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14. ABSTRACT We hypothesize that (1) prostate cancer cells that express cyclooxygenase-2 (COX-2), prostaglandin E2(PGE2) and interleukin-6 (IL-6) display enhanced bone targeting and (2) the level of expression of COX-2, PGE2 and IL-6 in established bone metastases determines the overall bone response, with lower vs. higher cytokine levels inducing osteoblastic vs. osteolytic responses, respectively. We utilize two human prostate cancer cell lines (MDA-PCa-2B that expresses low levels of COX-2 and PGE2 and produces osteoblastic lesions vs. PC-3 that expresses high levels COX-2/PGE2 and induces osteolytic mets). Over the past year, we demonstrated that (1) low levels of PGE2 stimulate preosteoblast cell growth, differentiation and Wnt signaling (2) Forced overexpression of COX-2 in MDA-PCa-2b cells induces the Wnt antagonist DKK-1 (3) PGE2 addition to PC-3 cells stimulates Dkk-1 (4) Forced overexpression of COX-2 in MDA-PCa-2B cells inhibits preosteoblastic cell growth in co-culture. Over the next and final year of the grant proposal we will determine the effects of COX-2/PGE2 expression in the two PCa cell lines on in vivo bone targeting and bone reaction					
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INTRODUCTION

The overall purpose of these studies is to investigate our hypothesis that dose-dependent expression of COX-2/PGE₂/IL-6 by prostate cancer cells influences both bone targeting and the bone response (osteoblastic vs. osteolytic) once prostate cancer cells reside in the bone microenvironment. Specifically, we hypothesize that low levels of COX-2/PGE₂/IL-6 expression favor an osteoblastic bone response whereas higher expression levels by prostate cancer cells produce osteolytic lesions. In order to prove these hypotheses, we proposed a series of in vitro and in vivo experiments, utilizing human prostate cancer cell lines that differentially express COX-2/PGE₂ and induce different bone reactions. Our lab and others have also demonstrated differential expression by human prostate cancer cell lines of inhibitors of Wnt signaling (i.e. Dkk-1). The Wnt signaling pathway is known to regulate osteoblastic differentiation and we have further investigated the interrelationships between Wnt inhibitor expression/COX-2/PGE₂ and the induction of an osteoblastic vs. osteolytic bone reaction by human prostate cancer cell lines.

BODY

Over the past year, we continued to investigate the effects of COX-2 and its major product, PGE₂, on the regulation of prostate cancer bone metastases. Our hypothesis is that dose-dependent expression of COX-2/PGE₂ by prostate cancer cells influences both bone targeting and the bone response (osteoblastic vs. osteolytic) once prostate cancer cells reside in the bone microenvironment. We postulated, specifically, that low levels of COX-2/PGE₂ expression/secretion favor an osteoblastic bone response (i.e. MDA-PCa-2b cells) whereas higher expression/secretion levels by prostate cancer cells (i.e. PC-3 cells) produce osteolytic lesions. We have published data demonstrating that PGE₂ (at doses > 1 μ M) and IL-6 promote osteoclastogenesis via the OPG/RANK/RANKL system (1). We determined the effects of COX-2 and PGE₂ on the growth of MC3T3 cells, which are mouse pre-osteoblasts. In light of recent reports indicating that the Wnt and BMP signaling pathways are key mediators of osteoblast differentiation, we expanded our original studies to include analysis of the dose-dependent effects of COX-2/PGE₂ on components of the Wnt signaling pathway in MC3T3 mouse preosteoblast cells. Finally, utilizing co-cultures of prostate cancer cells and MC3T3 cells, we determined the effects of altering COX-2/PGE₂ expression/secretion by the cancer cells on the growth and Wnt signaling activity in the preosteoblast cells.

1. PGE₂ exerts dose-dependent effects on MC3T3 pre-osteoblast growth and differentiation. PGE₂, at low doses (< 1 μ M) stimulated MC3T3 growth, and slightly increased their differentiation. At higher doses (> 1 μ M), PGE₂ inhibited both the growth and differentiation of the bone cells. These effects were dose-dependent with maximal inhibitory effects demonstrated at the highest dose of 10 μ M (Fig.1A&B). We further examined the effect of PGE₂ on canonical Wnt

signaling utilizing a T-cell factor luciferase (Tcf-Luc) reporter assay. As shown in Fig.1C., while low doses of PGE2 slightly increased Tcf-Luc activity, high doses of PGE2 significantly inhibited this measure of canonical Wnt signaling activity.

