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CpG-Oligodeoxynucleotide Inhibits Smad-Dependent Bone Morphogenetic Protein Signaling: Effects on Myeloma Cell Apoptosis and In Vitro Osteoblastogenesis

Nikolai N. Nørgaard,* Toril Holien,* Sofia Jönsson,*,† Hanne Hella,* Terje Espevik,* Anders Sundan,* and Therese Standal†*

The TLR9 agonist CpG-oligodeoxynucleotide (CpG-ODN) with a phosphorothioate backbone (PTO-CpG-ODN) is evaluated in clinical trials as a vaccine adjuvant or as treatment of cancers. Bone morphogenetic proteins (BMPs) regulate growth and differentiation of several cell types, and also induce apoptosis of cancer cells. Cross-talk between BMP- and TLR-signaling has been reported, and we aimed to investigate whether CpG-ODN influenced BMP-induced osteoblast differentiation or BMP-induced apoptosis of malignant plasma cells. We found that PTO-CpG-ODN inhibited BMP-2–induced osteoblast differentiation from human mesenchymal stem cells. Further, PTO-CpG-ODN counteracted BMP-2- and BMP-6–induced apoptosis of the human myeloma cell lines IH-1 and INA-6, respectively. In contrast, PTO-CpG-ODN did not antagonize the antiproliferative effect of BMP-2 on hMSCs or IH-1 cells. Inhibition of Smad-signaling and p38 MAPK-signaling indicated that apoptosis of IH-1 cells is dependent on Smad-signaling downstream of BMP, whereas the antiproliferative effect of BMP-2 on IH-1 cells also involves p38 MAPK-signaling. Together, the data suggested a specific inhibition by PTO-CpG-ODN on BMP–Smad-signaling. Supporting this we found that PTO-CpG-ODN inhibited BMP-induced phosphorylation of receptor-Smads in human mesenchymal stem cells and myeloma cell lines. This effect appeared to be independent of TLR9 because CpG-ODN and other ODNs with the ability to form multimeric structures inhibited Smad-signaling as efficiently as PTO-CpG-ODNs, and because knockdown of TLR9 by small interfering RNA in INA-6 cells did not blunt the effect of PTO-CpG-ODN. In conclusion, our results demonstrate that PTO-CpG-ODN inhibits BMP-signaling, and thus might provoke unwanted TLR9-independent side effects in patients. The Journal of Immunology, 2010, 185: 000–000.

During infection or tissue damage, pathogen- or host-derived nucleic acids may elicit an immune response (1). DNA derived from bacteria and DNA-viruses are recognized by TLR9. This receptor also recognizes and initiates an immune response toward complexes of self- DNA with other molecules such as anti-DNA Abs and HMGB1 (1–5). Originally, TLR9 was found to recognize unmethylated CpG motifs (6). Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs trigger TLR9 activation, and are commonly used as TLR9 agonists in vitro. These CpG-ODNs often have a phosphorothioate (PTO) backbone, which are resistant to endonucleases that degrade the phosphodiester bond of native DNA. Due to their potent immunostimulatory properties, CpG-ODNs are evaluated in clinical trials to treat different forms of cancer, either as single agent, in combination with other treatment or as vaccine adjuvants (7–9). Although TLR9-independent responses to CpG-ODNs have been described (10–12), still there is a general acceptance of CpG-ODN to be recognized mainly by TLR9.

Bone morphogenetic proteins (BMPs) regulate morphogenesis, growth, and differentiation of various tissues and organs (13). Besides their important role during normal processes such as bone formation and osteoblast differentiation, BMP-signaling also play a role in cancer. In some cancers, BMPs have been shown to promote survival of the malignant cells, whereas in multiple myeloma, BMPs are acting as apoptotic factors, at least in vitro (14–17).

Being members of the TGF-β superfamily, BMPs are recognized by three distinct type II receptors (BMPRII, ActRIIa, and ActRIIb) and at least four type I receptors (Alk1, Alk2, Alk3, and Alk6). Ligand binding to the receptors facilitates receptor heterimerization, so that the constitutively active type II receptors are able to phosphorylate the type I receptors. Activated type I receptor phosphorylates BMP-responsive Smad-effectors, Smad1/5/8, to promote nuclear transport together with Smad4. BMPs also activate Smad-independent signaling pathways such as the p38 MAPK pathway (18). Cross-talk between TLR- and BMP-signaling pathways has been described (19), so we therefore aimed to investigate whether PTO-CpG-ODN, as a TLR9 agonist, influenced BMP-induced osteoblast differentiation and BMP-induced apoptosis of malignant plasma cells.

