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Original article

Tumor-associated macrophages and angiogenesis: A statistical correlation that could reflect a critical relationship in ameloblastoma

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ABSTRACT

Neoplasm growth is determined not only by the tumor cells themselves, but also by the tumor microenvironment. Increased densities of macrophages and activation of angiogenesis have been identified as common events in the progression of several neoplasms. Ameloblastoma is one of the most frequent odontogenic tumors and an excellent model for the study of neoplasm progression due to the different clinical variants that it exhibits. Here, by immunohistochemical studies using antibodies against CD68 and CD34, we evaluated the density of macrophages and microvessels associated to 45 paraffin-embedded ameloblastomas. In solid/multicystic ameloblastoma (SMA), we observed significantly higher densities of both macrophages and microvessels than in unicystic (UA) and desmoplastic (DA) ameloblastomas. Likewise, higher densities of macrophages and microvessels were found in UA than in DA. Furthermore, a predominance of intratumoral and peritumoral macrophage infiltrates was seen in SMA, while in UA, both macrophages and microvessels were also detected in the wall of the cysts. In contrast, DA had scant macrophages and microvessels, mainly situated distant from tumoral cells. In addition, a high correlation between macrophage and microvessel densities was observed in the samples ($r = 0.9623$). Our results suggest that these two tumor microenvironmental elements could have an important role during ameloblastoma progression.

Introduction

The growth of human neoplasms appears to be determined by both tumor cells and components of the tumor microenvironment, including immune cells and microvessels present in the tumor area [1,27]. It has been proposed that both angiogenesis and inflammation are involved in the evolution of some tumors [19,27]. In some epithelial neoplasms, an evident relationship between inflammatory alterations and progression of malignancies has been demonstrated [10,22]. In colorectal cancer, it has been suggested that there is a functional association between the characteristics of the macrophage population and the metastatic process [12]. Moreover, tumor angiogenesis, triggered by stromal macrophages, appears to regulate the progression of endometrioid carcinoma [9]. Angiogenesis could also be involved in the singular conduct of several odontogenic lesions, including solid and unicystic ameloblastoma [1].

Tumor-associated macrophages promote tumor growth and angiogenesis through the paracrine effect, mediated by the signaling pathway of endothelial nitric oxide synthase, and the autocrine effect, which stimulates its own polarization toward a pro-angiogenic/immune-suppressive M2 phenotype [5]. Indeed, polarization of tumor-associated macrophages in an M2 phenotype and abnormal hypoperfused vessels have been identified as hallmarks of malignancy [25]. Consequently, the induction of macrophage polarization to a tumor-inhibiting M1 phenotype promotes antitumor immune responses and vessel normalization, decreasing tumor growth and metastasis [25]. Moreover, macrophages derived from tumor tissues express high levels of vascular endothelial growth factors (VEGFs), which regulates malignant progression through stimulation of leukocytic infiltration, tumor cell invasion and tumor angiogenesis [7,18,28].

Ameloblastoma, one of the most frequent epithelial odontogenic tumors, is still an important cause of aggressive surgeries as

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a consequence of its local invasiveness, which is apparently associated with several clinical/pathological factors [11,20]. This benign neoplasm can be used as an excellent model to analyze tumor aggressiveness and invasiveness, as well as tumor progression. There are three recognized clinical intraosseous ameloblastoma variants: unicystic (UA), solid/multicystic (SMA) and desmoplastic (DA) [2,3,11,24]. They are able to cause different degrees of esthetic and functional alterations, each with different outcomes of treatment. SMA has an aggressive behavior that contrasts with the more indolent behavior of UA and the still indefinite clinical evolution of DA [2,3,11,24]. Although ameloblastoma has been extensively studied, important questions remain regarding the reasons for the diverse clinical courses of its variants. Stromal and tumor areas exhibit variable degrees of tumor-infiltrating inflammatory cells in ameloblastoma [21]. Angiogenesis could also be involved in the particular conduct of SMA, as shown by a recent study reporting that this tumor exhibited a greater microvessel density than other odontogenic lesions analyzed [1].

Here we studied the density and distribution of macrophages and microvessels in different variants of ameloblastoma to test the hypothesis that alterations in the prevalence of ameloblastoma-associated macrophages correlate with changes in the vasculature existing in the tumor area, and may possible contribute to the progression of this odontogenic neoplasm.

