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Increased density of tumor associated macrophages is associated with decreased survival in advanced thyroid cancer

Mabel Ryder^{1,2}, Ronald A. Ghossein³, Julio CM Ricarte-Filho², Jeffrey A. Knauf^{1,2}, and James A. Fagin^{1,2}

¹Endocrinology Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, NY, New York.

²Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, NY, New York.

³Department of Pathology, Memorial Sloan Kettering Cancer Center, NY, New York.

Abstract

Thyroid cancers are infiltrated with tumor associated-macrophages (TAMs), yet their role in cancer progression is not known. The objectives of this study were to characterize the density of TAMs in well-differentiated (WDTC), poorly-differentiated (PDTC) and anaplastic thyroid cancers (ATC) and to correlate TAM density with clinicopathologic parameters. Immunohistochemistry was performed on tissue microarray sections from WDTC (n=33), PDTC (n=37) and ATC (n=20) using macrophage specific markers. Electronic medical records were used to gather clinical and pathologic data. Follow-up information of PDTC patients was available for 0–12 years. Nine of 33 WDTC (27%), 20/37 PDTC (54%) and 19/20 ATC (95%) had an increased density of CD68+ TAMs (≥ 10 per 0.28 mm²) ($p = 0.03$, WDTC vs. PDTC; $p < 0.0001$, WDTC vs. ATC; $p < 0.002$, PDTC vs. ATC). Increased TAMs in PDTC was associated with capsular invasion ($p = 0.034$), extrathyroidal extension ($p = 0.009$) and decreased cancer-related survival ($p = 0.009$) compared to PDTC with a low density of TAMs. In conclusion, the density of TAMs is increased in advanced thyroid cancers. The presence of a high density of TAMs in PDTC correlates with invasion and decreased cancer-related survival. These results suggest that TAMs may facilitate tumor progression. As novel therapies directed against thyroid tumor cell-specific targets are being tested, the potential role of TAMs as potential modulators of the thyroid cancer behavior will need to be considered.

Keywords

Tumor associated macrophages; Human thyroid cancer; CD68⁺ TAMs; Poorly-differentiated thyroid cancer; BRAF

Introduction

Infiltrating inflammatory cells are major constituents of tumor microenvironments, of which tumor associated-macrophages (TAMs) may comprise as much as 50% of the tumor mass. Understanding the functions of TAMs on tumor progression is complex due to the pleiotropic actions of macrophages on tumor progression (Lewis & Pollard 2006). Clinical studies of human breast (Bolat 2006; Leek *et al.* 1996; Tsutsui *et al.* 2005; Yu 2003), prostate (Lissbrant *et al.* 2000) and cervical cancers (Schoppmann *et al.* 2002) support a role of TAMs as tumor

Corresponding author's e-mail and contact information: Requests for reprints should be addressed to: Mabel Ryder, M.D. 1275 York Avenue, Box 296 New York, NY 10021 (646) 888–2713 Office (646) 888–2718 Fax ryderm@mskcc.org.

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promoters based on the association of increased density of TAMs with tumor vascularization, metastases and poor prognosis. Evidence for a key role of TAMs in cancer progression is further buttressed by the fact that selective depletion of TAMs in breast cancer mouse models disrupts transformation, progression and invasion (Lin *et al.* 2006). The abundance of TAMs in the various thyroid cancer histotypes is relatively understudied (Herrmann *et al.* 1994), and there is little information on their possible role on thyroid cancer progression. The goal of this study was to examine the presence of TAMs in several histological thyroid cancer grades and, in an unbiased manner, correlate the presence of increased TAMs with clinicopathologic outcomes. We found a strong association between TAM abundance and advanced histological grade. We further show that TAM density was strongly associated with tumor invasiveness and cancer-related mortality in PDTC.