In this study, we show that PTO-CpG-ODN inhibits BMP-induced osteoblastogenesis and BMP-induced apoptosis of myeloma cells. PTO-CpG-ODN inhibits BMP-induced phosphorylation of

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Abbreviations used in this paper: ALP, alkaline phosphatase; ARS, Alizarin Red S; BMP, bone morphogenetic protein; CpG-ODN, CpG oligodeoxynucleotide; hMSC, human mesenchymal stem cell; PI, propidium iodide; poly (I), polyinosinic acid; potassium salt; PTO, phosphorothioate; PO, phosphodiester.

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Materials and Methods

Osteoblast differentiation

hMSC were seeded in 24-well (17,000 cells/well) and 96-well (7000 cells/well) plates in MSCGM. Osteogenic differentiation was induced when the cells were 70% confluent by adding dexamethasone (10⁻⁸ M) (Sigma-Aldrich), β-glycerophosphate (10 mM) (Sigma-Aldrich), and L-ascorbic acid (0.05 mM) (Sigma-Aldrich) (referred to as differentiating media) with or without BMP-2 (300 ng/ml) (R&D Systems, Minneapolis, MN) and PTO-CpG-ODN or PTO-GpC-ODN. Media were renewed every 3–4 d. After 24 h of stimulation, the cells were pulsed with 0.75 μCi methyl-[³H] thymidine (NEN Life Science Products, Boston, MA) per well and harvested 18 h later with a Micromate 96 well harvester (Packard, Meriden, CT). β radiation was measured with a Matrix 96 counter (Packard). IH-1 cells were seeded in 96-well plates at a density of 30,000 cells/well and stimulated with BMP-2 (300 ng/ml), CpG-ODN (4 μM), p38 inhibitor SB203580 2 μM (Sigma-Aldrich), and Smad-inhibitor Dorsomorphin 5 μM (22) (Sigma-Aldrich) as indicated in the figure. After 6 h of stimulation, the cells were pulsed with 0.75 μCi methyl-[³H] thymidine per well and harvested 18 h later as described previously.

Real-time quantitative PCR

To quantify osteirx mRNA, hMSC were treated in osteogenic media in the presence of BMP-2 with or without CpG-ODN for 11 and 14 d. Then, total RNA was isolated and relative quantitative PCR was performed using LightCycler (Roche Applied Science, Rotkreuz, Switzerland) as described previously (21). Primers and annealing temperature were as follows: osteirx, forward 5′-ATGGGCATCTCTCTGTTGTA-3′ and reverse 5′-GG-GAGCAAGTACGATGGTA-3′; annealing temperature 64°C, extension time 27 s. GAPDH forward 5′-CACATGGAGAAGGC-3′ and reverse 5′-GAGCAGACATCGGGTAG-3′. Results were analyzed with LightCycler Software v.3.5 (Roche Applied Science) using the second derivative maximum method to set crossing points. Samples were run in duplicates. The ratio of target mRNA/GAPDH was in control samples (cell cultured in differentiation media only for 11 d) set to 1. hMSC were cultured for 1, 3, and 5 d in osteogenic media with or without BMP-2 and CpG-ODN before harvesting and isolation of total RNA using RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. First-strand cDNA was used as template in a RT-qPCR synthesis reaction using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) and random hexamer primers according to the manufacturer’s instructions. A total of 270 ng cDNA per sample was applied on a PCR array profiling the expression of 84 genes related to TGF-β/BMP-mediated signal transduction (SABiosciences). The PCR was carried out on StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the protocol from SABiosciences. The raw threshold cycle data were analyzed using the StepOne Software (Applied Biosystems), which uses the ΔΔCt calculation method to determine relative mRNA levels.

