PT-100, a Small Molecule Dipeptidyl Peptidase Inhibitor, Has Potent Antitumor Effects and Augments Antibody-Mediated Cytotoxicity via a Novel Immune Mechanism

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ABSTRACT

The amino boronic dipeptide, PT-100 (Val-boro-Pro), a dipeptidyl peptidase (DPP) inhibitor, has been shown to up-regulate gene expression of certain cytokines in hematopoietic tissue via a high-affinity interaction, which appears to involve fibroblast activation protein. Because fibroblast activation protein is also expressed in stroma of lymphoid tissue and tumors, the effect of PT-100 on tumor growth was studied in mice in vivo. PT-100 has no direct cytotoxic effect on tumors in vitro. Oral administration of PT-100 to mice slowed growth of syngeneic tumors derived from fibrosarcoma, lymphoma, melanoma, and mastocytoma cell lines. In WEHI 164 fibrosarcoma and EL4 and A20/2J lymphoma models, PT-100 caused regression and reduction of tumors. The antitumor effect appeared to involve tumor-specific CTL and protective immunological memory. PT-100 treatment of WEHI 164-inoculated mice increased mRNA expression of cytokines and chemokines known to promote T-cell priming and chemotraction of T cells and innate effector cells. The role of innate activity was further implicated by observation of significant, although reduced, inhibition of WEHI 164 and A20/2J tumors in immunodeficient mice. PT-100 also demonstrated ability to augment antitumor activity of rituximab and trastuzumab in xenograft models of human CD20+ B-cell lymphoma and HER-2+ colon carcinoma where antibody-dependent cytotoxicity can be mediated by innate effector cells responsive to the cytokines and chemokines up-regulated by PT-100. Although CD26/DPP-IV is a potential target for PT-100 in the immune system, it appeared not to be involved because antitumor activity and stimulation of cytokine and chemokine production was undiminished in CD26−/− mice.

INTRODUCTION

In addition to mutations that give rise to malignant transformation (1), tumor progression requires various responses on behalf of the host animal. These include the development of a tumor stroma derived from host fibroblasts (2), vascularization of the tumor (3, 4), and suppression of an effective immune response against tumor-associated antigens (5, 6). The growth of solid tumors is accompanied by tissue remodeling that appears to involve interactions between the tumor cells and the stroma (2). The reactive stromal fibroblasts are not transformed, but they are distinguished from normal fibroblasts by their production of certain extracellular matrix proteins and proteases (7, 8). Among the latter, fibroblast activation protein (FAP) appears to be consistently expressed as a cell-surface protein in the stroma of malignant solid tumors (9). The presence of FAP in two malignant melanoma cell lines (10, 11) also suggests that FAP expression can be consistent with the production of cytokines and chemokines known to promote T-cell priming and chemotraction of T cells and innate effector cells. The role of innate activity was further implicated by observation of significant, although reduced, inhibition of WEHI 164 and A20/2J tumors in immunodeficient mice. PT-100 also demonstrated ability to augment antitumor activity of rituximab and trastuzumab in xenograft models of human CD20+ B-cell lymphoma and HER-2+ colon carcinoma where antibody-dependent cytotoxicity can be mediated by innate effector cells responsive to the cytokines and chemokines up-regulated by PT-100. Although CD26/DPP-IV is a potential target for PT-100 in the immune system, it appeared not to be involved because antitumor activity and stimulation of cytokine and chemokine production was undiminished in CD26−/− mice.

FAP is a serine protease that is closely related to another type II integral membrane protein, CD26/DPP-IV (12, 13). The catalytic site in CD26/DPP-IV and FAP contains the characteristic catalytic triad of Ser630/624, Asp708/702, His740/734 (residues are numbered according to human CD26/DPP-IV and FAP respectively), and the active serine is situated in a nucleophile elbow in the sequence Gly-Trp-Ser-Tyr-Gly (13–15). Both enzymes cleave NH2-terminal dipeptides from polypeptides with the sequence NH2-Xaa-Pro/Ala, where Xaa can be any natural amino acid (12–14). In addition, FAP was found to possess gelatinase activity in vitro, and this observation gave rise to the notion that the enzyme might play a role in tissue remodeling by digesting type I collagen of the extracellular matrix (16, 17). Rat CD26/DPP-IV has also been reported to possess gelatinase activity (18), and although this activity is seldom attributed to CD26/DPP-IV, the ability of CD26/DPP-IV to form a complex with FAP at invadopodia of migratory fibroblasts has suggested a possible role for the coordinated activity of both enzymes (17). The amino boronic dipeptide, Val-boro-Pro (PT-100; Fig. 1), appeared to be an interesting drug candidate in this context. PT-100 competitively inhibits the dipeptidyl peptidase (DPP) activity of FAP and CD26/DPP-IV, and there is a high-affinity interaction with the catalytic site due to the formation of a complex between Ser630/624 and the boron of PT-100 (11, 19).

