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TITLE: Effects of Androgen Ablation on Anti-tumor Immunity

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**14. ABSTRACT**

Androgen Ablation (AA) constitutes the most common therapy for the treatment of advanced prostate cancer. While initially effective at reducing tumor burden, most patients recur with androgen insensitive disease. There exists a clear need to augment the clinical efficacy of hormone-based therapies, and immunotherapy of prostate cancer represents one such promising approach. Data indicate that AA affects the immune system both systemically as well as at the prostate. There is also growing evidence in the literature of the thymic reconstitution effects following AA. We had previously found that castration led to increase in tumor growth, possibly due to an increase in immune infiltrates. In the past year, we have repeated the experiments to determine the effects of castration on the induction and on the growth of existing tumors, examined the T cell infiltrates in the prostate and prostate tumors following castration and determined the effects of castration on the allo-stimulatory activity of DCs from lymph nodes. We conclude that AA alone does not generate an antitumor response of great significance. We propose to carry out vaccination using prostate-specific antigens in conjunction with AA to determine if AA can ultimately lead to a synergistic positive outcome for the immunotherapy of prostate cancer.
Introduction

Androgens are required for the normal growth, development and function of the prostate gland and also support the growth of prostate neoplasms. Since the early 1940s, androgen ablation (AA) therapy has been a standard treatment for advanced prostate cancer after Huggins and Hodges[1] made the observation that prostate tumors are partially responsive to androgen withdrawal. AA remains a palliative approach as it does not totally eliminate all the prostate cancer cells. While initially effective at reducing tumor burden, most patients eventually develop disease refractory to androgen withdrawal. The hormone refractory stage of prostate cancer represents the terminal stage of the disease as treatment options are limited and the median survival for such patients is approximately 1 year.

Androgens exert potent immunosuppressive effects and it has been shown that patients with advanced prostate cancer have dysfunctional cell-mediated immunity characterized by the predominance of a Th2 cytokine profile[2]. It was also shown that the dendritic cells (DC) from prostate cancer patients were functionally impaired and were less potent stimulators in an allogenic mixed leukocyte reaction[3]. Castration of mice stimulates B and T lymphopoiesis, thymic and bone marrow hyperplasia[4]. Immunotherapeutic approaches for the treatment of prostate cancer could be affected by the AA since the loss of androgens may influence the nature of the host immune response. This study aims to identify the effects of AA on anti-tumor immunity and to determine the mechanism behind any effects.
Body

This progress report describes the activities on the grant proposal from September 2004 to September 2005. In the past year, we have investigated the allo-stimulatory ability of dendritic cells (DCs) isolated from castrated and sham-operated mice in order to determine if AA leads to a non-specific augmentation on the immune response. We have also examined the prostate and tumor-infiltrating lymphocytes following AA. Technical issues raised by reviewers in last year’s progress report are addressed in the first part of this report. The proposal of a new direction for the grant is dealt with in the discussion section.

Technical issue 1: Isotype control data was not presented for the FACs analysis Figure 1 showing the CD80 expression on CD80-transfected TRAMP-C2 cells.

Figure 1. Overlay histogram of CD80 expression and isotype control staining on TRAMP-C2 and stably transfected CD80 clone by flow cytometry.

Red = Unstained
Green = CD80-TRAMP-C2 isotype control
Pink = CD80-TRAMP-C2 CD80 staining
Blue = TRAMP-C2 CD80 staining
Technical issue 2: Number of mice per group in Figures 3 and 4 were not included.

Figure 2. Effect of AA on existing B16 tumors

Figure 3. Effect of castration on existing TRAMP-C2 tumors
Body (continued)

Technical issue 3: Repeating the TRAMP-C2 in vivo experiments

The effect of AA had a paradoxical effect of increasing the tumor size in mice bearing the TRAMP-C2 and CD80-TRAMP-C2 tumors. The figures presented in the previous report were representative of 3 experiments. In addition, we show below that AA has the same effect even when mice were castrated 7 days or 21 days prior to tumor induction.