siRNA transfection

The myeloma cell line INA-6 was transfected by electroporation using the Nucleofector device (Amaxa Biosystems, Cologne, Germany). We used buffer K and program X-001 (Amaxa). For siRNA mediated knockdown, we used synthetic TL9R, activin A receptor type I, and nontargeting ON-TARGETplus SMARTpool siRNAs obtained from Dharmacon (Dygen, Rygge, Norway). The concentration of siRNA per transfection was 1 μM. Because of low transfection efficiency, we cotransfected the cells with the selection marker pcDNA3 CD4 (kind gift from Martin Janz, Berlin). After 18 h, we isolated transfected cells using Dynabeads CD4 (Dynal, Oslo, Norway). The cells were used for further studies when the level of TL9R protein was at the lowest, which was at 48 h posttransfection.

Western blotting

Nuclear extracts were prepared using Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Total cell extracts were prepared by lysing the cells in buffer containing 1% NP40, 150 mM NaCl, 50 mM Tris-HCl, a protease inhibitor mixture (Complete Mini, Roche, Basel, Switzerland) and 50 mM NaF and 1 mM NaVO₄ for
phosphatase inhibition. The protein concentration in each sample was determined using Micro BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer’s instructions. A total of 30–50 μg protein was loaded onto precasted 10–12% Bis-Tris Gels (Invitrogen, San Diego, CA) and subjected to SDS-PAGE. Gels were then blotted onto nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk in 0.1% Tween-20-Tris Buffered Saline pH 7.4 (TBST) for 1 h at room temperature. Anti–phospho-Smad 1/5/8 (Cell Signaling Technology, Beverly, MA), anti-TLR9 (Cell Signaling), and anti-GAPDH (AbCam, Cambridge, U.K.) were incubated overnight at 4˚C in 1% nonfat dry milk in TBST. Membranes were washed four times and incubated with appropriate secondary Ab for 1 h at room temperature. After four washes in TBST, the membranes were developed with ECL detection reagents (ECL, Amersham Biosciences, Arlington Heights, IL) according to manufacturer’s instructions and exposed to Hyperfilm ECL (Amersham Biosciences).

Apoptosis assay
The cells were treated with BMP-2, BMP-6, ODNs, the p38 inhibitor SB203580, or dorsomorphin, for 48 h as indicated in the figure legends, in RPMI 1640 containing 10% FCS. Cell viability and apoptosis were subsequently determined by flow cytometric analysis of annexin V-FITC binding and propidium iodide (PI) uptake (APOPTEST-FITC kit, Nexins Research, Hoeven, Netherlands).

Cell cycle analyses
Cell cycle analyses were performed as described (23). Briefly, ~300,000 hMSCs were treated for 24 h with BMP-2 (300 ng/ml) with or without CpG-ODN (4 μM) in mesenchymal stem cell growth media. The cells were thereafter harvested and resuspended in 50 μl 250 mM sucrose, 40 mM trisodiumcitratidihydrat, 5% DMSO, pH 7.6. Then 450 μl 30 μg/ml trypsin in 3.4 mM trisodiumcitratidihydrat, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Triis-Hcl pH 7.6 were added, and the cells were incubated at room temperature for 10 min before addition of 375 μl trypsin inhibitor 0.5 mg/ml, 100 μg/ml RNase A in 3.4 mM trisodiumcitratidihydrat, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Triis-Hcl pH 7.6. The cells were incubated for 10 more minutes at room temperature, then filtered (40 μM pore size) before 375 μl ice cold 3.4 mM trisodiumcitratidihydrat, 0.1% NP-40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Triis-Hcl pH 7.6 was added, and left on ice for 15 min. Cell cycle data were obtained by flow cytometry (FACS-LSRII, BD) and subsequently analyzed by the flow cytometry analysis software FlowJo (Tree Star, Ashland, OR).

Statistics
Statistical analyses were performed with SPSSX/PC computer program (SPSS, Chicago, IL). Results were considered statistically significant when p < 0.05.