The potential of PT-100 as an antitumor agent was intriguing in the light of our earlier observation that PT-100, apparently through its interaction with FAP, could stimulate hematopoiesis via the increased production of cytokines (including granulocyte colony-stimulating factor (G-CSF)) both in vitro in stromal cell supported human bone marrow cultures and in vivo in mice (11). The cytokines involved in the stimulation of hematopoiesis by PT-100 are also known to promote innate as well as T-cell-mediated antitumor responses. In particular, G-CSF gene transfection of the colon adenocarcinoma C-26 cell line has been shown to inhibit tumor take in mice and cause regression of an established tumor in sublethally irradiated mice inoculated with the transfected cells (20). The release of G-CSF by the tumor cells appeared to promote tumor infiltration by polymorphonuclear leukocytes expressing interleukin (IL)-1 and tumor necrosis factor (TNF)-α mRNA followed, sequentially, by macrophages and T cells.

Here, we report the potent antitumor effect of PT-100 in mice. PT-100 abrogates tumor growth as a single agent and augments antibody-dependent cell-mediated cytotoxicity. Similarly to hematopoietic stimulation, the antitumor activity did not require an interaction with CD26. The data suggest that, via the up-regulation of cytokine and chemokine expression in both the tumor and the draining lymph nodes, PT-100 can stimulate immune responses against tumors involving both the innate and adaptive branches of the immune system.

MATERIALS AND METHODS

Animals and Cell Lines. BALB/c, C56BL/6, DBA/2, and BALB/cnu/nu mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and Taconic (Germantown, NY). NOD.CB17-Prkdcscid1J and B6.129S7-Rag1tm1Mom and B6.129S7-Rag2tm1Wts1 mice were from The Jackson Laboratory, and C129S6 (B6)-Rag2tm1Wts1 mice were from Taconic. B6, BALB/c CD26−/− mice from the laboratory of Takeshi Watanabe were back-crossed to the BALB/c strain for seven generations to provide BALB/c CD26−/− mice. The CD26 mutation in these mice was identified as described previously (11, 21). WEHI 164, MM45T.Sp, MM52.T, EL4, A20/2J, B16-F10, P815, LL/2, Namalwa, and

Received 2/12/04; revised 4/10/04; accepted 5/12/04.

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Note: B. Jones and B. Wallner contributed equally to this work.

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LS180 cell lines (American Type Culture Collection, Manassas, VA) were propagated in vitro by serial culture.

**PT-100.** The amino boronic dipeptide PT-100 was synthesized at >95% purity as previously described (22), by Ash Stevens, Inc. (Riverview, MI), and Bristol-Myers Squibb (Princeton, NJ).

**Assays of PT-100 Antitumor Activity in Vitro.** Mice were injected s.c. with tumor cells in a shaved flank as follows: 8 × 10^3 B16-F10 cells or 4 × 10^4 EL4 cells in C57BL/6 mice; 4 × 10^4 WEHI 164, MM52.T, and MM45ST.Sp cells or 6 × 10^4 A20/23 cells in BALB/c mice; and 4 × 10^4 P815 cells in DBA/2 mice. A total of 1 × 10^5 Namalwa or 4 × 10^4 LS180 human cells was inoculated into nonobese diabetic (NOD) scid mice. Tumor-inoculated mice were given varying doses (5, 10, or 20 mice/treatment regimen as indicated in "Materials and Methods").

**Flow Cytometry.** Cell-surface expression of HER2/neu in the LS180 cells was determined by flow cytometry after immunofluorescent staining with human Fc receptor blocking (3G8, 3D3, 10.1) mAb and trastuzumab or normal human IgG (Sigma) followed by phycoerythrin-labeled antihuman IgG (G18-145). Likewise, CD26 expression in the EL4 cells was determined by staining with mouse Fc receptor blocking (2.4G2) mAb and phycoerythrin-conjugated rat IgG2a,κ (R35-95) or anti-CD26 mAb (H194-112), mAb were from Phar-Mingen. Data were collected from 10^5 viable cells and analyzed with WinList 3D (Verity Software, Topsham, ME) software.

**Gene Chip Analysis.** WEHI 164 tumor-inoculated mice were treated with PT-100 or saline from day 2 onward. Total RNA was purified by Trizol (Life Technologies, Inc.) extraction of lymph nodes and tumors harvested 2 h after administration of the first dose of PT-100 on day 4. cDNA was synthesized using T7-(dT)24 primers (AMT oligo, Biotech, Brighton, MA) and a Superscript cDNA kit, processed into cRNA by in vitro transcription (Invitrogen, Carlsbad, CA), and analyzed on Affymetrix (Santa Clara, CA) U74 gene chips according to the manufacturer’s instructions. Data were processed using Affymetrix Microarray Suite 5 and expressed as log2 signal ratios (PT-100 treatment/saline treatment) of mRNA expression.

