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Evidence of lymphangiogenesis in Warthin's tumor of the parotid gland

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Summary The details of the pathogenesis of cystadenolymphoma (Warthin's tumor) of the parotid gland are still unclear. Neovascularization is considered to be a pivotal factor for solid tumor progression and biological behavior of the tumor. Using double-labeling immunohistochemistry for LYVE-1 and CD34 (specific markers for lymphatic and vascular endothelial cells, respectively) this study analyzes lymphatic vessel density (LVD) and blood vessel density (BVD) in 10 Warthin's tumors and 10 pleomorphic adenomas of the parotid gland as well as in 5 normal parotid glands and 5 normal parotid lymph nodes. There was no significant difference in the intratumoral LVD and BVD among pleomorphic adenoma and normal parotid gland tissue. In contrast, the intratumoral LVD and BVD were significantly higher in Warthin's tumor than pleomorphic adenoma, normal parotid gland and parotid lymph node ($P < 0.0001$ versus $P < 0.004$). The increase in lymphatic vessels in Warthin's tumor suggests that epithelial tumor cells might promote lymphangiogenesis in this kind of lesions.
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Introduction

Cystadenolymphoma of the parotid gland (Warthin's tumor) is the second most common benign tumor of the parotid gland. It accounts for about 15% of all epithelial tumors of the parotid gland.[1] Warthin's tumors most commonly present as an asymptomatic, slowly growing mass usually affecting men in the 5th and 6th decades. In about 12% of the cases, there is bilateral tumor development, which is commonly synchronous. In about 6% of the cases multifocal lesions may be observed in one parotid gland.[2] Several studies showed that a significant number of patients suffering from Warthin's tumor are smokers, in contrast to patients with other salivary gland tumors.[2,3]

These well encapsulated lesions consist of an oncocytic epithelial cell component arranged in double layers and a variable stroma component with lymphocytic infiltrates

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and lymph follicles that correspond to the distribution of the lymphocytic infiltrations of a lymph node.[4] Up to now no agreement has been reached regarding the pathogenesis of this disease. In most surveys the assumption prevails that this tumor has its origin in heterotopic salivary parenchyma inclusions in the intra- and periglandular lymph nodes of the parotid gland.[5,6]

Since the epithelial tumor components, like the lymphocytic infiltrations, are polyclonal cell populations[7] this kind of tumor cannot be considered to be a true neoplasm. The clinical behavior of this lesion as the total lack of recurrence and malignant transformation of this tumor further supports this view. Because of the arguments against a true neoplastic origin of this entity, we classify this tumor in the group of tumor-like lesions.[8]

Histological quantitation of tumor angiogenesis and lymphangiogenesis is an important step in the understanding of the biology of these tumors. The aim of the present study was to analyze the intratumoral blood vessel density (BVD) as a measure of angiogenesis using CD34 staining, which is a specific marker for detection of all vascular endothelial cells, and lymphatic vascular density (LVD) as a measure of lymphangiogenesis using lymphatic vessels endothelial hyaluronan receptor-1 (LYVE-1) staining, which is a specific marker for detection of lymphatic vascular endothelial cells in comparison to pleomorphic adenoma of the parotid gland, normal parotid gland tissues and normal intraparotid lymph nodes.

Materials and methods

Patients and specimens

A total of 20 patients of similar age undergoing lateral parotidectomy for Warthin's tumor (2 females and 8 males, median age 54.2 years, range 51—56 years) and pleomorphic adenoma (6 females and 4 males, median age 52 years, range 50—56 years) of the parotid gland at our department were enrolled in the present study. The tumor size of both entities was approximately equal. The average size of Warthin's tumors at diagnosis, as determined at preoperative sonography, was 25.3 ± 4 mm (range 23—27 mm) in greatest diameter. Similarly, the average maximum diameter of pleomorphic adenoma was 24.8 ± 3 mm (range 22—28).

Archived formalin-fixed, paraffin-embedded tissue specimens from these tumors and tissue specimens of 5 normal parotid glands and 5 normal intraparotid lymph nodes obtained during parotidectomy were included for immunohistochemical studies.

Double immunostaining

From the tissue samples, 4 μm thin slices were cut, drawn upon 3-aminopropyltrietoxysilane (APES)-coated slides and dried overnight at 37 °C. Dewaxed tissue sections were pretreated with 10 mM citrate buffer (pH 6.0) at 121 °C for 15 min. After rinsing in 1/15 M phosphate buffered saline (PBS), the sections were immersed in 0.3% H_2O_2 in methanol for 20 min to block the endogenous peroxidase activity. They were incubated in 4% Block Ace solution (Dainippon Seiyaku, Osaka, Japan), and then in a mixture of anti-human LYVE-1 rabbit antibody (a kind gift from Prof. Ishii, Department of Pathology, Toho University, School of Medicine, Tokyo, Japan) (diluted 1:200) together with anti-human CD34 mouse antibody (diluted 1:50, DAKO, Glostrup, Denmark) at 4 °C overnight. After washing with PBS, they were treated with peroxidase-conjugated anti-rabbit goat IgG (Simple stain MAX-PO, Nichirei, Tokyo, Japan) for 1 h at room temperature and developed with diaminobenzidine (Dojindo, Kumamoto, Japan). Following a rinse in PBS, the tissues were then treated with a biotinylated anti-mouse rabbit IgG for 30 min, followed by alkaline phosphatase-conjugated streptoavidin for 30 min at room temperature (Histofine, Nichirei, Tokyo, Japan). The site of the immunoreaction was developed with Vector Red Substrate Kit (Vector Laboratories, CA, USA) before counterstaining with hematoxylin.

Quantification of blood and lymphatic vessels

Vessel counts were performed by light microscopy, as previously described with slight modifications.[9] The highly vascular areas were selected by low power scanning of the sections (magnification, 40x). The vessel density was determined in 10 high-power fields (HPF; 400×) of the tissue sections in areas with the highest vascularity (''hot spots''), and the results were recorded as the sum of all vessel counts per 10 HPF. LVD and BVD were expressed as the mean value of the counted microvessels.

All statistical tests were carried out using SPSS (version 11.5, SPSS Inc., Chicago, USA). The CD34 und LYVE-1 score between groups were compared with the Mann-Whitney test. *P*-values $\leqslant 0.05$ were considered to indicate statistical significance.

Results

In all tissues examined, the LYVE-1 positive lymphatic vessels were of irregular shape with a thin endothelial wall, whereas erythrocyte-filled blood vessels were not stained. The vast majority of the lymphatic vessels in normal parotid gland had wide open lumina and were mainly located in the interlobular connective tissue. They were in close proximity to the ducts and around the CD34 positive vascular area. Identification of the lymphatic vessels was confirmed by a combination of double immunohistochemical staining with antibodies to LYVE-1 and CD34, which revealed mutually exclusive expression of these markers on two vessel types. In the parotid lymph nodes, LYVE-1 expression was found on the endothelial cells of the lymphatic sinus and in some reticular cells. In all tissue specimens of pleomorphic adenoma and Warthin's tumor a diffuse expression of LYVE-1 and CD34 positive endothelial cells were present. These vessels were often similar in shape and size to the vessels of normal parotid gland and normal parotid lymph node. In both tumors, positive lymphatic vessels were variably observed within the intratumoral stoma next to blood vessels or dispersed between the tumor cells in close contact with them (Fig. 1).

We found significantly higher intratumoral LVD in Warthin's tumor (mean, 31; SD, ±4.6) as compared to normal parotid gland (mean, 3; SD, ±1.1), normal parotid lymph

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