Materials and methods

Sample selection

Samples of 29 SMA (64.45%), 11 UA (24.44%) and 5 DA (11.11%) previously fixed in 10% neutral formalin and paraffin-embedded were collected from two surgery services in Mexico City (Hospital de Especialidades, Centro Médico Nacional Siglo XXI; and Centro Médico Nacional 20 de Noviembre) and one in Cuba (Hospital Calixto García). The protocol was approved by the institutional committee of research and ethics under the registration number 1201. To analyze the morphologic aspects of each neoplasm and to evaluate both macrophages and microvessels associated to tumors, samples were sectioned, mounted on microscope slides, stained with hematoxylin and eosin (H&E), covered with synthetic resin and examined by optical microscopy (Nikon H550L, Yokohama, Japan).

Immunohistochemical assays

Tissue sections with a thickness of 2 μm were submitted to immunohistochemical studies as described [3]. Samples were treated with 0.1 M sodium citrate (pH 6.2) and Tween-20 for the unraveling of the epitopes. Endogenous peroxidases were blocked with 0.9% hydrogen peroxide, followed by incubation with 1% BSA in PBS. Then, samples were incubated for 45 min at 37 °C with a dilution of 1:50 of monoclonal antibodies: (i) against CD68 (Dako Corporation, Carpinteria, CA, USA) to detect macrophages, or (ii) against CD34 (Dako Corporation, Carpinteria, CA, USA) to detect endothelial cells of vessels. Afterwards, samples were incubated for 30 min at 37 °C with a biotinylated anti-mouse antibody, and finally for 30 min at 37 °C with the streptavidin/peroxidase complex (LSAB + Labeled streptavidin-Biotin, Dako Corporation, Carpinteria, CA, USA). The reaction products were visualized by incubation with 3,3′-deaminobenzidine–H_2O_2 substrate (Dako Corporation, Carpinteria, CA, USA). Sections were counterstained with Mayer's hematoxylin solution and visualized by optical microscopy (Nikon H550L, Yokohama, Japan). As the positive control we used an oral mucosa sample, and as the negative control we omitted incubation with the primary antibody.

Sample evaluation

We analyzed the slices treated with anti-CD68 or with anti-CD34 to detect the five fields at low power (10×) closest to the tumor cell that had the highest macrophage density (MD) or microvessel density (MVD), respectively. MD and MVD measurements were always performed with a magnification of 40×. The mean of the five fields was taken as MD or MVD for each case, while the mean of MD and MVD of all cases belonging to the same clinical category was taken as MD and MVD for that group. To corroborate visual counts, images from immunohistochemically treated sections were captured on a video camera (Nikon DS-Si1, Yokohama, Japan) coupled to the optical microscope (Nikon H550L, Yokohama, Japan). These images were processed and analyzed using the NIS-Elements software installed in a microcomputer. Any reactive endothelial cell or endothelial cell cluster clearly distinguishable from other adjacent tissue elements was counted as one microvessel, independently of the presence of a vessel lumen.

Statistical analysis

The statistical analysis of the results obtained was performed by taking a significance level of 5% ($p < 0.05$). The data from each clinical group were expressed as the mean value ± the respective standard deviation. We used the SigmaStat for Windows statistical software (version 3.5). The unpaired Student's *t*-test was performed to obtain statistical significance of variance between groups.

Results

Counts of macrophages and microvessels

To detect a potential relationship between the number and distribution of macrophages and microvessels in ameloblastoma, we evaluated sections from different cases of ameloblastoma stained with H&E and immunohistochemically treated with anti-CD68 and anti-CD34 antibodies. In the initial analysis, by staining samples with H&E, the richest inflammatory infiltrate and the highest MVD were detected in SMA, whereas the smallest inflammatory infiltrates and MVD were found in DA, generally next to tumor areas (Fig. 1A–C). These observations were confirmed by visual and computational counts. Data showed a higher MD and MVD in SMA than in UA and DA (Tables 1 and 2). Remarkably, we detected a high correlation coefficient between MD and MVD for all samples (Table 3). When we considered SMA, UA and DA independently, we obtained different, but also high positive correlation coefficients between MD and MVD for each group evaluated (Table 3).

