Growth Regulation of Human Prostate Cancer Cells by Bone Morphogenetic Protein-2

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Abstract
Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) family and have been identified as factors that stimulate bone formation in vivo. They turned out to be multifunctional molecules regulating the growth, differentiation, and apoptosis in various target cells. Some BMPs and their receptors (BMPRs) are expressed on prostate cancer cells. We have reported previously that BMPR-IB mRNA expression is highest in the prostate, a characteristic that is not shared by the other BMPRs, BMPR-IA and BMPR-II. However, the amounts of BMPR-IB mRNA were significantly low in prostate tissues after androgen withdrawal therapy. They were also low in prostate cancer cell lines. Semiquantitative RT-PCR showed that BMPR-IB mRNA was induced by androgen in the androgen-sensitive human prostatic cancer cell line LNCaP, whereas the expression of BMPR-IA and BMPR-II mRNAs was not affected by androgen. When the recombinant human BMP-2 was added to the LNCaP cells in the presence of androgen, cell growth was inhibited. In contrast, the growth rate was increased by the addition of the same ligand when the cells were cultured in the absence of androgen; under this condition, the amounts of BMPR-IB mRNA were decreased significantly. These observations showed that the amounts of BMPR-IB, but not those of BMPR-IA, were regulated by androgen and further suggest that BMPR-IA and BMPR-IB differentially modulate prostate cancer cell growth in response to BMP under different hormonal conditions; BMPR-IA elicits growth stimulation, and BMPR-IB conveys a negative regulatory signal in response to BMP.

Introduction
The most favored site of metastasis of prostate cancer is bone, where new bone formation is often induced at the metastatic foci (1, 2). However, molecular mechanisms involved in bone metastasis remain unsolved. The seed and soil theory permits metastatic tumor growth in certain organs because of the enhanced adhesion, chemotaxis, and growth at these sites (3–5). The selective spread of prostate cancer to bone, together with the consistent ability of prostate cancer to incite an osteoblastic reaction, suggests that there are bidirectional stimulatory paracrine pathways between prostate cancer cells and osteoblasts and bone stromal cells (6).

BMPs were originally extracted from bone as factors that induce cartilage and bone formation in vivo. The amino acid sequence revealed that they belong to the TGF-β superfamily (7, 8). A number of studies have demonstrated that BMPs stimulate proteoglycan synthesis in chondroblasts as well as alkaline phosphatase activity, collagen synthesis, and osteocalcin expression (9–11). In addition to the functions as a possible bone-inducing factor, BMPs have been shown to play important roles during vertebrate organogenesis (12). Furthermore, BMPs were implicated in the chemotaxis of monocytes (13), migration of osteoblasts (14), and differentiation of neural cells (15). BMPs and their receptors are widely distributed not only in the bone and cartilage but in other tissues. BMPs may constitute another example of a growth/differentiation factor family that shows a diverse function, depending on the target cells.

Three BMPRs have been identified: two type I receptors (IA and IB) (16, 17) and one type II receptor (18–20). The three molecules are members of the TGF-β transmembrane serine-threonine kinase receptor family. Among 15 BMP family members known to date, at least BMP-2, BMP-4, BMP-7, and growth differentiation factor-5 were shown to bind to BMPRs (21–23). It has also been reported that some of the BMPs can bind activin type I and II receptors (24). Effective ligand binding and signal transduction of BMPs are dependent on the optimal cooperation of one of the two type I receptors and the single type II receptor, which together form the necessary heteromeric receptor complexes (18–20).

Recently, we reported the cloning of the human BMPR-IB cDNA and the expression of BMPRs in various human tissues and cell lines. Among various human tissues, the highest amounts of BMPR-IB mRNA were found in the prostate. Although the level of mRNA of BMPR-1A and BMPR-1II was similar among cancerous and noncancerous prostate tissues, BMPR-IB expression was significantly low in cancerous and noncancerous prostate tissues obtained from the patients who received androgen withdrawal therapy. BMPR-IB mRNA expression was also low in the prostate cancer cell lines (25). Although the expression of some BMPs and BMPRs was detected in normal and cancerous prostate tissues and in some prostate cancer cell lines, the biological significance of BMPs in the development of prostate cancer remains to be established (26, 27).