Materials and Methods

Study Design

Immunohistochemical (IHC) analyses were performed on paraffin-embedded human tissue microarray sections from blocks of WDTC (n=33), PDTC (n=37), ATC (n=20) and corresponding non-neoplastic and non thyroiditis thyroid tissues (n=46) from WDTC (n=30) and PDTC (n=15) using two monoclonal antibodies which primarily label tissue macrophages: anti-CD68 KP-1 (pre-diluted according to the manufacturer; Ventana, Tucson, AZ), and anti-CD163 (1:100, Vector, Burlingame CA). Each tumor was represented by 3 tissue cores taken from randomly chosen fragments of the tumor (0.6 mm diameter per core). Of the 33 WDTC, 19 were classical PTC, 13 were follicular variant PTC (FVPTC) and 1 was a Hürthle cell carcinoma. Nine of 19 classical PTC and 0/13 FVPTC had an increased density of TAMs ($p = 0.004$). PDTC were defined as carcinomas showing follicular cell differentiation (at the histologic and/or IHC level i.e. positive for thyroglobulin) with tumor necrosis and/or ≥ 5 mitoses per 10 high-power fields (HPF, 400x). Positive controls included tonsillar tissue and giant cell tumors rich in histiocytes. The negative control consisted of the isotype mouse monoclonal control antibody (clone MOPC-1, Ventana), which showed no specific immunostaining. A single pathologist (R.A.G), who was blinded to the clinical assessments of each case, scored the immunostains by counting the number of CD68⁺ TAMs in each of the 3 tissue cores from each patient tumor sample (total core surface: 0.28mm²), and took the mean of three counts. Sections scored with ≥ 10 CD68⁺ TAMs/ 0.28 mm² were designated positive for a high density of TAMs and those with < 10 CD68⁺ TAMs/0.28 mm² were designated as negative.

Genotype

Genotyping for oncogenic *BRAF*^{v600e} was performed on WDTC and PDTC using 30 micron paraffin-embedded tissue sections. DNA was extracted using the Puregene DNA purification kit for 5–10mg tissue (Gentra). The presence of *BRAF*^{v600e} was determined by Sequenom analysis (primer and primer extension sequences are available upon request).

Statistics

Clinicopathologic data were recorded for each case in a blinded manner to the IHC staining results and approved by IRB protocol. Statistical analyses were performed using SPSS 14.0 for windows and GraphPad Prism 5.0. Noncontinuous variables were analyzed using Fischer's exact two-sided test and continuous variables were analyzed using unpaired two-sided T tests. Survival analyses were performed using Kaplan-Meier log-rank tests. Significance was defined as $p < 0.05$.

Results

TAM density in thyroid cancers of different histological grades

Of the 46 non-neoplastic thyroid tissue specimens, only 2 (4%) were positive for CD68 (mean number of CD68⁺ macrophages was <5). In tumors, the anti-CD68 antibody showed dense cytoplasmic staining of mononuclear and multinucleated giant type cells that morphologically resembled macrophages (small 'bean' shaped nuclei with a low nuclear: cytoplasmic ratio). In WDTC and PDTC, positively labeled cells were found within the lumen of the follicles (especially the multinucleated giant cells) and interspersed between the tumor cells. By contrast in ATC they were diffusely infiltrated throughout the core sections. The specificity of the stain was verified with anti CD163 (Figure 1). Nine of 33 WDTC (27%), 20 of 37 PDTC (54%) and 19 of 20 ATC (95%) were positive for an increased density of CD68⁺ TAMs (Table 1, $p=0.030$ WDTC vs. PDTC; $p<0.0001$ WDTC vs. ATC; $p=0.002$ PDTC vs. ATC). The mean number of CD68⁺ TAMs was <5 in WDTC, 9 in PDTC and 39 in ATC. In the majority of tumor samples from individual patients, there was a consistent number of TAMs between the 3 cores. Figure 1 shows representative sections of each histologic grade at low and high power magnifications.

Clinicopathologic correlations

As histologic grade is an important determinant of tumor behavior and clinical prognosis, it was important to explore the effect of TAMs on tumor progression within a particular histologic subtype, rather than comparing the impact of TAMs on clinicopathologic outcomes between grades. Although the number of WDTC with > 10 TAMs/0.28 mm² was comparatively low (9/33; 27%), there was no statistical correlation between presence of TAMs and extrathyroidal extension, capsular invasion and vascular invasion. However, the overall abundance of TAMs in the positive cases tended to be lower than that observed in the positive cases in PDTC, but the difference was not statistically significant. None of the patients with WDTC died of their disease (0/33) and few tumors had increased TAMs, whereas 18/20 patients with ATC died of thyroid cancer with a median survival of 3 months (data not shown), and all but one tumor had increased TAMs. Hence we could not explore the possible role of increased TAMs on the biological behavior and clinical outcomes in these tumor types. By contrast, patients with PDTC had variable clinical outcomes and 54% of these tumors had increased TAMs, making this histiotype the most suitable to examine the effects of increased TAMs on clinicopathologic parameters. Overall, patients with PDTC had a median cancer-related survival that ranged from 2.8 to 7.1 years. When stratified according to the presence of increased TAMs, PDTC with a higher density of TAMs were associated with increased capsular invasion ($p=0.034$), extrathyroidal extension (ETE) ($p=0.009$) and decreased cancer-related survival ($p=0.009$), compared to patients with a low number of TAMs (Table 2 & Figure 2). The mean cancer-related survival of patients with PDTC and increased CD68⁺ TAMs was 5.4 ± 0.8 years, compared to 9.7 ± 1.2 years for patients with a low abundance of TAMs ($p=0.009$). The mean cancer-related survival for patients with ETE was 5.7 ± 0.8 years compared to 11.0 ± 1.2 years for patients without ETE ($p=0.005$; Figure 2), consistent with published data that ETE is a negative prognostic marker in thyroid cancers (Andersen *et al.* 1995). When the TAM-related survival data was adjusted for ETE, the correlation between increased TAMs and decreased cancer-related survival was lost (data not shown). There were no differences between patients with or without increased CD68⁺ TAMs in gender, age, stage, or mitotic rate. Patients with increased CD68⁺ TAMs had a trend toward larger tumors, more extensive tumor necrosis, a higher frequency of distant metastases and increased FDG-avid PET (+) disease, but these comparisons were not statistically significant (Table 2).