**Reverse Transcription-PCR Analysis.** Total RNA extracted from cells, tumors or tissues with Trizol was used to synthesize cDNA with SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) according to the manufacturer’s instructions. cDNA was amplified with the following forward and reverse primers: FAP, 5′-CTCTGCGATATTCACTACAGGCAGACATAC-3′ and 5′-GAGAAATTGCATCTGGTCACAAACT-3′; CD3e, 5′-TCAAGGAAGCCGGCCGCATGC-3′ and 5′-AGTCTCTGAATCTCCCTGACCA-3′; and glyceraldehyde-3-phosphate dehydrogenase primers (Clontech, Palo Alto, CA). PCR reactions were performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA). Amplification (30–35 cycles) conditions with Platinum PCR Supermix (Invitrogen) according to the manufacturer’s instructions were as follows: denaturing, 94°C/15 s; annealing, 55°C/30 s; and extension, 72°C/30 s. Products were analyzed by agarose gel electrophoresis, ethidium bromide staining, and imaging with a Typhoon 9210 Imager (Amersham Pharmacia Biotechnology, Piscataway, NJ).

**ELISA.** Serum cytokine and chemokine concentrations were determined by Quantikine ELISA (R&D Systems, Minneapolis, MN).

**Statistical Analysis.** Standard errors and significance by Student’s t test were calculated using Microsoft Excel software (Redmond, WA).

**RESULTS**

**Inhibition of Tumor Growth by PT-100 in Mice in Vivo.** To screen mouse tumors for sensitivity to PT-100, mice received s.c. injections of syngeneic tumor cells and were given saline or PT-100 twice daily from day 2 after tumor inoculation and onward. Comparison of tumor sizes between control and PT-100-treated mice on days 15–20 indicated that PT-100 significantly (P < 0.005) inhibited the growth of WEHI 164, MM45ST.Sp, and MM52.T fibrosarcomas, EL4 and A20/2J lymphoid tumors, B16-F10 melanoma, and P815 mastocytoma (Table 1). A potent antitumor effect was indicated by test/control ratios of <0.4, corresponding to a >60% reduction in tumor size. Potential direct cytotoxicity of PT-100 was investigated in vitro cultures of the tumor cell lines that were tested in mice. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay of cell viability indicated that PT-100 had no effect on tumor cell growth in vitro at concentrations of 30 pg/ml to 30 μg/ml (data not shown).

**Growth Inhibition of Established Tumors by PT-100 in Vivo.** The effect of PT-100 on established tumors was investigated in mice inoculated with B16-F10, WEHI 164, and EL4 tumor cells by delaying PT-100 administration (p.o.) until palpable tumors were detectable in all of the mice on day 7 or 8 after s.c. injection of tumor cells. Additional growth of these tumors was significantly (P < 0.005) inhibited by treatment with PT-100 in all three models (Fig. 2). In addition to slowing tumor growth, treatment of EL4 tumor-bearing mice with PT-100 caused a dose-dependent regression in mean tumor size in all mice (Fig. 2C) and a loss of detectable tumors in some of the mice. This complete regression of EL4 tumors occurred by day 20 after tumor inoculation in 5 of 10, 4 of 10, and 3 of 10 mice receiving 20-, 30-, and 40-μg doses of PT-100, respectively; however, these...
mice relapsed at various times after day 30 when PT-100 treatment was stopped on day 19 or 24. Tumors reappeared either at the site of s.c. injection or as metastases in the axial or inguinal lymph nodes.

**Tumor Rejection by Early PT-100 Treatment in Vivo**

To investigate whether early treatment of tumors might achieve long-term survival, mice inoculated with WEHI 164 or EL4 tumor cells were given PT-100 p.o. twice daily from days 2 to 20. WEHI 164 tumor-inoculated mice were treated with a 5-H9262 g dose of PT-100, and EL4 tumor-inoculated mice were treated with a 20-H9262 g dose because previous investigation of dose responses indicated these were the optimal doses (data not shown). WEHI 164 and EL4 tumors grew progressively in saline-treated mice resulting in a control tumor incidence of 100%. Tumors also grew initially in PT-100-treated mice and reached a palpable size by day 7 after inoculation. Tumors persisted in PT-100-treated mice with minimal increase in size until day 15, at which time, mean tumor volumes of 0.08 ± 0.03 and 0.29 ± 0.04 c.c. were recorded for WEHI 164 and EL4 tumors, respectively. Thereafter, tumor regression leading to apparent rejection between days 15 and 30 was observed in 7 of 10 WEHI 164 tumor-inoculated mice and 13 of 20 EL4 tumor-inoculated mice (Fig. 3). In PT-100-treated mice that did not reject their tumors, tumor growth was significantly inhibited (WEHI 164, P < 0.005; EL4, P < 0.000005). Data from replicate experiments in which the loss of tumors was scored between days 15 and 30 indicated that PT-100 administration caused apparent rejection.

