Parathyroid hormone activates adhesion in bone marrow stromal precursor cells

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Abstract
The ability of parathyroid hormone (PTH) to enhance bone formation has recently been exploited in the treatment of osteoporosis. However, the underlying mechanisms are unknown. Osteoblasts, the bone-forming cells, derive from multipotential bone marrow stromal precursors called colony-forming units-fibroblastic (CFU-F) upon culture ex vivo. Adhesion of such stromal precursors to bone is likely to be an early event in the anabolic response of bone to PTH. To test this, we measured the number of CFU-F that could be extracted from murine bone marrow after administration of an anabolic dose of PTH. We found that a very early response is a dramatic reduction, starting within 2 h, in the number of CFU-F that could be extracted from murine bone marrow after administration of an anabolic dose of PTH. We found that a very early response is a dramatic reduction, starting within 2 h, in the number of CFU-F that could be extracted from murine bone marrow after administration of an anabolic dose of PTH. We then tested whether PTH has the ability to activate adhesion of CFU-F in vitro. For this, bone marrow cells were incubated in PTH for varying times. Non-adherent cells were then removed, and the adherent cells were incubated in PTH-free medium for 14 days to assess, as colony formation, the number of CFU-F that had adhered in the preceding period. We found that incubation in PTH caused a substantial increase in the number of CFU-F that adhered within 24 h. This increase was abrogated by peptidic inhibitors of integrins. The increase did not seem to be mediated through a PTH-induced increase in interleukin-6, since interleukin-6 had no effect on CFU-F numbers when substituted for PTH. Similarly, adhesion was unaffected by incubation of bone marrow cells in dibutyryl cyclic AMP, nor by inhibitors or donors of nitric oxide. However, activation of CFU-F in vitro by PTH was strongly inhibited by indomethacin and mimicked by prostaglandin E_2, and indomethacin reversed the PTH-mediated reduction of CFU-F that could be extracted from mouse bone marrow. These results suggested that PTH rapidly activates adhesion of CFU-F to plastic or bone surfaces. This activation may represent an early event in the anabolic response of bone cells to PTH.


Introduction
The adult human skeleton is continuously resorbed and renewed by the actions of osteoclasts and osteoblasts, cells that originate from precursors in the bone marrow. The maintenance of the skeleton requires the co-ordinated activity of these cells; loss of this co-ordination underlies many systemic and localized bone diseases, including osteoporosis (Jilka et al. 1992, 1996, Manolagas & Jilka 1995, Weinstein et al. 1998).

Parathyroid hormone (PTH) plays a major role in the balance between bone formation and bone resorption. Although the hormone is widely known as a stimulator of bone resorption, it is now well established that it also has strong anabolic effects on bone. Whether PTH induces predominantly resorption or formation depends upon the mode of administration: continuous exposure of the skeleton to high levels of PTH causes bone loss (Hock et al. 1989, Hock & Gera 1992, Ma et al. 2001), while intermittent (e.g. one to three times daily) exposure increases bone formation (Tam et al. 1982, Hock et al. 1989, Riond 1993). The mechanism through which the hormone induces bone loss has recently been clarified: PTH acts on osteoblastic cells to induce expression of the osteoclast-inductive ligand Receptor-activator of NFκB Ligand (RANKL) and suppress expression of osteoprotegerin, the soluble decoy receptor for RANKL (see Chambers 2000). However, despite the large body of evidence that intermittent administration of PTH is strongly anabolic and increases bone mass in humans (Reeve 1996, Neer et al. 2001), little is known of the mechanism by which it stimulates bone formation.

