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PRINCIPAL INVESTIGATOR: Dr. Gail Fraizer

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

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**ABSTRACT** (Maximum 200 Words)

The androgen pathway is central to prostate tumorigenesis. An increased risk of higher stage, more aggressive prostate cancer is associated with a more active androgen receptor (AR). We are investigating an innovative transcription based mechanism that represses AR activity *in vitro*. Our hypothesis is that the tumor suppressor gene, WT1, may play a role in prostate tumorigenesis mediated by repression of AR gene expression. To validate our AR promoter data we demonstrated that AR target gene down-regulation by WT1 is dependent on an intact DNA binding domain, is mediated by AR and is hormone dependent. Additionally we confirmed our RNA studies showing that WT1 protein expression patterns are inversely related to AR expression. Androgen responsive cell lines express AR but fail to express WT1, while androgen independent lines express WT1 and lack AR, suggesting a correlation with late-stage androgen independence. We have established stably transfected tumor cell lines and have tested the tumorigenicity of these lines with the intent of using them to establish a mouse model of prostate cancer progression. With the correlation of WT1 expression with higher grade disease and the potential to demonstrate WT1 repression of AR expression in mice, we will establish the role of WT1 in the development of androgen independence.
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INTRODUCTION

Prostate cancer is an increasingly important public health problem with nearly 200,000 cases diagnosed and 32,000 deaths expected in 2001. Yet the significance of latent, non-invasive prostate cancer and its relationship to the lethal invasive form is not understood. Delineating the mechanisms of progression of latent androgen dependent prostate cancer to invasive androgen independent prostate cancer is of fundamental importance. Additionally identifying the biomarkers of invasive androgen independent prostate cancer will allow the identification of appropriate therapy on an individual basis.

An increased risk of higher stage, more aggressive prostate cancer is associated with the inheritance of a more active androgen receptor (AR) (1-3). We are investigating an innovative transcription based mechanism that represses AR activity in vitro. One such regulator of the AR is the the tumor suppressor gene, WT1, a zinc finger transcription factor that modulates expression of growth factors, their cognate receptors and steroid hormone receptors among others. Our hypothesis is that WT1 may play a role in prostate tumorigenesis mediated by repression of AR gene expression.

Cultured prostate cancer cell lines which retain both the AR and its signaling pathways intact continue to retain sensitivity to androgen stimulation. Thus when these cells are injected into mice, they are able to grow and develop into tumors only if the hormone, androgen is available (ie, in male adult mice). As expected, androgen removal by surgical castration prevents tumor formation by these hormone dependent tumor cells, as does hormone removal (or ablation) by chemical methods. That is, if the hormone and receptor are unable to interact, then the signaling pathway will fail to be stimulated and tumor cell growth will be slowed if not prevented. The primary hypothesis of this work is that the development of invasive, androgen independent prostate cancer may be altered by a regulator, such as WT1, that blocks the normal androgen signaling pathway. With our establishment of stably transfected LNCaP lines expressing high levels of WT1, we have examined the tumorigenicity of WT1 expressing prostate cancer cells. Surprisingly we observed loss of tumorigenicity in LNCaP cells expressing either the transcriptionally active, wild-type WT1 or the inactive, mutant form of WT1. Since WT1 suppresses tumor cell growth independent of transcriptional repression of AR, this suggests that either alternative mechanisms of AR repression are active in vivo, or that suppression of tumor formation by WT1 does not require transcriptional repression of AR expression in vivo. Currently, we are examining the molecular mechanisms involved in our model of WT1-mediated suppression of prostate tumor growth to determine whether these mechanisms may also lead to the development of androgen independence.

