A Novel Design of Targeted Endocrine and Cytokine Therapy for Breast Cancer

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ABSTRACT

The aim of this study is to combine endocrine therapy [human prolactin (hPRL) antagonist, G129R] and immune therapy (interleukin 2 (IL2)) in the design of a fusion protein, G129R-IL2, to treat human breast cancer. This novel approach uses the specific interaction between the G129R and hPRL receptors (PRLRs), thus directly targeting the fusion protein to the malignant breast tissues that have previously been shown to contain high levels of PRLR. The localized bifunctional fusion protein is designed to block signal transduction induced by hPRL as well as to activate T lymphocytes near the tumor site. A bacterial expression system was used to produce G129R-IL2 fusion protein that maintained both G129R and IL2 activities as demonstrated by cell-based assays such as signal transducer(s) and activator(s) of transcription (STATs), phosphorylation, breast cancer cell proliferation, and T-cell proliferation. The anti-tumor activities of G129R-IL2 were demonstrated in vivo using a syngeneic model system with BALB/c mice and EMT6-hPRLR breast cancer cells. After daily injection (i.p.) of G129R-IL2 (100 μg/mouse) for 18 days, the tumor growth in the G129R-IL2-treated group was only one-third the size as compared with that of the control group. The growth rate in the G129R-IL2-treated group is also significantly slower than that of the group treated with G129R alone (200 μg/mouse/day). We hope that this novel bifunctional protein will contribute significantly to human breast cancer therapy.

INTRODUCTION

One of the leading causes of cancer death in women is metastatic breast cancer. The etiology of breast cancer is complex, but its rarity among males suggests a role of female sex hormones (1, 2). In addition to estrogen, more and more evidence supports the notion that hPRL is also intimately involved in breast cancer development (3–7). The following lines of evidence demonstrate the relationship between PRL and breast cancer: (a) PRL is synthesized by human breast cancer cells, which suggests its autocrine/paracrine role in the mammary gland (4); (b) PRLRs are up-regulated in the majority of malignant breast tissue (8); (c) PRL transgenic mice have high breast cancer rate (9); and (d) the inhibition of the binding of PRL to PRLR inhibits breast cancer cell growth (10).

hPRL is a single-chain, neuroendocrine, polypeptide hormone with 199 amino acids in its mature form. As a member of the GH family, PRL is primarily produced by the lactotrophs of the anterior pituitary gland in all vertebrates. The biological activities of PRL are mediated through specific membrane receptors known as PRLRs. The primary site of PRL action is the mammary gland. In this organ, PRL plays a decisive role in the stimulation of DNA synthesis, epithelial cell proliferation, and the promotion of milk production (11–15). The generation of PRL and PRLR gene knockout mice has unambiguously demonstrated that PRL and PRLR are key regulators in mammary development (12, 16).

In previous studies, Chen et al. (17–22) have developed a hGH antagonist by making a single amino acid substitution mutation at position 120 of the hGH molecule (hGH-G120R). The mutated hGH has been shown to block GH action both in vitro and in vivo (21) and has completed its Phase III clinical studies (23). By adopting a strategy similar to the development of the hGH antagonist, Goffin et al. (24) and our laboratory (10, 25–27) demonstrated that a single amino acid substitution mutation at position 129 of hPRL resulted in a hPRL-specific antagonist (G129R). We have demonstrated that G129R is able to inhibit human breast cancer cell proliferation via the induction of apoptosis (10). G129R has also been shown to inhibit tyrosine phosphorylation of oncogene STAT3 (26, 28) and to modulate transforming growth factor α/β levels in breast cancer cells (27). Furthermore, additive effects of hPRL-G129R and tamoxifen, which serves as an antiestrogen agent, have been

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3 The abbreviations used are: PRL, prolactin; hPRL, human PRL; CSS, charcoal-stripped serum; FBS, fetal bovine serum; GH, growth hormone; hGH, human GH; IL2, interleukin 2; hIL2, human IL2; PRLR, PRL receptor; IRMA, immunoradiometric assay; mAb, monoclonal antibody; STAT, signal transducer(s) and activator(s) of transcription; ATCC, American Type Culture Collection; RT-PCR, reverse transcription-PCR; TBS, Tris-buffered saline; MTS-PMX, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt, phenazine methosulfate.

