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In Vitro Osteogenic Response of Rat Bone Marrow Cells to bFGF and BMP-2 Treatments

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Basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) are actively pursued for stimulation of bone formation. To assess their promise for systemic therapy of osteoporosis, we ascertained the effects of bFGF and BMP-2 on bone marrow cells in vitro. Bone marrow cells were obtained from young (8 weeks) and adult (32 weeks) rats by femoral aspiration and were exposed to osteogenic medium (ie, basal medium with 10 mM β-glycerolphosphate and 100 nM dexamethasone) containing the growth factors. The cell viability in osteogenic medium was reduced after 3 weeks but not if the concentration of β-glycerolphosphate/dexamethasone was reduced to 3 mM/30 nM. Unlike BMP-2, bFGF at 2–50 ng/mL was capable of enhancing long-term cell viability. Continuous treatment of bone marrow cells for 3 weeks resulted in dose-dependent stimulation of mineralization by BMP-2, but not by bFGF, whose activity was optimal at 2–10 ng/mL. To explore the effect of short-term exposure, bone marrow cells were treated with growth factors for 1 week and subsequent mineralization was investigated. BMP-2 exposure increased the extent of mineralization, but bFGF was not effective after the short exposure. We concluded bFGF was more potent (ie, required lower concentration) for stimulating osteogenic parameters, but BMP-2 effects were lasting on the bone marrow cells.

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Protein growth factors are endogenous regulators of cells responsible for mineralized tissue mass and are being explored therapeutically for bone tissue engineering. The growth factors have been used in clinics by implantation with biomaterial carriers for bone repair at a site of administration.[14,30] The growth factors also have been administered systemically in preclinical models to stimulate bone deposition throughout the skeletal tissues.[19,28] The growth factors particularly are attractive for systemic stimulation of bone formation in patients with osteoporosis. Unlike the osteoporosis drug bisphosphonates that inhibit osteoclastic activity, growth factors have the potential to directly enhance skeletal integrity by stimulating deposition of new mineralized tissue by bone marrow cells (BMC). Basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) are two growth factors that act as a prototypical mitogen and morphogen, respectively.[5] The bFGF administered by intravenous route was shown to stimulate endocortical bone deposition in young (growing)[21] and ovariectomized rats,[12,22,36] the latter serving as a model of postmenopausal osteoporosis. Angiogenic and mitogenic effects of the bFGF have been attributed for the beneficial effects at bone sites.[12,21,22,36] Systemic administration of BMP-2 was attempted in two osteoporotic mouse models, which led to increased trabecular bone volume at femurs.[34]

Human-derived BMC are sensitive to bFGF and BMP-2 treatments, but the reported osteogenic effects of these growth factors are conflicting. Lecanda et al[13] reported a BMP-2 dependent matrix mineralization parallel with enhanced alkaline phosphatase (ALP) activity and increased deposition of the extracellular matrix proteins collagen-α(I), osteopontin, and decorin. Frank et al[3] used a medium containing bFGF/dexamethasone combination and reported differences in some osteogenic markers (BMP-2, bone sialoprotein, and osteopontin expression), but not in others (ALP activity, collagen-α(I) expression, and mineralization); bFGF treatment reduced colony formation and ALP activity of the BMC,[16] but acted as a mitogen

once successful BMC colonies were established.[15,16,24,35] Clonal differences in the osteogenic effect of bFGF on human BMC[3] and BMC derived from a murine model were observed.[6] bFGF alone (without dexamethasone) was incapable of stimulating osteogenic markers in one study,[15] but increased ALP positive colonies in another.[35] The BMC in these studies were obtained from individuals with varying ages, gender, and health status, so it is perhaps not surprising the reported effects were conflicting.

A better understanding of osteogenic effects of bFGF and BMP-2 is required to determine their potential in osteoporosis therapy. We proposed three aims for this study: to compare the dose-response relationships for the two growth factors to probe the relative potency of the proteins for osteogenesis; to determine the influence of a short (1-week) growth factor exposure duration to probe the lasting effects of the proteins' therapeutic action, and; to ascertain any changes in growth factor responsiveness with age.

MATERIALS AND METHODS

Three series of experiments were conducted for the three purposes of this study. In the first series of experiments where the relative potency of growth factors were investigated, BMC from young rats (8 weeks) were treated with bFGF (2–50 ng/mL) and BMP-2 (50–500 ng/mL) for a 3-week duration. In the second series of experiments where the effect of growth factor exposure duration was investigated, BMC from young rats were treated with bFGF (2–50 ng/mL) and BMP-2 (50–500 ng/mL) combinations for a 1-week period, after which the cells were maintained in medium without the growth factors for a 3-week period. In the third series of experiments in which the effect of age on osteogenic response was investigated, the second series of experiments were repeated but by using BMC obtained from adult rats (32 weeks old). The DNA content, ALP activity and mineralization as a function of time were determined in all experiments.

We used female Sprague–Dawley rats from Biosciences (Edmonton, Alberta, Canada). Six-week-old and 32-week-old rats were shipped to the University of Alberta and the animals were housed for an additional 2 weeks for acclimatization to laboratory conditions. The rats were allowed free access to food and water and were kept on a 12:12 hours of light:dark cycle. All animal procedures were performed according to guidelines of the Canadian Council on Animal Care and were institutionally preapproved before the study onset.

We used Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS; without phenol red), penicillin (10,000 U/mL solution), streptomycin (10,000 μg/mL solution), and heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY). All tissue culture plastic ware was from Corning (Corning, NY). Recombinant bFGF was obtained from Peprotech Inc (Rocky Hill, NJ). Recombinant BMP-2 was obtained from an *Escherichia coli* expression system. Its activity was reported in the literature.[10,29] A CyQUANT cell proliferation kit from Molecular Probes (Portland, OR) was used to quantitate DNA concentration in cell lysates. The tissue culture reagents dexamethasone, β-glycerol phosphate (β-GP), ascorbic acid, the ALP substrate p-nitrophenol phosphate (p-NPP), and p-nitrophenol (p-NP) were from Sigma (St. Louis, MO) and they were used without further purification. The Sigma Kit #567 (Sigma) was used for calcium quantitation in solution.

The rats were sacrificed by CO_2 asphyxiation, and the BMC were isolated from both femurs and pooled to obtain a single cell suspension. The cells were isolated using aseptic techniques in a biological safety cabinet. The marrow was removed by cutting the femur at distal and proximal ends with a bone cutter and then were aspirated using a 5-mL syringe with an 18G needle. The bone marrow was flushed out with approximately 15 mL of DMEM containing 10% FBS, 50 mg/L ascorbic acid, 100 U/mL penicillin, and 100 μg/L of streptomycin (referred as basal medium). The cells were centrifuged (600 g for 10 minutes) and suspended in a small volume of fresh basal medium, and the cell density was determined with a hemocytometer. The centrifugation was performed to remove endogenous molecules, which was previously suggested to influence establishment of BMC colonies.[31] The bone marrow aspirate was seeded in 25 cm^2 flasks in duplicate.

