowest and weakest VEGF expression in inner layer tumor cells (9.2± 11.1 and 60% score1). And the young aged patients (<20 yrs) express significant highest MvD around tumor islands (35.97±47.7), and slightly lower around the lining tumor cells in UAB than other age groups, (Table 2). Anyhow, there was no statistical correlation between VEGF expression and MvD on one hand and the sex and age on the other hand.

Regarding the WHO classification, there were significant differences in the degree of VEGF expression at the outer cell layer of Tumor Island among all Am variants. Yet follicular, plexiform and lining cells in UAB had higher expression than acanthomatous and basal, beside that 67% of plexiform cases were of moderate intensity. While the inner cells in acanthomatous subtype (the squamous metaplastic cells) express moderate positively and strong intensity which was significantly higher than follicular, UAB and basal. And the lining tumor cells of UAB were significantly less than outer layer of mural islands, but much higher than its inner layer cells, Table 3. There was significant negative correlation between VEGF expression in outer and inner cell layers (r=-0.52, P=0.005) and positive correlation with lining tumor cells in UAB (r=0.72, P=0.000).

On the other hand, there was no significant differences in MvD in almost all histological solid subtypes of Am. The only exception was the significant lower microvessel count reported around the lining tumor cells in UAB (13.07) in comparison to both follicular (20.6) and plexiform islands (40.1) and invasion islands of mural growth (19.25). However, still there are some differences in shape and size of these microvessels among histopathological subtypes. All solid variants of Am. Except plexiform showed mostly round and small vessels, unlike plexiform which showed elongated and medium sized vessels. While in UAB there were more elongated medium sized vessels around mural and lining tumor cells than follicular type but less than plexiform, Table 3.

Considering different morphological cellular pattern, basal cells show the highest VEGF positivity and intensity (87.52±13.4), followed by columnar cells (78.9±16.3). While cuboidal cells had relatively high VEGF expression and 68% of them were of moderate intensity (significant differed than columnar). On the other hand, the squamous metaplastic cells in acanthomatous subtype and granular cells in granular subtype showed moderate VEGF positively. However, the former had 75% of the cells with strong intensity in comparison to the later expression 78% moderate intensity and both of them were significantly lower than basal and columnar (granular cells were significant less than cuboidal and columnar and basal). VEGF expression was low markedly and predominantly weak in stellate reticulum cells (81% score 1) that differ significantly from all other cell pattern, Table 4.

Data analysis revealed that the induction for angiogenesis by VEGF and the shape of microvessels is significantly correlated, since the increase expression of this growth factor by tumor cells in outer layer cells in different Am variants and the lining cells in UAB is correlated with the increase of the number of round new blood vessels around Am. islands and around the lining tumor cells in UAB (r= 0.32, P=0.03) and (r=0.35, P=0.037) respectively.

The secretion of this growth factor by basal tumor cells in basal cell Am is well correlated with the number of new blood vessels formation (r= 0.52, P=0.05). On the other hand, the size and shape of newly formed microvessels in plexiform Am are greatly correlated with the type of the peripheral or outer layer cells. The increase in VEGF expression by the cuboidal peripheral cells in this subtype are correlated with the increase in the number of moderate size and elongated shaped blood vessels (r=0.7, P=0.02) and (r=0.9, P=0.01).

Discussion

For the past few years angiogenesis has been the field under extensive investigation. In the present study, VEGF was detected in Am in comparison to tooth germ in presecretory ameloblast cells and stellate reticulum cells, prior to the stage of enamel and dentin formation. The expression was highly positive with moderate intensity of staining in ameloblast, whereas in stelate reticulum was weak in positivity, suggesting that, angiogenesis, during tooth development might be regulated by odontogenic epithelial cells. Weak VEGF expression in the microvessels near the odontogenic epithelial cells suggesting that, this angiogenic factor induced by odontogenic tissue acts on endothelial cells via paracrine mechanism.

On the other hand, the mean value of microvessel density distribution in both dental follicle and papillae , confirm Scott and Symons evidence (18), that only during pre-secretary phase ameloblast draw their nutrition from the blood vessels of different sizes and shapes of the dental papilla.

The results of the present study suggested that, high VEGF expression increasing the possibility of Am tumor cells to invade the surrounding normal tissue, as it dose with the vast majority of human tumors (1,8,11,13,19,20,21). In fact it was not an unexpected finding.

VEGF expression was detected in all variant of ameloblastoma variants, significantly higher than the normal tissue of the tooth germ suggesting that VEGF production by odonogenic epithelial cells was up regulated in

association with neoplastic changes. Similar findings were reported by Kumamoto et al (14).