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observed (10). Taken together, the ability of G129R to inhibit breast cancer cell proliferation, especially its additive effects with tamoxifen, makes it potentially valuable as a therapeutic agent for the treatment as well as prevention of breast cancer.

Tumor immune therapy has been of great interest for many years (29, 30). IL2 has been one of the main cytokines used for treating cancer. IL2, originally called T-cell growth factor, is a Mf 15,000 glycoprotein encoded by a single gene on chromosome 4 in humans (31). Characteristics of IL2 that make it attractive in cancer therapy include its ability to stimulate T lymphocytes as well as natural killer cells (32). However, one of the disadvantages in using IL2 is that patients systemically receiving IL2 often experience serious side effects that limit the amount of IL2 that can be administered. This limitation of dosage in turn directly affects the efficacy of treatment (33, 34).

In this study, we explore the possibility of fusing G129R with IL2 in the hope of generating a bifunctional protein that will have a dual therapeutic effect (targeted endocrine and cytokine) in the treatment of breast cancer. The targeting ability of this novel fusion protein uses the highly specific interactions between ligand (G129R) and receptor (PRLR). After G129R binds to PRLR, it not only blocks the signal transduction inducted by PRL but also localizes IL2 at the tumor site, which will play a crucial role in T lymphocyte activation, thus leading to tumor cytotoxicity. Because IL2 would be concentrated by PRL but also localizes IL2 at the tumor site, which will play a crucial role in T lymphocyte activation, thus leading to tumor cytotoxicity.

The animals used for this study were 8–10-week-old female BALB/c mice (Jackson Laboratory; Bar Harbor, ME), which were housed in compliance with NIH guidelines.

Cloning of G129R-IL2 Fusion cDNA for Escherichia coli Expression. A two-step cloning procedure was used to generate a recombinant DNA encoding G129R fused to IL2. Primers corresponding to G129R (minus sequences encoding signal peptide and stop codon, and plus restriction sites of Ndel and BamHI: 5’-CAT ATG TTG CCC ATC TGT CCC GGC-3’ and 5’-GGA TCC GCA GTT GTT GTG GAT-3’) were used to amplify the G129R fragment from pCR3.1-G129R (10). Primers corresponding to hIL2 (minus sequences encoding signal peptide, and plus restriction sites of BamHI and XhoI: 5’-GGA TCC GCA CCT ACT TCA AGT CTC GAG TTA AGT TAG TGT GAT GAT-3’) were used to amplify the hIL2 fragment from hIL2 cDNA, purchased from ATCC. Both fragments were cloned into pCR2.1 TA cloning vector (Invitrogen, Inc., Carlsbad, CA) and sequenced. The fragments were reisolated by restriction digestion, purified, and ligated into the pET22b+ expression vector (Novagen, Madison, WI; Fig. 1).

