Systemic Interleukin 2 Therapy for Human Prostate Tumors in a Nude Mouse Model

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ABSTRACT

Once the regional lymph nodes become involved in prostate carcinoma, 85% of patients develop distant metastases within 5 years, and metastatic disease is difficult to treat. We have investigated the effect of systemic interleukin 2 (IL-2) treatment on metastatic prostate carcinoma using a xenograft tumor model. Cells from a PC-3/IF cell line, produced by intraperitoneal injection of human PC-3 prostate carcinoma cells, were injected in the prostate of Balb/c nude mice. Prostate tumors and para-aortic lymph nodes were resected, and tumor cells were recultured and passed in the prostate in vivo to produce new cell lines. On day 6 following prostatic injection of these cell lines, mice were treated with i.p. injections of IL-2 at 25,000–50,000 units/day for 5 consecutive days. The effect of IL-2 on tumor progression was assessed, and histological studies were performed on prostate tumor and lymph node sections. The tumor cell lines generated by serial prostate injection were tumorigenic and metastasized to regional para-aortic lymph nodes. Tumors of 0.4 cm were obtained by day 16 and grew to 1–1.5 cm by day 40 with metastasis to para-aortic lymph nodes. Following two to three weekly courses of 5 days of 25,000–40,000 units/day of IL-2, the growth of prostate tumors was inhibited by 94%. Higher doses of 50,000 units/day were toxic. Histologically, prostate sections showed vascular damage manifested by multifocal hemorrhages and an influx of lymphocytes and polymorphonuclear cells into disintegrating tumors and areas of necrosis containing numerous apoptotic cells. In contrast to control mice, para-aortic lymph nodes were not enlarged in responding mice. These findings suggest that systemic IL-2 therapy can induce an antitumor response in prostate tumors and control their growth and metastasis.

INTRODUCTION

Carcinoma of the prostate is the most common malignant tumor in men, with over 240,000 newly diagnosed cases and more than 40,000 deaths each year (1). For cancer still limited to the prostate gland, radiation therapy and surgery are both potentially curative treatment modalities; however, treatment of disseminated disease remains palliative (2, 3). In patients diagnosed with occult or small clinically palpable lesions, 30–35% develop metastases, and 20% die within 5–10 years. Once the regional lymph nodes become involved, 85% of patients develop distant metastases in 5 years (2, 4). Metastatic disease is most commonly treated by androgen blockade to eliminate testosterone production, but within an average of 18 months, the disease becomes hormone refractory, with an expected survival of less than a year (3). At this stage, no effective therapy is available: radiation therapy is only palliative, and conventional chemotherapy has failed. We propose to test a different approach using immunotherapy, which has been promising for metastatic malignancies that are resistant to conventional therapy, including surgery, hormonal treatment, and radiation therapy and chemotherapy. Immunotherapy uses activated immune cells and/or cytokines to enhance immune mechanisms directed against the tumor that may be present although ineffective in cancer patients (5). The lymphokine/cytokine IL-2, produced by activated T lymphocytes, is critical for immune responsiveness and has been extensively used in several clinical trials for the treatment of refractory malignancies (5, 6). Metastatic renal cell carcinoma and melanoma have been the most responsive to IL-2 infusions (6). Few basic research studies have tested the potential of immunotherapy, including that of IL-2, for advanced prostate cancer. s.c. tumors induced by injection of syngeneic prostate tumor cell lines in Dunning rats were not significantly inhibited by peritumoral s.c. injections of IFN-γ or TNF-α, but the combination of both was more effective (7). More recently, gene therapy approaches were studied for prostate cancer using the androgen-independent MatLy-Lu rat prostate carcinoma implanted s.c. in Dunning rats. Intradermal administration of IL-2 gene-modified tumor vaccines cured and induced immunological memory in some of the rats bearing s.c. tumors (8). However, this approach was less effective for the treatment of tumors orthotopically implanted in the prostate, causing only a slight increase in survival. Granulocyte macrophage colony-stimulating factor- or IFN-γ-secreting cells were less effective in this study (8) and induced a longer survival in ~30% of the mice in a separate study (9). Recently, the canarypox virus ALVAC vector was used to transfect the RM-1 mouse prostate cancer cells

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3 The abbreviations used are: IL-2, interleukin 2; TNF-α, tumor necrosis factor-α; CM, culture medium; CU, Cetus unit(s).
with cytokines or the costimulatory molecule B7-1. The transfection of RM-1 with TNF-α and IL-2 inhibited the growth of these cells implanted s.c. in C57BL/6 syngeneic mice (10). These observations suggest that prostate carcinoma might be responsive to IL-2 therapy and warrant further efforts to test the sensitivity of prostate carcinoma to IL-2.

