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of Systemic Anti-Tumor Immunity

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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	12
References.....	N/A
Appendices.....	13

INTRODUCTION

Our clinical trials program at Baylor College of Medicine has established that *in situ* HSV-*tk*+GCV gene therapy is safe and some signs of efficacy and immune cell stimulation were observed. Although to our knowledge there are no clearly defined tumor antigens for prostate cancer, our preclinical studies using *in situ* gene therapy protocols have led us to believe that until such antigens become available, the manipulation of antigen presenting cells as well as cytokine levels through gene transfer will allow for more efficient bridging between a localized limited anti-tumor immune response and widespread effective systemic immunity. Based on our preliminary data we proposed testing specific gene-modified cell-based immunotherapy protocols and evaluating these protocols for their ability to generate a systemic anti-metastatic immune response in preclinical models for prostate cancer that rely on specific and unique mouse prostate cancer cell lines with high metastatic activity. Developing strategies for more efficient antigen presentation and T-cell response will also facilitate the application of specific tumor antigen-based vaccines, once they become available. We have shown that IL-12+B7 transduced antigen-presenting cells (APC) can effectively induce an anti-tumor immunity; that IL-12+B7 modified prostate cancer cell vaccine strategies can generate a systemic anti-tumor immune response; and, that IL-12+B7 gene delivery has specific therapeutic effects against bone metastases. We proposed to test the efficacy of specific therapeutic approaches incorporating novel biological concepts of IL-12 gene modified cell-based therapy using preclinical models of metastatic prostate cancer. This report documents the progress we have made in the past one year (Jan 2002- Dec 2002). We have been testing *in situ* IL-12+B7 gene-modified antigen presenting cell (APC) protocols for anti-metastatic activities and testing the anti-metastatic activities of IL-12+B7 gene-modified APC therapy combined with IL-12+B7 gene-modified tumor or dendritic cell vaccines. Additional studies have begun to evaluate IL-12 gene-transduced bone marrow stem cell based therapy for its capacity to suppress bone metastases.

BODY

We have made substantial progress over the past year with regard to several aims proposed in the grant as delineated in the Statement of Work. We will discuss each of the three aims separately a below:

- Task 1.** To test *in situ* IL-12+B7 gene-modified antigen presenting cell (APC) protocols for anti-metastatic activities and to determine the specific effector cells involved in the response.
- a. Treat 178-2BMA orthotopic tumors with control Ad β -gal or AdmIL12/B7 infected macrophages, dendritic cells or a combination of both. Analyze therapeutic effect at fixed time. (80 host mice 20 donor mice) [Months 0-3]
 - b. Treat 178-2BMA orthotopic tumors with control Ad β -gal or AdmIL12/B7 infected macrophages, dendritic cells or a combination of both at the same time that they are injected with adenoviral vectors. Analyze therapeutic effect at fixed time. (80 host mice 20 donor mice) [Months 3-6]
 - c. Treat 178-2BMA orthotopic tumors with control Ad β -gal or AdmIL12/B7 infected macrophages, dendritic cells or a combination of both. Analyze therapeutic effect long term (time of survival). (120 host mice 30 donor mice) [Months 6-12]
 - d. Treat 178-2BMA orthotopic tumors with control Ad β -gal or AdmIL12/B7 infected macrophages, dendritic cells or a combination of both at the same time that they are injected

with adenoviral vectors. Analyze therapeutic effect long term (time of survival). (120 host mice 30 donor mice) [Months 12-18]

- e. Perform NK and CTL assays on splenocytes from selected subsets of treated animals from above tasks [Months 0-18]
- f. Determine the immune cell mediated mechanisms involved in the therapeutic activities observed in task 1a to 1d by repeating experiments in mice depleted for specific immune cell populations. (360 host mice 90 donor mice) [Months 12-36]

We have completed many of the experiments relevant to Task 1 sections a, c, and e. We have completed an extensive analysis of the therapeutic effect of macrophage transduced with AdmIL-12 in the 178-2 BMA orthotopic prostate cancer model system and a manuscript regarding these results is being drafted. We have used a vector with only the IL-12 genes for these studies because we have an upcoming clinical trial with the human IL-12 genes and because macrophage tested in this protocol appeared to have adequate expression of the B7 molecule (see Table I). An abstract describing the results was presented at the 2002 American Urological Association Annual meeting (see Appendix). These studies have demonstrated that *in vitro* AdmIL-12 gene-modified macrophages secreted IL-12 and had increased surface expression of MHC class I and II as well as F4/80 antigen compared controls of uninfected or Ad β -gal infected macrophages (Table I).

Table I *In vitro* characterization of transduced macrophage (moi 100)

Macrophage	IL-12 (pg/ml) 24 h / 48 h	% Positive / Mean Fluorescence Intensity				
		Control IgG	MHC Class I	MHC Class II	F4/80	CD80 (B7-1)
Uninfected	<4 / <4	2.8 / 3.3	12.4 / 8.5	8.3 / 23.1	69.1 / 6.4	19.3 / 3.9
Ad β Gal	<4 / <4	4.9 / 3.2	13.3 / 11.3	5.9 / 51.2	68.5 / 6.2	30.0 / 3.7
AdmIL-12	4100 / 12000	4.3 / 3.3	92.9 / 21.1	49.7 / 47.3	79.9 / 12.4	29.9 / 4.5

In vivo AdmIL-12 gene modified macrophages injected into orthotopic tumors established with 178-2BMA cells induced significant suppression of primary tumor growth and spontaneous lung metastases compared with controls. Mice with orthotopic tumors treated with AdmIL-12 gene-modified macrophages also survived significantly longer than controls. (Figure 1).

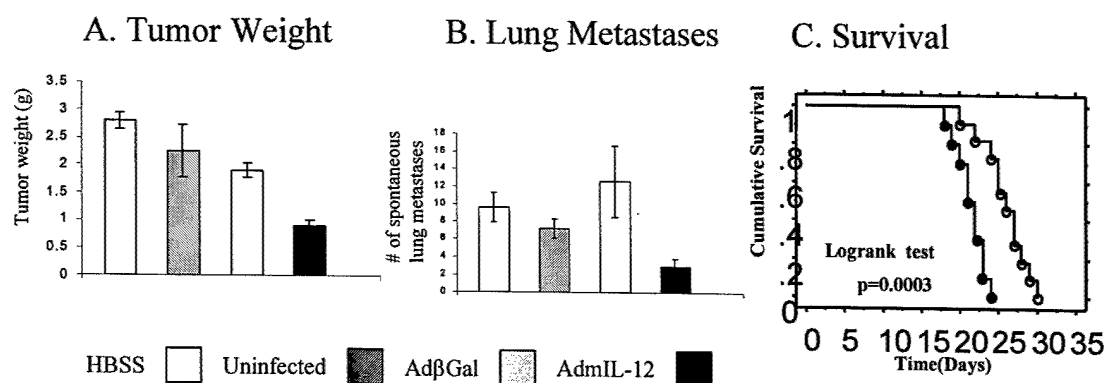


Figure 1. Macrophage injected in situ in orthotopic prostate cancer model. A. Suppression of tumor weight by AdmIL-12 transduced macrophage ($p < 0.0001$, vs HBSS). B. Suppression of spontaneous lung metastases ($p = 0.0032$ vs HBSS). C. Improved survival of animals treated with macrophages transduced with AdmIL-12 compared to Ad β Gal.