Results
CpG-ODN inhibits BMP-induced osteoblast differentiation
hMSCs cultured in osteogenic media (see Materials and Methods) will differentiate toward osteoblasts. The addition of BMP-2 to these cells enhances ALP activity, an early marker for osteoblast differentiation, as well as mineralization, a late marker. CpG-ODN almost completely blocked the effect of BMP-2 on both ALP activity (Fig. 1A) and mineralization (Fig. 1B). Furthermore, CpG-ODN blocked the BMP-2 induced expression of Osterix, an osteoblast-specific transcription factor expressed in fully mature osteoblasts (Fig. 1C). The inverted CpG sequence (GpC-ODN) in a similar manner inhibited BMP-induced ALP activity (Fig. 1D), suggesting that the observed effect may be TLR9 independent.

CpG-ODN inhibits BMP-induced apoptosis of myeloma cells
BMPs induce apoptosis of myeloma cells in vitro, and we further investigated whether CpG-ODN was able to antagonize the pro-apoptotic effect of BMP on myeloma cells. BMP-2 treatment of the IH-1 myeloma cell line for 48 h led to apoptosis measured by annexin/PI staining. CpG-ODN did not by itself significantly increase survival of the cells, but dose dependently counteracted

![FIGURE 1. CpG-ODN inhibits BMP-induced osteoblast differentiation. A. ALP activity of hMSCs treated for 3 or 7 d in differentiation media with or without BMP-2 (300 ng/ml) and CpG-ODN (4 μM) as indicated. B. Mineralization of hMSCs treated for 11 or 15 d in differentiation media with or without BMP-2 (300 ng/ml) and CpG-ODN (4 μM) as indicated quantified with ARS staining. C. mRNA expression of Osterix in hMSCs treated for 11 and 14 d in differentiation media in the presence of BMP-2 (300 ng/ml) with or without CpG-ODN (4 μM). D. ALP activity of hMSCs treated for 7 d in differentiating media with or without BMP-2 (300 ng/ml), CpG-ODN or GpC-ODN as indicated. Error bars show 1 SD of triplicate measurements (A, D) or 1 SD of duplicate measurements (B, C).](http://www.jimmunol.org/ by guest on October 30, 2017)
BMP-2–induced apoptosis (Fig. 2A). Different myeloma cell lines express different BMP receptors and the cell line INA-6 is responsive to BMP-6, but not to BMP-2 (15). Likewise, BMP-6 treatment of INA-6 cells for 48 h led to apoptosis as measured by annexin/PI staining. CpG-ODN by itself did not promote survival, but antagonized the apoptotic effect of BMP-6 (Fig. 2B). CpG-ODN at the same concentration inhibited BMP-6–induced apoptosis, suggesting that also the antiapoptotic effect of CpG-ODN in myeloma cells is TLR9 independent (Fig. 2C). Further, an acidic environment in the endosomes is a prerequisite for TLR9 signaling (24), but increasing pH in endosomes by ammoniumchloride did not influence the antiapoptotic effect of CpG-ODN, although it to some extent reduced the proapoptotic effect of BMP-6. The latter effect is probably due to reduced Smad signaling from endosomes (25) (Supplemental Fig. 1). Together, our data indicate that the antiapoptotic effect of CpG-ODN is TLR9 independent.

**CpG-ODN does not antagonize the effect of BMP on mesenchymal stem cell proliferation and cell cycling**

BMP-2 inhibits proliferation and promotes differentiation of mesenchymal stem cells. Although CpG-ODN counteracted the effect of BMP-2 on osteoblast differentiation, CpG-ODN did not antagonize the inhibitory effect of BMP-2 on proliferation as measured by [3H]thymidine incorporation (Fig. 3A). In fact, CpG-ODN by itself inhibited proliferation of hMSCs, and the effect of CpG-ODN was additive to the effect of BMP-2. As evident by cell cycle analysis, both CpG-ODN (75% cells in G0/G1) and BMP-2 (66% cells in G0/G1) promoted an accumulation of cells in G0/G1 phase compared with untreated cells (63% cells in G0/G1), and the combination of BMP-2 and CpG-ODN treatment was additive (80% cells in G0/G1) (Table I).

**CpG-ODN does not antagonize the effect of BMP on myeloma cell proliferation**

Although BMP-2 treatment for 48 h promotes apoptosis of IH-1 cells, there is no significant apoptosis after 24 h of BMP-2 treatment (data not shown). Thus, we treated IH-1 cells for 24 h to examine effect of BMP-2 and CpG-ODN on cell proliferation. BMP-2 treatment of IH-1 cells for 24 h inhibits proliferation measured by [3H] thymidine incorporation (Fig. 3B). Although CpG-ODN slightly increases proliferation, it does not counteract the antiproliferative effect of BMP-2 (Fig. 3B).