BODY

We have previously published that WT1 repression of the AR gene promoter construct (4) is mediated by DNA binding. Additionally we have published that WT1 represses the endogenous AR promoter in androgen responsive cells (5). Using in vitro assays we have demonstrated that this AR target gene down-regulation by WT1 is dependent on an intact DNA binding domain, is mediated by AR and is hormone dependent. We have previously demonstrated an inverse relation between expression of WT1 and AR mRNA and protein in prostate cancer cell lines. Androgen responsive cell lines express AR but fail to express WT1, while androgen unresponsive lines express WT1 and lack AR, suggesting a correlation of WT1 with AR defective, late-stage androgen independence. Thus, our hypothesis that WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells was tested both in vitro and in vivo assessing the significance of WT1 expression in prostate tumors and the mechanism of AR repression.
Published findings:

1. WT1 and AR expression are inversely correlated

2. WT1 repression of the AR promoter is mediated by DNA binding

3. WT1 repression of the AR pathway

These previous results demonstrated that: 1) WT1 and AR expression are inversely correlated in androgen target tissues, 2) WT1 protein binds at least two of the WT1 binding sites in the AR promoter in vitro, 3) WT1 directly binds and represses exogenously added AR promoter constructs in kidney and gonadal cells, and 4) WT1 repression of the endogenous AR promoter in kidney and gonadal cells interferes with the androgen signal transduction pathway causing down-regulation of AR target gene transcription.

Unpublished work:

**PROSTATE TUMOR SUPPRESSION MEDIATED BY WT1, A TUMOR SUPPRESSOR GENE**

This research has been directed towards developing a model of androgen-independent prostate cancer progression based on the hypothesis that WT1 contributes towards the progression of prostate cancer to androgen independence by modulating AR activity in prostate cells. This hypothesis has been supported by our recent findings demonstrating WT1 expression in both human and murine prostate tumor cells and by the development of prostate tumor lines expressing WT1 which fail to form tumors in nude mice (see Technical Objective III). We have engineered (by DNA transfection) prostate tumor lines expressing either the transcriptionally active form of wild-type WT1 or an inactive WT1 mutant form. The parental or unaltered LNCaP cell lines are androgen responsive, PSA expressing cells that contain mutant AR with slightly altered hormone affinity. Both the parental and the LNCaP control (or mock treated) cell lines were injected into nude mice leading to large tumor formation beginning at 4 weeks and peaking at 6–8 weeks after injection. This is in contrast to the suppression of tumor development following injection of cells engineered to express WT1. After a long lag-time (8-10 weeks) we began to see a few small tumors forming (peaking at 12-14 weeks). These subcutaneous tumors were severely reduced in both size and number compared to the tumors formed by injection of the parental or untransfected LNCaP cell lines. We expect to find they express AR in the absence of WT1.

**TECHNICAL OBJECTIVE I. To determine the pattern of WT1 expression in prostate tumors.**

Is WT1 protein expressed in prostate epithelial cells?

Previous analysis demonstrated WT1 expression in high grade and stage tumors. Gleason 5 grade tumors showed weak or no WT1 expression, while some Gleason 9 grade sections displayed focal epithelial cytoplasmic staining, sometimes accompanied by nuclear staining but often not. Normal prostate tissues and low grade PIN samples lacked WT1 expression (data not shown). However with continued analyses of patient samples we have now observed weak staining in both Gleason Grade 5 tumors and in non-prostate epithelial cells (data not shown). Using anti-WT1 Ab (C19, Santa Cruz) both dark brown staining nuclei and light brown staining cytoplasm are visible in prostate tumor epithelial cells. Primarily we observed focal cytoplasmic staining of epithelial cells of high grade prostate tumor sections (Gleason 9) and rarely staining of low grade tumor sections
(Gleason 5) with both cytoplasmic and nuclear staining (FIGURE 1, in appendix). Top panels shows low magnification (150x), while lower shows high power (200 and 300x).

**Is WT1 protein found in both nucleus and cytoplasm?** In an effort to validate the previously observed cytoplasmic staining of prostate tissue sections, we examined adult human and mouse kidneys. We observed nuclear staining restricted to podocytes, but also clear evidence of cytoplasmic staining in the adjacent tubular epithelial cells (FIGURE 2, in appendix). Similarly both cytoplasmic and nuclear staining has been observed with the monoclonal Ab (data not shown). Additionally, western blot analysis of nuclear extracts and cytoplasmic remnants (supernatant removed from nuclear pellets) showed the presence of WT1 protein in both the nuclear and cytoplasmic fraction of androgen insensitive cell lines: DU145, LNCaP-LN3, PC3, and PC3 sublines (data not shown). We confirmed the cytoplasmic location of WT1 protein using the same polyclonal anti-WT1 Ab (C19, Santa Cruz) in immunohistochemical analyses of prostate epithelial cell lines grown in 8-well chamber slides. We observed dark brown staining nuclei and light brown staining cytoplasm in the androgen insensitive cell lines (Data not shown). Specificity of staining was confirmed by treating Ab with WT1 blocking peptide prior to incubation of the polyclonal Ab with cell lines. While WT1 expression is not limited to the nucleus, its function (if any) in the cytoplasm is unknown.

