Antileukemic Activity of Flt3 Ligand in Murine Leukemia

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ABSTRACT

Flt3-ligand (Flt3-L) is an early acting costimulatory cytokine that has been shown to possess antitumor properties in murine solid tumor models. Flt3-L is a trans-membrane protein (tm) but can be proteolytically cleaved to a soluble form, which is also biologically active. In this study, the antitumor effect of both soluble and tmFlt3-L was evaluated in a mouse leukemia model. To mimic the multiorgan involvement characteristic of human leukemia, a factor-dependent cell line FDC.P1 was made leukemogenic by transfection with the human BCR/ABL gene. The resulting cell line, AW, expresses BCR/ABL RNA and protein. It maintains a similar in vitro growth rate as the parent cell line, but unlike the parent cell line, AW cells are factor independent and tumorigenic. Growth of FDC.P1 and AW cells are unaffected by the addition of soluble human Flt3-L to the culture medium. Also, AW growth is unaltered after transduction with a retroviral vector expressing the tm isoform of human Flt3-L (AW/tmFlt3-L). When 10^6 AW cells were i.v. injected into syngeneic DBA/2 mice, fatal leukemia developed in nine of nine (100%) mice within 4–6 weeks with involvement of the blood, bone marrow, spleen, and thymus. Systematic administration of soluble human Flt3-L (500 μg/kg/day) for 10 days protected mice from leukemia, with 11 of 17 mice tumor free at week 8 (64.7%). The tm isoform of Flt3-L also was protective. When 10^6 AW/tmFlt3-L cells were injected i.v. into mice, only 35.7% (5 of 14) developed leukemia versus 100% in control groups. Adoptive transfer of immunity was also demonstrated; T cells obtained from tumor-free animals conferred protection to 87% (seven of eight) naive mice challenged with AW cells. These results demonstrate that both soluble and membrane-bound human Flt3-L has antitumor activity in this leukemia model.

INTRODUCTION

Flt3-L is an early-acting costimulatory cytokine that regulates proliferation and differentiation of a number of blood cell lineages (1–3). Its receptor, Flt3, belongs to a family of receptors including other cytokines to stimulate proliferation and differentiation of hematopoietic stem cells and progenitor cells (7, 8). Flt3-L is an early-acting costimulatory cytokine that regulates proliferation and differentiation of a number of blood cell lineages (1–3). Its receptor, Flt3, belongs to a family of receptors including other cytokines to stimulate proliferation and differentiation of hematopoietic stem cells and progenitor cells (7, 8). In vivo and in vitro studies have shown that Flt3-L plays an important role in both multipotent stem and lymphoid cell differentiation (9–14).

Recently, Flt3-L was shown to induce expansion of functional DCs and NK cells in vivo (15–18). Soluble Flt3-L has been shown to possess antitumor activity in murine models (19, 20). In a solid tumor model, our laboratory has shown that transduction of cancer cells with retroviral vectors expressing Flt3-L induces tumor rejection and generates CD8^+ T cell-mediated tumor immunity to the parent cancer cells (20, 21). Because both DC and NK cells are important mediators in the immune system, the DC and NK cell stimulatory property of Flt3-L is one possible explanation for the tumor prevention properties observed (21).

Current models demonstrating the antitumor activity of Flt3-L use solid tumors. Such models provide a concentrated collection of tumor cells for an immune-mediated response and can also be affected by nonimmunological mechanisms affecting tumor growth, such as angiogenesis. To minimize the effect of these factors, we assessed the role of Flt3-L in a liquid tumor model. We developed a murine leukemia model by transfecting the human BCR/ABL gene into the factor-dependent cell line FDC.P1 cells. The resulting cell line, AW, is factor independent and tumorigenic (22, 23). This model provides a systemic leukemia similar to that seen in human. Using this model, we show that both soluble and tm isoforms of Flt3-L have antileukemia activity.

MATERIALS AND METHODS

Retroviral Vectors and Cell Lines. Retroviral vectors used in this study have been described previously (20) and are shown in Fig. 1. The LtmFlt3-L vector contains full-length cDNA sequence encoding the human tm isoform Flt3-L (tmFlt3-L; provided by Immunex Corp., Seattle, WA) and the neomycin phosphotransferase gene 3’ to the SV40 early promoter. The LNL6 retroviral vector, which contains the neomycin phosphotransferase gene, was used as a control (24).