**Table 1. PT-100 inhibition of mouse tumors in vivo**

<table>
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<tr>
<th>PT-100 (μg)</th>
<th>WEHI 164</th>
<th>EL4</th>
<th>A202J</th>
<th>B16-F10</th>
<th>P815</th>
<th>MM45T.Sp</th>
<th>MM52.T</th>
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<tr>
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<td>0.17</td>
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<tr>
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<td>0.17</td>
<td>NT</td>
<td>0.23</td>
<td>0.38</td>
<td>0.39</td>
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</tr>
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</table>

* Tumors were injected s.c. into syngeneic mice, and the indicated doses of PT-100 were administered p.o. and twice daily from day 2 after tumor inoculation onward. Control mice received saline. Means (n = 10) of tumor volumes recorded between days 15 and 20 were used to calculate the test/control volume ratios. Test/control volume ratios corresponding to significant (P < 0.005) inhibition of tumor growth by PT-100 are indicated in bold type.

* Not tested.

Fig. 2. Effect of PT-100 on growth of mouse tumors established in syngeneic mice. Mice were inoculated s.c. with B16-F10 melanoma cells, WEHI 164 fibrosarcoma cells, or EL4 lymphoma cells. When palpable tumors were apparent at 7 or 8 days after inoculation, mice received the following treatments. A, B16-F10 tumor-bearing C57BL/6 mice given saline (○), 10 μg of PT-100 (▲), or 40 μg of PT-100 (▲) from days 8 to 14 after tumor inoculation. B, WEHI 164 tumor-bearing BALB/c mice given saline (○), 5 μg of PT-100 (□), 10 μg of PT-100 (▲), or 40 μg of PT-100 (▲) from days 7 to 24 after tumor inoculation. C, EL4 tumor-bearing C57BL/6 mice were given saline (○), 5 μg of PT-100 (■) or 20 μg of PT-100 (▲) from days 7 to 19, or 30 μg of PT-100 (×) or 40 μg of PT-100 (▲) from days 7 to 24 after tumor inoculation. Saline and PT-100 were administered p.o., twice daily. Data represent, means ± SE (n = 10) of tumor volumes, and in C, tumor volumes in individual mice on day 20 are shown. At all doses tested, PT-100 significantly inhibited the growth of the three tumors: B16-F10, P < 0.0005 (day 15); WEHI 164, P < 0.005 (day 25); and EL4, P < 0.0005 (day 20). A representative experiment of at least two is shown for each tumor model. For the EL4 model, the responses to saline, 5 and 20 μg of PT-100, and the responses to 30 and 40 μg of PT-100 were determined in two independent experiments in which tumor growth in saline-treated control mice was similar.

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of 65 ± 16% (mean ± SD of percentage of tumor rejection in each of 12 independent experiments) of WEHI 164 tumors in BALB/c mice and 51 ± 13% (mean ± SD, 5 independent experiments) of EL4 tumors in C57BL/6 mice. In the WEHI 164 model, tumor regrowth did not occur during an additional 60-day period of observation. In the EL4 model, however, tumors reappeared, either at the site of inoculation or as lymph node metastases, in 35 of 90 mice that had earlier appeared to be tumor free, resulting in a 22% frequency of stable rejection. Thus, early PT-100 treatment resulted in tumor rejection and long-term survival of mice inoculated with WEHI 164 or EL4 tumors. In a third model, day 2-initiated treatment of B16-F10 tumors in C57BL/6 mice with 40-μg PT-100 doses caused significant inhibition of tumor growth but did not achieve tumor rejection (data not shown).

PT-100 Induces Specific Immunity to Tumors. The ability of PT-100 to stimulate a tumor-specific CTL response was investigated in mice that received s.c. injections of EL4 tumor cells and were given 20 μg of PT-100 p.o. twice daily from days 2 to 20. On day 22, specific CTLs were readily detectable after in vitro restimulation of spleen cells from PT-100-treated mice, whereas activity was inappreciable in spleen cells from tumor-inoculated control mice (Fig. 4A).

Tumor cytolyis appeared to be due to classical MHC class-I restricted CTL because the activity was strongly inhibited by addition of anti-CD8 but not anti-CD4 mAbs to the in vitro cytotoxicity assays (Fig. 4B).

