Local Administration of Granulocyte/Macrophage Colony-stimulating Factor Increases the Number and Activation State of Dendritic Cells in the Sentinel Lymph Node of Early-Stage Melanoma

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ABSTRACT

The initial tumor-draining lymph node, the sentinel lymph node, not only constitutes the first expected site of micrometastasis but also the first point of contact between tumor-associated antigens and the adaptive immune system. A tumor-induced decrease in the frequency and activation state of sentinel lymph node dendritic cells will impair the generation of effective antitumor T-cell responses and increase the likelihood of metastatic spread. Here, we demonstrate that intradermal administration of granulocyte macrophage-colony stimulating factor around the excision site of stage I primary melanoma tumors increases the number and activation state of dendritic cells in the paracortical areas of the sentinel lymph node and enhances their binding to T cells. We conclude that local treatment of melanoma patients with granulocyte macrophage-colony stimulating factor, before surgery, conditions the sentinel lymph node microenvironment to enhance mature dendritic cell recruitment and hypothesize that this may be more conducive to the generation of T-cell-mediated antitumor immunity.

INTRODUCTION

Dendritic cells are bone marrow-derived antigen-presenting cells that are critical to the initiation of T-cell–mediated immunity. In melanoma, skin-resident myeloid dendritic cells (i.e., the Langerhans cells) take up and transport tumor-associated antigens to tumor-draining lymph nodes (1, 2). To subsequently activate melanoma-specific T cells, the migrated Langerhans cells need to become activated (i.e., mature) and express high levels of costimulatory molecules, as well as appropriate chemokine receptors for their migration to the paracortical T-cell areas of the tumor-draining lymph node (3). Melanoma is the most immunogenic tumor identified to date; tumor-associated antigen-reactive T cells are detectable both in the blood and in tumor-draining lymph nodes from melanoma patients, and their frequency can be increased by specific vaccination (4–6). This intrinsic immunogenicity may make melanoma lesions particularly amenable to therapeutic approaches aimed at strengthening tumor immune surveillance. High numbers of sufficiently activated dendritic cells in the lymphatic tumor-drainage catchment area are very important in this regard. Dendritic cell development and activation can both be frustrated by inhibitory factors commonly associated with melanoma (2, 7, 8). The degree of such immunosuppression in tumor-draining lymph nodes is directly related to their distance to the primary tumor. Indeed, the first lymph node to directly drain the primary tumor, the so-called sentinel lymph node, is the preferential site of early metastasis (9–11) and shows the most pronounced immunologic down-regulation (12, 13). The frequency of paracortical sentinel lymph node-dendritic cell is reduced, and most sentinel lymph node-dendritic cell lack dendritic morphology and display lower expression levels of costimulatory molecules as compared with nonsentinel lymph node-dendritic cell (12, 13). Such a paralysis of dendritic cells in the first line of immunologic defense may well facilitate the metastatic spread of melanoma cells to more distal tumor-draining lymph nodes (12, 14). Local release of granulocyte/macrophage colony-stimulating factor (GM-CSF) has previously been reported to result in enhanced recruitment of activated dendritic cells to draining lymph nodes in a murine model (15). Here, we demonstrate that intradermal injection of GM-CSF around the excision site of primary melanoma tumors increases the number and activation state of dendritic cells in the paracortical areas of the sentinel lymph node. A concomitant increase in dendritic cell–T-cell clustering is indicative of intensified immune surveillance. These observations lend support to perioperative local administration of GM-CSF as a valuable immunoadjuvant option in the treatment of early-stage melanoma.

MATERIALS AND METHODS

Patients. In this single-blinded phase II study, 12 patients with stage I melanoma according to criteria of the American Joint Committee on Cancer (all patients with Breslow thickness \( \leq 1.5 \) mm, ages 18 to 70 years), who were scheduled to undergo a sentinel lymph node procedure, were assigned randomly to preoperative local administration of either recombinant human GM-CSF or saline. Patients who had undergone previous immunotherapy or chemotherapy were excluded, as well as patients receiving immunosuppressive medication or suffering from any autoimmune disorder. The study was approved by the medical ethical committee of the VU University Medical Center, and written informed consent was obtained from each patient before treatment. Patient characteristics are listed in Table 1.