The cells were allowed to attach and grow for a period of 7 days in 25-cm^2 flasks in duplicate. Afterwards the spent medium and unadherent cells were removed and the cell monolayer was washed with cold HBSS. The cells were trypsinized with 0.25%trypsin/EDTA, harvested and centrifuged (approximately 600 g), and subcultured on 25 cm^2 flasks for one additional week after 1:4 dilution of the obtained cell suspension. After this time period, the BMC was trypsinized, seeded in 24-well plates, and allowed to attach for 1 day in basal medium. The medium subsequently was changed to osteogenic media (basal–medium-containing FBS, ascorbic acid, penicillin, streptomycin, and supplemented with 10 mM β-GP and 100 nM dexamethasone). Where indicated, the full osteogenic medium was diluted by 3/10 and 1/10 with the basal medium. The growth factors were added to the medium at desired concentrations (0–50 ng/mL for bFGF and 0–500 ng/mL for BMP-2). The choice of the growth factor concentrations was based on bFGF[33] and BMP-2[27] studies that reported mitogenicity and differentiation effects, respectively, of rat BMC. The BMC were exposed to continuous bFGF and BMP-2 exposure for a 3-week period or a 1-week period followed by 3 weeks of culture in osteogenic medium without any growth factor supplements. In the former case, the medium was changed weekly with fresh addition of the growth factors. In the latter case, the medium was changed to the desired osteogenic media (without growth factors) on a weekly basis.

The cells in multiwell plates were harvested at predetermined time points to obtain quantitative measures of the chosen osteogenic parameters. In the case of continuous growth factor exposure, the cells were harvested after 1, 2, and 3 weeks of continuous growth factor treatment. In the case of short (1-week) growth factor exposure, the cells were harvested after 1, 2, and 3 weeks of the removal of the growth factors in media.

To determine ALP activity, BMC were washed with HBSS (×2) and lysed with 300 μL ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X; pH, 10.5). Two hours after lysis, 150 μL of lysed solution was added to 48-well plates, and 150 μL of 2 mg/mL ALP substrate (p-NPP) was added to the lysed cells to give a final concentration of 1 mg/mL pNPP. The changes in optical density ($\lambda_{absorbance}$: 405 nm) were determined in a multiwell plate reader at periodic intervals for up to 15 minutes. To determine the DNA content in wells, the remaining cell lysis solution was frozen at -20° C, thawed at a suitable time, and analyzed with the CyQUANT DNA kit according to the manufacturer's instructions using a fluorescent plate reader ($\lambda_{excitation}$ at 480 nm, $\lambda_{emission}$ at 527 nm). A DNA standard provided with the CyQUANT kit was used to determine the DNA concentrations in cell preparations.

The wells containing the lysed BMC were washed with HBSS (x2) and treated with 0.25 mL of 0.5 M HCl for 4 to 6 hours to determine calcification. The aliquots of the solutions were used to quantify the amount of dissolved calcium using the Sigma diagnostic kit ($\lambda_{absorbance}$, 574 nm). Manufacturer supplied reference solutions were used for a calcium standard curve.

In the first set of experiments BMC were first subjected to continuous bFGF and BMP-2 exposure for a duration of 3 weeks. The cells were treated with all combinations of bFGF (ie, 0, 2, 10, and 50 ng/mL) and BMP-2 (ie, 0, 50, 150, and 500 ng/mL) concentrations. In the second series of studies we used short-term (1 week) exposure of BMC to BMP-2 and bFGF and determined changes in the osteogenic parameters. The medium for these experiments was 3/10 osteogenic medium because it was sufficient to induce mineralization in continuous exposure experiments.

The relative ALP activity was expressed as the change in optical density of the wells per unit time (mAbs/minute), and further normalized with the DNA content of the wells. The level of calcification was summarized as the concentration of calcium (mg/dL) per well. All results were expressed as mean ± standard deviation (SD), and statistical differences ($p < 0.05$) among the study groups were analyzed by analysis of variance (ANOVA).

RESULTS

Comparative Response of BMC to Growth Factors

BMC in full OM retained their viability for 2 weeks in the absence of bFGF. However, a reduction ($p < 0.05$) in cell viability was observed after 3 weeks based on the reduced DNA content of the BMC (Fig 1A). The continuous bFGF exposure at 2–50 ng/mL resulted in better cell viability after 3 weeks, but did not affect the DNA content at previous assessment points (Fig 1A). In 3/10 and 1/10 OM there was no differences in cell numbers for bFGF treated cells for all three assessment points. There was no loss of cell viability after 3 weeks, as observed with full OM (in Fig 1A) . The ALP activity in the absence of bFGF increased from 1 week to 3 weeks (Fig 1B), but bFGF treat-

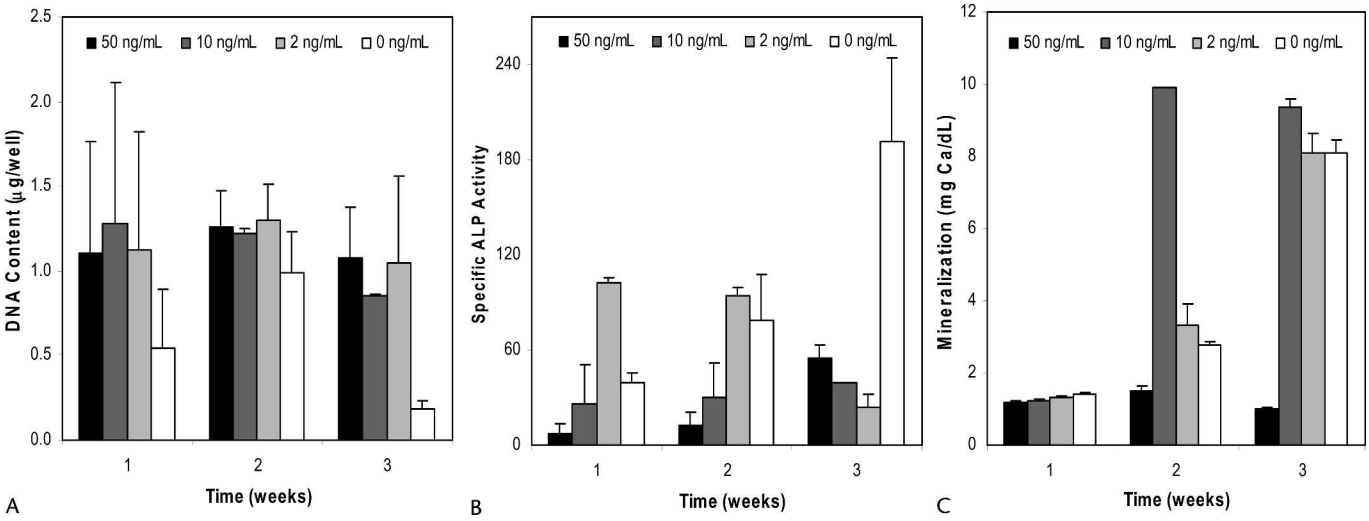

Fig 1A–C. Graphs show (A) changes in the DNA content, (B) ALP activity, and (C) mineralization after 1, 2, and 3 weeks of continuous bFGF treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and allowed to attach for 1 day. Basic fibroblast growth factor was then added to wells at indicated concentrations. The amount of DNA, ALP activity, and mineralization (per well) were assessed after 1, 2, and 3 weeks. (A) There were no apparent differences in DNA content for the bFGF treated BMC after 1 and 2 weeks, but a significant reduction of DNA content was observed after 3 weeks in the absence of bFGF treatment. (B) Basic fibroblast growth factor treatment of BMC, especially at the highest two concentrations (10 and 50 ng/mL, respectively), resulted in consistent reduction of ALP activity. (C) There was no mineralization in any of the BMC cultures after 1 week of culture, but significant mineralization was achieved after 2 weeks. Basic fibroblast growth factor at 10 ng/mL accelerated the extent of mineralization on Week 2, but the higher concentration of bFGF (50 ng/mL) resulted in a reduction of mineralization after 3 weeks.