**Apoptotic effect of BMP-2 is Smad dependent: Antiproliferative effect of BMP-2 is also dependent of p38MAPK signaling**

Although an inhibitor of Smad signaling (dorsomorphin 2 μM) counteracted the apoptotic effect of BMP-2, an inhibitor of p38 MAPK did not antagonize the apoptotic effect of BMP-2 in the IH-1 myeloma cell line (Fig. 4A), suggesting that the apoptotic effect of BMP is mediated by Smad signaling. In contrast, inhibition of p38 MAPK partly antagonized the antiproliferative effect of BMP-2 (Fig. 4B) suggesting that the effect of BMP-2 on proliferation is dependent on p38 MAPK signaling. Dorsomorphin also partly counteracted the antiproliferative effect of BMP-2, which indicates that antiproliferative effect of BMP-2 is dependent on both Smad and p38 MAPK signaling. However, it is hard to distinguish between inhibition of proliferation and apoptosis, and reduced [3H]thymidine incorporation may be due to some apoptosis of IH-1 already after 24 h of BMP-2 treatment. Therefore, it is possible that the effect of dorsomorphin is rather due to reduced BMP-2–induced apoptosis than reduced antiproliferative effect. However, because CpG-ODN profoundly counteracts BMP-2–induced apoptosis, but not the antiproliferative effect of BMP-2, we conclude that CpG-ODN has more prominent effect on BMP-Smad signaling than on BMP-p38 MAPK signaling.

**CpG-ODN inhibits BMP signaling**

To further study the effect of CpG-ODN on BMP signaling, we performed quantitative-PCR array for a number of genes in the TGF-β/BMP-signaling pathway. Of the 84 genes on this array, BMP-2 induced >2-fold increased expression of 12 of the genes after 1 d compared with cells in differentiating media only (Fig. 5). The expression of 11 of these 12 genes was reduced in cells treated with the combination of CpG-ODN and BMP-2 compared with cells treated with BMP only; supporting CpG-ODN directly interferes with BMP-signaling (Wilcoxon signed ranks test, CpG-ODN, and BMP-2 versus BMP-2 only, sum of negative ranks 113, sum of positive ranks 10, p = 0.004) (Fig. 5A). After 3 d of BMP-2 treatment, nine genes were induced >2-fold by BMP-2 compared with cells in differentiation media only at day 3 (Fig. 5B), and for eight of these genes, the expression was reduced (Wilcoxon signed ranks test, CpG-ODN, and BMP-2 versus BMP-2 only, sum of negative ranks 43, sum of positive ranks 2, p = 0.01). A similar gene expression pattern was observed at day 5, but the difference...
was not statistical significant (data not shown). In general, CpG-ODN did not seem to reduce gene expression, because of the 70 genes expressed after 1 d of treatment with osteogenic media only, 40 showed increased expression by CpG-ODN (Wilcoxon signed ranks test, CpG-ODN versus control, sum of negative ranks 938, sum of positive ranks 1547, \( p = 0.08 \)). Raw data from PCR array analyses are presented in Supplemental Table I.

**CpG-ODN inhibits Smad signaling**

A major signaling pathway downstream of BMP receptors is activated by phosphorylation of Smads 1/5/8, and we therefore investigated possible effects of CpG-ODN on Smad signaling in myeloma cell lines and hMSCs. hMSCs treated with BMP-2 showed presence of phosphorylated Smads 1/5/8, which was reduced in extracts from cells treated with CpG-ODN and BMP-2 (Fig. 6A).

Furthermore, nuclear extracts of the IH-1 myeloma cell line treated with BMP-2 showed presence of phosphorylated Smads 1/5/8, whereas levels of phosphorylated Smads were markedly reduced in nuclei of cells treated with CpG-ODN together with BMP-2 (Fig. 6B). CpG-ODN seemed to inhibit phosphorylation of Smads 1/5/8 by the type I receptor rather than inhibiting the translocation of phosphorylated Smads to the nucleus, because also the total amount of phosphorylated Smads 1/5/8 was reduced with BMP and CpG-ODN treatment compared with BMP alone (Fig. 6B).

