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Original Article

CD163$^+$/CD68$^+$ tumor-associated macrophages in angiosarcoma with lymphedema

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Abstract: Angiosarcoma of soft tissue is a group of aggressive malignancies with high mortality. However, molecular pathogenesis and therapeutic targets of angiosarcoma remain to be established. We explored the influence of M2-polarized tumor-associated macrophages (TAMs) on the formation of angiosarcoma. CD163$^+$/CD68$^+$ macrophages were determined by immunohistochemistry from a series of 38 samples, including 17 cases of angiosarcoma with lymphedema and 21 cases of lymphangioma. The number of CD163$^+$/CD68$^+$ macrophages in angiosarcoma was significantly higher than that in lymphangioma. VEGFc was universally expressed in both angiosarcoma tumor cells and CD163$^+$/CD68$^+$ macrophages. VEGFR3 was expressed only in angiosarcoma tumor cells. Our study indicates a potential role of TAMs in the development of angiosarcoma with lymphedema. The VEGF signaling pathway may thus serve as a potential target for treatment of angiosarcoma.

Keywords: CD68, CD163, angiosarcoma, lymphedema, tumor-associated macrophages (TAMs), VEGF

Introduction

Traditionally, malignant tumors of vascular origin (angiosarcomas) have been divided into hemangiosarcomas and lymphangiosarcomas on the basis of morphological criteria, which suggest blood or lymphatic origin of malignant endothelial cells. Studies have shown that malignant vascular tumors express mixed immunophenotypes of both lymphatic and blood endothelium [1, 2]. Therefore the general term angiosarcoma is more accurate in describing lymphangiosarcomatous vs. hemangiosarcomatous origin [3, 4].

Angiosarcoma of soft tissue is a very aggressive malignancy with high mortality [5]. However, the pathogenesis of angiosarcoma remains mysterious.

Malignant tumors are complex structures interacting with micro-environment for growth and invasiveness. However, the molecular mechanism in the tumor-associated immune microenvironment that drives invasion and metastasis of angiosarcoma has not been well established. These observations underscore an urgent need to identify new biomarkers with the potential to predict tumor development and progression, enable diagnosis at earlier stages of the disease, and facilitate early detection of disease recurrence or metastasis after treatment.

Macrophages are critical immune effector cells as one of the major components of tumor-infiltrating leukocytes. These cells play a key role, in carcinogenesis [6]. Macrophages that infiltrate and surround the tumor nest are defined as tumor-associated macrophages (TAMs) [7]. TAMs interact with neoplastic cells by releasing various cytokines which contribute to cancer initiation and progression. Emerging findings suggest that increased numbers of TAMs in various types of carcinomas are associated with a poor prognosis [8-10]. There are several known functional markers of TAMs. The presence of CD163 is a key factor to distinguish different TAMs. CD163, a member of the scavenger receptor cysteine-rich family, is involved in anti-inflammatory functions and predominantly expressed on M2 macrophages [11]. Accumulating evidence indicates that a high number of TAMs, as demonstrated by exclusive immunohistochemistry (IHC) with antibodies against CD163, are associated with an unfavorable prognosis in a variety of malignancies [12-14].

Table 1. Clinicopathologic features of patients with angiosarcoma

ID	Gender	Age (years)	Histo-pathological diagnosis
1	Female	21	(Left lower extremities) angiosarcoma, FNCLCC, grade 1
2	Male	23	(Left hand) angiosarcoma, FNCLCC, grade 2
3	Female	60	(Left upper limb) angiosarcoma, FNCLCC, grade 3
4	Female	52	(Left chest wall) angiosarcoma, FNCLCC, grade 2
5	Female	66	(Right foot) angiosarcoma, FNCLCC, grade 2
6	Female	38	(Right calf) angiosarcoma, FNCLCC, grade 2
7	Male	39	(Left calf) angiosarcoma, FNCLCC, grade 2
8	Female	65	(Right upper limb) angiosarcoma, FNCLCC, grade 2
9	Female	51	(Right upper arm) angiosarcoma, FNCLCC, grade 1
10	Female	62	(Left shoulder and back) angiosarcoma, FNCLCC, grade 2
11	Female	54	(Right arm) angiosarcoma, FNCLCC, grade 2
12	Male	63	(Right lower extremities) angiosarcoma, FNCLCC, grade 2
13	Female	15	(Left ankle) angiosarcoma, FNCLCC, grade 1
14	Female	65	(Left calf) angiosarcoma, FNCLCC, grade 1
15	Female	43	(Left upper arm) angiosarcoma, FNCLCC, grade 3
16	Female	47	(Left lower extremities) angiosarcoma, FNCLCC, grade 2
17	Female	58	(Left calf) angiosarcoma, FNCLCC, grade 2

However, CD68, the well-established generic macrophage marker, could not distinguish M1 or M2 subtypes from other infiltrated macrophages [15]. In this study, clinical significance of macrophages in angiosarcoma was evaluated with CD163 and CD68, and the association between the number of $CD163^+/CD68^+$ macrophages and VEGF was analyzed.