ment (especially at 10 and 50 ng/mL) reduced the specific activity at each assessment point (Fig 1B). Similar results were obtained with 3/10 and 1/10 OM. Abundant calcification was seen in 3/10 and full OM, and the level of mineralization progressively increased with culture time (Fig 1C). With bFGF there was an accelerated mineralization at 10 ng/mL concentration, but an increase of bFGF concentration to 50 ng/mL led to reduced mineralization after 2 and 3 weeks. There was no calcification with 1/10 OM at any of the bFGF concentrations or time points.

Similar to the results with bFGF treatment, BMC in full OM retained their viability for 2 weeks, but a reduction in cell viability was observed after 3 weeks of BMP-2 treatment (Fig 2A). The presence of BMP-2 did not influence the loss in viability in OM after 3 weeks (Fig 2A). The BMC in 3/10 and 1/10 OM retained their viability throughout the 3-week study period, and the BMP-2 treatment of BMC did not have any influence on the DNA content. The ALP activity was increased as a function of BMP-2 concentration when BMC was cultured in the full OM, but no clear effects were noted in other media (Fig 2B). Similar to the results with the bFGF treatment, abundant calcification was seen in 3/10 OM and full OM, but not in 1/10 OM throughout the 3-week study period. The BMP-2 treatment at the highest concentrations (150 ng/mL and 500 ng/mL) stimulated mineralization in the full OM, but a clear dose response effect of BMP-2 treatment on mineralization was clearest in the 3/10 OM (Fig 2C). This was the case for the 2-week and 3-week assessment points.

Short-term Exposure to Growth Factors

BMP-2 generally led to a reduced cell numbers, which was most evident in the highest concentrations of BMP-2 (500 ng/mL) after 2 (Fig 3B) and 3 weeks (Fig 3C). For this BMP-2 concentration, bFGF increased the DNA content of BMC in the concentration range tested ($p < 0.01$ in Fig 3A; $p < 0.05$ in Fig 3B; $p < 0.01$ in Fig 3C). There was no clear effect of bFGF and BMP-2 on ALP activity after 1 and 2 weeks (Fig 4A-C). BMP-2 did not lead to a reproducible increase in the ALP activity, and bFGF did not appear to consistently increase or decrease the measured ALP activity. Only on Week 3 did bFGF reduce the ALP activity in the presence of 50, 150, and 500 ng/mL BMP-2.

The effects of bFGF and BMP-2 treatment on BMC mineralization are summarized in Fig 5. As before, there was a progressive increase in the level of mineralization as a function of time. The extent of mineralization was increased with an increase in BMP-2 concentration, particularly after 1 (Fig 5A; $p < 0.05$) and 2 (Fig 5B; $p < 0.05$)

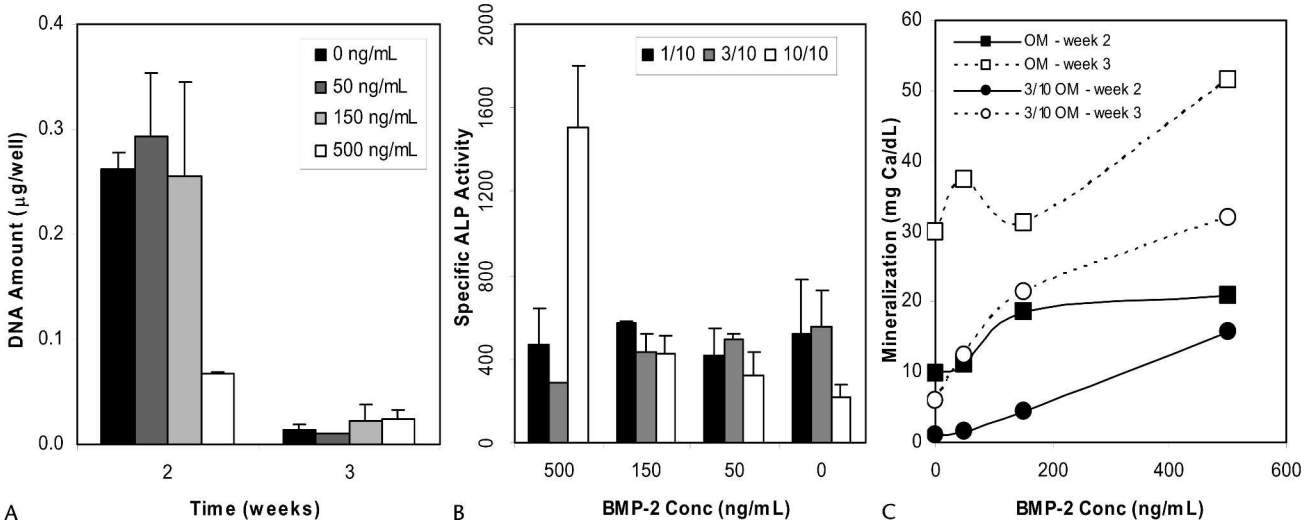

Fig 2A–C. Graphs show (A) changes in the DNA content, (B) ALP activity, and (C) mineralization after 2 and 3 weeks of continuous BMP-2 treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and were allowed to attach for 1 day. Bone morphogenetic protein-2 was then added to wells at indicated concentrations. The amount of DNA, ALP activity, and mineralization (per well) were assessed after 2 and 3 weeks. (A) A significant reduction of DNA content was observed after 3 weeks, irrespective of the BMP-2 concentration in the medium. (B) The BMP-2 treatment of BMC at 500 ng/mL in full osteogenic medium gave an increased ALP activity, but no effect of BMP-2 was observed in 3/10 and 1/10 osteogenic medium. (C) There was no mineralization in any of the BMC cultures after 1 week of culture (not shown), but significant mineralization was obtained after 2 weeks in full and 3/10 osteogenic media. The BMP-2 effect on mineralization was most obvious in 3/10 osteogenic medium, where the propensity of cultures to mineralize was low in the absence of BMP-2.

