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Basic Fibroblast Growth Factor in the Presence of Dexamethasone Stimulates Colony Formation, Expansion, and Osteoblastic Differentiation by Rat Bone Marrow Stromal Cells

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Abstract. Basic fibroblast growth factor (bFGF) is known to stimulate endosteal bone formation *in vivo* by a mechanism possibly mediated via osteoblast precursor cells present in the bone marrow. In high density cultures of primary bone marrow cells, and in the presence of glucocorticoids, bFGF stimulates the formation of a bone-like matrix; however, due to the dense nature of these cultures, the exact mechanism of action is unclear. In an adaptation of the fibroblastic colony formation unit assay, in which the bone marrow cells are grown in the presence of dexamethasone, β-glycerophosphate, and ascorbate, mineralized colonies are formed which stem from single mesenchymal precursor cells and grow in isolation from each other. Using this system we have been able to investigate the mechanism by which bFGF stimulates the formation of bone like tissue *in vitro*. We have shown that bFGF increases the formation of a calcified collagenous matrix *in vitro* by (1) increasing the total number of fibroblastic colonies formed, (2) increasing the proportion of differentiated colonies that synthesize collagen and calcify, and (3) stimulating the proliferation and collagen accumulation of the individual colonies. A maximal increase in total and differentiated colony numbers was seen after only 5 days exposure to bFGF, however, continued exposure to bFGF continued to increase the size and collagen content of the individual colonies. Bearing in mind the endosteal location of newly formed bone seen after treatment with bFGF, these processes may well play an active role in this effect.

Key words: Bone formation — Fibroblastic colony-forming unit — Osteoblast — Recruitment — Bone marrow stromal cells.

Postmenopausal, male, and senile osteoporosis are increasingly becoming major socioeconomic problems. Although the bone loss associated with these conditions can be prevented by hormone replacement therapy (HRT) or by treatment with blockers of bone resorption such as bisphosphonates, this is not always effective. For this reason there is a need for agents that stimulate bone formation and replace bone lost during the course of the disease [1]. At present there are only a few substances that stimulate bone formation in humans and laboratory animals. These include parathyroid hormone (PTH) [2, 3] and prostaglandin E_2 (PGE_2) [4] both of which stimulate the formation of cortical and trabecular bone. More recently, a number of other substances have been found to be anabolic for bone, in particular, bFGF which induces bone formation *in vivo* both after implantation [5] and systemic application [6–9]. bFGF is one of a family of at least seven fibroblast growth factors. It is a potent mitogen for many types of mesenchymal and epithelial cells *in vitro* and is synthesized by a wide variety of organs and cell types [10, 11]. This is also true for bone [12, 13] and it has been shown that the bFGF synthesized by osteoblast (OB) *in vitro* is subsequently bound to the extracellular matrix, suggesting that the bFGF in the bone matrix may be OB-derived [14].

A major problem in bone biology is that there are no satisfactory *in vitro* cell systems with which the mechanism of action of bone anabolic substances can be studied [1, 15]. Although their effects on OB cultures have been well characterized, these are not normally consistent with their actions *in vivo*. Treatment with bFGF, PTH, or PGE_2 *in vivo* results in an increase in collagen accumulation and OB numbers. However, in OB cultures *in vitro*, PTH or PGE_2 tend to inhibit proliferation and collagen synthesis [16, 17]. Similarly, although bFGF stimulates OB proliferation *in vitro*, alkaline phosphatase (APase) activity, collagen synthesis, and osteocalcin synthesis are all inhibited [18–21]. As increased bone formation *in vivo* is characterized by an increase in the number of mature OB and not by an increase in bone apposition rate [22], these *in vitro* results would tend to suggest that mature OB are not the major target cell for bone anabolic substances and that perhaps OB precursor cells may be better candidates.

Possible OB precursor cells may be the bone marrow stromal cells (BMSC) found in the marrow space adjacent to the trabeculae. The osteogenic potential of these cells has been well established both *in vivo* and *in vitro* [23, 24] and the osteoblastic differentiation of BMSC has been characterized [25–30]. BMSC also synthesize bFGF [31] and respond to bFGF with an increase in proliferation although, as with OB cultures, APase activity is also inhibited [32–34]. In the last decade, a number of cell culture systems have been established that recapitulate *in vitro* the process of bone formation [35, 36]. In particular, rat BMSC cultured in the presence of ascorbate, β-glycerophosphate, and dexamethasone form calcified nodules with biochemical, ultra-

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structural, and morphological properties similar to woven bone [37, 38] and it has been shown that bFGF enhances the ability of BMSC to form these calcified nodules [39, 40]. Using a similar system, we have recently shown that rat BMSC respond to treatment with PGE_2 *in vitro* with an increase in cell number, collagen accumulation, total APase activity, and calcium deposition and that this effect was due to an increase in colony formation rather than an increase in cellular activity per se [41]. Furthermore, we have shown that colony development is the product of three distinct processes: colony formation, expansion, and differentiation. In CFU-f cultures, these are to some extent modulated in isolation from each other and their individual contributions can be studied using a modification of the CFU-f assay [42]. These observations suggest that BMSC are possible target cells for bone anabolic agents and their effects may be mediated by changes in BMSC colony formation, expansion, and differentiation. Because of this, it was decided to use this system to investigate the actions of bFGF on by BMSC.

Materials and Methods

Materials

Materials were obtained from the following companies: culture media, Gibco (Paisley, Scotland); plasticware, Nunc (Frankfurt, Germany); fetal calf serum and bFGF (Eurobio, Frankfurt, Germany); sirius red F3BA, Aldrich Chemical Company (Deisenhofen, Germany). All other chemicals were from Sigma (Deisenhofen, Germany) and used without further purification.

