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A Fragment of the Hypophosphatemic Factor, MEPE, Requires Inducible Cyclooxygenase-2 to Exert Potent Anabolic Effects on Normal Human Marrow Osteoblast Precursors

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Abstract MEPE, 56.6 kDa protein isolated from tumors associated with hypophosphatemic osteomalacia, increases renal phosphate excretion and is expressed in normal human bone cells. AC-100, a central 23-amino acid fragment of MEPE, contains motifs that are important in regulating cellular activities in the bone microenvironment. Thus, we assessed in vitro effects of AC-100 on multipotential normal human marrow stromal (hMS) cells that have the capacity to differentiate into mature osteoblasts. Proliferation was quantified by [H^3]thymidine uptake and cell counting and differentiation by the levels of mRNA for the α2-chain of type I procollagen (*COL1A2*), alkaline phosphatase (*AP*), and osteocalcin (*OC*) measured using real time reverse transcriptase PCR (RT-PCR) and by the formation of mineralized nodules. AC-100 increased proliferation by 257 ± 89% ($P < 0.005$), increased gene expression of *COL1A2* by 339 ± 85% ($P < 0.005$), *AP* by 1,437 ± 40% ($P < 0.001$), and *OC* by 1,962 ± 337% ($P < 0.001$). In addition, it increased mineralized nodule formation by 81 ± 14% ($P < 0.001$) in a dose- and time-dependent fashion. In equimolar dosages, the parent compound, MEPE, had the full activity of the AC-100 fragment. AC-100 elicited a comparable response to both IGF-I and BMP-2 with respect to proliferation and differentiation of hMS cells. Using gene expression microarray analysis, we demonstrated that AC-100 increased (by ~3-fold) the mRNA for cyclooxgenase-2 (*COX-2*), an inducible enzyme required for prostaglandin synthesis. Moreover, NS-398, a specific inhibitor of COX-2 action completely blocked AC-100-induced increases in proliferation and differentiation. Thus, AC-100 has potent anabolic activity on osteoblast precursor cells in vitro and these effects require the induction of COX-2. J. Cell. Biochem. 93: 1107–1114, 2004.

Key words: COX-2; BMP-2; anabolic agent; bone formation

MEPE, 56.6 kDa protein isolated from tumors associated with hypophosphatemic osteomalacia, increases renal phosphate excretion and is expressed in normal human bone cells [Rowe et al., 1996, 2000]. MEPE has been cloned and expressed recombinantly [Petersen et al., 2000; Rowe et al., 2000; Argiro et al., 2001]. Human MEPE is a 525 amino acid residue protein with major structural similarities to a group of phosphoglycoproteins found in bone and tooth mineral matrix, including osteopontin, dentin matrix protein 1, and bone sialoprotein II [Rowe et al., 2000]. MEPE is expressed in cells of normal mineralized tissues including odontoblasts [Rowe et al., 2000], fully differentiated osteoblasts [Argiro et al., 2001], osteocytes [Petersen et al., 2000] and its concentration increases during osteoblast-mediated matrix mineralization [Argiro et al., 2001]. This raises the possibility that MEPE modulates bone formation independently of its actions on systemic phosphate homeostasis.

The central portion of human MEPE (residues 242–264) is conserved among species and contains motifs that are important in regulating cellular activities in the bone microenvironment, including an integrin-binding, a glycosaminoglycan-binding, and a calcium-binding

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motif. Based on these characteristics, the 242–264 fragment of MEPE was synthesized and was named AC-100. We report herein that this fragment has potent anabolic effects on normal osteoblast precursor cells in culture that are similar to those of insulin-like growth factor-I (IGF-I) in stimulating proliferation and to those of bone morphogenetic protein-2 (BMP-2) in stimulating differentiation. We also find that induction of cyclooxygenase-2 (COX-2) is required for these anabolic effects to become manifest.