It is believed that the osteoblasts derive from the proliferation and osteoblastic differentiation of multipotential bone marrow stromal precursor cells. These clonogenic cells, also called colony-forming units–fibroblastic (CFU-F) and more recently mesenchymal stem cells (Pittenger et al. 1999), can give rise under appropriate experimental conditions to a broad spectrum of fully differentiated connective tissues, including cartilage, bone,
adipose tissue, fibrous tissue and myelosupportive stroma (Friedenstein et al. 1974, Owen 1988, Bianco & Robey 2000). Bone marrow stromal precursors are quiescent under physiological conditions, but are 'spontaneously' and rapidly activated to adhere and proliferate under the conditions typically used for cell culture, to form colonies

Femora and tibiae were removed from 8- to 10-week-old (Poole, Dorset, UK) unless otherwise stated. Reagents and hormones were purchased from Sigma Materials and Methods

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in vitro (Castro-Malaspina et al. 1980, Piersons et al. 1985, Scott & Bertram 1995). The nature of the stimulus that activates these quiescent cells is unknown, but cells commonly respond to culture conditions with activation: it has been suggested that incubation of cells in vitro activates behaviour similar to that seen in wound healing (Friedman et al. 1994, Thyberg 1996, Iyer et al. 1999). Thus, incubation of bone marrow cells in vitro activates, and thereby reveals the presence of, the uncommitted precursors responsible for tissue replacement.

Two of the earliest events reported after PTH administration are c-fos expression in cells on and adjacent to bone surfaces (Lee et al. 1994), and a (non-proliferative) increase in the number of cells on bone surfaces (Dobnik & Turner 1995). This suggests that an early component of the anabolic action of PTH might be the adhesion to bone of cells from the bone marrow. A similar rapid increase in the number of cells on bone surfaces, which cannot be attributed to proliferation, occurs after induction of bone formation by mechanical stimuli (Chow et al. 1998b). To test whether this adhesion to bone is associated with loss of CFU-F in the bone marrow, we measured the number of CFU-F that could be extracted from murine bone marrow shortly after administration of an anabolic dose of PTH. We found that the number of CFU-F that could be extracted was substantially reduced, starting within 2 h of injection of PTH. We also found that PTH stimulated adhesion in vitro of CFU-F from untreated mice. These observations are consistent with a model in which an early component of the anabolic response of bone to PTH is the activation of adhesion in osteoblastic precursors.

Materials and Methods

Reagents and hormones were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated.

Cell culture

Femora and tibiae were removed from 8- to 10-week-old MF1 male mice and were aseptically dissected free of soft tissue. Epiphyses were removed and the diaphysis flushed with 15 ml α-modified minimum essential medium (αMEM), using a 20 ml syringe fitted with a 21 gauge needle. Bone marrow cells isolated from each animal were kept separately. Cells were disaggregated by repeatedly pipetting the cell suspension using a 20 ml syringe fitted with a 21 gauge needle. Nucleated cells removed were counted using a haemocytometer. The cells isolated from each animal were cultured separately with αMEM supplemented with 10% bovine calf serum (Autogen Bioclear, Calne, Wilts, UK), penicillin (100 IU/ml), streptomycin (100 µg/ml) and l-glutamine (2 mM). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide.

Effect of PTH in vivo on CFU-F formation in vitro

The animals used were 8- to 10-week-old MF1 male mice obtained from breeding colonies at St George’s Hospital Medical School, London. Each mouse was given one or more doses of either vehicle alone (1 mM HCl (BDH, Poole, Dorset, UK), 0·15 M NaCl (BDH) and 2% heat inactivated mouse serum) or PTH dissolved in vehicle (50 µg/kg body weight) by i.p. injection. In some experiments, indomethacin (7·5 mg/kg) or its vehicle was also injected. Mice were killed at intervals by cervical dislocation, and bone marrow cells were harvested as described above.

To examine the activation of adhesion of CFU-F isolated from PTH- and vehicle-treated mice, 1·5 × 10⁶ nucleated bone marrow cells/ml were inoculated into the wells (2 ml per well) of six-well plates (Greiner, Stonehouse, Glos, UK), and incubated for 14 days without feeding. On day 14 the cells were fixed with 10% neutral buffered formalin in PBS for 10 min. Total numbers of CFU-F were determined by staining with 0·1% toluidine blue (BDH) in PBS. Colonies large enough to be seen with the naked eye were defined as CFU-F and counted. Counts were performed ‘blind’ on coded plates. In some experiments, cells were also stained for alkaline phosphatase as described below.