BMP-6 treatment of INA-6 cells promoted phosphorylation of Smads 1/5/8, which, in a similar manner, was counteracted by CpG-ODN (Fig. 6C). Poly-A-ODN did not affect BMP-6 induced phosphorylation of Smads 1/5/8 in INA-6 cells to the same extent (Fig. 6C). Further, PO-CpG-ODN, and PO-polyA-ODN did not blunt Smad signaling, suggesting that the PTO backbone may play a role for the observed effect.

GpC-ODN are reported to be nonstimulatory with respect to typical TLR9 responses, and we found that GpC-ODN as efficiently as CpG-ODN inhibited phosphorylation of Smads 1/5/8, supporting that the effect is not dependent on TLR9 (Fig. 6D).

**CpG-ODN inhibits Smad signaling in a TLR9-independent manner**

To elucidate whether TLR9 mediated the effects of CpG-ODN, TLR9 expression in INA-6 cells were knocked-down by siRNA. TLR9 expression was successfully knocked-down (Fig. 7); however, this did not influence the effect of CpG-ODN (Fig. 7). Thus, the effect of CpG-ODN on Smad signaling seems to be independent of TLR9. We further tried to verify these findings by using HEK 293-Elam luc cells stably transfected with TLR9 and native HEK 293-ELAM luc cells, which do not express TLR9. However, neither of these cells responded to CpG-ODN by blunting Smad signaling (Supplemental Fig. 2). Thus, different cell types respond differently to CpG-ODN. It has been shown that some proteins unspecifically bind to PTO-modified ODN (8), but the experiments with HEK cells suggest that the inhibitory effect of CpG-ODN on Smad signaling is not merely due to unspecific binding of CpG-ODN to BMPs.
ODNs with the ability to multimerize inhibit BMP-induced apoptosis and Smad signaling

Because poly A with a PTO backbone had no influence on Smad phosphorylation, we assumed that not only the backbone but also the sequence and/or the structure of the ODNs might be of importance. To investigate this assumption more thoroughly, we tested the effect of different ODNs on BMP-induced apoptosis and Smad phosphorylation. We found that type A CpG (combined PTO and PO backbone with poly-G flanking 5' and 3' ends) and PO-CpG-ODN with a poly-G tail inhibited BMP-induced apoptosis (Fig. 8A) and Smad signaling (Fig. 8B) as efficient as PTO-CpG-ODN, whereas PO-CpG-ODN without poly-G tails or PO-CpG with poly-T tails did not (Fig. 8A,8B). Also, poly-G, either with PO or PTO backbone inhibited Smad signaling, in contrast to poly-A, which did not (Fig. 6C). Thus, neither backbone nor sequence seems to be important for the observed effects, but possibly rather the ability of the nucleotides to multimerize (forming G-tetrad) (26, 27).

It has been shown that PTO-CpG-ODNs (28–30), PO-CpG-ODNs with poly-G tails (31) and ODNs containing poly-G sequences (32, 33) are bound and taken up into cells by scavenger receptors. Thus, we tried to inhibit the effect of PTO-CpG-ODN by broadly specific competitive inhibitors to scavenger receptors such as dextran sulfate, fucoidan, and poly (I). Surprisingly, all these ligands in a similar manner as PTO-CpG-ODN inhibited BMP-induced apoptosis and Smad signaling (Fig. 8C,8D), suggesting that interactions between scavenger receptors and ODNs interfere with BMP-Smad signaling. Myeloma cells express both isoforms of scavenger receptor B, SR-B1, and SR-B2 (data not shown). Because malignant plasma cells are of the same cell lineage as B cells, and it was shown that, in B cells, both PTO-CpG-ODN and PTO-GpC-ODN signaled via SR-B1 (28), we specifically tried to investigate
whether a neutralizing Ab against SR-B1 and SR-B2 would blunt the effect of CpG-ODNs. However, neutralizing Ab treatment did not influence the effect of CpG-ODN on BMP-induced apoptosis or BMP-Smad signaling (data not shown). Importantly, scavenger receptors are a large group of receptors with redundant functions, so inhibition or knock-down of one type of scavenger receptor might be compensated for by a similar function of other scavenger receptors (34, 35). This experiment, however, does not exclude the possibility that SR-B or other scavenger receptors mediate the effects of PTO-CpG-ODN on BMP signaling.