Preliminary analysis of tumors indicated significantly increased infiltration of CD8⁺ T cells in those injected with AdmIL-12 gene-modified macrophages compared to controls. In a trial experiment

we measured splenocyte-derived cytotoxic natural killer (NK) cell activity and was tumor specific cytotoxic T lymphocytes (CTL). NK activity was enhanced on day two after AdmIL-12 gene-modified macrophage injection and on day fourteen tumor specific T lymphocytes were increased compared to Ad β gal infected macrophages (Figure 2). These experiments will need to be repeated to verify the results.

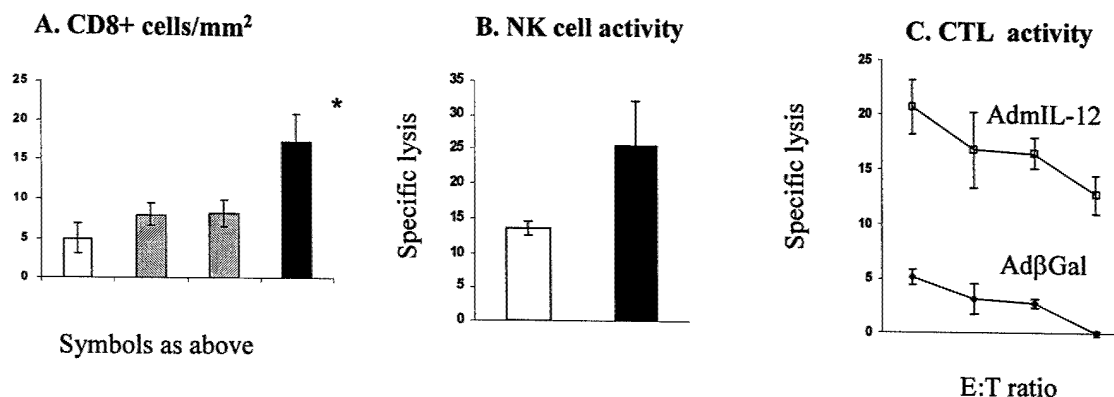


Figure 2. AdmIL-12 transduced macrophage induce immune response. **A.** Significantly increased infiltration of CD8+ T cells in orthotopic tumors ($p=0.0469$ vs HBSS). **B.** Increased NK cell lytic activity in splenocytes from animals treated two days previously with orthotopic AdmIL-12 transduced macrophages ($P=0.1360$, unpaired t test at E:T of 100:1). **C.** Increased tumor specific cell lytic activity in splenocytes from animals treated 14 days previously with orthotopic AdmIL-12 transduced macrophages ($P=0.0037$, unpaired t test at E:T of 100:1).

Trafficking studies suggest that intratumoral injected AdmIL-12 gene-modified macrophages could migrate to draining lymph nodes and the lung. We are in the process of analyzing these data and should complete these studies and have a manuscript ready for submission within the next year.

We have also made considerable progress on studies (also for tasks 1 a, c, and e) that have analyzed the effects of *in situ* delivery of dendritic cells (DC). Bone marrow derived DC were genetically engineered to express high levels of IL-12 with or without the costimulatory molecule B7-1, by *ex vivo* infection with recombinant adenoviral vectors (AdmIL-12 or AdmIL-12/B7). We used the 178-2 BMA orthotopic metastatic mouse prostate cancer model to test the effects of this immunotherapy protocol. DC were generated from bone marrow of syngeneic 129/Sv mice by culturing in the presence of GM-CSF and IL-4 then transduced (at 3000 moi) with replication-defective adenoviral vectors (CMV promoter) that transduced the control beta-galactosidase gene (DC/ β -gal), IL-12 (DC/IL-12) or IL-12/B7 (DC/IL-12/B7). In vitro characterization confirmed that this high moi was necessary for IL-12 production and upregulation of surface markers (table II).

Table II *In vitro* characterization of transduced dendritic cells (DC) (moi 3000)

DC	IL-12 (pg / 5 x10 ⁵ cells) 48 h	Mean Fluorescence Intensity			
		CD 11c	MHC Class II	CD80 (B7-1)	CD86 (B7-2)
Uninfected	0	32.4	10.0	26.0	64.7
DC/ β Gal	143	18.5	9.4	23.2	67.8
DC/IL-12	1032	17.8	11.1	28.8	70.7
DC/IL-12/B7	nd	18.0	31.6	37.4	100.1

DC/IL-12 produced high levels of biologically active IL-12, and was enhanced IL-12 production by co-existing cancer cell. FACS analysis showed that DC/IL-12/B7 expressed increased level of MHC II and was enhanced B7-1 expression.

Seven days after establishment of intraprostatic tumors, 1×10^6 of these transduced DCs were injected directly into the orthotopic tumors. Intratumoral injection of DC/IL-12 or DC/IL-12/B7 induced a significant suppression of primary tumor growth compared to that of DC/ β -gal ($p=0.0155$ and 0.0007 , respectively). Intratumoral injection of DC/IL-12 or DC/IL-12/B7 also showed a trend of reduction the number of spontaneous lung metastatic nodules in comparison to of DC/ β -gal ($p=0.0818$ and 0.0502 , respectively) (Figure 3).

In survival experiments, DC/IL-12 injection demonstrated a significant advantage ($p=0.001$), while DC/IL-12/B7 treatment did yield the longest survivor (Figure 3C).

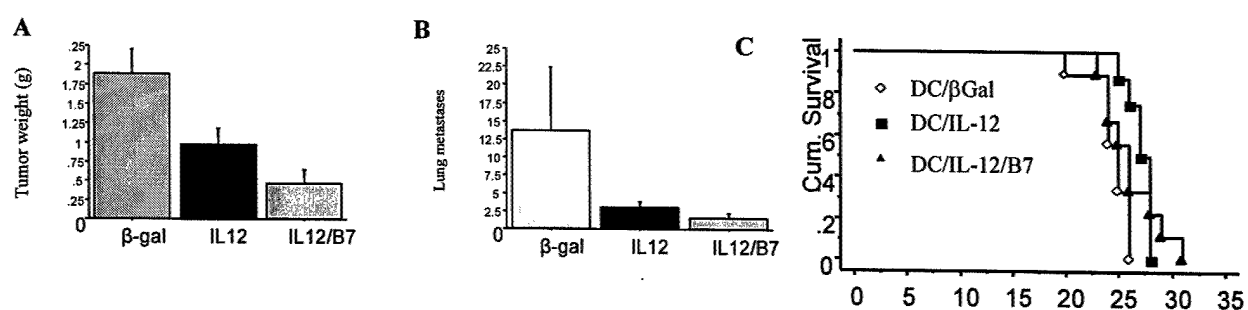


Figure 3. *In vivo* antitumor and antimetastatic effect of DC injected in situ in 178-2 BMA orthotopic model. **A.** Tumor weight 21 days after establishment of tumors, 14 days after DC injection. **B.** Spontaneous lung metastases. **C.** Increased survival of animals with DC/IL-12 ($p=0.001$ vs DC/ β Gal). Although the survival of the DC/IL12/B7 treated animals did not achieve statistical significance some of these animals lived longest.