Effect of PTH in vitro on CFU-F formation

Bone marrow was harvested from untreated mice and 1·5 × 10⁶ nucleated bone marrow cells/ml were plated as described above in six-well plates, with/without PTH (100 ng/ml). After 2 h of incubation at 37 °C, the cultures were gently agitated and non-adherent cells transferred to the adjacent well. After transfer, the remaining adherent cells were washed and 2 ml fresh αMEM without PTH was added. Plates containing non-adherent cells were again incubated to allow further cells to become adherent, before agitating the cultures and transfer of non-adherent cells to new wells. Thus, a series of pour-off wells was created, in which cells that had become adherent at various intervals were incubated for 14 days, without feeding, to enable the development of CFU-F-derived colonies.

To test the effect of potential agonists and antagonists of the effects of PTH on the adhesion of CFU-F, bone marrow cells were incubated, without PTH, for 2 h. Cultures were agitated. Non-adherent cells were harvested, adherent cells were discarded, and non-adherent
cells were incubated for 22 h in fresh wells containing PTH (100 ng/ml) with/without indomethacin (10⁻⁶ M), S-nitroso-N-acetylpenicillamine (SNAP; 0·3 mM), NG-monomethyl-£-arginine (L-NMMA; 1 mM), dibutyryl cyclic AMP (10⁻³ M), interleukin-6 (10 ng/ml), prostaglandin (PG) E₂ (10⁻⁶ M), Arg-Gly-Asp (RGD) or Arg-Gly-Gln (RGE) (both 10⁻⁴ M). Cultures were then agitated, non-adherent cells discarded, and medium replaced with αMEM. Incubation was continued to 14 days and colony numbers were counted.

**Staining for alkaline phosphatase activity**

For enzyme histochemistry of alkaline phosphatase, cells were fixed with 10% neutral buffered formalin in PBS for 10 min. Alkaline phosphatase activity was detected by incubating the cells for 30 min at 37 °C with veronal HCl buffer, pH 9·2, containing 10 mg/ml naphthol AS-B1 as substrate, 5% dimethylformamide and 0·5 mg/ml of fast red TR.

**Statistical analysis**

Values are expressed as means ± S.E.M. Statistical significance between PTH- and vehicle-treated groups were analysed with Student’s t-test; P<0·05 was considered to be significant.

**Results**

We tested the notion that an early component of the anabolic response to PTH is a reduction in number, through adhesion to bone surfaces, of bone marrow

**Figure 1** Effect of PTH in vivo on the number of CFU-F extractable from bone marrow. Mice were injected with a single dose of PTH (50 µg/kg) or vehicle, and bone marrow was taken for CFU-F assay at the stated time after injection. Histograms show the number of colonies formed per culture as percentage (± S.E.M.) of vehicle-injected mice (number formed per 10⁶ bone marrow cells from vehicle-injected animals (means ± S.E.M.): 2 h, 90·8 ± 4·6; 3 h, 95·7 ± 4·0; 6 h, 64·2 ± 6·2; 24 h, 78·6 ± 7·4). n=12 cultures per time-point. *P<0·05, reduction in CFU-F numbers was significant vs control.

**Figure 2** Effect of PTH in vivo on the number of CFU-F extractable from bone marrow. Mice were injected one to four times with PTH (50 µg/kg) or vehicle at 3-hourly intervals, and bone marrow was taken for CFU-F assay 24 h after the first injection. Histograms show the number of colonies formed per culture as percentage (± S.E.M.) of vehicle-injected controls (number of colonies formed per 10⁶ bone marrow cells from vehicle-injected animals (means ± S.E.M.): PTH × 1, 66·7 ± 4·2; PTH × 2, 46·2 ± 2·3; PTH × 3, 35·2 ± 3·1; PTH × 4, 35·9 ± 3·3). n=12 cultures per variable. *P<0·01 vs appropriate control groups.
stromal precursor cells. To do this, we measured the number of cells capable of forming fibroblastic colonies in vitro (CFU-F) in bone marrow from mice 24 h after a single intraperitoneal injection of 50 µg/kg bovine 1–34 PTH. This dose is at the lower end of the range of doses that induce bone formation without perturbation of calcium homeostasis. We found strong suppression (Fig. 1) by PTH of the number of colonies that formed during the 14 days of incubation. PTH administration significantly reduced the number of CFU-F that could be detected if mice were killed within 2 h of a single dose. The number of CFU-F detectable in bone marrow continued to decrease for at least 6 h, but there was a partial recovery by 24 h after the injection. The number of CFU-F detectable was suppressed to a comparable degree if measured 24 h after the first injection of multiple doses of PTH (Fig. 2).