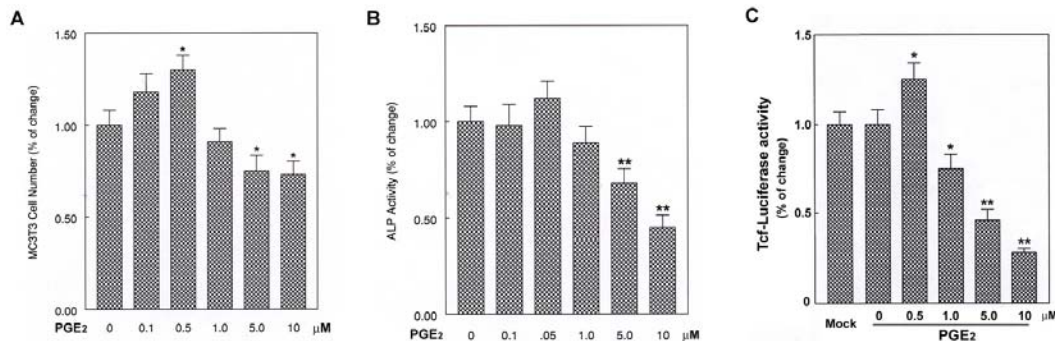


Fig. 1. Direct effects of PGE2 on preosteoblast cells. (A) *Cell growth*, MC3T3 cells were cultured in serum-free medium and treated +/- various doses of PGE2, as indicated, for 7 days. Cell numbers were counted using a hemocytometer. (B) *Differentiation*, MC3T3 cells were cultured in serum-free medium and treated +/- various doses of PGE2, as indicated, for 14 days. Alkaline phosphatase (ALP) activity in the lysates was assayed by the hydrolysis of *p*-nitrophenyl phosphate (Sigma) to *p*-nitrophenol. Absorbance was determined at 405 nm and compared with a *p*-nitrophenol (Sigma) standard titration curve. ALP activity was normalized to total protein content. (C) *Tcf-Luciferase reporter activity*. Cells were co-transfected with the Tcf reporter plasmid (or empty vector as mock control) and the β -galactosidase expression vector and treated with either vehicle or various doses of PGE2 for 24h. The resulting Tcf-luciferase activities were normalized to protein concentrations and β -galactosidase activity. Data are expressed as fold-induction compared with the vehicle control (100%), and represent the mean $\pm$ SE from three separate determinations. * $p < 0.05$, ** $p < 0.01$.

2. COX-2 and PGE2 regulated the production of natural inhibitors of Wnt signaling in prostate cancer cells. Osteoblast development is closely regulated by Wnt signaling (2). Naturally occurring Wnt inhibitors such as sFRP and DKK, play an important role in the modulation of Wnt signaling in bone. MC3T3 pre-osteoblast cells do not secrete significant amounts of either inhibitor. We demonstrate that PC-3 and PC-3ML human prostate cancer cells (which induce an osteolytic reaction *in vivo*) secrete high amounts of both Dkk-1 and sFRP-1. In contrast, MDA-PCa-2b human prostate cancer cells (which induce an osteoblastic bone reaction *in vivo*) do not secrete significant amounts of either inhibitor (Fig. 2). These data strongly support previous findings from our laboratory as well as other investigators indicating that the development and progression of prostate cancer metastases in the bone environment are modulated by interactions between prostate cancer epithelium and bone cells via components of the Wnt signaling system (3).

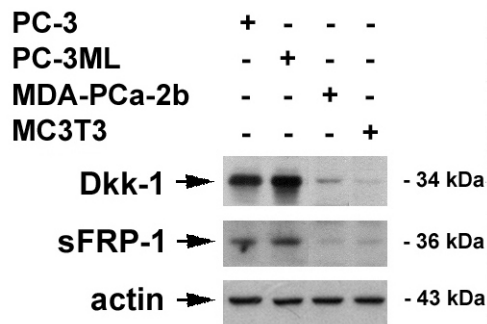


Fig.2. Protein expression of Dkk-1 and sFRP-1 in PCa and MC3T3 preosteoblast cell lines assayed by Western blotting. Cells were lysed and subjected to Western blot analysis. Protein content was assayed and 30µg total protein was loaded in each lane.

a) *Co-expression of COX-2 and DKK-1 in MDA-PCa-2b cells with forced expression of COX-2 protein.* We established several sub-lines of MDA-PCa-2b that stably express COX-2 and examined the expression of the, Dkk-1. As shown in Fig.3, the harvested clones that over-expressed COX-2 also expressed significant amounts of Dkk-1 (Fig.3A) indicating that COX-2 may regulate the production of this Wnt inhibitor, thereby modulating Wnt activity in a paracrine fashion in the bone microenvironment. These data may explain the observations that PC-3 cells express both COX-2 and high levels of Dkk-1, and induce osteolytic lesions in nude mice, whereas MDA-PCa-2b cells which induce an osteoblastic bone response express undetectable amounts of COX-2 and low levels of Dkk-1.

