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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 cells lines (MDA-Pca 2b, osteoblastic response, low cytokine expression and PC-3ML, osteolytic response, high cytokine expression). Over the past year, we have established a subline of MDA-PCa-2b that stably expresses COX-2 and secretes high levels of PGE2. Our data indicates that IL6 stimulates PGE2 secretion significantly only in the COX-2+ transfected MDA-Pca-2b cells. We compared wtMDA-Pca2b vs. COX-2 + transfectants with regards to their ability to stimulate the Wnt signaling pathway (a key mediator of osteoblastic differentiation) in an autocrine and paracrine (osteoblast cell line MC3T3). We demonstrate that forced expression of COX-2 and/or higher doses of exogenous PGE2 inhibits Wnt signaling in both MDAPCa2b cells and MC3T3 osteoblasts, whereas low PGE2 induces Wnt signaling. The mechanism of this dose-dependent effect of COX2/PGE2 involves modulation of Wnt receptor (LRP5/6), beta-catenin, and a natural Wnt inhibitor (DKK) expression in MC3T3 osteoblast cells grown in co-culture with MDA-PCa2b (wt vs. COX-2+) cells or with addition of varying doses of PGE2. The overall effect of COX-2/PGE2 was to stimulate Wnt signaling at low expression/dosing levels and inhibit Wnt signaling at high expression/dosing levels. A similar dose-response effect was observed with regards to BMP expression (another osteoblast stimulator) in that low COX-2/PGE2 stimulated BMP whereas high levels inhibited BMP in bone cells. We will continue our in vitro studies and begin in vivo studies in the coming year.					
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INTRODUCTION:

The overall purpose of these studies is to investigate our hypotheses 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. MDA-PCa-2b cells have low endogenous COX-2/PGE₂ expression and induce an osteoblastic bone response, whereas PC-3ML cells have higher basal COX-2/PGE₂ expression and induce osteolytic bone metastases

BODY:

Over the past year, we have successfully established a subline of MDA-PCa-2b cells (low endogenous COX-2/PGE₂ expression, produce blastic response in bone microenvironment) that was stably transfected to express higher levels of COX-2. We utilized and compared these COX-2+ cells to wt-MDA-PCa-2b cells in terms of their COX-2 expression, PGE₂ secretion and ability to stimulate osteoblast cells in co-culture (MC3T3 cells). We also demonstrated that IL-6 enhanced PGE₂ secretion only in the COX-2+ transfectants. Recent reports indicate that the Wnt and BMP signaling pathways are key mediators of osteoblast differentiation (1-5). We, therefore, studied the effect of co-culture with COX-2+ (vs. wt) MDA-PCa-2b cells on activation of the Wnt signaling pathway, expression of Wnt receptors and downstream signals (beta-catenin) as well as natural Wnt inhibitors such as secreted frizzled related protein-1 (sFRP) and DKK-1. We also measured the same endpoints in the osteoblast cells after addition of a range of doses of PGE₂. Finally, we determined the dose-dependent effect of PGE₂ and co-culture with COX-2+ MDA-PCa-2b cells on bone morphogenic protein (BMP) expression, another known osteoblast differentiating agent, in the MC3T3 osteoblast-related bone cells.

1. Establishment of a sub-line of MDA-PCa-2b that stably expresses COX-2.

The COX-2 expression vector, pcDNA-hCOX-2, containing the full-length of cDNA encoding the human COX-2 gene, and control vector (empty vector) were generous gifts from Dr. T. Hla (Dept. of Physiology, Univ. of Connecticut). Stable transfection was performed as described previously with modification. Briefly, 90-95% confluent cells were transfected in OPTI-MEM medium containing 7.8 µg of Lipofect-AMINETM2000 reagent (Invitrogen, Carlsbad, CA) and 3 µg of either the COX-2 expression plasmid, or mock plasmid. After 5 h, the LipofectAMINETM2000 reagent was removed, and the cells were refed with DMEM containing 10% FBS, and incubated for 24h. The cells were split in 1:10 ratio and incubated for another 24h prior to selection for stable transformants by G418 (600 µg/ml). The resistant clones were isolated and characterized for the expression of COX-2. Fig. 1a demonstrates that while parental MDA-PCa-2b cells do not express COX-2, stable transfection of COX-2 gene leads to expression of COX-2

protein in these cells detected by Western Blot analysis. Accordingly, the synthesis of prostaglandin E₂ (PGE₂), a major product of the COX-2-catalyzed pathway, is significantly upregulated in those transfectants (Fig.1b). In addition, treatment with interleukin-6 (IL-6) induced a significant increase in PGE₂ production in those cells expressing COX-2 (Fig.1c).