Cell Culture

Preparation of BMC. Pooled BMC were obtained from tibia and femur from four 125 g female Wistar rats, as described previously [41]. Briefly, the bones were removed under aseptic conditions and all soft adherent tissue removed. One end of the bones was removed, a hole was made in the opposing end with an 18-gauge syringe needle, and the cells were flushed out with 10 ml Dulbecco's Modified Eagle Medium (DMEM) containing 12% fetal calf serum (FCS), 10^{-8} M dexamethasone, and 50 μg/ml ascorbic acid (OB-DMEM). The cells were dispersed by repeated pipetting and a single cell suspension was achieved by forcefully expelling the cells through a 20-gauge syringe needle. Microscopic analysis of the cells confirmed the single cell nature of the cell suspension. The cells were then used as follows.

High Density BMC Cultures. For primary (1°) high density BMC cultures, 10^6 cells were plated out in 2 cm^2 wells in 0.75 ml of the above-mentioned medium and immediately challenged with bFGF. The medium was then first changed after 5 days for bFGF-free OB-DMEM plus 10 mM β-glycerophosphate, and thereafter three times weekly. The cultures were maintained for 14 days after which the cells were washed with phosphate-buffered saline (PBS), fixed by the addition of cold ethanol, and analyzed as described below.

CFU-f Assay. For the analysis of fibroblastic colony-forming unit (CFU-f), 10^6 BMC were plated out in 55 cm^2 Petri dishes in the above-mentioned medium with the relevant concentrations of bFGF. The medium was changed after 5 days for OB-DMEM plus 10 mM β-glycerophosphate and thereafter twice weekly. The cultures were maintained for 18 days after which the cells were washed with PBS and fixed by the addition of cold ethanol. After fixation, the cultures were stained sequentially for APase, calcium, and collagen-positive colonies and total colonies, as described below. After each staining, the culture dishes were photographed and destained. The colony numbers were then determined blindly directly from the photographs. Total fibroblastic colonies (Col-f) were considered to be derived from CFU-f. Colonies that also stained positive for APase, calcium, and/or collagen were termed Col-AP, Col-Ca, and Col-co, respectively, and were assumed to be derived from CFU-f with the ability to express APase, calcify, and/or synthesize collagen.

Nonadherent BMC. Nonadherent BMC were obtained by plating out 2×10^7 BMC in 10 ml OB-DMEM in a 55 cm^2 Petri dish and allowing the cells to adhere for 18 hours. The cells were then agitated, the nonadherent cells were removed, and the adherent cells were discarded. The recovery of nonadherent cells was normally greater than 95% of the original cell number. Aliquots (0.5 ml) of the nonadherent cell suspension, corresponding to 10^6 BMC minus adherent cells, were then used for CFU-f experiments.

Adherent BMC. Adherent BMC were investigated by plating out 10^6 BMC in 55 cm^2 Petri dishes in OB-DMEM. The cells were allowed to adhere for 18 hours after which the medium, together with nonadherent cells, was removed, filtered, and the cell-free medium was returned to the cells. The cultures were thereafter treated as described for the CFU-f assay.

Analyses

APase/Col-AP. APase was stained histochemically as described by Bancroft and Stevens [43]. The cultures were exposed for 30 minutes at 20°C to a solution of naphthol phosphate (0.05 mg/ml) in Tris (0.08 M, pH 7.5) containing fast red bb (1 mg/ml). The cultures were then washed under running water, air dried, and photographed, then destained with absolute ethanol and additionally fixed at this point with 5% formalin in order to give additional mechanical stability.

Calcium/Col-Ca. The fixed cultures were stained histochemically for calcium with 0.5% alizarin red pH 5 after which they were washed under running tap water and air dried. After analysis, the cultures were destained with 5% perchloric acid.

Collagen/Col-co. Total collagen was assessed using a modification of the method of Lopez-de Leon and Rojkind [44]. After fixation and demineralization, the cultures were stained with 1% sirius red F3BA in saturated picric acid for 18 hours after which excess sirius red was removed by washing under running tap water until no more sirius red could be eluted. To quantitate collagen, the dye was then eluted with 0.1 N NaOH/methanol (50:50), measured spectrophotometrically at 490 nm, and total collagen was determined by comparison with a standard curve. This method is based on the specific and quantitative staining of collagen by sirius red F3BA in saturated picric acid and correlates well with hydroxyproline analysis [44–47]. CFU-f cultures were similarly stained with sirius red F3BA, washed under running tap water, dried, and photographed. After analysis, the cultures were destained with methanol/0.2 N NaOH (50:50).

Proliferation/Col-f. Cell number was assessed by the method of Currie [48]. After fixation, the cells were washed with borate buffer (10 mM, pH 8.8), stained with 1% methylene blue in borate buffer for 30 min and then rewashed 3 times with borate buffer. CFU-f cultures were then photographed and the methylene blue positive colonies used as a measure of the number of CFU-f in the original cell suspension. To assess cell number, bound methylene blue was then eluted with 1% HCl in ethanol and the absorbance measured at 650 nm. Cell numbers could then be determined by comparing with a standard curve.

Statistical Analysis. All results are expressed as the means ± standard deviations. Statistical significance was determined using analysis of variance with a probability of less than 0.05 being considered significant. ''n'' denotes the number of replicate cultures from a representative experiment.