**TECHNICAL OBJECTIVE II. To determine if WT1 represses the androgen signalling pathway.** For a better understanding of the role played by WT1 in regulating the expression of AR and AR-target genes, we examined the effect of overexpression of WT1 in cells expressing a functional AR (293 kidney and TM4 Sertoli cells). In these assays the ability of WT1 to directly repress the endogenous AR promoter resulted in an indirect repression of an AR-target gene construct containing four copies of the ARE binding site (GGTACAnnnTGTTCT), the E1B TATA box and the luciferase gene (Reference 5, see attached in Appendix). WT1 over-expression in 293 and TM4 cells down-regulates AR and indirectly results in a dose-dependent reduction in ARE-activated luciferase activity. These experiments have been extended to prostate tumor cells, LNCaP, where we confirmed this indirect mechanism by dual labeling immunofluorescence assays of WT1 and AR expression in LNCaP cells transiently transfected with WT1 (not shown). Thus, WT1 repression of the endogenous AR promoter in kidney and gonadal cells interferes with the androgen signal transduction pathway causing down-regulation of AR target gene transcription.

WT1 does not directly act on the ARE-luciferase

Several explanations for the inverse relationship of WT1 and AR expression are possible. One is that WT1 binds AR and thus blocks binding of AR to its cognate binding site, the ARE. A second possible explanation is that WT1 may directly bind the ARE site and block AR from binding to the ARE. We then ruled out the latter possibility that WT1 was directly binding the ARE by transfecting cos cells with the ARE-luciferase target and WT1(-/-) without AR. As expected WT1 alone failed to
activate the ARE-luciferase target (right two bars), while cotransfection of the empty vector CB8+ and the AR in the presence of hormone R1881 (2nd bar from left) activated the ARE-luciferase target 3x. Likewise, in the presence of hormone (R1881) WT1 does not block AR protein from activating the ARE. We demonstrated that the AR expression construct is activated 5x by the exogenously added AR expression construct even in the presence of exogenously added WT1(-/-) expression construct (4th bar from left). Thus, WT1 repression of AR downstream targets cannot be mediated by protein-protein interactions in these co-transfection assays.

TECHNICAL OBJECTIVE III. Establish a mouse model for prostate cancer progression.

We have established stably transfected lines over-expressing the (-/-) isoform of wild-type WT1 or the DDS zinc finger mutant and have determined their growth characteristics and are using them to establish a mouse model of prostate cancer progression. While no obvious morphological differences have been observed between WT1, DDS and vector transfected lines, we do observe a slight growth suppression of the WT1 transfected lines. Thus, we anticipated a slight increase in the normally long tumor growth latency period in vivo (12). To date we have injected several LNCaP cell lines stably transfected with WT1-FLAG expression constructs (obtained from our collaborator, Dr. C. Roberts). We observed tumor formation within 4-8 weeks in nude mice inoculated subcutaneously with matrigel suspended cells (1:1). We have examined several cell lines of: LNCaP mock transfected, vector transfected, WT1 (-/-) and DDS (-/-) transfected cells.

The parental or unaltered LNCaP cell lines are androgen responsive, PSA expressing cells that contain mutant AR with slightly altered hormone affinity. Both the parental and the LNCaP control (or mock treated) cell lines were injected into nude mice leading to large tumor formation beginning at 4 weeks and peaking at 6-8 weeks after injection. This is in contrast to the suppression of tumor development following injection of cells engineered to express WT1. After a long lag-time (8-10 weeks) we began to see a few small tumors forming (peaking at 12-14 weeks). These subcutaneous tumors were severely reduced in both size and number compared to the tumors formed by injection of the parental or untransfected LNCaP cell lines. We expect to find they express AR in the absence of WT1.