Materials and methods

Tissue specimen

This retrospective study was approved by the Ethics Committee of Beijing Shijitan Hospital, Capital Medical University. Signed informed consent was obtained from each patient. Surgical tissue specimens of angiosarcoma (17 cases) and lymphangioma (21 cases) were randomly collected. Diagnosis of angiosarcoma and lymphangioma tissue was confirmed by histopathological examination.

Formalin-fixed paraffin-embedded (FFPE) specimens were used to prepare a series of tissue sections for immunohistochemical staining.

Immunohistochemistry

Primary and secondary antibodies were purchased from Zhongshan Biotechnology, China. Four-micron sections were incubated with the following primary antibodies: monoclonal mouse anti-human CD68 (KP1, 1:150), CD163 (10D6, 1:150), VEGFR3 (KLT9, 1:30), and polyclonal antibody rabbit anti-human VEGFc (1:75). The secondary antibody reagent kit PV-8000 was applied. The experimental steps were carried out according to the manufacturer's instructions.

Histopathologic examination

The intensity of immune infiltrates was assigned a semi-quantitative score from 0-3 (16) as follows: 0 = "none" (no immune infiltrates), 1 = "focal" (mostly perivascular in tumor with some intratumoral extension), 2 = "moderate" (prominent extension of immune infiltrates away from perivascular areas and amongst tumor cells), or 3 = "severe" (immune infiltrates obscuring tumor).

The staining intensities of VEGFc in both tumor cells and macrophage, as well as VEGFR3 in tumor cells were scored based on the following criteria (17): "0" represents no staining or faint staining intensity in 10% cells; "1+" represents faint staining in >10% of cells; "2+" represents moderate staining in >10% of cells; "3+" represents strong staining in >10% of cells. The tissue specimen was considered positive for VEGFc or VEGFR3 when the staining intensity score was 1+, 2+, or 3+, and negative when the score was 0.

Statistical methods

Statistical data were analyzed using SPSS version 20.0 software. Associations between tumor types and different biomarkers were examined by χ^2-test (2-sided). The significance level was set at a $P<0.05$.

Results

Clinicopathologic characteristics of angiosarcoma with lymphedema

This study was conducted in a cohort of 17 patients diagnosed angiosarcoma with lymph-

Table 2. Clinicopathologic features of patients with lymphangioma

ID	Gender	Age (years)	Histo-pathological diagnosis
1	Male	1	(Left and right pleural) lymphangioma
2	Female	62	(Thymus) lymphangioma
3	Male	40	(Left axilla) cystic lymphangioma
4	Female	14	(Right neck) lymphangioma
5	Male	37	(The penis) lymphangioma
6	Male	10	(Right thigh) lymphangioma
7	Female	14	(Left neck) lymphangioma
8	Female	16	(Left neck) lymphangioma
9	Male	47	(Left neck) lymphangioma
10	Female	43	(Thigh) lymphangioma
11	Male	12	(Retroperitoneal) lymphangioma
12	Female	49	(Right supraclavicular) lymphangioma
13	Male	50	(Left neck) lymphangioma
14	Female	48	(Left neck) lymphangioma
15	Male	36	(Thigh) lymphangioma
16	Male	62	(Neck and supraclavicular) lymphangioma
17	Male	9	(Right upper limb) lymphangioma
18	Male	26	(Left and right perididymis) lymphangioma
19	Female	33	(Left thigh) lymphangioma
20	Male	14	(Right axilla) lymphangioma
21	Female	35	(Left neck) lymphangioma

edema (**Table 1**) and 21 patients diagnosed lymphangioma (**Table 2**).

The patients diagnosed angiosarcoma with lymphedema age ranging from 15 to 66 years with a median age of 48.4 years. The lesion located in the extremities with primary or secondary lymphedema. Four, 11, and 2 patients, respectively, were diagnosed with Federation Nationale des Centers de Lutte Contre le Cancer (FNCLCC), grade 1, grade 2, grade 3 angiosarcoma. The patient diagnosed lymphangioma age ranging from 1 to 62 years with a median age of 31.3 years. The lesions were located in the extremities, neck, supraclavicular, axilla, pleural, penis, and retroperitoneal.