Distribution of macrophages and microvessels

In all SMA samples, we detected abundant macrophages located peri- and intratumorally (Fig. 2A). In UA, macrophages were generally found in the wall of the cyst, although a prominent number of them were also detected close to tumor cells (Fig. 2B). Although macrophage scantiness was evident next to tumor areas of the DA (Fig. 2C), scattered macrophage groups were found relatively distant from tumor tissue sections, but belonging to the same surgical specimen. Thus, the mean value of macrophages in five fields with close proximity to tumor cells was taken as the MD for this variant of ameloblastoma (Table 1). Location of microvasculature associated with ameloblastoma coincided with the presence of macrophages. In this sense, microvessels were mainly observed

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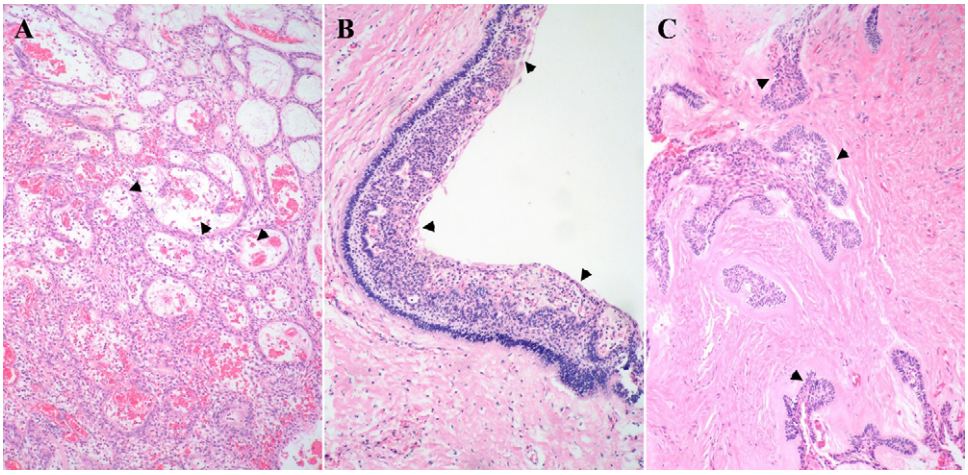

Fig. 1. Microphotographs of tissue sections showing H&E staining of different clinical variants of ameloblastoma. (A) Panoramic view of a solid ameloblastoma. Inflammatory cells appear near to a tumor area (arrowheads) (original magnification 10×). (B) Panoramic view of a unicystic ameloblastoma. Arrowheads show the cystic wall and the epithelium lining with tumor cells (original magnification 10×). (C) Panoramic view of desmoplastic ameloblastoma. Tumor cells are scattered in a highly hyalinized stroma (arrowheads) (original magnification 10×).

Table 1
Evaluation of macrophage and microvessel densities in clinical variants of amelobastoma.

Clinical tumor variant	Macrophage parameters		Microvasculature parameters	
	Range	MD ± SD[a]	Range	MVD ± SD[b]
SMA[c]	14.6–39.2	26.13 ± 7.23	12.8–34.2	24.04 ± 5.59
UA[d]	15.2–28.0	19.67 ± 4.10	14.2–24.4	18.31 ± 3.07
DA[e]	2.6–14.6	7.84 ± 5.03	0.2–9.6	3.64 ± 4.66

[a] Macrophage density ± standard deviation.
[b] Microvessel density ± standard deviation.
[c] Solid/multicystic ameloblastoma.
[d] Unicystic ameloblastoma.
[e] Desmoplastic ameloblastoma.

Table 2
Significance of the differences in macrophage and microvessel densities in clinical variants of ameloblastoma.

Clinical variants compared	*P*-value					
	MD[a]			MVD[b]		
	SMA[c]	UA[d]	DA[e]	SMA	UA	DA
SMA	–			–		
UA	0.00024	–		0.00098	–	
DA	0.00011	0.00085	–	0.00019	0.00294	–

[a] Macrophage density.
[b] Micro vessel density.
[c] Solid/multicystic ameloblastoma.
[d] Unicystic ameloblastoma.
[e] Desmoplastic ameloblastoma.

peritumorally and intratumorally in SMA (Fig. 3A). In UA, the location of microvessels was also peri- and intratumoral, as well as in the cystic wall (Fig. 3B). Although scantiness of microvessels in tumor areas was evident in DA, we found microvessel clusters distant from tumor cells, corresponding to areas with the highest presence of macrophages (Fig. 3C). Counts in areas close to tumor cells were evaluated for MVD in DA cases (Table 1).

Table 3
Correlation coefficients between macrophage and microvessel densities in clinical variants of ameloblastoma.

Clinical tumor variant	Correlation coefficient between MD[a] and MVD[b]
SMA[c]	0.9752
UA[d]	0.9551
DA[e]	0.9495
All samples	0.9623

[a] Macrophage density.
[b] Microvessel density.
[c] Solid/multicystic ameloblastoma.
[d] Unicystic ameloblastoma.
[e] Demoplastic ameloblastoma.