In this study, we demonstrate that among the three BMPRs, only BMPR-IB expression was up-regulated by androgen in an androgen-sensitive prostate cancer cell line, LNCaP. Culture of LNCaP cells with the rhBMP-2 in the presence of androgen resulted in the up-regulation of BMPR-IB mRNA and growth inhibition of the cells. Culture of LNCaP cells with rhBMP-2 in the absence of androgen, however, led to the down-regulation of BMPR-IB mRNA and growth stimulation. This is the first report that shows that the growth of prostate cancer cells was regulated by BMP, and that the growth was negatively regulated by BMP in androgen-sensitive prostate cancer cells through induction of BMPR-IB.
Materials and Methods

Cell Lines and Cell Culture. The human prostate cancer cell lines LNCaP, PC-3, and DU-145 were obtained from the American Type Culture Collection (Rockville, MD). The TSU-PR1 cell line was provided by Dr. Shori Kanoh (The University of Tsukuba, Tsukuba, Japan). All of the cell lines were routinely maintained in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin. For the steroid stimulation analysis, LNCaP cells were cultured in a serum-free medium for 3 days prior to the addition of synthetic androgen R1881 (New England Nuclear, Boston, MA) or 17β-estradiol (Sigma Chemical Co.).

RNA Preparation and Semiquantitative RT-PCR. Androgen dependence of BMPR mRNA expression was examined by semiquantitative RT-PCR based on the comparison with an internal reference, β-actin expression. The analyses used four prostate cancer cell lines that express (LNCaP) or do not express (PC-3, TSU-PR1, and DU-145) androgen receptor (Ref. 28; data not shown). The prostate cancer cell lines were stimulated by various concentrations of R1881 (0–1 μM) or 17β-estradiol (0–100 nM) for 24 h. Total RNAs were extracted by ISOGEN (Nippogene, Tokyo, Japan) according to the protocol recommended by the manufacturer. Likewise, total RNAs were extracted from LNCaP cells that were stimulated by the combination of 100 ng/ml rhBMP-2 (Genetics Institute, Inc., MA) with or without 1 nM R1881 for 48 h. The rhBMP-2 protein was donated by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan).

A 10-μg portion of total RNAs from the prostate cancer cell lines was reverse-transcribed by random primer and Super-Script reverse transcriptase (Life Technologies, Inc.) in a volume of 40 μl. The resulting cDNA (0.5 μl) was subjected to PCR with the primers described below. The RT-PCR reaction in the exponentially amplifying cycle allowed semiquantitative comparison of the mRNA expression. The expected sizes of the PCR products and the primer sequences used are as follows: hBMPR-IA, 1401 bp, 5'-GCATACTAATTGACATTTGCT-3' and 5'-TAGAGTTTCTCTCCCTTGAGG-3'; hBMPR-IB, 634 bp, 5'-GCAGCACAGACGGATAGT-3' and 5'-TCTCATGCCTCATCAACAC-3'; hBMPR-II, 694 bp, 5'-ACGGGAGAGAAGACGAGCT-3' and 5'-CTAGATCAAGAGGGTGCG-3'; BMP-2, 671 bp, 5'-TCATAAAACCTGCAACAGCCAACTCG-3' and 5'-GCTGTACAGCAGACCCAC-3'; PSA, 540 bp, 5'-GGTCGACACAGCCTGATCTCA-3' and 5'-CCACGATGGTGTCCTGATC-3'; β-actin, 313 bp, 5'-GACATCTCATGAAGATCCT-3' and 5'-GCGGATGTCCACGTCACACT-3'. These primers were designed from the sequences of human BMPR-IA (16), human BMPR-IB (25), human BMPR-II (20), BMP-2 (7), PSA (29), and β-actin (30). The amplification reactions were performed with an initial incubation step at 95°C for 3 min followed by 30 cycles each at 95°C for 45 s, 60°C for 1 min, and 72°C for 30 s. These cycles were followed by a final incubation step at 72°C for 10 min. The samples were subjected to electrophoresis in 2% agarose gel and stained with ethidium bromide. The RT-PCR analysis was repeated at least twice for each of the two independent experiments.