METHODS

Reagents

Chemically synthesized AC-100 was obtained from Acologix, Inc. (Emeryville, CA) and was ≥96% pure. Human recombinant BMP-2 was purchased from R&D Systems (Minneapolis, MN). IGF-I was purchased from Sigma Chemical (St. Louis, MO). Tissue culture reagents were purchased from either Sigma Chemical or Gibco Life Technologies, Inc. (Grand Island, NY). Tissue culture plastic ware was purchased from Corning, Inc. (Corning, NY). Molecular biology reagents for the cDNA production and Real Time PCR were purchased from Roche Molecular Biochemicals (Indianapolis, IN) and Bio Rad Laboratories (Hercules, CA). The RNA Capture Kit was purchased from Qiagen, Inc. (Santa Clarita, CA). The $1,25(OH)_2D_3$ and [^{3}H]thymidine were obtained from NEN Life Science Products (Boston, MA).

Cell Culture

Normal human mesenchymal stem (hMSC) cells and media were purchased from Clonetics/BioWhittaker (San Diego, CA). The hMS cells are non-transformed, multipotential stromal cells obtained from normal human bone marrow. When cultured in proprietary growth medium, they can be maintained for up to 10 passages without phenotype drift. With appropriate media, they can be differentiated into osteoblasts, adipocytes, chondroblasts, or myoblasts [Pittenger et al., 1999]. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C, and the cell population was expanded by culture with proprietary growth (MSCGM BulletKit) media (Clonetics/BioWhittaker). The growth medium was changed twice a week.

Assessment of Cell Proliferation and Differentiation

Proliferation of the hMS cells was assessed by [^{3}H]thymidine incorporation and by cell counting. Cells were plated in 48-well microtiter plates at a density of 2×10^4 cells/well. After 48 h at 37°C, the cells were washed twice in phosphate buffered saline (PBS) and incubated at 37°C in MSCGM BulletKit media in the absence or presence of 100 ng/ml BMP-2, 1,000 ng/ml AC-100, 10 nM IGF-I or vehicle for 0, 3, 6, 9, or 12 days. The dosages chosen for BMP-2 and IGF-I had been previously shown to be maximal in previous studies from our laboratory [Gori et al., 1999; Thomas et al., 1999]. The cells were then harvested by trypsinization and counted with a Coulter Counter (Coulter Electronics, Ltd., Luton, UK). To assess the rate of synthesis of DNA, 1 μCi of [^{3}H]thymidine was added for the last 24 h of incubation. Cells were harvested by trypsinization, and [^{3}H]thymidine was extracted by trichloroacetic precipitation and detected by scintillation counting.

hMS cell differentiation was induced by culture in BulletKit growth medium to which dexamethasone 10^{-8}M + $1,25(OH)_2D_3$ 10^{-8}M + β-glycerol-PO^4 10 mM + ascorbate-PO^4 100 μM was added (standard differentiation medium). For dose-response studies, the dosages of AC-100 were 0, 10, 100, 1,000, 5,000 ng/ml, and those for BMP-2 were 0, 1, 10, 100 ng/ml. For time-course studies, the dosage of AC-100 was 1,000 ng/ml, for BMP-2 it was 100 ng/ml, and for MEPE it was 20 μg/ml. As previously described [Gori et al., 1999], the extent of differentiation was determined by expression of the genes coding for α2 chain of type I procollagen (*COL1A2*), alkaline phosphatase (*AP*) and osteocalcin (*OC*).

Assessment of Steady State mRNA Levels by Real Time RT-PCR

Steady state levels of mRNA were assessed by real time RT-PCR, as previously described [Locklin et al., 2001]. This method provides quantitative data on relative changes on a given cDNA when amplified for a fixed number of PCR cycles. Measurements of mRNAs for *COL1A2*, *AP*, and *OC* were made using the Light Cycler (Roche Diagnostics, Indianapolis, IN) and measurements of mRNAs for bone morphogenetic protein-2 (*BMP-2*), *runx2* (also called *Cbfa1*), and *osterix* were made using the Bio Rad I-Cyler Real Time PCR Machine (Hercules, CA). Cells

were plated in 6-well microtiter plates at a density of 1×10^5 cells in the growth medium and cultured for 48 h at 37°C. They were then washed twice in PBS and cultured for 0, 2, 4, 6, and 8 days in the standard differentiation medium in the presence or absence of vehicle or of 100 ng/ml of BMP-2 or 1,000 ng/ml of AC-100. Total cellular RNA was isolated using the Qiagen RNA Capture Kit Mini Kit. cDNA was synthesized from 1.8 μg of total RNA using the Reverse Transcriptase, AMV Kit (Roche Diagnostics) per kit protocol. Aliquots of cDNA were amplified in a 20 μl PCR reaction mixture which contained 10 pmoles/μl of 5′ and 3′ oligo-primers, 3 μM $MgCl_2$, 1× FastStart DNA Master SYBR Green Enzyme mix with 2 μl of cDNA added per reaction. The primer sequences used for the PCR reactions are given in Table I.