Mice that had rejected WEHI 164 and EL4 tumors because of PT-100 treatment from days 2 to 20 were completely resistant to growth of the primary tumor cell type as indicated by the lack of tumor formation (Fig. 4, C and D) and survival of the mice over a 30-day observation period. Tumor resistance appeared to be immunologically specific because inoculation of different BALB/c-derived and C57BL/6-derived tumor cells resulted in equally rapid tumor growth regardless of whether the mice were naïve or had previously rejected primary WEHI 164 or EL4 tumors as a result of PT-100 treatment.

PT-100 Has Limited Efficacy in Immunodeficient Mice. The role of adaptive immunity in the antitumor effect of PT-100 was additionally investigated by comparing activity in immunocompetent versus immunodeficient mice. PT-100 had an insignificant effect upon EL4 and B16-F10 tumor growth in C57BL/6/RAg2−/− mice but did significantly inhibit the growth of WEHI 164 and A20/2J tumors in BALB/cRag1−/− and Rag2−/− mice (Fig. 5). Similar activity of PT-100 against WEHI 164 tumors was also observed in Rag mutant mice (data not shown). In marked contrast to immuncompetent mice, BALB/cRag1−/− mice failed to reject WEHI 164 tumors. Similarly, A20/2J tumor rejection was observed in 60% of BALB/c mice treated with PT-100, but rejection did not occur in Rag2−/− mice. The data suggest that, although PT-100 can have significant activity against certain mouse tumors in the absence of adaptive immunity, functional T cells are required for tumor rejection.

Increased Cytokine and Chemokine Expression in Tumor-Bearing Mice Treated with PT-100. PT-100 was previously shown to stimulate cytokine (11) and chemokine (G. T. Miller, unpublished data) production by cultured bone marrow stromal cells. It was therefore anticipated that the antitumor immune responses observed in PT-100-treated mice were induced via the up-regulation of certain cytokines and chemokines. Affymetrix gene arrays were used to compare cytokine and chemokine mRNA expression in WEHI 164 tumors and draining lymph nodes between control mice and mice given 5 μg of PT-100 twice daily from day 2 after tumor inoculation and onward. Examination of cytokine mRNA levels 2 h after the first administration of PT-100 on the third day of treatment (day 4) revealed that expression of IL-1α, IL-1β, IL-6, IFN-β, G-CSF, IL-1RA, and thrombospondin-1 mRNA was increased in lymph nodes of PT-100-treated mice, and expression of IL-1β, IL-6, and G-CSF mRNA was also increased in the tumors (Fig. 6A). PT-100 did not
appearance of the expression levels of GM-CSF, lymphotoxin, IL-2, IL-3, IL-4, IL-9, IL-12, IFN-γ, IFN-α, IL-18, TNF-α, TNF-β, TGF-β, and IL-15 mRNA at the time point investigated because the changes in mRNA levels in PT-100-treated versus control mice were <2-fold (data not shown). PT-100 treatment also increased mRNA expression of a wide array of chemokines in lymph nodes and a smaller subset in tumors (Fig. 6A). Of the chemokine mRNA levels evaluated, only those of SDF-1α/CXCL12, Angiopoietin-CXCL13, MRPs/CCL9/10, TECK/ CCL5, and PF4/CXCL4 appeared not to be increased by PT-100 treatment. These data indicate that PT-100 treatment increases the expression of mRNA for chemokines that collectively chemoattract and activate multiple immune effector cells (25, 26).

PT-100 administration to either normal or myelosuppressed mice was previously shown to increase serum levels of G-CSF (11); therefore, it seemed likely that the increased expression of cytokine and chemokine mRNA observed in the present study would be paralleled by increases in the serum levels of the corresponding proteins. Although it was not feasible to investigate serum levels of all of the cytokines and chemokines implicated in the PT-100 response by the mRNA analysis, ELISA of G-CSF, eotaxin/CCL11, macrophage inflammatory protein (MIP)-1α/CCL4, thymus and activation-regulated chemokine (TARC)/CCL17, and KC/CXCL1 indicated that PT-100 treatment caused highly significant increases (Fig. 6B).

PT-100 Enhances the Activity of Tumor-Specific Antibodies against Human Tumor Xenografts. The known biological activities (25, 26) of the cytokines and chemokines up-regulated by PT-100 in vivo suggested that PT-100 treatment might promote the antitumor activity of neutrophils, macrophages, or natural killer cells participating in antibody-dependent cell-mediated cytoxicity mediated by the CD20-specific mAb, rituximab, and the HER2-specific mAb, trastuzumab (27). The antitumor effect of PT-100 was therefore investigated in combination with each antibody in NOD scid mice inoculated s.c. with human Namalwa CD20+ B-cell lymphoma or LS180 HER2+ colon carcinoma cells.