Terminology. In these experiments the following terms are used in order to describe colony behavior. *Colony formation* refers to the total number of colonies seen at the end of the culture period; *recruitment* to the process by which CFU-f are induced to form

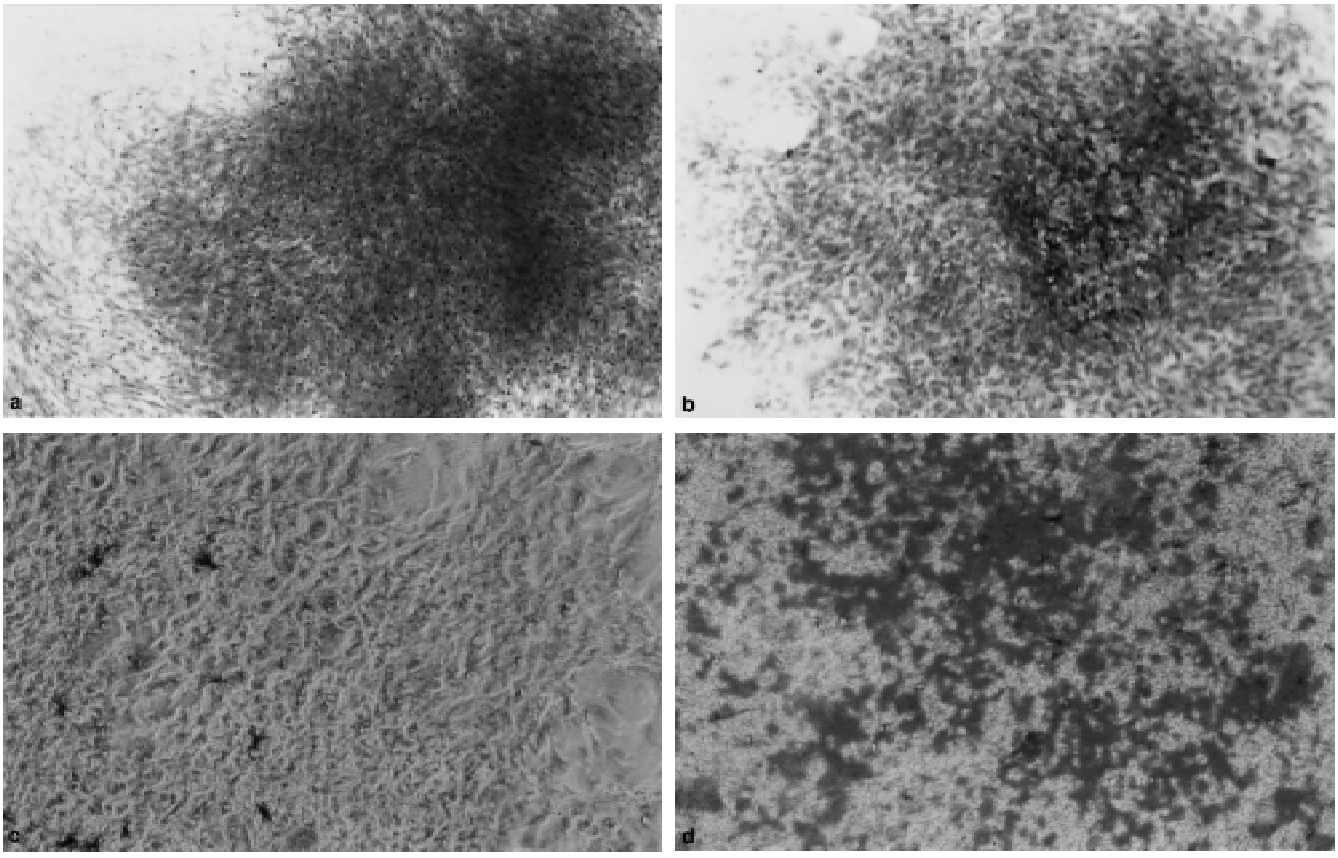

Fig. 1. Photomicrographs of BMC-derived fibroblastic colonies, magnification ×20. The BMC were cultured as described in Methods and exposed to either vehicle **(a, c)** or 10 ng/ml bFGF **(b, d)** for the first 5 days of the culture period. The cultures were stopped and fixed after 14 days and stained with either methylene blue **(a, b)** or sirius red **(c, d)**. The colonies in control cultures were largely fibroblastic, the cells had a characteristic spindle-shaped morphology **(a)**, and there was little evidence of a collagenous matrix or calcification **(b)**. In bFGF-treated cultures, many of the colonies took on an osteoblast-like phenotype, with the central cells adopting a cuboidal morphology whereas the peripheral cells remained spindle shaped **(c)**. In addition, a dense collagenous matrix was layed down which consequently calcified **(d)**.

colonies, e.g., the transition of nonadherent to adherent CFU-f or the indution of proliferation in quiescent CFU-f; *colony differentiation* to the ability of the colonies to express markers characteristic of OB, in this case, APase activity, collagen synthesis, and calcification. Not all cells in the colonies differentiate, however, as each colony stems from a single precursor cell, the expression of the osteoblastic phenotype means that the original CFU-f and/or one or more of its offspring have adopted the osteoblastic phenotype. *Colony expansion* refers to the size of the colony, in this case colony cell number, at the end of the culture period and is considered to be analogous to proliferation.