Production and Purification of G129R-IL2. BL21 (DE3) cells (Novagen) were transformed with pET22b-G129R-IL2 using the calcium chloride method. An E. coli BL21 (DE3) seed culture (200 ml) carrying the pET22b-G129R-IL2 plasmid was grown overnight at 37°C and was used to inoculate 4 liters of L-broth (Bio 101, Carlsbad, CA) containing 100 μg/ml ampicillin (Fisher Scientific, Fair Lawn, NJ). The culture was grown at 37°C with agitation until the A600nm reached 0.9, at which time 1 mm isopropyl β-thiogalactoside (IPTG; Alexis Biochemicals, San Diego, CA) was added to induce expression of T7 RNA polymerase; the culture was incubated for an additional 3 h. The cells were then harvested by centrifugation at 6,000 × g for 5 min and resuspended in 0.2 M NaPO4 (pH 8), 10 mM EDTA, 0.1 mg/ml lysozyme, and 0.5% Triton X-100, and incubated at 37°C for 1 h. The cells were disrupted by sonication using five 1-min pulses at 5 kHz applied with a Vibra-Cell Sonicator (Fisher Scientific). The insoluble inclusion bodies were recovered by centrifugation at 12,000 × g for 15 min at 4°C; resuspended in 0.2 M NaPO4 (pH 7), 5 mM EDTA, 1 mM urea, and 0.5% Triton X-100; recollected by centrifugation at 12,000 × g for 15 min; resuspended in 0.2 M NaPO4 (pH 8), 8 mM urea, and 1% v/v β-mercaptoethanol; and heated at 55°C for 10 min. Renaturation of the solubilized G129R-IL2 was performed by dialysis against decreasing concentrations of urea/TE buffer [20 mM Tris, 2 mM EDTA (pH 8.3)] for 4 days. The renatured protein was then filtered with 0.45 μm filters and purified using an anionic exchange column (Q-Sepharose) on a fast-performance liquid chromatography system (Amersham Pharmacia, Newark, NJ). The concentration of G129R-IL2 was determined using a hPRL IRMA kit (DPC, Inc., Los Angeles, CA), and its purity was determined via silver staining using the Silver Stain Plus kit (Bio-Rad Inc., Hercules, CA).

Verification of Fusion Protein Production via Western Analysis. Samples (200 ng) were analyzed using 4–15% SDS PAGE followed by Western blotting. After SDS-PAGE, the protein was transferred to ECL Hybond nitrocellulose (Amersham Pharmacia) at 16 W for 1.5 h. Blots were blocked with TBS containing 5% milk and 0.05% Tween 20 (blocking buffer)
Antitumor Effects of a hPRL Antagonist IL2 Fusion Protein

The cells were then incubated at room temperature for 30 min and washed three times with PBS containing 0.05% Tween 20 to remove any remaining hPRL receptor.

The optimal cell number/well was determined by titration experiments. The cells were allowed to settle and adhere overnight (18 h), and various concentrations of hPRL, G129R, or G129R-IL2 were added. The cells were incubated for an additional 24 h at 37°C in a humidified 5% CO₂ incubator. After incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit; Promega Corp., Madison, WI) was added to each well following the manufacturer’s instructions, and the plates were read at 490 nm using a BIO-RAD benchtop microplate reader (Hercules, CA).

All of the experiments were carried out in triplicate.

**HT-2 Cell Proliferation Assay.** Before each assay, HT-2 cells were washed three times in growth medium lacking IL2. The cells were counted, and ~5 × 10⁵ cells were transferred to each well of a 96-well plate. Dose-response curves were obtained by varying the concentration of IL2, G129R-IL2, PRL, or G129R-IL2 incubating for 24 h at 37°C. Cell proliferation assays (MTS-PMS; Promega) were performed in triplicate using the same procedure described in the previous section.

**In Vivo Studies of Antitumor Efficacy of G129R-IL2.**

Two experiments were conducted to determine the antitumor efficacy of G129R-IL2 fusion protein using EMT6-hPRLR cells and a BALB/c female mouse model. In the first experiment, eight eight-week-old female BALB/c mice were inoculated s.c. with 1 × 10⁶ EMT6-hPRLR breast cancer cells and randomized into two groups. One day after breast cancer cell inoculation, mice were injected (i.p.) with either 50 μg/mouse of G129R-IL2 or PBS every 24 h for 14 consecutive days. In the second experiment, 24 mice were given s.c. injections of 1 × 10⁷ EMT6-hPRLR breast cancer cells and were randomized into four groups. One day after breast cancer cell inoculation, mice were given injections (i.p.) of PBS, G129R (200 μg/mouse), or G129R-IL2 (100 μg/mouse or 200 μg/mouse, respectively) for 18 consecutive days. At the end of the experiments, the tumors were dissected and weighed. It should be pointed out that the original experimental design included a group of animals that was treated with 20 μg of free IL2 mixed with 20 μg of G129R/mouse. Because of the toxicity of IL2 to the mice, the mice died and the experiment ended. The data are expressed as mean ± SE, and the Student t test was used to analyze the statistical difference between groups.