We have investigated the effect of systemic injections of IL-2 for the treatment of prostate tumors using a metastatic prostate carcinoma tumor model established in nude mice. The PC-3 human prostate carcinoma tumor cell line, which is androgen independent (11), was heterotransplanted into the prostate of athymic nude mice. Previous studies have shown that intraprostatic injection of cells from PC-3M cell line, a variant of PC-3 cell line produced by intrasplenic implantation of PC-3 cells, caused the formation of prostate tumor and metastasis to regional para-aortic lymph nodes in Balb/c nude mice (12, 13). We have produced a new cell line designated PC-3/IF by implantation of PC-3 cells in the femur of nude mice. When PC-3/IF cells were injected in the prostate of nude mice, prostate tumors with metastasis to regional para-aortic lymph nodes were obtained, similar to the models described previously (12, 13). Sequential in vivo and in vitro passages of tumor cells obtained from prostate tumors and lymph nodes resulted in the generation of tumorigenic and metastatic cell lines. Prostate tumors, induced by intraprostatic injection of these cell lines, were treated with systemic IL-2 treatment. We found that IL-2 induced an antitumor response in tumors located in the prostate and was effective at controlling their growth and metastasis to lymph nodes.

MATERIALS AND METHODS

Tumor Model. The human prostate carcinoma PC-3 tumor cell line was purchased from American Type Culture Collection (Manassas, VA). PC-3 cells were cultured in CM, consisting of F-12 K nutrient mixture supplemented with 7% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 2 mM glutamine, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate (Sigma Chemical Co., St. Louis, MO), 10 mM HEPES buffer, 100 units/ml penicillin/streptomycin, 0.5 μg/ml fungizone, and 50 μg/ml gentamycin. A PC-3/IF cell line was generated in our laboratory from prostate tumors obtained in nude mice following intraprostatic injection of PC-3 cells according to a procedure described previously (14). The PC-3/IF cell line showed more aggressive growth in vivo than did the original PC-3 cell line4 and was used for prostate implantation. Tumor cells were washed twice in PBS, and a concentration of 4–5 × 105 cells in 20 μl of PBS was injected into the prostate of 4–6-week-old male Balb/c nu/nu nude mice (purchased from Life Sciences, St. Petersburg, FL, or University of South Florida, Tampa, FL). The prostate of anesthetized mice was exposed through a midline laparotomy incision and by retraction of the bladder and male sex accessory glands anteriorly. Injection of cells was performed with a 27-gauge needle inserted in the prostatic lobe located at the base of the seminal vesicles. The abdominal wound was sutured using a 4.0 chromic gut suture in a running fashion. Mice were housed and handled under sterile conditions in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The animal protocol was approved by Wayne State University Animal Investigation Committee.

