Human Adrenomedullin Up-regulates Interleukin-13 Receptor α2 Chain in Prostate Cancer *In vitro* and *In vivo*: A Novel Approach to Sensitize Prostate Cancer to Anticancer Therapy

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Abstract

Interleukin-13 (IL-13) receptor α2 (IL-13Rα2), a high-affinity IL-13 binding subunit and a tumor antigen, is amplified in a variety of human tumor cell lines and tumors *in vivo*. By cDNA microarray, we have shown that gene transfer of human and rat adrenomedullin (AM) up-regulates IL-13Rα2 in a human prostate tumor cell line. Here, we show that IL-13Rα2 mRNA and protein are also up-regulated in PC-3 prostate tumor cells by recombinant AM (rAM) and human synthetic AM peptide in a dose-dependent manner *in vitro* and *in vivo* in mouse prostate tumor model. The 8- to 10-fold up-regulation of IL-13Rα2 by rAM or AM peptide in prostate tumor cells *in vitro* and *in vivo* increased their sensitivity to IL-13PE cytotoxin consisting of IL-13 and a truncated form of *Pseudomonas* exotoxin. Immunodeficient mice with established prostate tumors transfected with AM or treated with AM peptide showed reduction in tumor size by intratumoral administration of IL-13PE in a dose-dependent manner. At the highest dose (three 100 μg/kg/d every alternate day), >70% reduction of tumor size was observed compared with controls (P 0.01). These results indicate that two completely unrelated hormones (AM and IL-13) are closely related to each other and that we have identified a novel role of AM in sensitizing certain types of prostate tumors to IL-13R–directed therapeutic agent.

Introduction

Interleukin-13 (IL-13) is a pleiotropic immune regulatory cytokine and the receptors for this cytokine are overexpressed on a variety of human cancer cell lines and primary tumors derived from renal cell carcinoma, malignant glioma, ovarian carcinoma, acquired immunodeficiency syndrome–associated Kaposi's sarcoma, and head and neck tumors (1–7). IL-13 receptors (IL-13R) are composed of two prominent proteins termed IL-13Rα1 (also known as IL-13Rα′) and IL-13α2 (also known as IL-13Rα; refs. 8,9). IL-13 binds to IL-13Rα1 chain with low affinity, whereas it binds to IL-13Rα2 chain with high affinity.

After binding to IL-13Rα1 chain, IL-13 recruits the predominant IL-4 binding protein (IL-4Rα) for signaling (1, 2, 9–11). IL-13Rα2 chain was shown to neither recruit any chain nor mediate signal transduction (12, 13). However, this chain is internalized after binding to ligand (10). Recent studies have shown that IL-13Rα2 mediates signaling through activator protein-1 pathway in murine macrophage cell line (14).

To target IL-13Rs, recombinant fusion proteins composed of IL-13 and a truncated form of a bacterial toxin, *Pseudomonas* exotoxin (IL-13PE38QQR and IL-13PE38), have been developed (15, 16). This immunotoxin is highly cytotoxic to tumor cells that express high levels of this receptor (5, 15, 17–21). Based on these studies, four phase I/II clinical trials were completed to determine the safety and tolerability of this agent in patients with recurrent glioblastoma multiforme (22).

Previous studies have shown the potential role of adrenomedullin (AM) in prostate carcinoma by generating a stably transfected human prostate carcinoma PC-3 cell line (23). AM, a potent hypotensive 52–amino acid amidated peptide, is a pluripotent factor isolated from renal pheochromocytoma tumors (24). Later studies identified the presence of AM in both human- and rat-derived prostate tissues, prostate carcinoma, and prostate cancer cell lines (25). AM is a tumor promotion factor (26, 27) and causes vasodilatation and bronchodilation, control hormone secretion, and renal homeostasis and regulates cell growth in cells derived from benign and malignant tumors (26, 28, 29).

To study the molecular mechanism of growth control in human prostate carcinoma by AM, we did cDNA microarray analysis of a PC-3 cell line transfected with AM. We identified ~ 100 genes that were up-regulated and involved in regulating cell cycle arrest, apoptosis, cytoskeleton, cell adhesion, the extracellular matrix, immune function, and transcription (30). Interestingly, we observed that IL-13Rα2 mRNA and protein were highly up-regulated (4- to 25-fold) as a result of AM gene transfer. However, the mechanism through which IL-13α2 is up-regulated is not known. It is also not known whether AM could up-regulate IL-13Rα2 *in vivo* and sensitize tumor to IL-13PE immunotoxin. It will be equally of interest to know if a synthetic peptide for AM could up-regulate IL-13Rα2 chain *in vitro* and *in vitro*.

In the present study, we have investigated the effect of purified recombinant AM (rAM), synthetic AM peptide, or gene transfection of AM on IL-13Rα2 in prostate cancer cells *in vitro* and *in vivo* in a mouse model. The biological function of up-regulated IL-13Rα2 protein was examined in a xenograft model of human prostate cancer. AM gene–transfected prostate carcinoma cells formed tumors in athymic nude mice, which were subsequently treated with IL-13PE immunotoxin, and the tumor growth was measured. A dramatic decrease in tumor size was observed.

