Parathyroid Hormone (1–34) Regulates Integrin Expression in Vivo in Rat Osteoblasts

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Abstract   Intermittent administration of parathyroid hormone (PTH) activates new sites of bone formation by stimulating osteoblast differentiation and function resulting in an increase in bone mass. Because integrins have been shown to play a crucial role in osteoblast differentiation and bone formation, in the present study, we evaluated whether human PTH (1–34) upon administration to rats, influenced integrin expression in osteoblastic cells isolated from the metaphysis and the diaphysis of rat long bones. Initial immunohistochemical evaluation of bone sections demonstrated that the osteoblasts expressed at least α_v, α_2, α_3, and $\alpha_5\beta_1$ integrins. Immunocolocalization studies for integrins and vinculin established that α_v, α_2, and $\alpha_5\beta_1$, but not α_3 integrins were present in the focal adhesion sites of osteoblasts attached to FN coated surfaces. Osteoprogenitor cells isolated from metaphyseal (but not diaphyseal) marrow of rats injected with intermittent PTH (1–34) exhibited greater α_v and reduced α_2 levels, with no apparent changes in α_3, and $\alpha_5\beta_1$ integrin levels, as assessed by immunohistochemistry, Northern, and Western blot analyses. However, these changes were not observed on the same cells treated with PTH in vitro. These observations suggest that integrin modulation by PTH is likely to be indirect and that selective phenotypic expression of integrin subtypes is part of te cascade of events that lead to PTH (1–34) mediated osteoblast differentiation. J. Cell. Biochem. 83: 617–630, 2001. © 2001 Wiley-Liss, Inc.

Key words: parathyroid hormone; integrins; osteoblasts and osteoprogenitor cells; osteoporosis

Intermittent subcutaneous administration of PTH increases bone formation with improved strength at axial and appendicular sites in rats, dogs, and humans [Reeve et al., 1980; Tam et al., 1982; Podbesek et al., 1983; Gunness-Hey and Hock, 1984; Tada et al., 1990; Dempster et al., 1993; Sato et al., 1997]. While the exact mechanism(s) of the enhanced bone formation is not precisely understood, PTH initiates a complex cascade of events that results in an increase in the number of functioning osteoblasts that lay down bone extracellular matrix [Dobnig et al., 1995; Onyia et al., 1995, 1997; Schmidt et al., 1995]. Several RGD containing proteins such as type I collagen, fibronectin (FN), osteopontin, vitronectin, bone sialoprotein, and thrombospondin are present in the extracellular matrix of bone, and interact with a subset of integrins present on the osteoblast cell surface [Lawler and Hynes, 1986; Oldberg et al., 1986; Dedhar et al., 1987; Oldberg et al., 1988; Gehron-Robey et al., 1992; Stein and Lian, 1993; Termine and Robey Gehron, 1996]. Integrins are heterodimeric adhesion receptors that anchor cells to extracellular matrix molecules and play key roles in a variety of cell functions such as cell migration and signal transduction [Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1991; Hynes, 1992; Clark and Brugge, 1995; Meredith et al., 1996]. In addition, integrins exhibit temporal and spatial variations during embryonic differentiation, and targeted disruption of various integrins results in abnormal

development [DeSimone, 1994; Gumbiner and Yamada, 1995; Hirsch et al., 1996; Hynes, 1996; Sastry and Horowitz, 1996; Beauvais-Jouneau and Thiery, 1997].

Previous studies have established the presence of α_v, α_1, α_2, α_3, α_4, and $\alpha_5\beta_1$ integrins on osteoblasts [Castoldi et al., 1997; Ganta et al., 1997; Hughes et al., 1993; Hultenby et al., 1993; Moursi et al., 1997]. Further, functional antibodies to select integrins reduced the formation of mineralized nodules in rat osteoblast cultures [Moursi et al., 1997]. Since PTH increases osteoprogenitor differentiation in vivo, we evaluated whether treatment of rats with anabolic doses of PTH (intermittent administration) resulted in altered integrin expression in osteoprogenitor cells. Immunohistochemical analysis of rat femurs demonstrated the presence of α_v, α_2, α_3 subunits, and $\alpha_5\beta_1$ integrin in the metaphyseal and diaphyseal marrow cells. Immuno-colocalization studies of integrins with vinculin indicated that of the various integrins expressed, only α_v, α_2 subunits, and $\alpha_5\beta_1$ integrins were present in focal adhesion sites of isolated osteoblasts in culture. Northern and Western blot analyses of osteoblasts isolated from PTH (1−34) treated rats revealed that α_v levels were increased and α_2 levels were decreased in the metaphyseal derived osteoblasts, but were unaffected in diaphyseal marrow derived osteoblasts. However, when isolated osteoblasts were treated with PTH (1−34) in vitro, α_v integrin subunit level remained unchanged. These results suggest that osteoblast differentiation by PTH is associated with selective expression and modulation of integrin subunits and that these indirect changes are selectively seen in osteoblasts derived from metaphyseal region of the bone.

