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ORIGINAL ARTICLE

Prognostic implication of types of tumor-associated macrophages in Hodgkin lymphoma

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Abstract To evaluate roles of tumor-associated macrophages (TAMs) for prognosis of classical Hodgkin lymphoma (CHL). Expression of markers for TAMs, CD68, HLA-DR, CD163, HLA-DR/CD68 (M1), and CD163/CD68 (M2) was immunohistochemically examined in 82 cases with CHL. Positively stained cells were counted and correlation of number of TAMs and patients' survival time was analyzed. Number of CD163+ cells and M2 cells was significantly correlated with shorter overall survival ($P<0.05$), while it was marginally significant for CD68+ cells ($P=0.0827$). HLA-DR+cells and M1 cells showed no significant correlation with overall survival. When confined to mixed cellularity subtype, number of M1 cells was correlated with favorable prognosis ($P<0.05$), while M2 did not ($P=0.7$). Older age and male sex were unfavorable factors for prognosis. At multivariate analysis, number of CD163+ cells, M2+ cells, and age were independent factors for poor overall survival ($P=0.03$, 0.02, and 0.01, respectively). CD163+ cells and M2 cells might work to be tumor promotive in CHL. M1 cells might be tumor suppressive in mixed cellularity type.

Keywords Tumor-associated macrophages · M1 type · M2 type · Hodgkin lymphoma · Immunohistochemistry · Double staining · Prognosis

Introduction

Hodgkin lymphomas (HL) are histologically characterized by the presence of mono-(Hodgkin cells) and/or multinucleated Reed–Sternberg cells (RS cells) scattering in various amounts and kinds of inflammatory cells. Two different biologic entities are recognized within HL: nodular lymphocyte predominance (NLP) and classical HL (CHL) comprising nodular sclerosis (NS), mixed

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cellularity (MC), lymphocyte-rich (LR), and lymphocyte depletion (LD) type. With the advance in the treatment for HL in the past few decades, 5-year survival rate in the favorable prognosis group reached to 98%, while that in the unfavorable group was at least 85% [1]. Assessment of stage of disease is a prerequisite for appropriate management of HL. For this, the Ann Arbor staging and the International Prognostic Factors Project on Advanced HL were established [2]. Even with advances in therapeutic modalities and accurate assessment of stage, however, prediction of outcome might be difficult in some cases of HL.

Roles of tumor-associated macrophages (TAMs) for tumor behaviors have attracted attention of researchers [3–7]. Recent study showed that estimation of amount of TAMs in lesional tissues, as revealed by immunohistochemical detection with CD68, could be used for prediction of prognosis of CHL [4]. TAMs recognize tumor-associated antigens and stimulate cytotoxic T lymphocytes to activate the anti-tumor immune responses. In contrast, some TAMs could induce immune tolerance and enhance progression and metastasis of tumors or stimulate angiogenesis and invasion into extracellular matrix [5, 6].

The most common marker used for immunohistochemical detection of TAMs is CD68, which is expressed in a wide range of monocytes/macrophages or dendritic cells. TAMs are polarized into two extreme forms, M1 and M2, which represent the major inflammatory components in many tumors. M1 TAMs secrete different types of chemokines and cytokines such as interleukin (IL)-1, IL-12, IL23, tumor necrosis factor-α (TNF-α), human leukocyte antigen (HLA)-DR, nitrogen intermediates and reactive oxygen. M1 TAMs are characterized by their ability to promote tumor regression response. On the other hand, M2 TAMs promote tumor progression and metastasis. M2 TAMs express considerable amount of CD206 (mannose receptor), CD204 (scavenger receptor A), IL-1, IL-10, CC ligand 22 (CCL22), and CD163 [7, 8].

To examine whether prognosis of patients with HL could be predicted based on the appearance of M1 or M2 TAMs, double staining with use of HLA-DR/ CD68 (M1 macrophages) and CD163/CD68 (M2 macrophages) were performed in 82 cases of CHL.

