Antitumor Activity and Immunotherapeutic Properties of Flt3-Ligand in a Murine Breast Cancer Model

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ABSTRACT

Flt3-Ligand (Flt3-L) is a stimulatory cytokine for a variety of hematopoietic lineages, including dendritic cells and B cells. The antitumor properties of Flt3-L were evaluated in C3H/HeN mice challenged with the syngeneic C3L5 murine breast cancer cell line. Eighty % of animals receiving 500 μg/kg/day of Chinese hamster ovary-derived human Flt3-L for 10 days were protected from tumor growth, whether the tumor challenge was administered on the first or fourth days of Flt3-L administration. The protection provided by soluble Flt3-L was transient. All tumor-free animals rechallenged 4 weeks after the primary challenge developed tumor. Transduction of C3L5 with retroviral vectors expressing human or murine Flt3-L did not influence in vitro growth or MHC expression but decreased in vivo tumor development to 0 and 10% of mice, respectively. This compares with tumor growth of 52% with interleukin-2 transduced C3L5 and over 85% with untransduced and control vector-transduced C3L5. Unlike animals treated with soluble Flt3-L, administration of Flt3-L as a tumor vaccine protected mice from a subsequent challenge with untransduced C3L5 in 60—78% of mice, compared to 8% of controls. Our initial work used the most common Flt3-L isoform, which is membrane bound but can undergo proteolytic cleavage to generate a soluble form. To evaluate the role of the various Flt3-L isoforms in preventing tumor formation, retroviral vectors encoding only the membrane-bound form or only the soluble isoform were evaluated in the C3L5 model. Tumor formation was similar with either isoform, preventing tumor formation in 80—90% of mice after the primary challenge and 88—89% after the secondary challenge. Splenocytes obtained 4 weeks after the secondary challenge conferred adoptive immunity to naive mice in 60% of animals. This initial report of antitumor activity by Flt3-L is consistent with its known stimulatory effect on antigen-presenting cells and suggests it may enhance the development of tumor vaccines.

INTRODUCTION

Flt3, also known as flk2 and Stk-1, is a type III tyrosine kinase membrane receptor first identified in hematopoietic stem and progenitor cells, including CD34+ bone marrow and cord blood cells, and is believed to play a role in the regulation of hematopoiesis. (1—3) The Flt3-L isoform is a cytokine with potent stimulatory activity. The murine and human homologues of Flt3-L have been cloned and share 72% amino acid homology (4—6). Human Flt3-L stimulates proliferation of cells expressing the murine Flt3-L receptor, has a similar biological activity on murine bone marrow cells as murine Flt3-L, and has a synergistic effect with other murine cytokines (7). Flt3-L alone has a weak stimulatory effect on in vitro progenitor cell colony growth but has additive or synergistic effects when combined with GM-CSF, granulocyte-CSF, IL-3, IL-11, IL-12, erythropoietin, stem cell factor (steel factor), and other cytokines (7—9). Flt3-L also appears to have a role in lymphopoiesis by supporting the proliferation of B-cell progenitors (8).

Recently, Flt3-L has been noted to possess a growth-stimulatory effect on dendritic cells (10). Because dendritic cells are important antigen-presenting cells, we hypothesized that tumor cells expressing Flt3-L may be useful in inhibiting tumor growth and in promoting tumor immunization. To test this hypothesis, we chose the C3L5 murine breast cancer cell model (11, 12). Animals were immunized with non-irradiated, vector-expressing tumor cell lines, and tumor-free animals were challenged 4 weeks after inoculation with parent C3L5 cells. The antitumor and immunizing activity of recombinant Flt3-L was compared with that of other cytokines with known antitumor activity. Because Flt3-L exists in a variety of isoforms, we constructed retroviral vectors that encode soluble and membrane-bound forms of Flt3-L and compared the antitumor activity of these constructs in our model. In this study, Flt3-L was found to possess antitumor properties, whether given systemically or in the context of a tumor vaccine. Interestingly, sustained antitumor immunity was observed only when Flt3-L was administered as a tumor vaccine.