The PCR product sizes were 228 bp for *OC*, 422 bp for *AP*, and 347 for *COL1A2*. The same amplification profiles were used for *COL1A2* and *OC*. The amplification was performed using 35 cycles of denaturation at 95°C for 3 s, with annealing at 56°C for 7 s and extension at 72°C for 20 s. For *BMP-2*, the amplification was performed using 35 cycles with an annealing temperature of 61°C. *Runx2* amplification was performed using 40 cycles with an annealing temperature of 56°C. *Osterix* amplification was performed using 40 cycles with an annealing temperature at 57°C and *GAPDH* amplification was performed using 35 cycles with an annealing temperature of 56°C. The PCR product sizes were 267 bp for *runx2*, 155 bp for *osterix*, 134 bp for BMP-2, and 500 bp for *GAPDH*. The cDNA aliquots were amplified via the iQ SYBR Green Supermix (Bio Rad) in a 25 μl PCR reaction mixture containing 10 pmoles/μl of 5′ and 3′ oligo-primers, 3 μM $MgCl_2$, with 5 μl of cDNA per reaction. The amplified DNA samples were quantified by the fluorescence readings from the I-Cyler and normalized to *GAPDH*.

TABLE I. Primer Sequences Used for the PCR Reactions

OC-U 5′-CTC ACA CTC CTC GCC CTA TT
OC-D 3′-GGT CAG CCA ACT CGT CAC A
AP-U 5′-TCA AAC CGA GAT ACA AGC AC
AP-D 3′-GGC CAG ACC AAA GAT AGA GT
COL1A2-U 5′-GTG TAA GCG GTG GTG GTT
COL1A2-D 3′-GCC CGG ATA CAG GTT T
BMP-2-U 5′-CCT GCT TCG CCA TCT CTC CGA G
BMP-2-D 3′-TTC CTC CTC CGT TTT CT TTT CCT TG
Runx2-U 5′-CCG CAC GAC AAC CGC ACC AT
Runx2-D 3′-CAG GTG AAA GGA GCC CAT TA
Osterix-U 5′-AAT TTG GTG GCG CTA GCC CT
Osterix-D 3′-AT ACC CGA GGA AAG TGG AC
GAPDH-U 5′-CCT GCT TCG CCA TCT CTC CGA G
GAPDH-D 3′-TTC CTC CTC CGT TTT CTT TTC CTT G

Formation of Mineralized Nodules

Cells were plated in 12-well microtiter plates at a density of 5×10^4 cells/well in the standard growth medium and cultured for 48 h at 37°C. The cells were then washed twice in PBS and cultured for 21 days in the presence standard differentiating medium alone or with BMP-2 (100 ng/ml), AC-100 (1,000 ng/ml), or MEPE (20 μg/ml). Media and drug were changed twice weekly. Mineralized nodules were identified by Von Kossa staining and counted using a Nikon Diaphot inverted microscope (Nikon Corp., Tokyo, Japan).

Assessment of Gene Expression by Microarray Analysis

We assessed the effect of AC-100 treatment on gene expression in the Clonetics hMSC cells using gene expression microarray analysis as previously described [Locklin et al., 2001]. Briefly, cells were plated in five T-150 vented flasks at a density of 1.0×10^6 cells per flask in growth medium and cultured at 37°C for 48 h. Flasks containing either vehicle alone or AC-100 (1,000 ng/ml) were cultured for 24 or for 48 h in standard differentiation medium. At each time point, the vehicle and treatment flasks were trypsinized, cells removed and total RNA was extracted for analysis via the Qiagen Rneasy Kit (Qiagen). Eight micrograms of total RNA were amplified and biotin labeled. The mixture was hybridized to a U95A human gene expression microarray (Affymetrix, Santa Clara, CA) containing probes for 12,600 human genes. The chips were then washed, stained with phycoerythrin-streptavidin and read with an Affymetrix scanner. Hybridization intensity values to mRNA frequency were calculated in molecules per million. Analysis of intensity values was conducted using Spotfire Decision Site (Somerville, MA) software.