Materials and methods

Patients

During the period from November 1999 to April 2010, a total of 4,800 cases with lymphoproliferative diseases were registered with the Osaka Lymphoma Study Group (OLSG), Osaka, Japan. Histologic specimens obtained by biopsy were fixed in 10% formalin and routinely processed for paraffin embedding. Histologic sections cut at 4 μm were stained with hematoxylin and eosin and immunoperoxidase procedure (ABC method). All of the histologic sections were reviewed by one of the authors (KA), and classified according to the WHO classification. The diagnosis of malignant lymphoma was confirmed in 3,826 (79.7%) of 4,800 cases. Of these 3,826 cases, 3,508 (91.7%) were NHL and 318 (8.3%) HL. Among HL cases, 26 cases were NLP and 260 CHL, which comprised 164 cases of MC, 77 NS, 32 LR, 15 LD, and unspecified in four. Eighty-two cases of CHL, in which adequate clinical data were available, were selected for the present study. They included 52 cases of MC, 20 NS, six LR, and four LD cases. Clinical characteristics of these cases are summarized in Table 1.

Immunohistochemistry (IHC)

Monoclonal antibodies used for IHC were CD20, CD3, CD30 and CD68 (Clone: PG-M1) (Dakocytomation, Glostrup, Denmark, 1:400, 1:50, 1:50 and 1:50, respectively), HLA-DR (Medical & Biological Laboratories, Nagoya, Japan, 1:50), and CD163 (Novocastra-Leica, Wetzlar, Germany, 1:50).

Immunohistochemical double staining of HLA-DR/CD68 and CD163/ CD68 was performed using En vision™ G|2 double staining kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions. The same method for antigen retrieval (Dako Pascal to heat) was employed in single and double staining. Primary antibodies HLA-DR or CD163 were labeled with brown color using DAB+and CD68 with red color by using permanent red. According to the previous study [6, 8], the average number of positively stained cells in six representative fields (0.146 mm^2) of tissue section, where the number of stained cells was the largest, was counted by the two investigators (NW and MAAZ) in a blind fashion. The number of Hodgkin–RS cells was rather similar in the fields selected and not counted. The cutoff point was set at the median number of positive cells.

Statistical analysis

Overall survival rates were calculated with the Kaplan–Meier method, and survival curves were compared by the logrank test. Multivariate analysis was performed with the Cox proportional hazard regression model.

Results

Clinical findings

Clinical findings are summarized in Table 1. Age of the patients in all CHL cases ranged from 15 to 85 (mean, 54) years with a male to female ratio of 55:27. Tumor stage was

Table 1 Clinical characteristics of 82 patients

Disease	Case no.	Age range (mean)years	Male/female	Stage (n=79)	
				I–II	III–IV
Mixed cellularity	52	16–84 (58)	17:9	27	24
Nodular sclerosis	20	17–78 (40)	13:7	12	6
Lymphocyte-rich	6	26–76 (55)	5:1	6	0
Lymphocyte-depleted	4	64–85 (74)	3:1	2	2
Total	82	15–85 (54)	55:27	47	32

evaluated in 79 cases according to the Ann Arbor staging system: 40.5% of the cases showed advanced diseases (Stages III and IV). Most patients with early stage (I and II) are treated with radiotherapy and/or combination chemotherapy, while those with advanced stage (III and IV) are treated with combination chemotherapy. The standard combination chemotherapy used with most of the patients is *ABVD* regimen (adriamycin, bleomycin, vinblastine, and dacarbazine).

Follow-up periods for survivors ranged from 8.8 to 115 (mean 47.5) months. Twenty-three (28%) patients died.

Histopathological findings

RS cells were positive for CD30 and either negatively or weakly positive for CD20 in two of 52 cases of MC, two of six cases of LR, and one of four cases of LD. Generally, M2 cells (CD163/CD68) predominated M1 cells (HLA-DR/CD68) in every subtype of CHL (Fig. 1). The number of single positive cells for HLA-DR (mean=150) was larger than that for CD163 (mean=104), and that was the smallest for CD68 (mean=72.2). The median number of TAMs was used as the cutoff value: number of TAM, clinicopathological parameters, and prognosis of patients are summarized in Table 2.

Univariate analysis

The number of CD163+ cells showed a significant correlation with shorter overall survival (P=0.03). In contrast, the number of HLA-DR+cells had no significant correlation with overall survival (P=0.6191), while CD68+ cells showed a marginal significance (P=0.0827). At double staining, the number of HLADR+/CD68+ (M1) cells showed no significant correlation with prognosis (P=0.255), while patients with a larger number of CD163+/CD68+ (M2) cells showed less favorable prognosis than those with smaller number (P<0.05) (Fig. 2).

