FLT3 Ligand Administration Inhibits Tumor Growth in Murine Melanoma and Lymphoma

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Abstract

Successful treatment of melanoma and lymphoma may result from the induction of specific antitumor immunity. Dendritic cells (DCs) are powerful antigen-presenting cells and show a remarkable capacity to stimulate antigen-specific T-cell responses. Administration of FLT3 ligand (FL) results in a reversible accumulation of functionally active DCs in both lymphoid and nonlymphoid tissues. Therefore, we evaluated the possible antitumor effect of FL in murine melanoma (B16 and CL8–1) and lymphoma (EL-4) models. In all experiments, tumor growth was significantly inhibited by FL administration. Analysis by immunohistochemistry revealed an increase in the DC accumulation within B16 and EL-4 tumors after treatment with FL. No change was observed for CL8–1 melanoma. These data suggest a potential role for FL in the immunotherapy of malignant skin tumors and possible DC involvement in this effect.

Introduction

In the last three decades, a steady increase in the incidence of cutaneous melanoma has been observed worldwide. This rate exceeds that of all other solid tumors with the exception of lung cancer in women (1). The treatment of patients with advanced melanoma stages remains discouraging. Durable clinical cures of patients with stage III or stage IV disease are extremely rare and can probably be attributed more to host defense mechanisms than iatrogenic intervention. Development of hematopoietic cells is regulated by soluble and membrane-bound factors. Some cytokine receptors contain intrinsic tyrosine kinase domains; others, like receptors for IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, or granulocyte macrophage colony-stimulating factor, lack such sequences. In 1991, a murine tyrosine kinase receptor was identified independently by two groups. Rosnet et al. (2) named it flt3; Matthews et al. (3) termed it flk-2 (fetal liver kinase 2). FLT3 is a member of the type III receptor tyrosine kinase family, which also includes the c-kit and c-fms receptors. Murine and human receptors share 86% structural homology. The genes encoding these molecules are located on chromosome 5 in mice and on chromosome 13 in humans (4). FLT3 is expressed in lymphohematopoietic tissues, placenta, brain, and a high proportion of leukemia and lymphoma cell lines (5). In hematopoietic tissues, expression is restricted to the CD34-positive progenitors, including cells with the capacity for long-term reconstitution of irradiated hosts. FLT3 has been used to identify and subsequently clone the corresponding ligand, FL (6, 7). FL is widely expressed in both mouse and human tissues. Murine and human FL are 75% homologous, and both ligands are fully active on cells bearing either murine or human receptors (8). The predominant form of FL is synthesized as a transmembrane protein from which the soluble form is generated, presumably by proteolytic cleavage (9). FL has little hematopoietic activity by itself, but synergizes with other cytokines, such as IL-3, IL-6, IL-7, IL-11, IL-12, and colony-stimulating factors (10). Recently, Maraskovsky (11) reported that administration of FL to mice resulted in increased numbers of functionally active DCs in bone marrow, gastrointestinal lymphoid tissue, liver, lymph nodes, lung, peripheral blood, peritoneal cavity, spleen, and thymus. We obtained similar results (12). FL could be an interesting candidate for in vivo immunotherapy of cancer, because approaches with DCs are usually hampered by low numbers of isolated cells of this type. Lynch et al. (13) have shown an effective treatment of murine fibrosarcoma by FL therapy. The aim of the present study was to investigate whether FL would have antitumor effects in skin tumors. Therefore, we chose a lymphoma model and two melanoma models. Using B16 and CL8–1 melanoma, we compared a poorly immunogenic and a highly immunogenic melanoma, respectively.

Materials and Methods

Mice. Male C57BL/6 mice were obtained from Taconic (Germantown, NY), acclimatized for at least 2 weeks, and used at 6–8 weeks of age. All animals were separated into groups of four mice each and maintained under a 12-h light/dark cycle at a temperature of 20°C–22°C. Food and water were available ad libitum. All animal procedures were approved by the University of Pittsburgh Animal Care Committee.

Tumor Cell Lines. CL8–1 melanoma and EL-4 lymphoma were kindly provided by Dr. Elieser Gorelik (University of Pittsburgh Cancer Institute). B16 melanoma was a gift from Dr. James C. Yang (National Cancer Institute). Cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Life Technologies, Inc., Grand Island, NY). Culture was maintained at 37°C in 5% CO2. All cell lines were mycoplasma free. FL. Lyophilized recombinant human Chinese hamster ovary cell-derived FL with a biological activity of 1.8 × 107 units/μg protein was a generous gift from Immunex Corp. (Seattle, WA). Reconstitution was performed in 0.01% MSA at a concentration of 0.1 mg/ml.

