Overexpression of a Dominant Negative Type II Bone Morphogenetic Protein Receptor Inhibits the Growth of Human Breast Cancer Cells

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Abstract

Bone morphogenetic proteins (BMPs) exert cell type-specific effects on cell proliferation. To clarify the role of the BMP pathway in human breast cancer cells, we used a dominant negative strategy with a truncated human type II BMP receptor (DN-BMPRII; amino acid 1–172) fused to the NH2 terminus of enhanced green fluorescent protein. Transient overexpression of DN-BMPRII interfered with BMP-2-induced Smad1 transcriptional activity and caused cells to accumulate in G1. Stable cell lines that constitutively overexpressed DN-BMPRII were resistant to BMP-2-induced Smad1 phosphorylation and proliferated much more slowly than control stable cell lines. These results suggest that BMPs interacting with type II BMP receptors contribute to the proliferation and/or survival of human breast cancer cells.

Introduction

BMPs are members of a large family of growth factors that includes activins and TGF-β. BMPs, activins, and TGF-β are structurally related and use similar signal transduction pathways that involve transmembrane receptors and Smad proteins (1). BMP signaling is initiated by the association between BMPs such as BMP-2 and the membrane-associated type I and II BMP receptors. On activation of the receptor complex by the cytokine, the type I receptor phosphorylates specific intracellular proteins named Smads, namely, Smad1, Smad5, and Smad8. Once phosphorylated, these receptor-associated Smads associate with the product of the tumor suppressor gene Smad4. The resulting hetero-oligomer is then translocated to the nucleus to modulate the expression of BMP-responsive genes, presumably by interacting with BMP response elements in the promoters of BMP target genes. The role of BMPs in development and endochondral bone formation is well characterized, but their role in cancer is somewhat controversial (2). Depending on the particular cell type, culture conditions, or dose of BMP used, BMPs have been shown to stimulate or repress the growth of cancer cells. In fact, BMP-2 has been shown to stimulate the growth of pancreatic carcinoma cells and prostate cancer cells (3, 4). Recently, BMP-2 was found to suppress apoptosis induced by tumor necrosis factor α or by serum deprivation (5, 6). On the other hand, BMP-2 clearly inhibits the growth of cancer cells of many origins including cells derived from thyroid, prostate, myeloma, and pancreatic cancers (3, 4, 7–9). Whereas the role of TGF-β in the control of breast cancer cell proliferation has been extensively studied, considerably less is known about the role of BMPs. We know that some BMPs among those cloned to date are expressed in human breast cancer cells. Indeed, BMP-2, BMP-3, BMP-5, and BMP-6 have been shown to be expressed in human breast cancer cells (10, 11). We and other laboratories have demonstrated that BMP-2 could inhibit the growth of estrogen-sensitive as well as estrogen-insensitive human breast cancer cell lines (12–14). Considering that there exist several different BMPs, that these exert opposite effects on cell proliferation, and that not all of the BMPs may be physiologically relevant in all cell types, we hypothesized that the best way to determine the role of BMPs in breast cancer cells would be to constitutively repress the BMP pathway. To this end, we designed DN-BMPRII and established T-47D cell lines that stably express the recombinant receptors. We found that transient or stable overexpression of DN-BMPRII blocks activation of Smad1 by BMP-2 and inhibits the growth of breast cancer cells. These results suggest that BMPs interacting with BMPR-Is contribute to the proliferation of human breast cancer cells.

Materials and Methods

Cells and Hormones. The cell lines used in these studies were obtained from the American Type Culture Collection (Manassas, VA). For routine culture, T-47D cells were grown as described previously (12). Recombinant human BMP-2 was purchased from R&D Systems (Minneapolis, MN). BMP-2 was resuspended in PBS buffer supplemented with 0.1% (w/v) BSA as carrier protein; 17β-estradiol was purchased from Steraloids (Wilton, NH).