Specific aim 1: Task 4: Comparing the growth of TRAMP-C2 and CD80-TRAMP-C2 tumors in castrated and sham-operated mice.

6-8 weeks old C57BL6/ mice were castrated or sham-operated prior to injection of $1 \times 10^5$ tumor cells.

Effect of AA on TRAMP-C2 induction

![Graph showing the effect of AA on TRAMP-C2 induction](image)

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Figure 4. Effect of AA on castrated of sham-operated mice

Figure 4 shows that castration 7 days prior to tumor induction had the effect of increasing the tumor size in both TRAMP-C2 and CD80-TRAMP-C2 tumor injections.
Body (continued)

Castration 3 weeks prior to induction

![Graph showing mean tumor volume over days post tumor induction.](figure5a)

Figure 5a. Effect of castration on TRAMP-C2 tumor induction

![Bar graph showing mean tumor mass.](figure5b)

Figure 5b. Effect of AA on tumor mass

We had earlier postulated that the increase in tumor size in the castrated animals could be due to an increase in immune infiltrates. Therefore, we decided to lower the tumor inoculum to $1 \times 10^5$ tumor cells to see if the infiltrates can control or eradicate the tumors. In figure 5a, the castrated mice had slightly smaller tumors compared to the sham-operated mice. However, the mean tumor mass (Figure 5b) at the end of the experiment between the 2 groups was not significantly different. This leads us to the conclusion that the effect of AA alone was not sufficient to generate an antitumor response of much significance.
Body (continued)

**Specific Aim 2:** Task 2: Validate method for quantifying CD3$^+$ cells infiltrating prostate and prostate tumors following AA using immunofluorescence.

Based on the hypothesis that the increase in tumor mass in castrated animals could be due to an increase in immune infiltrates, we decided to collagenase-digest the prostate tumors and run an analysis of the immune infiltrates using flow cytometry.

6-8 weeks old C57BL/6 mice were castrated or sham-castrated 7 days prior to injection of $5 \times 10^5$ TRAMP-C2 or CD80-expressing TRAMP-C2 cells. The tumors were subjected to collagenase digestion and the cells collected were stained with CD3 and CD8 and analyzed by flow cytometry. The below figures show the results of our analysis of the tumors from figure 4.

**T cell infiltrates in TRAMP-C2 tumors**

![Graph showing T cell infiltrates in TRAMP-C2 tumors](image)

**T cells infiltrates in CD80-TRAMP-C2 tumors**

![Graph showing T cells infiltrates in CD80-TRAMP-C2 tumors](image)

**Figure 6. Effect of castration on T cell infiltrates in prostate tumors**

An increase in CD3$^+$CD8$^+$ infiltrating T cells might account for the increase in tumor mass in the castrated mice bearing the TRAMP-C2 tumor but castration led to reduce numbers of CD3$^+$CD8$^+$ infiltrating T cells in the mice given the CD80-TRAMP-C2 tumors.
Body (continued)

We had proposed in last year's report to investigate by immunofluorescent staining if the increase in tumor size could be due to increase in immune infiltrates. The tumor sections shown below are representative of an immunofluorescent analysis of established TRAMP-C2 or CD80-TRAMP-C2 tumors from castrated or sham-operated mice.

![Fig. 7a TRAMP-C2 Sham-operated](image1)
![Fig. 7b TRAMP-C2 Castrated](image2)
![Fig. 7c CD80-TRAMP-C2 Sham-operated](image3)
![Fig. 7d CD80-TRAMP-C2 Castrated](image4)

The immunofluorescent staining data showed that there was more immune infiltrates in the mice that were castrated post tumor induction. Suggesting that there could be some modest augmentation in antitumor response following castration.
Specific Aim 1: Task 9: Develop methods for isolation of dendritic cells from mouse splenocytes

Over the course of the year, several methods of isolating DCs have been carried out. Successful isolation of DCs was measured by functional analysis of their allo-stimulatory ability. We decided upon using a discontinuous Optiprep gradient to isolate lymph node DCs eventually as they work best in the MLR assay.