We have preliminary evidence, that needs to be confirmed, suggesting that both DC/IL-12/B7 and DC/IL-12 treatments enhanced the systemic immune response using Natural Killer (NK) cell and Cytotoxic T Lymphocyte (CTL) activity assays (see Figure 4).

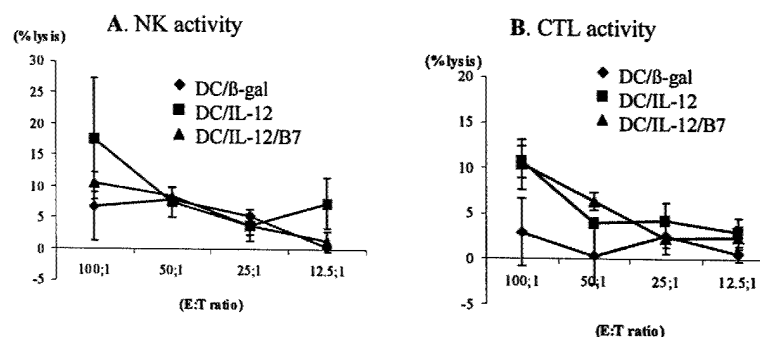


Figure 4. Immune activities after *in situ* DC. **A.** NK activity on day 3 after injection. **B.** CTL activity on day 7.

In these pre-clinical models, gene-modified macrophage and DC based intratumoral immunotherapy appears to be a good candidate for *in situ* gene-modified cell therapy in locally advanced prostate cancer based on tumor growth suppression, inhibition of metastasis and survival improvement.

The combination of gene-modified DC together with macrophage has not been undertaken at this time. The simultaneous treatment of orthotopic tumors with adenoviral vectors and gene modified APC's (task 1b&d) has not been pursued at this time. We have not yet obtained the mice necessary to undertake section f.

Task 2. To test the anti-metastatic activities of IL-12+B7 gene-modified APC therapy combined with IL-12+B7 gene-modified tumor or dendritic cell vaccines and determine the specific effector cells involved in the response.

- a. Treat animals with gene-modified tumor vaccine and 178-2BMA orthotopic tumors with optimal *in situ* APC treatment protocol determined in task 1 (AdmIL12/B7 infected macrophages, dendritic cells or a combination of both without or with concomitant *in situ* adenoviral vector injection). Analyze therapeutic effect at fixed time. (60 host mice 15 donor mice) [Months 24-30]
- b. Treat animals with tumor lysate pulsed IL-12+B7 gene-modified dendritic cell vaccine and 178-2BMA orthotopic tumors with optimal *in situ* APC treatment protocol determined in task 1 (AdmIL12/B7 infected macrophages, dendritic cells or a combination of both without or with concomitant *in situ* adenoviral vector injection). Analyze therapeutic effect at fixed time. (60 host mice 15 donor mice) [Months 24-30]
- c. Treat animals with IL-12+B7 gene-modified tumor vaccine and 178-2BMA orthotopic tumors with optimal *in situ* APC treatment protocol determined in task 1 (AdmIL12/B7 infected macrophages, dendritic cells or a combination of both without or with concomitant *in situ* adenoviral vector injection). Analyze therapeutic effect in survival experiment. (90 host mice 15 donor mice) [Months 30-36]
- d. Treat animals with tumor lysate pulsed IL-12+B7 gene-modified dendritic cell vaccine and 178-2BMA orthotopic tumors with optimal *in situ* APC treatment protocol determined in task 1 (AdmIL12/B7 infected macrophages, dendritic cells or a combination of both without or with concomitant *in situ* adenoviral vector injection). Analyze therapeutic effect at fixed time. (90 host mice 15 donor mice) [Months 30-36]
- e. Analyze systemic immune activities generated in aim 2a to d by NK and CTL assays [Months 24-36]
- f. Challenge long term survivors from aim 1c, 1d, 2c, and 2d with subcutaneous tumor cells. Establish T cell lines from protected animals. [Months 18-36]
- g. Deplete specific immune cell populations (as in task 1f) in selected long term survivors [Months 24-36]

We have already undertaken several experiments relevant to this specific task. We have established procedures for the vaccination protocol with dendritic cells in conjunction with the orthotopic 178-2 BMA prostate cancer cell model. Stimulation of an anti-tumor immune response by dendritic cells is critically dependent on their tightly regulated ability to produce IL-12. In this study, bone marrow derived DC were genetically engineered to produce high levels of IL-12 by *ex vivo* infection with a recombinant adenoviral vector (AdmIL-12). We used the mouse orthotopic model of prostate cancer (178-2 BMA) to test the effect of immunotherapy using these genetically modified DC. This cell line reproducibly metastasizes with high frequency to bone, lymph nodes and lung following orthotopic inoculation.

DC were infected with replication-defective adenoviral vectors (CMV promoter) that transduced the beta-galactosidase gene (DC/ β -gal) or IL-12 (DC/IL12) at 3000 MOI, and were pulsed with cell extracts from 178-2 BMA cells. DC/IL-12 produced high levels of biologically active IL-12 and DC/IL-12 pulsed with tumor extract showed significant higher secretion of IL-12 than unpulsed DC/IL-12. FACS analysis showed that DC/IL-12 expressed increased levels of MHC-I and II. (Table III).