Prognostic significance of appearance of CD68, HLA-DR, CD163, M1, or M2 TAMs was evaluated by stage. Overall survival rates in patients with low TAM (CD68, HLA-DR, CD163, M1, or M2) and high TAM group were estimated by early stage (stages I and II, 47 cases) and advanced stage (stages III and IV, 32 cases) with the

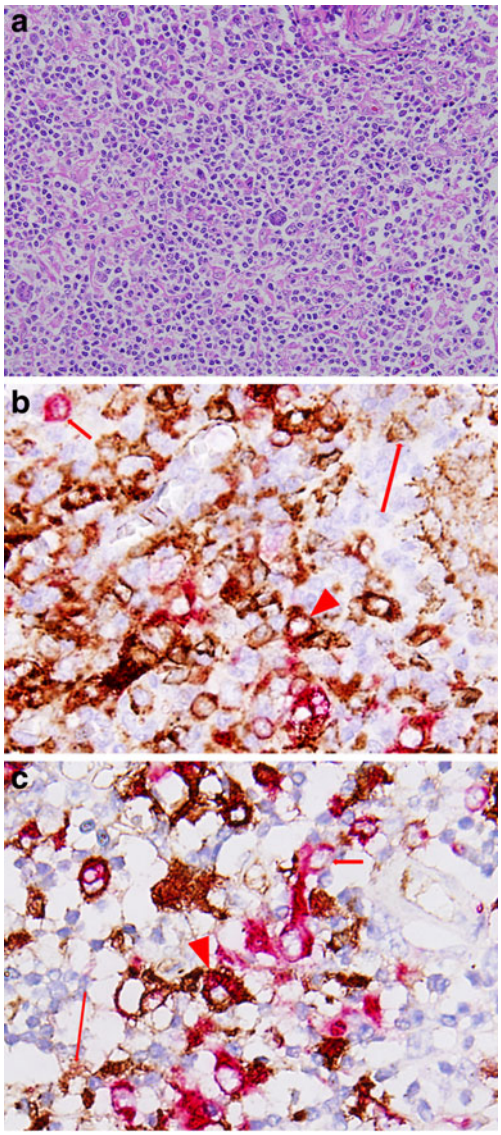

Fig. 1 Mixed cellularity subtype of classical Hodgkin lymphoma. **a** Multinucleated Reed–Sternberg cells (RS cells) are admixed with numerous inflammatory cells including macrophages, lymphocytes, eosinophils, and plasma cells. Hematoxylin and eosin stain. ×400. **b** Single positive cells for HLA-DR (brown) [*long arrow*], and CD68 (red) [*short arrow*], double positive cells for HLA-DR and CD68 (brown and red) [*arrow head*]. **c** Single positive cells for CD163 (brown) [*long arrow*] and CD68 (red) [*short arrow*], double positive cells for CD163 and CD68 (brown and red) [*arrow head*]

Table 2 Tumor-associated macrophages, clinicopathological parameters and prognosis of patients

	Cases no.	Mean of TAM* (range)	Median (Cutoff value)	Univariate analysis			Multivariate analysis
				Histological subtype			
				MC	NS	Total of all subtypes	
TAMs							
CD68	82	72.2 *(6–235)	60.3	0.5358	0.21	0.0827	0.0728
HLA-DR	82	150 *(4–797)	111.3	0.2578	0.506	0.6191	–
CD163	82	104.7* (3–427)	93.8	0.2824	0.198	0.03	0.0308
M1	82	17 *(0–101)	9.6	0.03862	0.151	0.255	–
M2	82	44.5 *(2–245)	31	0.6991	0.26	0.048	0.0258
Sex							
Male	55				P=0.0635		0.0728
Female	27						
Age			59 years				
≥59	44				P=0.0131		0.0131
<59	37						
Stage							
I–II	47				P=0.2071		–
III–IV	32						

TAM tumor-associated macrophages, *MC* mixed cellularity, *NS* nodular sclerosis, *LR* lymphocyte-rich, *LD* lymphocyte depletion

* Mean number of TAMs in six high power fields

Kaplan–Meier method, and survival curves were compared by the logrank test. Prognosis of high CD163 group was significantly worse than that of low CD163 group in early (P=0.036) but not advanced stages.

