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RESEARCH

Ectopic lymphoid structures in primary cutaneous melanoma

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Abstract Ectopic lymphoid structures have been described in several tumor types including metastatic lesions, but not primary tumors, of patients with melanoma. Here we present evidence of B-cell follicles in primary cutaneous melanomas, being present in 39 of 147 cases (27 %). B-cell clusters were associated with T lymphocytes, most of which belonging to $CD45RO^+$ memory T cells. A network of $CD21^+$ follicular dendritic cells was demonstrated in 8 of 22 cases studied (36 %). MECA-79^+ HEV-like venules were observed in the neighborhood of the follicles in the majority of cases, however, their presence was not confined to tumors hosting ectopic lymphoid structures. The appearance of B-cell aggregates did not show association with the outcome of the disease, although a trend for their higher prevalence was observed in thicker tumors. Our results show that neogenesis of lymphoid structures does occur in primary melanomas, albeit with lower frequency compared to that reported in metastases.

Keywords Ectopic lymphoid structures · Primary melanoma · B cells · T cells · Follicular dendritic cells · High endothelial venules

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Introduction

Priming of antitumor T-cell responses is generally believed to take place primarily in draining lymph nodes via cross presentation of tumor antigens by dendritic cells (DCs) capturing antigens at the primary tumor site and migrating to lymph nodes. However, experimental studies have shown that T-cell activation by dendritic cells may occur extranodally, at tumor sites [10, 21]. Tertiary lymphoid structures described in lung carcinoma, composed of B lymphocytes organized in follicles, adjacent T-cell clusters associated with mature DCs and high endothelial venules (HEV) [6, 7], are hypothesized to facilitate the interactions of tumor antigens, antigen presenting cells and T cells. Ectopic lymph node-like structures or B-cell follicles have been observed in a number of tumor types such as colorectal, breast and ovarian carcinoma [4, 5, 17, 19, 20]. In some of these cases the presence of $CD21^+$ follicular dendritic cells (FDCs) in B-cell follicles, resembling ectopic germinal centers, was also mentioned [4, 5, 7, 17]. Moreover, two recent studies found similar structures in melanoma metastases [3, 14]. In one of these works, Cipponi et al. also investigated samples from primary melanomas but failed to detect CD21L expression by RT-PCR and either $CD21^+$ FDCs or follicle-like structures by immunohistochemistry (in 25 and 10 samples, respectively) [3]. The authors concluded that primary melanomas do not host follicles, although many of them contained T-cell infiltrate, mature dendritic cells and

high endothelial venules, indicating an incomplete lymphoid neogenesis [3, 23].

In contrast to the above finding, in a recent publication on the prognostic impact of melanoma-infiltrating B lymphocytes [11], we reported the presence of lymphoid structures resembling follicles in a larger series of primary cutaneous melanomas. By immunohistochemical detection of CD20 expression, B cells clustered in dense aggregates were observed in 28 of 106 primary melanoma samples (26 %) [11]. The present study, extending the case number investigated, aimed at characterization of cell types involved in these cell clusters and at evaluating the association of their presence with clinicopathological parameters and the outcome of the disease.

Materials and methods

Patient characteristics

Archival tissue samples were obtained from 147 patients with primary cutaneous melanoma. In 101 of these patients, operated between 1980 and 2000 at the National Institute of Oncology or at the Institute of Dermato-Venerology, Semmelweis University, Budapest (97 stage I–II, 4 stage III), no sentinel node biopsy was performed, while in a second cohort of 46 patients operated between 1999 and 2001 at National Institute of Oncology, sentinel node biopsy proved negative in 30 and positive in 16 cases. None of the patients received any antitumor treatment prior to surgery. All surviving patients had follow-up data for at least 5 years. Clinicopathological characteristics are summarized in Table 1. Seventy-six patients had no metastases developed during the follow-up period, while 21 patients had metastases confined to regional lymph nodes, which were excised. Fifty patients developed distant visceral metastases. The 5-year survival of patients in both the nonmetastatic and the lymph node metastatic groups was 100 %, while only 3 patients developing distant visceral metastases survived for more than 5 years.