Table III *In vitro* characterization of transduced dendritic cells (DC) (moi 3000)

DC	Pulsed with 178-2 BMA cell lysate	IL-12 (pg / 5×10^5 cells) 48 h	Mean Fluorescence Intensity				
			CD 11c	MHC Class I	MHC Class II	CD80 (B7-1)	CD86 (B7-2)
Uninfected	NO	nd	13.6	9.6	51.8	12.0	26.0
Uninfected	YES	nd	13.9	11.7	49.5	12.7	23.2
DC/ β Gal	YES	404	11.6	17.8	58.9	17.7	28.8
DC/IL-12	YES	3268	13.2	22.2	67.0	28.0	37.4

At 3 days post orthotopic tumor inoculation, 1×10^6 cells of DC/ β -gal DC/IL-12 or HBSS were injected subcutaneously (sc) or intravenously (iv). Pulsed DC/IL-12 induced a significant suppression of primary tumor growth compared to control HBSS ($p=0.0168$) when given sc, whereas pulsed DC/IL-12 delivered iv had no effect on tumor growth (Figure 5A). We therefore used sc delivery to further characterize anti-metastatic effectiveness. Tumor lysate pulsed DC/IL-12 cells delivered sc significantly increased the number of animals free of spontaneous lung metastasis in comparison to HBSS, non-infected DC pulsed with tumor lysate or DC/ β -gal pulsed with tumor lysate (Figure 5B) ($p=0.0149$, 0.0078 and 0.0498 , respectively). In survival experiments, tumor lysate pulsed DC /IL-12 significantly improved survival compared to HBSS ($p=0.0433$). (Figure 5C)

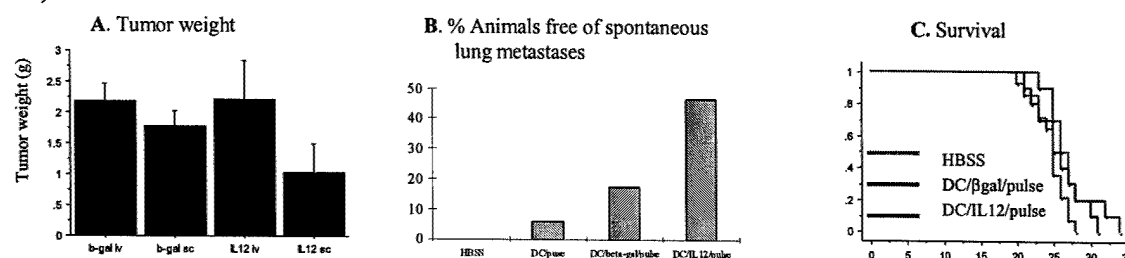


Figure 5. A. Tumor weight after iv or sc injection of 178-2 BMA cell lysate pulsed DC/ β Gal or DC/IL-12 cells. B. The percent of animals free of spontaneous lung metastases after sc delivery of HBSS, pulsed DC, pulsed DC/ β Gal or pulsed DC/IL12. C. Survival of animals that received sc HBSS, pulsed DC/ β Gal or pulsed DC/IL12.

We intend to evaluate the systemic immune response from similar DC experiments with splenocytes harvested from mice with orthotopic tumors treated with sc injection of tumor lysate pulsed DC/IL-12 versus controls. Lytic activity against YAC cells (NK activity) on day 4 after treatment will be compared with control splenocytes from mice with orthotopic tumors treated with sc injection of tumor lysate pulsed DC/ β gal. Lytic activity against 178-2 BMA cancer cells (CTL activity) on day 11 after treatment will be compared with control splenocytes from mice with orthotopic tumors treated with subcutaneous injection of pulsed DC/ β gal.

In this pre-clinical model, IL-12 gene-modified DC based immunotherapy appears to be a good candidate for adjuvant therapy post radical prostatectomy based on its effects on tumor growth suppression, inhibition of metastasis and improvement of survival.

Task 3. To test IL-12 gene-transduced bone marrow stem cell based therapy for its capacity to suppress bone metastases and to determine the cellular and molecular mechanisms involved in the response.

- a. Isolate bone marrow stem cells and transduce with retroviral vectors DFG-mIL12 or DFG-eGFP. Characterize IL-12 and GFP expression and *in vitro* growth. (20 donor mice) [Months 0-6]
- b. Determine efficiency of bone marrow stem cell repopulation of normal mice or mice previously treated with total body irradiation. (20 donor mice, 20 host mice) [Month 6-12]
- c. Evaluate metastatic potential of orthotopic 178-2 BMA tumors in mice with reconstituted bone marrow stem cells. (20 host mice) [Months 12-18]
- d. Evaluate anti-metastatic ability of gene-modified bone marrow stem cells administered concomitantly with orthotopic tumor formation through molecular and biochemical analyses. (20 donor mice 40 host mice) [Months 18-24]

We have made significant progress on this task and have recently submitted an abstract to the 2003 American Society of Gene Therapy Annual Meeting to describe these studies (see Appendix). We have determined that procedures for isolation and titering of the necessary retrovirus for these studies. We have had to adopt a modified protocol to purify sufficient quantities of retrovirus for the proposed *in vivo* studies. We have been able to obtain high titer ($> 10^6$ units/ml) of both vectors based on titrations using NIH3T3 cells and flow cytometric analysis of GFP or intracellular IL-12 positive cells. We have also determined that bone marrow stem cells can be isolated and infected with these retroviral stocks. We have had to modify the original protocol for bone marrow engraftment of recipient animals somewhat in that the host mice have not been irradiated to deplete their bone marrow. This has been necessitated because of logistic problems that will hopefully be resolved in the coming year. Despite this we have used as bone marrow stem cell donors Rosa mice that have constitutive β -gal expression to document that engraftment of normal mice can occur at a reasonable level.

In an preliminary experiment to address task 3d we established orthotopic tumors with 178-2 BMA cells and three days later injected the animals with retrovirus (DFG-eGFP or DFG-IL12) modified bone marrow stem cells (BM/GFP or BM/IL12). A summary of these results is presented in Table IV

Table IV

Treatment	Tumor weight (g)	Lung Mets	Spleen weight
HBSS	2.25 +/- 0.115	8.7 +/- 1.8	0.187
BM/ β Gal	2.215 +/- 0.32	5.6 +/- 2.0	0.214
BM/IL-12	1.944 +/- 0.13 (p=.28)	7.1 +/- 3.9 (p=0.7)	0.196

As is evidenced by these results the treatment effects were minimal. Therefore we evaluated a different model system to test the anti-metastatic effectiveness of modified bone marrow stem cells. We have adopted a protocol that enhances the metastatic activity detection compared to the orthotopic protocol as proposed and described above for tasks 1 and 2. In this modified protocol 5000 viable 178-2 BMA cells are injected via the tail vein three days prior to bone marrow stem cell inoculation. This model develops both lung and bone metastases that can be scored 21 days after injection. The animals were sacrificed at various time points and sera and specific tissues were collected and analyzed. Sera obtained from DFG-mIL-12 bone marrow treated mice showed a gradual increase in IL-12 (p40, ELISA) that reached a peak (0.89 ng/ml) at day 9. In contrast, IL-12 levels in the serum of DFG-eGFP bone marrow treated mice were significantly lower (0.11 ng/ml) throughout the time course. Flow cytometric analysis indicated that 21 days following the bone marrow cell injection, approximately 15% of peripheral blood cells stained lac-Z positive. Mice treated with DFG-mIL-12 transduced bone marrow cells had significantly fewer metastatic lung

colonies (mean=39) compared to mice treated with DFG-eGFP transduced bone marrow cells (mean=76, $P=0.0127$) or with HBSS (mean=67, $P=0.0155$). Histochemical analyses showed that 80% of the mice in the DFG-eGFP bone marrow treated group and 83% of the mice in the HBSS treated group had bone metastases. HBSS treated mice showed large metastatic tumor deposits that extended into the connective tissues surrounding the bone. In marked contrast, only 17% of the mice had bone metastases in the group treated with DFG-mIL-12 transduced bone marrow cells. (Figure 6)