Generation of PC-3/PI Cell Lines. Following prostate injection of PC-3/IF cells, mice were sacrificed at various time points and autopsied in sterile conditions in a laminar flow hood. Prostate tumors were resected, minced with scissors, and dissociated in HBSS with a syringe plunger. The cell preparations were filtered through a wire mesh, and cells were washed twice with HBSS. Cells were resuspended in CM, and the tumor cells were counted and cultured in flasks at 37°C in a humidified 5% CO2 incubator to yield a new cell line designated PC-3/PI-1 (PI, prostate implantation). PC-3/PI-1 cells were reimplanted in the prostate of nude mice for additional passage in the prostate, as shown in Fig. 1. This cycle of in vivo prostate passage followed by in vitro culture of prostate tumors was repeated three times and led to the generation of PC-3/PI-1, PC-3/PI-2, and PC-3/PI-3 cell lines (Fig. 1). We have established several PC-3/PI lines from separate mice, from prostate tumors obtained in each one of the three in vivo passages. Mice that developed prostate tumors showed enlarged regional para-aortic lymph nodes located along the spine. These lymph nodes were resected in sterile conditions, dissociated into a single-cell suspension, and cultured to yield new cell lines designated PC-3/PI-LN, as shown in Fig. 1. The number of cells recovered following dissociation of tumors was in the range of 2 × 105–5 × 106 cells for prostate tumors and 0.2 × 106–0.5 × 106 cells for lymph nodes from advanced prostate tumors (after day 40). To assess the doubling time of the cell lines generated from in vivo tumors, cells were plated in triplicate in six-well plates at a concentration of 50,000 cells per well and cultured in CM for 5 days. Cells were counted every 24 h, and doubling times were calcu-
Fig. 2 Histology of PC-3 prostate tumors. Prostate sections were stained with H&E. A, normal prostate morphology consisting of several glands (×25). B, benign gland in the prostate (arrowheads) surrounded by tumor cells on day 30 post-PC-3/PI tumor cell injection (×100). C, enlargement of tumor area in the prostate on day 21 post-tumor cell injection, showing poorly cohesive pleomorphic tumor cells with large vesicular nuclei and prominent nucleoli (arrows) and a mitotic figure (short arrow; ×100). D, para-aortic lymph nodes showing normal lymph node morphology on day 5 following intraprostatic tumor injection (×50). E, invasion of large tumor cells within sinusoids of para-aortic lymph nodes (arrows) on day 44 post-tumor cell injection replacing normal small immune cells in the lymph nodes (arrowheads; ×50) in contrast to lymph nodes not containing tumor cells shown in D at the same magnification (×50).

Tissue Preparation for Histology. At various time points following tumor cell implantation in the prostate, mice were sacrificed, and the prostate, seminal vesicles, para-aortic lymph nodes, and spine were processed for histology studies. The soft tissues were fixed in 4% paraformaldehyde. The spines were fixed in a solution of 10% paraformaldehyde, 30% ethanol, and 1.5% acetic acid for 2 days and then decalcified in 4% parafomaldehyde containing 5% trichloroacetic acid for 5 days. The tissues were embedded in paraffin and sectioned. Sections were stained with H&E.

Experimental Protocol. Five days following intraprostatic injection of PC-3/PI cells, treatment was initiated with recombinant human IL-2 (specific activity, 3 × 10⁶ CU/mg; 1 CU is equivalent to 6 units; generously provided by Chiron Corporation, Emeryville, CA). IL-2 at 12,500–25,000 CU in 0.5 ml of dextrose solution was injected i.p. twice a day for 5 consecutive days. This cycle was repeated for 1–2 additional weeks. The selection of IL-2 doses was based on previous IL-2 dose titration experiments (15). Control mice were injected i.p. with 0.5 ml of 5% dextrose solution. On day 30–34 post-PC-3 cell injection, mice were sacrificed, autopsied, and examined for gross tumors in the prostate. The prostates and para-aortic lymph nodes were resected and processed for histological studies.

Statistical Analysis. To compare the size of prostate tumors between treatment and control groups, the two-sided Student’s t test for independent samples was used.

RESULTS

Metastatic Prostate Tumor Model. Following injection of PC-3/IF cell line in the prostate of male Balb/c nude mice, prostate tumors of 0.5 cm were observed by day 35 at autopsy. Prostate tumors were resected, minced, and processed for single-cell suspension preparation and then recultured in vitro for production of a new cell line designated PC-3/PI-1. Serial injections of the PC-3/PI cell lines in the prostate resulted in the
generation of PC-3/PI cell lines (Fig. 1). All mice that developed prostate tumors had enlarged para-aortic lymph nodes (0.2–0.5 cm), which showed the presence of live tumor cells by microscopic observation. PC-3/PI-LN tumor cell lines were produced from these lymph nodes (Fig. 1). In vitro, the PC-3/PI and PC-3/PI-LN tumor cell lines had comparable doubling time of 11–14 h. In vivo, these new cell lines were tumorigenic and induced prostate tumors of 0.4 cm in size by day 16 (at autopsy), as compared to 0.5-cm prostate tumors obtained by day 35 following PC-3/IF cell injection. Already by days 16 and 23, several para-aortic lymph nodes were enlarged and readily detectable along the spine. Invasion of tumor in seminal vesicles was observed, and occasionally, liver or mesentry metastases were found. No metastases were detected in the lungs by India ink staining following a procedure described previously (15). Mice bearing prostate tumors became moribund by day 40 and, at autopsy, showed large prostate tumors of 1–1.5 cm. The presence of tumor in prostate was confirmed histologically. As compared to normal prostate morphology (Fig. 2A), invasion of tumor cells surrounding remaining prostate glands was observed in the prostate following PC-3/PI cell injection (Fig. 2B). The tumor in the prostate presented as a poorly differentiated epithelial neoplasm consisting of pleomorphic tumor cells with large vesicular hyperchromatic nuclei and prominent nucleoli with high mitotic rate (Fig. 2C). The para-aortic lymph nodes showed invasion of large numbers of tumor cells (Fig. 2E) as compared to normal lymph node morphology (Fig. 2D). Inguinal lymph nodes were often enlarged in prostate tumor-bearing mice, but no tumor cells were detectable in single-cell suspensions of these lymph nodes, by microscopic observation, culture, or histology. Histological observation of the spine did not show the presence of tumor cells, ruling out metastasis to the spine.

