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14. ABSTRACT

Prostate cancer (CaP) is the second most common cause of cancer death in North American men. CaP is characterized by stages that include aggressive forms that disseminate to other tissues. Tumors release factors that attract and activate cells of the immune system including macrophages. Exposure of macrophages to inflammatory stimuli results in the transcriptional activation of an anti-inflammatory phospholipase A2, platelet-activating factor acetylhydrolase (PAF-AH) that inactivates PAF and other bioactive phospholipids. PAF-AH expression is dramatically increased in CaP compared to normal prostate tissues. During the tenure of this Award we used in vivo and in vitro methodologies to investigate whether PAF and PAF-AH participate in the pathogenesis of CaP. We generated PAF-AH-deficient mice in a model of PCa (the TRAMP model) that recapitulates many aspects of human CaP. We established that deficiency of PAF-AH in mice decreases survival and increases disease severity. Secondly, we established that CaP cells respond to stimulation with PAF by increasing calcium transients, activating MAP kinases, and increasing cellular proliferation. These results identified a key role for PAF and PAF-AH in the pathogenesis of CaP and provide us with a framework on which we will build the next research phase which includes targeting this pathway to develop novel strategies to treat human CaP.

15. SUBJECT TERMS
BIOACTIVE LIPIDS ● PLATELET-ACTIVATING FACTOR ● KNOCKOUT MOUSE ● METASTASIS ● ANGIOGENESIS ● SURVIVAL ● VASCULARIZATION ● INFLAMMATION ● OXIDANTS ● OXIDIZED PHOSPHOLIPIDS ● PHOSPHOLIPASE A2 ● TUMOR GROWTH

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INTRODUCTION

Prostate cancer (CaP) is the most common malignancy in North American men and represents the second most common cause of cancer death. CaP is characterized by many different stages including very aggressive forms that disseminate to bone, lymph nodes, and other tissues. The host's immune system plays a central role in the ability of tumors to obtain nutrients and oxygen. Tumors release factors that attract and activate cells of the immune system including macrophages. Macrophages have well-defined roles as agents that participate in inflammation and that help us fight infections, but the biological significance of tumor-associated macrophages (TAMs) is unclear (Elgert et al., 1998). When macrophages are recruited to tumor sites complex cancer-macrophage interactions take place (Joseph and Isaacs, 1998; Satoh et al., 2003). The biological result of these interactions depends on the changes that occur within macrophages, the physical location reached by these cells as they encounter the tumor, and the type of malignancy. In some tumors, including CaP, macrophages can both stimulate and inhibit cancer growth and proliferation (Shimura et al., 2000). One of the phenotypic changes that take place when macrophages are exposed to specific stimuli is the transcriptional activation of a gene encoding a phospholipase A_2, platelet-activating factor acetylhydrolase (PAF-AH) (Stafforini et al., 1990). This enzyme has anti-inflammatory properties owing to its ability to hydrolyze a large group of bioactive lipids including platelet-activating factor (PAF), oxidized phospholipids and esterified isoprostanes with varied and potent biological functions (Stafforini et al., 2003; Stafforini et al., 2006). The levels of PAF-AH mRNA, which provides a mechanism to down-regulate bioactive lipid signaling, are altered during CaP progression compared to normal prostate tissues. The PAF-AH mRNA detected in these analyses likely originates from macrophages recruited to CaP sites. *The goal of this proposal was to investigate whether PAF-AH present in CaP sites reflected participation of this enzyme and its substrates in the pathogenesis of the disease.* To address this issue, we used mice (known as TRAMP mice) that spontaneously develop CaP owing to overexpression of an oncogene in the prostate gland exclusively. We made these animals deficient in PAF-AH by crossing them to our PAF-AH knockout animals. Using this and related model systems we established that PAF signaling increases the rate of tumor growth and likelihood of metastatic events during CaP.

