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Tumor-Associated Macrophages Are Related to Volumetric Growth of Vestibular Schwannomas

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Hypothesis: Tumor-associated macrophages contribute to vestibular schwannoma development.
Objective: An important clinical problem regarding vestibular schwannoma treatment is their variable growth rate. Tumor biological research can help to clarify this growth rate and may offer targets for therapy. Inflammation is an important biological process involved in the development of many solid tumors. Macrophages are major determinants of intratumoral inflammation. Macrophages can be divided into two groups; the M1- and M2-type macrophages. M2-type macrophages are associated with tumor-promoting processes like angiogenesis, tumor cell growth, and downregulation of the antitumor immune response. Both macrophages and angiogenesis can serve as targets for therapy. CD163 is a specific marker for M2-type macrophages. The goal of this study was to investigate if the expression of CD163 positive macrophages in sporadic vestibular schwannomas is associated with angiogenesis and tumor growth.
Methods: CD163 expression in 10 fast-growing vestibular schwannomas was compared with CD163 expression in 10 slow-growing vestibular schwannomas. Tumor growth was determined by comparing preoperative tumor volume measurements on MRI. The relation between macrophage expression and angiogenesis was evaluated by assessing microvessel density (CD31).
Results: CD163 expression and microvessel density were significantly higher in fast-growing vestibular schwannomas ($p < 0.001$ and $p = 0.019$, respectively). Tumors with higher CD163 expression contained significantly more microvessels ($p = 0.014$).
Conclusion: This study demonstrates that M2-type macrophages in vestibular schwannomas relate to angiogenesis and volumetric tumor growth. These results imply that the M2-type macrophage infiltrate contributes to progressive tumor growth, making it a potential target for pharmacologic therapy.
Key Words: Angiogenesis—CD163—Growth—Inflammation—Tumor-associated macrophage—Vestibular schwannoma.

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Vestibular schwannomas are benign tumors recapitulating the differentiation repertoire of the myelin-forming Schwann cells of the vestibular branch of the VIIIth cranial nerve in the internal auditory canal or the cerebellopontine angle. These tumors grow slowly and progressively, ultimately causing brainstem compression. Therapeutic management of vestibular schwannomas can be divided into 3 main strategies, that is, microsurgery, stereotactic radiotherapy, or serial radiologic observation, also known as the wait and scan policy. So far, unlike neurofibrosis Type 2–related tumors (1,2), sporadic vestibular schwannomas are not pharmacotherapeutically treated one of the main problems in determining the most suitable strategy is the large variability in growth rate these tumors can display. More understanding of this variable growth rate would be of great benefit when determining the most suitable therapeutic approach. This requires more insight into tumor biological factors affecting vestibular schwannoma growth. Studying vestibular schwannoma biology not only contributes to a better understanding of its growth pattern, it may also help to identify potential therapeutic targets.

Recently we have performed a pilot study on histopathologic characteristics of vestibular schwannoma growth, which indicated a significant correlation between CD68-positive macrophages and clinical tumor growth (3). So far, little is known about the mechanism and biological value of the inflammatory process in vestibular schwannomas. A possible explanation may come from research on other types of tumors (4–9). It is supposed that the inflammatory microenvironment has many tumor-promoting

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effects (10). One of the major determinants of the inflammatory infiltrate is macrophages (11). These so-called tumor-associated macrophages (TAMs) can, based on their immunological functions, roughly be divided into 2 different categories (12). The first category comprises the classically activated or M1-type macrophages. M1 macrophages have a defensive purpose; they are inflammatory and initiate cytotoxic responses against tumor cells and intracellular pathogens (13,14). Monocytes differentiate into M1 type macrophages in response to signals produced by bacterial products like lipopolysaccharides or by cytokines such as IFNγ (15). M1 macrophages are characterized by the expression of proinflammatory cytokines such as IL-12, IL-1, and IL6 (16). Upon activation, M1 macrophages start to produce nitric oxide and reactive oxygen species; they stimulate cytokine-induced cytotoxicity and promote natural killer and T-cell activity. Altogether these processes result in a strong antitumor effect. The alternatively activated M2 macrophages on the other hand possess different functions. Monocytes differentiate into M2 type macrophages in response to cytokines like IL-4 and IL-13 (17). M2 macrophages express scavenger receptors, such as CD163 (4,15,18,18–22), and are associated with production of, for instance, IL-10, IL-1b, and vascular endothelial growth factor (VEGF). M2 macrophages dampen antitumor inflammatory responses, participate in remodelling and repair of damaged tissues and stimulate angiogenesis (11,12,23). The combination of these functions results in a tumor-promoting effect. It should be noted that the classification of macrophages into M1 and M2 subtypes is an oversimplification of the actual macrophage population to increase the comprehensibility of macrophage differentiation.