Plasmids. The plasmids containing Smad cDNA fragments that were used as probes for Northern blots and in transfection assays were constructed as follows: the Smad1 cDNA (coding nucleotides 1–1398) was amplified from LNCaP cell RNA by reverse transcription-PCR using Pfu polymerase and subcloned in pcDNA3-Gal4 to generate pcDNA3-Gal4-Smad1. AU1 epitope-tagged Smad4 was subcloned in pcDNA3 as described previously (15). The pcMV5 ALK6Q203D plasmid was generated by site-directed mutagenesis of a plasmid expressing the wild-type receptor using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The pEGFPN3-BMPRII(1–172) plasmid was generated by reverse transcription-PCR from MDA-MB-468 cells using oligonucleotide primers corresponding to nucleotides 1–26 and 516–487 (codons 1–172) of the BMP-2 open reading frame. The primers contained restriction sites for cloning the BMPRII-PCR product into the BGII and Apal sites of pEGFP-N3 (Clontech, Palo Alto, CA) to generate a plasmid that expresses the DN-BMPRII fusion protein. Plasmid pcDNA3-Egfp-F, which encodes the EGFP protein fused to the HA epitope (YPYDVPDYASL) and bearing a COOH-terminal furin cleavage signal encoded by the 18 COOH-terminal aa of the human p21-ras protein, was constructed using PCR.

Transfections and Luciferase Assays. As indicated in Fig. 1B, 18–24 h after plating, T-47D cells were transfected with pG5-E1B-TATA-Luc reporter plasmid, pRL-null Renilla luciferase control vector, and expression plasmids. Transfections were performed using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). Equal amounts of DNA were added to each well by adding pcDNA3 vector as necessary. The transfected cells were then cultured for 24 h, and BMP-2 (0.4 nm) or an equivalent volume of BMP-2 vehicle (PBS-BSA, 0.1%) was added. The cells were lysed 20 h later, and the luciferase activities were measured. Data are expressed as the
normalized luciferase activity (firefly luciferase activity divided by Renilla luciferase activity) of transfected cells relative to the normalized luciferase activity (firefly luciferase activity divided by Renilla luciferase activity) of untransfected cells. The mean ± SE of five determinations from two independent experiments is shown.

**Results**

To study the role of the BMP pathway in the control of breast cancer cell proliferation, we designed a hybrid BMPR-II composed of the extracellular and transmembrane domains of BMPR-II (aa 1–172) expressed as a fusion to the NH2 terminus of EGFP (Fig. 1A). We hypothesized that this hybrid receptor, which does not contain the kinase domain, would exert a dominant negative effect on BMP signaling by sequestering BMPs into inactive receptor complexes. We chose to use DN-BMPRII because BMPR-II is expressed in human breast cancer cells, because BMPR-II has been shown to interact specifically with the BMP subfamily of the TGF-β superfamily, and because overexpression of truncated BMPR-II has been shown to block the BMP pathway in Xenopus laevis and in chick chondrocytes (11, 17–20).

To assess the efficacy of DN-BMPRII, we examined its effect on BMP-2-induced Smad1 activation in transiently transfected cells (Fig. 1B). We cotransfected expression vectors for Gal4Smad1 and Smad4 together with a luciferase reporter vector containing five repeats of the DNA-binding site for the DNA-binding domain of Gal4 (Gal4DBD). As shown in Fig. 1B, cotransfection of Gal4Smad1 and Smad4 increased luciferase activity 12-fold compared with the luciferase activity measured in cells that were transfected with a plasmid expressing Gal4DBD alone (compare Lane 1 with Lane 2). Moreover, BMP-2 treatment (0.4 nM) of cells transfected with Gal4Smad1 and Smad4 caused a further 2.3-fold increase in luciferase activity compared with untreated cells transfected with Gal4Smad1 and Smad4, thereby demonstrating that the transcriptional activity of this reporter system was activated by BMP-2 treatment (Lane 3). We then cotransfected the DN-BMPRII expression vector (pEGFPN3-BMPRII; 1–172) together with plasmids expressing Gal4Smad1 and Smad4. DN-BMPRII did not affect the transcriptional activity of Gal4Smad1 and Smad4 (Lane 4). However, DN-BMPRII blocked the stimulatory effect of BMP-2 on Gal4Smad1 and Smad4-induced promoter activity (Lane 5). To ensure that the inhibitory effect of DN-BMPRII was due to a specific blockade of the BMP pathway at a step upstream of type I BMP receptor activation, we overexpressed a constitutively active form of the type IB BMP receptor (ALK6Q203D) to bypass the dominant negative effect of DN-BMPRII. As shown in Lanes 8 and 9 of Fig. 1B,
ALK6Q203D completely reversed the dominant negative effect of DN-BMPRII. These results demonstrate that DN-BMPRII specifically blocks the BMP pathway.