Microvasculature morphology

Architecture of the microvasculature associated to ameloblastoma was delineated by CD34 immunostaining. SMA, UA and DA exhibited a differential mixture of two staining patterns (Fig. 3A–C). The first pattern was constituted by short and straight microvessels of regular frequency. This pattern was predominant in UA, particularly in the cystic wall (Fig. 3B). The other pattern was composed of a higher number of longer vessels with irregular morphology and frequency and with dilated or impossible to differentiate luminal spaces. This was a common pattern in SMA (Fig. 3A) and the principal pattern in the periphery of the DA. In the latter case, the microvasculature appears to be obliterated by hyalinized collagen

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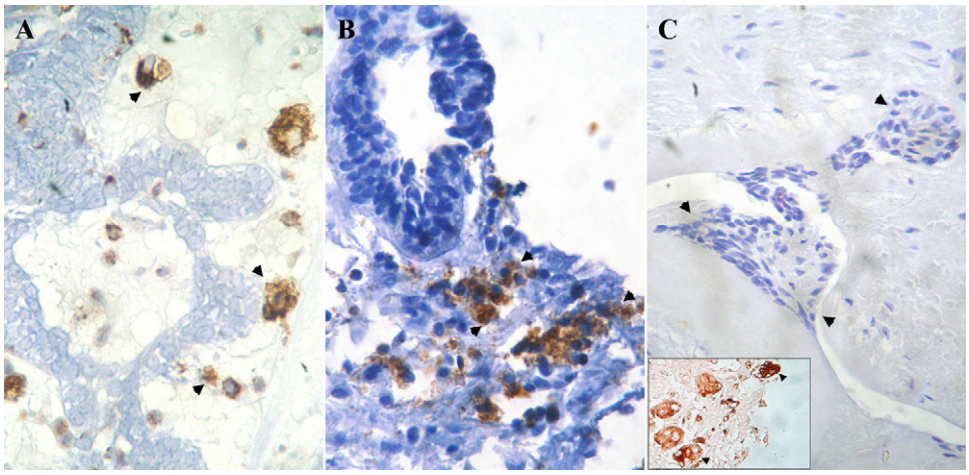

Fig. 2. Microphotographs of tissue sections showing immunoreactions to a monoclonal antibody directed against CD68 in different clinical variants of ameloblastoma. (A) View of a solid ameloblastoma with cells positive for CD68. Arrowheads show tumor infiltrating macrophages (original magnification 40×). (B) View of a unicystic ameloblastoma with cells positive for CD68. Arrowheads point to groups of positive macrophages located in the cystic wall (original magnification 40×). (C) Absence of intratumoral macrophages was evident in desmoplastic ameloblastomas. Arrowheads point to tumor cells (original amplification 40×). Inset shows some macrophages detected in the periphery of this lesion. Arrowheads point to some of these macrophages (original amplification 40×).

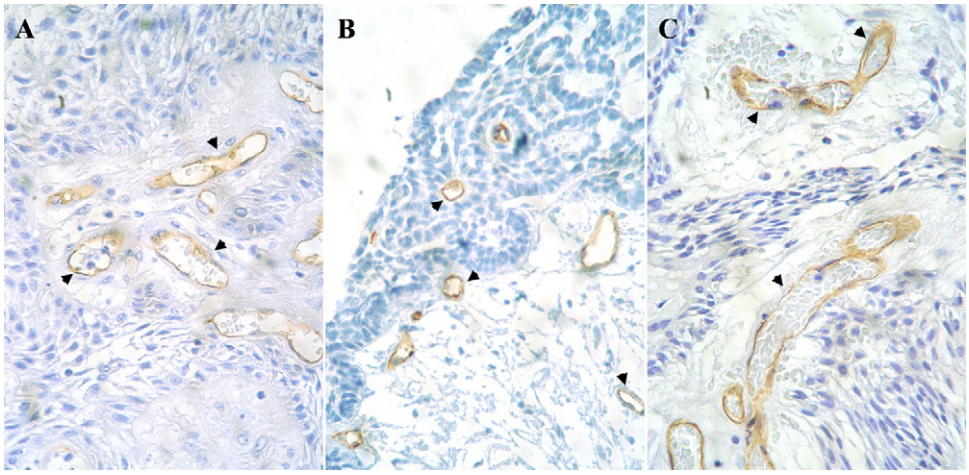

Fig. 3. Microphotographs of tissue sections showing immunoreactions to a monoclonal antibody directed against CD34 in different clinical variants of ameloblastoma. (A) Microvessels next to tumor in a solid ameloblastoma. Arrowheads point to long microvessels (original magnification 40×). (B) Microvessels in a unicystic ameloblastoma. Arrowheads point to short, uniform microvessels (original magnification 40×). (C) View of a desmoplastic ameloblastoma. Arrowheads show microvessels next to tumor cells (original magnification 40×).

in the tumor periphery and exiguous in the proximity of tumor cells (Fig. 3C).