Materials and Methods

Cell culture and reagents.

PC-3 prostate cancer cell line was purchased from the American Type Culture Collection and maintained in RPMI 1640 containing 10% fetal bovine serum (Biowhittaker), 1 mmol/L

HEPES, 1 mmol/L nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin (Biowhittaker).

rAM and AM peptide.

rAM was expressed from a clone of the PC-3 cell line after stable transfection of full-length AM (see Supplementary Materials and Methods for details). Synthetic AM (1–52) peptide was purchased from Bachem Labs and used after its reconstitution in sterile protease-free water.

Western blot analysis for AM detection.

For Western blot analysis, 10 μL of cell-free supernatants obtained from parental, mock-transfected (vector alone), and AM gene–transfected (AM stable transfected) PC-3 were boiled for 5 min in SDS sample buffer [2% SDS, 50 mmol/L Tris-Cl (pH 6.8), 0.1% bromphenol blue, 10% glycerol], electrophoresed, transferred on polyvinylidene difluoride membrane (Bio-Rad), and immunoreacted with anti-AM antibody (Bachem Labs) in TBS-Tween 20 buffer for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham).

Recombinant cytokines and toxins.

Recombinant chimeric fusion protein IL-13PE38 (IL-13PE immunotoxin or IL-13PE) was expressed in *Escherichia coli* and purified to >95% homogeneity in our laboratory as described previously (15, 16). Recombinant IL-4 and IL-13 were also produced as described (31). Recombinant IL-2 was a kind gift from Chiron Corp.

RNA extraction and reverse transcription-PCR.

Total RNA was extracted using the RNeasy RNA extraction kit (Qiagen) as per the manufacturer's instructions. The optimal reverse transcription-PCR (RT-PCR) conditions for IL-13Rα2 chain (32) and AM and the primers used in the amplification technique have been described previously (23). Real-time RT-PCR analysis for IL-13Rα2 and β-actin was done using a Bio-Rad Real-time iQ5 after designing gene-specific primers. The relative fluorescence intensity was determined by dividing the intensity of IL-13Rα2 mRNA band by the intensity of β-actin band and expressed as a ratio of relative fluorescence units (RFU; refs. 15, 30). The experiments were done in quadruplicate.

IL-13R binding studies.

Recombinant human IL-13 was labeled with ^{125}I (Amersham Research Products) using IODO-GEN reagent (Pierce) according to the manufacturer's instructions. The specific activity of the radiolabeled cytokine was estimated to range between 40 and 120 μCi/μg of protein. Binding experiments were done as described elsewhere (11).

Immunofluorescence assay.

Twenty thousand cells were cultured in the presence or absence of rAM in a chambered glass slide (Lab-Tek, Nalge Nunc International) for 48 h under similar conditions as described earlier (6). Additional three washes with PBS were included to remove culture

medium that contained AM or mock supernatant. This step minimized the possibility of interference of exogenous AM in the assay. In some experiments, PC-3 cells were preincubated with neutralizing rabbit anti-AM antibody (1:100 dilution; a generous gift from Dr. Ouafik, IFR Jean Roche, Marseille, France) for 1 h and subsequently cultured in the presence of rAM or AM peptide for an additional 48 h to evaluate the specificity of AM in up-regulation of IL-13Rα2. For controls, PC-3 cells were incubated with an equal quantity of rabbit serum in parallel. Indirect immunofluorescence assay (IFA) was done for IL-13Rα2 and AM protein expression as described above.

Cytotoxicity assay.

The cytotoxicity of IL-13PE was determined as described previously (17). For blocking experiments, the tumor cells were preincubated with IL-13, IL-4, or IL-2 (2 μg/mL) for 45 min before addition of IL-13PE. The cells were maintained for 5 more days and then viable cell population was measured by trypan blue exclusion dye technique.

Tumor xenograft studies.

PC-3 cells transfected with AM- or mock-transfected or control (5×10^6) were injected s.c. in the right flank of male athymic nude mice. When PC-3 control tumor xenografts reached a size of 20 ± 4 mm^2 within 1 wk, 100 ng AM peptide was injected intratumorally to avoid any systemic adverse side effects every alternate day three times in three sides of the tumor. Forty-eight hours after the last treatment, the animals were sacrificed and tumors were excised and preserved for detection of IL-13Rα2 mRNA by real-time RT-PCR and IL-13Rα2 protein by immunohistochemical (IHC) techniques.

To determine the sensitivity of PC-3 tumors to IL-13PE after up-regulation of IL-13Rα2 by AM transfection, IL-13PE (50 and 100 μg/kg) or PBS/0.2% human serum albumin (vehicle) was injected intratumorally when the tumor size has reached 20 ± 3.2 mm^2. Animals with vector only–transfected PC-3 cells served as controls. Treated and control animals were followed for 5 wk, at which point the experiment was terminated due to large tumor burden in control groups. The average values of three independent experiments were calculated and expressed as mean ± SD.

Statistical analysis.

An unpaired two-tailed *t* test was used to detect significant difference between experimental and corresponding control groups using JMP software package (Brooks/Cole-Thompson Learning). *P* values of 0.05 were considered significant.

