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14. ABSTRACT We have shown, in an alternative model system (of the mouse bladder) via collaborative research, that prostasin serine protease is capable of attenuating LPS-induced inflammatory gene expression response, specifically the mRNA expression of inducible nitric oxide synthase (iNOS), by potentially intercepting cytokine signaling at the cytokine receptor level. A new candidate protein, gp130, the signal transducer of the interleukin-6 receptor complex, has been proposed to be the 120-130-kDa tyrosinephosphorylated protein regulated by prostasin in the DU-145 and PC-3 cells, as presented in the original proposal. Efforts are underway to demonstrate the applicability of this new molecular mechanism in the prostate cancer cell line PC-3, which had been shown by others to respond to inflammatory challenges such as LPS and cytokines through upregulation of the iNOS gene expression, while over-expressing the gp130 signal transducer. This new working hypothesis directly related to the original proposal will potentially lead to better understanding of the role of inflammation in prostate cancer biology.					
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INTRODUCTION

The purpose of this project is to reveal the mechanism by which prostasin serine protease suppresses *in vitro* invasion of prostate cancer cells. The working hypothesis in the original proposal was that reduced tyrosine phosphorylation of either FAK or p130Cas (or both); and reduced protein expression of PKC α , were the signaling steps involved in prostasin's anti-invasion action. Four specific aims were proposed: 1. Identification of the 120-130-kDa tyrosine-phosphoprotein. 2. Prostasin's role in FAK-integrin signaling. 3. Prostasin's interaction with signaling proteins in caveolae. 4. Investigation of prostasin anti-invasion signal transduction by introducing active site-inactivated prostasin in prostate cancer cell lines DU-145 and PC-3.

Summary of report by Specific Aim:

- Specific Aim 1. Identification of the 120-130-kDa tyrosine-phosphoprotein
 - o In earlier reports on this Specific Aim, we had provided evidence that the 120-130-kDa tyrosine-phosphoprotein, whose level or level of phosphorylation was reduced following forced expression of prostasin serine protease in the DU-145 and PC-3 cell lines, was not the focal adhesion kinase (FAK), nor the p130Cas protein. Over the past six months of research, however, we have obtained new data to propose a new candidate protein for this 120-130-kDa tyrosine-phosphoprotein, namely "gp130", the signal transducer subunit of the interleukin-6 (IL-6) receptor complex. This new progress will be highlighted below in the BODY of this report.
- Specific Aim 2. Prostasin's role in FAK-integrin signaling
 - o We have performed the originally proposed experiments and additional experiments by the modified plans indicated in previous reports, using the DU-145 cell line derivatives uniformly expressing the human prostasin serine protease regulated by tetracycline. We have ruled out any effect of prostasin on FAK-integrin signaling *via* either binding of integrins or changing integrin expression (data not shown). The abandonment of pursuit of prostasin's role in this signaling pathway gives further indirect support of prostasin's potential role in intercepting IL-6 signaling, leading to its invasion suppression role (to be highlighted below under Specific Aim 1).
- Specific Aim 3. Prostasin's interaction with signaling proteins in caveolae
 - o Based on our previously reported results that prostasin down-regulated caveolin-1 and Src expression in the DU-145 cells, we planned and performed the following experiments during the no-cost extension year:
 - ENaC α -Src reciprocal immunoprecipitation from the DU-145 cells to show direct interaction of the two molecules. The results from these experiments were not conclusive as the quality of the ENaC α antibody was inadequate for such experiments.
 - Examination of ENaC (all three subunits) mRNA expression changes in DU-145 cells expressing prostasin or the active-site mutant (inducible). By using realtime PCR following reverse transcription (RT-rtPCR), we have found that expression of prostasin or its active-site mutant does not affect ENaC subunit mRNA expression.
- Specific Aim 4. Investigation of prostasin anti-invasion signal transduction by introducing active site-inactivated prostasin in prostate cancer cell lines DU-145 and PC-3.

- o This Specific Aim was designed as a service aim, the prostatic active-site mutant has been successfully generated and used in experiments described under the other Specific Aims.

BODY

Specific Aim 1: We will show that the 120-130-kDa tyrosine-phosphorylated protein being FAK or p130Cas, or both, using reciprocal immunoprecipitation/western blot analysis. We will confirm a role for prostatic in regulating cell movement by performing a motility assay.