In the 33 WDTC, 16 (48%) were positive for *BRAF*^{V600E}, 11 from tumors without increased TAMs and 5 from tumors with increased TAMs ($p=0.71$). Of the 37 PDTC, 6 were positive

for *BRAF*^{V600E}. This mutation was equally distributed between tumors with a low density of TAMs ($n=3$) and tumors with a high density of TAMs ($n=3$).

Discussion

Our study demonstrates, for the first time, that an increased density of TAMs is associated with tumor progression in advanced thyroid cancers. There is a remarkably strong correlation between increased TAMs and histologic grade, and between TAMs, tumor invasiveness and decreased cancer-related survival in PDTC. These results suggest that TAMs promote thyroid cancer progression. If correct, this represents a conceptually novel paradigm that has the potential to influence the development of experimental therapies for advanced disease.

The mechanisms by which TAMs are recruited to the tumor microenvironment in thyroid cancer are not known. Expression of oncoproteins involved in thyroid cell transformation may directly stimulate the recruitment of TAMs through increased expression of macrophage chemoattractants. Expression of oncogenic *BRAF* was found to be particularly potent at inducing their expression as compared to RET/PTC (Mesa, Jr. *et al.* 2006) suggesting that cancers with this mutation may be particularly prone to macrophage infiltration. In the present series, neither WDTC nor PDTC with *BRAF* mutations were preferentially associated with a high density of TAMs, indicating that other TAM recruiting factors are likely at play. However, because there was a low percentage of PDTC with *BRAF* mutations in this series, the relationship between TAMs and tumor genotype should be viewed as inconclusive in this tumor grade. Regardless of how they are recruited, TAMs secrete a rich repertoire of chemokines and growth factors that may exert paracrine effects on tumor cells to facilitate progression. For example, TAM-derived chemokines may directly enhance tumor cell growth (Mantovani 1994) and may indirectly influence thyroid cancer cell expression of chemokine receptors, such as CXCR4 (Castellone *et al.* 2004; Hwang *et al.* 2003), that are important for tumor spread. Secretion of matrix metalloproteases by TAMs can remodel the extracellular matrix, which in turn enables tumor cell mobility, migration and invasion at both local and distant sites (van Kempen & Coussens 2002). Decreased oxygen tension at necrotic sites may stimulate the recruitment of both phagocytic and angiogenic TAMs to scavenge debris and to stimulate tumor angiogenesis, respectively (Lewis *et al.* 2007). Activation of an angiogenic switch is an absolute requirement for tumor progression, and TAMs have been shown to trigger this process in breast cancer animal models (Folkman *et al.* 1989; Lin *et al.* 2006).

The remarkable functional plasticity of TAMs in tumor microenvironments may explain several observations in our study. The correlation between increased TAMs, capsular invasion, ETE and the trend toward distant metastases in PDTC suggests that TAMs are not merely prognostic markers of tumor progression, but may actively participate in the biology of the process. The fact that the density of TAMs and ETE are not independent variables for cause-specific survival suggests that these two events may indeed be functionally related. A study by Fiumara *et al.* found no association between the presence of TAMs and ETE, lymph node disease and distant metastases in WDTC (Fiumara *et al.* 1997). However, this study was limited to patients with low grade tumors that are generally associated with favorable outcomes. The presence of both increased TAMs and extensive tumor necrosis in all ATC (data not shown), and the trend towards this association in PDTC suggests that TAMs may also be involved in thyroid cancer angiogenesis. Indeed, Dhar *et al.* showed an association between increased TAMs and tumor vascularity in WDTC (Dhar *et al.* 1998).