Results

Expression of AM mRNA and immunoreactive protein in stable transfected PC-3 tumor cells.

The PC-3 cell line stably transfected with AM gene transcribed mRNA and translated and secreted AM protein in the cell-free supernatant. Real-time PCR showed a significant increase in mRNA expression ($P < 0.01$) and protein (see Supplementary Fig. S1*A* and B). The AM protein from two different AM clones immunoreacted with a highly specific

antibody and showed a single band of ~6 kDa in the Western blot analysis. Cell-free supernatants from either PC-3 parental or vector only–transfected PC-3 cells (mock transfected) did not show a positive band for AM. As the band intensities for AM protein at a seeding density of 0.5×10^6 cells per flask in four independent experiments were identical for clone AM1, we used this seeding density in all of our experiments to maintain consistent AM expression.

AM enhances IL-13Rα2 expression *in vitro* and *in vivo*.

AM peptide was added to tumor cell cultures at various concentrations every alternate day and incubated for 3 days to examine the effect of synthetic AM peptide on *IL-13Ra2* mRNA expression. By real-time RT-PCR studies, *IL-13Ra2* mRNA expression was increased in a dose-dependent manner and approximately 7- to 8-fold increased expression was observed in treated cells compared with untreated PC-3 cells ($P < 0.01$; Fig. 1*A*). Similar to mRNA, protein for IL-13Rα2 was also increased by AM peptide treatment (Fig. 1*B*).

For *in vivo* regulation, PC-3 tumor cells were injected s.c. in immunodeficient animals. Eighteen days later, some tumors received 100 ng AM peptide intratumorally, whereas some tumors were injected with excipient. As shown in Fig. 1*C*, AM significantly up-regulated *IL-13Ra2* mRNA expression. Similar to *in vitro* study, AM peptide up-regulated *IL-13Ra2* mRNA expression by about 7- to 8-fold ($P < 0.001$). Expression of β-actin mRNA remained uniform and unchanged in this tumor cell line, which indicated that AM did not alter the transcription of all genes.

To examine whether gene-transfected tumor cells maintained expression of *IL-13Ra2* mRNA *in vivo* when these cells were s.c. injected to develop tumors, we determined mRNA expression in PC-3 AM and PC-3 mock tumors by real-time RT-PCR. As shown in Fig. 1*D*, PC-3 AM explant tumors maintained significantly high level expression of *IL-13Ra2* mRNA 35 days after implantation ($P < 0.001$). PC-3 control or mock-transfected tumors did not show any up-regulation of receptor mRNA.

AM-transfected and control tumor cells were also analyzed for protein expression for AM and IL-13Rα2. As shown in Fig. 2*A*, PC-3 cells showed an intense immunofluorescence staining for IL-13Rα2 in green and AM protein in red in a stable PC-3 cell line transfected with AM. Approximately 90% of the cells were positive for AM and 82% were for IL-13Rα2 in a stable transfected PC-3 cell line. Similar to real-time RT-PCR results, mock-transfected cells showed a basal level for AM and IL-13Rα2. When two images were merged, IL-13Rα2 and AM showed a yellow immunofluorescence in 68% of AM-transfected cells (Fig. 2*A*), indicating that both red and green immunofluorescent proteins (AM and IL-13R*a*2) were colocalized. The remaining cells may have a minimal overlap of the two colors or these proteins coexpressed at levels below the detection limit of the assay. These results indicate that prostate tumor cells express IL-13Rα2 protein at a basal level and AM treatment up-regulates its transcription and translation in treated cells.

To examine the specificity of IL-13Rα2 up-regulation by AM, PC-3 tumor cells were preincubated with AM neutralizing antibody or isotype control antibody for 45 minutes before treatment with AM peptide or rAM and RT-PCR assay was done for *IL-13Ra2* As

shown in Fig. 2*B*, rAM and AM peptide up-regulated *IL-13Rα2* mRNA compared with control cells. Neutralizing antibody to AM decreased expression of *IL-13Rα2* mRNA induced by rAM as well as AM peptide. However, isotype control antibody had no effect.

The specificity of IL-13Rα2 up-regulation in AM-treated PC-3 cells was also evaluated at protein levels (Fig. 2*C*). PC-3 cells were incubated with AM in the presence of either neutralizing antibody to AM or isotype IgG control. The cells were then immunostained with either AM antibody or IL-13Rα2 antibody to detect the level of expression of these two proteins. Neutralizing antibody to AM decreased not only AM protein expression but also IL-13Rα2 protein levels. In contrast, isotype control IgG did not decrease expression of AM or IL-13Rα2 protein.

Radiolabeled binding and competition study.

Based on RT-PCR and immunofluorescence results, we predicted that radiolabeled IL-13 would specifically bind to AM gene–transfected PC-3 cells. Therefore, binding studies were done using ^{125}I-IL-13 in PC-3, PC-3 mock, and four clones of AM-transfected PC-3 cells. As shown in Fig. 2*D*, only four clones of PC-3 AM cells bound ^{125}I-IL-13 at high levels (P < 0.001). Total ^{125}I-IL-13 binding was inhibited by excess of unlabeled IL-13 indicating specificity of IL-13 binding. PC-3 control or mock-transfected cells showed no specific binding.