Discussion

We, in this study, show that CpG-ODN in a TLR9-independent manner antagonizes BMP-induced Smad signaling in both mesenchymal stem cells and multiple myeloma cell lines. Presence of CpG-ODN during BMP-induced in vitro osteoblastogenesis completely blunted the differentiation process, leaving the cells in a state of anergy. Also, presence of CpG-ODN protected two-myeloma cell lines from BMP-induced apoptosis.

BMPs regulate fundamental processes such as cell proliferation, differentiation, migration and apoptosis, and controls embryonic development and postnatal tissue homeostasis. Further, in many cancers, BMP-Smad signaling has an antitumor effect (36–39). The antagonistic effect of CpG-ODN on Smad signaling observed, in this study, thus raises important questions around the safety of CpG-ODNs in clinical use. In this study, we used a 24 mer PS-ODN containing three human-optimized immunostimulatory 6 mer CpG motifs made with a fully phosphorothioate backbone (CpG-ODN 2006, also known as CPG 7909, PF-3512676, or ProMune) (40, 41). This particular CpG-ODN is in phase I or phase II clinical trials to treat several forms of cancer, including breast cancer, chronic lymphocytic leukemia, cutaneous T cell lymphoma, non-Hodgkin’s lymphoma, and melanoma (7, 42). Although in general CpG-ODNs are reported to be well tolerated in patients, more severe hematological side effects have been observed. These adverse effects of CpG-ODN therapy include anemia, thrombocytopenia, and neutropenia (7). BMPs regulate hematopoiesis (43–45), including erythropoiesis and the differentiation of megacaryocytes (46, 47). The hematological side effects from CpG-ODN treatment can thus be due to TLR9-dependent activation of the immune system, or possibly also due to TLR9-independent side effects such as interference with BMP signaling. Further, our results indicate that CpG-ODN in single/combination therapy or as vaccine adjuvants should be avoided in treating patients with cancer cells susceptible to BMP-induced apoptosis, like multiple myeloma. In contrast, such treatment can be advantageous in cancers where BMPs have a pro-survival, proliferative, or invasive/metastatic effect (48–51). CpG-ODN potently antagonized BMP-induced osteoblastogenesis in vitro, but no adverse effects on bone homeostasis have been observed in preclinical or early clinical studies. Although there is a continuous bone remodeling in adults, the turnover is relatively slow, and effects on bone would possibly not be evident after long time (several years) treatment. Moreover, the effect of inhibiting BMP signaling in bone cells may be most critical during periods of extensive bone growth or fracture healing.

FIGURE 8. ODNs with the ability to form multimeric structures and scavenger receptor ligands inhibit BMP-induced apoptosis. A. Annexin V-FITC and PI binding were measured by flow cytometry in INA-6 cells treated for 48 h in RPMI 1640 with 10% FCS and 1 ng/ml IL-6 with BMP-6 (50 ng/ml) and different ODNs (2 μM) as indicated. B, Levels of phosphorylated Smads 1/5/8 in total cell extracts from INA-6 cells treated with BMP-6 (50 ng/ml) and ODNs (2 μM) as indicated for 1 h. C, Annexin V-FITC and PI binding measured by flow cytometry in INA-6 cells treated for 48 h in RPMI 1640 with 10% FCS and 1 ng/ml IL-6 with BMP-6 (50 ng/ml), PTO-CpG-ODN (2.5 μM), fucoidan (1 μg/ml), poly (I) (10 μg/ml), and dextran sulfate (20 μg/ml). D, Levels of phosphorylated Smads 1/5/8 in total cell extracts from INA-6 cells treated with BMP-6 (50 ng/ml), PTO-CpG-ODN (2 μM), fucoidan (1 μg/ml), poly (I) (10 μg/ml), and dextran sulfate (20 μg/ml) as indicated for 1 h. Percentages of viable nonstained cells are presented, and error bars represent 1 SD of duplicate measurements (A, C).
TLR9 is considered the major receptor for CpG-ODN, although a few studies have described TLR9-independent effects of CpG-ODN (10, 11, 28, 52). We found that PTO-CpG-ODN and PTO-GpC-ODN to the same extent inhibited BMP-induced osteoblast differentiation, BMP-induced apoptosis of myeloma cells and Smad-signaling downstream of BMP. Further, knockdown of TLR9 with siRNA did not influence the effect of PTO-CpG-ODN on BMP signaling, and ODNs with poly-G sequences inhibited BMP-Smad signaling independent of backbone. Together, these data strongly suggest that the effect of PTO-CpG-ODN on BMP-Smad signaling is independent of TLR9.