Upregulation of CD68+ macrophages in angiosarcoma

CD68 was localized within the cytoplasm of the macrophages and exhibited granular, brownish staining in angiosarcoma specimens (**Figure 1A**). There were no or very few CD68+ macrophages in the lymphangioma (**Figure 1A**). The levels of total CD68+ macrophages in angiosarcoma tissues were significantly higher than those in the lymphangioma tissue ($P<0.05$, **Table 3**).

Upregulation of CD163+ macrophages in angiosarcoma

CD163 immunoreactivity was characterized by a granular brownish pattern and membrane staining (**Figure 1B**). The expression level of CD163 was significantly higher in angiosarcoma than in lymphangioma ($P<0.05$, **Table 3**).

Expression of VEGFc or VEGFR3 in angiosarcoma

VEGFc immunoreactivity was localized within the cytoplasm of the macrophages and tumor cells in angiosarcoma (**Figure 2A**). VEGFR3 immunoreactivity was localized within the cytoplasm of the tumor cells in angiosarcoma (**Figure 2B**).

Discussion

Angiosarcoma represents less than 1% of all sarcomas. This disease can be either primary or secondary to chronic lymphoedema with cytogenetic differences between these two forms [18].

Lymphedema-associated cutaneous angiosarcoma was first described in 1948 by Stewart and Treves, also known as Stewart-Treves syndrome. This type of tumor develops on the lymphedematous limb or chest wall after mastectomy and axillary lymph node dissection [19]. Previous reports have described angiosarcoma development in patients with lymphedema secondary to congenital lymphedema, lymph node dissection, filarial infection, and chronic idiopathic lymphedema [20]. In the presence of lymphoedema, angiosarcoma can grow as plaques or cutaneous and subcutaneous nodules, single or multiple, which may coalesce, with an unknown etiology. The infrequent occurrence of this disease and the innocuous appearance of the tumor lead to delays in diagnosis and treatment. In addition, precise mechanisms for the development of angiosarcoma on the basis of lymphedema are unknown.

Persuasive evidence from clinical and preclinical studies demonstrated that macrophages