BODY

UPREGULATION OF PAF-AH IS RECAPITULATED IN THE TRAMP MODEL OF PROSTATE CANCER — We first generated an animal model suitable to test the hypotheses posed. This model capitalized on the availability of mice engineered to develop CaP and mice in which the PAF signaling pathway was exacerbated owing to impaired degradation. Once we obtained a suitable number of animals of the desired genotype, we investigated whether this model recapitulated the upregulation of PAF-AH observed in authentic human CaP samples. This was a key issue to address because the premise of the project was based on observations in human CaP that might not have been reproducible in a murne model of the disease. We found that PAF-AH activity increased during development in both serum and prostate samples isolated from TRAMP animals (**Fig. 1, A-B)**.

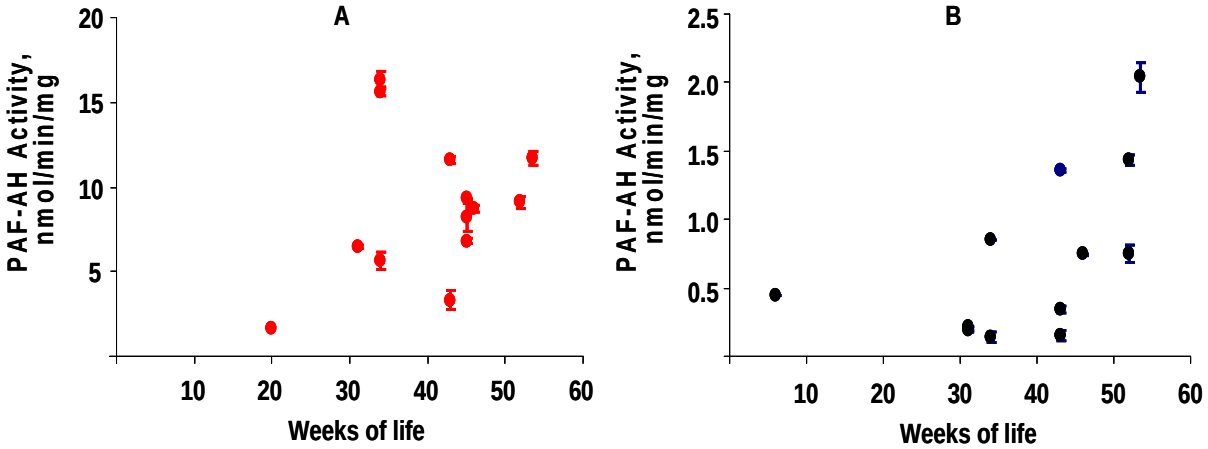


Fig. 1. PAF-AH activity in plasma and prostate tissues from TRAMP mice during CaP progression. *A, PAF-AH activity and protein levels were determined in plasma samples of TRAMP mice of various ages. B, Extracts from grossly-uninvolved dorsolateral prostate glands were assayed for PAF-AH activity and protein content.*

In the C57BL/6 background, cancer progression has been reported to be complete at 10-20 weeks of age and metastatic disease in the lymph nodes or lungs is present in 100% of the animals by 28 weeks of age (Gingrich et al., 1996). Thus, in this model, upregulation of PAF-AH expression in serum and in prostate tissues occurs with a relatively long latency period compared to that of tumor development. Since the PAF-AH activity determined in tissue extracts likely reflects contributions from calcium independent phospholipases A_2 including the intracellular paralog of PAF-AH, we determined the levels PAF-AH protein in uninvolved dorsolateral prostate samples and in prostate tumors, using immunoblot analyses. We found (**Fig 2, A-B**) that the expression of PAF-AH increased with time and that the tumors expressed higher levels of PAF-AH compared to prostate tissues that were uninvolved as judged by gross examination. Note the different amounts of protein subjected to electrophoresis in the experiments depicted in **Figs. 2, A-B**. In related studies we found PAF-AH expression in plasma remains constant throughout the life of the mouse (not shown). This result established that the TRAMP model recapitulated human CaP in terms of upregulation of PAF-AH expression. We then proceeded to develop the remaining tasks of the proposal by investigating if a causal relationship existed between PAF-AH expression and disease severity utilizing *in vivo* approaches that are described next.