Statistical Analysis

All values are expressed as the mean ± SEM. ANOVA was used for dose- and time-dependent differences and when comparing multiple groups with a single control. If the ANOVA was significant, the significance of individual points versus vehicle was assessed by 2-sample *t*-tests.

RESULTS

Unless noted otherwise, all results were significant for each of three independent experiments. A representative example of one of these is shown in the Figures.

AC-100 Increases Cell Proliferation

AC-100 treatment (1,000 ng/ml) increased [^{3}H]thymidine incorporation by hMS cells in a time-dependent fashion (Fig. 1). The increases over vehicle values were $257 \pm 89\%$ ($P < 0.005$) and were maximal at 9–12 days of treatment. The relative increases during the time-course after treatment with BMP-2 (100 ng/ml) ($P < 0.001$) and IGF-I (10 nM) ($P < 0.001$) were similar to and not significantly different from that after treatment with AC-100. The increases in cell number after AC-100 treatment were similar proportionally to the increases in [^{3}H]thymidine incorporation (data not shown). We have previously reported that 100 ng/ml of BMP-2 and 10 ng/ml of IGF-I induce maximal responses in marrow stromal cells [Gori et al., 1999; Thomas et al., 1999].

AC-100 and MEPE Increase Expression of Genes Associated With Osteoblast Differentiation

Treatment for 48 h with AC-100 increased mRNA levels dose-dependently over vehicle treatment for *COL1A2* ($P < 0.001$), *AP* ($P < 0.001$), and *OC* ($P < 0.001$) over the range from 0 to 5,000 ng/ml (Fig. 2). Maximal expression was achieved for all three bone-related genes at a dosage of 1,000 ng/ml. BMP-2 treatment over the range of 0–100 ng/ml also

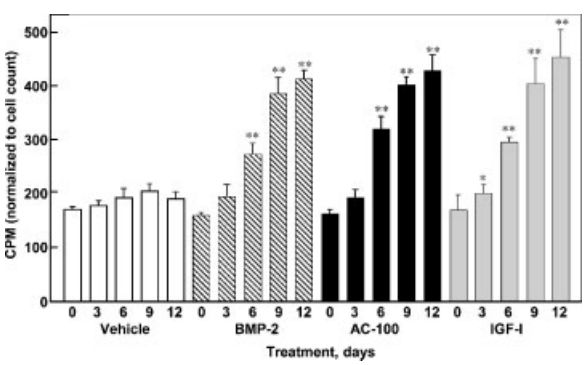

Fig. 1. Effect of AC-100 on hMS cell proliferation as assessed by [^{3}H]thymidine incorporation as compared with BMP-2 and IGF-I. Proliferation was increased (ANOVA $P < 0.001$) by all three mitogens in a time-dependent manner. For significance of individual values compared with vehicle, $^*P < 0.05$ and $^{**}P < 0.005$.