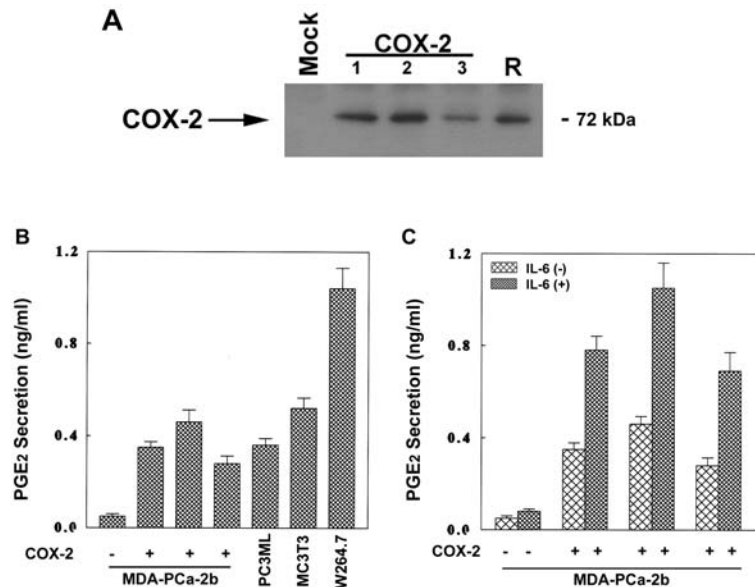


Figure 1. Stable transfection of MDA-PCa-2b cells with the COX-2 gene.

MDA-PCa-2b cells were cultured in DMEM containing 10% FBS. 90-95% confluent cells were transfected with either the COX-2 expression vector or mock plasmid as control in OPTI-MEM medium containing 7.8 μ g of Lipofect-AMINETM2000 reagent. After selection for stable transfectants by G418 (600 μ g/ml), resistant clones were isolated and characterized for the expression of COX-2. **A) COX-2 expression.** Cells transfected with either mock vector or COX-2 expression plasmid were culture in serum free

medium (SFM) for 2d. Total cell lysates were prepared and subjected to Western blot analysis using an anti-COX-2 antibody. 20 μ g protein was loaded in each lane. **R** is the COX-2 recombinant protein as a positive control. **B) PGE₂ production.** Cells transfected with either mock vector or COX-2 expression plasmid were culture in SFM for 2d. Medium was collected and subjected to ELISA for the detection of PGE₂ secretion. **C) IL-6 induced PGE₂ secretion in COX-2 transfected cells.** Cells transfected with either mock vector or COX-2 expression plasmid were cultured in SFM for 1d and treated with or without IL-6 (10ng/ml) for 2 additional days. Medium was collected and subjected to ELISA for the detection of PGE₂ secretion.

2. Expression of COX-2 in MDA-PCa-2b cells leads to decreased Wnt signaling.

The canonical Wnt signaling pathway plays a central role in lineage commitment, osteoblast development, and bone formation, which is a critical component that maintains the balance between osteoblast and osteoclast activities. We observed that expression of COX-2 decreased Wnt activity, as determined by a T cell factor (Tcf) luciferase reporter assay (Fig.2). In addition, we examined the effect of exogenous PGE₂ on Wnt activity in MC3T3, an osteoblast cell line. As shown in Fig.2, while low dose PGE₂ (0.5 μ M) stimulated Wnt activity, higher doses significantly inhibited Wnt signaling, demonstrating dose-dependent differential effects of PGE₂ on the Wnt signaling pathway.