Approved Statement of Work:

a). Reciprocal immunoprecipitation for the identification of FAK and/or p130Cas.

This task will be initiated at the onset of the project and is expected to be completed within months 1-6.

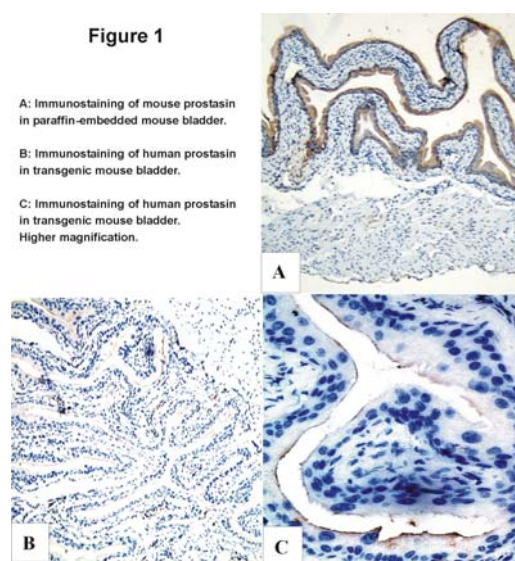
b). Cell migration assay

This task will be initiated at the onset of the project and is expected to be completed within months 1-6, however, the assay will continue to be employed in months 7-36 to serve the need of pathway investigation.

Outcome:

As stated in the Summary above, the work performed under this aim in earlier years had led to the conclusion that the 120-130-kDa tyrosine-phosphorylated protein in question was confirmed not to be FAK or p130Cas, as originally proposed.

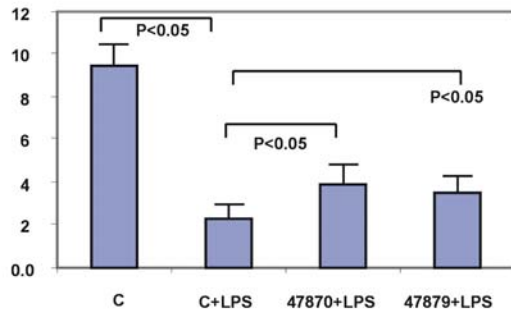
During the no-cost extension year (2004-2005), we have successfully generated a rabbit polyclonal antibody against the mouse prostatic homologue, by using methods that we have previously described (1). With this antibody as a tool, we were able to survey the tissue expression pattern of prostatic in the mouse, with the intent of identifying alternative tissue sites of expression and alternative models of research investigation to lead back to the identification of the 120-130-kDa tyrosine-phosphorylated protein in question.



After immunohistochemical analysis of various mouse tissues, we have identified the bladder as a high-expressor (Figure 1A). Expression of prostatic is mainly localized in epithelial cells. Because the uro-epithelial cells are the first defensive boundary against microbial pathogens, prostatic, an epithelial membrane-bound proteolytic enzyme, may play roles in the process or the defense against the urinary tract infections. We undertook a study of prostatic's effect on the bladder's inflammatory response using a transgenic mouse model expressing human prostatic in various tissues (under the control of *Rous sarcoma virus* 3'-LTR, known as the RSV promoter), including the bladder (Figure 1 B, and 1C). This work was carried out in collaboration with Dr. Julie Chao of the Medical University of South Carolina (Charleston, SC), whose laboratory created the model. Funding of this collaboration was partially provided by an NIH grant to the co-PI on this grant, Dr. Limei Chen.

Figure 2

Prostasin mRNA expression in mouse bladder



LPS treatment down-regulated mouse prostasin expression. Over-expression of human prostasin in the transgenic mice attenuated the mouse prostasin gene down-regulation by LPS. C: FVB mice; 47870 & 47879: prostasin transgenic mice; LPS: lipopolysaccharides; Y-axis: arbitrary unit.

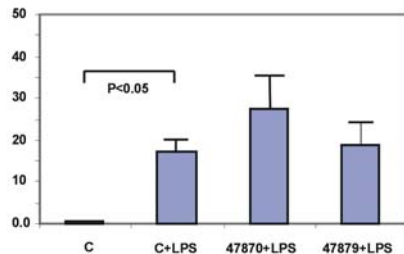
In this mouse model, we performed the following experiments: Mice were injected with lipopolysaccharides (20mg/kg, *i.p.*) and analyzed after 18 hours for gene expression changes of mouse prostasin, IL-6, tumor necrosis factor alpha (TNF α), and inducible nitric oxide synthase (iNOS) by Reverse-transcription/Real-time PCR (MyiQ, Bio-Rad). The animal experiments were approved by the IACUC of the University of Central Florida for Dr. Chen's NIH grant.

