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TITLE: Accelerated Tumor Cell Death by Angiogenic Modifiers

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Department of the Army position, policy or decision unless so
designated by other documentation.
Purpose: Because of the inherent stability of endothelial cells and the importance of
this cell type for the proliferation of both localized and disseminated cancers, anti-
angiogenic therapy is an attractive target for the investigation and further development
of novel therapeutic approaches to cancer metastasis. The purpose of this proposal is to
assess molecular mechanisms underlying synergy between squalamine and vascular
endothelial growth factor(s) (VEGF).
Table of Contents

Cover .................................................................................................................. 1
SF 298 ............................................................................................................... 2
Table of Contents ............................................................................................ 3
Introduction ..................................................................................................... 4
Body .................................................................................................................. 4-5
Key Research Accomplishments ...................................................................... 5
Reportable Outcomes ....................................................................................... 5
Conclusions ...................................................................................................... 5
References ......................................................................................................... 6
Appendices ......................................................................................................... 6
ABSTRACT

Purpose:
Because of the inherent stability of endothelial cells and the importance of this cell type for the proliferation of both localized and disseminated cancers, anti-angiogenic therapy is an attractive target for the investigation and further development of novel therapeutic approaches to cancer metastasis. The purpose of this proposal is to assess molecular mechanisms underlying synergy between squalamine and vascular endothelial growth factors (VEGF).

Scope:
Angiogenic modifier including VEGF and withdrawal of androgen were used to “injure” the endothelial cells in an effort to enhance squalamine induced tumor shrinkage in animal models of human prostate cancer. The cellular mechanisms underlying tumor cell death in these models will be evaluated.

Major Findings/Progress:
We have completed in vitro studies to show synergism between squalamine and VEGF in promoting the death of human prostate cancer cell lines. This synergism appears to be dependent upon the prostate cancer cell background in which LNCaP and C4-2 but not PC-3 and DU-145 cells are highly sensitive to squalamine-VEGF synergy in inducing cell kill. This synergistic action between squalamine and VEGF appears to interfere with the ability of VEGF to stimulate prostate cell attachment to substratum, laminin. Our next goal is to find out the particular downstream pathway that mediates extracellular matrix-intracellular signaling cascade that may be the underlying molecular mechanism that triggers squalamine-VEGF synergism, thus causing human prostate cancer cell death in vitro. We have confirmed such synergism in vivo in two tumor models: 1) the LNCaP prostate cancer progression model and 2) CWR22 model.

Subject Terms:
Prostate cancer metastasis, angiogenic modifiers, squalamine, vascular endothelial growth factor or VEGF, animal model for prostate cancer, cell death or apoptosis

Introduction:
Cancer growth requires the supply of oxygen and nutrients. Although endothelial cells are responsible for forming new vasculatures for the delivery of oxygen and nutrients and removal of metabolic waste from tumors, it becomes apparent that similar molecular mechanisms may be operating between endothelial and tumor cells.

Body:
The emphasis of the current proposal is three fold. First, we propose to extend our in vitro observations of squalamine-VEGF synergism to the in vivo mouse model of human prostate cancer using both LNCaP progression and CWR22 models to evaluate if androgen-independent progression of such models may be affected by the administration of squalamine in castrated mice. Second, we propose to evaluate the cellular response of prostate cancer cells to VEGF and squalamine, focusing on the cytoskeletal organization changes in response to squalamine and
VEGF. Possible alterations of cell surface integrin isotypes that may modify integrin-matrix interaction will also be evaluated. Third, we propose to evaluate the downstream intracellular signaling pathways that may be altered by squalamine and/or VEGF. A comparative study will be conducted to evaluate signal transduction in LNCaP progression model and the androgen-independent DU-145 and PC-3 cells. Signal cascade through the VEGF receptor, focal adhesion kinases, FAK, Pyk2, and MAP kinase associated ERK and JNK pathways will be studied in relation to apoptosis of prostate cancer and endothelial cells in vitro.

**Key Research Accomplishments**

- We have observed synergy between VEGF and squalamine in vitro in cultured human prostate cancer cell lines. Results of these studies indicated that such synergism may be cell background dependent. For example, LNCaP and C4-2 but not PC-3 or DU-145 cells are highly sensitive to squalamine-VEGF synergy in cell kill in vitro. This cell kill appears to be dependent upon VEGF elicited downstream cell signaling, because such synergism can be effectively blocked by the co-presence of VEGF antibody.

- We have evaluated squalamine effect in animal models by the application of another angiogenic modifier, the removal of testicular androgen through castration, which enhances the destruction of endothelial cells. Using both LNCaP and CWR22 models, we have observed the synergism between castration and squalamine, which caused remarkable cell death and tumor shrinkage in these models. We found that such synergism between squalamine and castration only occurred if animals were castrated early, rather than later, upon squalamine application.

These results together imply that at the time of castration, squalamine could have a beneficial effect in causing accelerated tumor cell death in patients with androgen-independent prostate cancer.

**Reportable Outcomes:**


- A review on molecular mechanism of cotargeting tumor stroma and epithelium has been accepted for publication in J. of Cellular Biochemistry, 2001.

Both of these references are attached with this report.

**Conclusions:**

- Synergism between squalamine and VEGF was demonstrated in selective prostate cancer cell lines.

- Vascular endothelial cell destruction in castrated animals can be applied as an additional angiogenic modifier to synergize with squalamine-induced tumor cell kill. The “timing” of squalamine application is extremely important, and is highly effective at the time of castration when vascular endothelial cell destruction is maximized, inducing tumor cell death in both LNCaP progression and CWR22 human prostate tumor models.
References:
None

Legends and Figures

Figure 1 – Synergism between squalamine and VEGF was found in selective prostate cancer cell lines in vitro. LNCaP and its androgen-independent C4-2 derivative were highly sensitive to this synergistic interaction between VEGF and squalamine, whereas the androgen-independent PC-3 and DU-145 cells were not.

Figure 2 – Squalamine application to animals castrated early was shown to be highly effective in eliminating the growth of LNCaP tumors in vivo. Squalamine appeared to be highly effective only when it was applied immediately after castration in animals bearing LNCaP tumor in vivo.

