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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Sentinel lymph node (SLN) biopsy allows identification of the first-draining lymph node from the site of primary tumors. Identification of tumor in these nodes predicts the metastatic potential of tumors. We tested the hypothesis that SLNs are the primary site of antigen specific T cell activation. And, the activation state of dendritic cells (DC) in SLNs is predictive of the immune status of tumors. Paraffin embedded SLNs from breast cancer patients were analyzed by immunohistochemistry to determine the maturation state of DC. Our results demonstrated that tumor-free SLNs contained higher numbers of mature CD83+ DC compared to tumor-containing SLNs (p=0.01) suggesting that an active immune response was occurring in these nodes. In addition, tumor-free SLNs were more likely to contain cells expressing either IL-12 or IL-10 (p=0.07 and 0.01, respectively) compared to tumor-containing nodes. The expression of IL-12 indicates T cell activation via a Th1 pathway, while the presence of IL-10 is suggestive of immunosuppression via a Th2 response. Examination of T cell responses in these patients will confirm whether immune activation or suppression has occurred. With this information we will allow dissect the components of the immune response leading to tumor regression, thus allowing the development of successful immunotherapy.				
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INTRODUCTION: Sentinel lymph node (SLN) biopsies allow the identification of the major draining lymph node from the site of a primary tumor. In breast cancer the identification of tumor cells in these nodes predicts the positive metastatic potential of the tumor. Therefore, this technique is used as a diagnostic procedure to determine the necessity of regional lymph node dissection (1,2). We tested the hypothesis that the sentinel node is the primary site of tumor specific T cell activation. And, the activation state of the dendritic cell, the major antigen presenting cell (APC), within the SLN, is predictive of the breast cancer patient's immune response to their tumor.

From animal models of tumor metastasis the primary draining lymph node has been shown to be a site of T cell activation (3). These nodes, although often tumor-negative by immunohistochemistry (IHC) and PCR, are sites of active tumor specific T cell proliferation. The results of these animal studies suggest that mature peptide loaded APC are located in these nodes.

Dendritic Cells (DC) are critically involved in the activation of both CD4+ and CD8+ T cells thus initiating the primary immune response (4). In the case of tumors, DCs are present in the infiltrating population of lymphoid cells but their numbers do not correlate with overall survival. We hypothesized that DC are in fact the major determiners of the immune response to tumors. The activation state of the DC in the tumor draining lymph node (SLN) is critical to the outcome of the tumor. It has been proposed that DC deliver the polarizing signal that determines the fate of Th0 cells; i.e. whether they develop into TH1 or Th2 cells (4,6). Central to this hypothesis is the production of IL-12 by DC. IL-12 pushes naïve Th0 cells to a TH1 pathway leading to tumor destruction by activating a cytotoxic T cell response. The production of IL-12 by DC is inhibited by the suppressive cytokine IL-10 (6,7). IL-10 presence results in the development of a Th2 response and unresponsiveness to the tumor (6,7).

For these studies mature dendritic cells were defined as CD83⁺, HLA class II^{high}, CD1a⁺, while immature DC were defined as CD83⁻, CD1a⁺, and HLA class II^{low}. CD83 is a membrane glycoprotein which has been shown to be expressed on blood dendritic cells that co-express the highest level of HLA class II molecules (4,8).

Specific Aims: 1) To determine the activation state of the DC in breast cancer sentinel lymph node biopsies (SLNB). Our underlying hypothesis was that tumor-free SLN would contain immunologically mature CD83+ DC while tumor-containing SLN would contain immunologically immature DC. 2) To identify the cytokines present in SLNB. Our hypothesis was that the tumor-free SLN would contain cells expressing IL-12 suggesting a Th1 type immune response predominated while the tumor-containing SLN would contain few IL-12 expressing cells. In addition the expression of the immunosuppressive cytokine IL-10 should predominate in the tumor-containing SLN suggesting that T cell activation was suppressed. 3) To determine if the mature DC, present in these SLNB, are peptide loaded and capable of stimulation T cell proliferation. Our hypothesis was that activated tumor specific T cells would be found in the lymph nodes of patients with tumor-free SLN. The immune status of the DC within the SLN would correlate with the existence of functional tumor specific T cells in the draining lymph nodes.

Experimental Design

Study Population: SLN biopsy tissue of women, between the ages of 25 and 75 years, who had

Table 1. Comparison of patients with tumor-free versus tumor-containing SLN.

	Tumor- Free SLN	Tumor-Containing SLN
Age	56 yr (37 – 71)	55 yr (26 – 87)
Estrogen Receptor (%Positive)	86%	79%
Progesterone Receptor (%Positive)	66%	75%
Tumor size (mean±SD)	1.6 cm ± 0.8	1.4 cm ± 1.1
Stage I	14%	9%
II	50%	52%
III	36%	35%
Total	25	25

SLN biopsies done at a M.D. Anderson Cancer Center between 1998 and 2001 were included in this study. These patients were all diagnosed with breast cancer and had the SLN biopsy done as part of their treatment. Paraffin embedded SLN tissues were examined from 50 patients; including 25 tumor-free SLN and 25 tumor-containing SLN. The tumor status of the SLN was determined by H&E staining as well as IHC. All samples were banked in the Breast Cancer Tissue Core, Department of Pathology, MDACC. Table 1 compares the clinical features of patients with tumor-free and tumor-containing SLN biopsies.