MC subtype was the major group of this study, and statistical analysis revealed significant correlation between the number of M1 cells and prognosis of patients (P<0.05), while M2 did not (P=0.6991). Patients with a larger number of M1 cells showed more favorable prognosis than those with a smaller number. In NS subtype, any significant correlation between the number of TAM and prognosis was not found. Older age and male sex were unfavorable factors for prognosis (P=0.013 and P=0.06, respectively). There was no significant correlation between stage of disease and prognosis (P=0.2).

Multivariate analysis

The number of CD163+ cells, CD163+/CD68+ (M2) cells, and age proved to be independent factors for prognosis of HL (P=0.0308, 0.0258, and 0.0131, respectively). While CD68+ cells and sex showed marginal significance (P=0.0728).

Discussion

Hodgkin lymphoma (HL) is a disease principally involving lymphoid tissues, and characterized by the presence of RS cells admixed with various amounts of reactive inflammatory cells. The incidence of HL varies according to geographical location, age, and the socioeconomic class. It comprises less than 10% of all malignant lymphomas in Japan, but is more common in Western countries (40–45%). Males predominate females in most of the countries. The incidence increases at the fourth decade of life in Japan, while showing a bimodal pattern in Western countries, with first peak at 15–35 years and second at over 55 years old. The incidence of MC type is highest in Japan, while NS is the commonest in Western countries [9, 10].

It is well known that TAMs represent a major component of inflammatory cells in many kinds of tumors such as lymphomas, melanoma, and carcinomas [3–8]. Several studies suggested the association between number of TAMs and the prognosis of patients with tumors. Dual role of TAMs, either tumor regressive (M1) or progressive (M2), is considered to be generated by their immune responses against tumors [11–13]. Expression of CD68, HLA-DR, and CD163 is commonly used for identification of TAMs, although these molecules are also expressed in other kinds of cells, i.e., CD68 in Langerhans cells and immature CD1a-positive dendritic cells, HLA-DR in peripheral lymphocytes, and CD163 in some dendritic cells. [14–16]. Then, we performed double immunohistochemical staining of HLA-DR/CD68 and CD163/ CD68 to specifically identify the M1 and M2 TAMs.