Specific Aim 1: Task 10: Isolate DC from castrated and sham-operated C57BL/6 mice and test for ability to support MLR response by allogeneic DBA/2 splenocytes.

6-8 weeks old male C57BL/6 mice were castrated or sham-operated and sacrificed 3 weeks post surgery. Their inguinal, brachial, superficial cervical and deep cervical lymph nodes were harvested and DCs isolated using a discontinuous Optiprep gradient. The DCs were irradiated at 3000 rads and co-incubated with splenic lymphocytes from DBA/2 mice isolated using Lympholyte-M gradient at a 1:10 ratio. Figure 8 shows the proliferation of DBA/2 responder lymphocytes in a MLR using lymph node DCs from castrated or sham-operated C57BL/6 mice.

![Figure 8. Proliferation of DBA/2 responders in a MLR using irradiated DCs from castrated (CLN) or sham-operated (SLN) mice. Each column is representative of the lymph node DCs pooled from 2 mice.]

There was no significant differences in the ability of the DCs castrated and sham-operated mice to support proliferation of the DBA/2 responders.
A recent report from Sutherland et al.[5] showed that androgen blockade in very old mice (18-24 months old) led to complete restoration of thymic structure. To determine if the lack of differences in the castrated and sham-operated mice was due in fact to using young mice, we purchased 7-9 months old retired C57BL/6 breeder mice and repeated the MLR assay using these mice. We also decided to carry out the remaining tasks in the proposal using the 7-9 months old mice. 7-9 months old mice are considered to be middle aged mice.

**Figure 9.** Proliferation of DBA/2 responder lymphocytes in a MLR using irradiated DCs from castrated or sham-operated C57BL/6 mice at a stimulator:responder ratio of 1:10. Each column represents the lymph node DCs taken from 1 individual mouse.

From the data obtained, we still did not find differences in the allo-stimulatory ability of lymph node DCs from castrated or sham-operated middle-aged mice (7-9 months old).
Specific Aim 2: Task 4: Quantitate CD3⁺ cell infiltration in prostates of castrated and sham-operated mice, 21 days following surgery.

7-9 months old C57BL/6 mice were castrated or sham-operated and sacrificed 3 weeks post surgery. Their prostate glands were harvested and subjected to collagenase digestion. Flow cytometry was used to quantitate the amount of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ cells in the prostate.

![Graph of %CD3⁺CD4⁺ cells](image1)

Figure 10a. CD3⁺ CD4⁺ infiltrating cells in the prostate of 7-9 months old C57BL/6 mice

![Graph of % CD3⁺CD8⁺ cells](image2)

Figure 10b. CD3⁺ CD8⁺ infiltrating cells in the prostate of 7-9 months old C57BL/6 mice

From the above figures, no significant differences in the percentage of infiltrating CD3⁺ CD4⁺ or CD3⁺ CD8⁺ cells could be found.
Specific Aim 2: Task 3 and 5: Simultaneous quantitation of CD3+ cell infiltration measurement of BRDU incorporation in prostates of castrated and sham-operated mice, untreated or treated with anti-CTLA-4 or hamster IgG antibody.

In this experiment, we used 7-9 months old C57BL/6 mice and we also tried to look at simultaneously quantitating CD3+ cell infiltration measuring BRDU incorporation in prostates of castrated and sham-operated mice using flow cytometry and intracellular staining for BRDU. Level of BRDU incorporation was measured by the mean fluorescent index (MFI) of CD3+ CD4+ or CD3+ CD8+ cells.