Results

Effect of bFGF on Colony Formation by Total BMC

When BMC were cultured in the presence of 10^{-8} M dexamethasone, 50 μg/ml ascorbate and 10 mM β-glycerophosphate, fibroblastic colonies were formed, a proportion of which expressed markers characteristic of the osteoblastic phenotype, and the addition of bFGF stimulated the expression of these markers (Figs. 1, 2). In control cultures, the majority of the colonies remained fibroblastic and the cells displayed a characteristic spindle-shaped morphology (Fig. 1a) and did not lay down a collagenous matrix or calcify (Fig. 1b). In cultures treated with bFGF, an increasing number of the colonies took on an osteoblast-like phenotype with the cells at the center of the colonies adopting a cuboidal morphology whereas those at the periphery remained spindle shaped (Fig. 1c). In addition, a dense collagenous matrix was layed down which consequently calcified (Fig. 1d). In the absence of bFGF, a total of 184 ± 37 colonies were formed and of these, 84 ± 9 stained positive for APase activity. Under these conditions very few of the colonies synthesized collagen or calcified, giving rise to 37 ± 6 and 2 ± 0.6 Col-co and Col-Ca, respectively (Fig. 3a). The addition of bFGF caused an increase in all four types of colonies with a peak being seen at a concentration of 10 ng/ml at which 443 ± 20 Col-f, 278 ± 7 Col-AP, 275 ± 12 Col-Ca, and 318 ± 19 Col-co were formed (Fig 3a).

A differential concentration dependency was seen with regard to the formation of the different types of colonies. Both Col-f and Col-AP showed a concentration-dependent increase in numbers after addition of bFGF, with a statistically significant increase being seen at 0.1 and 0.01 ng/ml for Col-f and Col-AP, respectively (Fig.3a). On the other hand, no effect was seen on Col-Ca and Col-co numbers until 1 ng/ml when colony numbers jumped from baseline levels to almost maximal levels of 224 ± 22 for Col-Ca and 278 ± 10 for Col-co (Fig.3a). When the relative frequencies of the various types of colonies were analyzed it was found that the percentage of Col-AP did not vary significantly

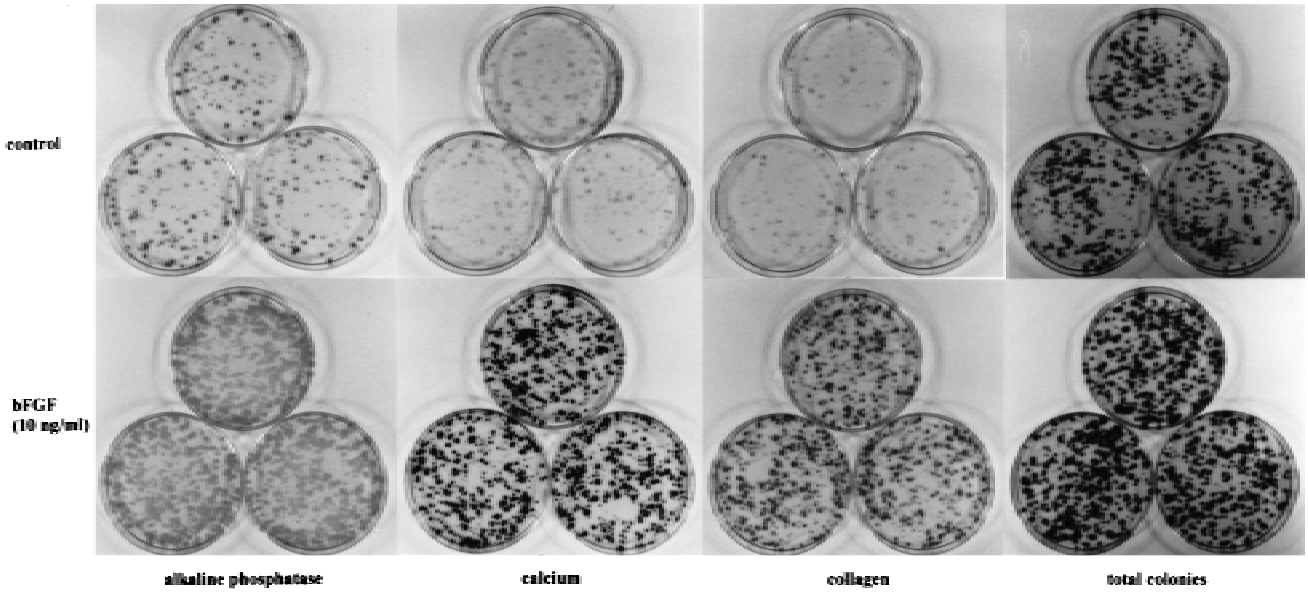

Fig. 2. Effect of bFGF on BMC fibroblastic colony formation and differentiation. BMC were cultured as described in Methods and exposed to either vehicle or 10 ng/ml bFGF for the first 5 days of the culture period. The medium was then replaced with fresh, bFGF-free medium and thereafter changed twice weekly. After 18 days the cultures were stopped, fixed, and stained consecutively for APase (Col-AP), calcium (Col-Ca), collagen (Col-co), and total colonies (Col-f).

throughout the concentration range. The numbers of CFU-AP remained within 45–62% with an average of 54% (Fig. 3b). In contrast, in control cultures and in cultures treated with 0.01 and 0.1 ng/ml bFGF, only 2% and 20% of the colonies were calcium- and collagen-positive, respectively, whereas the addition of 1, 10, or 100 ng/ml bFGF to the colonies increased the percentage of Col-Ca and Col-co to approximately 60% and 72%, respectively (Fig. 3b). The occasional finding of greater numbers of CFU-Ca than CFU-AP, especially in cultures treated with bFGF, was due to a counting artefact. As only the center of the colonies tended to calcify, in cultures with large numbers of colonies the CFU-AP sometimes merged together whereas the CFU-Ca were still discernible as separate colonies, thus giving rise to greater apparent numbers of CFU-Ca.