It has been reported that continuous prolonged incubation of bone marrow cells from untreated mice in PTH does not change the number of CFU-F that develop (Isogai et al. 1996). We confirmed that when bone marrow cells from untreated mice were incubated in PTH for 7–14 days, there was no effect on the number of CFU-F (data not shown). This suggests that PTH has actions on CFU-F in vivo that are not detected by the in vitro assay. The rapid increase in cell numbers on bone surfaces after anabolic stimuli (Dobni & Turner 1995, Chow et al. 1998b) suggests that activation of bone formation by PTH is likely to involve the adhesion of cells from the marrow space to bone surfaces. If PTH activates the adhesion of CFU-F, this activation might not have been detected in the above experiments because CFU-F become activated ‘spontaneously’ during the 14-day incubation period (Castro-Malaspina et al. 1980, Piersma et al. 1985, Scutt & Bertram 1995). Under these circumstances, activation of CFU-F by PTH might nevertheless become detectable if the baseline of ‘spontaneously’ activated CFU-F is removed. Therefore, to determine whether PTH activates adhesion, we removed ‘spontaneously’ activated CFU-F by repeatedly transferring non-adherent bone marrow cells to fresh wells after short periods of incubation. The cells that adhered during each period were detected as colony formation after 14 days of incubation in the absence, except in the final suspension of non-adherent cells, of PTH. We found (Fig. 3) that while many of the progenitors of CFU-F adhered relatively quickly, many adhered only after more prolonged incubation. This pattern of development of adhesiveness in the progenitors of CFU-F has been previously noted (Scutt & Bertram 1995). PTH increased the

![Figure 3](image-url)
number of CFU-F that adhered between 8 and 24 h. It appears that this ability of PTH to stimulate adhesion of CFU-F is obscured in continuous cultures by the large numbers of CFU-F that adhere ‘spontaneously’ due to the extraction procedure or the culture environment, within the first 2 h of incubation. PTH appeared to induce the adhesion of additional CFU-F that had not been so activated.

We tested the effect of several agents that are candidates for being mediators of the actions of PTH on bone cells. For these experiments, we facilitated detection of the ability of PTH to activate adhesion by discarding bone marrow cells that adhered ‘spontaneously’ in the first 2 h (Fig. 4). Thus, bone marrow cells were incubated in agent or vehicle for 2 h. Non-adherent cells were then transferred to fresh culture wells and incubated for a further 22 h. Cells that remained non-adherent were discarded, and incubation of adherent cells was continued for 14 days in agent-free culture medium. We found no effect by interleukin-6 (10 ng/ml; a cytokine induced in osteoblasts by PTH), dibutyryl cyclic AMP (10^{-2} M; a second messenger for PTH action), SNAP (0.3 mM; a nitric oxide (NO) donor) or L-NMMA (1 mM; an inhibitor of NO synthase) on the adhesion of CFU-F (data not shown). However, indomethacin (10^{-6} M) significantly suppressed both ‘spontaneous’ and PTH-induced adhesion of CFU-F (Fig. 5). Moreover, we found that PGE_{2} (10^{-5} M) had a similar effect on the adhesion of CFU-F (Fig. 6). This action was not additive with that of PTH.