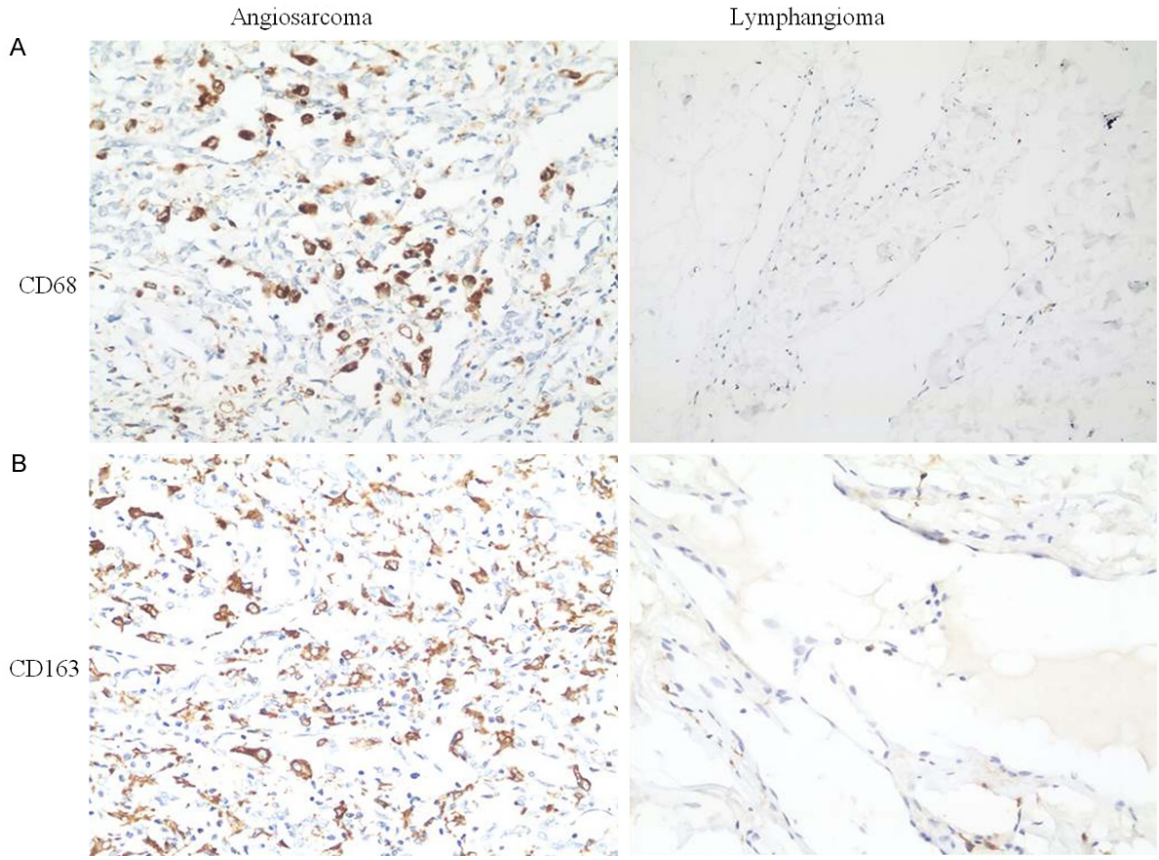

Figure 1. Immunohistochemical staining of CD68 and CD163 in patients with angiosarcoma and lymphangioma. A. Immunohistochemical staining with anti-CD68 for tissue specimens of angiosarcoma and lymphangioma. B. Expression levels of CD163 in angiosarcoma and lymphangioma.

Table 3. Expression of CD68 and CD163 in patients with angiosarcoma andlymphangioma

Disease	Patients (n)	CD68 score				*P*	CD163 score				*P*
		3	2	1	0		3	2	1	0	
Angiosarcoma	17	2	8	7	0		5	10	2	0	
Lymphangioma	21	0	2	13	6	0.001	0	7	14	0	0.00065

could promote cancer initiation, progression, and metastasis. Tumor associated macrophages (TAMs) influence tumor progression to different extents depending on tumor types [21]. Macrophages invade massively osteosarcoma tissues [22-24] and establish an immune-tolerant environment during tumor growth [23, 25].

Our study identified a large number of CD68+/CD163+ macrophages in angiosarcoma with lymphedema. While in lymphangioma, there were no or very few CD68+/CD163+ macrophages. These results suggested a critical role for CD68+/CD163+ macrophages in development of angiosarcoma with lymphedema.

Lymphatic injury may contribute to excessive production of proangiogenic cytokines through vascular endothelial growth factor (VEGF) signaling pathway. Indeed, VEGF is overexpressed in most angiosarcomas [26]. VEGF-C-expressing TAMs are involved in peritumoral lymphangiogenesis and subsequent dissemination in human cancer [27].

Our study found that both CD68+/CD163+ macrophages and tumor cells highly expressed VEGF-C in patients with angiosarcoma. Tumor cells also highly expressed VEGFR3 in angiosarcoma. These results indicated that VEGF-C/VEGFR3 signal pathway might promote the development and progression of angiosarcoma.

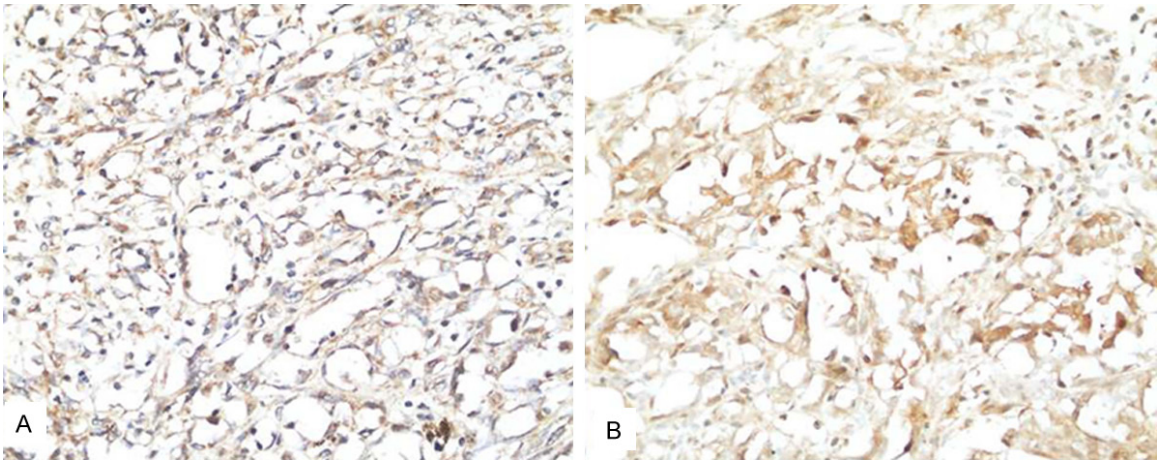

Figure 2. Immunohistochemical staining of VEGF-C and VEGFR3 in angiosarcoma. A. VEGF-C immunoreactivity was localized within the cytoplasm of the macrophages and tumor cells. B. VEGFR3 immunoreactivity was localized within the cytoplasm of the tumor cells.

In conclusion, our study demonstrates a positive association between expression of CD68+/CD163+ macrophages and carcinogenesis of angiosarcomas.

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Disclosure of conflict of interest

None.

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