In vivo regulation of IL-13Rα2 by AM.

To investigate regulation of IL-13Rα2 by AM *in vivo* and IHC and *in situ* hybridization (ISH) studies were performed on paraffin-embedded tissue sections obtained from PC-3 mock–transfected and PC-3 AM–transfected tumors. PC-3 AM tumor explants showed moderate immunostaining for IL-13Rα2 compared with weakly positive staining (±) in isotype sections or PC-3 mock explants (see Supplementary Fig. S2*A*). Percent positive fields were found to be 58% in PC-3 AM tumors and 8% in PC-3 mock tumor sections. Isotype control sections did not show any appreciable fluorescence and served as a negative control in the IHC assay. ISH for *IL-13Rα2* mRNA was a more sensitive assay and indicated that *IL-13Rα2* mRNA was overexpressed in AM xenograft tumors compared with mock tumor tissues. A sense riboprobe for *IL-13Rα2* gene served as a negative control in ISH experiments, which did not show any staining (see Supplementary Fig. S2*B*).

Enhanced sensitivity of AM-PC-3 tumor cells to IL-13PE *in vitro* and *in vivo*.

To determine the functional activity of overexpressed IL-13Rα2 protein by AM in tumor cells, we tested the cytotoxic activity of IL-13PE by measuring cell viability in control, AM-transfected, and AM peptide–treated PC-3 prostate tumor cell lines. Tumor cell lines were incubated in the presence of different concentrations of IL-13PE and cell viability was measured after 5 days in treated and untreated control tumor cells. IL-13PE was only slightly cytotoxic to PC-3 parental and mock-transfected control cell lines. However, AM peptide treatment of PC-3 cells resulted in a significant decrease in cell viability compared with untreated control cells (P < 0.05; Fig. 3*A*). Similarly, in AM-transfected PC-3 cells, a significant decrease in cell viability (P < 0.01) was observed by IL-13PE treatment (Fig. 3*B*). The IC$_{50}$ (concentration of IL-13PE that decreases 50% cell viability) was ~5.0 ng/mL

in PC-3 AM–transfected cells compared with PC-3 mock cells (>1,000 ng/mL; Fig. 3*B*). This increase in sensitivity was highly specific to IL-13PE as only IL-13 but not IL-4 or IL-2 could block the cytotoxic activity of IL-13PE in PC-3 AM–transfected cells (data not shown).

To determine the significance of up-regulated IL-13Rα2 chain *in vivo* in mouse model of human prostate cancer, PC-3, PC-3 mock–transfected, and AM-transfected prostate tumor cells were s.c. implanted in nude mice. Beginning day 12 of implantation, tumors were treated with 100 ng AM peptide every alternate day for 3 days, and then 2 days later, some tumors were injected with IL-13PE (50 or 100 μg/kg/d) every alternate day for 3 days. Mice bearing mock tumor or AM-transfected tumors were intratumorally injected with IL-13PE (50 or 100 μg/kg/d) every alternate day for 3 days and followed for tumor growth. As shown in Fig. 4*A*, both doses of IL-13PE decreased AM-treated tumor growth significantly compared with excipient treated mice (*P* < 0.05). In sharp contrast, IL-13PE did not have any effect on growth of mock-transfected tumors (Fig. 4*B*). All mice showed similar growth of tumors whether treated with 50 or 100 μg/kg/d IL-13PE or excipient control.

IL-13PE also caused significant inhibition of AM-transfected tumor growth in a dose-dependent manner (*P* < 0.05 and 0.01, respectively; Fig. 4*C*). At the highest dose (100 μg/kg/d), the tumor size remained smaller at all time points of monitoring compared with 50 μg/kg IL-13PE dose. At day 29 after IL-13PE treatment, tumor size in treated group (38 ± 4.0 mm^2) was ~75% lower compared with excipient treated tumor (220 ± 18.0 mm^2; *P* < 0.01). PC-3 tumors in control groups increased to 220 ± 18.0 mm^2 in size at day 45, at that time the experiment was terminated due to large tumor burden in control group.

Discussion

We show that exogenous AM protein or a synthetic peptide up-regulates IL-13Rα2 mRNA and protein in prostate tumor cells *in vitro* and *in vivo* in an animal model of human prostate tumor. IL-13α2 overexpression by AM was highly specific as neutralization of AM by antibody diminished IL-13Rα2 mRNA and protein levels. Interestingly, overexpression of IL-13Rα2 protein by AM sensitized tumor cells to an exclusively selective receptor–directed cytotoxic agent such as IL-13PE. IL-13PE immunotoxin mediated enhanced cytotoxicity to prostate tumor cells *in vitro* when treated with AM peptide or stably transfected with *AM* gene. This cytotoxicity was highly specific as an excess of IL-13 but not IL-4 or IL-2 neutralized IL-13PE–mediated cytotoxicity. Similar to *in vitro* studies, IL-13PE also mediated antitumor effects against AM peptide–treated or AM-transfected PC-3 tumors *in vivo*. These results provide proof of principle that AM-regulated *IL-13Rα2* gene makes a functional receptor on tumor cell surface *in vivo* for receptor-directed therapy by IL-13PE. Partial response to this approach in >70% of AM-transfected PC-3 tumor-bearing mice may be improved by designing effective delivery of this immunotoxin in tumor.