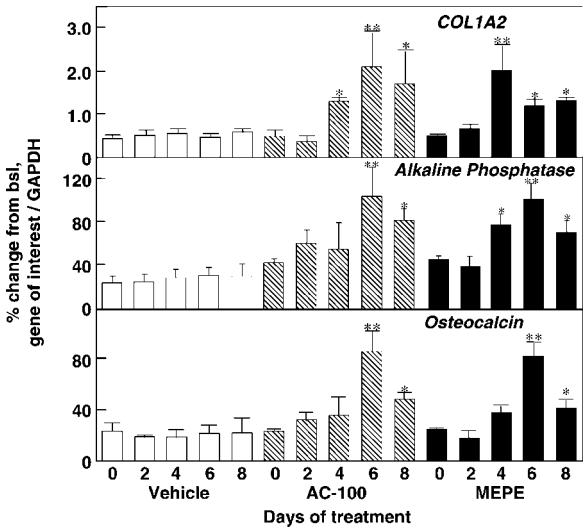

Fig. 2. Time course for the comparative effects of AC-100 and the parent compound, MEPE, on differentiation of hMS cells along the osteoblast pathway. Steady-state levels of mRNA for *COL1A2, AP,* and *OC* were increased in a time-dependent manner (ANOVA $P < 0.001$) during treatment with either compound and responses between the two did not differ significantly. For significance of individual values compared with vehicle, $^*P < 0.05$ and $^{**}P < 0.005$.

increased the three bone-related genes in a dose-dependent fashion (data not shown). The pattern of increases with BMP-2 did not differ significantly from the response after AC-100 treatment for expression of *COL1A2* or *AP* but was less ($P < 0.001$) for *OC*, a gene expressed late in osteoblast differentiation [Stein et al., 1990]. The expression of these genes after AC-100 treatment also was time-dependent ($P < 0.001$) for all three genes and reached a maximum at 6 days over vehicle of $339 \pm 85\%$ for *COL1A2*, of $1{,}437 \pm 117\%$ for *AP*, and $1{,}962 \pm 377\%$ for *OC*. The response following BMP-2 treatment was similar to that of AC-100 for *COL1A2* and *AP* but was substantially less for *OC* ($P < 0.001$) (data not shown). Finally, the pattern of the time-course after treatment with an equimolar dosage of MEPE (20 μg/ml), the parent compound, was not significantly different from that of AC-100 (1,000 ng/ml) for any of the three bone-related genes (Fig. 2).

We also assessed the effect of treatment with AC-100 (1,000 ng/ml) and BMP-2 (100 ng/ml) on induction of *runx2* [Ducy et al., 1997] and *osterix* [Nakashima et al., 2002]—early genes involved in osteoblast differentiation—at 0, 0.5, 2, 6, 12, 24, and 48 h. After AC-100 treatment, early values (<6 h) for *runx2* mRNA decreased in two of three experiments and increased at 12–

48 h in all three experiments. The increases were small (6–23%) but significant ($P = 0.02$–<0.001). Changes in *osterix* mRNA had a similar pattern with small (11–15%) but significant ($P = 0.02$–<0.001) increases at 12–48 h in all three experiments. The changes after BMP-2 treatment were even smaller and were significant for *runx2* mRNA in only one of three experiments but were significant for *osterix* in all three experiments. Finally, we measured the expression of *BMP-2* after AC-100 treatment and found that it did not change (data not shown).

AC-100 and MEPE Increase Mineralized Nodule Formation

The hallmark of cultured cells with the mature osteoblast phenotype is the ability to form mineralized nodules [Aubin and Triffitt, 2002]. After 21 days of culture in the standard differentiation media, the number of mineralized nodules per well was increased by $27 \pm 5\%$ for BMP-2 (100 ng/ml) ($P < 0.001$), by $81 \pm 14\%$ for AC-100 (1,000 ng/ml) ($P < 0.001$), and by $70 \pm 12\%$ ($P < 0.001$) for MEPE 20.0 μg/ml ($P < 0.001$). AC-100 and MEPE treatment had similar effect, but both peptides stimulated mineralized nodule formation more than did BMP-2 ($P < 0.001$) (Fig. 3).

Induction of COX-2 Is Required for the Anabolic Effects of AC-100

We used gene expression microarray technology to study the mechanism of AC-100 action. Only mRNAs that were changed by AC-100 treatment by 2-fold or more were considered. The only mRNA that was changed by this extent at both time intervals was COX-2: it was increased at 24 h by 3.6-fold and at 48 h by 2.2-fold.