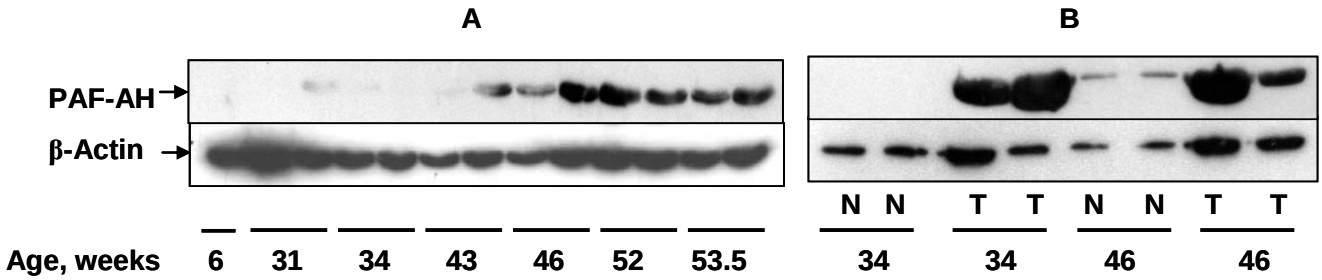


Fig. 2. PAF-AH expression in grossly uninvolved prostate and prostate tumor tissues from TRAMP mice during CaP progression. *A, Dorsolateral prostate gland extracts from TRAMP mice (75 μg) were subjected to immunoblot analysis using anti-PAF-AH and anti-β-actin antibodies. B, Same as A) using extracts (7.5 μg) from grossly-uninvolved prostates (N) or tumors (T) from TRAMP mice.*

GENETIC ABLATION OF PAF-AH DECREASES SURVIVAL IN A MURINE MODEL OF PROSTATE CANCER. In fulfillment of experiments proposed in the original proposal we utilized mice deficient in the expression of the secreted (plasma) form of PAF-AH. These animals develop normally but have enhanced susceptibility to inflammation, a feature also observed in PAF-AH-deficient human subjects (Stafforini et al., 2003; Stafforini et al., 1999; Stafforini et al., 1996). To investigate whether PAF-AH functionally affected CaP, we generated TRAMP mice harboring three different genotypes at the PAF-AH locus. We found (**Fig. 3**) that haploinsufficiency and complete deficiency of PAF-AH had a significant impact on survival.