MATERIALS AND METHODS

Retroviral Vector. Vectors used in this study are shown in Fig. 1. Fragments of cDNA sequence encoding human Flt3-L and specific isoforms of murine Flt3-L (provided by Immunex Corp., Seattle, WA) were inserted into the retroviral vector pLXSN originally developed by A. Dusty Miller. The vectors L(hFlt3-L)SN and L(mFlt3-L)SN were shuttle packaged through the ecotropic packaging cell line GP + E86 (13) into the amphotropic PA317 packaging cell line (14). Three additional vectors were obtained through a collaboration with Genetic Therapy, Inc. (Gaithersburg, MD) and used in this study (Fig. 1). G1Na and LXSNN retroviral vectors are control vectors containing the neomycin resistance gene alone. G1NaSV2G contains the NeoR gene and the human IL-2 gene (titer, 4.2 × 10^5; ELISA activity, >2000 pg/ml IL-2). The G113SvNa vector contains the NeoR gene and the human IL-1β gene (titer, 2.4 × 10^5; ELISA activity, 482 ng/ml).

Cell Lines and Transduction. Retroviral vectors were introduced by supernatant transduction into the syngeneic murine breast cancer cell line C3L5 (kindly provided by Dr. P. K. Lala, University of Western Ontario, London, Ontario, Canada; Refs. 11 and 12). One million C3L5 cells were incubated with vector at a multiplicity of infection of 1:2 for 2 h in the presence of Polybrene (8 μg/ml). C3L5 cells containing vector were obtained by selection in G418 (400 μg/ml) for 10—14 days. C3L5 cells were maintained in DMEM supplemented with 2 mmol/liter glutamine, 10% FCS, and 1 mmol/liter sodium pyruvate and incubated at 37°C, 5% CO₂. Cell lines used in this study tested negative for the presence of Mycoplasma and replication-competent amphotropic retrovirus.

In Vivo Studies. To assess the effect of i.p. administered human Chinese hamster ovary-derived Flt3-L (Ref. 15: 500 μg/kg daily for 10 days) on the growth of cytokine-transduced C3L5, mice were challenged with 1 × 10⁶ C3L5 cells injected s.c. into the anterior chest wall of C3H/HeN female mice.
(primary challenge). Animals were assessed for evidence of local tumor growth. Tumors were measured weekly in two dimensions, and tumor size was calculated using the formula: volume (width^2 X length)/2. If animals were tumor-free at 4 weeks, they were challenged with 1 X 10^6 C3L5 cells. Animals received tumor challenge on the first (day 0) or fourth (day 4) day of Flt3-L administration. As shown in Fig. 2, all mice injected with control vehicle (PBS) developed tumors by week 2, whereas four of five mice in the Flt3-L group were tumor free at 4 weeks. Tumor-free animals were then injected with 5 X 10^3 C3L5 cells in the contralateral chest wall and monitored for tumor growth. Each of the four animals in the day 0 and day 4 groups developed tumors, indicating a lack of immunity (data not shown).

Cytokine Expressing Retroviral Vector in C3L5. To assess the effectiveness of Flt3-L in a tumor vaccine, populations of cytokine-secreting C3L5 cells were generated using the retroviral vectors shown in Fig. 1. Prior to in vivo evaluation, the in vitro growth of vector-transduced cells was assessed to determine potential effects on growth from cytokine expression. As shown in Fig. 3, the growth rates of all vector-transduced C3L5 populations were comparable to untransduced C3L5, suggesting the cytokines studied did not influence cell growth. The one exception noted was C3L5 expressing IL-1β, which demonstrated reduced proliferation after transduction.

Vector-transduced cells were also evaluated for production of human Flt3-L and human IL-2 using assays described previously (16, 17). The parent C3L5 cell line does not produce detectable levels of soluble hFlt3-L or human IL-2 when evaluated by ELISA. Cells

**RESULTS**

**Soluble Flt3-L Administration.** The antitumor activity of soluble Flt3-L was evaluated using the murine breast cancer cell line C3L5. Mice received daily i.p. administration of human Flt3-L (500 μg/kg/ day) for 10 days. This dose was selected because it has no apparent toxicity in mice (15), is known to mobilize murine progenitor cells (15), and stimulates murine dendritic cell proliferation (10). Primary tumor challenge consisted of 1 X 10^6 C3L5 cells. Animals received tumor challenge on the first (day 0) or fourth (day 4) day of Flt3-L administration. As shown in Fig. 2, all mice injected with control vehicle (PBS) developed tumors by week 2, whereas four of five mice in the Flt3-L group were tumor free at 4 weeks. Tumor-free animals were then injected with 5 X 10^3 C3L5 cells in the contralateral chest wall and monitored for tumor growth. Each of the four animals in the day 0 and day 4 groups developed tumors, indicating a lack of immunity (data not shown).