Triple-Technique Sentinel Lymph Node Procedure and GM-CSF Administration. On day 0, patients underwent a triple-technique sentinel lymph node procedure as described previously (11, 16). In short, a lymphoscintigraphy was performed at least 4 hours before surgery to determine the lymphatic drainage pattern in a dynamic fashion. Just before surgery, a blue inert dye was injected adjacent to the site of the primary melanoma excision. During surgery, guided by the blue staining of the draining lymphoid tissues and a handheld gamma probe, the sentinel lymph node was positively identified, removed, and, after isolation of viable sentinel lymph node cells, examined meticulously by the pathologist (17). Both patient groups received four daily intradermal injections directly adjacent to the scar of the primary melanoma excision, from day –3 until day 0 just before surgery, with either 3 \( \mu \)g/kg body weight recombinant human GM-CSF (Leucocax, Schering Plough, Maarsen, the Netherlands), dissolved in 1.0 mL of saline or 1.0 mL of plain saline.

Isolation of Viable Sentinel Lymph Node Cells. Immediately after removal, sentinel lymph node cells were collected in sterile ice-cold complete medium, composed of Iscove’s modified Dulbecco’s medium supplemented with 25 mmol/L HEPES buffer (BioWhittaker/CAMBREX, Verviers, Belgium) with 10% FCS, 50 IU/mL penicillin-streptomycin, 1.6 mmol/L L-glutamine, and 0.05 mmol/L \( \beta \)-mercaptoethanol. Before routine histopathological examination of the sentinel lymph node, viable cells were isolated with a previously described cytological scraping method (18). In short, after measuring the size...
of the sentinel lymph node, it was bisected cross-wise with a surgical scalpel, and the cutting surface of the sentinel lymph node was scraped 10 times with a surgical blade (size no.22; Swann Morton Ltd., Sheffield, United Kingdom). Sentinel lymph node cells were rinsed from the blade with medium containing 0.1% DNase I, 0.14% Collagenase A (Boehringer Mannheim, Mannheim, Germany), and 5% FCS, incubated for 45 minutes at 37°C, and subsequently placed in PBS with 5 mmol/L EDTA for 10 minutes on ice. Finally, the sentinel lymph node cells were washed twice in complete medium, counted, and additionally processed.

Flow Cytometry. Freshly isolated sentinel lymph node cells were directly stained with antibodies labeled with either phycoerythrin or FITC and analyzed by flow cytometry at 100,000 events per measurement, as described previously (18). Monoclonal antibodies against CD1a, CD14, CD86 (PharMingen, San Diego, CA), CD40, and CD83 (Immunotech, Marseille, France) were used.

Immunocytochemistry. Cytopsin preparations were acetone-fixed and stained immunocytochemically as described previously (18). Monoclonal antibodies against CD1a, CD3, CD14, CD86 (Becton Dickinson, San Jose, CA), CD40, CD83 (Immunotech), and S100 (DAKO A/S, Glostrup, Denmark) were used.

The number of positively stained dendritic cells and the number of T cells clustered per dendritic cell were determined with an interactive video morphometry system (Q-PRODIT; Leica, Cambridge, United Kingdom). The outer border of each cytopspot was demarcated at a 100-fold magnification, and 40 fields of vision were randomly selected in an automated manner for subsequent evaluation (19). The total number of CD3+ T cells was counted in these 40 fields of vision and used to correct for cell density of the cytopspots of each patient. In each field of vision, the number of dendritic cells was counted on the basis of positive staining of specific markers and dendritic cell morphology. Furthermore, numbers of T cells directly clustered to the dendritic cells were counted. Results are listed as total number of dendritic cells, normalized per 600 CD3+ T cells (i.e., the mean number of T cells detected per 40 fields of vision), and a mean number of T cells clustered per dendritic cell.