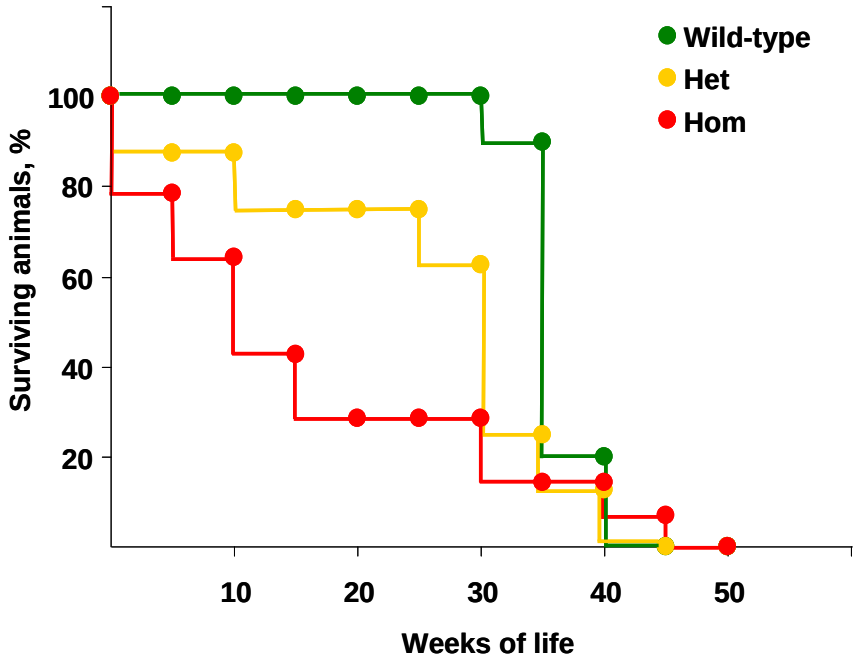


Figure 3. Partial and complete deletion of PAF-AH decreases the survival of TRAMP mice. *TRAMP animals that were wild-type, partially-deficient, or homozygous deficient for PAF-AH were allowed to develop until they died spontaneously or reached criteria for euthanasia.*

This observation suggested a functional association between PAF-AH expression and one or more parameters important in prostate tumorigenesis. The precise nature of these signals is unknown, but our results suggested that accumulation of substrates inactivated by PAF-AH such as PAF and OxPL contributed to the observed effects. We previously reported that PAF-AH expression is transcriptionally-activated by PAF and OxPL (Cao et al., 1998). Thus, our results support the hypothesis that the upregulation of PAF-AH in CaP is a compensatory mechanism engaged in response to increased levels of PAF/OxPL.

EXPRESSION OF THE PAFR IN PROSTATE TISSUES AND IN CELL LINES. The ability of PAF and OxPL to elicit signals requires expression of PAFR and coupling to G-proteins. We investigated the expression of PAFR in prostate tissues and in cell lines. First, we found that PAFR is moderately upregulated at the mRNA level in human CaP, and that its upregulation is associated with disease progression (**Fig. 4A**). Second, we found that three CaP cell lines derived from TRAMP mice express primarily a monomeric (48 KDa) form of PAFR (**Fig. 4B**).
In addition, we detected larger species whose molecular mass was 96 KDa and that appeared to have resulted from dimerization of PAFR or formation of a complex with another protein(s) or receptor(s) (**Fig. 4B).** Thirdly, extracts from dorsal (D) and ventral (V) prostate glands from TRAMP mice also expressed PAFR in monomeric (48 KDa) and 96 KDa forms (**Fig. 4C**). Deletion of the PAF-AH gene did not significantly affect the expression levels of monomeric PAFR. In contrast, the 96 KDa form was upregulated primarily in the dorsal prostate of PAF-AH deficient mice (**Fig. 4C**).

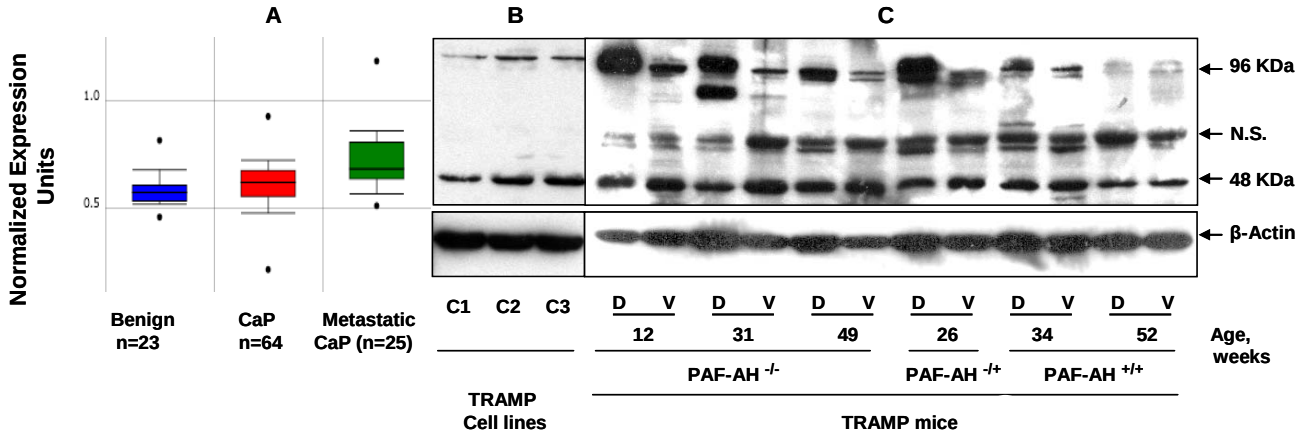
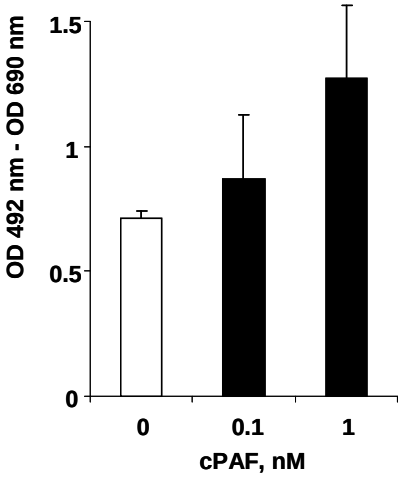