Figure 3 – Synergistic interaction between squalamine and castration was also noted in CWR22 model where squalamine application at the time of castration eliminated the growth of CWR22 tumors in animals (panel A). Although the tumor volume appears to decrease upon the treatment by squalamine, the tumor appears to persist because of the secretion of serum PSA in this model (panel B).

These results indicate that it is also possible that squalamine may induce prostate cancer differentiation by promoting PSA synthesis and production in CWR22 models.

Appendices:
I. Figure 1
II. Figure 2
III. Figure 3
VI. Curriculum Vitae
PROLIFERATION OF PROSTATE TUMOR CELL LINES IN THE PRESENCE OF SQUALAMINE, VEGF, AND ANTIBODIES AS MEASURED BY THYMIDINE INCORPORATION

Figure 1
Serum PSA After Castration + Squalamine
(Applied at Time of Castration)

- Control 1
- Control 2
- Tx Animal 1
- Tx Animal 2
- Tx Animal 3
- Tx Animal 4

Serum PSA After Castration + Squalamine
(Applied After PSA Rebound ≥ 10 ng/ml)

- Control 1
- Control 2
- Tx Animal 1
- Tx Animal 2

Figure 2
Tumor Volumes of Squalemine-Treated and Control CWR22 Tumor

Figure 3A
Serum PSA (ng/ml)

Time

PSA Changes in Squalarine-Treated and Control CW22 Tumors
Molecular Basis of Co-targeting Prostate Tumor and Stroma

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Abbreviation: Androgen Independent (AI), human osteocalcin (hOC), bone sialoprotein (BSP), osteoprotegerin (OPG), osteonectin (ON), osteopontin (OPN), prostate-specific antigen (PSA), insulin-like growth factor binding protein (IGFBP), transforming growth factor beta (TGFβ), parathyroid hormone related protein (PTHrP), insulin-like growth factor (IGF), androgen responsive elements (AREs), androgen responsive element enhancer core (AREc), androgen receptor (AR), electromobility shift assay (EMSA), osteoblast-specific element (OSE), extracellular matrix (ECM), bone morphogenic proteins (BMPs), matrix metalloproteinases (MMPs), tisseur inhibitor of metalloproteinase (TIMP)
Abstract

Hormone-refractory prostate cancer is one of leading cause of cancer death in Northern American men. The lethal phenotypes of human prostate cancer are characterized by progression to androgen-independence (AI) and a propensity to form osseous metastases. In ~80% of cases, prostate cancer colonizes bone and elicits a characteristic osteoblastic reaction. The bone metastases are initially sensitive to androgen deprivation treatments, but with time the cancer will eventually progress into an AI stage for which there is currently no effective treatment. Once initial hormonal therapy has failed, median survival of prostate cancer patients with bone metastases is less than 1 year (Tu, et al. 2001). Novel therapeutic and preventive strategies are needed to decrease morbidity and mortality of this disease.

In this review, we describe a novel gene therapy strategy in which tissue-specific and tumor-restrictive promoters are employed to drive the expression of therapeutic genes to co-target both the stromal and epithelial compartments in the treatment of prostate cancer bone metastases (Matsubara, et al. 2001). The rationale behind this strategy is based on the evidence that reciprocal stromal-epithelial interactions are important for prostate tumorigenesis and that the osteomimetic properties of prostate cancer cells contribute to prostate cancer progression (Koeneman, et al. 1999).

Regulation of tissue-specific and/or tumor-restrictive promoters, prostate-specific antigens and human osteocalcin, in androgen-independent bone metastatic prostate cancer cells.

Despite the common occurrence of prostate cancer, little is known about the underlying mechanisms responsible for prostate cancer growth, androgen-independent
progression, and acquisition of bone metastatic potential. To gain new insights into the molecular mechanisms of AI progression, our lab focused on defining the transcriptional regulation of human osteocalcin (hOC) and prostate specific antigen (PSA) expressions in AI prostate cancer cell lines. A strategy was outlined to identify the transcription factors involved in regulating the critical regions of the promoters of these genes, and their roles in tumorigenesis (Yeung, et al. 2000; Yeung, et al. 2001). The ultimately goal of our study is to devise strategies for expressing therapeutic genes under the control of tissue-specific promoters to treat prostate cancer bone metastasis effectively.

**PSA.** Due to its tissue-specific expression pattern, PSA is the most widely used serum marker for the diagnosis and management of prostate cancer. Since the expression of PSA is tightly regulated by androgen, an increase in serum PSA in androgen-depleted patients could indicate the development AI tumors. In addition to being dysregulated during cancer progression, PSA, a serine protease, is believed to contribute significantly at the molecular level to prostate cancer progression and metastasis via its protease activity. For example, the cleavage of IGFBP [Cohen et al., 1992] and TGFβ by PSA could increase the bioavailability of the mitogen IGF; and activating the latent form of growth factor TGFβ, enhancing matrix turnover, tumor angiogenesis and immune evasiveness. Moreover, the inactivation of PTHrP by PSA [Cramer et al., 1996] could tip the balance toward osteoblastic reactions in prostate cancer bone metastasis.

PSA is one of the most widely studied prostate genes. Numerous groups focused on the androgen regulation of PSA have identified several androgen-responsive elements (AREs) [Cleutjens et al., 1996; Huang et al., 1999] and a tissue-specific enhancer core (AREc) [Cleutjens et al., 1997] in the promoter. To identify transcription factors that
activate PSA promoter independent of androgen, we performed promoter deletion studies and identified two AI regulatory regions (AREc and pN/H), which suggested the involvement of two distinct pathways in androgen-independent regulation of PSA expression in AI C4-2 cells [Yeung et al., 2000]. One pathway clearly involved androgen receptor (AR) because the binding of AR to the AREs within the AREc appears to be prerequisite for the high activity of the AREc and PSA promoter in C4-2 cells. It is plausible that AR is activated through growth factor mediated signaling pathways independent of androgen. Growth factors like IGF-1, EGF and KGF were reported to induce AR-mediated gene transactivation in the absence of androgen [Culig et al., 1994]. These growth factors could signal through the PKA [Nazareth and Weigel, 1996] and/or PKC [Sadar, 1999] cascade in which AR activity is enhanced either by modification of AR itself [Ikonen et al., 1994] or by enhancing the interactions between AR and its coactivators [Yeh et al., 1999]. The fact that AREc is highly tissue specific and contains other cis-elements suggests [Cleutjens et al., 1997] that in addition to AR, other prostate specific co-activators are also involved in the AI regulation of AREc in C4-2 cells. Activated AR is one of the key transcription factors that interact with other prostate-specific transcription factor(s), and together they associate with the AREc and assemble into a highly active AREc enhanceosome complex [Huang et al., 1999] in C4-2 cells. The aberrant activation of AR and/or its co-activators by growth factors could provide growth advantage to cancer cells in an androgen-depleted environment, and this could be one of the important mechanisms that contribute to prostate cancer progression.