By measuring the total cell number per dish and dividing this by the number of Col-f, the average cell number per Col-f could be calculated. Similarly, using the total amount of collagen per dish and dividing by the number of Col-co, the average amount of collagen per Col-co could be calculated. It was found that although bFGF increased Col-f numbers concentration dependently, with a statistically significant effect being seen at 0.1 ng/ml (Fig. 4a), no effect on average Col-f cell number was seen until 1 ng/ml when maximal levels were seen (Fig. 4a). In contrast, although the numbers of Col-co showed an all or nothing response with 1 ng/ml bFGF giving rise to an almost maximal response (Fig. 4b), total collagen levels and average collagen levels per Col-co were concentration related with 1 ng/ml giving rise to 50–60% of the levels produced by 10 ng/ml (Fig. 4b).

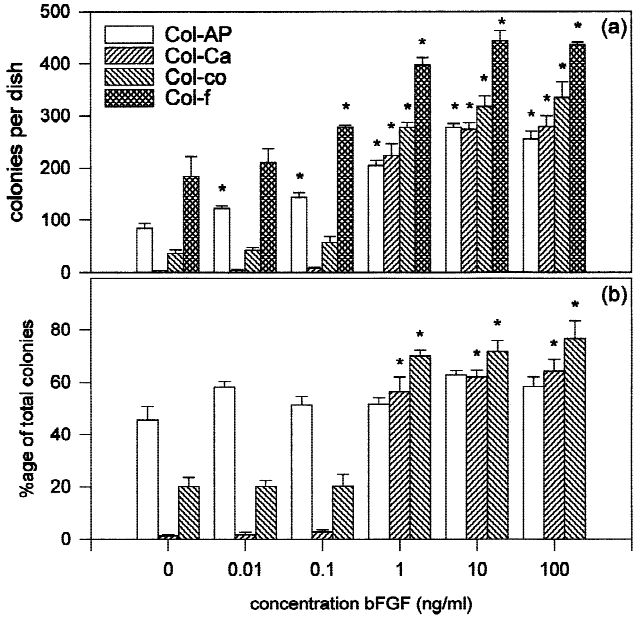

Fig. 3. Effect of bFGF on BMC fibroblastic colony formation and differentiation. **(a)** BMC were cultured as described in Figure 2 and the numbers of Col-AP, Col-Ca, Col-co, and Col-f were determined. **(b)** Data from Figure 3a with levels of Col-AP, Col-Ca, and Col-co expressed as a percentage of Col-f numbers. Results are expressed as means ± SD; n = 3. *Significant difference relative to untreated controls, $P < 0.05$.

Origin of Extra Colonies

To ascertain whether the extra colonies induced by bFGF stemmed from the nonadherent fraction or the adherent fraction of the BMC, three populations of cells were investigated; (1) total BMC, (2) adherent BMC, and (3) nonadherent BMC. The cells were then challenged with 10^{-7} M PGE_2, 10 ng/ml bFGF, or both together. It was found that both PGE_2 and bFGF stimulated colony formation in total, and nonadherent BMC with the two together had an additive

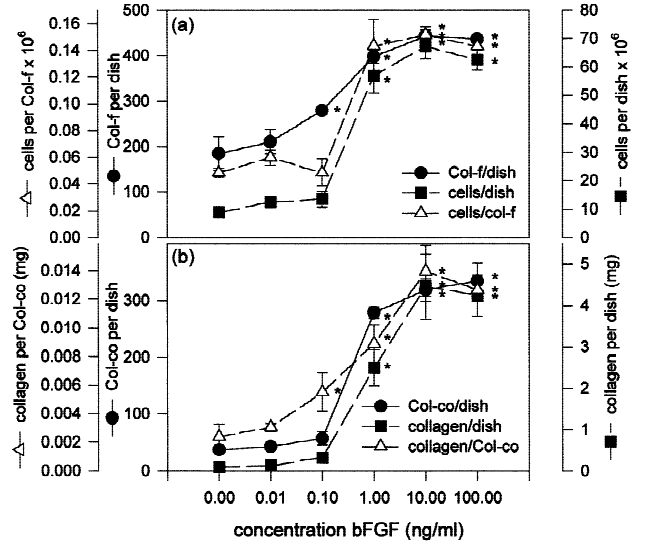

Fig. 4. Effect of bFGF on BMC fibroblastic colony cell number and collagen accumulation. **(a)** The BMC were cultured as in Figure 3 and total cell number was determined. Using this data together with the numbers of Col-f, the average colony cell number was determined. **(b)** In the same cultures, total collagen was determined and using this data together with Col-co numbers, the average amount of collagen per Col-co was calculated. Results are expressed as means ± SD; n = 3. *Significant difference relative to untreated controls, $P < 0.05$.