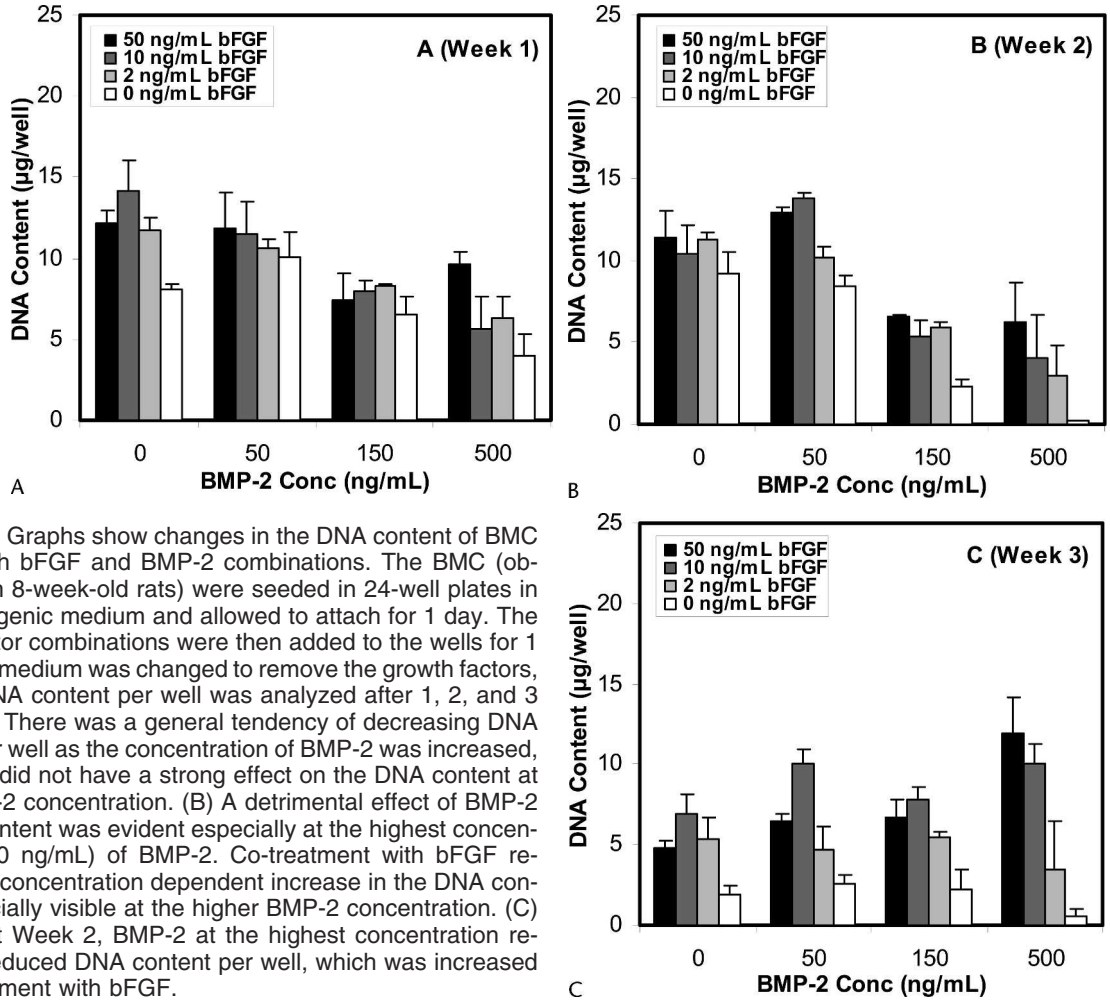

Fig 3A–C. Graphs show changes in the DNA content of BMC treated with bFGF and BMP-2 combinations. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the DNA content per well was analyzed after 1, 2, and 3 weeks. (A) There was a general tendency of decreasing DNA content per well as the concentration of BMP-2 was increased, and bFGF did not have a strong effect on the DNA content at each BMP-2 concentration. (B) A detrimental effect of BMP-2 on DNA content was evident especially at the highest concentration (500 ng/mL) of BMP-2. Co-treatment with bFGF resulted in a concentration dependent increase in the DNA content, especially visible at the higher BMP-2 concentration. (C) As seen at Week 2, BMP-2 at the highest concentration resulted in reduced DNA content per well, which was increased by co-treatment with bFGF.

weeks of stopping the growth factor treatment. After 3 weeks, BMC underwent mineralization even without the BMP-2 treatment. No effect of bFGF was seen on the level of mineralization, as the extent of mineralization was generally the same for a given BMP-2 concentration.

Growth Factor Effects on BMC from Older Rats

BMP-2 treatment (in the concentration range tested) did not affect the cellular DNA amount (Fig 6). Similarly, bFGF did not affect the DNA content of the BMC. The DNA content of the BMC derived from the older rats was generally lower than the DNA content of BMC from younger rats (compare Figs 3A vs. 6A, 3B vs. 6B and 3C vs. 6C). The ALP activity of BMC from older rats was not dependent on BMP-2 or bFGF treatment on Week 1 (Fig 7A). The bFGF treatment of BMC was stimulatory on Week 2 (Fig 7B), especially for lower concentrations of BMP-2 (0 ng/mL and 50 ng/mL). Similarly, bFGF was stimulatory on ALP activity for the lower concentrations (0, 50, and 150 ng/mL) of BMP-2 on Week 3 (Fig 7C).

We found a clear effect of BMP-2 on the extent of mineralization (Fig 8). As BMP-2 concentration increased there was a general increase ($p < 0.01$ at Week 1, Week 2 and Week 3) in the extent of mineralization at all assessment times. At the highest concentration of BMP-2 there was no clear effect of bFGF on the extent of mineralization after 1, 2, and 3 weeks. With 150 ng/mL, cotreatment with bFGF increased the extent of mineralization in a dose dependent manner. Mineralization at 0 mg/mL and 50 ng/mL BMP-2 was minimal after 1 and 2 weeks (Fig 8A and 8B), and the extent of mineralization on Week 3 were not different between these two groups (Fig 8C).

DISCUSSION

We investigated the responsiveness of BMC to two prototypical growth factors, bFGF and BMP-2. The culture conditions were optimized to allow cellular attachment and growth during the initial phase of BMC attachment and proliferation. Dexamethasone and β-GP had to be