**Flow Cytometry.** Class I and class II MHC antigens were evaluated using the phycoerythrin-conjugated anti-mouse H-2Kk and FITC-conjugated anti-mouse I-Ak (PharMingen, San Diego, CA). Simultest Control γ/β2 FITC/phycoerythrin antibodies (Becton Dickinson, San Jose, CA) were used as the isotype control. For cytometric analysis, 5 X 10^5 cells were washed in PBS, pelleted, and incubated with the appropriate antibody for 20 min on ice. Antibody-exposed cells were washed once in PBS with 1% human serum albumin and then resuspended in a volume of 0.5 ml and analyzed on a FACScan (Becton Dickinson Immunocytometry Systems). Membrane-bound Flt3-L was evaluated using a human Flt3 receptor-human Fc fusion protein (4). The receptor-Fc protein was incubated with cells for 30 min at room temperature. Cells were washed twice with PBS containing 2% PBS + 0.1% sodium azide and incubated with biotin-conjugated anti-human Fc (Caltag, Burlingame, CA) for 30 min. Tertiary staining was performed with streptavidin-conjugated with TRICOLOR (Caltag) and analyzed on a FACScan.
transduced with G1NaSvi2G vector produced 551 pg of IL-2/10^6 cells/24 h. C3L5 cells transduced with the L(hFlt3-L)SN vector, which encodes a transmembrane isoform of human Flt3-L that is expressed on the cell surface and can be proteolytically cleaved to generate a soluble form (18), produces 6058 pg of hFlt3-L/10^6 cells/24 h. Using a human Flt3 receptor-human Fc hybrid protein, membrane-bound human Flt3-L was also detected on transduced C3L5 cells (Fig. 4). Murine Flt3-L ligand production was not assessed in this study, due to the lack of an anti-murine Flt3-L antibody.

**Primary Tumor Challenge.** To determine whether tumor formation would be affected by cytokine gene expression, C3H/HeN female mice received s.c. injections of 1 × 10^6 cytokine-transduced C3L5 cells. Animals were followed weekly for 4 weeks for evidence of tumor growth. Palpable tumors were measured weekly in two dimensions, and the results of this analysis are shown in Fig. 5A. C3L5 cells expressing IL-2, murine Flt3-L, hFlt3-L, and human IL-1β prevented tumor growth in 40, 90, 100, and 100% of animals, respectively. A two-tailed Fisher’s Exact test was used to compare the proportion of tumor-bearing mice in each group to control. No significant differences were noted in the number of animals developing tumors in the untransduced and G1Na control groups (P = 1). Significantly less tumor growth was noted in cytokine-expressing C3L5 groups (P < 0.001 for all groups).

**Tumor Immunization.** To determine if tumor-free animals injected with cytokine-expressing C3L5 were immunized against the parent tumor, we performed a secondary challenge with untransduced C3L5 cells 4 weeks after the primary tumor challenge. Naive mice were used as a challenge control. Our findings, shown in Fig. 5B, demonstrate that the majority of animals injected with C3L5 cells expressing murine Flt3-L, hFlt3-L, and IL-2 are immunized to a subsequent challenge with untransduced C3L5 cells (P < 0.01 for all groups when compared to control). Interestingly, although C3L5 cells transduced with IL-1β did not form tumors in vivo (Fig. 5A), this did not result in immunization against a subsequent challenge with the untransduced C3L5 (Fig. 5B).

**Membrane-Bound versus Soluble Flt3-L Isoforms.** The murine Flt3-L gene gives rise to three functional isoforms, which can be membrane bound, soluble, or both (19). To evaluate the relative role of Flt3-L isoforms in preventing tumor growth, we constructed retroviral vectors producing either a strictly membrane-associated form or a purely soluble Flt3-L [L(hFlt3-L)SN and L(Ex6)SN, respectively; Fig. 1]. As shown in Table 1, both isoforms significantly decreased primary tumor formation (P < 0.001) and immunized mice against a second challenge of the parent C3L5 cell line (P < 0.01) when compared to control C3L5. As shown in Table 2, inhibition of tumor growth by Flt3-L vectors can be overcome by increasing the dose of Flt3-L-transduced C3L5 cells. There was no clear difference between either isoform with regard to preventing tumor growth or slowing tumor development.