In view of this, we tested the ability of indomethacin to neutralize the ability of PTH to reduce the number of CFU-F that could be extracted from murine bone marrow. To do this, mice were injected three times at 3-hourly intervals with PTH and/or indomethacin or vehicle. Bone marrow was extracted 24 h after the first injection and assessed for the number of CFU-F. We found (Fig. 7) that PTH caused a significant reduction in the number of CFU-F. This was prevented by indomethacin. Additionally, indomethacin caused a significant increase in the number of CFU-F that developed in control cultures. The increased adhesion induced by PTH appeared to be mediated by integrins. Thus, the PTH-induced increase in the adhesion of CFU-F was abrogated by RGD, but not by the inactive analogue RGE (Fig. 8).

It has been reported that PTH can increase the proportion of colonies that develop osteoblastic characteristics. However, we found that the proportion of colonies that contained alkaline phosphatase-positive cells was unaffected by incubation in PTH for the short period required for activation of adhesiveness (data not shown). Moreover, the proportion of CFU-F that contained
alkaline phosphatase-positive cells did not differ significantly in bone marrow extracted from PTH-treated vs control mice (data not shown).

Discussion

It is well established that intermittent administration of PTH leads to increased bone formation in vivo, although the mechanisms through which this occurs and the target cells have not been identified. Bone marrow contains uncommitted mesenchymal stem cells that are capable, when appropriately stimulated, of differentiation into osteoblasts, the cells that form bone (Friedenstein et al. 1968, Owen 1988, Pittenger et al. 1999, Bianco & Robey 2000). Osteoblasts themselves are incapable of proliferation, and it is considered that increased bone formation occurs through activation of precursors, that exist adjacent to bone surfaces, to proliferate and differentiate into osteoblasts. Under basal conditions these putative precursors, CFU-F, are in a quiescent, non-cycling state in vivo, but become 'spontaneously' activated for adhesion and proliferation upon incubation in vitro (Castro-Malaspina et al. 1980, Piersma et al. 1985, Scutt & Bertram 1995). The data presented in this report indicate that a very early response to the administration of PTH to mice is a dramatic reduction in the number of CFU-F, the uncommitted precursors of osteoblasts, that can be extracted from bone marrow. Furthermore, when bone marrow from untreated mice was incubated in PTH, we noted a marked increase in the number of CFU-F that became adherent within 24 h in vitro. This suggests that activation of adhesion in CFU-F is one of the earliest responses of bone cells to PTH.
If bone marrow stromal cells respond to PTH with activation of adhesion in vivo as they do in vitro, then our results could be explained through increased adhesion of bone marrow cells to bone or other surfaces or cells, which makes them unavailable for extraction with the bone marrow. This might be analogous, for example, to the depletion of blood cells caused by activation of adhesion (Furukawa et al. 1993, Busyman et al. 1996). Activation of adhesion is a common prelude to the homing and migration of multiple cell types. Unfortunately, there are no comparable markers whereby the fate of stromal bone marrow precursors can be monitored, although our finding that adhesion was inhibited by the integrin antagonist RGD suggests that increased adhesion of CFU-F to plastic by PTH is mediated by integrins. Such a mechanism is consistent with the observation that multiple integrins are expressed by stromal precursor cells, and mediate the adhesion of these cells to substrates (Gronthos et al. 2001). Whatever the mechanism, the adhesion might represent a component of the rapid (non-proliferative) increase in the number of cells noted on bone surfaces after anabolic stimuli (Dobning & Turner 1995, Chow et al. 1998).

Previous analyses of the number of CFU-F that can be obtained from bone marrow after PTH administration have been contradictory. Numbers of CFU-F have been reported either not to change (Jilka et al. 1999, Sakai et al. 1999) or to increase (Nishida et al. 1994). However, these assessments were all performed after prolonged PTH administration, after which time any precursors lost to bone surfaces are likely to have been regenerated and new steady-states are likely to have become established.

We found that activation of adhesion of CFU-F in both control and PTH-containing medium was significantly inhibited by indomethacin. Moreover, PGE2 induced a similar activation of adhesion of CFU-F. The combination

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**Figure 7** Effect of indomethacin on the ability of PTH in vivo to reduce the number of CFU-F extractable from bone marrow. Mice were given three intraperitoneal injections of vehicle or PTH (50 µg/kg) and/or indomethacin (indo; 7.5 mg/kg). Bone marrow cells were harvested 24 h after the first injection, and incubated in 3 x 2 plates at 1.5 x 10^6 cells/ml. After 2 h, non-adherent cells were harvested, and incubated in fresh wells for a further 14 days. Cultures were fixed and stained, and the number of CFU-F formed by this non-adherent cell population was counted. *P<0.01 vs control and PTH plus indomethacin. n=6 cultures per variable.