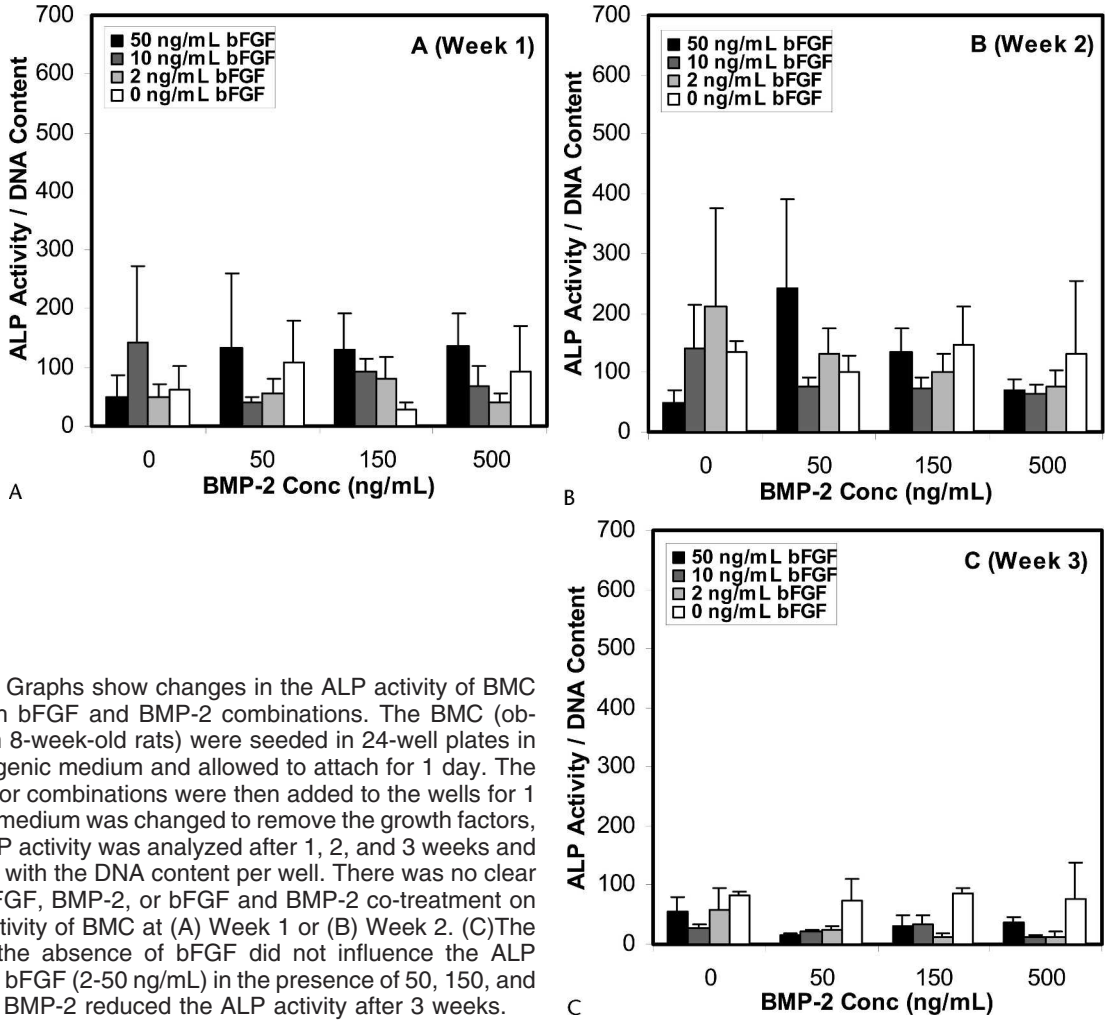

Fig 4A–C. Graphs show changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. There was no clear effect of bFGF, BMP-2, or bFGF and BMP-2 co-treatment on the ALP activity of BMC at (A) Week 1 or (B) Week 2. (C)The BMP-2 in the absence of bFGF did not influence the ALP activity, but bFGF (2-50 ng/mL) in the presence of 50, 150, and 500 ng/mL BMP-2 reduced the ALP activity after 3 weeks.

eliminated from the initial plating medium because of their inhibitory effects on cell growth.[6] Although the synthetic glucocorticoid dexamethasone and β-GP combination is routinely used for osteogenic differentiation, detrimental effects of dexamethasone and inorganic phosphate have been reported.[18] Inorganic phosphate (in the form of $H_2PO_4^-$, the likely hydrolysis product of a β-GP) was found to induce apoptosis,[18] and dexamethasone was shown to decrease the initial colony establishment in rat[1,26] and human BMC dose dependently.[16] We found such a detrimental effect of the osteogenic supplements previously and identified β-GP as the primary source.[6] Full osteogenic medium (10 mM β-GP) gave reduced levels of cell numbers (ie, DNA content), and diluting the concentrations of the osteogenic supplements in the media alleviated the loss in cellular viability (ie, loss in DNA content). An osteogenic medium with 3 mM/30 nM β-GP/dexamethasone combination did not have any apparent detrimental effects on cell viability for a 4-week culture period (1 week of growth factor exposure + 3 weeks of subsequent culture without growth factors), except when the cells were exposed to the highest concentration (500 ng/mL) of BMP-2. The differences in viability observed in this study with 3 versus 10 mM β-GP were consistent with the differences in viability obtained by using human bone-derived osteoblastlike cells.[18] Given the stimulatory effect of high concentration of BMP-2 on mineralization, it is likely that the cell viability might have been reduced as a result of extracellular mineralization. The bFGF was found to be beneficial in increasing viable cell numbers (based on DNA amounts recovered per well), presumably because of the mitogenic effect of bFGF on a subpopulation of BMC. It must be stressed that the beneficial effect of bFGF on cell numbers was observed under some conditions, and was not universal (eg, no benefit of bFGF was seen in Fig 1A).

As one would expect, results from in vitro cell cultures should be interpreted with caution. This study reported