**Protection from Tumor Challenge by Adoptive Transfer of Immunity.** The mechanisms by which tumor rejection was mediated were initially evaluated using cytotoxicity assays described previously (20). Coculture of chromium-labeled C3L5 cells and splenocytes from animals immunized with untransduced and vector-transduced C3L5 cells failed to demonstrate significant levels of cytotoxicity. These results were seen using freshly explanted splenocytes or splenocytes restimulated in vitro for 1 week with irradiated C3L5 cells prior to cytotoxicity testing (data not shown). Because this in vitro assay may not accurately reflect the in vivo environment, we chose to evaluate adoptive transfer of splenocytes from immunized animals. These
ANTITUMOR ACTIVITY OF Flt3-LIGAND IN BREAST CANCER

**DISCUSSION**

Flt3-L is a recently identified cytokine that demonstrates stimulatory activity for myeloid and lymphoid lineages (7–9). The recently identified dendritic cell and B-cell growth-promoting activity of Flt3-L prompted us to evaluate the activity of Flt3-L as an antitumor agent. Based on our interest in breast cancer (21, 22), we used the murine breast cancer model C3L5 and found that i.p.-administered Flt3-L prevented tumor formation in the majority of animals. Despite the antitumor activity observed after the primary tumor challenge, systemic administration of Flt3-L did not provide long-lasting immunity at the dose schedule evaluated.

Expression of Flt3-L by murine breast cancer cells did not affect their in vitro growth but did prevent tumor formation in vivo. In addition, tumor-free animals treated previously with Flt3-L-expressing C3L5 cells are protected from a second challenge with the parent C3L5 cells. This is in contrast to IL-1β-transduced C3L5 cells, which prevented tumor growth in vivo but did not immunize animals against a secondary challenge. IL-1β-transduced C3L5 cells demonstrated decreased in vitro growth that may have contributed, at least in part, to the lack of tumor growth in vivo. Tumor formation after a secondary challenge in IL-1β-C3L5-treated mice and in the occasional control mouse that did not develop tumor after the primary challenge (Fig. 5) suggests that inoculation of tumor cells alone is insufficient to elicit immunity to C3L5.

A number of investigators have demonstrated tumor immunization using cytokine-expressing tumor cells. Previously tested cytokines have a variety of stimulatory and inhibitory activities and include agents such as GM-CSF, IL-2, IL-4, IL-6, IL-7, tumor necrosis factor, and the interferons (23–27). In the present model, human and murine Flt3-L prevented primary tumor growth in the majority of animals, whereas IL-2 protected approximately 50% of animals. Flt3-L is the first early-acting cytokine with known stimulatory activities of dendritic and pre-B cells to exhibit tumor-immunizing properties.

The primary translation products of the human and murine Flt3-L genes are transmembrane proteins that can undergo proteolytic cleavage, generating a soluble protein (4, 6). These cDNAs were used to generate the L(hFlt3-L)SN and L(mFlt3-L)SN vectors. In addition, alternative splicing of Flt3-L mRNA can generate isoforms that are purely soluble or only membrane bound (18, 19). Inclusion of intron

![Figure 5](https://example.com/figure5.png)

Fig. 5. Tumor growth after primary and secondary challenge with vector-transduced C3L5 cells. In A, the primary challenge consisted of $1 \times 10^4$ vector-transduced C3L5 cells s.c. into the chest of C3H/HeN mice. Animals were followed for 4 weeks for evidence of tumor growth. In B, a portion of tumor-free mice received a secondary challenge with $5 \times 10^5$ untransduced C3L5 cells into the contralateral chest. Treatment groups consisted of untransduced cells (C3LS) or C3L5 cells transduced with the GlNa vector (Neo), the Gll12SVNa vector (IL-1), the Gll12SVt2G vector (IL-2), the L(hFlt3-L)SN vector (hFlt3-L), or the L(muFlt3-L)SN vector (mFlt3-L). Control for the secondary challenge consisted of untransduced C3L5 cells in naive mice. The numbers presented along the X-axis indicate the number of animals in each group.