Fig. 4 Expression of PAFR in human and mouse CaP cells and tissues. *A, Human PAF-AH mRNA levels in benign prostate specimens and in CaP and metastatic CaP samples. Data reproduced from Oncomine ™. B, Total cell lysates from TRAMP-C1, C2, and C3 cells (50 μg) were subjected to immunoblot analyses using anti-PAFR and anti-actin antibodies. C, Extracts (50 μg) of dorsal (D) and ventral (V) prostate tissues from TRAMP mice of various ages and PAF-AH genotypes were treated as in B. N.S.: non-specific.*



To investigate whether PAFR expressed in CaP cells was functional, we subjected TRAMP-C2 cells to a non-hydrolyzable analog of PAF (carbamoyl PAF, cPAF). We found (**Fig. 5**) that treatment of TRAMP-C2 cells with 1 nM cPAF, a concentration that reflected the levels of the natural ligand, increased the rate of proliferation with relatively fast kinetics compared to other cancer cell lines [(Melnikova et al., 2006), **Fig 5**]. These results demonstrated the functional integrity of PAFR expressed in TRAMP cells and supported the hypothesis that stimulation of the PAF axis contributes to prostate tumorigenesis.

Fig. 5. PAF stimulates cellular proliferation in CaP cells. *TRAMP-C2 cells were exposed to cPAF for 24 hours and cellular proliferation was assessed by the XTT assay.*

SIGNALING EVENTS. In the next series of experiments we investigated the expression and/or state of activation of select molecules previously identified as downstream effectors of PAF-mediated effects. PAF mediates angiogenic responses, in part, by increasing the expression of vascular endothelial growth factor (VEGF) (Ahmed et al., 1998). VEGF is considered to be the most important contributor to induction and maintenance of the neovasculature in tumors (Kim et al., 2002). We investigated whether CaP cells express VEGF using a model system that consisted of three TRAMP cell lines derived from a prostate tumor from a single 32-week-old TRAMP mouse (Foster et al., 1997) and protein extracts from CaP tumors. The antibody we utilized (sc-507, Santa Cruz Biotechnology) was raised a region of VEGF-A (amino acids 1-140) that shares sub-domains of identity with other members of the VEGF superfamily, including VEGF-B,-C, and –D. Thus, it is likely that this antibody recognizes multiple members of the VEGF superfamily. Our results suggested that at least two forms of VEGF [-A (M r= 21KDa) and –C (Mr = 46.5 KDa)] are expressed in TRAMP cell lines **(Fig. 5)**. Tumors from TRAMP-PAF-AH$^{-/-}$ transgenic mice expressed immunoreactive species whose size was consistent with the presence of VEGF-C (Mr = 46.5 KDa) and -D (Mr = 40.9 KDa). These results suggested that when the function of PAF-AH is ablated and ligands of PAFR accumulate, enhanced synthesis and release of VEGF-C and -D by CaP cells stimulate angiogenesis and/or lymphangiogenesis.