The mechanism of up-regulation of IL-13Rα2 chain by AM in prostate tumor cells is not known. Preliminary studies indicate that AM directly interacts with *IL-13Rα2* gene and that AM stabilizes IL-13Rα2 transcripts and prolongs its translation. In addition, it has been shown that human tumor cell lines that express the IL-13Rα2 chain are highly susceptible to

the cytotoxic effect of IL-13PE *in vitro* and *in vivo* in animal models of human cancers (10, 19). However, tumor cell lines that expressed low levels of IL-13R were dramatically less sensitive to this targeted cytotoxin (33). We later showed that when tumor cells were transfected with IL-13Rα2 chain, they showed enhanced sensitivity to IL-13 cytotoxin compared with wild-type or mock-transfected tumor cells (34, 35). As normal immune cells, endothelial cells, or other cells do not express IL-13Rα2, IL-13 cytotoxin is slightly or not cytotoxic to these cells (17, 32). These observations suggest that IL-13Rα2 chain provides a unique target for receptor-directed cancer therapy and combination of pretreatment with AM followed by IL-13 cytotoxin therapy may lead to better antitumor activity.

It is possible that pretreatment of host with AM may also up-regulate IL-13Rα2 in normal cells, leading to toxicity to IL-13 cytotoxin administration. However, *in vivo* administration of AM in tumors did not cause toxicity in animals. It is to be noted that rAM or AM peptide does not seem to induce IL-13Rα2 expression in cells that do not naturally express this chain (results not shown). Most normal cells, such as B cells, T cells, monocytes, and endothelial cells, do not express basal levels of IL-13Rα2 chain (1, 2, 5). Similar to normal immune cells, other prostate cancer cell lines, such as DU145 or LNCaP, also do not express basal levels of IL-13Rα2 chain and therefore do not show up-regulation of IL-13Rα2 and sensitivity toward IL-13PE. Lack of constitutive expression of IL-13R expression in prostate cancer cell lines may represent heterogeneity in IL-13R in prostate cancer. In this regard, our results are similar to previous studies, which showed that IL-13Rα2 chain is not expressed by all tumors. Thirty percent to 72% of head and neck, ovarian, and brain tumor samples express high levels of IL-13Rα2 chain, whereas others express none or modest levels of IL-13Rα2 chain (2, 5, 6, 15, 17, 18, 21, 36). Although a large number of prostate cancer samples need to be evaluated for IL-13R expression, our results suggest testing IL-13R expression in clinical biopsy samples before enrolling subjects in clinical trials involving AM-directed and IL-13R–directed cytotoxin/immunotoxin or gene therapies. Based on the knowledge of basal level expression of IL-13R, we may be able to predict "a priori" which patients will respond to IL-13R–targeted therapy and which patients will not. Future studies will address these important issues. In addition, we are studying other tumor types in which AM can up-regulate IL-13Rα2.

In conclusion, we have identified AM, a new class of tumor sensitizer for IL-13R–targeted anticancer therapy, in certain types of prostate cancers. IL-13R–directed therapy may be useful in prostate cancers with low expression of IL-13Rα2 after AM treatment followed by IL-13PE cytotoxin-based therapy. Finally, as recent studies have identified IL-13Rα2 chain as a tumor rejection antigen (37), AM may also be useful as a sensitizer for cancer vaccines for localized cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Grant support:

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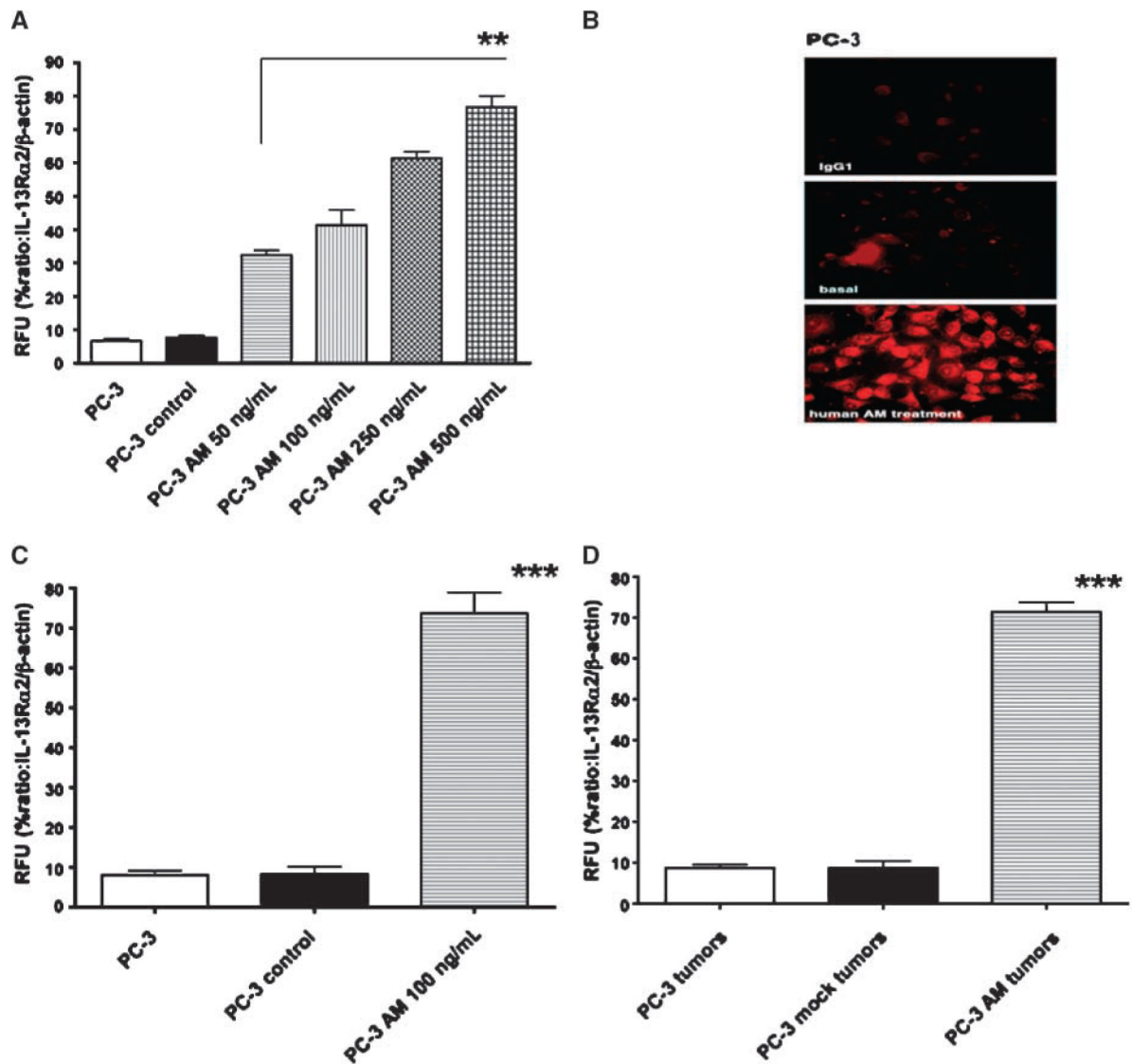
Figure 1.
Up-regulation of *IL-13Rα2* mRNA and protein by AM *in vitro* and *in vivo*. *A*, total RNA from PC-3 or PC-3 control or PC-3 cells treated with various concentrations (50–500 ng/mL) of AM peptide was analyzed by real-time RT-PCR. RFU of *IL-13Rα2* and *β*-actin was calculated and normalized from three independent experiments. The data are statistically significant (**, *P* < 0.01). *B*, AM peptide–treated PC-3 cells were immunostained by IFA for IL-13Rα2 protein detection. *IL-13Rα2* mRNA expression by real-time PCR was evaluated in PC-3 xenograft tumors treated with AM peptide from three independent experiments (*C*) and in AM-transfected PC-3 tumors compared with PC-3 or PC-3 mock–transfected tumors (*D*). The results are statistically significant (***, *P* < 0.001).
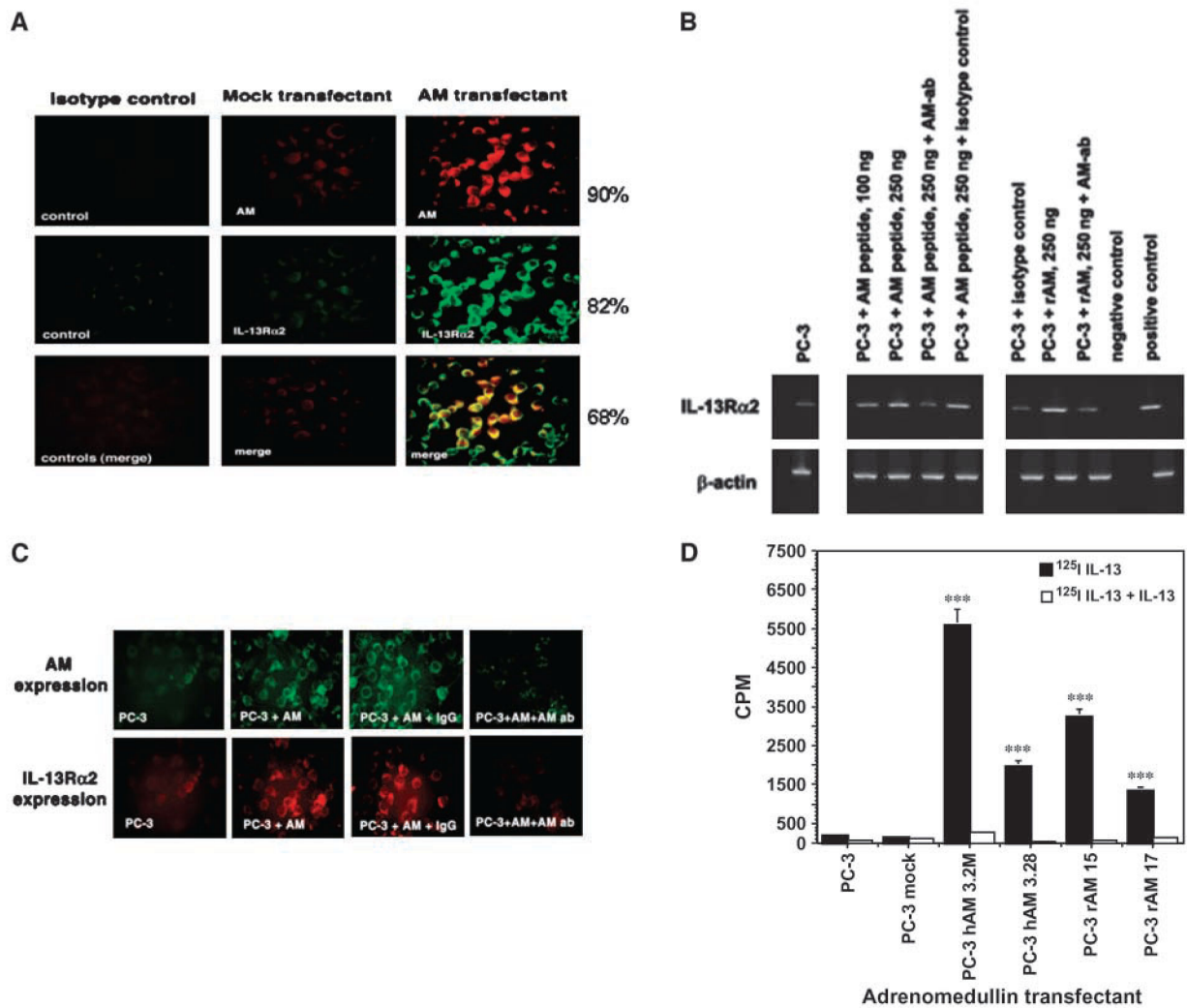
Figure 2.
Specificity of up-regulation of IL-13Rα2 by AM peptide and rAM. *A*, PC-3 cells were stained with either isotype control or polyclonal antibody to AM or monoclonal antibody to IL-13Rα2 as described in Materials and Methods. AM was detected using TRITC-conjugated anti-rabbit IgG to develop red fluorescence, whereas IL-13Rα2 was detected using FITC-conjugated anti-mouse IgG green fluorescence. The colocalization of both proteins in all three tumor cell lines was analyzed by merging the same field for TRITC- and FITC-immunostained red and green fluorescent tumor cells. Magnification, ×400. *B*, RNA was extracted from AM peptide–treated PC-3 cells in the presence of AM antibody or isotype IgG and RT-PCR analysis was done for *IL-13Rα2* mRNA expression. *β*-Actin expression was used as a housekeeping gene. RNA from H9 cells served as negative control and PM-RCC as positive control for *IL-13Rα2* expression. *C*, PC-3 cells were treated with AM in the presence or absence of antibody to AM or isotype IgG. The cells were stained with anti-AM and anti-IL-13Rα2 antibody. Magnification, ×400. *D*, receptor binding assay was done in PC-3, PC-3 mock, and AM-expressing PC-3 clones by incubating with ^{125}I-IL-13 (black columns) or with 200× cold IL-13 (*white columns*) for competition. Bound