First, the mouse prostasin mRNA expression was found to be down-regulated during LPS-induced inflammation (Figure 2).

Because LPS-induced inflammatory gene expression response is enhanced by cytokines such as TNF α and IL-6 *via* a positive feedback mechanism (2), we confirmed that our LPS injection did result in local gene expression up-regulation of these two cytokines in the bladder (Figure 3 and Figure 4). And this up-regulation of cytokine gene expression was not impeded by prostasin transgene expression.

Figure 3

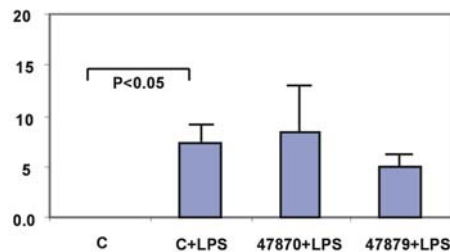
TNF- α mRNA expression in mouse bladder



TNF- α mRNA expression was up-regulated by LPS. Over-expression of prostasin in the bladder did not affect up-regulation of TNF- α by LPS. C: FVB mice; 47870 & 47879: prostasin transgenic mice; LPS: lipopolysaccharides; Y-axis: arbitrary unit.

Figure 4

IL-6 mRNA expression in mouse bladder



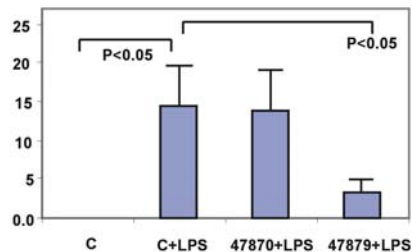
IL-6 mRNA expression was up-regulated by LPS. Over-expression of prostasin in the bladder did not affect up-regulation of IL-6 by LPS. C: FVB mice; 47870 & 47879: prostasin transgenic mice; LPS: lipopolysaccharides; Y-axis: arbitrary unit.

A classical response-gene of LPS-induced inflammation is the iNOS (2), and in our animal models, this gene's expression provided the most sensitive readout/phenotype, associated with the expression of prostasin (Figure 5). In a low prostasin-expressing line of the transgenic mice (47870), iNOS up-regulation upon LPS-induction was unimpeded, but in a high prostasin-expressing line (47879). The impediment of LPS-induction of the iNOS mRNA by prostasin was dramatic, from the level of ~15 fold (unimpeded control) to a level of ~3 fold. To determine whether the bladder iNOS mRNA expression change was a systemic or local effect of prostasin expression (because the prostasin transgene is expressed in a non-tissue-specific manner), we

injected mouse bladder with liposome-DNA (plasmid expressing human prostaticin), and this intravesicular gene delivery resulted in a similar impediment of iNOS mRNA induction by LPS (Figure 6).

Figure 5

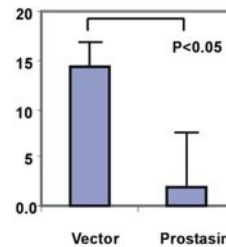
iNOS mRNA expression in mouse bladder



iNOS mRNA expression was up-regulated by LPS. Over-expression of prostaticin in one line of transgenic mice (47879) attenuated up-regulation of iNOS by LPS, but not in the other line (47870, with low-level expression of the human prostaticin transgene in the bladder).
C: FVB mice; 47870 & 47879: prostaticin transgenic mice;
LPS: lipopolysaccharides; Y-axis: arbitrary unit.

Figure 6

iNOS mRNA expression in bladder after gene delivery



Liposome-plasmid DNA complex was injected intravesically into mouse bladder, animals were then treated with LPS (i.p.). Up-regulation of iNOS expression was reduced in mice receiving prostaticin plasmid as compared to mice receiving the vector plasmid. Expression of the human prostaticin mRNA in mouse bladder was confirmed by RT-PCR (data not shown).