**Overexpression of DN-BMPRII Alters the Cell Cycle Distribution of Human Breast Cancer Cells.** We next examined the effect of DN-BMPRII on the proliferation of T-47D cells, T-47D cells were transiently transfected with plasmids expressing EGFP-F or DN-BMPRII. Four days after transfection, the cells were harvested, and the cell cycle distribution of EGFP-positive cells was determined by flow cytometric analysis (Table 1). EGFP-F was chosen as control because it is targeted to the membrane, as is DN-BMPRII, and, like other membrane-targeted proteins, it is resistant to the fixation process needed for cell cycle analysis. As shown in Table 1, the proportion of T-47D cells in the G1 phase of the cell cycle was significantly lower in cells expressing EGFP-F than in cells expressing DN-BMPRII (67.5 ± 2.6% versus 77.3 ± 3.7%, P < 0.05). On the other hand, the proportion of T-47D cells in the S phase of the cell cycle was significantly higher in the EGFP-F-expressing cells compared with DN-BMPRII-expressing cells (11.5 ± 1.2% versus 5.3 ± 1.3%, P < 0.05). No significant difference in the distribution of cells in the G2/M phase of the cell cycle was observed between the two groups. These results show that transient overexpression of DN-BMPRII causes T-47D cells to accumulate in the G1 phase of the cell cycle.

**Characterization of Cell Lines Expressing DN-BMPRII.** The inhibitory effect of transiently expressed DN-BMPRII on the cell cycle progression of T-47D cells was unexpected, considering that BMP-2 was reported previously to inhibit T-47D cell proliferation (12). We therefore stably transfected DN-BMPRII into T-47D cells to further analyze the effect of DN-BMPRII on the growth of human breast cancer cells. As control for these experiments, we established T-47D cell lines that stably express wild-type EGFP. T-47D cells were transfected with EGFP or DN-BMPRII expression plasmids, and clones that integrated the plasmids were selected with neomycin (750 μg/ml). We isolated and amplified a number of clones that displayed green fluorescence when exposed to UV light. Two control clones and two clones expressing DN-BMPRII were selected for further analysis. We determined the corresponding levels of EGFP proteins by immunoblotting using α-tubulin as loading control (Fig. 2A). On the basis of the levels of EGFP-BMPRII fusion protein, clone DN-BMPRII.1 expressed approximately 10 times more DN-BMPRII than clone DN-BMPRII.2. The two clones of EGFP-expressing cells that served as controls for subsequent experiments expressed intermediate levels of EGFP that were comparable with those of clone DN-BMPRII.1. To verify that the DN-BMPRII fusion protein was properly localized at the cell membrane, we analyzed its localization by fluorescence microscopy in stable T-47D cell lines. As shown in Fig. 2B (top panel), T-47D cells expressing EGFP displayed fluorescence localized throughout the cell. On the other hand, in cells transfected with the pEGFPN3-DN-BMPRII(1–172) expression vector, the fluorescence was localized mainly at the cytoplasmic membrane (Fig. 2B, bottom panel), showing that the DN-BMPRII fusion proteins were correctly located in the cell.