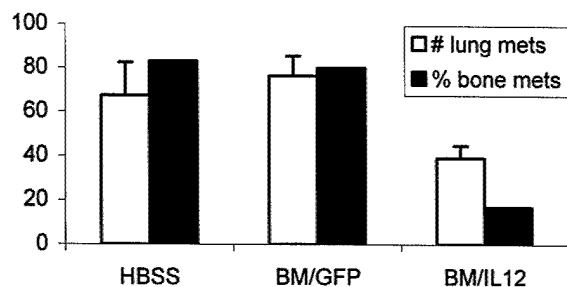


Figure 6. 129/Sv mice were injected with 5000 178-2 BMA cells via tail vein and three days later with 10^6 modified bone marrow BM cells in 100 μ l of HBSS or HBSS alone. The retrovirus DFG-eGFP or DFG-IL12 modified bone marrow stem cells were derived from 129/Sv Rosa mice. The number of lung metastases was determined 17 days later after staining with Bouin's solution. The % of animals with bone metastases was determined from H&E stained sections.

Therefore, systemically delivered bone marrow cells genetically engineered to produce IL-12 are effective against pre-established metastases in this model system of prostate cancer metastases.

We have therefore made significant progress with the preliminary experiments for tasks 3a-c. We are extremely encouraged by these results and will focus our efforts in the coming budget year to confirm these preliminary studies, refine the model system to make it potentially more clinically relevant (i.e., using irradiated hosts) and establish the procedures suitable for further documentation of the molecular and biochemical mechanisms for task 3d. We anticipate repeating these experiments with irradiated host mice in the coming year as this becomes logistically possible.

KEY RESEARCH ACCOMPLISHMENTS

1. Macrophage modified to express IL-12 can suppress tumor metastasis when injected into orthotopic tumors
2. Dendritic cells modified to express IL-12 or IL12/B7 can suppress tumor metastasis when injected into orthotopic tumors
3. Dendritic cells modified to express IL-12 can suppress tumor metastasis when injected subcutaneously in animals with orthotopic tumors
4. Bone marrow stem cells modified to express IL-12 can suppress metastasis when injected intravenously in animals with pre-established metastases.

REPORTABLE OUTCOMES

[each abstract attached in appendix]

Sub-cutaneous injection of bone marrow derived dendritic cells engineered to produce interleukin-12 induces anti-tumor activities in an orthotopic mouse prostate cancer model. Takashi Saika, Nobuyuki Kusaka, Takefumi Satoh, Vladimir B. Mouraviev, Guang Yang, Terry L. Timme and Timothy C. Thompson. Abstract (#214) presented at American Urological Association Annual Meeting, 2002.

Macrophages transduced with adenoviral vectors to express IL-12 suppress tumor growth and metastasis in pre-clinical models of prostate cancer. Takefumi Satoh, Takashi Saika, Shin Ebara, Nobuyuki Kusaka, Guang Yang, Jianxiang Wang, Vladimir B. Mouraviev, Guangwen Cao, Terry L. Timme and Timothy C. Thompson. Abstract (#199) presented at American Urological Association Annual Meeting, 2002.

Antimetastatic effects of IL-12 gene-modified bone marrow cells in a mouse model of metastatic prostate cancer. Hongyu Wang, Guang Yang, Takefumi Satoh, Nobuyuki Kusaka, Xiarong Ji, Terry L. Timme, Tetsuo Fujita, Taoyan Men, and Timothy C. Thompson. Abstract submitted to American Society of Gene Therapy Annual meeting, 2003.

CONCLUSIONS

The lack of effective therapy for locally invasive and metastatic prostate cancer dictates the necessity for intensive focus on the development of novel and effective therapeutic approaches for this important malignancy. Prostate cancer seems particularly well suited for direct *in situ* gene therapy strategies. Clinical trials have demonstrated the safety of *in situ* gene therapy and it appears likely that *in situ* suicide gene therapy such as HSV-*tk* + GCV will prove effective in the cytoablation of localized prostate cancer in specific subsets of patients. This gene therapy approach will also be useful in establishing a foundation upon which to build more specific and comprehensive *in situ* gene therapy approaches using multiple genes that will induce and sustain anti-tumor immunity based on optimized antigen presentation, the generation of a Th1 response, and the specific activation of CTL activities. Since there are currently no well-defined tumor antigens for prostate cancer, it is difficult to devise specific vaccine type gene-based strategies for the treatment of this disease. However, our preclinical studies using *in situ* gene therapy protocols have led us to believe that until such antigens become available, the manipulation of antigen presenting cells as well as cytokine levels (e.g., IL-12) through gene transfer will allow for more efficient bridging between a localized limited anti-tumor immune response and widespread effective systemic immunity. Developing strategies for more efficient antigen presentation and T-cell response will also facilitate the application of specific tumor antigen-based vaccines, once they become available. Thus far we have demonstrated that IL-12+B7 gene-modified macrophages and dendritic cells and their placement within primary tumors can lead to antitumor cytotoxic effects. We have shown that subcutaneous IL-12+B7 gene-modified tumor cell vaccines can lead to a systemic anti-tumor immune responses against prostate cancer. Finally, we have shown that optimization of systemic IL-12 levels through gene vector injections can enhance specific immune responses that suppresses bone metastasis. The further development of these novel findings in regard to bridging a localized and systemic anti-tumor immune response will likely lead to rapid progress in clinical gene therapy treatment, as our institution has ongoing clinical protocols into which specific modifications can be incorporated. Finally, our studies will also generate important reagents with which to identify true tumor antigens and thus hopefully make even more progress toward developing gene-based immunomodulatory therapeutic approaches for the treatment of metastatic prostate cancer.

In controls, the percentage of cells in the G1 phase increased from 76.4 to 88.8 and the percentage of cells in the S phase decreased from 8.9 to 3.7 after IL-6 treatment. In concordance with proliferation results, IL-6 did not cause a change in cell cycle distribution in the LNCaP-IL-6+ subline. IL-6 receptor expression gradually decreased during long-term IL-6 treatment. IL-6 caused a ligand-independent activation of the AR in LNCaP-IL-6+ cells and the levels reached 30% of those induced by androgen. In contrast to control LNCaP cells, LNCaP-IL-6+ cells express IL-6 mRNA. In their supernatants, mean IL-6 concentration of 358.7 pg/ml was measured. Interestingly, IL-6 secretion was not influenced by IL-1.