MATERIALS AND METHODS

Animals

Most of the studies were done using 4−6 week old (75−100 gm), Sprague−Dawley male rats (Harlan, Indianapolis, IN). The rats were fed ad libitum with Purina chow [calcium 1%, phosphate 0.61%, PMI Feeds, Inc. St Louis, MO] and water. Rats were given a daily dose (80 µg/kg in saline containing 2% heat inactivated rat serum) of hPTH (1−34) (Bachem, Torrance, CA) by subcutaneous injection for 5 days. At the end of the injection, rats were sacrificed and the bones were used for isolation of cells described

in various studies or processed for sectioning followed by immunohistochemical staining.

In one study (Tables II and III, Fig. 8), six-month old (270 gm), virgin Sprague−Dawley female rats were used. The rats were maintained on a 12-h light/dark cycle at 22°C with ad lib access to food (TD 89222 with 0.5% Ca and 0.4% P, Teklad, Madison, WI) and water. Bilateral ovariectomy was performed except where indicated and the rats were dosed daily with biosynthetic hPTH (1−34) (LY333334, Lilly, Indianpolis, IN) for 12 weeks starting one month after ovariectomy. The groups included (1) sham-operated control (Sham), (2) ovariectomized (ovx) control, and (3) ovx rats treated with hPTH (1−34). For PTH treatment, rats were given hPTH (1−34) (in physiological saline, 2% heat inactivated rat serum) by subcutaneous injection (0.3, 3, and 30 µg/kg). The animal protocols were approved by the Lilly Animal Care and Use Committee.

Quantitative Computed Tomography (QCT)

The effects of PTH on bone mineral content and density were evaluated by QCT in ovx rats. After the final dose of treatment, rats were euthanized by CO_2 inhalation, the left tibiae were removed, cleaned, and preserved in polystyrene vials filled with 50% ethanol/saline (V/V) at 4°C. The proximal metaphysis of excised tibiae were scanned at room temperature by pQCT using voxel dimensions of $0.148 \times 0.148 \times 1.2$ mm and analyzed using Dichte software (Norland/Stratec, Ft. Atkinson, WI) as described [Sato, 1995].

Immunohistochemical Evaluation of Bone Sections

Femurs from young (75−100 gm) male rats were fixed in 4% paraformaldehyde at 4°C (twice for 24 h each), washed with phosphate-buffered saline (PBS) (twice for 4 h each), followed by further incubations in 70% ethanol at 4°C (twice for 24 h each). The femurs were decalcified at 4°C for 1 month in 10% ethylene diamine tetracetic acid (EDTA), pH 7.5, replacing the EDTA solution every 48 h. The decalcified femurs were dehydrated overnight in a Miles Tissues Tek Vip Processor (Miles Scientific) and embedded in Paraplast. Longitudinal sections (7 µm) were cut on a Leitz 1512 microtome and transferred to precleaned slides (Fisherbrand Superfrost/Plus, Fisher Scientific, Pittsburgh, PA), deparaffinzed in xylene

(2 times 5 min each) and rehydrated by treating twice for 2 min in sequence, in the following reagents: 100% ethanol, 90% ethanol, 70% ethanol and distilled water. The endogenous peroxidase activity was inactivated by treating with 3% H_2O_2 in methanol for 20 min at 25°C. The sections were treated with normal goat sera to block non-specific binding, then incubated with 1:40 dilutions (in normal goat sera) of polyclonal anti-rat α_2, α_3, α_v, or anti-human $\alpha_5\beta_1$ antibodies (Chemicon, Temecula, CA), followed by detection with an avidin–biotin-peroxidase detection system (ABC Elite, Vectastain, Vector, Burlingham, CA). Controls included sections treated with either primary antibody alone or secondary antibody alone. The sections were incubated with diaminobenzidine solution [0.6 mg/ml of 0.05 M Tris/HCl pH 7.4, 0.3% H_2O_2 (Sigma, St. Louis, MO)] for 5–10 min. The sections were rinsed in water, counterstained with Hematoxyllin (Tissue Path, Biochemical Science Inc., Bridgeport, NJ), and fixed in 0.06% ammonium hydroxide (in water), followed by two treatments (5 min each) of 70% ethanol, 90% ethanol, 100% ethanol and xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Photomicrographs were taken with a Zeiss microscope.