Immunohistochemistry. Paraffin sections were mounted on Superfrost Plus glass slides and dried overnight at 37°C. After deparaffinization, the tissue sections were hydrated through decreasing (v/v) percentages of etomidate, and endogenous peroxidase was blocked with 0.1% hydrogen peroxide in methanol. Tissue sections were pretreated with 10 mmol/L citrate (pH 6) in an autoclave for 21 minutes at 121°C [for CD14 (1:25), CD83 (1:25), and an isotype-matched control antibody] or in a microwave at 100°C for 10 minutes [CD1a (1:25) and CD68 (1:400; DAKO A/S) ]. All antibodies (except CD68) were applied and incubated at room temperature for one hour. Detection and visualization were performed with the DAKO Chemmate Envision detection kit (DAKO A/S) according to the manufacturers’ instructions. For the CD68 antibody, an automated immunostainer (Ventana, Tucson, AZ) was used for all incubation, detection, and visualization steps according to the manufacturer’s instructions. Sections were counterstained with hematoxylin, dehydrated, and mounted. Tonsillar tissue sections were used as positive control samples.

Statistical Analysis. Differences between patient study groups were analyzed using the two-sample Mann-Whitney U test and considered significant when P < 0.05.

RESULTS

Clinical Observations. No significant differences in patient characteristics were observed between the two study groups (Table 1). Administration of recombinant human GM-CSF was well tolerated by all patients. Apart from minor musculoskeletal pain, mild fever, and general tiredness after the first injection in some patients, no significant side effects or skin abnormalities were observed. The sentinel lymph node contained no metastatic tumor cell deposits in any of the patients after routine pathological examination. The sentinel lymph nodes in the GM-CSF group were significantly larger in volume as compared with the control group (Table 1). Also, higher sentinel lymph node cell yields were obtained after scraping the cutting surface of the sentinel lymph node from GM-CSF-administered patients, although this did not reach significance in comparison to the control group.

Table 1  Patient and SLN characteristics in the GM-CSF and saline control groups

<table>
<thead>
<tr>
<th></th>
<th>GM-CSF</th>
<th>Control</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Sex (male:female)</td>
<td>4:2</td>
<td>3:3</td>
<td>NS</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>56 ± 11</td>
<td>57 ± 15</td>
<td></td>
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<tr>
<td>Breslow thickness (mm; mean ± SD)</td>
<td>1.04 ± 0.33</td>
<td>0.84 ± 0.27</td>
<td>NS</td>
</tr>
<tr>
<td>Yield scraping (×10³; mean ± SD)</td>
<td>1668 ± 296</td>
<td>759 ± 146</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Positive SLN</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
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* Volume: height × width × length.
Abbreviations: NS, not significant; SLN, sentinel lymph node.
Distribution and Morphology of Sentinel Lymph Node-Dendritic Cells In situ. Immunohistochemical staining for the dendritic cell markers CD1a and CD83 revealed colocalization of both markers in the paracortical regions (i.e., T-cell areas) of the sentinel lymph node (Fig. 1A). CD1a+ and CD83+ sentinel lymph node cells had a distinct irregular dendritic cell morphology (Fig. 1A). In contrast to CD1a+ and CD83+ dendritic cells, cells positive for the monocyte/macrophage markers CD14 and CD68 preferentially colocalized in the marginal sinuses of the sentinel lymph node and displayed a more rounded macrophage-like morphology (Fig. 1B). In none of the GM-CSF–administered patients, hyperplasia of lymphoid follicles or paracortical areas was observed, nor did the distribution patterns of the CD1a+/CD83+ dendritic cells or the CD14+/CD68+ macrophages inside the sentinel lymph node differ between the GM-CSF and the control group (data not shown).