VEGF was mainly located in the outer layer tumor cells in all Am histological subtypes, and was suggested to act on endothelial cells via paracrine mechanism, since endothelial cells themselves express much less VEGF than odontogenic epithelial cells. The reasonable explanation for this phenomena can be based on the theory that indicated any solid growing mass that enlarged more than $2mm^3$may undergo necrosis if not get good vascularization. In this condition angiogenic mechanism is mandatory for elaboration of the required vascular supply. This process is thought to involve the recruitment of the neighboring host mature vasculature to begin sprouting new blood vessel capillaries that grow and subsequently infiltrate the tumor mass (2,22). This accrued a convincing evidence reported in our study concerning the association of VEGF over expression with the MvD. Similar strong correlation was shown in other studies in oral SCC (23,24).

The high levels of VEGF expression in different outer cellular patterns in Am tumor cells, could be explained as these tumor cells might produce VEGF not only for vessel sprouting, but, also for the use as an autocrine growth factor, since previous studies reveal the existence of VEGF receptors in cancer cells in head and neck SCC (25,26).

Characterizing the tumor microvasculature on the basis sample provides important prognostic information in many malignancies as oral SCC (17,27), such studies demonstrate a significant correlation between MvD and tumor aggressiveness. Furthermore, Eberhard et al (28) considered that CD34 monoclonal antibody expression as the most reliable method for quantifying tumor vasculature. Accordingly, what we gain from our measurement of microvessels's count size and shape in different histological types was correlated with the inductive signals for angiogenrsis represents by VEGF positivity and intensity of staining expressed by certain cells in the outer layer cells of tumor islands and according to the architecture of the tumor tissue.

To further elucidate this observation, although , non significant difference in VEGF expression or MvD was found between follicular and plexiform Am subtypes, microvessels were numerous, rounded and small in follicular Am lined by columnar cells. Whereas, elongated, dilated, medium sized in plexiform Am lined by cuboidal cells which exhibited a moderate intensity of staining. Basal cells Am showed diffused expression of VEGF in most tumor cells, despite the MvD in this type was lower than that in other subtypes. This seems to fit the concept that, these basal cells are stem cells favoring the infiltrative and aggressive behavior of this Am subtype.

In the above mentioned subtypes of Am, VEGF expression in both positivity and intensity was markedly decreased in the inner layer cells, and moderately in squamous metaplastic cells acanthoumatous subtype and granular cells in granular cell Am. This reduction of VEGF expression is related to the terminal differentiation and regressive changes of these tumor cells. Conversely, the outer cells of tumor islands expressed VEGF strongly. This valuable observation could explain the aggressive behavior of odontogenic epithelium of Am and coordinates with the finding of Alon et al (29), they consider VEGF as a survival factor.

Further important issue must be addressed here in making such a claim, that mural invasive islands of unicystic Am showed over expression of VEGF in the outer cells which may act in a paracrine and autocrine manner for angiogenic stimulation and it is one of the factors responsible for its aggressive and infiltrative behavior.

Conclusion

VEGF was highly expressed by Am outer cells of the tumor islands and low in the inner tumor cells and the highest induction for angiogenic activity by ameloblastic tumor tissue was observed in mural islands of unicystis Am. The squamous metaplastic cells of acanthomatous subtype, significantly differed in VEGF expression from the inner cells of other Am subtypes, which render this subtype more aggressive in angiogenic,

The neovascularization in connective tissue stroma surrounding the tumor islands, detected by CD34 monoclonal antibody, described mainly as round, small microvessels surrounding follicular tumor islands and lining epithelial calls and mural tumor islands of unicystic Am, whereas elongated medium sized microvessels were observed in plexiform subtype.

Neither the VEGF nor MvD were correlated with sex or age. However, MvD was correlated to the VEGF expression which indicates a direct effect of this growth factor on endothelial cells in a paracrine manner. And The aggressive, infiltrative potential of mural invasion islands of unicystic Am is reflected by the increase MvD in the surrounding stroma, which is significantly higher than MvD surrounding the lining tumor cells.

The present study indicates that the aggressive locally invasive biological behavior of this benign odontogenic tumor is mainly attributed to angiogenic activity expressed by VEGF. The increase in the inductive effect of VEGF to increase MvD around the tumor islands aids to provide more nutritional supply to the tumor growth, which in turn avoid death and enhance the proliferation of the tumor cells.