In both Namalwa and LS180 xenograft models, tumor growth did not differ significantly between untreated mice and mice receiving control antibodies (s.c.) and saline (p.o.), and the tumor growth curves in saline-treated mice have been omitted in Fig. 7 for simplicity. As previously reported (28), growth of the human CD20+ B-cell lymphoma line, Namalwa, in NOD scid mice was inhibited by a 75-mg/kg dose rituximab injected i.p. on days 3, 5, and 7 after tumor inoculation (Fig. 7A), and increasing the dose to 150 or 300 mg/kg did not result in any greater inhibition of tumor growth (data not shown). The effect on tumor growth of a 5-μg dose of PT-100 administered from day 2 onward was similar to that of rituximab. When administered in combination, PT-100 and rituximab resulted in an ~2-fold greater reduction in tumor growth, and this represented a significant (P < 0.05) improvement over the antitumor effect of either agent alone (Fig. 7A).

Cells of the LS180 human colon carcinoma cell line were shown to express HER-2 by cell-surface immunofluorescent staining with trastuzumab and phycoerythrin-labeled anti-human IgG: 49.7 versus 2.5 linear mean fluorescent units in cells stained with phycoerythrin-anti-IgG alone. In NOD scid mice, LS180 tumors were quite resistant to treatment with 1–60 mg/kg doses of trastuzumab (data not shown), and 10 mg/kg had optimal, albeit weak, activity. LS180-inoculated NOD scid mice were given five injections of a 10-mg/kg dose of trastuzumab (i.p.) at 3-day intervals from days 4 to 16, and 5 μg of PT-100 were administered (p.o.) twice daily from day 2 onward. Neither treatment with trastuzumab nor PT-100 alone caused significant inhibition of tumor growth; however, the combination resulted in significantly reduced (P < 0.005) tumor sizes on days 15, 18, and 21 (Fig. 7B).
tumors, respectively. FAP and CD26 mRNAs were found to be coexpressed in tumors, draining lymph nodes, and spleens (Fig. 8A). In C57BL/6 mice bearing EL4 tumors, FAP and DPP-IV/CD26 mRNAs were also coexpressed in lymphoid tissue, but only FAP mRNA appeared to be present in the tumors. These findings, together with the determination that FAP mRNA was not expressed by WEHI 164, B16-F10, or EL4 tumor cells propagated in tissue culture (Fig. 8B), were consistent with previous studies that FAP expression is induced in the reactive fibroblasts of tumor stroma by the growth of malignant tumors that do not themselves express the protein (29). In agreement with previous studies of restricted expression of FAP in normal tissues (9, 11), FAP mRNA was detected by reverse transcription-PCR in whole embryonic tissue and bone marrow but not in testis and stomach (Fig. 8C). In addition, FAP mRNA was found to be expressed in normal lymph nodes. Similarly, CD26 mRNA was found not to be expressed in these tumor cells in vitro. Even EL4 lymphoma cells, which due to their T-cell lineage might have been expected to express CD26 (30), did not do so, as confirmed by immunofluorescence with CD26-specific mAb H194-112 (data not shown).

Data previously obtained in CD26-deficient mice suggested that PT-100 might exert its effect on hematopoietic tissue through an interaction with FAP rather than CD26 (11). The involvement of CD26 in the antitumor effect of PT-100 was therefore investigated in BALB/c CD26−/− mice inoculated with WEHI 164 tumor cells. As described above for CD26-expressing wild-type mice, administration of PT-100 starting on day 2 after tumor inoculation resulted in tumor rejection, and this was apparent in both CD26−/− and CD26+/+ mice (Fig. 9A). Comparison of the tumor rejection frequency, 9 of 10 in CD26−/− mice versus 5/10 in CD26+/+ mice, indicated that the interaction of PT-100 with CD26 was not required for the antitumor effect. Because the activity of PT-100 appeared to involve the up-regulation of cytokines and chemokines, this response was compared between BALB/c CD26−/− and CD26+/+ mice by ELISA of serum levels of G-CSF, KC/CXCL1, Eotaxin, MIP-1β, and TARC. Again, CD26 did not appear to be required for the cytokine and chemokine response to PT-100, and interestingly, the G-CSF, KC/CXCL1, eotaxin/CCL11, and TARC/CCL17 dose response curves suggested that CD26−/− mutant mice were significantly more sensitive to PT-100 (Fig. 9B).

**DISCUSSION**

PT-100 had a potent antitumor effect in mice inoculated with syngeneic tumor cell lines derived from fibrosarcoma, lymphoma, or melanoma. The antitumor effect differed from that of cytotoxic agents such as the taxanes (31) or cisplatin (32) in that PT-100 had no effect on tumor cell growth or viability in vitro cell cultures, even at concentrations that exceeded those achievable in vivo by active doses. PT-100 instead exerts its antitumor activities through the induction of cytokines and chemokines that are known to promote innate and adaptive immune responses against the tumor in vivo.

