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Presence of mature DC-Lamp$^+$ dendritic cells in sentinel and non-sentinel lymph nodes of breast cancer patients

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Abstract

Aim: Our study examined differences in the presence of mature, DC-Lamp$^+$ DC in the SLN and non-SLN according to the extent of metastatic involvement.
Patients and methods: Paraffin blocks of the SLN and non-SLN from patients with primary breast cancer who had undergone SLN biopsy and axillary dissection were separated into three groups: (Group A) no tumor cell involvement in the SLN and non-SLN; (Group B) isolated tumor cells or micrometastases in the SLN, and tumor cell-free non-SLN; and (Group C) macrometastases in the SLN. One section of all the SLN and non-SLN was examined with immunohistochemistry using an anti-DC-Lamp-antibody. The densest area occupied by the DC-Lamp$^+$ cells on each slide was quantified and recorded by an electronic imaging system. In this regard, the SLN and non-SLN were compared within the patients of each group using the Wilcoxon signed rank-test ($p < 0.05$).
Results: One hundred and fourteen SLN and 1258 non-SLN from 79 patients were examined. A significantly larger area was occupied by the DC-Lamp$^+$ cells in the SLN compared to the non-SLN in Groups A ($p = 0.024$) and B ($p = 0.009$), whereas no significant difference was found within Group C ($p = 0.107$).
Conclusions: This study suggests that the DC-dependent immune response is altered during the process of metastasis formation and is primarily activated before and during formation of micrometastasis.

Keywords: Sentinel lymph node; Breast cancer; Dendritic cells; Immunology; Micrometastases

Introduction

The sentinel lymph node (SLN) has become increasingly attractive for immunologic research. The SLN, as the lymph node with the highest probability for metastatic involvement,[1] is a promising site for immunologic studies to investigate immunological alterations during the development of lymphatic tumor spread.

Available studies have already described evidence for immunologic alterations of the SLN compared to the non-SLN in patients with breast cancer and melanoma. Whereas some authors have described evidence for immunosuppression in the SLN as a pre-condition for metastatic involvement,[2–4] other authors have described upregulation of the parameters that are usually associated with an enhanced immune response.[5–8] Table 1 gives an overview of the available studies on that topic.

Summarizing the available data, there is accumulating evidence that the SLN is a site of altered immunoactivity. However, the significant differences among the evaluated parameters and the variable extent of metastatic lymph node involvement in the study patients rendered the

Abbreviations: SLN, sentinel lymph node; DC, dendritic cells; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection; FFPE, formalin-fixed paraffin-embedded.

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Table 1
Summary of studies on immunologic aspects in SLN and non-SLN

Author (year)	Disease	Design	Main results
Cochran et al. (2001)[2]	Melanoma	SLN *vs.* non-SLN	Reduction of IDC in the paracortical area of SLN
Huang et al. (2000)[3]	Breast cancer	SLN* *vs.* non-SLN*	Reduction of S-100$^+$ HLA class II-expression$^+$ IDC in the paracortical area of the SLN; predominance of immature, non- or poorly differentiated DC
Essner and Kojima (2001)[4]	Melanoma	SLN− *vs.* non-SLN−	Reduction of CD80, CD86, CD40, CTLA-4, and CD28 in the SLN
Leong et al. (2002)[6]	Melanoma	SLN−*vs.* non-SLN−	Upregulation of IL-10, IFN-γ, IL2, GM-CSF in the SLN
		SLN mic *vs.* non-SLN−	No increase of cytokine secretion in the SLN
Poindexter et al. (2004)[5]	Breast cancer	SLN − *vs.* SLN+	Increase of IL-10 in SLN; no difference with respect to CD3, CD1a, CD83, MHC class II, and IL-12
Lee et al. (2005)[7]	Melanoma	SLN* *vs.* non-SLN*	Increase of IL-10, IFN-γ, and IDO mRNA in the SLN
		SLN *vs.* non-SLN−	No difference
		SLN (rhGM-CSF+) *vs.* SLN (rhGM-CSF−)	Larger T-cell and DC-areas; increased DC-density
Botella-Estrada et al. (2005)[8]	Melanoma	SLN* *vs.* non-SLN*/SLN− *vs.* non-SLN−	Increase of tyrosinase−, COX-2−, IFN-γ, IL-10−, GM-CSF-mRNA in SLN
		SLN mic *vs.* SLN−/non-SLN−	Increase of GM-CSF mRNA in SLN mic *vs.* non-SLN−
		SLN− *vs.* non-SLN−	Increase of S-100$^+$ and CD1a$^+$ DC
Movassagh et al. (2004)[12]	Melanoma	SLN+ and non-SLN− *vs.* SLN+ and non-SLN+	Increase of DC-Lamp$^+$ cell density in the SLN of patients with tumor-free non-SLN−