The other pathway mediated by pN/H appears to involve an unknown 45-kDa prostate specific transcription factor (p45). Through DNaseI footprinting and linker-
scanning mutagenesis approaches, we showed that p45 binds to a 17 bp site (RI) in the pN/H region and regulates the basal PSA promoter activity. Even though the RI element shares high homology with the SP-1 consensus site, p45 migrates differently from the Sp-1 transcription factors in EMSA, so it does not seem to belong to the Sp-1 transcription factor family. Furthermore, the observed absence of RI-p45 complex in PC3 cells and a higher amount of p45 complex in C4-2 than in LNCaP suggest that the expression of p45 is cell type-specific. Since the addition of androgen could not enhance pN/H activity, p45 does not seem to be regulated by androgen or AR. The fact that C4-2 nuclear extract consistently showed a higher level of RI-p45 complex in EMSA implies that increased association of p45 to RI site is a possible mechanism by which PSA promoter is activated in C4-2 cells in an AR and androgen independent manner [Yeung et al., 2000]. Further efforts to identify p45 may provide additional information on the AI growth and metastasis of prostate cancer cells. For example, P45 may regulate prostate cancer growth by binding to the promoters of the growth-related genes that contain the RI cis-element. The identification of p45 could also be of great clinical significance. Since there is a higher level of p45 in AI prostate cancer cells, new diagnostic approaches based on screening for the level of p45 in tumor cells could provide valuable information for staging the cancer and determining the progression and metastatic potential of the disease.

\textit{hOC}. Osteocalcin (OC) is one of the major non-collagenous bone matrix proteins expressed in bone. OC expression is transcriptionally regulated by vitamin D and was thought to be limited to cells of the osteoblast lineage. Many regulatory elements have been mapped to the proximal region of the OC promoter. These include OSE1, OSE2
and AP-1/VDRE. OSE1 and OSE2 were first identified in mouse OC promoter and they are associated with osteoblast-specific transcription factors, OSF1 and Runx2, respectively. Vitamin D receptors were shown to bind the VDRE in the proximal promoter, and its activity is tightly regulated by the members of the AP-1 family that bind the contiguous AP-1 site. In proliferating osteoblasts, c-Fos and c-Jun heterodimers were shown to block the binding of VDR and suppress the rat OC promoter activity, while the expression of Fra2 and JunD in the post-proliferated osteoblasts induces rat OC promoter activity by facilitating VDR/RXR binding [Lian et al., 1998].

Human prostate cancer cells with a propensity to metastasize to the skeleton and prostate cancer tissue specimens with increased Gleason scores reveal that prostate cancer cells synthesize, secrete and/or deposit large amounts of non-collagenous bone matrix proteins such as OC, osteopontin (OPN), osteoprotegerin (OPG), osteonectin (ON) and bone sialoprotein (BSP). In a recent study, we demonstrated that OC protein was prevalently expressed in primary prostate cancer (85%), in prostate cancer lymph node (100%) and in bone metastasis specimens (100%) [Chung and Zhau, 2001; Matsubara, et al. 2001]. Since OC was not expressed in normal human prostate gland, the predominant expression of OC and other bone matrix proteins in advanced prostate cancer imply a role in prostate cancer survival and growth in the bone environment. OC secreted by prostate cancer cells can complex with ECM and calcium, serving as a chemoattractant for recruiting osteoblasts and/or osteoclasts, which initiate bone remodeling [Glowacki and Lian, 1987]. This may contribute to the osteotrophic characteristics of prostate cancer bone metastasis. The discovery of hOC expression in prostate cancer specimens has opened new windows on biology and therapy of prostate
cancer bone metastasis. In an effort to understand the osteomimetic properties of prostate cancer cells, we used PC3 cells to investigate the regulation of OC expression. PC3 cells are AI, obtained from bone metastatic lesions of a prostate cancer patient. PC3 cells share with mature osteoblasts the unique feature of synthesizing and depositing a large amount of OC. In our study, we demonstrated three groups of transcription factors, Runx2, JunD/Fra-2 and Sp-1, responsible for the high hOC promoter activity in PC3 cells by binding to the OSE2, AP-1/VDRE and OSE1 elements, respectively. Furthermore, the functional hierarchy of OSE1, OSE2 and AP-1/VDRE was established in the regulation of hOC promoter activity (OSE1>AP-1/VDRE>OSE2) in PC3 cells. We also generated an artificial hOC promoter consisting of dimers of the three elements with significantly higher activity than the wild type promoter. Among the three groups of transcription factors, the expression levels of Runx2 and Fra-2 are higher in the OC-positive PC3 cells and osteoblasts, compared to the OC-negative LNCaP cells. Interestingly, unlike the mouse OC promoter, the OSE1 site in hOC promoter is regulated by the members of Sp-1 family instead the osteoblast specific factor, Osfl [Yeung, et al. 2001]. Therefore, by expressing the osteoblast-specific transcription factor Runx2 and differentially up-regulating the prominent Ap-1 factor, Fra2, in mature osteoblasts, PC3 cells have acquired the phenotypes of osteoblasts. The interplay and coordination among these transcription factors provides the molecular basis for AI prostate cancer cells behaving like mature osteoblasts. The balance and activity of these transcription factors is significant in conferring the osteolytic/osteoblastic phenotype of prostate cancer cells frequently observed in metastatic skeletal lesions.
Osteoblast-specific transcription factors and prostate cancer bone metastasis