CONCLUSIONS: Long-term treatment of prostate cancer cells with IL-6 generates a cell line which confers growth advantage in vitro. These cells are considered partially resistant to IL-6. Although they are not growth inhibited, AR activation by IL-6 is preserved. LNCaP-IL-6+ cells acquire an ability to constitutively secrete IL-6 which could influence prostate cancer progression, most probably by regulation of immune response or angiogenesis.

213

THE ROLE OF NUCLEAR MATRIX HIGH MOBILITY GROUP PROTEIN HMGI(Y) IN CHROMOSOMAL REARRANGEMENTS IN HUMAN PROSTATE CANCER CELL LINES

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INTRODUCTION AND OBJECTIVES: Chromosomal instability is a characteristic of the lethal phenotype in human prostate cancer metastatic lesions (Cancer Res. 56:3091-3102, 1996). With proteomic analysis, we have identified changes in specific nuclear matrix proteins that are present in human prostate cancer cell lines. Here we focus on the nuclear matrix protein HMGI(Y) in the human prostate cancer cell lines (LNCaP, DU145, PC-3) that may be correlated with chromosomal instability. Increased expression of HMGI(Y) has been reported to be correlated with higher Gleason grades (Cancer Res. 53:5512-5516, 1993) and with increased metastatic potentials of rat prostate cancer cell lines.

METHODS: Human prostate cancer cell lines PC-3 and LNCaP were analyzed by Spectral Karyotyping™ (SKY) to assess DNA rearrangement and chromosomal instability. The expression of HMGI(Y) was examined by RT-PCR, immunoblot and immunohistochemistry. Mouse HMG-I was transfected into LNCaP and its expression was confirmed by immunoblot and immunohistochemistry. SKY analysis was also performed on the transfected clones after 4 to 8 months of continuous passage.

RESULTS: SKY analysis by ourselves and others shows that the ratio of the structural aberrations per chromosome in LNCaP, DU145 and PC-3 is approximately 1:2:4 respectively. Likewise, the immunoblot shows that HMGI(Y) is localized to the nuclear matrix and a similar ratio of the expression of HMGI(Y) protein is observed of 1:2:4 respectively. In addition, immunohistochemistry shows nuclear staining by anti-HMGI(Y) antibody weakest in LNCaP, medium in DU145 and strongest in PC-3. The number of additional non-clonal, unbalanced chromosomal rearrangements in LNCaP cells transfected with HMG-I assessed by SKY analysis was 12/20 cells compared to 2/20 mock transfectants and 0/20 parental cells. This increased chromosomal instability as a direct result of the transfection of HMG-I strengthens its association with DNA rearrangements. This mechanism is also supported by the reported association of HMGI(Y) with homologous recombination complexes, such as Holiday junctions, as well as A-T rich sequences such as nuclear matrix DNA attachment regions (MARs/SARs).

CONCLUSIONS: This study suggests that HMGI(Y) might play an important role in the DNA rearrangement which is common feature of the lethal phenotype of prostate cancer.

214

SUB-CUTANEOUS INJECTION OF BONE MARROW DERIVED DENDRITIC CELLS ENGINEERED TO PRODUCE INTERLEUKIN-12 INDUCES ANTI-TUMOR ACTIVITIES IN AN ORTHOTOPIC MOUSE PROSTATE CANCER MODEL

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INTRODUCTION AND OBJECTIVES: Dendritic cells (DC) mediated immunotherapy has potential benefits as adjuvant therapy following radical prostatectomy for prostate cancer. Stimulation of an anti-tumor immune response by dendritic cells is critically dependent on their tightly regulated ability to produce interleukin-12 (IL-12). In this study, bone marrow derived DC were genetically engineered to produce high levels of IL-12 by *ex vivo* infection with a recombinant adenoviral vector (AdCMVIL-12). We used a mouse orthotopic model of prostate cancer (178-2BMA) to test the effect of immunotherapy using these genetically modified DC.

METHODS: DC were generated from bone marrow of 129/Sv mice by culturing in the presence of GM-CSF and IL-4. After 7 days culture, DC were infected with replication-defective adenoviral vectors (CMV promoter) that

transduced the beta-galactosidase gene (DC/β-gal) or IL-12 (DC/IL12) at 3000 MOI. At day 8, DC were pulsed with the cell extracts from 178-2BMA cells, which were derived from a bone metastasis that developed *in vivo* from a *ras+myc-*initiated, p53 null primary mouse prostate cancer using the mouse prostate reconstitution model system. This cell line reproducibly metastasizes with high frequency to bone, lymph nodes and lung following orthotopic inoculation. At 3 days post orthotopic tumor inoculation, 1x10⁶ cells of DC/β-gal DC/IL-12 or HBSS were injected subcutaneously (s.c.) or intravenously (i.v.).

RESULTS: DC/IL-12 produced high levels of IL-12 and DC/IL-12 pulsed with tumor extract showed significant higher secretion of IL-12 than unpulsed DC/IL-12. FACS analysis showed that DC/IL-12 expressed increased levels of MHC-I and II. DC/IL-12 with pulsing s.c. induced a significant suppression of primary tumor growth compared to control HBSS (p=0.0168), whereas DC/IL-12 with pulsing i.v. showed no effect in tumor growth. DC/IL-12 with pulsing s.c. significantly reduced the incidence of spontaneous lung metastasis in comparison to HBSS s.c., non-infected DC with pulsing s.c. or DC/β-gal with pulsing s.c. (p=0.0149, 0.0078 and 0.0498, respectively). In survival experiments, DC/IL-12 s.c. significantly improved survival compared to HBSS s.c. (p=0.0433).

CONCLUSIONS: In a pre-clinical model, IL-12 gene-modified DC based immunotherapy was shown to be a good candidate for adjuvant therapy post radical prostatectomy based on its effects on tumor growth suppression, inhibition of metastasis and improvement of survival.

215

ANDROGEN-INDEPENDENT GROWTH OF HUMAN PROSTATE CANCER CELLS IS MEDIATED BY MUTANT P53

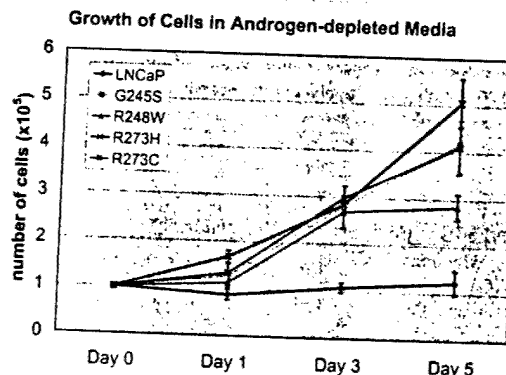
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INTRODUCTION AND OBJECTIVES: To date, the question why does metastatic prostate cancer (CaP) become androgen-independent (AI) has remained unanswered. p53 mutations occur at a rate of 70% in hormone-refractory CaP, suggesting that these mutations may be involved in the progression of CaP to AI growth. However, no direct evidence exists linking p53 mutations to AI growth. We have used the androgen-dependent LNCaP cell line, and its stable sub-lines transfected with p53 mutant alleles to study this phenomenon.