Cells and Cell Culture

For most of the study described here, primary metaphyseal and diaphyseal derived osteoblast cultures established from the femurs and the humeri of rats were used. In one study, a rat osteosarcoma cell line, ROS17/2.8, was used. Metaphyseal-derived and diaphyseal marrow cells were isolated as before [Onyia et al., 1997]. In brief, the bones from various treatments were resected, the connective tissue was removed and the bones were washed twice with α-minimal essential media (α-MEM) + 10% fetal bovine serum (FBS) + 5% penicillin (Pen) and streptomycin (Strep)), followed by extensive rinsing with Hank's Balanced Salt Solution (Mg^{2+}/Ca^{2+}-free). To isolate metaphyseal osteoblasts, the distal epiphysis was removed, about 3 mm of the metaphyses subjacent to the growth plate were cut off, minced, and digested for 1 h at 37°C with of 0.25% trypsin in Mg^{2+}/Ca^{2+}-free Hank's Balanced Salt Solution (0. 6 ml/each femur and humerus). After neutralization with 1 mg/ml of soybean trypsin inhibitor (Sigma, St. Louis, MO), cells were filtered through a 70 μM cell strainer (Becton-Dickinson, Lincoln Park,

NJ) centrifuged, and plated in α-MEM + 10% FBS + 1% Pen/Strep. After 1 week in culture, these cells displayed osteoblast features (type I collagen, osteocalcin, alkaline phosphatase expresion), exhibited cAMP response to PTH 1−34 challenge, and demonstrated spontaneous mineralization in vitro [Dobnig et al., 1995; Onyia et al., 1997]. For the isolation of the diaphyseal osteoblasts, both ends of the femur were cut off and the bone marrow was flushed out with PBS (Mg^{2+}/Ca^{2+}-free), using a syringe. The cells were centrifuged and plated in α-MEM + 10% FBS + 1% Pen/Strep. The non-adherent population was removed by aspiration after 24-48 h and the cultures were fed with α-MEM + 10% FBS + 1% Pen/Strep. The media were changed every second day thereafter. After 1 week in culture, these cells demonstrated osteogenic potential (alkaline phosphatase, type I collagen, and osteocalcin, type I PTH receptor, but no cAMP response to PTH). Only the first passage cells were used for all studies described.

ROS 17/2.8 cells were cultured and maintained in DMEM/F-12 + 10% FBS (v/v) and were used near confluency. These cells exhibit osteogenic potential in vitro (osteocalcin and alkaline phosphatse expression) and in vivo (ability to form mineralized nodule in rats) (Onyia et al., 1999).

Immunohistochemical Evaluation of Integrins in Isolated Cells

Cells grown to near confluency were serum-deprived by treating them for 24 h with α-MEM + 1% FBS + 1% Pen/Strep, were washed twice with PBS (Mg^{2+}/Ca^{2+}-free) and released by EDTA treatment (Versene 1:5,000, GibcoBRL, Grand Island, NY). Cells were kept in suspension for 1 h at 37°C in α-MEM containing 2% BSA, and were then plated onto Chamber Slides (4101 Falcon, NJ) previously coated with FN (10 μg/ml in PBS, at 4°C for 16 h). After 1 h, the media were removed, the attached cells (more than 90% of seeded cells) were fixed with 2.5% paraformaldehyde/PBS solution at 4°C for 30 min, permeabilized with digitonin (0.0025%, w/v) at room temperature for 10 min, and incubated with 2% nonfat dry milk (in PBS) for 20 min at room temperature [Foerster et al., 1993]. The fixed cells were treated first with 1:20 dilution of anti-vinculin monoclonal antibody (Clone AL-125, ICN, Costa Mesa, CA), then with 1:200 dilution of goat anti-mouse IgG

conjugated to rhodamine (Southern Biotechnology Associates, Birmingham, AL). The same cells were further subjected to integrin staining by incubation with polyclonal anti-integrin antibodies, (1:40 dilution) followed by a fluorescein isothiocynate conjugated goat anti-rabbit IgG (1:200, TAGO, Burlingame, CA). Finally, the cells were mounted with glycerol/water and an anti-fading reagent (Slow-Fade, Molecular Probes, Eugene, OR), observed under a Zeiss microscope equipped for epifluorescence and photographed. All antibody dilutions were done in 1% nonfat dry milk in PBS.