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Table (1) : VEGF expression and intensity with micro vessel density in tooth germ

VEGF	Pre ameloblast cells		Stellate reticulum cells	
	%	score(2)	%	score1
	64.33±3.82	100%	20±4.46	100

CD34 microvessels	count	Shape		Size	
		Round	elongated	Small	medium
Dental follicle	6.64±1.14	6.65±1.12	0	0	6.65±1.14
Dental papilla	9.07±0.83	7.06±2.49	3.05±1.11	6.24±0.99	2.87±1.16

Table (2) : VEGF expression (positivity & intensity score) and CD34 positive microvessels count, shape and size in relation to sex and age

Am No.		Total 50	Male 32	Female 18	≤ 20 13	21-40 25	≥ 41 12
Outer +ve	VEGF	81.7±15.6	83.9±13	79.5±18	84.1±14.5	77.9	85.7±12.7
Score %	1	0	0	0	0	0	0
	2	26	10	15	0	27	0
	3	74	90	85	100	73	100
Inner +ve	VEGF	31.9±15	14.3±12	29.3±15	27.5±12	21.8±15.8	9.2±11
Score %	0	0	23	14	0	18	40
	1	52	70	29	50	55	60
	2	35	0	43	0	27	0
	3	13	7	14	50	0	0
lining +ve	VEGF	74.9±14.8	69.1±14	82.7±14	74.1±15.8	72±14	79.7±12.5
Score%	1	0	0	0	0	0	0
	2	52	60	38	43	58	42
	3	48	40	62	56	42	58
CD34 +ve arroud island	count		25.4±31	16.86±6.7	35.9±47	18±9.5	16.5±4
	Round		19.5±9	16.4±5	24.8±8	16.5±8	16.2±4
	Long		12±12	1.4±1	15.8±15	9.8±11	1±0.4
	Small		15.9±9	14.3±4.7	18.7±11	14.7±6.9	12.6±4.7
	Medium		11.6±14	6.4±4.7	15.4±20	7.5±8	8.2±4.6
CD34 +ve around lining	count		13±4	13.1±4	11.6±2.6	13±3.8	13.6±4.6
	Round		12.7±4.5	12.7±4.2	11.6±2.6	11.8±4.6	12.9±4.5
	Long		9.6	1.7±1	0	5±6	3
	Small		11.6±4.6	11.8±5	11.6±2.6	10.8±3.7	11.6±7
	Medium		4.9±1	3.6±1.8	0	3.6±1.6	5.5±0.4

Table (3) :VEGF expression (positivity & intensity score) and CD34 positive microvessels count, shape and size in different histologic types of ameloblastoma

Am No.		Total 50	Folicular 11	Plexiform 6	Acanth 3	Basal 2	Desmo 1	Granu. 1	Unicystic amelobastoma 26	
Outer+ve	VEGF	81.7±15	89.4±18.1	87.5±16.4	76.3±3	75±21.2	85	65	93.8±12	
Score %	1	0	0	0	0	0	0	0	0	
	2	26.2	18	67	0	0	0	100	22	
	3	73.8	82	33	100	100	100	0	78	
Inner +ve	VEGF	31.9±15	39±15	35±14.1	55±13	20	25	23	26.2±13	
Score%	1	52.2	75	0	0	100	100	100	100	
	2	34.8	25	100	0	0	0	0	0	
	3	13	0	0	100	0	0	0	0	
lining +ve	VEGF	74.9±14							74.9±14	
Score%	1	0							0	
	2	52							52	
	3	48								
		Total	Folicular	Plexiform	Acanth	Basal	Desmo	Granu	mural	lining
CD34 +ve vessels	Count	22.4±25	20.6±10.5	40.1±67.4	16.7±1	16.4±12.4	38.8	8.4	19.2±5.9	13±4
	Round	18.4±8.	19.4±9.7	12.2±16.4	15.9±2	14.8±10.1	38.8	8.4	18.8±4.7	12.4±4.3
	Long	10.3±1	1.6±0.5	15.5±14.9	2.4	0	0	0	8.4±6.8	4.3±4
	Small	15.3±8.2	15.8±10.1	3±1.9	16±2.6	15.2±10.7	25.8	8.4	17.6±5.7	11.7±4.7
	Medium	9.7±11	7.22±2.4	17.5±22	2	2.4	13	0	7.4±5.2	4.1±1.6

Table (4) :VEGF expression (positivity and intensity) in various cellular patterns