Responsiveness of Prostate Tumors to IL-2 Therapy.

The prostate tumor model described above was used to assess the responsiveness of prostate tumors to systemic treatment with IL-2. In two separate experiments, mice were injected in the prostate with $5 \times 10^6$ cells from either the PC-3/PI-2-LN or PC-3/PI-3 cell line. The growth of these cell lines in vivo and in vitro was comparable. On day 6 post-cell injection, mice were treated with two or three weekly courses of 4–5 days of IL-2 at 25,000–50,000 CU/day, administered i.p. in 5% dextrose solution. Mice were sacrificed on day 30 or 34 and autopsyed. In experiment I, mice were treated for 4 days with IL-2 at 50,000 CU/day but showed signs of toxicity, including rashes and weight loss. After 2 days of rest, IL-2 treatment was continued, with a lower dose of 25,000 CU/day for 5 consecutive days, which was not toxic. This two-cycle IL-2 treatment induced a marked inhibition in tumor growth resulting in a 98% decrease in tumor size, as compared to untreated control mice (Table 1). On the basis of these findings, mice were treated in experiment II with IL-2 at 40,000 CU/day for 4 days, followed by 2 days of rest, and then with two additional weekly treatments of 30,000 CU/day for 5 consecutive days. These doses and schedule were well tolerated by the mice, with no apparent toxicity. Following this treatment, the size of prostate tumors was 90% significantly smaller than that of the control tumors ($P = 0.04$; Table 1). These data show that two to three cycles of nontoxic doses of IL-2 treatment markedly inhibited the growth of prostate tumors in seven of eight mice, resulting in a prostate size comparable to that of a normal mouse prostate (about $2 \times 2 \times 1$ mm). A preliminary experiment, in which only one course of 5 days of 50,000 CU/day of IL-2 treatment was given, showed less effectiveness.

Striking histological changes were observed in the prostate of IL-2-treated mice. In some mice, areas of necrosis were found in the prostate without detectable tumor cells. In other mice, remaining tumor areas were still observed in the prostate but were associated with areas of necrosis. Areas of necrosis and disintegrating tumor were heavily infiltrated by polymorphonuclear cells consisting mostly of neutrophils (Fig. 3A). In areas of necrosis and in the midst of remaining tumor cells, numerous apoptotic cells were identified by H&E staining (Fig. 3, A and B). Some tumor areas showed a massive influx of lymphocytes penetrating the tumor (Fig. 3C). Multifocal hemorrhages associated with inflammatory infiltrates were found in areas of disintegrating tumor (Fig. 3D). These findings, indicative of tumor destruction, were not observed in untreated tumors (Fig. 2, B and C). Interestingly, the regional para-aortic lymph nodes were of normal size in IL-2-treated mice, in contrast to enlarged lymph nodes observed in mice from the control group treated with 5% dextrose solution. Tumor cells were not detected by histology in para-aortic lymph nodes from IL-2-treated mice, with the exception of mouse 1 (experiment II; Table 1), which had a prostate tumor that was larger (252 mm³) than those of the other treated mice.