**RESULTS**

**Construction of pET22b-G129R-IL2 Expression Vector.** G129R-IL2 cDNA was cloned into the pET22b (+) expression vector as shown in Fig. 1. The G129R and IL2 cDNA sequences were found to be identical to those reported in GenBank, except for a single codon mutation (GGC to CGG), which resulted in Gly to Arg mutation at position 129 of hPRL (accession no. XM 035558). Two amino acids, Gly and Ser, were found to be identical to those reported in GenBank.

**Production of G129R-IL2 Fusion Protein.** The G129R-IL2 fusion protein was produced in the form of inclusion bodies. After refolding and ion exchange column purification, the yield of fusion protein was ~2 mg/liter as determined by the Bradford protein assay and PRL IRMA analysis. The purified protein was analyzed by 4–15% SDS PAGE followed by silver staining (Fig. 2A), and the identity of the Mf 38,000 fusion protein was further confirmed by Western analysis using antiserum against hIL2 (accession no. XM 035511) or hPRL, respectively (Fig. 2B and 2C).
HT-2 Cell Proliferation Assay. An HT-2 cell proliferation assay was used to determine whether or not the IL2 portion of the fusion protein was functional. Fig. 3A demonstrated a dose response of IL2 in the proliferation of HT-2. The stimulatory effect of G129R-IL2 fusion protein on HT-2 cell proliferation was similar to that caused by IL2 alone (Fig. 3B). G129R or hPRL alone had no effect on HT-2 cell proliferation (Fig. 3C). The EC50 values for the G129R-IL2 and IL2 were ~1 ng/ml.

STAT Assay. Fig. 4A demonstrates a dose response of STAT5 phosphorylation in T-47D cells induced by hPRL. STAT5 phosphorylation was detected at a maximum level for 100 ng/ml hPRL (Fig. 4A). G129R (Fig. 4B) and IL2 (Fig. 4C), on the other hand, were inactive in this assay. To determine the antagonistic effects of G129R-IL2, T-47D cells were treated with a constant concentration of hPRL (100 ng/ml) and various concentrations of G129R or G129R-IL2, fusion protein (50 ng/ml to 1 μg/ml). It can be seen that at a 1:5 ratio (hPRL: G129R), STAT5 tyrosine phosphorylation is significantly decreased (Fig. 5A); and at a 1:10 ratio (hPRL:G129R), STAT5 tyrosine phosphorylation is almost completely inhibited (Fig. 5A). Fig. 5B demonstrates that G129R-IL2 fusion protein inhibits STAT5 phosphorylation induced by hPRL to nearly the same extent as G129R; therefore, the G129R portion of the fusion protein is functional.

Generation of EMT6-hPRLR Cells. The tumor cell line used for the in vivo studies was the EMT6 mouse mammary tumor cell line. Because this cell line has nondetectable PRLR mRNA as determined by RT-PCR (Fig. 6A, Lane 2), EMT6 cells were transfected with hPRLR cDNA to generate an EMT6-hPRLR stable cell line. Fig. 6A (Lane 4) shows the results of RT-PCR that demonstrate the expression of hPRLR mRNA in the EMT6-hPRLR cell lines. The hPRLR mRNA expression level in the EMT6-hPRLR cell line selected was still found to be much lower than that of T-47D cells (Fig. 6A).

Once the EMT6-hPRLR cell line was established, the effects of hPRL, G129R, and G129R-IL2 on cell proliferation of this cell line were investigated. When equal numbers of cells (15,000) were treated with 500 ng/ml hPRL, G129R, or G129R-IL2, the stimulatory effects were seen from only the cells treated with hPRL; whereas both G129R and G129R-IL2 demonstrated inhibitory effects on EMT6-hPRLR cell proliferation (Fig. 7A). More importantly, G129R or G129R-IL2 (1:10) competitively inhibited the proliferative effects induced by hPRL (Fig. 7B).