Besides OC, the expression of other bone matrix proteins such as OPG, OPN, BSP was also found in bone metastatic prostate cancer cells. It appears that a switch of gene transcription occurs in prostate cancer bone metastasis that allows the cancer cells to acquire an osteoblast phenotype and presumably leads to their colonization in the skeleton. The roles of these bone matrix proteins are unclear, but based on their functions in bone development and remodeling, one can postulate that OPN expression in prostate cancer cells could facilitate their adhesion and migration [Denhardt et al., 2001] to the bone matrix and participate in subsequent bone “pitting” and steps involved in osteoid mineralization. Since BSP has been shown to be crucial for the expression of osteoblastic phenotypes in cultured bone marrow cells [Mizuno et al., 2000], overexpression of BSP by metastatic prostate cancer cells could enhance their attachment to osteoblasts and osteoclasts and stimulate osteoblast differentiation. The expression of OPG in prostate cancer cells may lead to the overall repression of osteoclast activity and a shift of bone remodeling toward osteoblast activity in bone metastasis [Simonet et al., 1997]. Therefore, the osteomimetic properties of prostate cancer cells in theory could allow them to invade, adhere, survive and grow better in the bone microenvironment [Koeneman et al., 1999]. Recent study using the osteotropic prostate cancer cells (C4-2B) demonstrated that in addition to having an osteoblastic phenotype, C4-2B cells could produce hydroxyapatite mineral in vitro; and stimulate osteoblasts to initiate mineralization in the bone [Lin et al., 2001]. The increased expression of bone matrix proteins by prostate cancer cells in skeletal metastatic sites may underlie the predilection...
of prostate cancer for bone and explain the mineral formation found in osteoblastic lesions.

Even though tumor metastases in patients with prostate cancer are predominantly osteoblastic, evidence from osteoblastic metastasis animal model indicates that an initial phase of bone resorption precedes new bone formation [Yi et al., 2000]. The importance of osteoclast activity in osteoblastic metastasis was further supported by recent findings that bisphosphonates (an inhibitor of osteoclast activity) could effectively alleviate bone pain in patients with metastatic prostate cancer [Adami, 1997]. Since bone is an abundant source of growth factors such as TGFβ, bFGF, IGFs, PDGFs, and BMPs, osteoclastic bone resorption could release and/or activate growth factors from the bone matrix, which could serve as paracrine mediators to stimulate tumor growth [Nakase et al., 1994]. Conceivably, prostate cancer cells could modify the bone microenvironment in a reciprocal manner by secreting soluble factors to promote osteoclasts activity, which in turn makes the bone stroma conducive for the growth and survival of prostate cancer cells. Therefore, the reciprocal interactions between prostate cancer cells and their supporting stroma are essential in promoting cancer progression, invasion and metastasis.

Fra2 is one of the transcription factors that may be involved in bone metastasis. We showed that a higher level of Fra2 was found in AI, bone metastatic prostate cancer cells compared to non-tumorigenic LNCaP prostate cancer cells. It is not clear how Fra2 is upregulated in prostate cancer cells. However, growth factor like TGFβ was known to increase the expression and activity of AP-1 factors. The binding of TGFβ to its cell surface receptor could trigger a cascade of signaling pathways, including the MAPK pathways. MAPK pathways were indicated to regulate both the amounts and
transactivating activity of the AP-1 factors in a stimulus-specific manner. As an oncoprotein, high level of Fra2 could contribute in numerous ways to increase proliferation, invasion and metastasis of cancer cells during disease progression. For example, over expression of Fra2 reportedly represses the tissue inhibitor of metalloproteinases (TIMP1) promoter activity by forming heterodimers with JunD, which are less active than JunD homodimers [Smart et al., 2001]. The repression of TIMP1 could allow the accumulation of MMPs and lead to increased invasiveness of the tumor. Furthermore, avian primary cells transformed by Jun/Fra2 showed increased anchorage independent growth by their ability to form colonies in soft agar [van Dam et al., 1998]. Therefore, these results are consistent with the notion that enhanced level of Jun/Fra2 confers cell motility and invasiveness.

Runx2 is an osteoblast-specific factor highly expressed in the bone metastatic prostate cancer cells. The expression of this osteoblast-specific factor is believed not only to impart the osteomimetic characteristics of prostate cancer cells, but it also has a significant role in carcinogenesis. BMPs and TGFβ are two of the growth factors shown to activate the transcription of Runx2 in bone cells [Bae et al., 2001]. As a member of the TGFβ family, BMPs are known to bind receptors and to induce a cascade of events leading to phosphorylation of Smad proteins. Upon phosphorylation, Smad translocate into the nucleus to interact with numerous transcription factors, which then regulate gene expression. Smad5 is believed to be responsible for activating the Runx2 gene transcription [Bae et al., 2001]. Other local factors such as hedgehogs [Yamaguchi et al., 2000] and IGF-1 [Yeh et al., 1997] were shown to synergistically enhance BMPs actions. For example, recombinant Shh and conditioned media collected from Shh-or Ihh-
overexpressing chicken embryonic fibroblasts can increase BMP2, which induces the downstream OC mRNA level [Kinto et al., 1997]. Therefore, hedgehog-signaling pathway may act cooperatively with the BMP signaling pathway in regulating the transcription of Runx2.

Some of the early gene targets of BMP signaling pathway include the N- and E-cadherins. Recombinant human BMP-2 (rhBMP2) rapidly and transiently increases N- and E-cadherin mRNA and protein levels in human osteoblasts. In addition, the N- and E-cadherin antibodies have inhibitory effects on both the basal and induced Runx2 mRNA levels, and abolish the rhBMP2-induced OC mRNA levels [Hay et al., 2000]. The importance of cell-matrix interaction in the regulation of Runx2 expression is further demonstrated by the fact that α2-integrin-collagen interaction is required for activation of Runx2 and induction of osteoblast-specific gene expression. Induction of matrix synthesis is accompanied by a dramatic increased in the binding of Runx2 to OSE2, suggesting that ECM synthesis up-regulates and/or activates Runx2. Furthermore, blocking of integrin-type I collagen binding prevents activation of the OC promoter by ascorbic acid and suppresses binding of Runx2 to OSE2 site [Xiao et al., 1998]. It is not clear how ECM activates Runx2; it is possible that a post-translational pathway or accessory factor(s) are involved in the regulation. Like the AP-1 factors, the MAPK pathway also regulates Runx2. An activated recombinant MAPK was shown to phosphorylate a Runx2 fusion protein in vitro [Xiao et al., 2000]. Therefore, the phosphorylation of Runx2 through the MAPK pathway is essential for responsiveness of osteoblasts to ECM signals and contributes for osteoblast-specific gene expression.
Evidence from tooth development study indicated that Runx2 regulates key epithelial-mesenchymal interactions that control advancing morphogenesis and differentiation of the epithelium. Runx2 expression in the mesenchyme is intimately associated with epithelial-mesenchymal interactions during tooth development and is affected by epithelial signals. Runx2 expression in the mesenchymal is controlled by signals emanating from the epithelium. In turn Runx2 regulates the expression of mesenchymal molecules that act reciprocally on epithelium to control the differentiation of the enamel organ [D'Souza et al., 1999]. It is plausible that the growth factors release from the bone matrix upregulate the expression of Runx2 in prostate cancer cells, as a result triggering a "ping-pong" mechanism (Fig 1) in which the high level of Runx2 allows expression of gene products in the cancer cells that could modify the surrounding stroma and eventually lead to enhance tumor growth.