Celluar type	No.	Positivity	Score intensity %		
			1	2	3
Columnar	36	78.9±16.3		19	81
Stellate reticulum	16	21.6±18	81	19	
Cuboidal	19	68.7±16		68	32
Squamous	6	57.7±23.5	22	25	75
Granular	9	40.7±15.3		78	
Basal	19	87.5±13.4		16	84

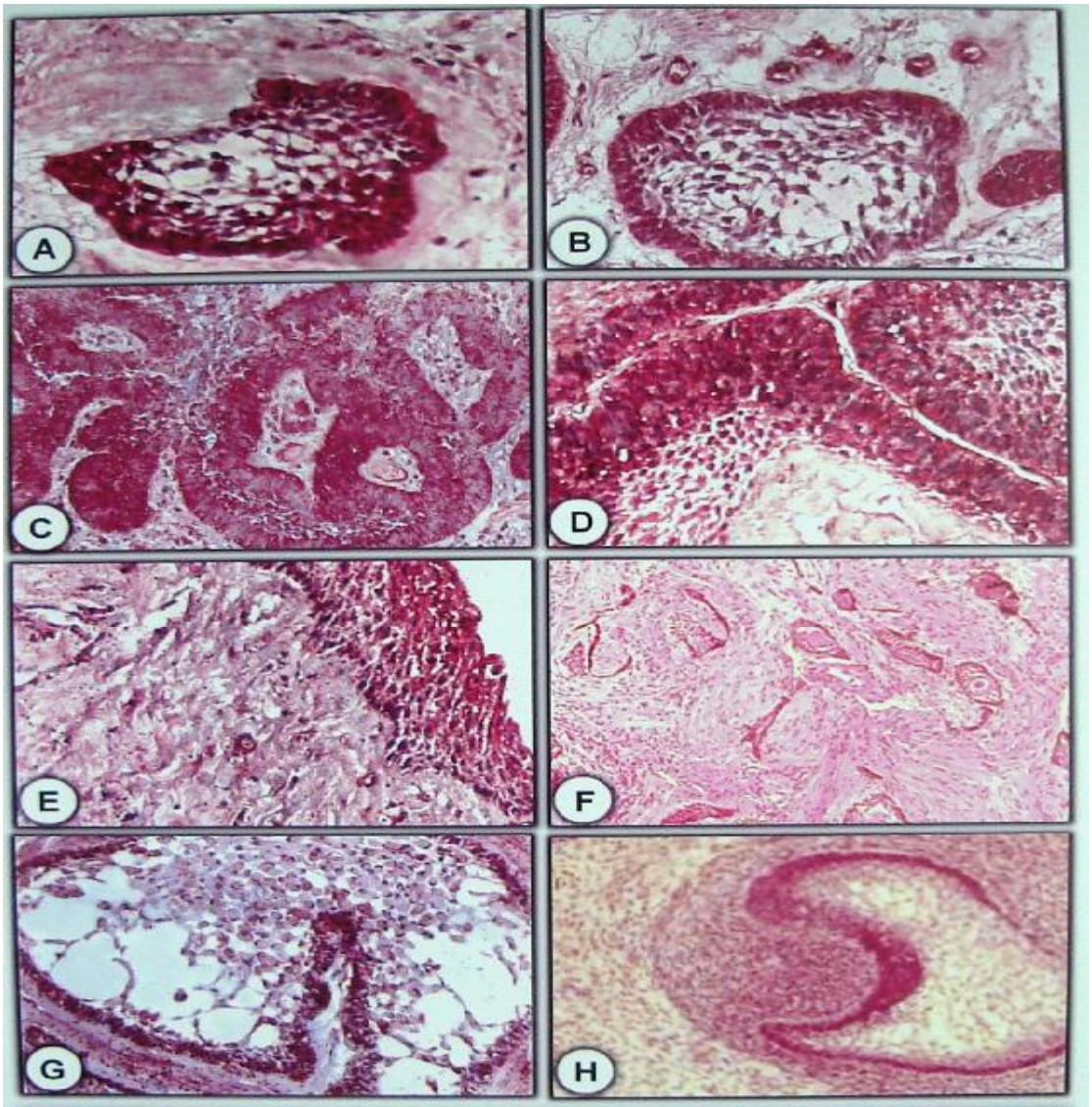

Figure (3-7): Immunohistochemical detection of anti VEGF antibody in different ameloblastoma subtypes (X200) (A) Mural islands in UAB (B) Follicular islands with surrounding positive small rounded microvessels (C) Plexiform (D) Acanthomatous subtype (E) Lining of UAB with surrounding small microvessels (F) Desmoplastic (G) Granular subtype (H) Tooth germ(X100).