There are presently vigorous efforts by many research groups to develop novel therapies for thyroid cancer that specifically interfere with signaling pathways activated by mutated oncoproteins. The findings in this paper may impact these efforts in at least two ways. First, if TAMs are important drivers of the biological behavior of advanced tumors, therapies that

do not target them may not be effective. Second, there is evidence that certain cytokines, such as tumor necrosis factor- α , which may be expressed by macrophages, can induce resistance to drugs that target the BRAF-MEK-ERK pathway by inhibiting apoptosis following BRAF inhibition (Gray Schopfer *et al.* 2007).

In summary, our results demonstrate that increased TAMs in high grade thyroid cancers are associated with invasive cancers and decreased cancer-related survival. This study underscores the importance of expanding our understanding of thyroid cancer progression from one that is narrowly focused on intrinsic oncogenic pathways to investigations that more accurately encompass the whole tumor microenvironment, including tumor-promoting TAMs.

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Figure 1.

Immunostain for tumor-associated macrophages (TAM) markers in tissue microarrays. The signal is golden brown. A: Very low density of CD68 + TAM per tissue core (0.28mm^2) in well differentiated papillary carcinoma, follicular variant B: High power of A (arrow indicates isolated TAM). C: Moderate density of CD68 + TAM in poorly differentiated thyroid carcinomas. D: High power of C (arrow indicates TAM). E: Very high density of CD68 + TAM in anaplastic carcinoma. F: High power of E (arrow points to CD68 negative anaplastic bizarre carcinoma cell surrounded by TAM). G: Same case as E,F showing very high density of CD163 + TAM. H. High power of G (arrow points to CD163 negative anaplastic bizarre carcinoma cell surrounded by TAM).

Figure 2.

Top: Kaplan-Meier log-rank cancer-related survival analyses of PDTC patients with or without increased TAMs ($p=0.009$). Bottom: Kaplan-Meier curve for patients with and without ETE at initial surgical presentation ($p=0.005$)

Table 1

Correlation between the density of CD68⁺ TAMs and tumor grade in human WDTC, PDTC & ATC. IHC was performed on tissue microarray sections composed of 0.6mm core biopsies. Positive cases were defined as 10 or more CD68⁺ TAMs per core (0.28 mm²).

Tissue Type	# of Cases	# Positive Cases (%)	Mean # CD68 ⁺ TAMs/ 0.28 mm ²	<i>p</i> value
Well-differentiated thyroid cancers (WDTC)	33	9 (27%)	<5	<0.0001 ^b 0.030 ^a
Poorly differentiated thyroid cancers (PDTC)	37	20 (54%)	9	0.002 ^c
Anaplastic thyroid cancers (ATC)	20	19 (95%)	39	

^aWDTC compared to PDTC

^bWDTC compared to ATC

^cPDTC compared to ATC

Table 2

Comparison of clinical and histopathologic parameters in patients with a low versus a high density of CD68⁺ TAMs in PDTC patients.

Variables	Density of CD68 ⁺ TAMs		<i>p</i> value
	Low (<i>n</i> = 17)	High (<i>n</i> = 20)	
Age	63 +/-4 (range 16–85)	61 +/- 4 (range 25–93)	0.760
Gender			
Male	4	9	0.300
Female	13	11	
Stage^a			
I	3	0	0.419
II	1	1	
III	4	2	
IV	9	12	
Tumor size, (cm)	4.3+/- 0.4 (<i>n</i> = 17)	5.4 +/- 0.6 (<i>n</i> = 14)	0.135
Necrosis^b			
Focal	8	4	0.149
Extensive	7	12	
Mitosis, # per high power field	4.0 +/-2.3 (<i>n</i> =12)	8.2 +/- 7.0 (<i>n</i> =16)	0.168
Capsular invasion			
Absent	5	0	0.034
Present	4	7	
No capsule	5	6	
Extrathyroidal extension			
Absent	10	2	0.009
Present	6	14	
Extrathyroidal vascular invasion			
Absent	8	4	0.128
Present	5	10	
FDG-PET			
No uptake	9	5	0.063
Positive uptake	4	12	
Distant metastases			
None	5	2	0.197
Present	9	15	

Variables	Density of CD68 ⁺ TAMs		<i>p</i> value
	Low (<i>n</i> = 17)	High (<i>n</i> = 20)	

^a Staging based on American Joint Committee on Cancer Staging manual, 6th edition.

^b Two tumors with a low TAM density and 3 tumors in the high TAM density group had no evidence of tumor necrosis For each variable, where n does not equal the total number of cases in the TAM group, there was insufficient or missing data from some cases in that particular category and therefore some cases were not included in the analysis.