The aim of this study was to investigate if the expression of M2 type macrophages in sporadic vestibular schwannomas is associated with angiogenesis and tumor growth.

MATERIALS AND METHODS

Multiple studies on different types of tumors indicate that the majority of tumor-associated macrophages are of the M2 type and highly express CD163 (19–22), making it an excellent marker to study M2 macrophages. We performed immunofluorescent stains against CD163 on formalin-fixed paraffin-embedded vestibular schwannoma tissue. To establish the relation between macrophage activity and vestibular schwannoma growth, we analyzed and compared the expression pattern of this marker in tumor samples of 10 radiologically observed fast-growing and 10 radiologically observed slow-growing tumors. To study the association between M2-type macrophage expression and angiogenesis the degree of CD31 positive microvessels was evaluated.

Patient Selection

Patients were retrospectively selected from the vestibular schwannoma database at the Leiden University Medical Center. The selection involved a group of consecutive patients surgically treated for a histologically proven vestibular schwannoma from October 2006 to December 2011. Two separate patient cohorts were composed. The first cohort comprised 10 cases of radiologically observed evident slow-growing tumors. The second cohort comprised 10 cases of radiologically observed evident fast-growing tumors. Decision for surgical treatment was based on clinical symptoms (e.g., tinnitus, vertigo, and hearing loss), the presence of increase of tumor size on sequential MRI scanning and patients' personal preference. Patients diagnosed with NF2 were excluded from this study.

All patient samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines of the Code for Proper Secondary Use of Human Tissue in The Netherlands (Dutch Federation of Medical Scientific Societies).

Tumor Measurement

All tumor measurements were conducted on T1-weighted gadolinium enhanced MRI examinations and carried out by one and the same author. Volume measurements were performed with a contour measurement method using Vitrea View software (Vital Imaging; Minnetonka, MN, USA) (Fig. 1). For validation purposes, tumor volume was measured twice for each MRI scan. The mean of these 2 measurements was used for further evaluation of tumor growth. Tumor growth rate was determined by comparing tumor volume on 2 sequential MRI scans and expressed in milliliters per year.

Enzymatic and Fluorescent Immunostainings

Immunohistochemical stainings were conducted on 4-μm thick slides obtained from formalin-fixed and paraffin-embedded vestibular schwannoma tissue. All stainings were performed on one single tumor block per tumor sample. For CD31 (DAKO), enzymatic immunohistochemical reactions were carried out according to standard laboratory methods (24). In brief, heat-induced citrate buffer antigen retrieval was performed using microwave treatment of all slides after dewaxing and rehydration, followed by blocking of endogenous peroxidase with 3% H2O2/methanol. Primary antibodies were incubated over night at room temperature. Slides were incubated with poly-HRP-anti mouse/rabbit/rat I 69 (immunologic, DVPO 110HRP). Visualization was carried out in a 3.3'-diamino-benzidine-tetrahydrochloride (DAB) solution after the addition of hydrogen peroxide 30% shortly before application. All washing procedures were performed in phosphate-buffered saline (PBS). Slides were counterstained with hematoxylin.

For CD163 immunofluorescent (Novocastra, Newcastle Upon Tyne, UK) stains were performed. After Tris EDTA (pH 9.0) heat induced-antigen retrieval, the primary antibody was incubated

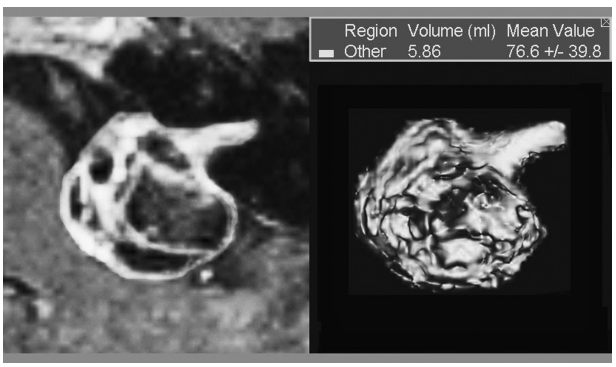

FIG. 1. Example of volume measurement of left-sided vestibular schwannoma performed with the contour measurement method on a T1-weighted gadolinium enhanced MRI scan.

over night, followed by incubation with a secondary fluorescent antibody (Alexa goat-anti-mouse IgG1 AL-647 (Invitrogen).