In a recapitulation of the significance and relevance of this collaborative study to the prostate cancer research supported by this grant, we underscore the following points:

- The accessibility of the mouse bladder as an alternative site for investigating prostaticin's cellular signaling mechanism has provided us with a highly sensitive molecular readout/phenotype, *i.e.*, mRNA expression of iNOS induced by LPS (or, during inflammatory response).
- This readout/phenotype provided a further advantage, that we are now able to begin dissecting a potentially complex network of signaling molecules and events triggered by prostaticin expression. Specifically, the iNOS mRNA induction by LPS is mediated by two mechanisms, one coming straightly from the TLR4 receptor complex signaling, and the second, a feedback up-regulation, coming from the cytokine receptor signaling, *e.g.*, IL-6 and/or TNF α , which are first induced by TLR4 receptor complex signaling (3).
- The fact that IL-6 and TNF α induction remained unimpeded with prostaticin expression and that iNOS induction was impeded but not wiped out by prostaticin points to a mechanism of prostaticin action at the feedback loop (*i.e.*, intercepting the cytokine actions), but independent of the initial LPS events (*i.e.*, *via* the TLR4 receptor complex).
- Relevance to prostate cancer: It has long been established that IL-6 plays a significant role in prostate cancer biology (4), and the PC-3 cell line has served as a good model of investigating the molecular mechanisms involved in IL-6 signaling in prostate (4).
- Most important, the PC-3 cell line is responsive to LPS (5), IL-6 (6), and TNF α (7), with iNOS being one of the response genes at a 1.8-fold sensitivity. In our original proposal, PC-3 cells forced to express prostaticin exhibited a reduced level or level of phosphorylation of a 120-130-kDa tyrosine-phosphorylated protein (while the cells were cultured on Matrigel). We now suggest a new candidate for this 120-130-kDa tyrosine-phosphorylated protein, the gp130 signal transducer subunit of the IL-6 receptor (IL-

6RB) (8), which is expressed in prostate cancer cell lines relevant to this project (*i.e.*, DU-145 and PC-3), but not in normal prostate epithelial cells (9).

- This new working hypothesis is easily testable with the following experiments. We will challenge the PC-3 cells, expressing prostasin or transfected with a control plasmid, or the active site-inactivated mutant (Specific Aim 4 results), with LPS, IL-6, or TNF α , and monitor the readout/phenotype in the form of iNOS mRNA expression by realtime PCR following RT (as described above). If prostasin expression impedes the iNOS induction, especially when IL-6 alone is used as the inducer, we will determine if the level or level of phosphorylation of gp130 is affected, by immunoprecipitation and western blotting. We believe that these are highly attainable goals and the outcome will significantly enhance the impact of the overall grant's value.

Finally, the Cell Migration assays proposed had been completed and reported on previously in the annual reports.

Specific Aim 2: We will examine whether prostasin re-expression resulted in a down-regulation of integrin using northern blot and/or RT-PCR-Southern blot, and western blot analyses and whether prostasin forms a direct link with $\beta 1$ and $\beta 3$ integrins using reciprocal immunoprecipitation/western blot analysis.

Approved Statement of Work:

a). Determination of whether prostasin re-expression resulted in a down-regulation of integrin molecules at either the mRNA or protein level.

This task will be initiated at the onset of the project and is expected to be completed within months 1-12. The methods and reagents established in this task, however, will continue to serve the project in months 13-36.

b). Determination of whether prostasin forms a direct link with the $\beta 1$ and $\beta 3$ integrins

This task will be initiated at the onset of the project and is expected to be completed within months 1-15. The methods and reagents established in this task, however, will continue to serve the project in months 16-36.

Outcome:

Both of the planned experiments had been performed and we have conclusively ruled out a direct role of prostasin in integrin function or expression.

Specific Aim 3: We will address the potential interactions between prostasin, Src, PKC α , and caveolin-1 by purifying caveolae and localizing the proteins biochemically by means of reciprocal immunoprecipitation/western blot analysis.

Approved Statement of Work:

a). Purification of caveolae and localization of prostasin in caveolae

This task will be initiated at the onset of the project and is expected to be completed within months 1-15. The methods and reagents established in this task, however, will continue to serve the project in months 16-36.

b). Examination of interaction between prostasin and caveolar proteins

This task will be initiated at the onset of the project and is expected to be completed within months 1-15. The methods and reagents established in this task, however, will continue to serve the project in months 16-36.

c). Interaction of prostasin with caveolin-1, and/or Src, and/or PKC α in a time-dependent manner

This task will be initiated in month 16 and is expected to be completed within months 16-36.