Increased Frequency and Activation State of Sentinel Lymph Node-Dendritic Cells upon Intradermal Administration of GM-CSF. Flow cytometric analysis showed a significantly increased frequency of CD1a+ sentinel lymph node-dendritic cells to be associated with GM-CSF administration, whereas no such association was apparent for the frequency of CD14+ monocytes/macrophages (Fig. 2A). Mean percentages of CD1a+ sentinel lymph node-dendritic cells were 0.68% (range, 0.31 to 1.03%) and 0.15% (range, 0.02 to 0.35%) for the GM-CSF and the control group, respectively (P < 0.05). Of note, no CD1a/CD14 double-positive cells were detected, indicating that these markers defined two distinct myeloid populations in the sentinel lymph node, i.e., CD1a+ dendritic cells and CD14+ macrophages. These data clearly demonstrate a specific stimulatory effect of local GM-CSF on the migration and recruitment of dendritic cells to the draining lymph nodes. The CD1a+ sentinel lymph node-dendritic cells were selectively gated and analyzed for expression levels of dendritic cell activation markers (Fig. 2B). Expression levels of the maturation marker CD83 and of the costimulatory markers CD86 and CD40 were all significantly increased in the GM-CSF group as compared with the control group (Fig. 2C), demonstrating an enhanced activation state of the sentinel lymph node-dendritic cell. Of note, neither in the GM-CSF–administered patients, nor in the patients receiving saline injections could any CD1a+/CD83+ sentinel lymph node-dendritic cell be detected, indicating the absence of immature Langerhans cells in the sentinel lymph node. Immunocytochemical analysis was in agreement with the flow cytometric data, showing significant and comparable increases in the frequency of CD1a+ sentinel lymph node-dendritic cells after intradermal recombinant human GM-CSF administration: from on average, one dendritic cell per 600 CD3+ T cells in the control group (equalling ~0.15%) to 6 to 10 dendritic cells per 600 T cells (1 to 1.5%) in the GM-CSF group (P < 0.05, Fig. 3A). Similar and significant increases were observed for the dendritic cell activation markers CD83, CD86, CD40, and S100 (all at P < 0.05) but not for the macrophage/monocyte marker CD14 (Fig. 3A). Equal counts for CD1a and the dendritic cell activation markers reflect the mature state of the CD1a+ sentinel lymph node-dendritic cells. On the sentinel lymph node cytopsots, clear

![Fig. 2. Flow cytometric analysis reveals increased frequencies and activation state of CD1a+ dendritic cells in melanoma sentinel lymph nodes upon local GM-CSF administration. A, percentages of CD1a+ and CD14+ cells in sentinel lymph node single-cell suspensions in patients who received four consecutive daily intradermal injections of GM-CSF or of saline placebo (saline control). Horizontal bars represent mean percentages. B, CD1a+ sentinel lymph node-dendritic cells were gated [see dot plot scatter diagrams (CD1a/iso)] and analyzed for the expression of maturation markers (see the corresponding histograms for isotype control, CD83, CD86, and CD40). Expression of the indicated markers is listed as mean fluorescence (MFL). Data are shown from two representative patients, one receiving saline injections (control) and one receiving GM-CSF. C, MFL intensities obtained with isotype control monoclonal antibodies or with monoclonal antibodies against CD83, CD86, or CD40 on CD1a+ sentinel lymph node-dendritic cells in patients receiving injections with saline placebo (control, n = 6) or with GM-CSF (GM-CSF, n = 6). Error bars represent SEM. All significant differences (P < 0.05) are indicated by asterisks.](cancerres.aacrjournals.org)
sentinel lymph node-dendritic cell/T-cell clusters (determined on the basis of CD3 positivity; data not shown) were observed in the samples from the patients that had received intradermal recombinant human GM-CSF (Fig. 3B). Quantitation showed significantly higher numbers (four on average) of T cells in direct contact to sentinel lymph node-dendritic cells in GM-CSF-administered patients than in patients that had received intradermal saline injections (one T-cell per sentinel lymph node-dendritic cell on average; Fig. 3C). Again, this T-cell clustering effect was found to be dendritic cell specific because the numbers of T cells in direct contact to CD14+ macrophages was not increased by GM-CSF administration (Fig. 3C).