Scavenger receptors are a broad class of transmembrane cell surface gp recognizing a diverse group of ligands including DNA, lipoproteins and phospholipids derived from pathogens or apoptotic cells (34, 35). Because PTO-CpG-ODN, PTO-GpC-ODNs, and ODNs with the ability to form G-tetrads have been shown to interact with scavenger receptors (28–33) and because we observed that several scavenger receptor ligands with broad specificity to a similar extent as PTO-CpG inhibited BMP-Smad signaling, we hypothesize that ligands to scavenger receptors in general might interfere with BMP signaling. CpG-ODN without poly-G tail and with a PO backbone did not interfere with Smad phosphorylation at all, so the effect of PTO-CpG-ODN is probably, at least partly, due to the phosphorothioate backbone. Supporting this are findings that short single-stranded PTO-ODNs can bind scavenger receptors in vitro (30). Synthetic ODNs with a PO backbone are susceptible to nucleases, and for this reason, CpG-ODN with a PTO backbone is preffered in clinical trials (7). The data presented in this study suggest that PTO-CpG-ODN inhibit Smad-signaling in vitro. Whether pathogen-derived DNA or host-derived native DNA (in complex with proteins) has similar effects on Smad phosphorylation is at present not known. Further, in this study, we have shown this effect in mesenchymal stem cells and malignant plasma cells, two very different types of cells. HEK 293 cells, on the other hand, did not respond in the same manner to PTO-CpG-ODN. This cell type dependency possibly might be due to a different repertoire of scavenger receptors on different cell types. Moreover, whether this TLR9-independent mechanism plays a role in other types of cells, for example, innate immune cells, is at present not known.

A major signaling route initiated by BMPs is the Smad pathway, but BMPs also activate non-Smad signaling pathways such as the TGF-β activated kinase 1, leading to phosphorylation of p38 MAPK (18, 53). Although CpG-ODN inhibited Smad-signaling and antagonized BMP-induced osteoblastogenesis and BMP-induced apoptosis of myeloma cells, it did not interfere with the antiproliferative effect of BMP on myeloma cells or mesenchymal stem cells. Experiments performed here with inhibitors of p38 MAPK and Smad pathway suggest that the apoptotic effect of BMP-2 on myeloma cells is dependent on Smad signaling, the inhibitory effect of BMP-2 on myeloma cell proliferation is mediated also by the p38 MAPK pathway. Thus, CpG-ODN in a specific manner inhibits BMP-induced Smad signaling, but not BMP-induced p38 MAPK signaling. It is therefore unlikely that CpG-ODN acts by preventing interaction of BMP-2 with its receptor.

Recently, CpG-ODN with a PTO backbone was shown to protect cancer cells from TRAIL-induced apoptosis by inhibiting binding of TRAIL to its receptor (54). Further, we have, in this study, shown that CpG-ODN with a PTO-backbone prevents BMP-induced apoptosis of malignant plasma cells, and BMP-induced osteoblastogenesis by inhibiting Smad signaling in a TLR9-independent manner. Thus CpG-ODN used in clinical trials to treat cancer and as vaccine adjuvants interferes with important apoptotic pathways in cancer cells and with an important pathway regulating growth and differentiation of several cell types. In conclusion, in future clinical trials, CpG-ODN with a stabilized phosphodiester sequence should be preferred over CpG-ODN with a PTO backbone to reduce unwanted backbone-related side effects of PTO-CpG-ODN.

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Disclosures

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References