**Overexpression of DN-BMPRII Alters the Cell Cycle Distribution of Human Breast Cancer Cells.**

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<thead>
<tr>
<th>Overexpressed protein</th>
<th>G1 phase</th>
<th>S phase</th>
<th>G2/M phase</th>
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<tbody>
<tr>
<td>EGFP-F</td>
<td>67.5 ± 2.6</td>
<td>11.5 ± 1.2</td>
<td>21.0 ± 3.0</td>
</tr>
<tr>
<td>DN-BMPRII</td>
<td>77.3 ± 3.7*</td>
<td>5.3 ± 1.3*</td>
<td>17.5 ± 2.8</td>
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* P < 0.05 versus EGFP-F by statistical analysis using Student’s t test.
We reported previously that BMP-2 up-regulates p21 protein levels in T-47D cells (12). We therefore examined p21 levels in cultures of cells expressing EGFP or DN-BMPRII after a 24-h exposure to BMP-2 (0.4 nM) or vehicle. As shown in Fig. 3B, BMP-2 up-regulated p21 protein levels by an average of 16.4-fold (range, 5.4–24.5-fold) and 4.2-fold (range, 3.1–5.8-fold) in clones EGFP.1 and EGFP.2, respectively. Interestingly, in the absence of BMP-2, p21 protein levels were higher in cells expressing DN-BMPRII compared with those in cells expressing EGFP. Moreover, BMP-2 had minimal effects on p21 protein levels in both clones expressing DN-BMPRII. In fact, BMP-2 up-regulated p21 protein levels by an average of 2.0-fold (range, 1.8–2.3-fold) and 1.1-fold (range, 0.9–1.4-fold) in DN-BMPRII.1 and DN-BMPRII.2 cells, respectively. These results show that DN-BMPRII blocks the BMP pathway in human breast cancer cells overexpressing DN-BMPRII.

Stable Overexpression of DN-BMPRII Inhibits T-47D Human Breast Cancer Cell Proliferation. The results of transient expression experiments indicated that overexpression of DN-BMPRII inhibited cell proliferation. To determine whether stable expression of DN-BMPRII can modulate the proliferation of human breast cancer cells, we compared the proliferation of cells that express DN-BMPRII with that of cells that express EGFP. Cells from EGFP and DN-BMPRII clones were grown for up to 9 days in culture medium containing the potent natural estrogen 17β-estradiol (1 nM), and cell proliferation was assessed by measuring the DNA content of each well, which is a reliable indication of cell number. As shown in Fig. 4, cells expressing EGFP alone proliferated more rapidly than cells expressing DN-BMPRII. In fact, after 9 days in culture, the number of cells of clones EGFP.1 and EGFP.2 increased 7.9 ± 3.2 and 6.7 ± 0.6 times, respectively, compared with day 0. On the other hand, the number of cells expressing DN-BMPRII only increased by 20% (DN-BMPRII.2) to 60% (DN-BMPRII.1) during the same period. These results indicate that both transient and stable overexpression of DN-BMPRII inhibit the growth of T-47D human breast cancer cells.

Discussion

The results presented in this article provide exciting new information on the role of BMPs in the control of human breast cancer cell proliferation. In this article, we show that blocking the BMP pathway by overexpression of DN-BMPRII inhibits the proliferation of T-47D cells.

Because the identity of the BMPs expressed in T-47D cells is not known and because BMPR-II is specific to the BMP pathway, we thought that the best approach to determine the role of BMPs in these breast cancer cells was to overexpress DN-BMPRII. We confirmed that the BMP pathway was effectively blocked in cells that express DN-BMPRII by testing the effect of BMP-2 on two known BMP-responsive parameters. Cells that overexpress DN-BMPRII were refractory to BMP-2-induced Smad1 activation and phosphorylation. The inhibitory effect of DN-BMPRII was potent, as demonstrated by the fact that the effects of BMP-2 were blocked as effectively in cells expressing relatively high levels of DN-BMPRII as in cells expressing 10-fold lower levels of DN-BMPRII.