The IL-3-dependent hematopoietic line FDC.P1 (25) was maintained in RPMI 1640 supplemented with 10% FCS and 10% WEHI-3 CM (26). The BCR/ABL transformed FDC.P1 cells were named AW. AW and retrovirally transduced cell lines, AW/LNL6 and AW/tmFlt3-L, were maintained in RPMI 1640, 10% FCS, without WEHI-3 CM. Murine IL-3-dependent hematopoietic cell line Ba/F3/tFlt3, a subline of Ba/F3 that ectopically expresses human Flt3 receptor, was cultured in RPMI 1640 supplemented with 10% FCS and 0.1 ng/ml murine IL-3 (27). All cells used in this study were kept in continuous culture for a maximum of 2 months. Cell viability was determined by trypan blue exclusion.

Transfection and Transduction. The AW cell line was generated by transfection of FDC.P1 cells with the human BCR/ABL gene. pGDeNeo, a plasmid created by removal of the neomycin phosphotransferase gene from the pGD210 plasmid (28) using the ClaI restriction enzyme contains the P210^{eas} cDNA driven by the myeloproliferative sarcoma virus long terminal repeat and was linearized with NdeI prior to electroporation. Electroporation was carried out on Gene Pulser (Bio-Rad, Richmond, CA) using published methods (29). WEHI CM was removed from culture medium 48 h after transfection, and AW cells were the population of IL-3-independent cells generated after culture of 2 weeks.

Retroviral vectors were introduced by supernatant transduction into the AW cells. Briefly, 10^9 AW cells were incubated with vector at 37°C for 2 h in the presence of Polybrene (8 μg/ml). These cells were cultured for 24 h and then placed in selection for 14 days using G418 (400 μg/ml active compound).

Minimal Residual Disease Assay. Two assays were used to assess minimal residual disease. A single-cell suspension of 10^9 marrow, spleen, or thymic mononuclear cells was plated in RPMI (10% FBS) without WEHI. Assays were scored as positive if viable cells were detected after 3 weeks of culture initiation. The second assay used RT-PCR. Total RNA was isolated using TRI-Pure isolation reagent from 10^5 cells. cDNA was synthesized using random primers. Nested PCR primers were described previously (30). First-round and second-round primers are 5’-AGCATGGCCTTCAGGTGCACAGCCGCAACGGCAA-3’/5’-TCCTGACTATGAGCGTGCA-3’ (Flt3-L) and 5’-GTTCCTGATCTCCTCTGACTATGAGCGTGCA-3’/5’-TCAGACCCTGGAGTTTCTCGTGGT-3’ and amplify 390- and 299-bp fragments, respectively. Amplification of murine β-actin was used as an internal control.

Flow Cytometry. Surface expression of Flt3-L in AW/tmFlt3-L cells was measured by flow cytometry. Briefly, AW and AW/tmFlt3-L cells were washed twice with PBS and stained with either rat anti-human Flt3-L antibody (Immunex Corp.) or isotype-matched control antibody (PharMingen, San Francisco, CA).

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3 The abbreviations used are: Flt3-L, Flt3-ligand; DC, dendritic cell; NK, natural killer; tm, trans-membrane; IL, interleukin; CM, conditioned medium; RT-PCR, reverse transcription-PCR; PE, phycoerythrin.
Development of a Murine Leukemia Model. Hariharan et al. (22) has shown previously that FDC.P1 cells transfected with the BCR/ABL gene cause tumors when injected s.c. and i.p. into mice. To test the antileukemia effect of Flt3-L, we developed a murine leukemia model using a similar strategy. FDC.P1 cells (8 × 10^6) were electroporated with the BCR/ABL gene and selected in medium containing only 10% FCS. The resulting cell population (AW) was factor independent, whereas the culture of parental FDC.P1 without IL-3 resulted in no viable cells after 7 days of culture (data not shown). RT-PCR (Fig. 2A) and immunoblotting (Fig. 2B) confirmed expression of BCR/ABL mRNA and protein in AW cells. Expression of the BCR/ABL gene did not alter the expression of T-cell, B-cell, and myeloid cell surface markers (CD4, CD8, B220, and Gr-1, respectively; data not shown).