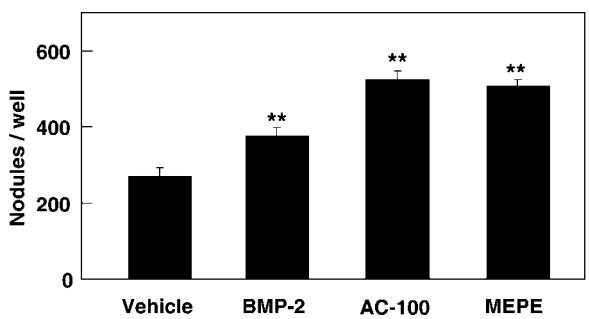

Fig. 3. Comparative effects BMP-2, AC-100, and MEPE in inducing formation of mineralized nodules after 21 days of treatment in differentiating media. Both AC-100 ($P < 0.0001$) or MEPE ($P < 0.0001$) increased mineralized nodule formation by approximate 2-fold increase whereas although BMP-2 treatment also increase nodule formation ($P < 0.001$), the increase was only half of that with AC-100 or MEPE and was significantly less ($P < 0.001$) than either. For significance of individual values compared with vehicle, $^{**}P < 0.005$.

We then assessed whether COX-2 activity was required for the anabolic action of AC-100 on the hMS cells by determining whether its effect could be attenuated by co-treatment with NS-398, a specific inhibitor of COX-2 activity [Bakker et al., 2003]. Six days of treatment with AC-100 (1,000 ng/ml) alone increased proliferation ($P < 0.001$) (Fig. 4) and the expression of bone-related genes ($P < 0.001$) (Fig. 5) in hMS cells whereas combined treatment with AC-100 plus NS-398 resulted in values that were virtually identical with those obtained by treatment with NS-398 alone.

DISCUSSION

The focus of this report is on the action of AC-100, the synthetic 242–264 fragment of MEPE, on osteoblast lineage cells. We demonstrated that both MEPE and AC-100, had potent anabolic actions in vitro on hMS pre-osteoblastic cells that were obtained from normal marrow and were not transformed. Moreover, because the hMS cells are early uncommitted osteoblast precursors that still are capable of differentiating into other lineages [Pittenger et al., 1999], we could assess the full range of osteoblast differentiation. Treatment with AC-100 resulted in large increases in hMS cell proliferation and in expression of *COL1A2*, *AP*, and *OC*, characteristic marker genes for osteoblast differentiation. We also showed that AC-100 significantly increased the expression of the early genes—*runx2* and *osterix*—but the increases were small and of uncertain physiological importance. Treatment with AC-100 for 21-days also increased the formation of mineralized nodules, the hallmark of the fully differentiated osteoblast.

In side-by-side studies, we found that the anabolic action of the AC-100 on the hMS cells was comparable to that of IGF-I and BMP-2 for proliferation and that of BMP-2 for differentiation, two of the most potent of the growth factors [Jones and Clemmons, 1995; Canalis et al., 2003].

Hayashibara et al. [2004] has reported that AC-100 has anabolic effects on bone and we confirm that in this study using different in vitro

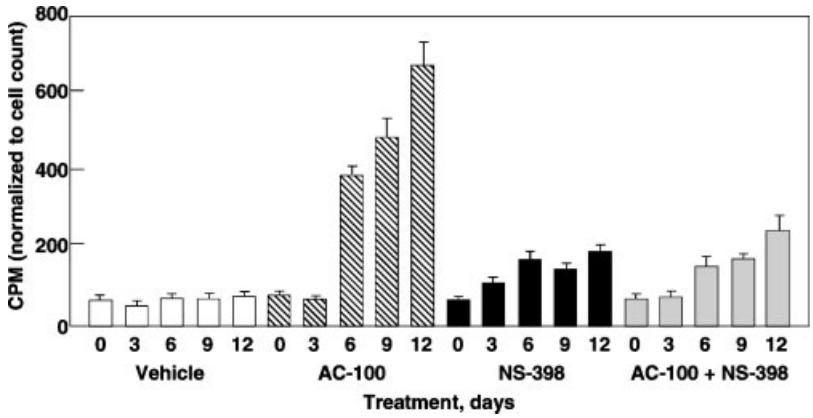

Fig. 4. Effect of co-treatment with the specific COX-2 blocker, NS-398 (0.144 ng/ml), on the stimulatory effect hMS cell proliferation after 8 days of treatment with AC-100 (1,000 ng/ml). AC-100 resulted in large, time-dependent increases (ANOVA $P < 0.0001$) whereas the effect of treatment with combined AC-100 and NS-398 were not different from the effect of NS-398 alone.