Cell Growth Assay. In RPMI 1640 containing 10% FBS, LNCaP, PC-3, TSU-PR1, and DU-145 were seeded at 5000, 1000, 1000, and 1000 cells/well, respectively, into 96-well plates and cultured for 24 h. In serum-free conditions, these cells were seeded at 5000 cells/well into 96-well plates and cultured for 24 h. Then the same volume of RPMI 1640 was added either with or without 200 ng/ml rhBMP-2. In androgen challenge tests, LNCaP cells were seeded at 5000 cells/well and cultured in a serum-free RPMI 1640 for 3 days followed by the addition of the same volume of RPMI 1640 containing 200 ng/ml rhBMP-2 either with or without 2 nM R1881. The final concentrations
We have reported previously that the mRNA expression of BMPR-IB, TSU-PR1, and DU-145, addition of rhBMP-2 did not lead to change when the compound was added, and after 90 mm of incubation at 37°C, absorbance at 490 nm of the ligand, BMP-2, was examined by the same semiquantitative RT-PCR. The BMP-2 mRNA expression was not changed by 1 nM R1881 in LNCaP or PC-3 cells (Fig. 3). These results suggest that only BMPR-IB expression is uniquely regulated by androgen in the BMP/BMPR system and contributes to the growth effect of rhBMP-2 in the androgen-sensitive LNCaP cells.

The induction of the BMPR-IB mRNA in the LNCaP cells was dose dependent. When examined under various concentrations of R1881 (0–1 μM) and 17β-estradiol (0–100 nM), the amounts of BMPR-IB mRNA were increased over the 0–1 nM range of R1881 (Fig. 4a). The PSA was used as a positive control, because its expression has been shown to be induced by androgen (33). Interestingly, both PSA and BMPR-IB mRNA were up-regulated by β-estradiol in the semiquantitative RT-PCR analysis (Fig. 4b). It has been shown that 17β-estradiol binds to the androgen receptor in LNCaP cells, because the cells carry a mutation in the androgen-binding domain of the androgen receptor, resulting in the alteration of the ligand-binding specificity and steroid-induced transactivation (34, 35).

Regulation of the Amounts of BMPR mRNAs by Androgen and rhBMP-2. The status of BMPR expression in the LNCaP cells showing the opposite growth response to BMP-2 (Fig. 2) was examined by semiquantitative RT-PCR. RNAs were extracted from aliquots of the cells analyzed in Fig. 2, where the LNCaP cells in a serum-free medium were treated with 100 ng/ml rhBMP-2 and/or 1 nM R1881. When only rhBMP-2 was added to the medium, the levels of the mRNA of BMPRs were not changed on day 2. The addition of the synthetic androgen up-regulated the BMPR-IB expression (Fig. 5, fifth lane from left), although the degree of the induction was lower than that stimulated by 1 nM R1881 only (Fig. 5, fourth lane from left). By contrast, the expression of BMPR-IA and BMPR-II was unaffected by any combination of rhBMP-2 and R1881. The same results were obtained on day 5 (data not shown).