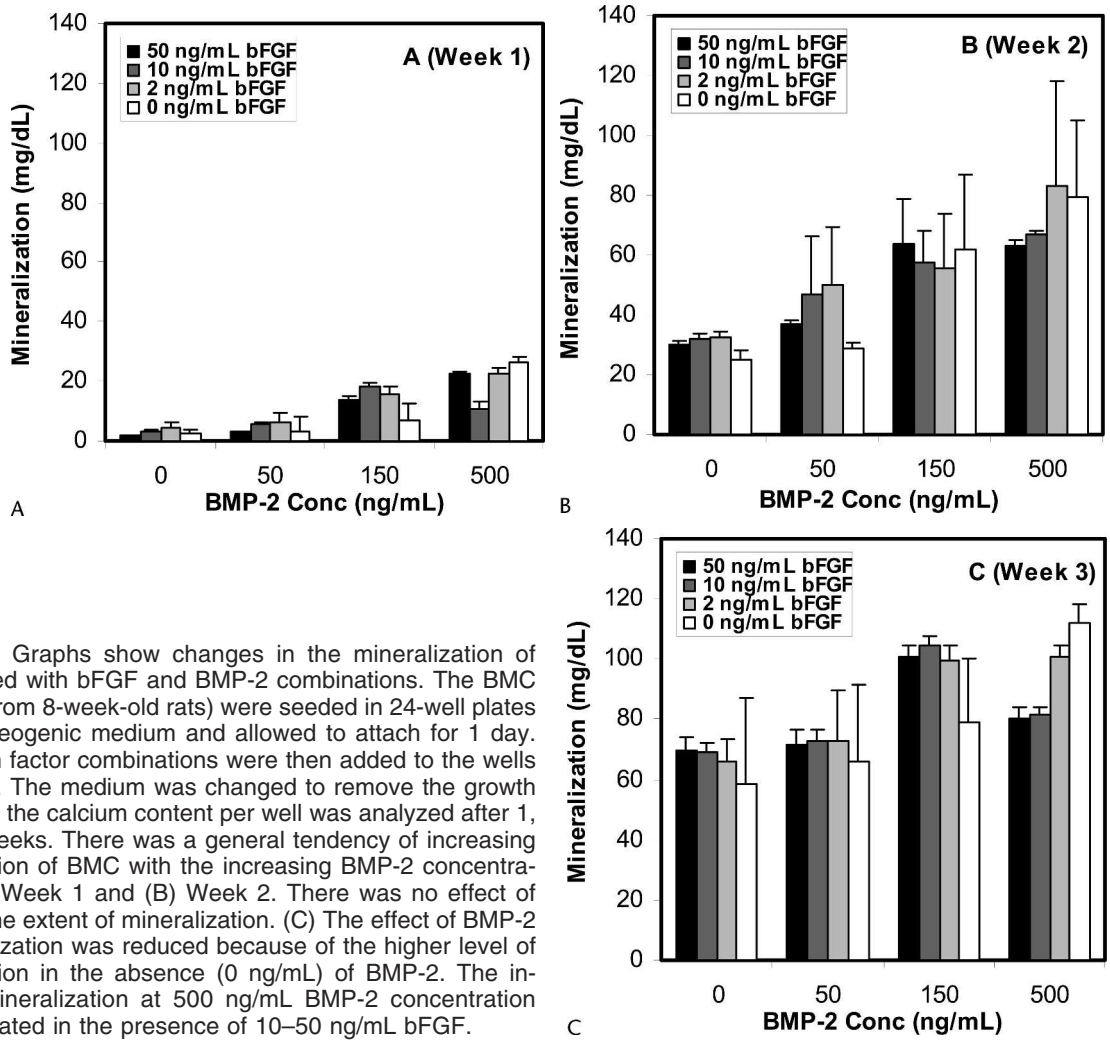

Fig 5A–C. Graphs show changes in the mineralization of BMC treated with bFGF and BMP-2 combinations. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. There was a general tendency of increasing mineralization of BMC with the increasing BMP-2 concentration at (A) Week 1 and (B) Week 2. There was no effect of bFGF on the extent of mineralization. (C) The effect of BMP-2 on mineralization was reduced because of the higher level of mineralization in the absence (0 ng/mL) of BMP-2. The increased mineralization at 500 ng/mL BMP-2 concentration was attenuated in the presence of 10–50 ng/mL bFGF.

cellular responses in the absence of two critical variables present in the endogenous bone marrow environment. One variable not incorporated into our cultures is the soluble factors (e.g., hormones and other peptide-based regulators) that may modulate the cellular responses to bFGF and BMP-2 exposure. The other variable is the three-dimensional nature of the cellular environment *in vivo*. It was recently recognized that BMC grown within a 3-dimensinal configuration displays a differential response to growth factors, such as BMP-2[7] and Transforming Growth Factor-β,[9] as compared to the cells grown on two-dimensional surfaces. The results reported in our culture system should be considered as a first line approximation to the possible in vivo effects of the growth factor response.

Basic fibroblast growth factor and BMP-2 were capable of stimulating mineralization under certain culture conditions, but differences in their effects were evident. Irrespective of the duration of exposure, a clear dose-response was consistently obtained for BMP-2, which gave a progressively higher amount of mineralization as its concentration was increased. The BMP-2 appeared to act as osteogenic signal throughout the concentration range tested. It increased the mineral deposition without affecting cell numbers. This was consistent with the literature on the bioactivity of this morphogenetic protein.[5,23,27] The bFGF exhibited an optimal concentration of approximately 10 ng/mL for stimulation of mineralization for continuous exposure, after which a detrimental effect on mineralization was observed. It is likely that bFGF might directly inhibit the osteogenic activity of differentiated osteoblasts at higher concentrations. bFGF may also increase the population of other lineage cells at higher concentrations because multiple cell lineages share the same pluripotent precursor cells as the osteoblasts in the bone marrow environment.[25] Further studies will be needed to differentiate between the two possibilities. BMP-2 was stimulatory on

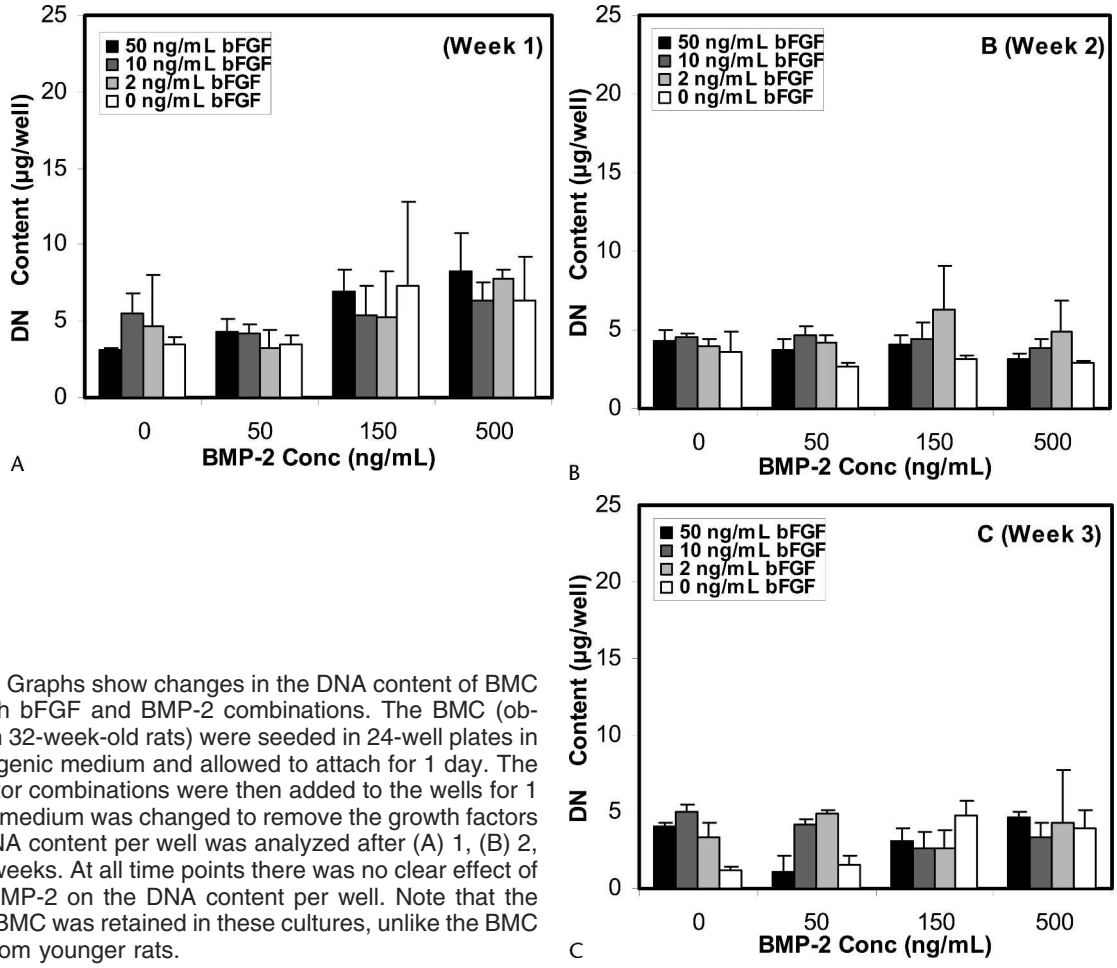

Fig 6A–C. Graphs show changes in the DNA content of BMC treated with bFGF and BMP-2 combinations. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the DNA content per well was analyzed after (A) 1, (B) 2, and (C) 3 weeks. At all time points there was no clear effect of bFGF or BMP-2 on the DNA content per well. Note that the viability of BMC was retained in these cultures, unlike the BMC obtained from younger rats.