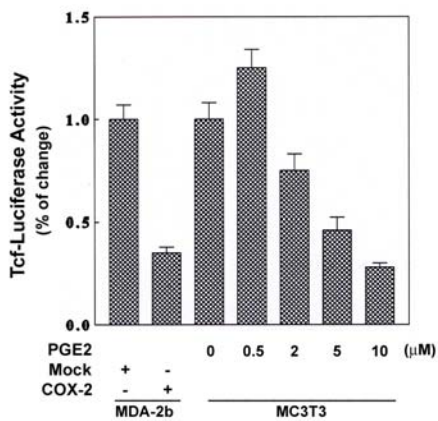


Fig. 2. Effects of COX-2 expression and exogenous PGE₂ on Tcf luciferase activity. MDA-PCa-2b cells stably transfected with either mock vector or COX-2 expression plasmid were culture in DMEM without treatment. MC3T3 cells were cultured in SFM and treated with or without various doses of PGE₂ for 2d. Both cell lines were transiently co-transfected with the Tcf-luciferase (Luc) reporter construct, pGL3-OT (a gift from Dr. Aaronson, Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine), and the β -galactosidase (Gal) expression vector. Cell lysates were prepared and luciferase activity was assayed and quantitated using a TD-20e luminometer. The resulting Luciferase activities

were normalized to β -Gal activity and protein content. Data shown are means $\pm$ SE of two independent assays.

3. Regulation of PGE₂ and COX-2 on the components of Wnt signaling pathway.

We further determined the effects of COX-2 and various doses of PGE₂ on LRP5/6, the co-receptor of Wnt signaling, and expression of β -catenin, a critical element in the Wnt signaling pathway. In addition, the effects of COX-2/PGE₂ on DKK-1 and soluble frizzled related protein-1 (sFRP), two natural Wnt inhibitors, were determined. Fig.3 demonstrates that transfection of the COX-2 gene inhibited the expression of both LRP5/6 and β -catenin in MDA-PCa-2b cells. In contrast, COX-2 expression had no effect on sFRP production. COX-2 transfection increased DKK-1 expression, which can decrease Wnt activity in COX-2 expressing MDA-PCa-2b cells. The effects of various doses of PGE₂ on the same Wnt components in MC3T3 cells were also tested. While low dose PGE₂ did not modulate these components of the Wnt signaling pathway, higher doses of PGE₂ inhibited LRP5/6 and β -catenin expression, and increased DKK-1 production (Fig.3), consistent with the effect of forced COX-2 expression reported above.

4. Low dose PGE₂ increases BMP2 expression.

BMP signaling plays critical roles in bone cell development and bone formation. Recent studies demonstrate a close interconnection between BMPs and Wnt signaling in many cell systems, including embryonic stem cells. We examined the effects of COX-2/PGE₂ on BMP2 expression. As shown in Fig.3 (Low panel), neither COX-2 nor higher doses (i.e., 2, 5, 10 μ M) of exogenous PGE₂ affect BMP2 expression, however, low dose (0.5 μ M) of PGE₂ upregulated BMP2 expression, consisting with the hypothesis that the effect of low-dose PGE₂ is primarily anabolic.