Immunohistochemical detection of immune cell types

Three-μm sections from formalin-fixed, paraffin-embedded cutaneous melanoma samples were used. Immunohistochemistry was performed as described earlier [11, 12], using monoclonal antibodies against CD3, CD20cy, CD45RO (all from Dako, Glostrup, Denmark), CD21 (BioGenex, Fremont, CA), AID (Invitrogen Corporation, Camarillo, CA), or DC-LAMP (CD208) (Beckman Coulter-Immunotech, Marseille, France), followed by polymer-conjugated secondary antibody (SS^{TM} One-Step Polymer-HRP IHC Detection System, BioGenex). For staining with anti-MECA-79 rat monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX), biotinylated anti-rat IgG (Vector Laboratories, Burlingame, CA) was used as secondary reagent, followed by Streptavidin HRP (Chemicon International, Temecula, CA). Antibody binding was visualized with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories), then the slides were counterstained with hematoxylin.

Statistical analysis

Associations of the presence of B-cell follicles with clinicopathological parameters were evaluated using the χ^2 test. Analysis of survival was performed by the Kaplan–Meier method, and the statistical analysis was carried out by the Mantel–Cox test. Statistics were calculated using the BMDP Statistical Software Pack.

Results

By immunohistochemical examination of 147 primary melanoma samples, we found B-cell aggregates, ranging in size from approximately 0.1 to 0.8 mm, in 39 cases (27 %). No difference was observed in the presence of these structures between the two patient cohorts (see in Materials and methods), or between stage I–II and stage III cases.

Samples found to contain B-cell aggregates were further studied in order to characterize cell types associated to these structures. CD3$^+$ T lymphocytes, most of which belonging to memory T cells expressing CD45RO (Fig. 1), were regularly found to be associated with B-cell clusters. The presence of CD21$^+$ follicular dendritic cell network (Fig. 1) was demonstrated in 8 of 22 cases studied (36 %). In some cases a few cells expressing AID (activation-induced cytidine deaminase, the enzyme responsible for class switch recombination and somatic hypermutation of immunoglobulin genes) could also be observed (Fig. 2). Morphologically, these cells resembled interfollicular large B cells that were shown to express AID in secondary lymphoid organs as well as in ectopic lymphoid tissues in Sjögren syndrome [2, 16]. Mature dendritic cells expressing the DC-LAMP marker were also found associated with the T-cell clusters in some cases, but they were also observed outside the ectopic lymphoid structures. Furthermore, MECA-79 staining revealed HEV-like venules in the vicinity of follicles in the majority of cases studied (14/18, 78 %) (Fig. 1), but their presence was not restricted to tumors with ectopic lymphoid structures.

Analyzing the appearance of B-cell aggregates with regard to clinicopathologic parameters revealed a higher prevalence in tumors of axial location, especially in those of the head and neck region (6/10, 60 %). Furthermore, a trend for higher frequency of cases with follicle-like structures was observed in thicker melanomas, while no significant association with other patient or tumor characteristics was noted (Table 1).

Table 1 Association of the presence of B-cell follicles with clinicopathologic parameters

	No. of patients	Presence of B-cell aggregates	Significance
		No. (%)	
All patients	147	39 (27)	
Age			
≤53 years	72	16 (22)	
>53 years	75	23 (31)	n.s.
Sex			
Male	66	22 (33)	
Female	81	17 (21)	n.s.
Location			
Extremities	61	10 (16)	
Axial (trunk+head or neck)	86	29 (34)	p=0.0190
Histological type[1]			
SSM	91	21 (23)	
NM	51	18 (35)	n.s.
Thickness (mm)			
≤2.0	55	9 (16)	
2.01–4.0	55	16 (29)	
>4.0	37	14 (38)	p=0.0630
Ulceration			
Present	64	21 (33)	
Absent	83	18 (22)	n.s.
Metastatic pattern (5-year)			
Nonmetastatic+LN metastatic	97	26 (27)	
Visceral metastatic	50	13 (26)	n.s.
Survival			
>5 years	100	27 (27)	
<5 years	47	12 (26)	n.s.