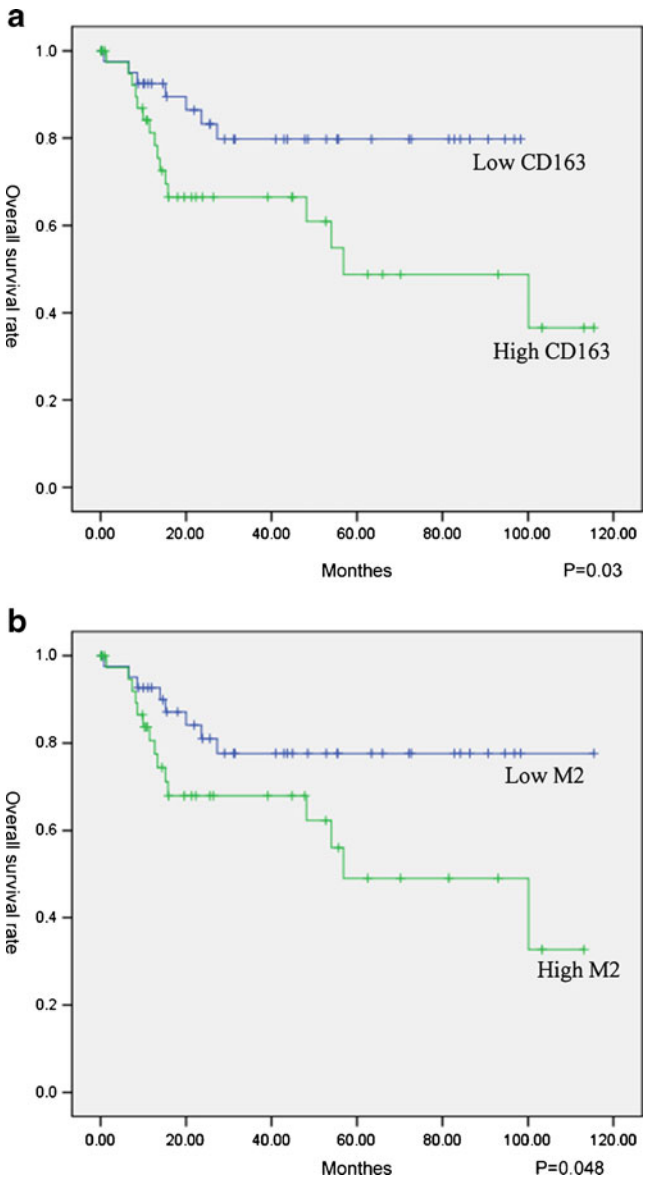

Fig. 2 **a** The overall survival rate in patients with higher number of CD163+ cells was significantly worse than that of lower number (p=0.03). **b** The overall survival rate in patients with higher number of CD163+/CD68+ cells was significantly worse than that of lower number (p<0.05)

At the single immunostaining, the number was highest for HLA-DR+cells, while lowest for CD68+ cells. The number of cells positive for CD68+ was smaller than that positive for CD163+ is in agreement with the previous studies [7, 17, 18], showing count numbers of tumor-infiltrating CD68+ macrophages were lower than those of CD163+ macrophages in HL and other malignancies such as malignant melanoma and leiomyosarcoma. At the double immunostaining, the number of M2 (CD163+/CD68+) cells was higher than that of M1 (HLA-DR+/CD68+) cells. These findings suggest that HLA-DR+cells were less labeled with CD68 than CD163, or HLA-DR might be expressed in a wider range of cells other than TAM. In some cases, CD163 expression seems to be relatively confined to CD68+ TAMs, because only a few cells were single positive for CD163+ at double staining.

A number of CD163+ cells or M2 type TAMs in the tumor tissues were associated with unfavorable prognosis of patients (P=0.03 and P=0.048 respectively). The number of CD68+ cells showed a marginal significance for prognosis in the present study (P=0.0827). These results are roughly in agreement with the recent studies. Steidl et al. [4] reported a strong association between the increased number of CD68+ cells and the poor prognosis of CHL defined by the disease-specific survival in both univariate and multivariate analyses but not by progression-free survival. They used gene expression profiling in an independent cohort of 166 cases of CHL. Kamper et al. [17] reported that the number of CD68+ or CD163+ cells correlated with the shorter event-free survival at the univariate analysis, while CD68, but not CD163, was significantly correlated with overall survival at the multivariate analysis. They constructed the tissue microarray, and then 288 cases were evaluated immunohistochemically. The slight discrepancies found between the present study, Steidl's, and Kamper et al.'s studies might be due to differences in methodologies employed, number of cases examined, or different characteristics of diseases themselves.

Multivariate analysis revealed that the number of CD163+ cells and M2 cells was an independent factor for unfavorable prognosis in CHL. In contrast, the number of HLA-DR+cells had no significant correlation with overall survival (P=0.6191). Older age proved to be an independent factor for poor prognosis of CHL (P=0.0131) at both univariate and multivariate levels. Steidl et al. [4] and Kamper et al. [17] also reported that older age correlated with the adverse prognosis. While male sex showed marginal significance for poor prognosis (P=0.06). Kamper et al. reported a significant correlation of sex with overall (P=0.05) but not with the event-free survival, but Steidl et al. reported no significant correlation with sex.

Interestingly, the number of M1+ cells but not HLA-DR+ cells was significantly correlated with favorable prognosis of patients with MC type (P=0.03), which comprised 63.4% of the current cases. This finding showed the importance of studying characteristics of CHL by each histological subtype. There have been no reports describing effects of M1 cells for prognosis of patients with CHL. Kamper et al. [17] reported the higher expression of CD68 and CD163 in TAMs of MC compered to NS subtypes.

In conclusion, the present study suggests practical implication for counting M2-TAMs, as defined CD163+ or CD163+/CD68+ cells in prediction of prognosis of patients with CHL. The number of M1-TAM correlated with prognosis of patients with MC type, suggesting further

study on prognostic roles of M1, M2-TAMs in each histological subtype of CHL.

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