CPM was measured in the cell pellet on a gamma counter. *Columns*, mean of duplicate determination; *bars*, SD. The experiment was repeated thrice. The values are statistically significant (***, *P* < 0.001).
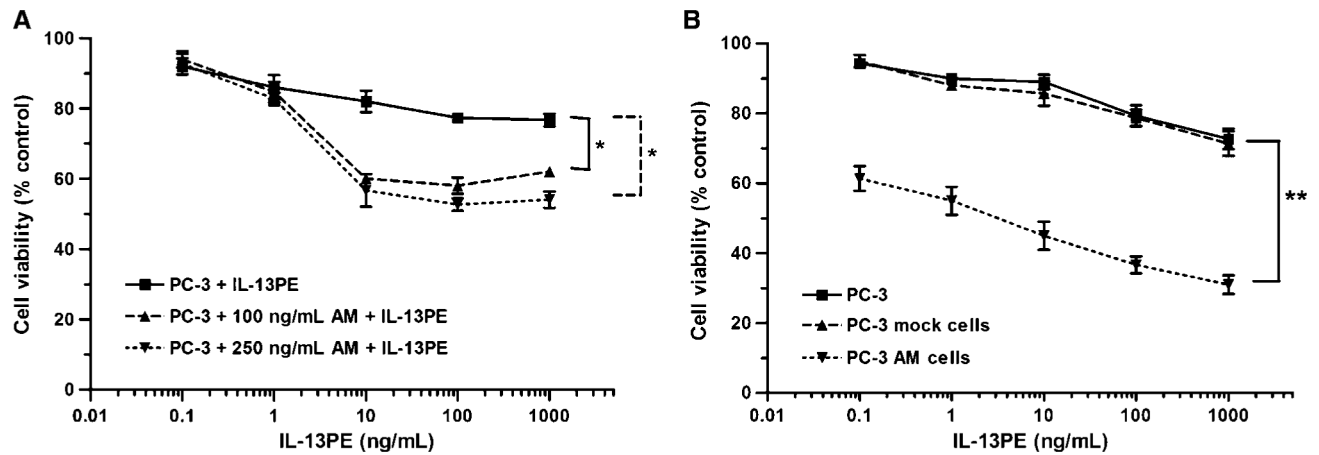
Figure 3.