While the results of over-expression of WT1 in prostate tumor cell lines clearly showed that WT1 suppresses tumor formation, these results did not reveal the mechanism of suppression. Since a major function of WT1 protein is to bind DNA and repress RNA transcription, the tumor suppression data initially suggested that tumor suppression was mediated by repression of AR transcription by WT1, preventing androgen dependent tumor cell growth. However, the prostate tumor cell lines stably expressing the mutant form of WT1 also appeared to suppress tumor growth in vivo, that is, we unexpectedly discovered a discordance between transcriptional repression and tumor suppression. This unusual mechanism of tumor growth suppression cannot readily be explained, as the mutant form of WT1 is unable to bind DNA at the AR promoter and unable to repress AR expression. Thus we hypothesize that some other mechanism of AR repression may have occurred, possibly via altered protein-protein interactions.
Principal Investigator Fraizer, Gail C.

Currently we are comparing the “suppressed” or “delayed” tumors established in mice injected with prostate cancer lines stably expressing WT1 and the rapidly growing large tumors established in mice injected with the control (or mock treated) LNCaP cell lines. This comparison of tumor characteristics, as well as, WT1 and AR expression in these “revertant” or late developing tumors is nearing completion and a manuscript is in preparation and is expected to be submitted soon.

KEY RESEARCH ACCOMPLISHMENTS

We have developed a novel mechanism of blocking prostate tumor cell growth in mice by taking advantage of a regulatory mechanism involving the tumor suppressor gene, WT1, a transcription factor able to block the androgen signaling pathway. Specifically, cultured prostate cancer cell lines expressing high levels of WT1 suppress tumor formation in immunoincompetent (or nude) mice. We have engineered these prostate cancer cell lines so that they are stably expressing either the transcriptionally active isoform of WT1 or its mutant counterpart, that is unable to bind and repress AR. This mouse model for prostate cancer development will allow me to address several fundamental questions central to prostate cancer diagnosis and therapy.

Summary of research accomplishments to date:

1. WT1 protein is strongly expressed in androgen insensitive prostate cancer cell lines, but not in androgen responsive prostate cancer cell lines.
2. WT1 protein is focally expressed in both nucleus and cytoplasm of high grade human prostate tumor sections and in poorly differentiated mouse tumors.

3. LNCaP cell lines engineered to express wild-type and mutant WT1 have been established and injected into mice.

4. Tumor incidence, volume and time to develop have been determined for androgen sensitive "parental" LNCaP cell lines and control (mock treated) LNCaP cell lines.

5. Early development and resorption of small masses, and the eventual recurrence and outgrowth of a few delayed or "suppressed" tumors has been observed for the WT1 transfected LNCaP cell lines.

6. Histopathology of early developing masses has been initiated, with early "suppressed" or "resorbed" tumor injection sites demonstrating no evidence of tumor development.

7. Immunohistochemistry and histopathology of late developing tumors is in progress, with large tumor masses demonstrating excessive hemorrhagic regions.

REPORTABLE OUTCOMES:

Academic Appointment (with promotion to tenure track):
Assistant Professor, Department of Biological Sciences, Kent State University, OH

Funded Collaborative Grants:
Medical and Health Research NWO, P.I. Guido Jenster, Erasmus University Rotterdam, Netherlands

Manuscripts Published and Submitted:


Fraizer, G., De La Cerda, J., Diaz M., "Wilms' Tumor Suppressor Gene WT1 Suppresses tumor formation by Prostate Cancer Cells" (Manuscript in Preparation).

Presentations and Published Abstracts:

Invited Seminars:
Department of Biological Sciences, *Kent State University*, Kent, OH, 3/7/01, "Can the tumor suppressor, WT1, disrupt the androgen signaling pathway in prostate cells?"