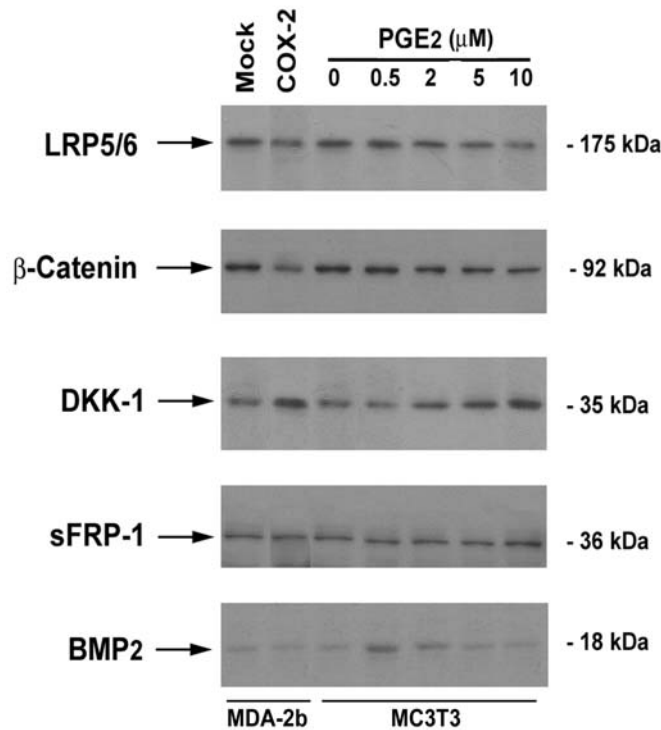


Fig. 3. Effects of COX-2 expression and exogenous PGE2 on the components of Wnt signaling pathway and BMP2 expression. MDA-PCa-2b cells stably transfected with either mock vector or COX-2 expression plasmid were culture in SFM without treatment. MC3T3 cells were cultured in SFM and treated with or without various doses of PGE2 for 2d. Total cell lysates were prepared and subjected to Western blot analysis using antibodies against various antigens as indicated. 20μg protein was loaded in each lane.

KEY RESEARCH ACCOMPLISHMENTS

1. Establishment of a subline of the human prostate cancer cell line MDA-PCa-2b stably transfected to express high levels of COX-2 and secrete high levels of PGE₂.
2. Established a luciferase reporter assay for the measurement of Tcf activity (downstream signal target of the Wnt pathway in osteoblast cells).
3. Grew co-cultures of prostate cancer cells (MDA-PCa-2b, wt or COX-2+) with osteoblast-related cells (MC3T3) and measure effects of COX-2 expression and PGE2 secretion by prostate cancer cells on Wnt signaling in the osteoblast cells.
4. Demonstrated that IL-6 addition enhanced PGE2 secretion selectively in the COX-2+ transfected MDA-Pca-2b cells.

5. Determined the dose-dependent effects of PGE₂ on Wnt signaling in osteoblast cells.
6. Determined the effect of COX-2 expression in prostate cancer cells, as well as the dose-dependent effect of PGE₂ on osteoblast expression of Wnt receptors, beta-catenin, and naturally occurring Wnt inhibitors (sFRP and DKK).
7. Established the dose-dependent effects of PGE₂ on BMP expression in osteoblast cells. Determined the effect of co-culture with COX-2 expressing PCa cells on BMP expression in osteoblast cells.

REPORTABLE OUTCOMES

Two related abstracts have been submitted to

- 1) AACR Annual Meeting, March 2006 (late-breaking abstract)
- 2) Endocrine Society Annual Meeting, June 2006

We are in the process of writing-up these findings for submission for publication.

CONCLUSIONS:

We have successfully transfected MDA-PCa-2b cells to overexpress COX-2 and secrete high amounts of PGE₂. We demonstrated interactions between the IL-6 and COX-2/PGE₂ signaling pathways by determining that IL-6 addition increased PGE₂ secretion selectively in the COX-2+ transfected MDA-PCa-2b cells. We have grown these cells in co-culture with osteoblast cells and demonstrated that high COX-2 expression induces various mediators of the Wnt signaling pathway in the osteoblasts. We have also demonstrated dose-dependent effects of PGE₂ on Wnt and BMP expression that are consistent with our original hypothesis. Specifically, we demonstrate that low levels of PGE₂ induce Wnt signaling (via a combination of effects including increasing Wnt receptor and beta-catenin expression and decreasing the natural Wnt inhibitor DKK) whereas high levels of PGE₂ have the opposite effect. Finally, we observed dose-dependent effects of PGE₂ on BMP expression in osteoblasts, again confirming that low levels of PGE₂ promote osteoblastic differentiation whereas high levels inhibit this parameter. In the next year, we will begin *in vivo* studies with the mock vs. COX-2+ transfected MDA-PCa-2b cells, continue our co-culture studies (PCa with osteoblasts) in the presence or absence of IL-6, and stably transfect PC3-ML cells to knockout COX-2 expression (using a COX-2 dominant-negative plasmid).

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