Table 1

<table>
<thead>
<tr>
<th>Tumor growth in mice challenged with C3LS cells containing retroviral vectors expressing two isoforms of Flt3-L</th>
</tr>
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<tbody>
<tr>
<td>Primary challenge</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>C3L5</td>
</tr>
<tr>
<td>C3L5/Neo vector</td>
</tr>
<tr>
<td>C3L5/Flt3-LSN</td>
</tr>
<tr>
<td>C3L5/Ex6SN</td>
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Table 2

<table>
<thead>
<tr>
<th>Tumor growth in C3LS cells expressing the soluble (Ex6) and membrane-bound (5H) forms of murine Flt3-L</th>
</tr>
</thead>
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<tr>
<td>Tumor dose</td>
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<tr>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Week 1</td>
</tr>
<tr>
<td>C3L5</td>
</tr>
<tr>
<td>2 ± 1</td>
</tr>
<tr>
<td>697 ± 106</td>
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</table>

* NT, not tested.
C3L5 cells, do not express detectable amounts of membrane-bound formation, evaluation of the infiltrating cells at the sight of the tumor for tumor formation and the rate of in vivo tumor growth are also cleavage and was used to generate the L(5H)SN vector. Alternate cells for tumor recognition, a requirement that could explain the lack mechanisms, such as an in vivo enhancement of antigen presentation by 5 produces a hydrophobic domain that does not undergo proteolytic cleavage and was used to generate the L(Ex6)SN vector. Alternate splicing of exon 6 leads to a premature stop codon before the transmembrane region and was used to generate the L(Ex6)SN vector. Both the soluble and membrane-bound forms of Flt3-L are biologically active, but the relative biological significance of either isoform is unknown (4). A wide variety of hematopoietic cell lines express the membrane-bound form of Flt3-L (28, 29), but other cells, including C3L5 cells, do not express detectable amounts of membrane-bound Flt3-L. We observed that either Flt3-L isoform introduced into C3L5 cells prevents tumor growth and confers long-lasting immunity that can be adaptively transferred to naive mice. The C3L5 dose required for tumor formation and the rate of in vivo tumor growth are also similar in this model. These findings suggest that both isoforms possess equivalent antitumor properties in this model. The precise mechanisms by which Flt3-L-expressing tumor cells confer tumor immunization are unknown. Immunizing properties have been described for a wide variety of cytokines (23–27), and the breadth in structure and function of these cytokines suggests that a variety of pathways may lead to tumor immunization. Although the immunizing properties of Flt3-L may be a direct effect on T lymphocytes, its superiority to IL-2 in our study suggests that other mechanisms, such as an in vivo enhancement of antigen presentation by dendritic cells, may be responsible for the development of T cell-mediated immunity. The low level of class I and class II MHC expression on C3L5 cells may require the recruitment of accessory cells for tumor recognition, a requirement that could explain the lack of in vitro cytotoxicity noted in our work but successful tumor rejection by adoptive transfer of splenocytes. Our findings are consistent with previous reports of tumor-immunizing activity in cytokines, the physiological activity of which has been ascribed to non-T-cell hematopoietic lineages (e.g., IL-6 and GM-CSF; Refs. 23 and 26). In particular, GM-CSF has been found to be superior to a wide variety of cytokines in the B16 melanoma model, including IL-2 (23). Because increasing concentrations of C3L5/Flt3-L cells lead to tumor formation, evaluation of the infiltrating cells at the sight of the tumor challenge may also provide an insight into the local effect of Flt3-L in this model. The B-cell growth properties of Flt3-L warrant evaluation of humoral immunity. Beyond its potential as a tumor vaccine, we hope that C3L5/Flt3-L may prove useful in determining the immunological properties of Flt3-L.

Table 3 Adoptive transfer of immunity in mice challenged with C3L5 cells

<table>
<thead>
<tr>
<th>Spleen source</th>
<th>Splenocyte protection</th>
<th>Total T-cell protection</th>
<th>CD4 cell protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3L5(5H)SN</td>
<td>2/5</td>
<td>2/5</td>
<td>4/5</td>
</tr>
<tr>
<td>C3L5(Ex6)SN</td>
<td>2/5</td>
<td>2/5</td>
<td>4/5</td>
</tr>
<tr>
<td>Control splenocytes</td>
<td>5/5</td>
<td>5/5</td>
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</tr>
<tr>
<td>Naive mice</td>
<td>3/3</td>
<td>3/3</td>
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* K. Cornetta, unpublished data.

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