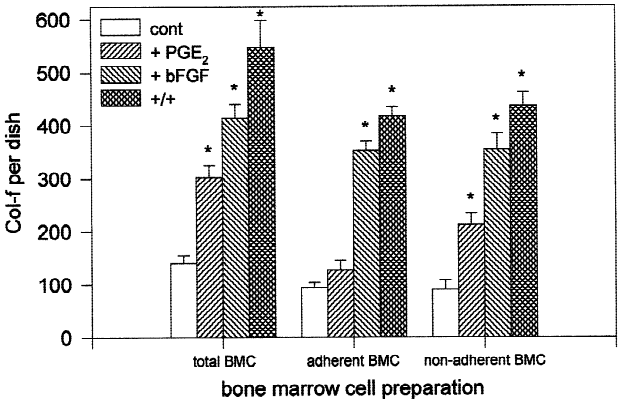

Fig. 5. Does bFGF act on adherent or nonadherent BMC? Total, adherent, and nonadherent BMC were prepared as described in Methods and then challenged for the first 5 days of the culture period with 10^{-7} M PGE_2, 10 ng/ml bFGF, or both together. After 10 days, the cultures were stopped and Col-f was determined. Results are expressed as means ± SD; n = 3. *Significant difference relative to untreated controls, $P < 0.05$.

effect. On the other hand, PGE_2 had no significant effect on colony formation by adherent BMC whereas bFGF stimulated colony formation to 374% of control levels (Fig. 5). Similarly, challenging adherent-BMC with bFGF produced a concentration-dependent increase in the numbers of all types of colonies with a maximum being seen at about 10 ng/ml and a significant increase being seen at 0.01 ng/ml for Col-AP, Col-Ca, and Col-co and 0.1 ng/ml for Col-f. Treatment with 10 ng/ml bFGF increased the numbers of Col-AP from 176 ± 5 to 275 ± 7, Col-Ca from 32 ± 12 to 246 ± 4, Col-co from 29 ± 12 to 282 ± 10, and Col-f from 235 ± 13 to 377 ± 8 (data not shown).

Effect of Varying Length of Exposure to bFGF on Colony Formation and Differentiation

When total BMC as exposed to 5 ng/ml bFGF for various lengths of time, it was found that the numbers of all types of colonies were increased to maximum levels after exposure for the first 5 days. Longer exposure to bFGF had no supplementary effect (Fig. 6a). Extending the exposure to bFGF, however, from 5 to 18 days caused a gradual supplementary increase in cells per dish and cells per Col-f to a maximum of 158% and 149% of the values after 5 days exposure, respectively (Fig. 6b). Similarly, collagen per dish and collagen per Col-co were also increased by extending the exposure from 5 to 18 days to 278% and 276%, respectively, of the values obtained after 5 days exposure (Fig. 6c). When the BMC were treated with 5 ng/ml bFGF for 1-5 days, numbers of Col-f increased steadily, reaching a maximum after 4 day exposure. Parallel to this, the average colony cell number increased steadily with the length of exposure to bFGF (Fig. 7).

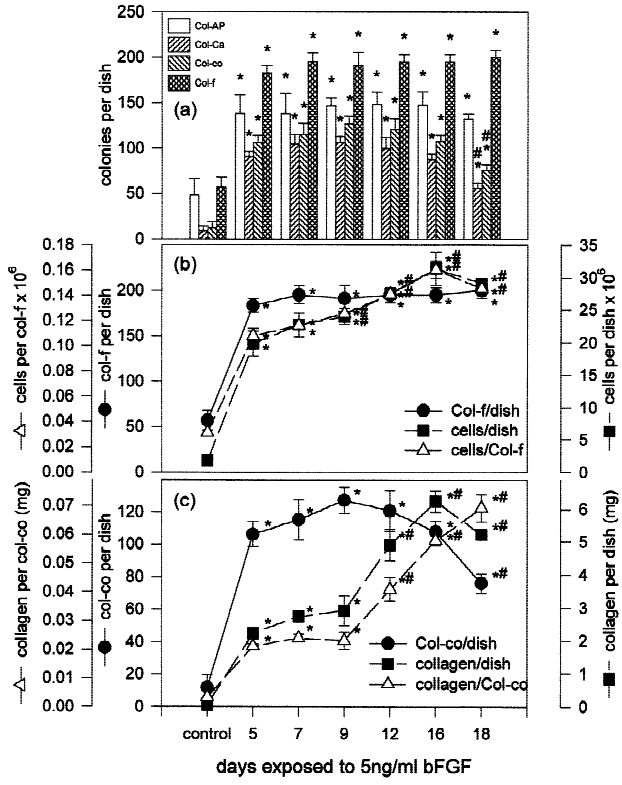

Fig. 6. Effect of length of exposure to bFGF on BMC colony formation and differentiation. BMC were cultivated as in Figure 3 except that the cells were exposed to 5 ng/ml bFGF for differing lengths of time from the begining of the culture period. The cultures were continued for a total of 18 days and then stopped and **(a)** Col-AP, Col-Ca, Col-co, and Col-f, **(b)** average colony cell number, and **(c)** the average amount of collagen per Col-co were determined. Results are expressed as means ± SD; n = 3. *Significant difference relative to untreated controls and #relative to cultures treated with bFGF until day 5, $P < 0.05$.

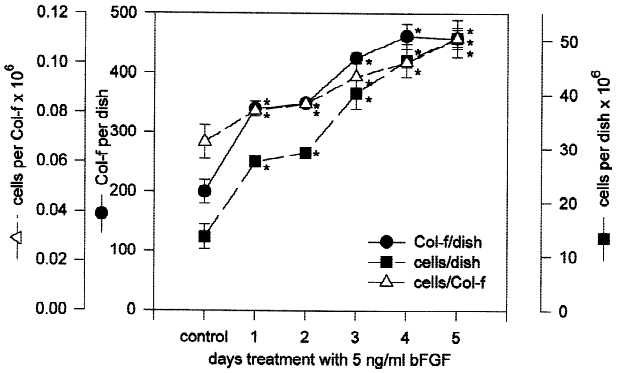

Fig. 7. Effect of length of exposure to bFGF on BMC fibroblastic colony cell number. The BMC were cultured as in Figure 6 except that the cultures were treated with bFGF for up to 5 days. Col-f, total cell number, and average colony cell number were determined. Results are expressed as means ± SD; n = 3. *Significant difference relative to untreated controls.