Department of Biological Sciences, *Wright State University*, Dayton, OH, 2/12/01

Department of Urology Research Seminar, *U.T. M.D. Anderson Cancer Ctr.*, TX, 7-7-99; "Role of WT1 in prostate cancer progression"

**Abstracts:**

**Fraizer, G., De La Cerda, J., Diaz M.**, Tumor formation by Prostate Cancer Cells is Suppressed by WT1 " American Association for Cancer Research, San Francisco, 2002 (submitted 11-7-01)

Gail Fraizer, Miguel Diaz, Annamaria Zaia, Ryuji Shimamura, Grady Saunders


**Development of Cell Lines**

WT1-FLAG stably transfected LNCaP cell lines have been established, as have DDS- mutant WT1-FLAG and FLAG-vector transfected LNCaP lines.

**CONCLUSIONS**

If WT1 transcriptionally represses AR gene expression in WT1-producing prostate tumor cells, two possible predictions can be made. 1) Overexpression of a tumor suppressor and subsequent repression of the AR pathway could lead to tumor suppression. Or 2) If suppression of AR confers a growth advantage selecting AI tumor cells, then we would predict an outgrowth of aggressive hormone refractory tumor cells causing tumor progression. In the latter case, growth advantages conferred by the repression of the AR by WT1 lead to androgen independence. The hypothesis that WT1 can mediate androgen independent tumor progression was tested using both in vitro and in vivo approaches and we assessed both the significance of WT1 expression in prostate tumors and the mechanism of AR repression. The first two specific aims were performed concurrently and in the recent work accomplished the third specific aim using the stably transfected LNCaP lines were able to establish a mouse model of prostate cancer. We are currently determining whether any tumors established by stably transfected LNCaP lines will progress to androgen independence. This will support our hypothesis that WT1 represses AR expression in vivo. Together work has demonstrated that WT1 alters the androgen signaling pathway and thereby the progression of prostate tumors to androgen independence.

**References**

3. Jenster, G., deRuiter, P.E., vanderKorput, H., Kuiper, G., Trapman, J., and Brinkmann, A.O. Changes in the abundance androgen receptor isotypes: effects of ligand treatment, glutamine-


APPENDIX
Figure Legends

Figure 1. WT1 expression in prostatic epithelium
Antibody staining of paraffin embedded tissue sections from patients with high grade prostate cancer. These tissues were obtained beyond that needed for diagnosis and were removed with informed consent. Slides were incubated with polyclonal WT1 Ab (C19, Santa Cruz) and HPO-conjugated anti-rabbit Ab. Tissues were counterstained with DAB and bluing solution and then mounted. Slides were examined and photographed digitally at 150x (A, B) to 300x (C,D) magnification. These sections displayed focal epithelial cytoplasmic staining, sometimes accompanied by nuclear staining but often not. Both dark brown staining nuclei and light brown staining cytoplasm are visible in prostate tumor epithelial cells.

Figure 2. WT1 expression in Normal Kidney
Antibody staining of paraffin embedded tissue sections from normal adult mice were incubated with polyclonal WT1 Ab (C19, Santa Cruz) and HPO-conjugated anti-rabbit Ab. Tissues were counterstained with DAB and bluing solution and then mounted. Slides were examined and photographed digitally at 150x (A, B) magnification. We observed dark brown staining nuclei and light brown staining cytoplasm in both murine glomerulii and renal tubules. Specificity of staining was confirmed by treating Ab with WT1 blocking peptide prior to incubation of the polyclonal Ab (data not shown). Antibody staining of paraffin embedded tissue sections from patients undergoing nephrectomy were obtained beyond that needed for diagnosis and were removed with informed consent. Slides were incubated with polyclonal WT1 Ab (C19, Santa Cruz) and HPO-conjugated anti-rabbit Ab. Tissues were counterstained with DAB and bluing solution and then mounted. Slides were examined and photographed digitally at 300x (C,D) magnification. These sections displayed nuclear staining restricted to podocytes, but also clear evidence of cytoplasmic staining in the adjacent tubular epithelial cells (not shown). Similarly both cytoplasmic and nuclear staining has been observed with the monoclonal Ab (data not shown).
WT1 expression in prostatic epithelium

Fig1

A.  
B.  
C.  
D.
WT1 expression in normal kidney

Fig 2
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