[1] ALM (4) and LMM (1) cases are not shown. n.s., not significant; SSM, superficial spreading melanoma; NM, nodular melanoma; ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; LN metastatic: metastases only in regional lymph nodes

Evaluating the prognostic relevance of the presence of B-cell clusters using Kaplan-Meier analysis yielded no significant association with survival of the melanoma patients, and there was no difference in the metastatic pattern or in 5-year survival rate of patients with or without lymphoid follicles (Table 1).

Discussion

The results of the present work confirmed our previous finding [11] that primary skin melanomas contain ectopic lymphoid structures in approximately one-quarter of cases. In a recent investigation Cipponi et al. failed to detect such structures by immunohistochemistry in 10 cases of primary melanoma [3]. Parameters of the tumors used in that study were not included in the article. It is possible that, beside the difference in sample size, differences in the distribution of tumors according to Breslow index could account for the discrepancy between the two studies since we found a trend for higher prevalence of follicle-like structures in thicker melanomas. It has been proposed that only cases belonging to the rare subset of desmoplastic/neurotropic melanomas are known to contain lymphoid structures [3]; in this regard it is worth noting that all primary tumors with B-cell follicles felt into the common histologic subtypes of superficial spreading or nodular melanomas in our study.

Similarly to observations on other tumor types and on melanoma metastases [3–7, 14, 17, 19, 20], B-cell clusters in primary melanoma were associated with T lymphocytes, and a network of $CD21^+$ follicular dendritic cells was demonstrated in about one-third of samples studied. In some cases, DC-$LAMP^+$ mature dendritic cells could also be noticed within the T-cell clusters. Their presence, however, was not restricted to the ectopic lymphoid structures, unlike in the case of lung cancer, described by Dieu-Nosjean et al. [7]. In lung cancer, high endothelial venules were also found exclusively associated with ectopic lymphoid tissues [6]. In our study MECA-79 staining showed HEV-like venules in the neighborhood of the follicles in the majority of primary melanoma cases studied, however, their presence could also be demonstrated in lymphocyte-rich areas of tumors that did not host ectopic