Experimental Design. Mice were injected s.c. on day 0 with 1 × 105 B16 melanoma cells (n = 8), 3 × 105 CL8–1 melanoma cells (n = 8), or 1 × 105 EL-4 lymphoma cells (n = 8). All injections were performed into the shaved right flank in a total volume of 100 μl. Starting 24 h later, mice were injected daily s.c. with either 0.01% MSA or 20 μg of FL in 0.01% MSA for 10 consecutive days. All treatment injections were performed into the shaved left flank in a total volume of 200 μl. Tumor growth was monitored daily. All experiments were repeated independently.

Statistical Analysis. Data analysis was done using SigmaStat software (Systat, San Rafael, CA). The differences between the mean tumor sizes in different groups in dynamic were analyzed using two-way repeated-measure ANOVA. Data are presented as the means ± SE of the mean.
Immunohistochemistry. On day 11 after tumor cell administration, one animal/group was sacrificed by cervical dislocation, and immunohistochemistry of the tumors was performed using 6-μm cryostat sections. After application of the avidin/biotin blocking kit SP-2001 (Vector Laboratories, Inc., Burlingame, CA), the following monoclonal primary antibodies were applied for 1 h: (a) rat antimore mouse CD8 and CD86 (both from PharMingen, San Diego, CA); and (b) NLDC-145 (Serotec, Washington, D. C.). After two washes in PBS, the sections were incubated for 30 min with biotinylated secondary mouse antirat antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The color reaction was developed for 7 min using the peroxidase chromogen kit (AEC; Biomega Corp., Foster City, CA). All sections were lightly counterstained with hematoxylin. The number of positive cells/tissue section was determined semiquantitatively: 0, a completely clean field with no positive staining; +, weak but definitely positive staining of scattered cells; ++, strongly positive staining, with at least 50% of the cells being positive; and +++, a staining intensity between + and ++++. All three stainings for each tumor were performed by independent investigators.

**Results**

FL Inhibits Tumor Growth in B16 Melanoma, CL8-1 Melanoma, and EL-4 Lymphoma. Fig. 1 summarizes the tumor growth data of two independent experiments with B16 melanoma (Fig. 1A), CL8-1 melanoma (Fig. 1B), and EL-4 lymphoma (Fig. 1C). The duration of each experiment was 19 days for melanoma and 17 days for lymphoma. All experiments showed a significant difference between control and treatment groups ($P < 0.001$). In conclusion, these data show that administration of FL inhibits tumor growth in murine melanoma and lymphoma models.

**FL Causes Intratumoral Increase of NLDC-145-positive Cells in B16 Melanoma and EL-4 Lymphoma but not in CL8-1 Melanoma.** Table 1 summarizes the intratumoral staining patterns obtained by immunohistochemistry with the antibodies NLDC-145, CD86 (B7.2), and CD8. There was a significant FL-induced increase in staining intensity for NLDC-145 in B16 and EL-4 tumors in all samples collected on day 11 after injection of tumor cells (Fig. 2). Control tumors were negative, whereas FL-treated tumors showed weak (B16) or moderate (EL-4) staining with this antibody. CL8-1 tumors were strongly positive in all samples, with no differences between FL- and MSA-treated tissue. No differences in staining intensity were observed for either CD86 or CD8 in all tumor sections analyzed. In conclusion, these data suggest that FL therapy results in increased accumulation of DCs in certain murine tumors, and that DCs may be involved in the early antitumor effect of FL.