b) *Modulation of Dkk-1 expression by COX-2 inhibitors and PGE2 addition.* We examined the expression of Dkk-1 in mock- and COX-2-transfected MDA-PCa-2b cells +/- various doses of PGE2, and in PC-3 cells +/- meloxicam, a selective COX-2 inhibitor. As shown in Fig.3B, Dkk-1 expression was upregulated by PGE2 and down-regulated by a COX-2 inhibitor in MDA-PCA-2b and PC-3 cell lines, respectively.

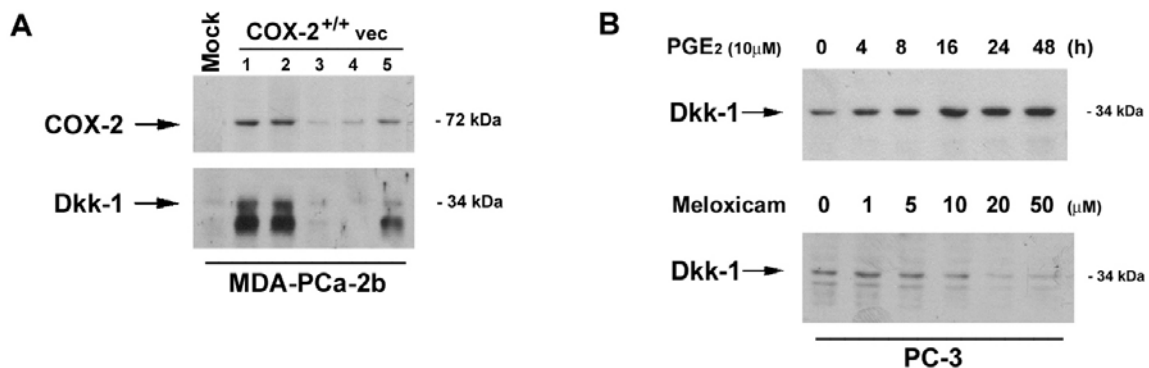


Fig. 3. COX-2 and PGE2 increase Dkk-1 protein expression in human prostate cancer cell lines.. (A) *Forced expression of COX-2 increases Dkk-1 and sFRP-1 protein levels in MDA-PCa-2b cells.* MDA-PCa-2b cells were stably transfected with the COX-2 expression vector. After selection with G418, the resultant clones were harvested and characterized by Western blot analysis. (B)

Effects of PGE2 and Meloxicam on Dkk-1 expression in PC-3 cells assayed by Western blot analysis.

3. Forced expression of COX-2 in MDA-PCa-2b cells suppressed MC3T3 preosteoblast cell growth and differentiation via inhibition of Wnt activity.

To further determine the effects of COX-2/PGE2 on PCa-induced bone metastases, we utilized an *in vitro* co-culture system in which MC3T3 preosteoblast cells were plated on the bottom of a 12-well cluster plate and MDA-PCa-2b cells were plated in the inserts containing 0.4 μ M holes for communication. Growth rates, cell differentiation [assayed by alkaline phosphatase (ALP) activity] and Wnt signaling activity (assayed by Tcf-luc reporter assay) of the bone cells were determined. As demonstrated in Fig.4, we observed a small increase in the all three parameters when bone cells were co-cultured with mock-transfected MDA-PCa-2b cells. However, significant inhibition of those parameters was observed when the same bone cells were co-cultured with an MDA-PCa-2b subline with forced expression of COX-2 protein. These data are consistent with our previous findings that COX-2 increases the production of Dkk-1 and sFRP-1, two Wnt inhibitors, which in turn suppresses Wnt activity in preosteoblast cells, thereby inhibiting both their growth and differentiation.

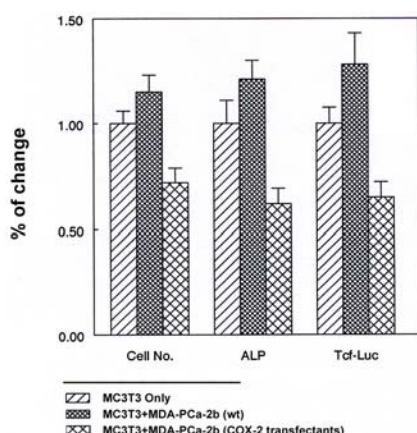


Fig. 4. Forced expression of COX-2 in MDA-Pca-2b cells inhibits preosteoblastic cell growth, differentiation and Tcf-luciferase reporter activity in co-culture. MC3T3 cells were grown alone or co-cultured with either mock-transfected MDA-PCa-2b cells, or COX-2 transfectants for 7d. Cell numbers and ALP activity were assayed. Wnt signaling activity was determined by Tcf-Luciferase reporter assay and the results were normalized by protein content and b-gal activity. * p<0.05, ** p<0.01.