methodologies. In their study, it increased indices of bone formation in mouse calvaria both in organ culture and following local injection in vivo. However, Gowen et al. [2003] reported that mice with targeted disruption of *mepe*, the product of which they have named OF45, have increased bone formation, decreased age-related trabecular bone loss, and form significantly more mineralized nodules ex vivo. It is unclear, at present, how to reconcile our findings and those of Hayashibara et al. [2004] with those of Gowen et al. [2003]. Given the complex nature of mineral metabolism, it is possible that several regulatory mechanisms to be involved. Therefore, a simple knockout of the MEPE gene may result in compensatory effects on other factors involved in either phosphate and/or bone metabolism. It is also possible that MEPE acts physiologically on bone in concert with a number of other factors that modulate its overall effect in vivo. Whether MEPE is cleaved by bone cells to produce AC-100 or other active fragments that mediate the effect of MEPE on bone is not presently known.

Our findings that both MEPE and AC-100 increase the formation of mineralized nodules in culture differs from that of Rowe et al. [2004] who found that MEPE inhibited mineralization in a mouse 2T3 osteoblastic cell line when stimulated with BMP-2 in culture. They attribute the impairment of mineralization to the action of the ASARM-sequence on the COOH-terminal of MEPE that is distinct from the AC-100 sequence. The ASARM peptide presumably binds and sequesters Ca^{2+}, thereby inhibiting mineralization [Rowe et al., 2004]. The mineralized nodule assay reflects two processes—the ability of osteoblasts to secrete a mineralizable matrix (part of the phenotype of the mature osteoblast and a reflection of the extent of osteoblast differentiation) and the actual mineralization process which requires available calcium and phosphate [Aubin and Triffitt, 2002]. The differences in results are likely the result of several items, including some methodological differences. We used a concentration of β-glycerol phosphate of 10 mM, employed a single dose of MEPE of 20 μg/ml, and exposed the cells to MEPE for 21 days from seeding to about 70% confluence to induce mineralization. In contrast, Rowe et al. used a concentration of β-glycerol phosphate of 5 mM/ml, employed a range of much smaller doses of MEPE of from 10 to 800 ng/ml, and added the β-glycerol phosphate only when the cells had reached confluence and then maintained it for up to 26 days. The cells used in this report were primary, human osteoblastic cells derived from bone marrow, whereas the cells used by Rowe et al. are a murine osteoblastic cell line. It is possible that MEPE (and/or AC-100) acts differently on cells at different stages of differentiation, as is the case with other molecules such as the TGF-βs. Also, under the conditions of our experiment we were able to induce mineralization with MEPE alone whereas for Rowe et al. it was necessary to use co-treatment with BMP-2. These differences likely resulted in conditions that allowed us to emphasize the effect of MEPE on inducing osteoblast differentiation and Rowe