**DISCUSSION**

In this randomized trial, we have shown that short-term local administration of GM-CSF significantly increases the number and activation state of dendritic cells in the sentinel lymph nodes of stage I cutaneous melanomas. Although the study of nonsentinel lymph nodes in these patients would have provided valuable information on the specificity of the observed effects for sentinel lymph nodes, we were bound by restrictions from the local ethical committee and could not perform these comparative studies. Previous reports, however, clearly demonstrated a preferential decrease in sentinel lymph node-dendritic cell frequency and maturation state as early as stage I of melanoma development (12, 13). The fact that these inhibitory effects on dendritic cell activation and migration to the tumor-draining lymph nodes are directly related to the proximity of the primary tumor indicates the causative agents to be tumor-derived. Data from earlier studies suggest that interleukin 10 and gangliosides may be good candidates in this regard (7, 8). Clearly, the crippling of dendritic cell functions in the principal nodes involved in immune surveillance will frustrate specific T-cell activation and likely increase the chance of tumor immune escape and metastasis.

GM-CSF has powerful stimulatory in vivo effects on dendritic cell recruitment, activation, and survival (20). This makes it a prime candidate to test its modulatory effects on sentinel lymph node-dendritic cell numbers and on their activation state in a clinical setting. Evidence that systemic and long-term GM-CSF administration can increase tumor-draining lymph node-dendritic cell frequencies and improve clinical outcome was recently reported by us in patients with locally advanced breast cancer (21). Here, we demonstrate that in a relatively short period (four injections in 4 days) and in a localized fashion (intradermal injections around the tumor excision scar), the frequency of sentinel lymph node-dendritic cells can be quadrupled, and their phenotypic maturation state increased (as determined by a quantitative flow cytometric method). This indicates the applicability of GM-CSF administration in the treatment of early-stage melanoma. Previous reports of suppressed sentinel lymph node-dendritic cells in stage I melanoma (12, 13) suggest that this GM-CSF–induced up-regulation of both sentinel lymph node-dendritic cell frequency and maturation state signifies a restoration of dendritic cell functions to more normal levels. Increased numbers of CD83+ dendritic cells infiltrating the paracortical areas of the sentinel lymph node upon GM-CSF administration indicate an enhanced migration of mature dendritic cells to the sentinel lymph node T-cell zones, which has previously been reported to be CCR7 mediated (22, 23). Interestingly, no CD1a+/CD83- immature dendritic cells were present in the single-cell suspensions of either the GM-CSF- or the saline-treated sentinel lymph node-DCs (magnification, ×400). Results are shown for a representative control and GM-CSF–treated patient.

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cell clusters at a given time. Thus, local peritumoral GM-CSF administration might lead to enhanced tumor-associated antigen presentation and T-cell recruitment and activation in the sentinel lymph node. Indeed, our observation of GM-CSF–induced increases in sentinel lymph node size and cell numbers may also result from this enhanced T-cell attraction and retention by activated sentinel lymph node dendritic cells. In keeping with this, we have found evidence for specific recruitment of tumor-specific CTLs to sentinel lymph nodes. Peritumoral treatment with GM-CSF may be expected to facilitate the activation of tumor-specific CTLs, which was recently suggested to offer protection from the outgrowth of micrometastases (22). Nevertheless, the finding of GM-CSF–induced increased numbers of tumor-specific CTLs and a possible diminished outgrowth of micrometastases in the sentinel lymph node has yet to confirm this hypothesis. To this end, we are planning a larger prospective study, which should include patients with melanomas between 1 and 4 mm of Breslow thickness, who are not eligible for adjuvant therapy but who are at risk for occult nodal metastasis (16). Particularly these patients may benefit from local GM-CSF treatment because nodal metastases, despite offering a ready source of tumor-associated antigens, may be expected to interfere with dendritic cell maturation and migration to the sentinel lymph node (26) and to thus interfere with proper antitumor CTL activation. GM-CSF may counteract these detrimental effects and prevent additional metastatic spread and outgrowth.

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