METHODS: LNCaP cells were stably transfected with 4 dominant-negative mutant p53 alleles. All cell lines studied were grown in androgen-depleted media for 5 days. Viable cells were counted at 1, 3 and 5 days. Western blotting was used to examine protein levels of AR, Bag1 and phosphorylated Akt in untreated and 5-day androgen-depleted cell lysates, while ELISA was used to measure PSA levels.

RESULTS: Parental LNCaP cells did not grow under androgen-depleted conditions. However, the p53-transfected stable sub-lines were able to grow (see figure). Upon androgen-depletion, the levels of AR decreased in the mutant p53 sub-lines compared to the parental cells, which was accompanied by attenuated receptor activity, since decreased PSA levels compared to parental LNCaP cells was also observed. Bag1L levels increased under androgen-depleted conditions in 3 of the mutant p53 sub-lines as compared to the parental line. In contrast, phosphorylated Akt levels increased in both the parental and mutant p53-transfected sub-lines.

CONCLUSIONS: Mutant p53 mediates AI growth of CaP cells, potentially through a mechanism involving Bag1 but not Akt.



METHODS: The scheme for detecting hAR mutations is as follows: the hAR fragment such as DBD/LBD region is amplified and co-transferred into ADE2-containing yeast with a gapped hAR expressing plasmid lacking this region. The yeast will form red, pink or white colonies dependent on the hAR mutation and the ligand in the medium. White colonies in DHT medium and red in medium with progesterone (P) or other non-androgen ligands indicate wt hAR or mt hAR with wt function; white in both DHT and P medium indicate GOF mt hAR; red in DHT is LOF mt hAR. We generated 44 of the 55 published CaP-derived mutant (mt) ARs that have been published.

RESULTS: Table 1, below, summarizes the transactivational profiles for these mutants along with physiological levels of five steroids and the expected serum level of flutamide. The assay allows all 44 mutations to be analyzed in a single plate, which greatly helps with issues of interassay variation. Seven (16%) had loss of function, three (7%) had wild type function, and 14 (32%) had partial function and 20 (45%) had gain of function. The results absolutely mimic those found in mammalian cells. However, due to the difficulties using mammalian cells to date in the world only 6 mutants have been so analyzed.

CONCLUSIONS: We have developed a rapid method of determining functional activity of all hAR missense mutations with different ligands. This is the first step in developing new individualized hormonal interventions for patients with metastatic prostate cancer.

Table 1. Summary for ligand-stimulated transactivational activity of 44 CaP-derived AR mutants

	No ligand	DHT 10 ⁻⁹ M	DHEA 10 ⁻⁶ M	E2 10 ⁻¹⁰ M	PG 10 ⁻⁸ M	HC 10 ⁻⁵ M	Flut 10 ⁻⁵ M	Csdx 10 ⁻⁵ M
Activated	0	37	20	3	4	2	3	1
Not activated	44	7	24	41	40	42	41	42

DHT: (1-3 nM); DHEA: dehydroepiandrosterone (0.3-11 µM for DHEA-S); E2: estradiol (0.04-0.2 nM); PG: progesterone (0.3-3nM); HC: hydrocortisone (0.1-0.5 µM); Flut: flutamide (~8 µM); Csdx: casodex.

198

C-ERBB-2 MEDIATED PACP SIGNALING TO THE MAP KINASE PATHWAY IS INVOLVED IN ANDROGEN-INDEPENDENT PSA SECRETION IN HUMAN PROSTATE CANCER CELLS

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INTRODUCTION AND OBJECTIVES: Although most prostate cancers are initially androgen dependent, they become eventually androgen-independent following androgen ablation therapy. However, even under the androgen-deprived environment, the circulated level of prostate-specific antigen (PSA), an androgen-regulated marker, in hormone refractory prostate cancer patients is constitutively up-regulated through a yet unknown mechanism. Our previous studies have clearly demonstrated that highly tyrosine phosphorylated c-ErbB-2, which subsequently activates the proliferation signal, can lead to an androgen-independent phenotype of prostate cancer. Furthermore, this phosphotyrosine level of c-ErbB-2 is at least in part regulated by the cellular form of prostatic acid phosphatase (PACP), a prostate-unique protein tyrosine phosphatase. Thus, we propose a functional role of cellular PACP and the c-ErbB-2 signal pathway in androgen-independent PSA secretion.

METHODS: The LNCaP cell model including different passages of cells was used for the experiments. High passage C-81 cells are androgen-independent cells lacking endogenous PACP expression. The levels of PSA in the conditioned media of different LNCaP cells under various conditions were determined by Western blot analyses. The secreted level of PSA was normalized to the cell number of each experiment.

RESULTS: In a steroid-reduced condition, C-81 cells secreted a higher level of PSA than C-33 parental cells. The restored expression of cellular PACP in C-81 cells reduced PSA secretion. The elevated expression of c-ErbB-2 or mitogen-activated protein kinases (MAPK) by cDNA transfection resulted in an increased PSA secretion by C-33 cells. Conversely, the expression of a dominant negative mutant of c-ErbB-2 protein in C-81 cells correlated with a lower level of PSA secretion in comparison with control cells. Moreover, the inhibition of c-ErbB-2 or MAPK by their specific inhibitors, AG 825, 879 or PD98059, led to a decreased secretion of PSA.

CONCLUSIONS: Our data indicated that the c-ErbB-2 mediated PACP signaling to the MAPK pathway is involved in androgen-independent PSA secretion in human prostate cancer LNCaP cells.

199

MACROPHAGES TRANSDUCED WITH ADENOVIRAL VECTORS TO EXPRESS IL-12 SUPPRESS TUMOR GROWTH AND METASTASIS IN PRE-CLINICAL MODELS OF PROSTATE CANCER

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INTRODUCTION AND OBJECTIVES: Macrophages (Mφ) have multiple immunological functions including phagocytic activities and antigen presentation. After differentiation from the pool of monocytes and entry into tissues, Mφ can produce various cytokines, and tumor-associated Mφ have the potential to mediate tumor cytotoxicity and to stimulate antitumor lymphocytes. In this study, we compared the efficacy of mIL-12 gene-modified Mφ to control βgal gene-modified Mφ for intratumoral injection therapy.