Discussion

Although bFGF has not been nearly so well studied as other bone anabolic substances such as PTH or PGE_2, it is evident that systemic application of pharmacological doses of bFGF has a stimulatory effect on bone formation [6–9] and induction [5]. Significantly, after treatment with bFGF, new bone formation is only observed on the endosteal and trabecular bone surfaces, with none being seen on the periosteal surface, and in some cases, the marrow cavity is almost filled with newly formed cancellous bone. This would suggest that the target cells for this effect are the OB precursors present in the bone marrow. Consistent with this, results from high density cultures of BMC in this laboratory (data not shown) and the work of others [39, 40] show that bFGF can enhance the ability of BMSC to form a bone-like matrix *in vitro*.

However, these studies were performed using what we have termed ''high density cultures''. Although these cultures can be used to demonstrate the effects of various substances on the formation of a bone-like matrix *in vitro*, due to the heterogenous nature of the cell population it is hard to interpret the data in terms of what is happening at the cellular level. This is because at least three different processes are taking place simultaneously, i.e., recruitment, differentiation, and proliferation, and the individual contributions of these cannot be assessed in these cultures. For example, in a mixed population of cells such as those derived from rat bone marrow, if APase activity is increased we cannot say if this is due to (1) an increase in specific APase activity in preexisting OB, (2) an increase in the proliferation of APase-positive cells, or (3) the recruitment of mesenchymal precursor cells to the osteoblastic lineage. In CFU-f cultures these three parameters can to some extent be measured in isolation from each other in the same cultures. This can be achieved by measuring recruitment of mesenchymal precursor cells as the total number of colonies (i.e., colony formation), differentiation as the percentage of total colonies expressing the OB phenotype, and proliferation as the size of the individual colonies. Using the CFU-f assay, we have shown that bFGF can increase the formation of a bone-like matrix *in vitro* by a number of discrete mechanisms.

bFGF increased total colony formation with a significant increase being seen at concentrations as low as 0.1 ng/ml. We have previously shown that, in the presence of physiological concentrations of glucocorticoids, PGE_2 can increase total colony formation by causing the transition from nonadherent to adherent mesenchymal precursors present in BMC [41, 49]. This was not the case for bFGF. Both PGE_2 and bFGF increased colony formation in cultures of total BMC and nonadherent BMC. bFGF, however, also increased total colony numbers in adherent BMC cultures in which all nonadherent cells had been removed. This would suggest that bFGF promotes the proliferation or survival of adherent mesenchymal cells (CFU-f) that under normal conditions would have remained quiescent or died and consequently not have formed colonies. In addition, it was found that addition of bFGF after day 3 of the culture period had no effect on colony formation, suggesting that the bFGF responsive CFU-f had already become apoptotic or unresponsive by this time point and that bFGF may be acting as a survival factor (data not shown). Like PGE_2, bFGF also stimulated colony formation from nonadherent BMC which would suggest that bFGF can also induce the transition from nonadherent to adherent mesenchymal precursors. However, as about 20% of the nonadherent BMC spontaneously adhere and form colonies in the absence of PGE_2 or bFGF, it is also possible that bFGF is acting through these although it is unlikely that this could account for the observed increase in colony formation.

bFGF stimulates the osteogenic differentiation of colonies. In control cultures, although a significant proportion expressed APase, almost no cultures calcified and only a small number accumulated collagen. The addition of bFGF caused an increase in all types of colony. However, whereas the percentage of Col-AP remained fairly constant throughout the concentration range in both total and adherent BMC cultures, the percentage of Col-Ca and Col-co were increased until at 10 ng/ml, 62% of the colonies were calcium positive and 71% were collagen positive compared with 62% being APase positive. This would suggest that a fixed percentage of CFU-f have the ability to express APase regardless of the growth factor environment and that the presence of bFGF, or some other growth factor, is required for the synthesis of collagen and/or subsequent calcification. We are at present developing an automated method for determining the phenotype of each individual colony in a CFU-f culture. Preliminary results suggest that colonies can be APase positive, collagen positive, APase/collagen positive or calcium/collagen/APase positive. This is in agreement with the findings of other workers. Satomura and Nagayama [38] showed that mineralized nodules formed in rat BMSC cultures were characterized by a high APase activity and a dense collagenous matrix. Malaval et al. [30] showed that in addition to these proteins, mineralized colonies are also characterized by the expression of a number of other bone-related proteins including osteonectin, thrombospondin, and osteocalcin. However, this study is not so much focussed on the colonies themselves but on the pharmacology of their formation and the effect of bFGF on the population of mesenchymal precursor cells as a whole. By using the CFU-f assay we can obtain data that is normally obtained using high density cultures. However, it is also evident that a number of different colony types are present and by quantitating the changes in their phenotypes, we can analyze the mechanism of action of bone anabolic agents such as bFGF on the whole population.