In Vivo Studies of the G129R-IL2 Fusion Protein. Pharmacokinetic studies of G129R-IL2 were first conducted to determine the effective dose needed. Eight-week-old female BALB/c mice were given injections i.p. of either 25 μg/mouse...
In our preliminary animal studies, eight female BALB/c mice were inoculated s.c. with $1 \times 10^6$ EMT6-hPRLR breast cancer cells and randomized into two groups. Each animal then received daily injections of G129R-IL2 (50 µg/mouse). We found that the serum concentration of fusion protein was maintained at $\sim$30 ng/ml, which reduced the growth of EMT-6-PRLR in mice ($115 \pm 55$ mm$^3$ versus $238 \pm 75$ mm$^3$ in control group). Although because of small sample numbers, no statistical difference could be found in tumor volume nor in final tumor weights between the two groups, it provided dose reference for our main animal studies.

Twenty-four female BALB/c mice were inoculated s.c. with $1 \times 10^6$ EMT6-hPRLR breast cancer cells and randomized into four groups. Fig. 10 demonstrates that the tumor growth was similar between the groups treated with G129R (241 ± 45 mm$^3$; 200 µg/day/mouse) and a high dose of G129R-IL2 (223 ± 41 mm$^3$; 200 µg/day/mouse); however, mice that were given injections of 100 µg of G129R-IL2 showed the best response, in which the average tumor volume was approximately one-third of that in the control group ($125 \pm 25$ mm$^3$ versus $305 \pm 55$ mm$^3$).

**DISCUSSION**

Recent advances in the understanding of the immune system and in defining tumor antigens have motivated the development of many new strategies using immune therapy in cancer treatment (36–38). There is ample evidence that cancers express tumor-specific antigens and that hosts have T cells that can respond to these antigens (39, 40). However, it is likely that tumor cells are poor antigen-presenting cells because they do not provide second signals, which are needed for full T-cell activation (40). Therefore, the major effort in tumor immune
therapy is focused on how to augment weak host immune responses to tumor antigens, such as exogenously administering cytokines to the patients. Among the many cytokines used, IL2 has been demonstrated to yield promising results (36–38). IL2 is the principal cytokine responsible for the progression of T lymphocytes from the G1 to S phase of the cell cycle. It is mainly produced by CD4+ T cells and in smaller quantity by CD8+ T cells (41). With the help of recombinant DNA technology, recombinant hIL2 has been used in vivo to treat patients with advanced renal cell carcinoma and melanoma (33, 34). The aim of such an approach is to generate tumor-reactive lymphocytes in cancer patients. However, it has been reported that cancer patients receiving systemic hIL2 often experience potentially life-threatening side effects that limit the total amount that can be administered, which in turn directly affects the efficiency of treatment (33, 34). The major efforts regarding the use of IL2 in tumor therapy, therefore, have been concentrated on how to balance the side effects and the effective dose. By increasing the specificity of administered IL2 (via the targeting of IL2 precisely to the tumor sites), it is possible to dramatically increase the therapeutic effects of hIL2 while significantly decreasing its side effects.

Recently an alternative approach for using the binding specificity of antitumor mAbs to direct cytokines to tumor sites has been introduced (40–46). This novel approach combines the unique targeting ability of mAbs with the activities of cytokines and, therefore, achieves an effective concentration of IL2 in the tumor microenvironment. The targeted IL2 therapy has been shown to be able to completely eradicate disseminated pulmonary and hepatic murine melanoma metastases in immunocompetent syngeneic mice (42, 43) and has also generated promising clinical results (47). These findings demonstrate that targeted IL2 can provide an effective tool in cancer immunotherapy and establish the missing link between T-cell-mediated cytotoxicity and objective clinical response. There are several obvious advantages of this targeted IL2 therapy. First, an mAb-IL2 fusion protein does not have to reach all of the target cells to achieve the maximum effects because it is not a direct cytotoxic reaction (46, 48). Second, it has been shown that the induction of a cellular immune response using the mAb-targeted IL2 approach facilitates the eradication of established s.c. melanoma metastases, even if the tumor displays substantial antigen heterogeneity (47). Most importantly, the therapeutic effect of targeted IL2 therapy is associated with the induction of a long-lived and transferable, protective tumor immunity. In addition, this mAb-targeted IL2 therapy is also different from, and advantageous to, the ex vivo transfer of cytokine genes because it concentrates IL2 in the tumor environment in a nonpersonalized way that makes this approach clinically more feasible (42–50).