Microscopic Analysis

CD163 stained slides were analyzed using a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany). This analysis method has been described with regard to macrophage expression analysis before (22). Slides were scanned using a fixed laser-filter. Alexafluor 647 (red) was exited at 633 nm and detected with a 650-nm-long pass filter. On the digitalized scans, the fluorescent signal was represented by an artificial color (red). Per scan the microscope was focused to pick up the maximum amount of fluorescent signal. All images were 1,024 × 1,024 pixels, stack size 521.2 × 521.2 μm, and a 25× objective was used (Fig. 2, A and B).

All assessments were carried out blinded with regard to clinical tumor characteristics. Per tumor slide, 5 randomly chosen scans were obtained. These scans were saved in a JPEG format. Using ImageJ (National Institutes of Health, Bethesda, MD, USA) images were converted to a binary configuration. All images were uniformly thresholded and ''despeckeled'' to minimize overexposure effects and to reduce background noise. The remaining number of positive, red pixels was calculated and expressed as the area fraction, which served as the score for macrophage expression. The average count of the 5 scans per tissue slide was used for statistical analysis.

Microvessel density was determined using the Chalkley point overlap technique. This technique allows for rapid analysis with a relatively low interobserver variability and has been described in detail before (25,26). In brief, CD31-stained slides were scanned for vascular hot spots. Using an ocular grid with 25 random points, these hot spots were scored at a 200× magnification. The grid was orientated to permit the maximum number of points hitting the stained microvessels (Fig. 2C). The Chalkley count was the average of the maximum number of points hitting a microvessel in the 3 most prominent vascular hotspots per tumor slide.

Statistics

The difference in CD163 expression and microvessel density in the slow versus fast-growing tumors was determined with the Mann-Whitney *U* test. Two tests were performed to evaluate the relationship between CD163 expression and microvessel density. For the first test, the total patient cohort (n = 20) was divided into 2 groups with regard to the degree of CD163 expression. The Mann-Whitney *U* test was used to compare the degree of microvessel density in tumors displaying lower CD163 expression with the degree of microvessel density in tumors displaying higher CD163 expression. The second test was a Spearman correlation test comparing CD163 expression and microvessel density. For all statistical tests, a level of significance of $p < 0.05$

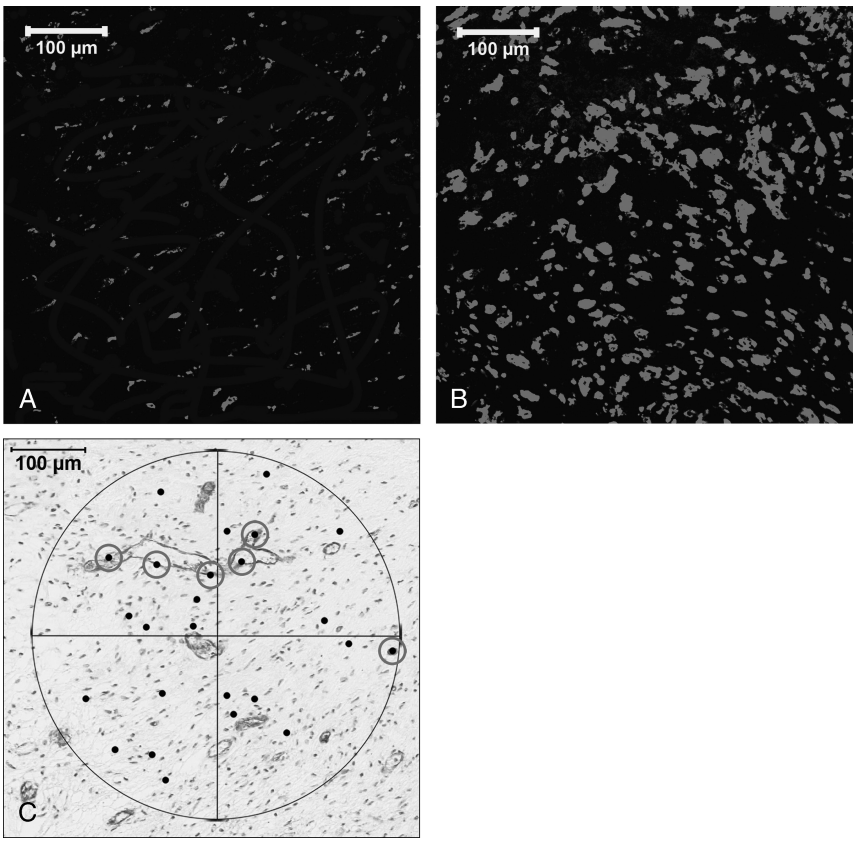

FIG. 2. CD163 immunofluorescent stain for macrophages in vestibular schwannoma, (*A*) low expression and (*B*) high expression. Original magnification, ×250. Example of the Chalkley count for microvessel density (*C*). The *circled grid points* hit CD31 stained microvessels. Original magnification, ×200.

was taken into account. All calculations were performed using SPSS Inc. software, version 16.0.