Antibodies: The following antibodies were used: anti CD3, Biogenex; anti HLA Class II, DAKO; anti CD83, Immunotech; anti CD1a, Immunotech; anti IL-10, R&D Systems; and anti IL-12, R&D Systems. Optimal concentrations were determined empirically and all antibodies were tested for staining of cytoplasts of PBMC derived immature DC (PBMC stimulated with GMC-SF and IL4 for 6 days), or mature DC (PBMC stimulated with GMC-SF and IL-4 for 6 days followed by TNF α for 24 hr).

Immunohistochemistry: Sections, roughly 3 μ m thick, were cut from tissue blocks of formalin fixed paraffin embedded SLN. Immunocytochemical staining of deparaffined, fixed slides was performed after antigen retrieval by heating (Microwave) in citrate buffer. Slides were incubated with biotinylated goat anti mouse IgG followed by Avidin:Biotinylated peroxidase Complex (ABC reagent, Vector labs). The peroxidase was developed by 3-amino-9 ethylcarbazole 9 (AEC, red color, Vector Labs) and counter stained with Gill's hematoxylin (Vector Labs). Isotype matched antibodies were used as negative control antibodies. The positive control antibody for each group of slides was anti CD3.

Slides were scored following a method described by Bell et. al (5) by counting positively stained cells in 5 fields per slide under high power (40x). Results were recorded as mean $\pm$ standard deviation of the counts in these 5 fields. All slides were counted blindly with results confirmed by a second reading by a breast cancer pathologist.

Statistical Analysis: Statistical significance was determined by a Student's t test of two samples with unequal variance and two-tailed distribution. A p value $\leq$ 0.05 considered significant.

RESULTS: Table 2 lists the results of the IHC studies. Because of the localized distribution of DC within the SLN, results are reported as the mean of the total cells counted in 5 fields under high power magnification (40X). All slides were counted blindly and 20% of these slides were counted again by a breast pathologist. The second set of counts confirmed the initial results. These results show that tumor-free SLN contain higher numbers of CD83+ DC than tumor-containing SLN ($p < 0.05$). In addition, higher numbers of IL-10 and IL-12 positive cells were found in the tumor-free nodes ($p = 0.01$ and 0.07 , respectively). There was no statistical difference in the expression of CD1a+ cells in the tumor-free or tumor-containing SLN.

Shown in figure 1 are the results of IHC on

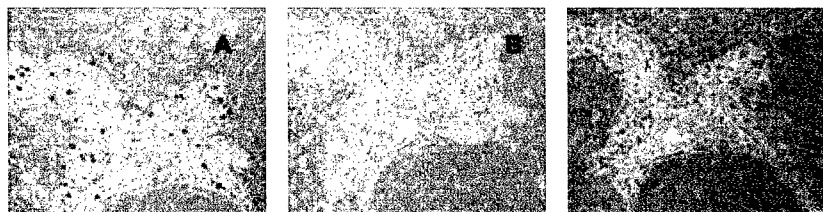
Table 2. Results of immunohistochemistry

	Tumor-free SLN*	Tumor-containing SLN*	P**
CD83	11.9 $\pm$ 10.8	5.8 $\pm$ 4.7	0.0132
CD1a	54.4 $\pm$ 25.1	56.4 $\pm$ 33.6	0.8060
IL-10	12.0 $\pm$ 14.8	3.3 $\pm$ 5.9	0.0107
IL-12	4.2 $\pm$ 5.4	1.9 $\pm$ 2.9	0.0695

* Mean $\pm$ standard deviation of the number of positive cells per high powered field

** Student t-test

Figure 1. CD83 (A), CD1a (B), and HLA class II (C) expression in a tumor-free SLNB.



patient #30, a representative example of a tumor-free SLN as determined by H&E (not shown). Staining for CD83 (Fig. 1A) was positive, both in terms of numbers of cells as well as intensity of staining. When attempts were made to localize the staining it was apparent that these cells were also positive for MHC Class II (Fig. 1C) but did not stain with anti CD1a (Fig. 1B). Within the same area of the CD83+ cells were cells producing IL-12 and IL-10 (data not shown). Analysis to determine if there was a relationship between numbers of CD83+ DC and numbers of IL-10 or IL-12 producing cells showed there was an association, although moderate, in tumor-free nodes but not tumor-containing SLN for both cytokines.