It was noted in a previous work, that significant numbers of Col-Ca and Col-co were also present in the control cultures [41]. This difference was found to be due to differ-

ences in the preparation of the BMC. In the previous work, in order to assess the affect of PGE_2 on BMC under conditions as close to physiological as possible, the cells were used ''as obtained'' (i.e., directly from the bone together with all growth factors, etc. originally present). In the present work, to avoid problems with endogenous bFGF, the cells were first washed and resuspended in fresh medium. Replacement of the ''washings'' caused the numbers of Col-Ca and Col-co to return to their original levels (data not shown). This would suggest that bFGF was present in the BMC conditioned medium, however, we have no data to support this and we cannot rule out the possibility that other cytokines have similar activity.

bFGF stimulated the proliferation of individual colonies. By measuring the total number of cells in the cultures we could calculate the average number of cells per Col-f. It was found, in both total BMC and adherent BMC cultures, that although total colony numbers, and correspondingly total cell numbers, were increased at concentrations of bFGF as low as 0.1 ng/ml, no significant effect was found on the number of cells per colony until 1 ng/ml. This is in agreement with the findings of Thomson et al. [33] who used subcultures of BMSC, thus eliminating the effect of colony formation, and who also found no effect on BMSC cell number until 1 ng/ml. This could mean that colony initiation and proliferation are independent of each other although it is also possible that our method of assessing proliferation was not sensitive enough to detect changes in cell number at low bFGF concentrations. The latter possibility is supported by the findings of Noff et al. [39] who measured ^{3}H-thymidine uptake and found an effect at concentrations as low as 0.01 ng/ml bFGF.

bFGF also stimulated the amount of collagen accumulated by individual Col-co. Using a similar approach to that used for proliferation, total collagen was calculated and from this the average amount of collagen accumulated per Col-co could be calculated. This has the advantage that only colonies that are synthesising collagen are included in the calculation whereas normally, results are expressed in terms of total cell number or total protein even though a large and variable number of the cells are not involved in collagen synthesis. The numbers of Col-co and the levels of collagen per Col-co were increased by concentrations of bFGF 1 ng/ml and above. In addition to these early effects on colony differentiation, long-term exposure to bFGF also increased average colony size and collagen accumulation. As just 5 days exposure to bFGF produced maximal numbers of all types of colony even though significant levels of collagen are not detected in these cultures until at least 10 days [41], this would suggest that the effects of bFGF on the differentiation of precursor cells are not necessarily linked to the direct control of collagen synthesis by more differentiated osteoblastic cells.

Although this approach of using average colony cell number or collagen content provides a method for assessing the effects of bone-active agents on colony expansion or collagen synthesis, it does also has a number of advantages. It is known that the distribution of colony sizes is heavily skewed with a large number of small colonies and relatively few larger ones so that mean plus or minus standard deviations are not the most appropriate statistical parameters for describing these data. However, when the colonies are heavily calcified it is not possible to count the individual cells, and when large numbers of colonies are present it is simply not practical. In addition, the cell density varies greatly between colonies and when the colonies are induced to differentiate (e.g., by the addition of agents such as bFGF) they take on a three-dimensional nature. This means that assessing colony size in terms of two-dimensional paramters such as surface area or diameter would also be unreliable. Therefore, the only method available to us at present is to measure total cell numbers from which we can calculate the average colony cell number. This argument is also true for collagen, however, apart from measuring each individual colony for collagen synthesis, we have no choice but to measure total collagen from which we can calculate average collagen per colony. It would of course be advantageous to express the results in terms of collagen per cell. However, as we cannot calculate the size of the colonies that synthesise collagen, we cannot say whether the increase in collagen accumulation is due to a specific increase in collagen synthesis or simply to increased proliferation. As mentioned above, we are at present developing a method by which the stained colonies are analyzed densitometrically and each colony is identified and quantitated in terms of cell number and the bone- related markers used here. This should answer many of the questions raised above and also allow us to assess the colonies in terms of APase activity and calcification which is, at present, not possible.

The increase in Col-f numbers and Col-f cell numbers is largely in agreement with reports from other groups describing an increase in proliferation in bone cells after treatment with bFGF [19–21, 32, 34, 39, 40]. On the other hand, the effect of bFGF on osteogenic differentiation is more complex, with both stimulatory and inhibitory responses being reported. One reason for this may be differences in culture conditions. Investigations finding a bFGF-induced decrease in collagen synthesis by MC3T3 cells or fetal rat calvaria or APase activity in rat BMSC have all been performed in the absence of glucocorticoids [18–21, 34]. On the other hand, rat BMSC cultured in the presence of glucocorticoids, as in this study, respond to bFGF treatment with an increase in collagen accumulation, APase expression, osteocalcin synthesis, and calcification [39, 40]. Consistent with this, when BMC were cultured in the absence of dexamethasone, bFGF had no effect on colony formation and, although a small proportion of the colonies were APase positive, none were calcified or synthesized collagen (data not shown). We have previously argued that the presence of physiological levels of glucocorticoids is necessary for the actions of bone anabolic agents [42]. Consistent with this is the finding that PGE_2, known to be bone anabolic *in vivo*, causes an IGF-independant stimulation of thymidine incorporation and collagen synthesis in fetal rat calvaria or long bones *in vitro* only in the presence of physiological concentrations of glucocorticoids, [50–53]. The presence of physiological concentrations of glucocorticoids are also necessary for the PGE_2-induced recruitment of BMSC to the OB lineage [41]. Similarly, although it is known that IL-1 is bone anabolic under certaing circumstances *in vivo* (54–56), in isolation, both IL-1 and cortisol inhibit collagen synthesis in mouse calvariae whereas IL-1, in the presence of cortisol, stimulates collagen synthesis [57].

In conclusion, bFGF can increase the amount of bone-like tissue in calcifiying CFU-f cultures by increasing colony formation, differentiation, expansion, and collagen accumulation. Using this particular approach, it may be possible to investigate the mechanisms of action of other bone anabolic agents and to develop new ones.

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