RESULTS

Details on patient characteristics are listed in Table 1. No significant differences in distribution of age, sex, and duration of preoperative radiologic follow-up were observed in the 2 patient cohorts. As expected, there was a significant difference in tumor growth rate and tumor volume between the 2 groups.

Immunofluorescent staining for CD163 was performed on tissue sections obtained from 10 fast-growing and 10 slow-growing sporadic vestibular schwannomas. All tumors displayed a certain degree of immunopositivity for CD163. CD163 expression was significantly higher ($p < 0.001$) in fast growing tumors compared with slow-growing tumors (Fig. 3A). The degree of microvessel density was also significantly higher in fast-growing tumors ($p = 0.019$) compared with slow-growing tumors (Fig. 3B). The degree of microvessel density was significantly higher ($p = 0.014$) in tumors displaying high CD163 expression (Fig. 3C), and the Spearman correlation test showed a significant positive relation ($p = 0.024$; $r = 0.50$) between CD163 expression and microvessel density.

DISCUSSION

Many, if not all, solid tumors contain a certain degree of inflammation (27). Macrophage activity is a major determinant of the intratumoral inflammatory microenvironment (11). As mentioned earlier, these tumor infiltrating macrophages, especially the alternatively activated M2 type macrophages, have been associated with tumor progression by stimulating angiogenesis, tumor cell growth and down regulation of the antitumor immune response. Although the majority of studies on macrophages in human tumors support this tumor-promoting model, it should be noted that a number of studies reported contradicting results in which high macrophage counts were associated with better prognosis (28–30). Presumably, different types of tumors give rise to different types of inflammatory infiltrates resulting in different effects on tumor development.

In this study, we have compared the expression of the M2 macrophages in a group of 10 slow-growing tumors with the expression of this marker in a group of 10 fast-growing tumors. We also compared the degree of microvessel density between these 2 groups, and finally, we investigated the relationship between macrophage expression and microvessel density. It should be taken into account that the outcomes of these comparisons remain observations of association. There is always a possibility that these findings are epiphenomena of a larger biological growth dynamic and, therefore, not directly linked to one another.

We used volumetric measurements to assess tumor size because this technique is more precise than the conventional 2-dimensional tumor measurements (31,32) Tumor growth rate was measured by comparing tumor volume on serial preoperative MRI scans and expressed in mil-

TABLE 1. *Patients' characteristics*

Case	Sex	Age (yr)	Tumor volume (ml)	Growth rate (ml/yr)	Preoperative magnetic resonance imaging follow-up (mo)
Slow-growing group					
L3721	M	60	0.35	0.06	13
L3742	F	54	0.27	0.21	7
L3773	F	52	0.22	0.07	6
L3774	F	50	0.88	0.19	27
L3775	M	53	0.69	0.17	10
L3779	F	51	1.46	0.18	32
L3780	F	56	0.74	0.25	11
L3781	F	44	0.53	0.19	12
L3787	M	58	0.52	0.18	12
L3797	F	60	2.13	0.23	12
Mean (±SD)		53.8 (±4.96)	0.77 (±0.59)	0.17 (±0.06)	14.2 (±8.46)
Fast-growing group					
L3725	F	62	3.26	1.33	11
L3731	F	81	14.41	4.41	19
L3733	F	76	11.66	8.84	10
L3740	F	52	2.27	2.50	7
L3741	F	71	7.25	1.83	21
L3745	F	56	5.62	2.18	6
L3746	M	46	30.73	43.98	4
L3792	F	75	6.92	3.43	21
L3793	F	39	7.32	5.52	10
L3805	F	39	23.05	2.90	9
Mean (±SD)		59.7 (±15.65)	11.24 (±9.16)	7.69 (±12.94)	11.8 (±6.26)
Difference fast versus slow (p)	0.26[a]	0.41[b]	*<0.0001*[b]	*<0.0001*[b]	0.31[b]

F indicates female; M, male; SD, standard deviation.
[a]Chi-square test.
[b]Mann-Whitney U test, results at $p \leq 0.05$ are shown in italic.