**Discussion**

B16 melanoma is a poorly immunogenic tumor of spontaneous origin that exhibits highly invasive metastatic ability and does not express either MHC class I or II antigens. The CL8–1 melanoma is a class I transfected B16 tumor, therefore with decreased tumorigenicity (14). EL-4 lymphoma is a chemically induced thymic lymphoma cell line that is syngeneic to C57BL/6 mice. In the present study, all three cell lines were used to evaluate the possible antitumor effect of FL administration. FL is a widely expressed glycoprotein with a $M_r$ of 15,000–18,000 (15). It was originally characterized as hematopoietic growth factor. Recently, it has been shown that FL administration induces the generation of functionally active DCs in several different murine tissues (11, 12). DCs are highly efficient antigen-presenting cells that initiate immune responses such as the sensitization of T cells restricted by MHC molecules. Because DCs have been shown to elicit antitumor responses in vivo (16), DC-based immunotherapy of cancer is currently being investigated in clinical trials. Isolation of adequate numbers of DCs has been difficult and is the main obstacle to these attempts. The most effective cytokine and media combination for the ex vivo generation of high numbers of active DCs remains to be identified. Increasing the numbers of DCs in vivo may represent an alternative approach. Recently, Lynch et al. (13) reported that FL can induce antitumor responses in vivo in a murine fibrosarcoma model. In the present study, this original finding has been confirmed using melanoma and lymphoma models. s.c. injections of 20 μg of FL for 10 consecutive days resulted in tumor growth inhibition of both B16 and CL8–1 melanomas, as well as EL-4 lymphoma. In all experiments, there was a significant difference in tumor growth between the control and treatment group. For immunohistochemical evaluation of intratumoral DCs, we stained tumor sections with the NLDC-145 monoclonal antibody. Over the decade since its discovery in 1986 (17), NLDC-145 has become established as a histochemical and flow cytometric marker for mouse DCs in a variety of tissues. In B16

**Table 1 Intratumoral immunohistochemistry**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Control</th>
<th>FL</th>
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<tbody>
<tr>
<td>B16 melanoma</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>NLDC-145</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CL8-1 melanoma</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>NLDC-145</td>
<td>++++</td>
<td>+++</td>
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<tr>
<td>CD8</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>EL-4 lymphoma</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>NLDC-145</td>
<td>++</td>
<td>++</td>
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<td>CD8</td>
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*++, weak but definitely positive staining of scattered cells; +++, a staining intensity between + and ++++. Strongly positive staining, with at least 50% of the cells being positive; 0, a completely clean field with no positive staining.*

**Fig. 1.** Administration of FL inhibited tumor growth in three different murine models. C57BL/6 mice received $10^5$ B16, $3 \times 10^5$ CL8–1, or $10^5$ EL4 tumor cells followed by 10 injections of FL (10 μg/day/mouse). For each tumor, 2 independent groups of mice were injected (a total of 16 mice for each tumor). Two-way repeated-measure ANOVA revealed that in all tested models, FL significantly inhibited the growth of tumors.
Fig. 2. Administration of FL resulted in a significant increase in NLDC-145-positive cells, presumably DCs, within B16 melanoma (B) and EL-4 lymphoma (F) compared with control samples obtained from nontreated mice (A and E, respectively). No differences were found between CL8–1 melanoma samples obtained from control or FL-treated animals (C and D, respectively).

melanoma and in EL-4 lymphoma, FL administration increased the number of NLDC-145-positive cells, whereas no difference in staining intensity was found for CL8–1 melanoma. This tumor contains significantly more DCs than B16 or EL-4 before FL administration. One could speculate that higher immunogenicity correlates with higher intratumoral numbers of DCs. Lynch et al. (13) assumed that the generation of the immune response to the tumor is the result of augmented generation of DCs by FL treatment. This hypothesis is likely but still needs to be proven. Because FL was effective in all three models and, at the same time, all three tumors contained different amounts of NLDC-145-positive cells before and even after FL treatment, one could speculate that DCs are not the only cell type mediating early antitumor effects after administration of FL. In all three tumors, we found no difference in staining intensity for CD8. Using adoptive transfer of splenocytes, Lynch et al. (13) reported that FL-induced fibrosarcoma regression seemed to be specifically mediated, at least in part, by CD8-positive cells. Additional studies of the mechanism of antitumor activity of FL are necessary and should include functional assays. Natural killer cells are another candidate for mediating early antitumor responses to FL. The results presented in this study may have implications for the therapy of human skin tumors. At the current rate of increase in melanoma incidence, those born in the United States in the year 2000 will have a 1:90 probability of developing this tumor during their lifetime (18). The rising incidence of melanoma, combined with the inadequacies of therapy currently available for metastatic disease, is frustrating. Perhaps the
administration of FL in combination with other cytokines such as IL-2, IL-12, or IL-15 or with tumor antigen may represent a successful treatment option for additional preclinical and clinical trials.

References

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