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. VEGF-C 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].
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.

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 χ²-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 lymphedema. The histopathological features of these patients are summarized in Table 1.
CD163+/CD68+ tumor-associated macrophages in angiosarcoma with lymphedema

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).

**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 lymphedema 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 lymphedema, 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

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Histo-pathological diagnosis</th>
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<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>1</td>
<td>(Left and right pleural) lymphangioma</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>62</td>
<td>(Thymus) lymphangioma</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>40</td>
<td>(Left axilla) cystic lymphangioma</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>14</td>
<td>(Right neck) lymphangioma</td>
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<td>5</td>
<td>Male</td>
<td>37</td>
<td>(The penis) lymphangioma</td>
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<tr>
<td>6</td>
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<td>10</td>
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<td>49</td>
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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.
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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