Discussion

Tumor progression to the stage of metastasis may result partly from the selection of certain primary tumor cell clones that are competent for survival, invasion, and growth at secondary sites and may be regulated through the action of the growth factors available there. Prostate cancer frequently metastasizes to the bone. BMPRs were found expressed in the prostate cancer tissues and prostatic cancer cell lines (25), whereas BMPs were abundant in the bone matrix (8). In this report, we showed that rhBMP-2 inhibits the growth of androgen-sensitive LNCaP cells, but not that of androgen-insensitive prostate cancer cells (PC-3, TSU-PR1, and DU-145), in a medium containing 10% FBS (Fig. 1). Interestingly, the growth effect of rhBMP-2 on LNCaP cells shifted from stimulatory to inhibitory action by the addition of androgen in the serum-free medium (Fig. 2). The semiquantitative RT-PCR analyses showed that only the BMP-2 mRNA expression among three BMPRs was induced by the androgen stimulation, resulting in the up-regulation of BMPR-IB (Figs. 3 and 5). Because the signaling specificity could occur through interaction of BMP-2 on the growth of LNCaP cells may be related to the status of BMPR expression, which was examined by semiquantitative RT-PCR. Fig. 3 shows that the BMPR-IB mRNA was up-regulated by androgen in LNCaP cells, which express the androgen receptor, but not in the PC-3 cells, which do not express the androgen receptor. In contrast, the levels of BMPR-IA and BMPR-II mRNA were not altered by androgen in the androgen receptor-positive LNCaP cells or in the androgen receptor-negative PC-3 cells. The same results were observed in other androgen receptor-negative cell lines, DU-145 and TSU-PR1 (data not shown). The androgen dependence of the expression of the ligand, BMP-2, was examined by the same semiquantitative RT-PCR. The BMP-2 mRNA expression was not changed by 1 nM R1881 in LNCaP or PC-3 cells (Fig. 3). These results suggest that only BMPR-IB expression is uniquely regulated by androgen in the BMP/BMPR system and contributes to the growth effect of rhBMP-2 in the androgen-sensitive LNCaP cells.

Fig. 2. The effect of rhBMP-2 on the proliferation of LNCaP cells with or without androgen. LNCaP cells were cultured in RPMI 1640 only (a) or with 1 nM R1881 added to RPMI 1640 medium (b). Each cell was treated with 100 ng/ml of rhBMP-2, and the growth of growth rates (Fig. 1). However, when the LNCaP cells were grown in a serum-free medium, addition of the BMP-2 resulted in growth inhibition, when both the 1 nM R1881 and 100 ng/ml rhBMP-2 were added to the serum-free medium. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega Corp.) as described (31). Each day, the tetrazolium compound was added to the serum-free medium.
BIFUNCTIONAL GROWTH EFFECTS OF rhBMP-2 IN LNCaP CELL LINE

Fig. 3. Androgen dependence of mRNA expression of BMPRs in prostate cancer cells. Semiquantitative RT-PCR using androgen-sensitive LNCaP cells or androgen-insensitive PC-3 cells is shown. The cells were maintained in serum-free medium for 3 days before the addition of 1 nM R1881 for 24 h.

a. R1881

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Fig. 4. Dose-dependent induction of BMPR-IB mRNA expression in LNCaP cells. LNCaP cells were cultured in serum-free medium for 24 h containing various concentrations of R1881 (0–1 μM; a) or 17β-estradiol (0–100 nM; b). The RT-PCR reaction in the exponentially amplifying cycles allowed semiquantitative comparison of gene expression. Only the PCR products at representative cycles were shown: BMPR-IB, cycle 26; PSA, cycle 30; and β-actin, cycle 22.
cells were cultured in the serum-free RPMI 1640 for 48 h in the presence of rhBMP-2 (100 ng/ml) with or without 1 nm R1881. The RT-PCR reaction in the exponentially amplifying cycles allowed semiquantitative comparison of gene expression. Only the PCR products at representative cycles were shown: BMPR-IA and II, cycle 25; BMPR-IB, cycle 28; and $\beta$-actin, cycle 20.

BMPR-II with one of the two type I receptors, it is possible that growth signaling of BMP-2 through BMPR-IB is growth suppressive and that through BMPR-IA is growth stimulative. It is possible that the BMP-2 affects the prostatic cells in different ways, depending on the balance of the BMPR-IB and BMPR-IA expression on the cells; BMP-2 may stimulate prostatic cell proliferation in an androgen-deprived condition, whereas the same ligand may induce growth suppression and promote differentiation in the presence of androgen in vivo.