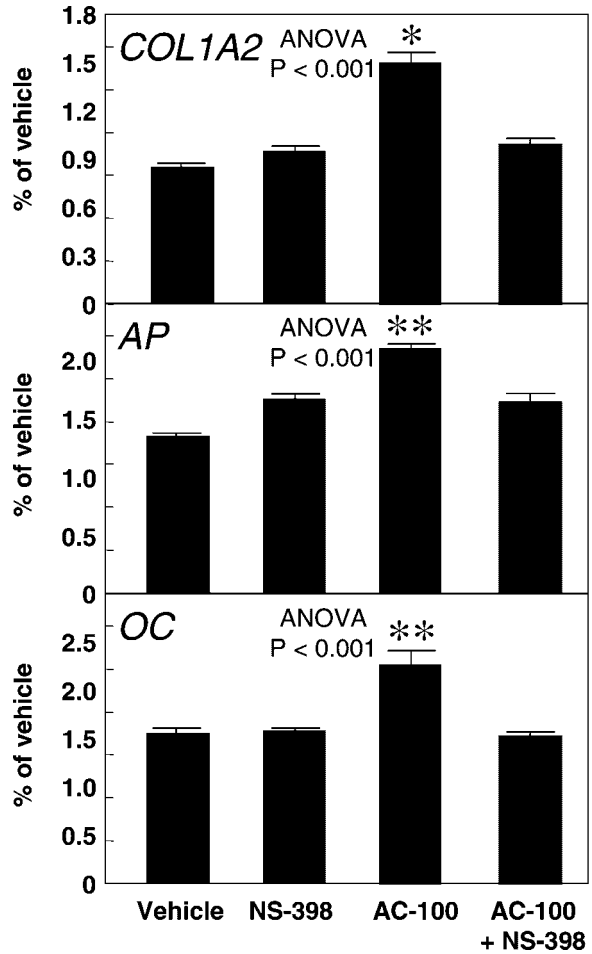

Fig. 5. Effect of co-treatment with the specific COX-2 inhibitor, NS-398 (0.144 ng/ml), on the stimulatory effect on expression of osteoblast differentiation genes (*COL1A2, AP,* and *OC*) by hMS cells after 6 days of treatment with AC-100 (1,000 ng/ml). After demonstrating significant differences among groups by ANOVA, we found that the AC-100 treatment group was higher by 2-sample *t*-test than the NS-398 treatment group (*$P < 0.01$; **$P < 0.001$). The effect of treatment with combined AC-100 and NS-398 were not different from the effect of NS-398 alone.

et al. to emphasize the effect of MEPE on mineralization per se.

We used gene expression microarray technology to study the mechanism by which AC-100 induces its anabolic action on hMS cells. In a mRNA extract obtained at 24 and 48 h after AC-100 treatment, we found that *COX-2* was the only mRNA whose expression was increased by >2-fold at both intervals. Action of the enzyme, cyclooxygenase, is required for prostaglandin synthesis [Pilbeam et al., 2002]. The enzyme has a constitutive form, COX-1, and an inducible form, COX-2, that is generally associated with inflammation or other stresses [Pilbeam et al., 2002]. We then assessed whether the induction of COX-2 is required for AC-100 to exert an anabolic effect on hMS cells by evaluating the effect of co-treatment with NS-398, a specific inhibitor of COX-2 activity [Bakker et al., 2003]. NS-398 co-treatment completely abrogated the AC-100-induced increases in hMS cell proliferation and differentiation. Although the increase in mRNA levels over control in the AC-100 alone treatment group was highly significant, it was less than we had observed in our earlier studies. The reason for this is unclear, but, since the hMS were normal non-transformed cells, it could be the consequence of a higher passage number for these later studies.

Our finding that induction of COX-2 was required for the anabolic action of AC-100 raises the possibility that it may act by stimulating the autocrine or paracrine production of prostaglandins. Several prostaglandins, but particularly prostaglandin E_2, are potent stimulators of osteoblast formation and differentiation in vitro [Flanagan and Chambers, 1992] and treatment of rats increases bone mass substantially [Jee and Ma, 1997; Suponitzky and Weinreb, 1998]. Prostaglandins are also believed to be the mediators of the increased bone formation induced by mechanical loading in vivo, an effect that can be blocked by specific antagonists of COX-2 activity [Forwood, 1996; Bakker et al., 2003]. Finally, several studies have demonstrated that both general cyclooxygenase inhibitors [Ro et al., 1976; Gerstenfeld et al., 2003] and specific COX-2 inhibitors [Simon et al., 2002; Gerstenfeld et al., 2003] impair fracture healing in experimental systems.

Because its anabolic actions of AC-100 on hMS cells in vitro are equivalent to those of IGF-I or BMP-2, AC-100 may have potential as an anabolic drug to increase bone mass. Despite its high potency, BMP-2 cannot be used to treat osteoporotic patients because it results in the formation of bone at the site of subcutaneous injection [Canalis et al., 2003]. In contrast, in a phase I study in human volunteers, AC-100 had no adverse local effects (Y. Kumagai, D. Rosen: Personal communication). However, further pre-clinical studies are needed to define the potential of AC-100 as a therapeutic agent, especially an assessment of the effects of AC-100 treatment on bone mineral density and bone histologic structure of small animals and the full delineation of the signaling pathway responsible for its anabolic action.

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