*Potential use of tissue-specific and tumor-restrictive promoters in co-targeting stromal-epithelial interactions in prostate cancer.*

Due to the poor response rate of previously treated patients with relapsed prostate cancer to conventional radiotherapy, surgery, or chemotherapy, our laboratory has examined some of the unique biological characteristics associated with prostate cancer and its relationship with prostate or bone stromal cells in the effort to formulate novel targeting strategies. Traditional therapy for prostate cancer has targeted only the malignant epithelial cell. Because of the osteomimetic properties of prostate tumor epithelial cells in bone, we proposed a novel co-targeting strategy incorporating an adenoviral gene therapy approach to the treatment of both localized and metastatic prostate cancers. This approach involves the use of bone matrix protein promoters, such
as OC to drive the expression of therapeutic genes co-targeting tumor epithelium and its supporting stroma to maximize tumor cell-kill.

Gene therapy is a newly developed technology based on understanding the genetic and molecular defect of disease. In dealing with cancer, toxic gene therapy is most commonly employed clinically. This kind of therapy involves suicide gene/prodrug systems, in which a suicide gene is delivered to cancer cells to activate a non-toxic prodrug in cells and thus selectively kill the tumor cells while sparing surrounding normal cells from tissue damage.

The concept of delivery and expression of therapeutic genes to tumor cells through the use of tissue-specific promoters has been well recognized. This approach decreases the adverse effects of the therapeutic genes on normal cells and increases the specificity and efficiency of gene transfer to tumor cells. Attractive approaches for the treatment of bone metastasis could be developed through our understanding of the molecular mechanism underlying the acquisition of osteomimetic properties by prostate cancer cells. The discovery of common bone matrix proteins synthesized by both prostate cancer cells and osteoblast cells, raise the possibility of employing the promoters of these proteins to drive the expression of therapeutic genes in both prostate cancer and bone stromal compartments for therapeutic gains. In this context, our laboratory has developed an OC-based toxic gene therapies for the treatment of prostate cancer metastasis. We envision the use of OC promoter to drive the expression of therapeutic genes in proliferative cellular compartments to eradicate the growth of both prostate cancer cells and their supporting stroma. This approach could achieve a higher degree of efficacy than the conventional approaches of blocking bone proliferation by
bisphosphonate or halting the growth of prostate cancer cells with non-selective chemotherapeutic agents or radiation. The effectiveness of this form of gene therapy can be explained by the uniqueness of the OGC promoter, which presumably drives the expression of toxic genes in at least three cellular compartments: tumor epithelium, bone stroma, and vascular endothelial pericytes (Chung and Zhau, 2001). The rationale for co-targeting bone stroma and epithelium was supported by our preliminary data showing that the growth of prostate cancer cells was enhanced when they were co-cultured with bone stroma cells. The destruction of bone stromal cells alone was sufficient for killing the co-cultured prostate cancer cells. Furthermore, a recent study by Tu et al [Tu et al., 2001] demonstrated that bone targeting with chemoinduction plus Strontium 89 (Sr89) significantly improved patient survival in comparison to Sr89 or chemoinduction alone. The improved survival of patients subjected to co-targeting of bone stroma and epithelium by chemoinduction and Sr89 dramatizes the advantages of co-targeting, and supports our strategy of co-targeting tumor epithelium and bone stroma by gene therapy, which could eventually result in improved survival of patients with metastatic bone disease.

Summary

The study of tissue-specific promoters such as PSA and hOC in AI prostate cancer cells has led to the identification of some of the transcription factors (AR, p45, Runx2, and Fra2) that are dysregulated during cancer progression. Although the roles of these transcription factors in tumorigenesis are unclear, it is feasible to use approaches like ribozyme and antisense to block the expression of these transcription factors and determine their respective effects on tumor growth and invasion. Once the functions are
established, we believe effective strategy employing tissue-specific and tumor-specific promoters to control gene expression in selective cell types could be designed to target these transcription factors for cancer gene therapy.

In the past few years, our laboratory has successfully developed a human prostate cancer skeletal metastasis model for the study of the biology and therapeutic targeting stromal-epithelial interactions. We believe the inter- and intra-cellular communication loops between prostate epithelium and its supporting stroma provide additional attractive therapeutic targets for prostate cancer treatment. Results from preliminary animal experiments support the concept that maximum prostate tumor destruction may be achieved by targeting both tumors and their supporting stroma compartments. The development of bone matrix protein promoters, such as OC, driving gene expression in both prostate epithelial and stromal compartments has allowed future explorations into prostate cancer/stroma interactions and signal cascades involving growth factor/growth factor receptor and cell-matrix interactions. Since bone matrix proteins have been implicated in prostate cancer progression, OC promoter could be valuable for delivering genes into both epithelium and stroma compartments to block the expression/secretion of critical factors that affect the growth and survival of cancer cells in vivo. Refining our understanding of the regulation of PSA and bone matrix proteins in prostate cancer cells at the molecular level could facilitate the future development of new molecular targets for the prevention and treatment of not only prostate cancer skeletal metastasis but also localized and invasive prostate cancers.