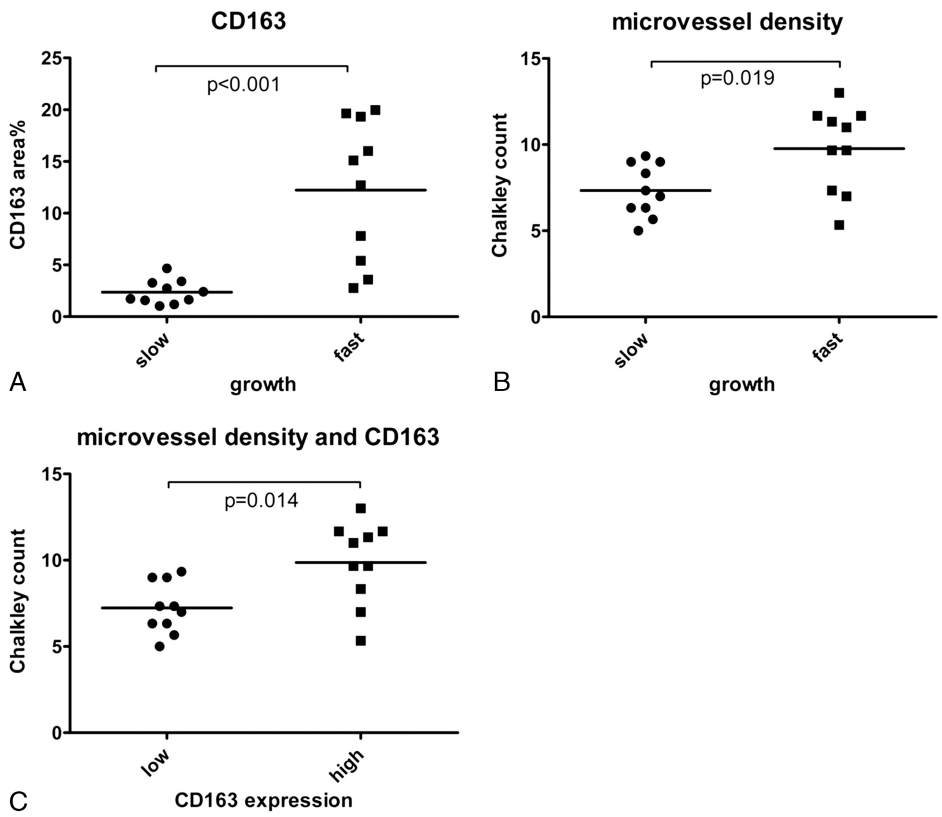

FIG. 3. CD163 expression in slow- versus fast-growing tumors (Mann-Whitney *U* test) (*A*). Microvessel density in slow- versus fast-growing tumors (Mann-Whitney *U* test) and microvessel density in tumors with low versus high CD163 expression (Mann-Whitney *U* test).

liliters per year. As demonstrated in Table 1, the group of fast-growing vestibular schwannomas contained significantly larger tumors than the group of slow-growing vestibular schwannomas. For this reason, our findings might not only be based on tumor growth rate alone, and they may be a related to overall tumor size as well.

In this study, we only included evidently slow-growing tumors and evidently fast-growing tumors. In reality a significant proportion of vestibular schwannomas display a more intermediate growth rate. However, because of the explorative nature of this study, we chose to leave out this portion of intermediate growing tumors to maximize the chances of demonstrating or ruling out significant differences between the patient groups.

The degree of CD163-positive macrophages was significantly higher in the group of fast-growing tumors. These results indicate that M2 macrophages are associated with fast-growing vestibular schwannomas. The fact that the fast-growing tumors also displayed a higher degree of microvessel density combined with the fact that there was a significant relationship between macrophage expression and microvessel density supports the concept that M2 macrophages play a substantial role in vestibular schwannoma biology.

Several therapeutic strategies targeting macrophage activity at different levels are currently under investigation. There are studies aiming to inhibit the attraction and induction of M2 macrophages (23,33,34), whereas others investigate the possibility of redirecting macrophages with a tumor promoting M2 phenotype toward macrophages with an antitumor M1 phenotype (35). If these strategies prove to be effective, tumor-associated macrophages might become novel therapeutic targets for the treatment of vestibular schwannomas. However, before the inflammatory process can be considered a realistic target in vestibular schwannoma therapy, more extensive research on tumor-related effects of macrophages and their interactions with other elements of the immune system is required.

In conclusion, this study demonstrates that M2-type macrophage expression in vestibular schwannomas is associated with angiogenesis and volumetric tumor growth. These results imply that the M2-type macrophage infiltrate may contribute to the progression of these tumors making it a potential target for future pharmacotherapeutical therapy.

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