In colonic epithelial cells PAF activates mitogen-activated protein kinase modules such as p38 MAPK, JNK and ERKs (Wang and Chakrabarty, 2003). We observed that deletion of PAF-AH did not alter the extent of JNK or p38 MAPK activation in TRAMP mice (not shown). ERK was constitutively-activated in the three TRAMP lines (**Fig. 6**). In TRAMP mice, ERK phosphorylation seemed to occur at an earlier time in tumors from PAF-AH deficient mice compared to wild-type TRAMP animals (**Fig. 6**). These results suggested that activation of the PAF axis contributes to increased growth through the ERK pathway in CaP cells.

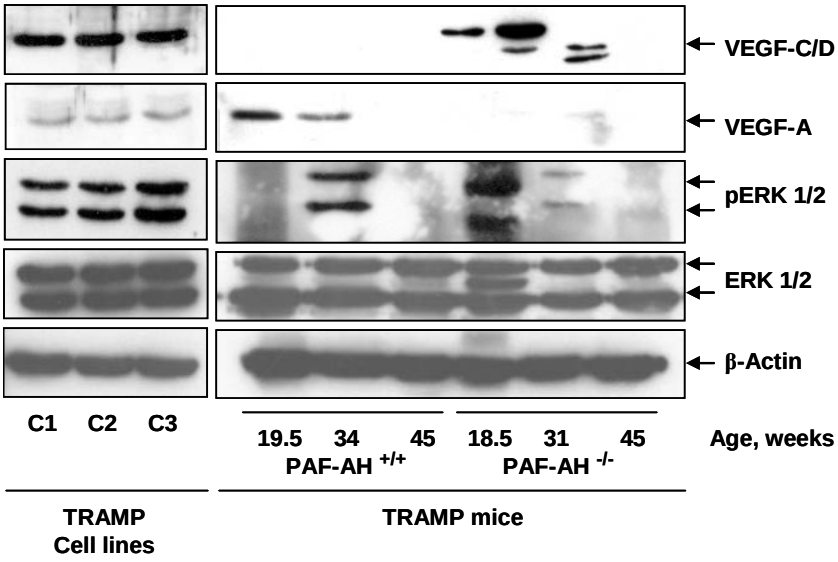


Fig. 6. Expression of VEGF and pERK in TRAMP cell lines and in tumors from TRAMP mice. *Total cell lysates from TRAMP-C1, C2, and C3 cells (50 μg, left panel) or extracts of prostate tumors excised from TRAMP mice of various ages and PAF-AH genotypes (35 μg, right panel) were subjected to immunoblot analyses using antibodies directed against the proteins indicated.*

KEY RESEARCH ACCOMPLISHMENTS RELEVANT TO THIS REPORT

- Established dissection, biochemical and immunological assays necessary for the execution of the study
- Generated genetically-engineered animals with the genotypes required for the execution of the study
- Investigated expression of PAF-AH in the plasma of TRAMP transgenic animals
- Obtained evidence for modulation of PAF-AH expression in the prostate gland during CaP development
- Observed decreased lifespan of mice that lack expression of PAF-AH during CaP development.
- Initiated analysis of tumor formation within the context of PAF-AH deletion and generated initial data suggesting increased severity of tumor formation and metastasis induced by PAF-AH deficiency.
- Complemented animal studies with molecular analyses in prostate epithelial and CaP cells. These included demonstration of functional PAF receptor expression, altered levels of VEGF upon deletion of PAF-AH *in vivo* and ERK activation in response to PAF-AH deletion
- Generated the framework upon which the PI crafted a new proposal that was submitted to the Department of Defense as an application for an IDEA Award.

REPORTABLE OUTCOMES

Presentations: This work has been presented on several occasions in our institution where it is open for discussion and subject to input and criticism from colleagues, trainees, and students. Moreover, portions of this work were presented by the PI to scientists at the Oswaldo Cruz Foundation in Rio de Janeiro, Brazil.