**DISCUSSION**

Recently, tumor models were developed for metastatic prostate carcinoma, using heterotransplantation of human prostate cancer cell lines into the prostate of immunodeficient athymic nude mice or severe combined immunodeficient mice, to
address basic questions in prostate cancer metastasis (12-14). Fidler et al. (12) showed that orthotopic implantation of PC-3M human prostate carcinoma cells in the prostate of nude mice resulted in tumor growth in the prostate and metastasis to regional paraaortic lymph nodes (12). They also demonstrated the feasibility of producing new variant cell lines with increased metastatic potential by culturing cells from prostate tumors and lymph nodes and reinjecting these new cell lines into the prostate (13). We have followed the same strategy and isolated tumor cell lines from the prostate tumors (PC-3/PI) and metastatic regional lymph nodes (PC-3/PI-LN), using an initially tumorigenic PC-3/IF cell line produced by passage of the original PC-3 cells in the femur of nude mice in vivo. Serial in vivo passages of these new cell lines in the prostate of nude mice led to the generation of cell lines that demonstrated increased kinetics of tumor growth in the prostate and metastasis to paraaortic lymph nodes. Prostate tumors of 0.4 cm and enlarged paraaortic lymph nodes were detectable by 16 days post-cell injection. Metastasis to regional paraaortic lymph nodes was demonstrated by isolation of tumor cell lines following culture of lymph node cell suspensions and by histology. This metastatic PC-3/PI prostate tumor model in athymic nude mice was comparable to the models described previously (12, 13) and was selected to study immunotherapy approaches as natural killer cell function is preserved in these mice (16). We found that PC-3 tumors localized in prostate were responsive to systemic IL-2 therapy. Compared to control mice, a significant average inhibition of 94% ($P = 0.03$) in the growth of prostate tumors was documented when two to three weekly cycles of 5-day nontoxic doses of 25,000–40,000 CU/day of IL-2 were administered. The majority of IL-2-treated mice showed no enlarged paraaortic lymph nodes and tumor cells were not detected histologically. These data suggest that IL-2 controlled the growth of prostate tumors and metastasis to regional lymph nodes. One cycle of 5 days of IL-2 was not sufficient to induce a measurable response, indicating that repeated cycles of IL-2 treatment were needed to induce a marked antitumor response. IL-2 efficacy was limited by toxicity of the treatment because higher doses of 50,000 CU/day were toxic and had to be discontinued. The antitumor response mediated by IL-2 on prostate tumors was confirmed histologically. Prostate sections from IL-2-treated mice showed vascular damage with multifocal hemorrhages and immune cell mobilization at the vicinity of the tumor. A massive influx of lymphocytes and polymorphonuclear cells, mostly neutrophils, was observed in disintegrating tumor areas and necrotic areas. These necrotic areas also contained many apoptotic cells indicative of tumor cell death. These findings suggest destruction of tumor cells by IL-2 therapy and are indicative of an antitumor response mediated by IL-2 in prostate tumors. Similar observations were made in our previous studies, in a model of renal carcinoma lung metastases, documenting that IL-2 produced vascular changes in the lung microenvironment and tumor nodules, which resulted in transient multifocal microscopic hemorrhages and mononuclear cell mobilization (17). IL-2-induced vascular damage is well documented and is responsible for the toxic effects of IL-2 therapy, including the vascular leak syndrome and the accumulation of extracellular fluids (18). IL-2 therapy was found to be associated with endothelial cell activation, leading to vascular leakiness of macromolecules (18). Similar to our findings in lung tumor nodules treated with IL-2 (17), apoptotic cells were also observed in IL-2-treated prostate tumors, which may result from
the effect of cytotoxic cytokines produced by IL-2-activated immune cells. Although T-cell function is impaired in nude mice, natural killer cells and macrophages are functional and may be responsible for the anti-tumor response mediated by IL-2 in the prostate tumors. Both cell types can be activated by IL-2 to mediate cytotoxic activity against tumor cells or produce cytokines including IFN-γ or TNF-α, which cause tumor cell apoptosis (19). Alternatively, as shown previously (20, 21), IL-2 may activate the prethymic T cells present in nude mice into functional T helper and cytotoxic T cells, which could lead to a mouse T-cell response against the human tumor cells. Previous studies have shown that intradermal administration of IL-2 gene-modified tumor vaccines can cause regression of Dunning rat prostate tumors implanted s.c. but not of tumors implanted in the prostate (8). Our studies demonstrate that systemic administration of IL-2 can mediate an antitumor activity against human prostate carcinoma tumors located in the prostate of nude mice. Our data indicate that IL-2 treatment was effective at controlling the growth of prostate tumors and metastasis to lymph nodes. Although the therapeutic effect of IL-2 is limited by dose-dependent IL-2 toxicity, our studies suggest that IL-2 may be an active agent in prostate cancer and should be considered for use in clinical trials.

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