Previous work by our laboratory and others suggested that BMPs inhibit the proliferation of human breast cancer cells (11–14). However, we report here that inhibition of the BMP pathway through overexpression of a dominant negative form of BMPR-II actually represses T-47D proliferation. The inhibitory effect of DN-BMPRII on T-47D cell proliferation was also independent of the levels of DN-BMPRII protein because both clones we studied showed comparable proliferation rates. This finding suggests that BMPs may stimulate, rather than inhibit, breast cancer cell proliferation.

To address this hypothesis, we subsequently cultured T-47D cells in...
medium containing a soluble ALK3\textsuperscript{BMPR-IA} receptor (BMPR-IA/ALK3/Fc chimera), which competes with endogenous BMP receptors for BMP binding and therefore blocks their activation. Although the growth-inhibitory effect of BMP-4 on T-47D cells was specifically and completely abrogated by addition of soluble ALK3 receptor to the culture medium, the receptor had no effect (neither stimulatory nor inhibitory) on T-47D cell proliferation in the absence of BMP-4 (data not shown). These results suggest that the growth-inhibitory effect of DN-BMPRII is not due to interference with BMPs that bind ALK3. We presently know that BMP-2, BMP-4, and BMP-7 bind ALK1, which means that these BMPs do not stimulate the growth of T-47D cells (21). However, because BMPRII also interacts with ALK2 and ALK6, BMPs interacting with these type I receptors could potentially induce cell growth with BMPRII.

It is unlikely that the growth inhibition induced by expression of DN-BMPRII would be due to interference of DN-BMPRII with other signaling pathways. In fact, the reversal of the dominant negative effect of DN-BMPRII by ALK6Q203D overexpression shows that this dominant negative effect is specific to the BMP pathway.

Because cells that overexpressed DN-BMPRII have been cultured for weeks, we cannot exclude that the differences observed in growth between DN-BMPRII- and EGFP-expressing cells are not due to a differentiation process caused by constitutive expression of DN-BMPRII. However, the decrease in the number of cells in S phase observed in T-47D cells transiently expressing DN-BMPRII shows that DN-BMPRII inhibits proliferation rapidly after transfection and confirms the growth-inhibitory effect of DN-BMPRII.

The inhibitory effect observed when DN-BMPRII is expressed in T-47D cells could be due to the inhibition of the p38 MAP kinase pathway. It is now admitted that BMPs can mediate some of their effects through the p38 MAP kinase pathway (22, 23). In fact, an interesting article published by Nohe et al. (24) showed that a truncated BMPRII receptor similar to the one we used in our experiments (but lacking the COOH-terminal EGFP protein) could specifically block the p38 MAP kinase pathway but not the Smad1 pathway. In our model, DN-BMPRII blocked Smad1-induced transcription. This difference may be attributed to differences in the endogenous type I receptors expressed in each cell line. In addition, Nohe et al. (24) used transiently transfected cells to study Smad1 phosphorylation, whereas we used cells that stably express DN-BMPRII.

Interestingly, a study using BMPRII mutants that have been found in patients suffering from primary pulmonary hypertension showed that transient transfection of normal murine breast epithelial cells (NMuMG) with plasmids expressing these mutants stimulated the proliferation of these cells (25). These differences could be attributed to the different molecular mechanisms responsible for the dominant negative effects of truncated versus mutated BMPRII receptors, interspecies differences, and/or differences between normal and malignant tissues. Additional studies will be necessary to assess this issue.

The observation that DN-BMPRII inhibits breast cancer cell proliferation could potentially have a profound impact on the design of clinical applications involving the use of BMPs. In fact, previous reports suggested that activation of the BMP pathway could be a useful approach in the treatment of breast cancer. This position will have to be reevaluated in light of the findings reported herein. These results will prompt us to investigate the expression of BMPs and their receptors and their respective roles in the growth of human breast cancer cells, both in their natural environment and at sites of metastasis.

Acknowledgments

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