![CD4 plots](image)

Figure 11a. Percentage of infiltrating CD3+ CD4+ in prostates of castrated and sham-operated mice untreated or treated with either anti-CTLA-4 or hamster IgG isotype control antibody. (n=5 per group)

![CD3+CD4+ plots](image)

Figure 11b. Mean fluorescent index of BRDU staining in CD3+ CD4+ cells
Figure 12a. Percentage of infiltrating $\text{CD}3^+ \text{CD}8^+$ in prostates of castrated and sham-operated mice untreated or treated with either anti-CTLA-4 or hamster IgG isotype control antibody. (n=5 per group)

Figure 12b. Mean fluorescent index of BRDU staining in $\text{CD}3^+ \text{CD}4^+$ cells

From the data obtained, the simultaneous quantitation of T cell infiltrates and BRDU incorporation is not optimal using flow cytometry. The quantitation of T cell infiltrates following anti-CTLA-4 treatment will be repeated on its own without trying to measure the proliferative capacity of these infiltrates by BRDU incorporation.

**Specific Aim 3:** To determine the effects of AA on circulating leukocytes and immune responsiveness in prostate cancer patients.

We have just received IRB approval to start collecting patient samples for the study. (See attached IRB approval letter page 21-22)
Key research accomplishments

1. Repeated *in vivo* experiments investigating effects of castration on the induction and the growth of existing TRAMP-C2 and CD80-TRAMP-C2 tumors.
2. Quantified CD3$^+$ cells infiltrating prostate and prostate tumors using flow cytometry and immunofluorescent staining.
3. Investigated effects of castration on allo-stimulatory effects of dendritic cells in a MLR assay.
4. Obtained IRB approval to start collecting patient samples for Specific Aim 3.
Reportable outcomes

Manuscripts, abstracts, presentations:

Poster presentation at AAI meeting 2005, San Diego Convention Center, April 2-6 2005
PSCA vaccination induces a prostate cancer protective immune response in the absence of autoimmunity
Garcia Hernandez ML, Koh YT, Hubby B and Kast WM.

Patents and licenses applied for and/or issued:
None

Degrees obtained that are supported by this award:
None

Development of cell lines, tissue or serum repositories:
None

Informatics such as databases and animal models, etc:
None

Funding applied for based on work supported by this award:
None

Employment or research opportunities applied for and/or received on experiences/training supported by this award:
None
Conclusions

We have repeated the in vivo experiments investigating the effects of AA on the induction and on the growth of existing TRAMP-C2 and CD80-TRAMP-C2 tumors. The results show that castration had the effect of increasing the tumor size. We postulated based on FACs analysis of T cell infiltrates in the prostate tumors (Fig. 6 and Fig. 7) that the increase in tumor size could be due to an increase in infiltrates and reduced the tumor inoculum to determine if the infiltrates could then control or eradicate the tumor. Although the castrated mice had slightly smaller tumors (Fig. 5a), the mean tumor mass at the end of the experiment was not significantly different. This leads us to the conclusion that AA alone cannot generate an antitumor response of much significance.

We have decided to use flow cytometry to quantitate the amount of T cell infiltration in the prostates following AA and CTLA-4 blockade since we are not equipped with a laser scanning cytometer. While we were able to determine the percentages of T cell infiltrates in the prostates our initial experiment trying to quantitate the proliferative capacity of the T cell infiltrates in the prostate was unsuccessful. Castration led to slightly higher levels of CD3+ CD4+ cells (Fig. 12a) but not CD3+ CD8+ cells (Fig. 12b). We will repeat the CTLA-4 blockade experiments to determine if it could augment the amount of T cell infiltrates in castrated mice.