We now show that i.v. injection of BCR/ABL-expressing FDC.P1 cells into syngeneic DBA/2 mice results in lethal leukemia characterized by leukocytosis with circulating leukemic blasts, splenomegaly, thymus enlargement, and development of hind limb paralysis (Table 1 and data not shown). RT-PCR of spleen, thymus, and bone marrow for BCR/ABL confirmed the presence of leukemic cells in AW-injected mice. Table 1 showed the analysis of live mice 4 weeks after the injection of 10^6 AW cells. Injection of the parental cell line FDC.P1 (10^6) did not cause leukemia after 12 weeks of observation. The survival of mice challenged with AW cells was dose dependent, with lethal disease developing in a median of 6 weeks after injection of 10^6 cells and 10^6 cells causing leukemia in 4 weeks (data not shown).

To evaluate the intrinsic immunogenicity of the AW cells, we immunized naive mice with 10^6 irradiated (3500 rads) AW cells.

### RESULTS

| BCR/ABL-expressing FDC.P1 cells (AW) cause systemic acute leukemia |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | BCR/ABL mRNA    | Protein         | BCR/ABL mRNA    | Protein         | BCR/ABL mRNA    | Protein         |
|                                | Expression (%)  | Expression (%)  | Expression (%)  | Expression (%)  | Expression (%)  | Expression (%)  |
| Untreated                       | 75 ± 5          | 80 ± 5          | 70 ± 5          | 80 ± 5          | 75 ± 5          | 80 ± 5          |
| AW                              | 95 ± 5          | 95 ± 5          | 90 ± 5          | 95 ± 5          | 90 ± 5          | 95 ± 5          |
| FDC.P1                          | 90 ± 5          | 90 ± 5          | 85 ± 5          | 90 ± 5          | 85 ± 5          | 90 ± 5          |

*p < 0.05 by unpaired Student’s t test. Analysis of blood cell counts, organ weight, and BCR/ABL expression in five mice treated with 10^6 AW or FDC.P1 cells, showing that AW cells cause lethal leukemia, whereas FDC.P1 cells do not.
injected s.c. One week later, mice were challenged with $10^4$ AW cells i.v., and four of four animals developed tumor. In a subsequent experiment, 10 mice were vaccinated with $10^6$ irradiated AW cells on days 1, 4, and 8 with AW challenge ($10^4$ cells) on day 11. All 10 animals developed leukemia in the time frame seen with unimmunized animals challenged with AW cells (data not shown), indicating that AW cells are poorly immunogenic.

Systemic Administration of Soluble Human Flt3-L Reduced Leukemia Development in Mice Challenged with AW Cells. To evaluate the effect of antitumor activity of soluble Flt3-L on leukemia development, DBA/2 mice were challenged with a single injection of $10^5$ AW cells during a 10-day course of Flt3-L administration. The treatment group received i.p. Flt3-L at 500 μg/kg/day for 10 consecutive days, whereas the control group received PBS for 10 days. Leukemic challenge was given on the fourth day of the 10-day course. As depicted in Fig. 3, 16 of 17 (94.1%) Flt3-L-treated mice were alive at 8 weeks, compared with 1 of 9 (11.1%) in the control group. At 8 weeks, all PBS-treated mice had evidence of leukemia, whereas 11 of 17 (65%) in the Flt3-L-treated group were disease free, as assessed by RT-PCR and in vitro culture of bone marrow, spleen, and thymus. The differences between Flt3-L and PBS treatment were significant ($P < 0.05$) by unpaired Student’s t test. These results demonstrate that soluble Flt3-L can protect mice from leukemia.

AW Cells Transduced with a Retroviral Vector Expressing the tm Isoform of Flt3-L (tmFlt3-L) Have Biological Activity. To assess whether tmFlt3-L has antileukemia activity, AW cells were transduced with L(tmFlt3-L)SN vector or LNL6 vector as described in “Materials and Methods.” L(tmFlt3-L)SN vector encodes a tm isoform of human Flt3-L that is expressed on the cell surface and can be proteolytically cleaved to generate a soluble form in breast cancer cell line C3L5 (20). Expression of the tm isoform of human Flt3-L on infected AW cells (AW/tmFlt3-L) was confirmed by surface staining and flow cytometry (Fig. 4A). Transduction of AW cells with retroviral vectors did not alter their in vitro growth characteristics (Fig. 4B) and phenotype (data not shown). The secretion of the soluble isoform of Flt3-L was not detectable.
in AW/tmFlt3-L cells in contrast to other cancer cell lines we have studied (20). It is known that expression of the protease required for proteolytic cleavage of tmFlt3-L is not expressed in all cell types, and our findings indicate that it is not expressed in AW cells.