In our previous studies, we have demonstrated that G129R was able to inhibit breast cancer cell proliferation via the induction of apoptosis both in vitro (10, 26–27) and in vivo (25). In this study, we used a strategy similar to that of mAb-IL2 to design a novel G129R-IL2 fusion protein that is targeted specifically to human breast cancer. The targeting ability of this novel fusion protein involves the highly specific interactions between the ligand (G129R) and receptor (PRLR), therefore, concentrating IL2 at the cancerous breast tissue in which PRLR levels have been shown to be elevated (8). We hypothesized that once the G129R-IL2 fusion protein reaches the malignant mam-

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**Fig. 5** Inhibition of STAT5 phosphorylation by G129R or G129R-IL2 in T-47D human breast cancer cells. T-47D cells were treated with the indicated concentrations of hPRL, G129R, and G129R-IL2 or with combination as indicated. Total protein was extracted from cells and analyzed via 4–15% gradient SDS-PAGE followed by Western blotting with antisera against either STAT5 or Phospho-STAT5 as indicated in each panel. A, the competitive inhibition of STAT5 phosphorylation by G129R. B, the competitive inhibition of STAT5 phosphorylation by G129R-IL2.

**Fig. 6** Confirmation of the expression of hPRLR in EMT-6-hPRLR cells. A, RT-PCR analysis of hPRLR mRNA level using total RNA isolated from EMT6 or EMT6-hPRLR cells. RT-PCR products were analyzed on a 1% agarose gel as indicated. Arrow, a 400-bp fragment. B, results of radioreceptor binding assay on three breast cancer cell lines. Specific binding of PRL receptor was measured using the formula: [(cpm of total binding − cpm of nonspecific binding)/cpm of total binding] × 100.

**Cell Lines**
mary tissues, it will elicit dual therapeutic effects: the G129R portion of the fusion protein will specifically block PRLR, inhibiting the autocrine/paracrine effects of endogenous PRL; and the IL2 portion of the fusion protein may elicit a T-cell-mediated antitumor cytotoxicity reaction in situ, as in the case of mAb-IL2 studies.

To express the G129R-IL2 fusion protein, several different cloning strategies were used. Eukaryotic expression systems were not effective and resulted in very low yields, which made it impractical for in vivo studies. Ultimately, the bacterial expression vector pET22b/H11001 was used to produce relatively large quantities of the G129R-IL2 fusion proteins. Although the yield was far from ideal when compared with the yield of G129R, the low yield of production may be, in part, attributable to the presence of five pairs of Cys residues (three pairs in hPRL and two pairs in hIL2) in this novel protein. Only a small portion of the protein was found to be able to refold properly and eluted from Q-Sepharose columns in low-salt fractions (0.15 mM NaCl). G129R-IL2 fusion protein in these fractions is fully active in cell-based assays. More than 60% of the fusion protein eluted from the Q-Sepharose columns in high-salt fractions (>1 M NaCl) was nonfunctional as tested by STAT5 and HT-2 assay. We believe that proteins in the high-salt fractions represent fusion protein with mismatched disulfur bonding, which results in nonfunctional conformations.