BMC after a short time (1 week) and continuous exposure, but the effects of bFGF were evident only after continuous treatment of BMC. There were no effects after the short-time exposure. This indicated a lack of memory effects in the culture system utilized in this study. The stimulatory effect of bFGF on osteogenic colony formation (based on increased calcium positive, collagen positive, and ALP positive colonies form the BMC aspirates) has been reported to take least 5 days of continuous BMC exposure.[31] A clear distinction was the concentration difference required between the two growth factors. The well established morphogen BMP-2 exhibited its full osteogenic effect at 500 ng/mL, but bFGF required a much lower concentration (approximately 10 ng/mL) to exhibit its osteogenic effect. This aspect of bFGF action is beneficial when one considers the need to administer substantial amounts of the growth factor for systemic stimulation of bone formation. The lower the dose required for an effect on BMC, the lower the side effects observed at extraskeletal sites are likely to be.[17]

Differences in their mechanisms of action make bFGF and BMP-2 likely to act on different cellular pathways involved on osteogenesis, and they may act synergistically when codelivered at skeletal sites. The bFGF and BMP-2 combination has been shown to act synergistically in several systems, including osteogenic differentiation of BMC in culture,[5] bone deposition by the growth factor treated and transplanted BMC in vivo,[5] and de novo osteoinduction after intramuscular[4] and subcutaneous[32] delivery of the growth factor combination. The cotreatment regimen used in our study was intended to reveal if there was such a synergistic effect on BMC. No such effect from bFGF was observed on the BMP-2 responses (ie, ALP activity and mineralization) of BMC from young rats. A synergistic effect was seen on the BMC of older rats. This was evident on the BMP-2 induced ALP activity and mineralization for lower BMP-2 concentrations (50 ng/mL and 150 ng/mL, respectively; Fig 8), and not for the higher concentration of 500 ng/mL. It was indicative of the synergistic effect manifesting itself only within a narrow

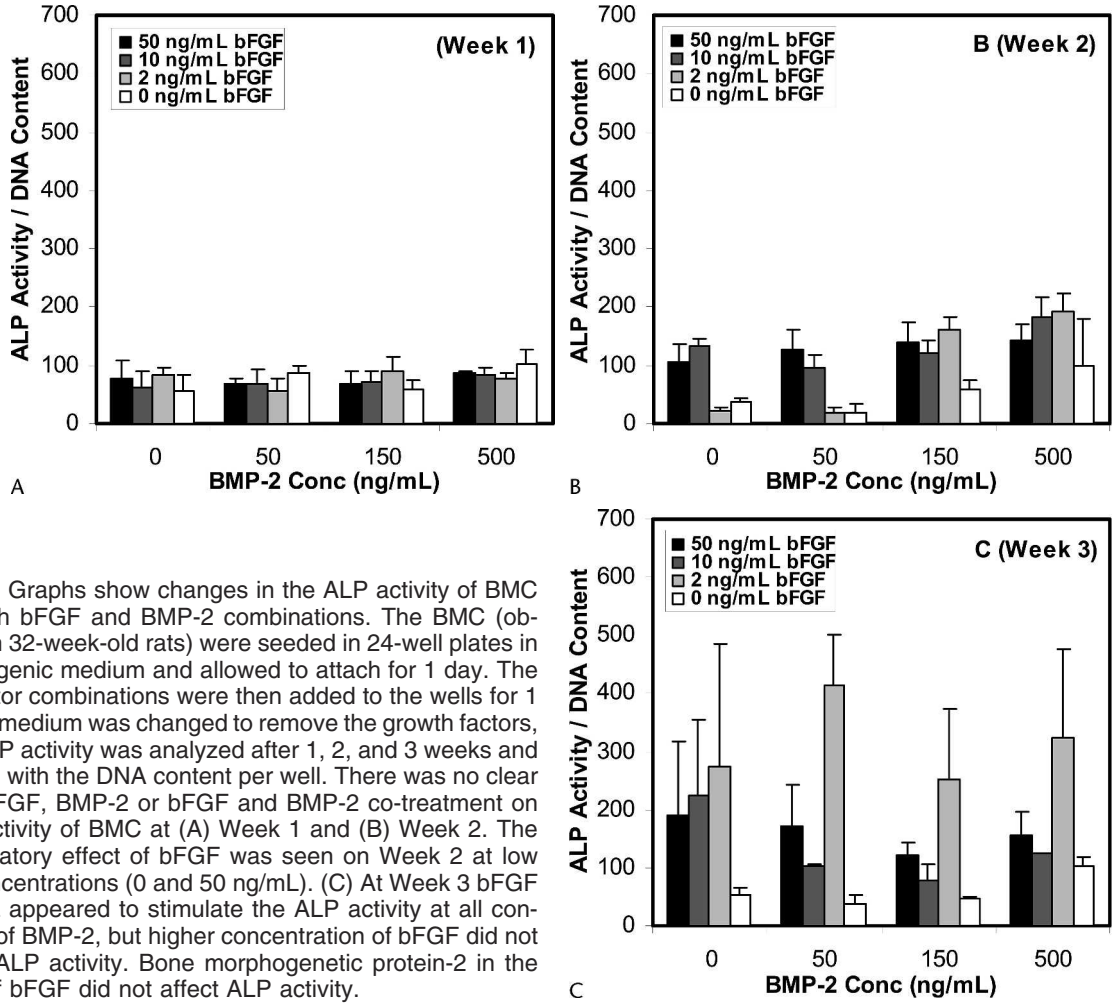

Fig 7A–C. Graphs show changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. There was no clear effect of bFGF, BMP-2 or bFGF and BMP-2 co-treatment on the ALP activity of BMC at (A) Week 1 and (B) Week 2. The only stimulatory effect of bFGF was seen on Week 2 at low BMP-2 concentrations (0 and 50 ng/mL). (C) At Week 3 bFGF at 2 ng/mL appeared to stimulate the ALP activity at all concentration of BMP-2, but higher concentration of bFGF did not affect the ALP activity. Bone morphogenetic protein-2 in the absence of bFGF did not affect ALP activity.

range of growth factor concentrations, and may not necessary extend to all concentrations of the growth factors. This also occurred when bFGF and BMP-2 were co-delivered subcutaneously in a rat ectopic model. Low doses of bFGF were stimulatory on BMP-2 induced ALP activity and de novo bone deposition, but high concentrations of bFGF were inhibitory.[4] It is likely that such biphasic interactions might also occur when BMC are exposed to the systemically administered growth factors. Our experiments may help identify the appropriate concentrations of growth factors for an optimal stimulation of bone deposition.