Abstract: An abstract was submitted and selected for poster presentation in the upcoming IMPaCT meeting to take place September 5-8, 2007 in Atlanta, GA.

Animal models: As a consequence of our work, a new line of mice has been generated that harbors the TRAMP transgene developed by Dr. Greenberg and his group and in which one or two PAF-AH alleles are deleted. These animals constitute excellent tools to probe the role of PAF-AH and the PAF signaling pathway in prostate carcinogenesis.

CONCLUSIONS

The results we have generated during the tenure of this grant used both cellular and *in vivo* approaches. Our studies suggested that the PAF signaling pathway contributes to the pathogenesis of CaP by modulating cellular growth and metastatic events. In the absence of PAF-AH, the severity of the CaP increases and is accompanied by a higher incidence of metastatic events and faster tumor growth. Conversely, phenotypic alterations that lead to elevated expression of PAF-AH and thus result in silencing of signals elicited by bioactive lipid mediators through the PAF receptor may inhibit growth and/or promote apoptosis. In the setting of CaP, this phenotype is consistent with a model of recruitment and activation of macrophages by inflammatory stimuli, resulting in the localized release of a PAF-AH activity which may serve to blunt the actions of bioactive lipids such as PAF. Our data strongly support out initial hypothesis, that is, that PAF-AH deficiency exacerbates CaP and that the increased levels of this enzyme observed in human CaP and in animal models of the disease represent a compensatory mechanism in response to accumulated PAF and other ligands of the PAF receptor. The approaches we utilized were complementary in nature, included both human and murine systems, utilized an animal model with proven resemblance to the human disease, and were internally consistent. The impact of our work at the present moment lies primarily on having identified a novel player in CaP pathogenesis. The implications of this work relate to our contribution to understanding the etiology of this disease and the possibility that this pathway may offer a number of interventional approaches.

"So what" section: the experimental design of our studies was based on the need to define what types of patients would potentially benefit the most from this work. The use of the animal model we developed and utilized here ensured that various phases of CaP development were represented. This feature allowed us to establish that PAF signaling is likely to play a role during early stages of CaP and that the results of PAF-mediated signaling increase the rate of metastatic progression. This information will help us define the subset of CaP patients who would benefit the most from potential targeted therapies in the future. There may also be diagnostic applications generated from the results of these studies. Because it is possible to measure the levels of PAF-AH in blood and presumably in prostate biopsies, we will be able to identify the levels of this protein in patients using clinical assays and genetic tests. This will allow us to investigate if altered functioning of this gene can become one of the tools in our diagnostic kit and whether PAF or PAF-AH can be used to assess clinical CaP and predict risk. Thus, the clinical applications that could result from the work proposed here have both diagnostic (assessment of gene and protein status in CaP patients) and interventional (*i.,e*, administration of recombinant protein or receptor antagonists) applications. Therapies aimed at inhibiting CaP growth or spreading by targeting this pathway alone or in combination with others, could be developed. For example, the use of gene-modified macrophage therapy treatment using intratumor injection of PAF-AH-transduced macrophages could be considered as a complement to existing therapies using IL-12-transduced macrophages (Satoh et al., 2003). Finally, new potential targets for intervention within this lipid-signaling pathway can be identified. For example, inhibition of the synthetic enzymes involved in PAF biosynthesis would offer an additional attractive possible target for intervention. And, inhibition of lipid oxidative reactions that can generate ligands for the PAF receptor such as oxidized phospholipids, could also be contemplated as a potential interventional approach.

REFERENCES

Ahmed, A., Dearn, S., Shams, M., Li, X. F., Sangha, R. K., Rola-Pleszczynski, M., and Jiang, J. (1998). Localization, quantification, and activation of platelet-activating factor receptor in human endometrium during the menstrual cycle: PAF stimulates NO, VEGF, and FAKpp125. Faseb J *12*, 831-843.

Cao, Y., Stafforini, D. M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1998). Expression of plasma platelet-activating factor acetylhydrolase is transcriptionally regulated by mediators of inflammation. J Biol Chem *273*, 4012-4020.