METHODS: Peritoneal exudate Mφ were infected adenoviral vector containing mIL-12 (AdmIL-12) or βgal (AdvCMV/βgal) 24hr before use. HBSS uninfected Mφ, βgal gene-modified Mφ, mIL-12 gene-modified Mφ were injected directly into orthotopic tumors (metastatic 178-2 BMA) at day 7 after tumor cell inoculation. At day 21, primary tumors and spontaneous lung metastases were evaluated. In some experiments animals were maintained until moribund to analyze survival.

RESULTS: FACS analysis showed an increase in the number of cells positive for MHC class I and II antigen as well as increased surface staining for F4/80 antigen in mIL-12 gene-modified Mφ compared to uninfected or AdvCMV/βgal gene-modified Mφ. mIL-12 gene modified Mφ induced significant suppression of primary tumor growth (884mg) compared with βgal gene-modified Mφ (1901mg) or uninfected Mφ (2257mg) ($p=0.0128$ and $p=0.0004$, respectively). mIL-12 gene-modified Mφ also demonstrated a significant suppression of spontaneous lung metastasis (mean 3.0) compared with the βgal gene-modified Mφ (12.6) or uninfected Mφ (7.2) ($p=0.0003$ and $p=0.046$, respectively). A significant survival advantage was demonstrated for mIL-12 gene-modified Mφ compared with control βgal gene-modified Mφ injected animals (21.3 vs. 25.7 days, respectively, $p=0.0003$). A significant infiltrate of tumor associated CD8+ T cells was demonstrated with mIL-12 gene-modified Mφ compared to βgal gene-modified Mφ ($p=0.0227$). Trafficking studies confirmed that intratumorally injected mIL-12 gene-modified Mφ could migrate to the draining lymph node and lung.

CONCLUSIONS: We have demonstrated that IL-12 gene-modified macrophages can be introduced into the prostate and that this novel approach has therapeutic activities in pre-clinical models of prostate cancer. Hopefully, this therapeutic approach will be useful for specific clinical applications.

200

SOYBEAN ISOFLAVONE, GENISTEIN, INDUCES GLUTATHIONE PEROXIDASE IN HUMAN PROSTATE CANCER CELL LINES, LNCaP AND PC-3

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INTRODUCTION AND OBJECTIVES: Epidemiological studies have revealed that the incidence of prostate cancer is much lower in Asian countries compared with Western countries. One of explanations of these differences is attributed to the difference of life styles, especially dietary tendency. Genistein, soybean isoflavone, has attracted attention in the prevention of prostate cancer for its various biological effects. These include induction of apoptosis, its estrogenic activities, inhibition of DNA topoisomerase activities and other critical enzymes involved in signal transduction, inhibition of angiogenesis, anti-oxidants activities, and so on. In the current study, we investigated the effect of genistein on prostate cancer cell lines, LNCaP and PC-3.

METHODS: Effect of genistein on proliferation of prostate cancer cells was determined by MTT assay. To understand multiple functions of genistein, cDNA microarray analysis was performed to obtain gene expression profiles of genistein treated LNCaP cells. Gene expression levels and enzyme activities were assessed by quantitative real-time PCR and enzyme assay.

RESULTS: MTT assay showed that genistein inhibited LNCaP and PC-3 cells proliferation in dose-dependent manner. DNA microarray study demonstrated that the gene expression of most genes including apoptosis inhibitor (survivin), topoisomerase (DNA) II, alpha, mitogen-activated protein kinase 6, fibronectin and meltrin gamma, was down-regulated in LNCaP cells after genistein treatment. On the contrary, the gene expression of glutathione peroxidase 1 gene was most up-regulated. Quantitative real-time PCR showed that glutathione peroxidase 1 gene expression was significantly up-regulated accompanied by the elevation of glutathione peroxidase enzyme activity both in LNCaP and PC-3 cells. However, gene expression levels of other antioxidant enzymes including superoxide dismutase, catalase and glutathione S-transferase showed no significant differences.

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Title: Antimetastatic Effects of IL-12 Gene-modified Bone Marrow Cells in a Mouse Model of Metastatic Prostate Cancer

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Recombinant interleukin-12 (IL-12) is a potent immunomodulatory cytokine that has been shown to exert strong antitumor and antimetastatic effects in various mouse models. Based on our previous studies demonstrating that IL-12 gene therapy has specific effects against bone metastases, we used a retroviral vector-mediated gene-modified cell therapy approach to overexpress IL-12 in adult hematopoietic stem cells to target and achieve long term transgene expression in bone marrow and potentially other sites where metastases develop. 129/SvRosa mice were treated with 5-fluorouracil six days before isolating bone marrow cells. Red blood cells were removed from the bone marrow by ficoll-paque TM plus centrifugation. After pre-stimulation with 20 ng/ml IL-3, 50 ng/ml IL-6, 100 ng/ml stem cell factor, 20 ng/ml granulocyte-colony stimulating factor (G-CSF) and 50 ng/ml Flt-3 Ligand for 24 hours, bone marrow cells were cultured in 6-well plate coated with retronectin (25 µg/well). The murine IL-12 transducing retroviral vector, DFG-mIL-12, or control vector, DFG-eGFP, was used for bone marrow cell infection. Bone marrow cells were infected once daily for three consecutive days with high titer ($>10^6$ IU/ml) supernatant.

Transduction efficiency of mouse bone marrow cells (10-30%) was determined by intracellular murine IL-12 expression or eGFP expression. DFG-mIL-12 or DFG-eGFP transduced bone marrow cells (10^6 /mouse) were injected via tail vein into recipient 129/Sv mice that harbored metastases previously established by i.v. injection of 178-2 BMA mouse prostate cancer cells three days prior to treatment. The animals were sacrificed at various time points and sera and specific tissues were collected and analyzed. Sera obtained from DFG-mIL-12 bone marrow treated mice showed a gradual increase in IL-12 (p40, ELISA) that reached a peak (0.89 ng/ml) at day 9. In contrast, IL-12 levels in the serum of DFG-eGFP bone marrow treated mice were significantly lower (0.11 ng/ml) throughout the time course. Flow cytometric analysis indicated that 21 days following the bone marrow cell injection, approximately 15% of peripheral blood cells stained lac-Z positive. Mice treated with DFG-mIL-12 transduced bone marrow cells had significantly fewer metastatic lung colonies (mean=39) compared to mice treated with DFG-eGFP transduced bone marrow cells (mean=76, $P=0.0127$) or with HBSS (mean=67, $P=0.0155$). Histochemical analyses showed that 80% of the mice in the DFG-eGFP bone marrow treated group and 83% of the mice in the HBSS treated group had bone metastases. HBSS treated mice showed large metastatic tumor deposits that extended into the connective tissues surrounding the bone. In marked contrast, only 17% of the mice had bone metastases in the group treated with DFG-mIL-12 transduced bone marrow cells. Therefore, systemically delivered bone marrow cells genetically engineered to produce IL-12 are effective against pre-established metastases in this model system of prostate cancer metastases.

Disclosure: None

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