KEY RESEARCH ACCOMPLISHMENTS

1. Determination of dose-dependent effects of PGE2 on preosteoblastic cell growth, differentiation and Wnt signaling with confirmation of our hypothesis that low doses of PGE2 stimulate preosteoblastic bone cell growth and differentiation whereas high PGE2 doses inhibit these activities via effects on Wnt signaling in the bone cells.
2. Establishment of a correlation between osteoblastic vs. osteolytic effects of various human prostate cancer cells and their expression levels of COX-2/PGE2 and Wnt inhibitors.

3. Characterization of our newly established sublines of the MDA-PCa-2b human prostate cancer cell lines that were stably transfected to overexpress COX-2/PGE2 with regards to its expression of the Wnt inhibitor Dkk-1 and the effect of this forced expression on preosteoblastic cell growth, differentiation and Wnt signaling in co-culture.

REPORTABLE OUTCOMES

Publications (2006)

1. Liu X.H., Kirschenbaum A., Yao S., and **Levine A.C.** Interactive Effect of Interleukin-6 and Prostaglandin E2 on Osteoclastogenesis via the OPG/RANKL/RANK System. *Ann. N.Y. Acad.Sci* 1068:225-233, 2006
2. Liu X.H., Kirschenbaum A., Yao S., Aaronson S.A., Liu G. and Levine A.C. Androgen-induced Wnt signaling activation in osteoblasts is critical for prostate cancer cell growth in the bone microenvironment. *Oncogene* (**Revised Manuscript Submitted December 2006**)
3. Liu X.H., Kirschenbaum A., Yao S., and Levine A.C. The role of interleukin-6/GP-130 signaling pathway in bone metabolism. In: ***Vitamins and Hormones***, Volume 74, Chapter 14: 341-355, 2006.

Abstracts (2006)

1. Liu X-H, Kirschenbaum A, Yao S, and **Levine AC**. Androgen-induced Wnt signaling activation in osteoblasts is critical for prostate cancer cell growth in the bone microenvironment. Annual Meeting of American Association of Cancer Research (AACR), Washington D.C., April 2006.
2. **Levine AC**, Kirschenbaum A, Yao S., Liu, X-H. Androgens differentially regulate Wnt signaling and osteoblast differentiation in stage-specific bone-related cell lines. 88th Annual Meeting of the Endocrine Society, Boston, MA, June 2006.

CONCLUSIONS

We originally hypothesized that COX-2/PGE2 expression/secretion levels in human prostate cancer (PCa) cell lines would correlate with bone metastatic targeting and bone reaction. Specifically, we hypothesized that low levels of COX-2/PGE2 would promote osteoblast growth and differentiation whereas high expression levels by prostate cancer cells would favor osteolytic bone metastases. During the first year of these studies, we recognized an association between Wnt signaling in bone cells and osteoblast growth and differentiation. We further investigated the possibility that COX-2/PGE2 expression by PCa cells would modulate osteoblast growth and differentiation via secreted Wnt-inhibitory factors such as Dkk-1. Over this past year, we have successfully completed both our original *in vitro* studies and the added *in vitro* work regarding the possible interactive role of Wnt signaling in these processes. Specifically, we have proved our hypotheses that low dose PGE2 promotes osteoblast growth and differentiation whereas higher doses inhibit these processes. We demonstrated

that these dose-dependent effects of PGE2 correlate well with changes in Wnt signaling in bone cells and that modulation of COX-2/PGE2 expression in PCa cells results in differential expression of Wnt inhibitors. MDA-PCa-2b cells with forced expression of COX-2/PGE2 secrete higher levels of the Wnt inhibitor, Dkk-1 than mock-transfectants. Finally, we have shown that preosteoblasts grown in co-culture with COX-2- vs. mock-transfected MDA-PCa-2b cells are growth and differentiation-inhibited by the high PGE2-secreting COX-2-transfected cancer cells. In the next and final year of this proposal, we will complete the *in vivo* studies determining the effects of modulation of PGE2 secretion by prostate cancer cells on bone targeting and bone reaction.

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