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Reference:


Figure 1. A “Ping-Pong” Mechanism of Transcription Factor Activation Through Stromal-Epithelial Interaction: Potential Contributors to Prostate Cancer Bone Metastasis. The homeostasis of localized prostate tumor is maintained by the constant stimulation from the prostate stroma and growth factors released from ECMs which determines the proliferative and differentiative status of tumor epithelium. At this stage, tumor epithelium is considered as androgen-dependent and expressed low levels of OC transcription factors, RUNX2 and Fra2, and bone matrix proteins, OC, OPN, BSP, and ON. Upon prostate cancer progression to androgen-independent and metastatic state (such as bone metastasis), a surge of intracellular autocrine and paracrine growth factor signaling results in an elevation of OC transcription factor activity (e.g., increased RUNX2 and Fra2) and elevated level of OC, BSP, OPN, and ON. The enhanced RUNX2 and Fra2 is responsible for an overall stimulation of osteoblast proliferation, which then supports tumor growth and survival in the skeleton through greatly increased autocrine and paracrine loops. The co-targeting concept is building upon the observation of increased osteomimetic properties by AI and metastatic prostate cancer cells so that replication-competent Ad vectors can be designed to exert cytotoxic effects on both tumor epithelial and bone stromal cell compartments.
Targeting angiogenic pathways involving tumor–stromal interaction to treat advanced human prostate cancer

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Key words: prostate cancer, stromal–epithelial interactions, anti-angiogenesis, intermittent androgen suppression

Abstract

Interfering with and preventing tumor angiogenesis is an attractive therapeutic approach for treating cancer metastases. This commentary presents treatment strategies that may enhance the effectiveness of anti-angiogenic therapy by selectively targeting newly sprouting and immature vessels, inhibiting the production of angiogenic factors, and disrupting extracellular matrices. We propose several clinical paradigms, including hormonal ablation, intermittent androgen suppression, chemotherapy, and radiation therapy, that ‘injure’ nascent vasculature and interrupt the cancer cell–stromal relationship, thereby potentiating the efficacy of experimental anti-angiogenic agents. These stromal–epithelial interactions play an important role in the development, proliferation and dissemination of prostate cancer, as well as guiding the processes of tumor neovascularization. Successful utilization and targeting of tumor angiogenesis requires an increased understanding of tumor cell–stromal cell–endothelial cell relationships, most notably the intricate intracellular signalling cascades mediated by growth factors and the extracellular matrix.

Introduction

Despite advances in prevention and early detection, refinements in surgical technique, and improvements in adjuvant radio- and chemotherapy, the ability to cure many men with prostate cancer remains elusive. This is especially apropos to the successful management of metastatic and recurrent hormone-refractory disease. Clinical protocols using either androgen deprivation therapy or chemotherapeutic agents have shown some promise in treating advanced prostate cancer [1,2]. Unfortunately, the proportion and durability of complete remissions have been limited and new therapeutic approaches are desperately needed.

Stromal–epithelial interactions are paramount to the development, proliferation, and spread of prostate cancer. Studies in our laboratory have established that a bi-directional relationship between tumor cells and their surrounding stroma contributes to the growth and dissemination of prostate cancer [3–5]. These mesenchymal–epithelial interactions are responsible for maintaining the functional integrity of the normal adult prostate gland. Irregularities in the constituents of the stromal–epithelial milieu or aberrations in their interactions can induce genomic instability, enhance tumor cell proliferation, and drive both metastatic spread and progression to a hormone-refractory state. Consequently, novel therapeutic protocols are being developed that target not only prostate tumor epithelial components, but surrounding stromal and extracellular matrix (ECM) elements as well.

For a prostate cancer to grow and metastasize, endothelial cells from this surrounding stroma must be recruited to form an endogenous microcirculation to support the developing neoplastic mass [6,7]. Similar angiogenic processes are necessary at sites of metastasis if disseminated tumor cells are to become securely entrenched and, subsequently, propagate. Although prostate cancer cells produce inherent pro-angiogenic signals, integration of downstream signaling involving soluble factors and stromal and ECM components are critical to promoting and maintaining neovascularization [8].
Stromal–epithelial cell injury may potentiate anti-angiogenic therapy

We have recently demonstrated that squalamine, an anti-angiogenic sterol isolated from shark liver, has potent anti-prostate cancer activity [11]. When squalamine was applied concomitantly with androgen withdrawal in a human prostate cancer xenograft model, an absolute and lasting eradication of both PSA and subcutaneous tumors was achieved. No such result was observed in intact tumor-bearing animals or in tumor-bearing animals treated with squalamine post-castration, yet subsequent to the appearance of androgen independent lesions. Immunohistochemical staining of responding and non-responding tumors indicated that combined squalamine and castration substantially diminished integrin αvβ3 expression. Additional histologic data established that squalamine’s actions were most potent in preventing proliferation of the freshly sprouting, phenotypically immature blood vessels that, we believe, emerge in the tumor tissue as it acquires hormone independence. This finding corroborated prior studies which have determined that squalamine’s efficacy is significantly enhanced when tumor vasculature and cancer cells are ‘injured’ immediately antecedent to its application [12,13].

Accordingly, we have developed two theories to explain the powerful anti-angiogenic as well as anti-prostate cancer effects of coincident androgen ablation and squalamine administration. First, androgen deprivation, may so effectively ‘stun’ the stromal–epithelial environment that normally prevalent and active pro-angiogenic factors are diminished, inactivated, or eradicated. At this juncture, the diminution of angiogenic forces may be sufficient enough that the otherwise ineffectual squalamine (if used in the absence of hormone-ablation) is consequently rendered potent. Alternatively, during the immediate post-orchiectomy rejuvenated proliferative phase (when stromal-mediated and autonomous epithelial tumor cell growth is actively acquiring hormone independence) the cellular and vascular architecture may become somewhat pliable and plastic, predisposing these precarious cells to either anti-angiogenic or cytotoxic effects of squalamine.

These interesting observations and speculations have led us to develop the following hypothesis: that the effectiveness of anti-angiogenic agents can be increased when applied coincident with other therapeutic interventions that injure components of the stromal–epithelial milieu. This premise is particularly plausible considering that most pro-angiogenic factors are located within the stromal milieu, either as soluble or ECM-bound factors, which affects survival of tumor epithelium.