Several members of the TGF-β superfamily are primarily known as growth inhibitors of various cell types (36–38), whereas at least the archetype of the family, TGF-β1, is known to either inhibit or stimulate growth, depending on the target cells and their conditions (39). The molecular basis for the bifunctionality is yet to be established, although many studies have focused attention on the difference in the receptor species; ligand binding assay and affinity labeling of the receptor have demonstrated alterations of the receptor subtype expressed on the cell surface, depending on the cell density (40), cell cycle phases (41), or ligand concentration (42). More recently, it has been demonstrated that TGF-β stimulated the proliferation of adult lung fibroblasts but inhibited the growth of fetal lung fibroblasts. Because a dominant-negative form of TGF-β type II receptor blocked not only TGF-β-induced mitogenic action upon adult lung fibroblasts but also TGF-β-induced growth inhibition of fetal lung fibroblasts, TGF-β type I receptors might be involved in the mechanism of multiple effects of TGF-β (43).

To our knowledge, the present study is the first report to demonstrate a bifunctionality of BMP-2, another member of the TGF-β family. Moreover, our data suggest that the level of the BMPR-IB expression regulated by androgen is a molecular mechanism for the bifunctional growth effects of BMP-2.

It has been demonstrated that BMP-2, BMP-4, BMP-7, and growth differentiation factor-5 can bind to BMPRs, which require the cooperation of one of the two type I receptors and the type II receptor for optimal ligand binding and signal transduction (21–23). It was also shown that BMP-2 binding was more efficient to the BMPR-IB and BMPR-II complex than to the BMPR-IA and BMPR-II complex (18). In addition to that, heterodimers of BMP-2 and BMP-7, and BMP-4 and -7 have much higher activity than either homodimer (12, 44). In the prostate tissues and some prostate cancer cell lines, at least BMP-2, BMP-4, and BMP-7 mRNAs were detected (26, 27). The complex nature of the varied affinity and specificity of BMP ligands to their receptors implicates a significant functional diversity of the BMP/BMPR system in the prostatic cells in vivo. Which receptor combinations occur in vivo, whether different type I receptors trigger distinctive downstream pathways, and whether competitive interaction for receptor binding between ligands occurs in vivo are a few of the many questions to be answered regarding the BMP/BMPR system. Moreover, the complexity of the multihormonal control of the prostatic cell growth has been documented by the presence of other androgen-induced changes of the mRNA and protein expression of growth factors and receptors; androgen could increase TGF-α and epidermal growth factor (EGF) receptor expression in an androgen-dependent human prostate cancer cell line ALVA101 (45). Androgen withdrawal such as in the rat castration model resulted in decreased EGF, insulin-like growth factor (IGF), and basic fibroblast growth factor, whereas TGF-β, EGF receptor, insulin-like growth factor receptor, and TGF-β receptor were increased (46, 47). In contrast to those receptors, BMPR-IB mRNA expression was uniquely down-regulated following androgen ablation.

For over the last 50 years, androgen ablation has been the standard treatment for prostate cancer. In most of the cases, however, prostate cancer cells eventually lose androgen dependence and metastasize to the bone, where the cancer induces new bone formation (2). Several different and sometimes conflicting molecular mechanisms have been proposed for the loss of androgen dependence in advanced prostate cancers, suggesting multiple modes of the hormone independence, including the loss of androgen receptor expression and amplification or mutation of the androgen receptor gene (48–51). However, it has been an enigma why the skeletal metastases are often the only sites of disease progression at the time of failure of endocrine therapy (6). Our study suggests that one of the possible mechanisms involves BMP-2 acting as a positive growth regulator under the condition where the androgenic signal has been ablated either by therapy or by the change of the cellular signaling circuitry, such as the loss of androgen receptor expression. Further study is required to elucidate the in situ expression of BMPs and BMPRs in prostate cancer tissues and their metastatic bone sites.

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References


* Unpublished data.
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