Discussion

An adequate vascular system is critical for transporting oxygen and nutrients and eliminating metabolic waste products, and thus for maintaining tissue viability and growth [26]. Under physiological conditions, a balance of pro- and anti-angiogenic factors prevails in tissue microenvironments. Nevertheless, in pathological conditions, such as tumor growth, an excessive angiogenesis is usually present [26]. Indeed, it has been hypothesized that tumor growth rates depend on capillary density and metabolic level [13].

Although angiogenesis is a complex process and an improved evaluation for events involved in it may include the immunostaining of VEGF and its receptors, in this study we analyzed MVD by using antihuman antibodies against CD34, because this parameter is considered "the gold standard" for the quantification of this process [14]. Our results show that SMA has a higher MVD than UA and DA. In concordance, when angiogenesis was assessed in several odontogenic lesions, SMA registered the highest density [1]. Similarly, a recent study analyzing angiogenesis through VEGF in benign and malignant ameloblastomas showed that expression of the growth factor and the MVD counts were higher in these lesions than in their non-neoplastic counterpart, tooth germs [15]. All these results suggest that increased angiogenesis could be a contributing factor in the higher risk of recurrence and local aggressive conduct of SMA [11,24]. In this context, it has been described that VEGF expression and MVD counts increased in the following order: primary ameloblastoma, recurrent ameloblastoma and malignant ameloblastoma [6].

Positivity for VEFG was mainly detected in both normal and neoplastic odontogenic epithelial cells [15], although it has been demonstrated that this growth factor is also produced by macrophages [18]. Thus, macrophages can stimulate tumor angiogenesis through VGEF [18]. Indeed, a positive correlation between high vascular grade and increased macrophage counts has been detected in several tumors, such as breast cancer [16], endometrioid carcinomas [9] and hepatocellular carcinoma [23].

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We also observed differences in the number of macrophages in the diverse clinical variants of ameloblastoma, particularly near tumor cells. In addition, our results show a high positive correlation between MVD and MD, independently of the ameloblastoma clinical variant included in this study. Similarly, a positive correlation between MD and MVD in both primary endometrioid carcinomas and their corresponding metastasis was established [9]. In addition, in hepatocellular carcinoma tissues, macrophage and microvessels counts were significantly higher than those in paracarcinomatous, and a positive correlation between MD and MVD has been demonstrated in these tissues [23].

In addition to quantitative differences, we observed subtle differences in the distribution of macrophages and microvessels among SMA and UA, and more marked differences between these two clinical ameloblastoma variants and DA. Peritumoral and intratumoral arrangements were detected in both SMA and UA, but in DA the scantiness of both microvessels and macrophages close to tumor areas is noteworthy. This can be considered a typical feature of DA, which differs strikingly from conventional ameloblastomas in anatomical location, radiographic features and expression of tumorigenic markers [2,4,8,17]. Indeed, DA is a unique lesion that was recently recognized as a separate clinical variant of ameloblastoma, and whose true aggressiveness is only beginning to be characterized [2,4,8,17]. Similar to DA, microvessels were found to be scattered and dilated in plexiform ameloblastoma [15].

Although tumor progression is a complex process involving numerous participants, the results of the current contribution support the idea that angiogenesis, promoted at least in part by macrophages, could contribute to the modulation of the different forms of ameloblastoma and therefore may help account for the heterogeneous clinical behavior of the same. Furthermore, the fact that differences were detected in the distribution of inflammatory infiltrates in colorectal cancer tumors, corresponding to differential clinical findings, supports the idea that angiogenesis promoted by macrophages participates in ameloblastoma [12].

In conclusion, our observations suggest that increased angiogenesis correlates with a higher number of macrophages in the inflammatory infiltrate in the diverse forms of ameloblastoma. In this sense, differences were found in both density and distribution of microvessels and macrophages, suggesting that they could have a role in the heterogeneous behavior of this odontogenic tumor. The present study represents a step forward in the understanding of both the biology of ameloblastoma and the potential role of macrophages and angiogenesis in tumor progression and aggressiveness. Future studies, particularly with a greater number of cases of DA, are required to further explore the role of angiogenesis in ameloblastoma development.

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