Tumor–stromal interactions and angiogenesis

The outcome of a patient with prostate cancer ultimately depends upon the tumor’s capacity for unhindered growth, local invasion, and the establishment of distant metastasis. Local factors, produced by mesenchyme, epithelial cells, or as a consequence of bi-directional mesenchymal–epithelial interactions between prostate tumor and stromal cells, are necessary for such proliferative, invasive, and migratory events [14].

Numerous cytokines and growth factors have been implicated in either enhancing or impairing a given prostate tumor’s inherent tumorigenic and metastatic phenotype [15]. While some act directly upon the tumor cells, others influence prostate tumor cell proliferation by modulating their interactions with the extracellular matrix interactions through either soluble or matrix-associated signaling. This can significantly alter tumor cell heterogeneity with the propensity of selecting androgen-independent and metastatic variants.

Angiogenesis

Angiogenesis refers to the formation of new blood vessels from pre-existing, nascent vasculature. It is a multistep sequential process involving the recruitment and proliferation of endothelial cells, their subsequent migration to the tumor mass, morphogenesis into a tubular structure, and maturation into a stable structure [16,17]. It is important to note that the structure of tumor vessels differ from those of normal tissues, especially with regard to cellular composition, tissue integrity, vascular permeability, and regulation of cell proliferation and apoptosis [18]. It is presumed that these many differences may impart selective susceptibility of tumor vessels to the effects of anti-angiogenic agents.

The establishment and maintenance of such a vascular supply is imperative to prostate carcinogenesis and involves the cooperation of a variety of molecules either constituting or inhabiting the ECM. A variety of
Recent studies report that up to 50 percent of patients who were thought to have organ-confined lesions were discovered to be understaged subsequent to surgery [43-45]. As a result, the majority of men with prostate cancer will eventually develop disseminated disease [46]. In addition to causing severe pain and morbidity, such metastatic disease is the primary cause of death in men with prostate cancer [47]. Androgen ablation therapy is the most widely accepted therapy for men with metastatic cancer. Because of its limited duration, however, certain chemotherapeutic agents have been incorporated in the treatment of advanced, hormone-refractory disease. Furthermore, radiation therapy (with or without hormonal therapy) is commonly used to treat locally-invasive lesions that are felt to be incurable by surgical means.

With regard to prostate cancer, androgen ablation therapy, chemotherapy, and radiation therapy share two common traits. First, by themselves, they customarily behave as temporizing agents resulting in disease remission, but are generally ineffective in curing advanced disease. Second, in addition to directly damaging prostate cancer cells, each of these treatment modalities induces injury to the surrounding stroma and extracellular matrix. In fact, studies demonstrating improved outcomes in men with prostate cancer after treatment with combined radiation and androgen ablation therapy attribute these findings, in part, to the resultant interference with the stromal–epithelial relationship [48]. Furthermore, p53, which can act as a radiosensitizer, may enhance the efficacy of radiation therapy via its anti-angiogenic properties, such as inducing expression of the anti-angiogenic ECM component, thrombospondin-1 [49,50]. Because of their individual and independent abilities to injure components of the extracellular milieu, androgen ablation therapy, chemotherapy, and radiation therapy are ideal therapeutic approaches to investigate for use in concert with anti-angiogenic agents to treat men with, or at risk for developing, advanced prostate cancer.

Taking all three of these treatment modalities into consideration, we can now reiterate our hypotheses as to how androgen ablation therapy, chemotherapy, and radiation therapy may induce sufficient injury to the stromal–epithelial environment that the effects of the subsequent utilization of anti-angiogenic agents would be potentiated. First, damage to the homeostatic cellular components of the stromal–epithelial milieu might decrease, or even completely suppress, the secretion of soluble pro-angiogenic factors and intracellular signaling of pro-angiogenic components, inhibiting many of the steps required for neovascularization. Such alterations in this constituency could greatly increase the sensitivity of the tumor to anti-angiogenic therapy. Second, injury to the vascular endothelial cells within the stroma could result in the immediate destruction of the tumor vasculature (resulting in the cessation of blood flow to the cancer cells) or weaken the vessels sufficiently that their susceptibility to attack by a second (anti-angiogenic) agent is increased. Furthermore, damage to established vessels might induce the formation of new vasculature to nourish and sustain both the tumor mass and the surrounding tissues, or alter the phenotype incipient vasculature so that it acquires the more immature characteristics of newer vessels. It is generally accepted that these younger, more immature vessels are most-susceptible to anti-angiogenic insult [51]. Third, immediately following intervention, some stromal and epithelial tumor cells are likely to overcome their injuries and begin to initiate repair pathways, in which they recapitulate a phenotypically younger and more unstable configuration. As the cellular architecture becomes increasingly precarious, these cells are more apt to be affected by the anti-angiogenic agents.

**Androgen ablation therapy**

Our data from the LNCaP-castrate xenograft model confirmed that squalamine’s actions were most potent on the freshly sprouting, immature blood vessels that, we believe, developed during a rejuvenated proliferative phase of those prostate tumor cells acquiring hormone independence. The effect was independent of serum VEGF levels. There is ample evidence that androgen application can stimulate vasculogenesis whereas androgen deprivation can inhibit neovascularization, allegedly the result of increased or decreased VEGF production (respectively). Folkman has demonstrated that VEGF production by LNCaP cells is under tight regulation by androgen and that androgen withdrawal inhibited hypoxic induction of VEGF [52]. Isaacs and associates have demonstrated that the activity of Linomide, an oral anti-angiogenic agent which has demonstrated effectiveness in suppressing human prostate cancer in preclinical animal studies, was potentiated by concurrent androgen ablation, presumably due to down-regulation of VEGF [30].

It was recently demonstrated that prostate gland growth in a rat model was regulated by the vascular
of anti-angiogenic agents. By cyclically applying and withdrawing androgen, intermittent androgen blockade systematically injures tumor and vascular cells over a prolonged period of time. This repeated stress should prime them for the actions of an anti-angiogenic agent, resulting in the destruction of both tumor cells and the surrounding stroma.