Outcome:

We have previously reported that prostasin expression in the DU-145 cell line reduced expression of caveolin-1 and Src, as well as PKC α (as shown in the original proposal). The molecular mechanisms of these phenotypes remained, however, unclear. Our attempts at addressing interactions of the potential caveolar interaction of prostasin with the molecules that have been down-regulated by it have been unsuccessful, as previously reported. But the new candidate pathway of IL-6 or other cytokine signaling may help shed new light in this aim because the lipid rafts (containing caveolin-1) are hypothesized to be involved in cytokine (e.g., IL-6) signaling (10). If the experiments described under Specific Aim 1 above proves that the key signaling molecule for prostasin is indeed the IL-6B/gp130, *i.e.*, the “120-130-kDa tyrosine-phosphorylated protein”, the caveolar related phenotypes that we have observed under this aim will be easily strung together in a mechanistic way.

Specific Aim 4: We will introduce into DU-145 and PC-3 cells a serine active site-inactivated prostasin to determine whether the cells' invasiveness is still reduced or unchanged. The signaling pathways delineated from the investigations in the first three Specific Aims will be re-examined in cells expressing a serine active site-inactivated prostasin.

Approved Statement of Work:

a). Examination of invasiveness for cells (DU-145 and PC-3) that are transfected with a plasmid containing an active site-inactivated prostasin cDNA

i). Construction of prostasin mutant (Ala-prostasin) cDNA plasmid: months 1-6.

ii). Establishment of DU-145 and PC-3 cells expressing Ala-prostasin: months 7-12.

b). Examination of the signaling pathways for cells (DU-145 and PC-3) expressing the Ala-prostasin

This task will be initiated in month 16 and continue on through the project period (month 36)

Outcome:

Please refer to the Summary under INTRODUCTION (Page 4).

KEY RESEARCH ACCOMPLISHMENTS

- A new and sensitive assay (realtime PCR following RT to detect mRNA expression changes of iNOS) has been established for investigating and dissecting prostasin's molecular signaling pathways and events.

REPORTABLE OUTCOMES

- **Manuscripts published:**

Narikiyo T, Kitamura K, Adachi M, Miyoshi T, Iwashita K, Shiraishi N, Nonoguchi H, Chen LM, **Chai KX**, Chao J, Tomita K. Regulation of prostasin by aldosterone in the kidney. *J Clin Invest*. 2002 Feb;109(3):401-8.

Chen LM, Zhang, XC, **Chai, KX**. Regulation of prostasin expression and function in the prostate. *Prostate*. 2004, Apr 1;59(1):1-12.

Chen MQ. Chen LM. **Chai KX**. Androgen regulation of prostasin gene expression is mediated by sterol-response element-binding proteins and SLUG. *Prostate*. In press, 2005

- **Meeting abstracts:**

Chai, KX. Prostasin serine protease is a potential metastasis suppressor. *CaPCURE 9th Annual Scientific Retreat*. Washington, D.C., September 20-22, 2002

Chen MQ. Chen LM. **Chai KX**. Androgen regulation of prostasin gene expression is mediated by sterol-response element-binding proteins and SLUG. *The 15th Annual Symposium of the Society for Basic Urologic Research*. Miami Beach, Florida, December 1-4, 2005

Chen LM. Chen MQ. **Chai KX**. Expression of prostasin and its role in lipopolysaccharide-induced bladder inflammation. *The 15th Annual Symposium of the Society for Basic Urologic Research*. Miami Beach, Florida, December 1-4, 2005

- **Patents:**

Inhibitors of Prostasin 60/379,469 - filed 5/10/02, Inventors: **Karl X. Chai** and Limei Chen

CONCLUSIONS Prostasin may be acting through intercepting cytokine-receptor signaling to inhibit tumor cell motility and invasion, the key mediator of these events may be the IL-6 receptor signal transducer subunit gp130. This new working hypothesis will be tested out in our continued research.

“SO WHAT” Establishing and understanding the role of prostasin in attenuating LPS or cytokine induced inflammatory response will enable us to delineate a more clear picture of how inflammation affects the prostate health and how this process may be related to prostate cancer. Novel molecular mechanisms identified in these previously unknown pathways affecting prostate cancer will be exploited for drug development and therapy.

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