SLN: sentinel lymph node(s); IDC: interdigitating dendritic cell(s); DC: dendritic cell(s); SLN−: SLN without metastases; non-SLN−: non-SLN without metastases; SLN*: no information on SLN metastatic status; non-SLN*: no information on non-SLN metastatic status; SLN mic: SLN with micrometastasis; SLN+: SLN with metastasis; SLN (rhGM-CSF+): SLN harvested after peritumoral injection of rhGM-CSF; SLN (rhGM-CSF−); SLN harvested without peritumor injection of rhGM-CSF; IDO: indoleamine 2,3-dioxygenase.

available results difficult to compare. It is therefore unclear whether immunosuppression is a general phenomenon in any tumor-draining SLN or whether it is specific only for those patients that develop metastases. In order to clarify this question, we initiated a study to compare the immunologic differences between the SLN and non-SLN with respect to the extent of metastatic involvement.

In this context, dendritic cells (DC) were of particular interest. Activated DC have been shown to play a major role in the functioning of the lymphatic immune system[9,10] and seem to be specifically altered in the SLN.[3,5,7,8] Amongst several markers to identify mature and immature DC, the expression of DC-Lamp, a lysosome-associated membrane glycoprotein characterizing mature DC[11] was reported to be inversely associated with the formation of lymph node metastases in melanoma patients.[12] We therefore chose DC-Lamp$^+$ cells as a surrogate marker for immunoreactivity in our study.

Patients and methods

Patients

The study was based on the paraffin blocks of 114 SLN and 1258 non-SLN from 79 patients with breast cancer who were surgically treated in the Department of Surgery and Surgical Oncology at the Robert Rössle Clinic, Charité, University Medicine Berlin, Campus Buch. Until 2003, all patients who underwent curative resection of primary breast cancer had a complete axillary lymph node dissection (ALND). Since 1995, a sentinel lymph node biopsy (SLNB) was also performed with the patient's consent in order to evaluate the sensitivity of the SLNB to detect lymph node metastases. After verification of the efficacy of SLNB to indicate the need of axillary treatment and its recommendation by official guidelines[13] all patients with a primary tumor size up to 2 cm underwent ALND only if the SLN was involved with tumor. All the patients enrolled in this study had undergone both SLNB and back-up ALND. Patients, who had undergone neo-adjuvant therapy, prior axillary treatment (irradiation or surgery), or patients with bulky axillary diseases, were excluded.

All relevant clinico-pathologic data were included into a database. Micrometastases (MIC) and isolated tumor cells (ITC) were defined according to Hermanek et al. and the sixth edition of the TNM classification of malignant tumors.[14,15]

Methods

SLNB detection techniques

All patients underwent SLNB using the radio-colloid technique as the standard detection method: after obtaining informed consent, 0.5–2.0 ml Tc^{99m}–nanocolloid (Nanocoll©) was injected around the tumor the day before surgery. In patients with non-palpable lesions, the radio-colloid was

injected around the tumor under ultrasound guidance until December 2000. After January 2001, patients with non-palpable lesions underwent sub- or retro-areolar injections. Seventeen hours later, lymphoscintigraphy was performed using a γ-camera. Intraoperatively, the SLN was detected by the guidance of a hand-held γ-probe and selectively excised by a small incision and careful preparation. In the case of difficult or failed SLN-identification using the radio-colloid method (e.g., weak or absent nuclide enrichment in the SLN), the blue dye technique using Patent blue© (Guerbet, Roissy, France) was applied as an additional option, at the discretion of the surgeon, according to the guidelines of the German Society of Senology.[13] All lymph nodes revealing radioactivity or blue staining were excised. Lymph nodes that were neither radioactive nor blue were defined as non-SLN.

Histopathologic lymph node examination

Lymph nodes up to 10 mm in diameter were bivalved; larger lymph nodes were lamellated in 2–3 mm sections and processed to paraffin blocks. After haematoxylin and eosin (H&E) staining, the sections underwent routine assessment. If the processed lymph node did not reveal metastasis, it underwent step sections with levels of about 250 μm until sampled completely. A pair of serial sections was cut, 1 μm in thickness each. One of these sections was stained by H&E. If no tumor cells were found by H&E-staining, the other sections underwent immunohistochemical staining with a pancytokeratin antibody (MNF116, DAKO©, Hamburg, Germany) (Fig. 1). This approach ensures that metastases with a maximum diameter of at least 250 μm will be identified with a 100% probability.[16]

Patient groups

Patients from the database (*vide supra*) were selected for the study if all SLN and non-SLN were available. Subject to tumor cell involvement of the SLN and non-SLN, the patients were assigned to the following groups: (Group A) SLN and non-SLN without evidence for tumor cells; (Group B) SLN involved by micrometastases or isolated tumor cells (according to Ref. 15) and non-SLN without evidence of tumor cells; and (Group C) SLN involved by macrometastases and involved or uninvolved non-SLN. The distribution of patients and the number of lymph nodes according to the different groups is depicted in Table 2.