The HT-2 proliferation analysis and STAT assays indicated that properly refolded G129R-IL2 fusion protein retained its IL2-like activity, namely stimulation of T-cell proliferation, as well as G129R-like activities, namely inhibition of STAT5 phosphorylation and inhibition of breast cancer cell proliferation. Although the fusion protein was functional in vitro, the real challenge was to determine whether this fusion protein could function in vivo. Pharmacokinetic results indicated that the blood clearance of G129R-IL2 fusion protein is much slower than that of either G129R alone or IL2 alone. The serum concentration of G129R-IL2 remained at 20–30 ng/ml after daily injection (50 μg/mouse/day). These data are very significant because previous studies have shown that the half-life of G129R or hIL2 are less than 2 h because of small molecular sizes. Moreover, the serum concentration of G129R or IL2 was not detectable 24 h after injection with a dose up to 200 μg/mouse. We believe that the significantly prolonged serum half-life of G129R-IL2 could not be explained merely by the increase in size of the fusion protein. It was reported that IL2 is able to bind to α-macroglobulin in serum (51), therefore, prolonging its serum half-life. This unique feature of IL2 might help to prolong the half-life of the G129R-IL2 fusion protein.

The concentration of G129R-IL2 used in our in vivo studies was similar to the dose used in hGH antagonist clinical studies (5–10 mg/kg of body weight) and is also in the range of G129R used alone in our recent in vivo studies with human breast cancer cell xenografts in nude mice (25). It is noteworthy that the concentrations of fusion protein used in our in vivo studies

Fig. 7 Inhibition of EMT6-hPRLR cell proliferation by G129R or G129R-IL2. EMT6-hPRLR cells were treated with hPRL, G129R, G129R-IL2, or in combination as indicated. In A, PRL induces cell proliferation of EMT6-hPRLR cells, whereas both G129R and G129R-IL2 have inhibitory effect on the proliferation of EMT6-hPRLR cells. In B, G129R or G129R-IL2 is able to competitively inhibit the stimulatory effect of hPRL on EMT6-hPRLR cells. The inhibitory effect of G129R-IL2 is significantly greater than that of G129R (P < 0.05).

Fig. 8 Pharmacokinetic studies of G129R-IL2 in Balb/c mice. BALB/c mice were given injections (i.p.) of either 25 μg or 50 μg of G129R-IL2, and serum samples were collected via tail vein bleeding at time intervals indicated. The serum concentration of G129R-IL2 was determined via the hPRL IRMA kit.
Twenty-four Balb/C mice were given injections of 1/11003 IL2 (100/9262 groups and treated with PBS, G129R (200 hPRLR cells. After tumor inoculation, mice were randomized into four groups of 100-μg/day/mouse is better than that of the 200-g/day/mouse group. We believe that the discrepancy between these results is attributable to the toxic reaction caused by the high dose of G129R-IL2 (200 μg/day/mouse). This speculation was supported by the observation that there is a body weight loss during the treatment period in the high-dose group (Fig. 9).

We also directly compared the inhibitory effects of G129R and G129R-IL2 in cancer cell proliferation assay (Fig. 7) as well as the growth of xenografts (Fig. 9). In both cases, G129R-IL2 showed stronger inhibitory effects than G129R alone. We assume that the better in vivo results are attributable to the effects of targeted IL2 and prolonged serum half-life of G129R, although additional studies regarding the immune response in vivo after the administration of G129R-IL2 are needed. We do not have a good explanation for the difference between G129R and G129R-IL2 in the inhibition of EMT6-hPRLR cell proliferation. We speculate that G129R-IL2 is probably more stable in cultured media as compared with G129R and, therefore, results in better inhibitory effects.

In conclusion, the data presented here demonstrate that the fusion of G129R and IL2 results in a novel, bifunctional protein, G129R-IL2. This novel fusion protein is able to act as a PRLR antagonist as well as a T-cell growth factor. With a relatively long serum half-life, daily injection of G129R-IL2 at a dose of 100 μg/mouse resulted in significant inhibition of breast tumor growth in vivo. Additional in vivo studies regarding the fusion protein’s biological activities using natural breast cancer cells are needed to evaluate its bifunctional properties. We believe that this targeted endocrine-immune design provides a novel and effective approach to human breast cancer treatment.

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