Cytotoxicity of IL-13PE to AM-treated tumor cells *in vitro* and AM-transfected *tumors in vitro*. *A*, PC-3 cells treated with two different concentrations of AM peptide (100 and 250 ng/mL) for 48 h. Cells were then treated with various concentrations of IL-13PE for 20 h. Cell viability was measured after 5 d using trypan blue exclusion assay in hemocytometer. *Points*, mean percent viable cells; *bars*, SD. Untreated cells served as control. The experiment was repeated thrice and statistically significant differences ($P < 0.05$) were compared with control and AM-treated PC-3 cells. *B*, PC-3 control, PC-3 mock–transfected, and PC-3 AM–transfected tumor cells were incubated with various concentrations of IL-13PE. The viable cells were counted after 5d. *Points*, mean percent viable cells; *bars*, SD. Untreated cells served as 100% control. The results are statistically significant (**, $P < 0.01$).
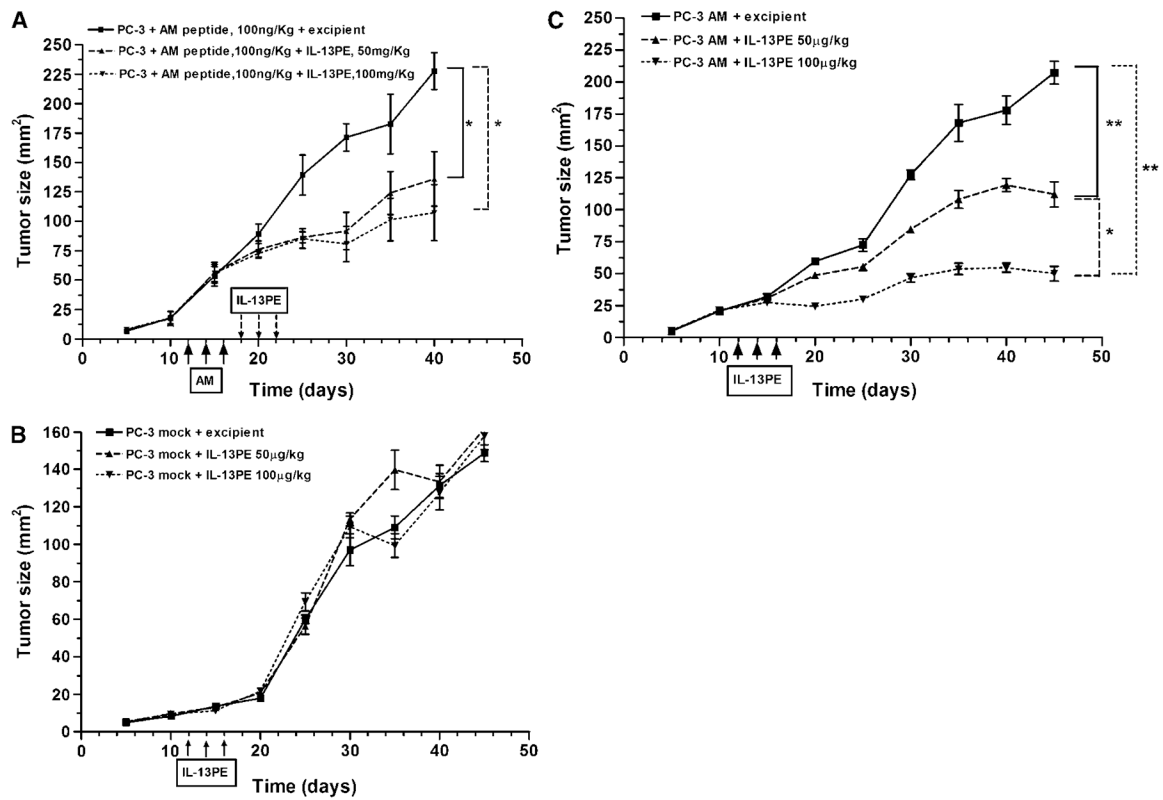
Figure 4.

Antitumor activity of IL-13PE in AM-treated or AM-transfected PC-3 prostate tumors. *A,* nude mice were implanted with 5×10^6 PC-3 tumor cells s.c. When tumors were established, AM peptide at a fixed dose (100 ng/kg) was injected directly into tumors every alternate day for three injections. Two days after AM treatment, these tumors were then injected with two different doses of IL-13PE (50 and 100 μg/kg) every alternate day for 3 d. Tumor sizes were measured. *Points,* mean of six animals per group for both doses; *bars,* SD. *, $P < 0.05$. Similarly, mock tumors (*B*) and PC-3 AM tumors (*C*) were treated with IL-13PE. The differences in tumor sizes were compared for statistical significance (*, $P < 0.05$; **, $P < 0.01$) between these groups of mice. *Points,* mean of six animals per group; *bars,* SD.