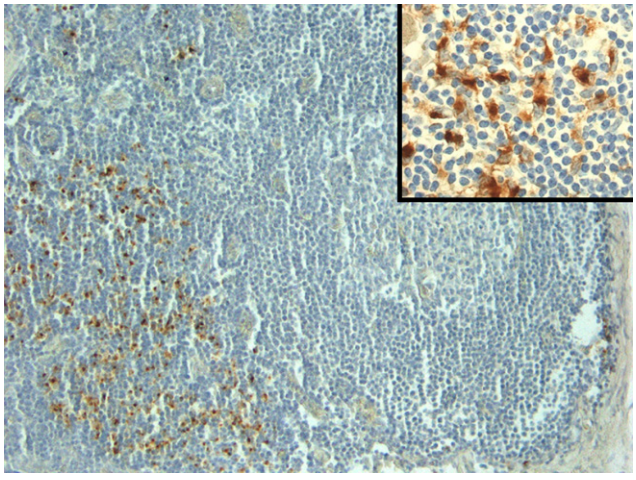

Figure 1. Immunohistochemical staining of DC-Lamp$^+$ mature dendritic cells, all located predominantly in the interfollicular area.

Immunohistochemical staining of SLN and non-SLN for immunologic studies

One serial section was taken from each formalin-fixed paraffin-embedded (FFPE) block of all SLN and non-SLN for immunohistochemical staining with the DC-Lamp-antibody using the avidin–biotin peroxidase complex system, as previously described.[11] In selected cases, we performed double staining with CD3 (pan-T-cell-marker; DAKO) in order to identify the localization of the DC-Lamp$^+$ cells in the lymph nodes. Furthermore, pairs of correlative sections (1 μm in thickness) underwent staining with CD1a (a marker for immature DC; Immunotech, Marseille, France) in order to determine the proportion of mature and immature DC semi-quantitatively.

Evaluation by the pathologist

All results that described staining patterns or morphologic features of the slides were evaluated under the guidance of a pathologist who specialized in cancer pathology. The cancer pathologist also selected the photomicrographs for the computer-assisted image analysis.

Quantitative evaluation of the DC-Lamp$^+$ DC expression by a Zeiss imaging system

The Zeiss imaging system "Axio Imager" (Zeiss, Jena, Germany) was used for quantitative evaluation. Photos of all lymph node slides were captured digitally at a 25× magnification and saved on a hard disc. In each slide, the spot with the highest accumulation of DC-Lamp$^+$ cells was selected for the quantitative analysis. The area occupied by the DC-Lamp$^+$ DC was digitally registered electronically by the imaging system. The relationship between the stained and unstained area of the registration field was recorded as the "area occupied" by the DC-Lamp$^+$ cells in the selected lymph nodes.

Table 2
Number of patients and lymph nodes according to the different groups

	Group A	Group B	Group C
Number of patients	28	25	26
Lymph nodes examined	499	269	463
SLN examined	33	20	42
Non-SLN examined	466	249	421

A: all lymph nodes without evidence for tumor cells; B: micrometastases or isolated tumor cells according to Ref. 15 in SLN, non-SLN without evidence of tumor cells; C: macrometastases in SLN, involved or uninvolved non-SLN.

Data processing and statistics

All data sets were tested for normal distribution. The non-parametric Wilcoxon signed rank-test was used to test the differences in regard to the area occupied by DC-Lamp$^+$ DC between the SLN and non-SLN in the different groups of patients.

Results

One thousand three hundred and seventy-two lymph nodes, consisting of 114 SLN and 1258 non-SLN from 79 patients were examined. No differences between the groups were encountered with respect to age, a history of contralateral breast cancer, tumor growth pattern, histology, grading of the malignancy, lymphovascular invasion, and vascular invasion.

Morphologic location of CD3$^+$, DC-Lamp$^+$ cells

Double staining with CD3 and DC-Lamp in five tumor cell-free lymph nodes confirmed that the DC-Lamp$^+$ cells, as well as the CD1a$^+$ cells, were invariably located in the T-cell zone.