These data were also analyzed in terms of the clinical features of the tumor as summarized in Table 1. When samples were divided according to their tumor grade and then the expression of CD83, CD1a IL-10 and IL-12 compared between the tumor-free and tumor-containing SLN the difference in the number of CD1a positive cells was statistically significant in those patients with Grade III tumors (Table 3). Tumor-containing SLN contain significantly higher numbers of CD1a, immunologically immature DC compared to tumor-free SLN. These results support our hypothesis that tumor specific T cells are activated in the tumor-free SLN that effect the metastasis of the tumor. Or, an alternative explanation is that the larger, grade III, tumor somehow inhibits the maturation of DC.

We compared the expression of CD83, CD1a, IL-10 and IL-12 in SLNB from these breast cancer samples to lymph nodes from uninvolved normal breast tissue. Using the same methodologies as

Table 3. Comparison by Tumor Grade

	Grade I			Grade II			Grade III		
	Tumor free	Tumor containing	p	Tumor free	Tumor containing	p	Tumor free	Tumor containing	p
CD83	4.5± 7.7	9.1± 4.6	0.44	10.2±11.6	4.9± 4.4	0.17	12.9± 8.3	5.8± 5.2	0.06
CD1a	57.9± 9.7	59.0±40.6	0.97	60.0±34.7	48.7±29.5	0.41	49.6±14.4	80.0±22.8	<0.01
IL-10	10.9±18.9	4.9± 5.5	0.64	9.5± 9.8	5.3± 7.6	0.26	9.4± 8.3	0.6± 1.2	0.02
IL-12	1.9± 1.8	4.7±6.4	0.54	3.9± 4.8	0.8± 1.5	0.07	2.4± 4.4	3.1± 2.4	0.31
n	3	3		11	12		8	8	

described above slides from five normal paraffin embedded lymph nodes were examined. We found that the expression of these markers in normal lymph nodes was not statistically different from their expression in the tumor-free SLNB (Table 3). These results suggest that the immune response in the tumor containing nodes were suppressed compared to that of both tumor free nodes and normal lymph nodes. In order to confirm this result more normal lymph nodes need to be examined.

NOTE: The experiments described above completed the work outlined in Aims one and two of this grant. We are now beginning the work as outlined in Aim 3. Since this work involves the use of fresh human tissue it cannot be started because it requires further review and approval from both the IRB of our institution as well as review and approval by the Human Subjects Research Review Board of the U.S. Army Medical Research and Material Command. We are now in the process of submitting the protocol for review to the M. D. Anderson Institutional Review Board.

Key Research Accomplishments:

- Established collaboration with the M.D. Anderson Cancer Center Breast Pathology Tissue Bank thus allowing access to archived tissue samples.
- Established methodology to successfully stain paraffin embedded samples with anti CD83 monoclonal antibody thus allowing the determination of the number of mature DC in these samples.

- Collected preliminary data necessary to provide sufficient justification to continue the study using human tissue samples. These data were required before IRB approval would be given for the use of human tissue samples in the experiments outlined in Aim 3 of the grant.

Reportable Outcomes:

Manuscripts

Poindexter, N., Hunt, K., Grimm, E., and A. Sahin. Increased numbers of mature CD83+ dendritic cells found in tumor-free versus tumor-containing sentinel lymph nodes of breast cancer patients. Manuscript in preparation.

Abstracts

Poindexter N.*, Hunt K., Grimm E., Sahin A. Increased numbers of mature CD83+ dendritic cells found in tumor-free sentinel lymph nodes from patients with breast cancer. 2002 AACR National Meeting, San Francisco, April, 2002.

Poindexter N.*, Hunt K., Grimm E., Sahin A. Comparison of the immune response in tumor-free versus tumor containing sentinel lymph nodes of breast cancer patients. To be presented at the Era of Hope Meeting, Orlando, September, 2002.

Funding Applied For

The Immune Response in Breast Cancer Sentinel Lymph Nodes. Principle Investigator Nancy Poindexter, Ph.D. Idea Award submitted to the U.S. Department of the Army, June 11, 2002.

Personnel supported by this work:

Ms. Sandra Kinney, Research Technician, Department of Bioimmunotherapy, M.D. Anderson Cancer Center, Houston TX.

Conclusions: Tumor-free SLNs contained higher numbers of mature CD83+ DC compared to tumor-containing SLNs ($p=0.013$). Tumor-free SLNs were more likely to contain cells expressing either IL-12 or IL-10 ($p=0.07$ and 0.01 , respectively) compared to tumor-containing nodes. These results suggest that an active immune response is occurring at these sites as evidenced by the significant number of immunologically mature DC in the tumor-free SLN.

These data generated, through this funding, provide the basis for the hypothesis of our future experiments that the SLN is the primary site of tumor specific T cell activation. Lack of activation of T cells within the SLN is the result of inhibition of DC maturation in the primary tumor. We propose that the SLN is not immunosuppressed, as has been reported (9), but rather the primary tumor microenvironment inhibits the maturation of DCs. In conclusion, understanding the influence of the breast tumor on DC maturation and function will lead to more rational design of vaccines and immunotherapies. We will be able to design treatments that will utilize the mature DC present in the SLN or include cytokines that provide the final signal for DC maturation.

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