Based on the proliferation of DBA/2 responder cells, castration did not lead to an improved capacity of DCs from young mice (Fig. 10) or middle aged mice (Fig. 11) to stimulate lymphocytes in the allo-reaction. Thus far, our results do not support a significant augmentation of the immune response following AA. Androgens have been shown to lead to thymocyte apoptosis[6] and castration leads to restoration of thymic architecture in really old mice[5]. It could be possible that the augmentation of immune responses following AA can only be observed in really old mice and in older men in the clinical setting. The oldest mice that we can currently obtain from our suppliers are only in their middle age (7-9 months). We could house the mice and wait for them to age to carry out the experiments but these results would not be available until next year. However, this information is still important in deciding the group of patients that might benefit from the combination of AA and immunotherapy.

Tasks 6 through 10 of Specific Aim 2 deals with TCR Vβ spectratyping. A report by Roden at al. [7] has showed that castration does not affect the TCR Vβ spectratyping and does not favor the expansion of any particular TCR clones. We have decided not to continue to focus our efforts on investigating the effects of AA on the immune system based on non-antigen specific responses. Ultimately the goal of the work is to determine if AA can be used synergistically with immunotherapy for the treatment of prostate cancer. The work of Hsueh et al. [8] showed that androgen blockade in young mice (7-8 weeks old) enhanced the response to a irradiated B16 tumor vaccine. In our laboratory, we have developed several prostate-antigen specific vaccines using different vaccination approaches that we intend to use to test if castration leads to an increase in the response to prostate-specific vaccines. The vaccination approaches we intend to use include the Venezuelan equine encephalitis virus replicon particle (VRP) as an RNA vector[9, 10]
and the gene gun that delivers DNA intradermally. Our laboratory has previously identified several prostate-specific antigens such as the murine six-transmembrane epithelial antigen of the prostate (STEAP), murine prostate stem cell antigen (PSCA), and murine prostate-specific membrane antigen (PSMA)[11] that can be used as potential targets for prostate cancer immunotherapy. Unlike the popular prostate-specific antigen (PSA) that is found in humans but has no known orthologue in mice, STEAP and PSCA and PSMA are found in both mice and humans. In addition, STEAP and PSCA are expressed on the surface of normal and prostate cancer cells and not secreted, making them even more attractive as potential immunotherapy targets. We will be diverging our efforts into carrying out experiments to determine if AA can ultimately improve the responses to prostate-specific vaccines.
References


Date: 8/24/2005
To: Eila C Skinner, M.D.
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From: Vice Chair - IRB
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TITLE OF PROPOSAL:
4P-04-1 IMMUNOLOGIC EFFECTS OF ANTIANDROGEN THERAPY FOR PROSTATE CANCER IN HUMANS

Action Date: 8/18/2005     Action Taken: Approved
Committee: Vice Chair - IRB

Note:
Your correspondence dated 8/1/05 (received on 8/5/05) and attachments
were reviewed by Dr. Anglin on 8/18/05. The proposed changes qualify
for expedited review according to 45CFR46.110 (b) 2) minor changes in
previously approved research during the period (of one year or less) for
which approval is authorized. The proposed changes were APPROVED.

The Revised Informed Consent Document, dated 8/1/05, was
APPROVED.

Based on expedited review of your response, contingencies of 4/21/05
have been fully satisfied.

Informed consent must be obtained by the investigator or person
authorized to obtain informed consent from all research subjects or their
legally authorized representatives. You must ensure that all project
personnel involved in the process of consent/assent are trained properly
and are fully aware of their responsibilities relative to the obtainment of
informed consent/assent according to the IRB guidelines and applicable federal regulations.

The IRB office has stamped the approved informed consent form(s) for use in this research project. It should be photocopied, as appropriate, onto the correct letterhead for the hospital or institute. You may not use this informed consent form document to consent new subjects after its expiration date. A photocopy of this IRB approved informed consent form document(s) bearing this stamp must be used for consenting and/or reconsenting the study subjects. The study subject must sign and date the informed consent document. The person obtaining informed consent must also sign the study consent form at the time consent is obtained. One copy of the informed consent should be given to the study subject, one copy placed in the medical record, and the investigator should retain one copy.