The establishment of specific immunological memory in mice that had rejected WEHI 164 or EL4 tumors as a result of PT-100 treatment and the increased priming of tumor-specific CTL elicited by PT-100 in the EL4 model implicate T-cell immunity in the antitumor effect. This hypothesis is supported by the observation that antitumor activity was reduced or abolished, depending upon the tumor model, in congenitally immunodeficient mice. Knowledge of the biological activities of the cytokines and chemokines up-regulated by PT-100 in tumors and draining lymph nodes suggests that the stimulation of tumor-specific T-cell immunity results from increased efficiency of antigen presentation by dendritic cells and greater clonal expansion of antigen-specific T cells (33, 34). The cytokines IL-1, IFN-β, and IL-6 are known to increase the efficiency of the interactions between T cells and dendritic cells leading to T-cell responses. IL-1 and IFN-β can promote B7 costimulation of T cells via paracrine pathways that trigger dendritic cell maturation (35, 36), and IL-6 has recently been shown to abrogate the suppressive effects of CD4+ CD25+ regulatory T cells on antigen-specific T-cell activation (37). The development of tumor immunity also requires efficient trafficking of antigen-presenting and primed T cells between the tumor and draining lymphoid tissue (34). The up-regulation of monocyte chemotactic protein (MCP)-1, MCP-2, and MCP-3, interferon-γ inducible protein (IP)-10, and monokine induced by interferon-γ (MIG) by PT-100 in tumor tissue demonstrated here was highly relevant in this regard because it had been shown previously that the MCP chemokines target monocytes and CD4+ and CD8+ T cells via the CCR3 receptor, and IP-10 and MIG target activated T cells via the CXCR3 receptor (25, 38). The cytokines and chemokines up-regulated by PT-100 in vivo can also promote tumor cytotoxicity and tumor infiltration by the effector cells of innate responses (25, 38). Innate effector cells such as natural killer cells, macrophages, neutrophils, and eosinophils could therefore contribute to the activity of PT-100 observed in immunodeficient mice. In addition, PT-100 induced the expression of thrombospondin-1, which is a potentially angiostatic agent (39), in lymph...
nodes and the chemokines IP-10/CXCL10 and MIG/CXCL9 in tumor tissue, where they have the potential to cause apoptotic death of vascular endothelial cells via an interaction with an alternatively spliced variant of CXCR3 (40). The degree to which the potential antiangiogenic activity of cytokines and chemokines contributes to the antitumor effect of PT-100 in vivo is currently uncertain. Although IP-10/CXCL10 and MIG/CXCL9 belong to the angiostatic subset of CXC chemokines characterized by the absence of the Glu-Leu-Arg motif, other CXC chemokines induced by PT-100 possess the motif and have been shown to promote angiogenesis (41). Analysis of chemokine mRNA expression in WEHI 164 tumors indicated that PT-100-induced expression of proangiogenic KC/CXCL1, MIP-2/CXCL2, and ENA 78/CXCL5 predominated over expression of antiangiogenic IP-10/CXCL10 and MIG/CXCL9. Because WEHI 164 tumors were extremely sensitive to PT-100, the data suggest that angiogenic effects may not play a major role in the antitumor activity of PT-100 in this particular tumor model. However, the net effect of PT-100 on angiogenesis could vary depending on tumor type. To address this question, the cytokine and chemokine responses to PT-100 will need to be analyzed and correlated with markers of angiogenesis across a panel of different tumor types in vivo.

The potential of PT-100 to stimulate both innate and antigen-specific T-cell responses suggested that the antitumor effect involves an initial attack on the tumor by innate effector cells followed by the development of T-cell immunity. Such a mechanism is attractive, given the role chemokines up-regulated by PT-100 appear to play in linking innate and adaptive immunity (34) and the role of innate effector cells such as natural killer cells in promoting tumor-specific immunity (42). In addition, the observation that PT-100 augmented the effect of rituximab and trastuzumab on the growth of CD-20⁺ Namalwa B-cell lymphoma and HER2⁺ LS180 carcinoma cell lines in NOD scid mice supports the hypothesis of a link between the PT-100 effect and the antitumor responses of macrophages, neutrophils, and natural killer cells that contribute to antibody-dependent cell-mediated cytotoxicity (27). It should be noted, however, that PT-100 could have a strong antitumor effect even when innate activity was not apparent. This was indicated by highly significant activity of PT-100 against EL4 and B16 tumors in immunocompetent mice compared with an insignificant effect in immunodeficient mice. The data in these two tumor models support the notion that PT-100 can directly stimulate T-cell immunity and are in agreement with the recent observation that PT-100 can stimulate CTL responses against synthetic peptide antigens (G. Cole, unpublished data).