**Chemotherapy and radiation therapy**

External beam radiotherapy, as well as brachytherapy, is frequently applied to the treatment of local and locally-advanced prostate cancer. Furthermore, radiation therapy is commonly used to treat symptomatic metastases. Radiation induces significant injury to both tumor and stromal cells [61,62]. There is often scarring and destruction of nascent vasculature, as well as damage to the surrounding stromal and ECM components. In both situations, much akin to androgen ablation therapy, the stromal-epithelial milieu initially experiences significant injury with damage to stromal cells as well as pro-angiogenic signals. With time, however, these cells attempt to repair the radiation-induced damage, and the stromal-epithelial compartments undergo neovascularization with the potential to support a tumor recurrence. Similarly, applying taxol and estramustine, two currently used agents in prostate disease and as a paradigm for other chemotherapeutic regimens, will similarly induce injury of both endothelial and epithelial tumor cells [63,64]. We propose that the application of an anti-angiogenic agent coincident with radio- or chemotherapy induced injury could inhibit stromal neovascularization and prevent tumor recurrence.

**Concluding remarks**

Anti-angiogenic agents have shown promise in several preclinical studies of prostate cancer. In our own experience squalamine, an aminosterol with anti-angiogenic properties, has demonstrated effectiveness when applied concomitantly with castration. As many of the pro-angiogenic influences present in prostate carcinogenesis reside with the stromal–tumor cell–ECM environment, it is not surprising that castration, which induces widespread damage within the stroma (in addition to having direct cytotoxic effects), would potentiate the activity of an anti-angiogenic agent. In this commentary, we speculate that the potency of anti-angiogenic agents will be most pronounced when applied in conjunction with other therapeutic modalities that maximally injure the stromal and ECM. For now, this includes intermittent androgen suppression, radiation therapy, and chemotherapy, but may, with time, be applicable to gene therapy and novel molecular approaches being developed for the treatment of both localized and advanced human prostate cancer.

**References**


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1987-1995  Member, Institution Recombinant DNA Subcommittee
1992-1993  Member, Institutional Faculty Classification Committee
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Editorships & Editorial Boards:
2000-present  Editorial Board, The Prostate
1997-present  Editorial Board, Prostate Cancer and Prostatic Diseases
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1994-present  Managing Editor, Urologic Oncology
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American Journal of Physiology and Pharmacology Biochemical and Biophysical Acta
Biochemical Pharmacology
Biology of Reproduction
Blood
British Journal of Cancer
Canadian Journal of Physiology
Cancer
Cancer Research
Developmental Pharmacology and Therapeutics
Drug Metabolism and Disposition
Endocrinology
International Journal of Cancer
International Journal of Oncology
Pharmacology and Experimental Therapeutics
Proceedings of the National Academy of Sciences
Journal of Andrology
Journal of Biological Chemistry
Journal of Cell Biology
Journal of Clinical Investigation
Journal of Pharmacology
Journal of Steroid Biochemistry
Journal of Urology
Molecular and Cellular Biochemistry
Molecular Endocrinology
Molecular Pharmacology
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Chairman, Gordon Research Conference on Reproductive Tract Biology

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Society Memberships:

1985-1995 American Society of Andrology
1972-present American Pharmacology and Experimental Therapeutics
1985-present Society of Chinese Bioscientists in America
1986-present American Association for Cancer Research
1986-present American Urological Association
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Research Focus:

- Tumor and microenvironment interaction that dictates cancer growth and progression
- Novel genetic and molecular targeting strategies for the treatment of localized and disseminated prostate cancer
- Profiling of tumor cell growth and gene expression under 2-D and 3-D growth conditions and in animal models
- Function of transcriptional factors that regulate tumor cell growth, differentiation and metastasis
- Normal and neoplastic prostate biology and development

Patents:

Issued
1998 5,728,815: Bone and prostate-derived protein factors affecting prostate cancer growth, differentiation, and metastasis
1998 5,772,993: Osteocalcin promoter-based toxic gene therapy for the treatment of calcified tumors and tissues
1999 5,874,305: Androgen Repressed Metastatic Human Prostate Cancer Cell Line
2000 6,159,467: In vivo suppression of osteosarcoma pulmonary metastasis with intravenous osteocalcin promoter-based toxic gene therapy

Pending
Identification, cloning and characterization of a novel gene
Viral processing techniques
Bone cancer therapy
Gene expression directed by a novel PSA promoter
Bone matrix protein promoters targeting of prostate cancer
Fetal urogenital sinus expressed sequences
Current Grant Support:


Department of Defense Prostate Cancer Initiative Idea Award, Principal Investigator. DAMD 17-00-1-0526. "Accelerated tumor cell death by angiogenic modifiers," $375,000 – 06/01/00 to 05/30/03

National Aeronautics and Space Administration, Principal Investigator. NRA-97-HEDS-02-126. "Modeling prostate cancer skeletal metastasis and gene therapy," $657,000 – 02/01/99 to 09/30/02

National Institutes of Health, Principal Investigator. CA 85555-01. "Ad-OC-TK/Val gene therapy clinical correlates," $500,000 – 05/01/00 to 04/31/02

DirectGene, Inc. (Industrial), Principal Investigator, "Tissue-specific and tumor-restricted promoter-directed therapeutic gene expression in pre-clinical models of human prostate cancer," $300,000 – 10/01/00 to 09/30/01

Eluysis (Industrial), Co-Principal Investigator. "Use of novel tumor-specific antigens to treat and diagnose cancer," $283,000 – 07/15/99 to 07/31/01

Kluge Foundation, Principal Investigator. "Molecular staging and therapy," $1,000,000 – 1998-present

National Institutes of Health, Sub-Contract with Johns Hopkins University. "SPORE in prostate cancer," $85,000 – 1992-present

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Introgen Therapeutics, Principal Investigator. "Brachygene therapy for prostate cancer," $90,000 – 02/01/00 to 02/01/01

National Cancer Institute, Subcontract. Cell Culture Core Facility, $58,062 – 02/00 to 01/01

CaPCURE Corporation, Principal Investigator. "Gene therapy strategy to enhance the targeting of prostate cancer bone metastasis using controlled tumor-specific promoters," $100,000 – 01/01/00 to 12/31/00

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Daiichi Pharmaceutical Corporation, Principal Investigator. #04-91-02631. "Effects of sulphated polysaccharide peptidoglycan complex tecogalan on the Growth of Human prostate cancer in vivo," $165,000 - 05/04/94 to 05/04/97

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National Institutes of Health, Co-Principal Investigator. "Training of academic urologists," Total Direct Costs: $696,000 / 5 years - 07/15/91 to 06/30/96

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Bibliography:

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