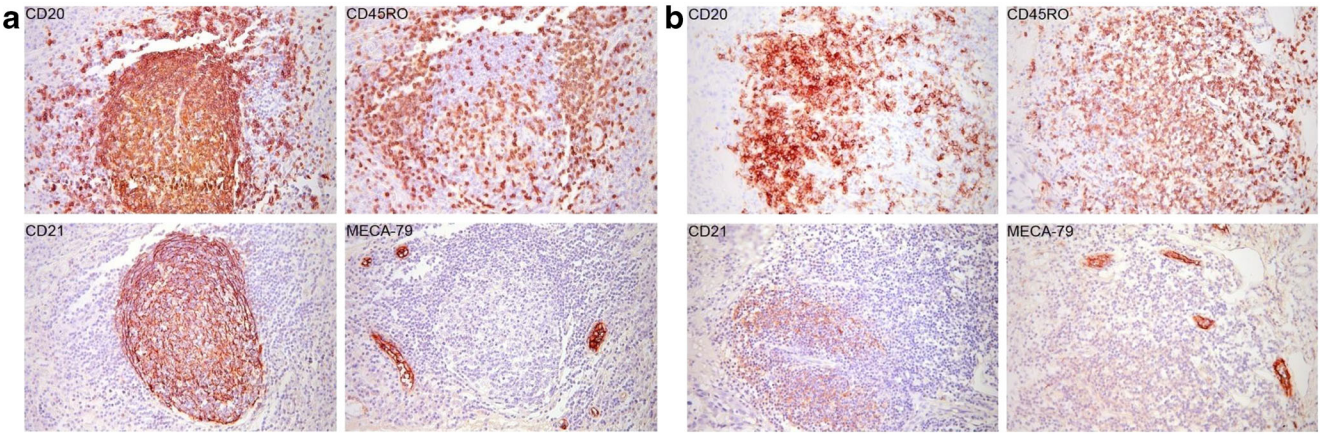

Fig. 1 Representative ectopic lymphoid structures in primary melanomas (**a**: Breslow 5.6 mm, nodular melanoma; **b**: Breslow 2.0 mm, superficial spreading melanoma). Immunohistochemical staining for CD20, CD45RO, CD21 and MECA-79. Pictures were taken using 10x objective

lymphoid structures, similarly to others' observations on primary melanoma [3, 13].

The functional and clinical significance of ectopic lymphoid structures in tumors is at present not clear. With regard to functionality, it has been suggested that ectopic lymphoid structures in tumor tissues may play a role in the in situ development of antitumor immune response, but few studies provided proofs for their functional significance. Cipponi and coworkers, analyzing the repertoire of rearranged immunoglobulin genes in microdissected follicles, demonstrated clonal amplification, somatic hypermutation and isotype switching, characteristics of antigen-driven B-cell response [3]. On the other hand, oligoclonal B-cell expansion has been documented in studies applying immunoglobulin gene analysis on tumor tissues from several types of cancer, irrespective of the presence of ectopic lymphoid structures [5, 17, 18].

Few studies have addressed the question how the appearance of these structures influence the outcome of the disease. In primary colorectal carcinoma and metastatic melanoma the presence of lymph node-like structures, and an associated 12-gene chemokine gene signature, correlated with overall survival; these analyses, however, included only limited numbers of patients (10 and 21 cases, respectively) [4, 14]. In the study of Dieu-Nosjean et al. on pulmonary cancer, the density of DC-LAMP$^+$ mature dendritic cells, which were exclusively detected in the T-cell areas of intratumoral lymphoid structures, was associated with clinical outcome [7]. The amount of mature DCs was found to correlate with favorable prognosis in several tumor types, however, in most cases no selective homing to ectopic lymphoid structures has been noted, although they were predominantly located in T-cell rich areas at the invasive margin of the tumors, forming clusters with T lymphocytes [1, 8, 15, 20, 22]. In our previous study on primary cutaneous melanoma, high peritumoral density of DC-LAMP$^+$ cells was also correlated with longer survival [12], a finding corroborated by another research team [9]. These cells were mostly found present within lymphocytic infiltrates [9, 12], however, no preferential location to organized lymphoid structures was noticed [12]. Furthermore, although the density of B lymphocytes proved of prognostic importance in our study on primary melanomas [11], the presence of B-cell aggregates showed no significant association with the patients' survival.

Taken together, it appears that neogenesis of lymphoid structures does occur in primary melanomas, albeit with lower frequency compared to that reported in cutaneous metastases. The presence of B-cell follicles did not show association with the outcome of the disease, although a trend for their higher prevalence was observed in thicker tumors. The potential application of determining the presence of ectopic lymphoid structures in predicting clinical response to immune-based therapies awaits further investigation.

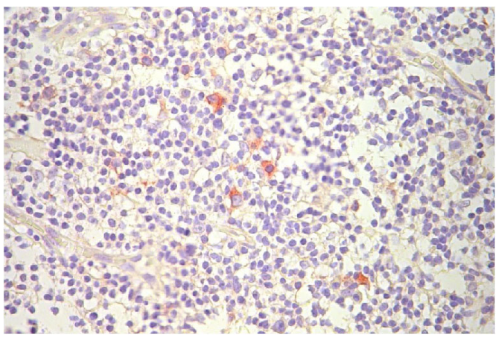

Fig. 2 AID staining of a few cells within the ectopic lymphoid structure shown in Fig. 1b. The picture was taken using 20x objective

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