Correlation of CD1a$^+$ and DC-Lamp$^+$ cells

Staining 22 correlative step sections from tumor cell-free non-SLN with DC-Lamp and CD1a revealed, by semi-quantitative analysis, that DC1a$^+$ cells were consistently less frequent than the DC-Lamp$^+$ cells, but correlated to the number of DC-Lamp$^+$ cells.

Quantitative comparison between SLN and non-SLN in regard to the mean area occupied by DC-Lamp$^+$ cells according to the extent of metastatic infiltration (Groups A–C)

A significant increase in the mean area occupied by the DC-Lamp$^+$ cells in the SLN compared to the non-SLN was found in Group A ($p = 0.024$) and Group B (overall, $p = 0.009$; subgroup B2, $p = 0.015$; Table 3); no difference was found within Group C.

DC distribution pattern dependence on the extent of tumor involvement

In contrast to tumor-free lymph nodes or lymph nodes with low-volume metastases, lymph nodes with macrometastases generally provided less space for immunocompetent cells, like DC, depending on the extent of tumor burden. Nevertheless, the distribution pattern of DC-Lamp$^+$ cells in the lymph nodes was unchanged by the extent of tumor involvement and was the same for SLN and non-SLN.

Table 3
Comparison between SLN and non-SLN with respect to the mean area occupied by DC-Lamp$^+$ DC (%) according to the extent of metastatic infiltration (Groups A–C) (intra-individual comparison)

Group	Lymph node	Number of patients	Mean (%)	Standard deviation (%)	*p*-Value
A	SLN	28	1.10	1.74	0.024
	Non-SLN	28	0.37	0.37	
B	SLN	25	1.56	1.94	0.009
	Non-SLN	25	0.69	0.57	
C	SLN	26	0.84	1.18	0.107
	Non-SLN	26	0.34	0.31	

A: all lymph nodes without evidence for tumor cells; B: micrometastases or isolated tumor cells according to Ref. 14 in SLN, non-SLN without evidence of tumor cells; C: macrometastases in SLN, involved or uninvolved non-SLN.

Statistical power

The power of the results in Table 3 is 60.2% for the comparison (SLN *vs.* non-SLN) within Group A, 69.4% for Group B, and 68.1% for Group C.

Discussion

The results indicate that the SLN is a site of increased DC-Lamp$^+$ cell presence in patients with no or low-volume metastases. No difference between SLN and non-SLN was found in patients with macrometastases.

Our results in relation to the extant literature

The SLN as a site of enhanced immunoactivity is a controversial issue. Whereas several studies from the study group of Cochran et al.[2] described a reduced presence of mature DC and simultaneously a predominance of immature DC in the SLN,[2,3,7] other studies found an increased number of mature DC and enhanced levels of cytokine-mRNA-expression in the SLN compared to the non-SLN.[13–16] Although the differences among the studies are not fully understood, one major reason may be the fact that previous studies examined only a limited number of randomly selected SLN and non-SLN per patient[2] and, thus may not be representative of the entire lymph node region. Also, some studies did not consider the nodal status and compared tumor-involved with tumor-free lymph nodes.[3] Examining all SLN and non-SLN of each patient and with respect to the extent of metastatic involvement, our study seemed to be a comparably strong indicator for the assumption that the SLN is a center of immunoactivation in nodal negative patients and patients with low-volume metastases.

Open questions

It is, however, still undetermined whether the enhanced level of DC-Lamp$^+$ cell presence is the reason for a potent immune system that is able to avoid macrometastases or

whether it is the consequence of a tumor that is unable to initiate immunosuppression. DC, as mediators of immunity, are known not only as initiators of the immune response, but also as inducers of immune tolerance. Movassagh et al.[12] and Laguens et al.[17] indicated that a high number of DC-Lamp$^+$ cells in lymph nodes is correlated with a lower rate of lymph node macrometastases, whereas Treilleux et al.[18] found the presence of DC-Lamp$^+$ cells in primary tumors to be associated with an increased frequency of lymph node metastases. Although still not fully understood, the presence of DC-Lamp$^+$ cells at least reflects a site of an intensified interaction between the tumor and the immune system.

Perspective

We are aware of the fact that our conclusions are based on a relatively small number of patients in each group with a moderate statistical power of 60–70% for the main results. Therefore, this study should be considered as an exploratory analysis, which does not allow far-reaching generalizations, but may give rise for a larger, prospective and planned study. It may also be of interest to combine histomorphologic and immunohistochemical techniques with dynamic imaging[19] or molecular imaging[20,21] to further elucidate the cell-to-cell interactions between the primary tumor and the lymph nodes.

Conflict of interest

Concerning manuscript no. EJSO-D-07-00172R1

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There is no conflict of interest to declare, particularly not from a commercial funding group or any sponsor.

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