To confirm that tmFlt3-L on the surface of AW/tmFlt3-L cells has biological activity, we examined the ability of AW/tmFlt3-L to induce tyrosine phosphorylation of the human Flt3 receptor on the surface of Baf3/Flt3 cells. Incubation of AW/tmFlt3-L with Baf3/Flt3 for 5 min induced stronger tyrosine phosphorylation of Flt3 on Baf3/Flt3 than soluble Flt3-L (Fig. 5). Neither AW nor AW/LNL6 cells stimulated tyrosine phosphorylation of Flt3 on Baf3/Flt3. None of AW derivative cell lines expressed Flt3 receptor (data not shown). In Baf3/Flt3 cells, there is some background tyrosine phosphorylation of Flt3. This is mostly attributable to low level expression of murine Flt3-L by Baf3/Flt3 cells, which can cross-react with human Flt3-L.4 These results demonstrate that tmFlt3-L on AW/tmFlt3-L cells is biological active.

tmFlt3-L-transduced AW Cells Have Reduced Tumorigenicity, and Naive Mice Were Protected from Tumor by Adoptive Transfer of Immunity. We next examined whether tmFlt3-L transduction of AW cells would have any effect on their ability to cause leukemia. DBA/2 female mice received i.v. injections with 10⁴ AW/tmFlt3-L cells, AW/LNL6 cells, or AW cells. As shown in Fig. 6, tmFlt3-L has a statistically significant effect on survival. Mice received AW/tmFlt3-L had delayed onset of leukemia compared with mice that received AW cells. Nine of 14 (64%) mice that received AW/tmFlt3-L were alive after 12 weeks, whereas 0 of 14 (0%) were alive in the control group. Mice alive that survived AW/tmFlt3-L cell challenge appeared to be leukemia free; no leukemia cells were detected by in vitro culture assay of bone marrow, thymus, and spleen cells from these mice (data not shown).

To measure whether AW/tmFlt3-L-injected mice develop immunity to AW cells, adoptive transfer of T cells was performed. T cells were obtained from mice 14 weeks after injection with AW/tmFlt3-L. Splenocytes from untreated mice were used as control. As shown in Fig. 7, T cells from AW/tmFlt3-L-treated mice provided a significantly higher protection in mice (87%; seven of eight) challenged with the parent AW cells as compared with T cells from naive mice (0%; 0 of 10).

DISCUSSION

Flt3-L have been shown to have antitumor activity in murine solid tumor models (19,20). Our studies show that Flt3-L is the most potent cytokine among those possessing antitumor activity in a murine breast cancer model (20,21). We now demonstrate the therapeutic efficacy of Flt3-L in a mouse leukemia model. Similar to observations in localized solid tumor, systemic administration of soluble Flt3-L inhibited the development of malignancy. We also demonstrate that Flt3-L-transduced leukemia cells promote tumor rejection and lead to antitumor immunity that can be conferred on naive mice by adoptive transfer of T cells.

Flt3-L stimulates proliferation and differentiation of a wide variety of hematopoietic cells including primitive progenitor cells, dendritic cells, B cells, and NK cells (7–14). Human and murine Flt3-L share 72% amino acid homology, have cross-species reactivity (2,3,7,31,32), and have similar immunizing properties in a murine breast cancer model (20). The primary translation products of Flt3-L are tm proteins

4 K. Brasel, personal communication.
that can be proteolytically cleaved to generate a soluble isoform (1, 3). Not all cells expressing tmFlt3-L generate the soluble isoform because activity of the protease required for cleavage of the tm form varies among different cell types (7). It appears that the protease is not active in AW cells because only tmFlt3-L was detected. In our leukemia model, expression of the soluble form on AW cells was not required for immunizing activity. This is consistent with our finding in murine breast cancer, using retroviral vectors that provided selective expression of the tmFlt3-L isoform (using an isoform that lacks the proteolytic cleavage site), had similar antitumor activity compared with vectors that produced both tmFlt3-L and soluble Flt3-L (20).