Elgert, K. D., Alleva, D. G., and Mullins, D. W. (1998). Tumor-induced immune dysfunction: the macrophage connection. J Leukoc Biol *64*, 275-290.

Foster, B. A., Gingrich, J. R., Kwon, E. D., Madias, C., and Greenberg, N. M. (1997). Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res *57*, 3325-3330.

Gingrich, J. R., Barrios, R. J., Morton, R. A., Boyce, B. F., DeMayo, F. J., Finegold, M. J., Angelopoulou, R., Rosen, J. M., and Greenberg, N. M. (1996). Metastatic prostate cancer in a transgenic mouse. Cancer Res *56*, 4096-4102.

Joseph, I. B., and Isaacs, J. T. (1998). Macrophage role in the anti-prostate cancer response to one class of antiangiogenic agents. J Natl Cancer Inst *90*, 1648-1653.

Kim, Y. M., Hwang, S., Pyun, B. J., Kim, T. Y., Lee, S. T., Gho, Y. S., and Kwon, Y. G. (2002). Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. J Biol Chem *277*, 27872-27879.

Melnikova, V. O., Mourad-Zeidan, A. A., Lev, D. C., and Bar-Eli, M. (2006). Platelet-activating factor mediates MMP-2 expression and activation via phosphorylation of cAMP-response element-binding protein and contributes to melanoma metastasis. J Biol Chem *281*, 2911-2922.

Satoh, T., Saika, T., Ebara, S., Kusaka, N., Timme, T. L., Yang, G., Wang, J., Mouraviev, V., Cao, G., Fattah el, M. A., and Thompson, T. C. (2003). Macrophages transduced with an adenoviral vector expressing interleukin 12 suppress tumor growth and metastasis in a preclinical metastatic prostate cancer model. Cancer Res *63*, 7853-7860.

Shimura, S., Yang, G., Ebara, S., Wheeler, T. M., Frolov, A., and Thompson, T. C. (2000). Reduced infiltration of tumor-associated macrophages in human prostate cancer: association with cancer progression. Cancer Res *60*, 5857-5861.

Stafforini, D. M., Elstad, M. R., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1990). Human macrophages secret platelet-activating factor acetylhydrolase. J Biol Chem *265*, 9682-9687.

Stafforini, D. M., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (2003). Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. Crit Rev Clin Lab Sci *40*, 643-672.

Stafforini, D. M., Numao, T., Tsodikov, A., Vaitkus, D., Fukuda, T., Watanabe, N., Fueki, N., McIntyre, T. M., Zimmerman, G. A., Makino, S., and Prescott, S. M. (1999). Deficiency of platelet-activating factor acetylhydrolase is a severity factor for asthma. J Clin Invest *103*, 989-997.

Stafforini, D. M., Satoh, K., Atkinson, D. L., Tjoelker, L. W., Eberhardt, C., Yoshida, H., Imaizumi, T., Takamatsu, S., Zimmerman, G. A., McIntyre, T. M., *et al.* (1996). Platelet-activating factor acetylhydrolase deficiency. A missense mutation near the active site of an anti-inflammatory phospholipase. J Clin Invest *97*, 2784-2791.

Stafforini, D. M., Sheller, J. R., Blackwell, T. S., Sapirstein, A., Yull, F. E., McIntyre, T. M., Bonventre, J. V.,

Prescott, S. M., and Roberts, L. J., 2nd (2006). Release of free F2-isoprostanes from esterified phospholipids is catalyzed by intracellular and plasma platelet-activating factor acetylhydrolases. J Biol Chem *281*, 4616-4623.

Wang, H., and Chakrabarty, S. (2003). Platelet-activating factor activates mitogen-activated protein kinases, inhibits proliferation, induces differentiation and suppresses the malignant phenotype of human colon carcinoma cells. Oncogene *22*, 2186-2191.

APPENDICES

NOT APPLICABLE