We also compared the response to growth factors when BMC from derived from young or older rats. The osteogenic potential of bone marrow environment generally declines with age.[2,8,20] This was similar to the DNA assessment in our study, which indicated lower cell numbers emanating from the BMC of older rats. Although we observed a similar level of specific ALP activity in young and old rats, the mineralization with BMC from old rats was less than the mineralization with BMC from younger rats. The BMC of old rats did not calcify in the absence of BMP-2 (compare Figs 5, 8), and the best calcification with 500 ng/mL BMP-2 concentration was approximately fivefold less with the BMC from older rats after 3 weeks of culture. bFGF did not appear to affect the mineralization of BMC from young rats, but was stimulatory in increasing the mineralization for BMC of older rats, especially at lower concentrations of BMP-2. Our results were similar to Kotev-Emeth et al,[11] who used an animal model similar to ours (Sprague-Dawley rats of 1.5 months versus 9 months). They observed a decrease in in vitro cell growth for the 9-month-old rats, but BMC from these rats were more responsive to bFGF compared with the BMC from younger rats, which did not exhibit a bFGF response.[11] The bFGF stimulation of bone-like modules (Alizarin-Red stained area) were also more substantial in the aged rats.[11] These observations suggest that osteogenic

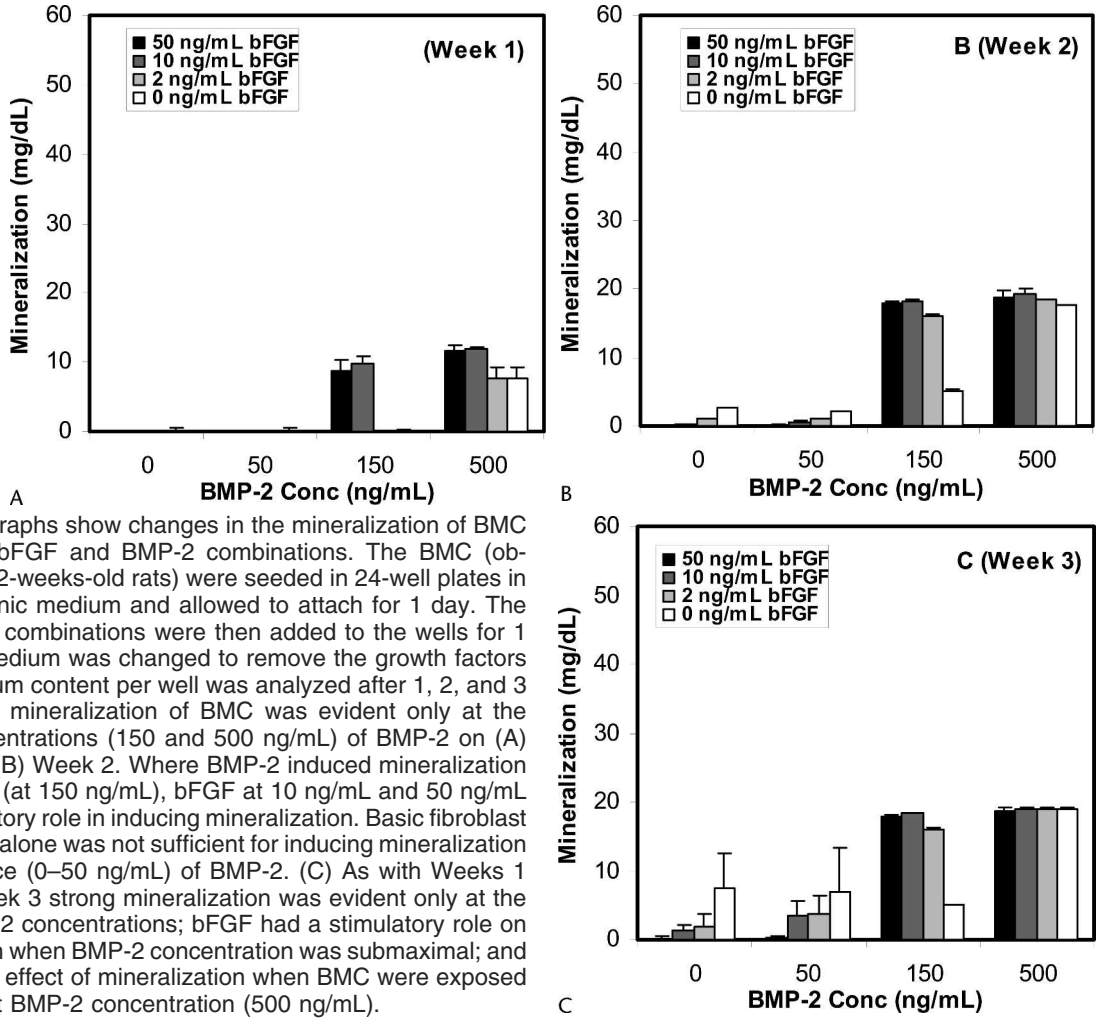

Fig 8A–C. Graphs show changes in the mineralization of BMC treated with bFGF and BMP-2 combinations. The BMC (obtained from 32-weeks-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. Clear mineralization of BMC was evident only at the highest concentrations (150 and 500 ng/mL) of BMP-2 on (A) Week 1 and (B) Week 2. Where BMP-2 induced mineralization was retarded (at 150 ng/mL), bFGF at 10 ng/mL and 50 ng/mL had a stimulatory role in inducing mineralization. Basic fibroblast growth factor alone was not sufficient for inducing mineralization in the absence (0–50 ng/mL) of BMP-2. (C) As with Weeks 1 and 2, at Week 3 strong mineralization was evident only at the highest BMP-2 concentrations; bFGF had a stimulatory role on mineralization when BMP-2 concentration was submaximal; and bFGF had no effect of mineralization when BMC were exposed to the highest BMP-2 concentration (500 ng/mL).

factors might be more beneficial for BMC at an advanced age. It will be interesting to see if this is true for BMC even for older rats, as an equivalent age for onset of osteoporosis is older than the 8-month-old to 9-month-old rats.[11]

Our findings indicated that osteogenic supplements β-GP/dexamethasone at 10 mM/100 nM were detrimental on BMC viability after 3 weeks of culture. bFGF was beneficial in increasing cell viability under mineralizing conditions, presumably by stimulating cell proliferation. Whereas continuous or short-term exposure to BMP-2 resulted in enhanced mineralization in either conditions, bFGF exerted its stimulatory effect in a narrow range of concentrations (approximately 10 ng/mL) and only after continuous exposure to BMC. The clinical utility of the latter growth factor, therefore, is likely to require continuous administration, whereas BMP-2 administration could be shorter duration. Co-treatment of BMC with BMP-2 and bFGF did not indicate a synergistic role of the bFGF on BMP-2 induced ALP activity and mineralization with BMC from young rats, but it was evident with BMC from older rats for certain lower concentrations of BMP-2. The BMC from older rats is more representative of osteoporosis patients, and this result indicates the possibility of utilizing the synergistic effects of bFGF and BMP-2 in a clinical scenario. Our results indicated differences in the osteogenic response depending on the age of rats from which the BMC were obtained, and concentration and the exposure duration of the growth factors. It will be important if such effects could be observed in vivo (ie, if the cells in the native bone marrow environment display such differential effects after growth factor treatments). Our future studies are designed with this end in mind.

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