**Figure 8** The effect of RGD/RGE peptides on adhesion of CFU-F to plastic. Bone marrow cells were incubated at 1.5 x 10^6 cells/ml in 3 x 2 plates. After 2 h, adherent cells were discarded and non-adherent cells were removed and placed in fresh wells and incubated for 22 h in vehicle, RGD or RGE (both 10^{-7}M), and/or PTH (100 ng/ml). Non-adherent cells were then removed and adherent cells were incubated in medium for a further 13 days, before counting CFU-F. n=6 cultures per variable. *P<0.05, significantly different from control cultures.
of PGE₂ and PTH did not appear to be additive. This supports a role for PGs in the action of PTH. PGs have previously been shown to activate adhesion in CFU-F in vitro (Scutt & Bertram 1995). Thus, our results are consistent with a model in which PTH activates adhesion through induction of PG synthesis in bone marrow cells. It is of interest that PG shares the ability of PTH to stimulate bone formation in vivo and, like PTH, has been found to be essential for the anabolic response of bone to mechanical stimulation (Chow & Chambers 1994, Fox et al. 1996).

Incubation of bone marrow cells in PTH has been reported to increase the proportion of CFU-F that develops osteoblastic characteristics (Isogai et al. 1996, Miao et al. 2001). We found that the proportion of colonies that developed alkaline phosphatase positivity, a classic marker for osteoblastic differentiation in these colonies, was unaffected by PTH. Presumably, in our cultures, exposure to PTH was too brief to influence commitment. Thus, activation and lineage commitment in bone marrow stromal precursors are distinct processes. This suggests a model in which CFU-F are activated by PTH but their subsequent behaviour can be modified by other environmental cues. PTH might have a more general ability to activate responsiveness without commitment in bone cells. Consistent with this, we recently found that the osteogenic response of rat bone to mechanical stimulation does not occur in thyroparathyroidectomized rats, but is restored if a single dose of PTH is administered at the time of mechanical stimulation. Thus, PTH appears to facilitate the response of bone cells to anabolic stimuli (Chow et al. 1998a). This raises the possibility that one explanation for the ability of PTH to induce both resorptive and anabolic responses in vivo might be through activation of the responsiveness of bone cells to stimuli such as those for bone resorption and bone formation.

It is likely that activation of CFU-F is not the only mechanism by which PTH stimulates bone formation. PTH has also been shown to stimulate the replication of osteoblastic progenitors (Macdonald et al. 1986, Nishida et al. 1994, Onishi et al. 1997). The activation of precursors that we have described might facilitate this by enhancing bone cell responsiveness to mitogenic agents that are present in culture medium. A second mechanism by which PTH has been reported to act is through direct stimulation of matrix synthesis in bone lining cells, reported by some (Dobnić & Turner 1995, Leaffer et al. 1995) but not all (Jilka et al. 1999) authors, and PTH has been reported to prevent osteoblast apoptosis (Jilka et al. 1999). Matrix synthesis and suppression of apoptosis are characteristic responses to anabolic stimuli; PTH might similarly enhance the responsiveness of bone cells to underlying anabolic stimuli. However, increased matrix synthesis and suppression of apoptosis do not readily explain the rapid increase in numbers of cells on bone surfaces that is likely to be a major component of the anabolic response observed after PTH treatment. Our results suggest that a crucial component of the anabolic response of bone to PTH is the activation of previously quiescent osteoblastic precursors. If so, the assays we have described might identify other agents capable of inducing bone formation.

Our results also raise the intriguing possibility that there might be analogous factors for other tissues and they might reflect a general mechanism by which quiescent tissue stem cells are activated for tissue renewal. The assay we have used provides an approach to detect other stem cell activation factors, in bone and in other tissues.

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