Because Flt3-L has known stimulatory activity in hematopoietic cells, we evaluated the effect of soluble and tmFlt3-L on the growth of the leukemic AW cells. In vitro growth of AW cells was not altered. Expression of tmFlt3-L from our retroviral vector led to detectable protein on the surface of transduced cells (as assessed by flow cytometric staining). The tmFlt3-L was biologically active because AW/tmFlt3-L cells induced tyrosine phosphorylation of Flt3 receptor in Baf/Flt3 cells.

In this study, we found that both soluble and tm isoforms of Flt3-L prevent development of leukemia in the majority of mice. In addition, tumor-free animals treated previously with tmFlt3-L-expressing AW cells conferred adoptive immunity to naive mice, implying that leukemia-specific immunity developed in tumor-free AW/tmFlt3-L-treated mice. Immunizing properties have been described for a wide variety of cytokines including IL-2, IL-4, IL-12, IFN-γ, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor (33–37). The wide variety in structure and function of these cytokines suggest that several pathways may lead to tumor immunization. Because Flt3-L has been shown to mobilize and stimulate hematopoietic progenitor cells (11, 14, 38) and to stimulate DC and NK cells (15, 16, 18, 39), the antitumor activities of Flt3-L are potentially mediated through DC and NK cells (21, 40, 41). Recent studies by our group indicated that tumor rejection involves NK cells because in vivo depletion of NK cells negates the antitumor activity of Flt3-L (21). This confirms a previous report by Peron et al. (40), suggesting Flt3-L may stimulate the antitumor activity of NK cells.

We have previously addressed the mechanism permitting adoptive transfer of immunity after immunization with Flt3-L-expressing tumor cells. In a murine breast cancer model, naive mice were protected from tumor challenge after receiving unfractionated splenocytes or splenic T cells but not splenic CD4-positive T cells, consistent with a CD8-mediated immunity (20). We have now shown that expression of Flt3-L on liquid tumor cells can also promote tumor rejection and elicit T cell-mediated immunity.

The leukemia model developed for this work has a number of interesting properties. Hariharan et al. (22) and others has shown previously that expression of BCR/ABL in FDC.P1 cells abrogated the need for exogenous growth factors (22, 23). BCR/ABL transforms the nontumorigenic FDC.P1 cells, leading to tumor nodules when cells are injected s.c. and ascitic tumors when injected i.p. (22). We now demonstrate that i.v. injection of BCR/ABL-expressing FDC.P1 cells leads to a systemic leukemia with infiltration of bone marrow, spleen, thymus, and peripheral blood. The initial indication of overt leukemia is most often hind leg paralysis, and autopsy revealed infiltration of the central nervous system with leukemic cells. This model may be suitable for evaluating the treatment of systemic leukemia, including the efficacy of treatment in protected sites such as the central nervous system. Another advantage of this model is the ability to detect minimal residual disease. We and others have described the sensitivity of the PCR in detecting the BCR/ABL translocation, which can detect one BCR/ABL-expressing cell in one million cells that do not express this oncogene (42). The ease with which these cells can be rescued from organs by placing splenocytes or bone marrow cells in cultures allows screening of a large number of cells for the presence of leukemia. These methods extend the relative period of observation beyond the 8-week assay period. Although it is possible that PCR-negative animals may harbor leukemic cells that will eventually lead to overt leukemia, the current system of PCR detection combined with culture assays for residual leukemia improves the sensitivity of disease detection, compared with animal observation, by many orders of magnitude. The ability to adoptively transfer immunity also suggests that the animals vaccinated with AW/tmFlt3-L are leukemia free.

In summary, we developed a BCR/ABL-expressing murine leukemia model with similar characteristics to human acute leukemia. Our data indicate that both soluble Flt3-L and tmFlt3-L protect mice from tumor challenge after receiving unfractionated splenocytes or splenic T cells but not splenic CD4-positive T cells, consistent with a CD8-mediated immunity (20). We have now shown that expression of Flt3-L on liquid tumor cells can also promote tumor rejection and elicit T cell-mediated immunity.
challenged with BCR/ABL-expressing leukemia cells. Transduction of leukemia cells with tmFlt3-L generates antileukemia immunity against parent leukemia cells. We believe that antitumor effect of Flt3-L in this BCR/ABL-expressing leukemia model will be useful in human leukemia immunotherapy.

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