PT-100 treatment resulted in tumor rejection in 50–70% of mice inoculated with WEHI 164, EL4, or A20 tumor cells when administration was started before establishment of a palpable tumor mass. Although tumor growth inhibition was highly significant in all of the mice treated, rejection frequencies and the duration of tumor-free survival varied between tumor models. Stochastic events clearly influence the efficacy of PT-100 treatment. The greater magnitude of the antitumor effect obtained when PT-100 was administered earlier rather than later in the course of tumor progression probably reflects a balance between the rate of tumor growth and the time required for PT-100 to induce optimal immune effector mechanisms. It is also
Tumor volume (c.c.)

Days after tumor inoculation

Fig. 7. Effect of PT-100 and tumor-specific antibodies on growth of human tumor cell xenografts in nonobese diabetic scid mice. Mice received s.c. injections of Namalwa B-cell lymphoma cells (A) or LS180 colon carcinoma cells (B) and were given control antibody plus saline ( ), control antibody plus PT-100 ( ), antitumor antibody plus saline ( ), or antitumor antibody plus PT-100 ( ). Treatment (p.o.) with 5 μg of PT-100 or saline was started on day 2 after tumor inoculation and continued twice daily until the end of the experiment. Tumor-specific antibody treatments (i.p. as indicated by ) were rituximab (75 mg/kg) on days 3, 5, and 7 ( ) or trastuzumab (10 mg/kg) on days 4, 7, 10, 13, and 16 ( ). Equivalent doses of control antibodies, either normal human IgG ( ) or rituximab ( ), were administered according to the same schedules. Data represent means ± SE (A, n = 5; B, n = 10) of tumor volumes. In each experiment, tumors treated with antitumor antibody plus PT-100 were significantly ( , P < 0.00005 to P < 0.005; B, P < 0.05 to P < 0.0005, depending on time of measurement) smaller than tumors treated with control antibody plus saline at all time points. Treatment of Namalwa tumors with rituximab plus PT-100 resulted in significantly (P < 0.05) greater inhibition of tumor growth from day 11 onward than treatment with either rituximab plus saline or normal IgG plus PT-100. Likewise, treatment of LS180 tumors with trastuzumab plus PT-100 resulted in significantly (P < 0.005) greater inhibition from day 15 onward than treatment with either trastuzumab plus saline or rituximab plus PT-100. For each xenograft model, data are representative of two experiments.

Fig. 8. Expression of DPP-IV/CD26 and fibroblast activation protein (FAP) mRNAs in mouse tumors and tissues. A, syngeneic mice received s.c. injections of WEHI 164, B16-F10, or EL4 tumor cells, and 2 days later, tumors, draining lymph nodes, and spleens were excised for analysis of DPP-IV/CD26 and FAP mRNA expression by reverse transcription-PCR as described in “Materials and Methods.” Expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was assayed as a control for RNA integrity.

B, reverse transcription-PCR analysis of the tumor cells propagated in vitro. C, day 17 whole mouse embryo (E17), bone marrow (BM), lymph node (LN), testis (Te), and stomach (Stm) from normal mice without tumors were analyzed by reverse transcription-PCR.
immune regulation. In the present study, FAP mRNA expression was shown to be constitutive in lymphoid tissue and bone marrow in vivo and to be induced in PT-100-sensitive solid tumors derived from mouse tumor cell lines that did not express the protein in vitro cell cultures. The hypothesis that FAP is actively involved in tumor progression is additionally supported by a previous study indicating that FAP expression in transfected HEK293 cells could increase their tumorigenicity in scid mice (15). Polyclonal anti-FAP antibodies that could inhibit the DPP activity of FAP in vitro were also found to suppress HT-29 adenocarcinoma growth in vivo, thereby supporting the involvement of the proteolytic activity of FAP in tumor progression (15). The antitumor activity of PT-100 additionally supports a role for DPP activity in tumor growth. The up-regulation of cytokines and chemokines early during the course of PT-100 administration in vivo suggests that, in addition to the digestion of extracellular matrix (16, 17), DPPs such as FAP might also play a role in suppressing cytokine and chemokine production in tumor or lymphoid tissue. This could occur via NH2-terminal cleavage that modulates the biologically active half-life of polypeptide signaling molecules that might regulate cytokine and chemokine production in either an autocrine or paracrine fashion. For example, the potential biological activities of CD26/DPP-IV include the regulation of immunity by the cleavage of certain chemokines and the regulation of blood glucose by cleavage of glucagon-like peptide-1 or glucose-dependent insulino...


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*Cancer Res* 2004;64:5471-5480.

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