Integrin mRNA Levels by Northern Blot Analysis

The integrin mRNA levels were evaluated under three conditions: (a) freshly isolated metaphyseal derived and diaphyseal marrow cells cells, (b) culturing the same cells for 1 week, then replating for 1 h into 175 cm^2 tissue culture flask, coated with FN (100 µg/plate), and (c) ROS 17/2. Six cells cultured for 1 week. Rats (75–100 gm) were injected with PTH 1−34 (80 µg/kg) once daily for three days, and the metaphyseals and diaphyseal marrow derived cells from 8−12 femurs were either plated into tissue culture dishes or utilized directly for isolation of mRNA. The final cell density before RNA isolation varied from 9,000 cells/cm^2 (metaphyseal) to 21,000 cells/cm^2 (diaphyseal marrow-derived). For experiments involving FN coated dishes, confluent cells were released by EDTA and then added on to a 175 cm^2 flask coated with 100 µg of FN. Cells (freshly isolated or confluent cultures scraped off the flasks) were placed in ice-cold PBS, and centrifuged. The pellet was lysed in 6 ml of buffer (4.0 M guanidinium HCl, 50 mM Tris pH 7.0, 10 mM EDTA, 0.5% sarcosyl, 0.5% β-mercaptoethanol), and the DNA was sheared with an 18 G needle. The solution was then applied onto 4 ml of a solution containing 5.7 M cesium chloride + 100 mM EDTA, pH 7.0, and centrifuged in a SW-41 rotor at 32 K rpm, 20°C for 21 h. The supernatant fraction was removed, the RNA pellet was suspended in 20 mM Tris, pH 7.6, containing 100 mM NaCl, 0.1% SDS, 1 mM EDTA, precipitated overnight with 100% ethanol (2.5 times the volume) at −20°C, washed once with 70% ethanol (−20°C), and dissolved in DEPC water. The purity and concentration were established by determining the absorbance at 260 and 280 nm. RNA was solubilized in a buffer containing 50% formamide, 2.2 M formaldehyde, MOPS buffer, (20 mM MOPS + 5 mM sodium acetate + 1.0 mM EDTA), mixed with ethidium bromide (10 ng/µl) and blue marker (10% ficoll 400, 0.2% bromophenol blue), incubated for 10 min at 65°C, and was immediately cooled by placing it on ice. RNA samples from various treatments were resolved by electrophoresis on 1.2% denaturing agarose gels (in 2.2 M formaldehyde, MOPS buffer). After electrophoresis (MOPS buffer, 130 V for 3 h), RNA samples were transferred to a nylon membrane (Ambion, Austin TX) in 10×SSC buffer (1.5 M NaCl, 0.15 M Na Citrate) via Turboblotter (Schleicher & Schuell, Keene, NH), prehybridized for 30 min, and then hybridized in NorthernMax prehyb/hyb buffer (Ambion, Austin TX) at 42°C overnight. The cDNA probes were cloned by PCR from liver cDNA (rat liver quick-clone cDNA, Clonotech, Palo Alto, CA), based on published sequences (Table I). The integrin and GAPDH probes were labeled by PCR using 15 µl of [α-^{32}P] dCTP, (3,000 mCi/mmol, Amersham, England), 4 µM each of dGTP, Dttp, and dATP, 10 ng of integrin fragment, and 0.5 µM oligonucleotides. The filters were washed with a buffer containing 0.1×SSC buffer + 0.1% SDS and exposed to X-ray film (Reflection, NEN, Boston, MA) at room temperature. The relative band intensity was determined by densitometric scanning of the autoradiographs using a Fluor-S Multiiimager and Quantity One image acquiisition and analysis software (Bio-Rad, Hercules, CA).

Western Blot Analysis of Integrins

Cells were scraped in ice-cold PBS and pelleted by centrifugation. The cell pellet was lysed in RIPA buffer (50 mM Tris, pH 8.0, +150 mM NaCl, +1% NP-40, +0.5% sodium deoxycholate, +0.1% SDS containing a cocktail of proteinase inhibitors; Complete Proteases inhibitor cocktail, Boehringer Mannheim, Indianapolis, IN). The lysate was incubated for 45 min on ice and was clarified by centrifugation at 10,000g for 10 min at 4°C. The supernatant fraction was boiled in sample buffer, electrophoresed on an SDS polyacryamide gel (7% resolving/3% stacking), and transferred to 0.45 µ nitrocellulose filter (Pharmacia, San Francisco, CA). The membrane was blocked with 5% nonfat dry milk in PBS for 1 h at 25°C to block nonspecific binding and then incubated with rabbit primary antibodies (α$_v$ at 1:1,000 dilution and

Target	Forward primer	Reverse primer	PCR product size (bp)	Genbank Accession #
GADPH	5′-CAGGGCTGCCTTCTCTTGTGA-3′	5′-TGGTCCAGGCTTTCTTACTCC-3′	966 [a]	M17701
Alpha$_v$	5′-AATGTCGTAATGGAGAATGTA-3′	5′-AACCCGAAGAAGGCTGGAACT-3′	396 [b]	X79003
Alpha$_5$	5′-GATGAGGAACAGTGAACCGAAGG-3′	5′-AGCAAAAGCAGGATAGAGGACAA-3′	369	S58528

[a]Fort et al., 1985.
[b]Shinar et al., 1993.

α_2 at 1:500 dilution) for 1 h at room temperature. After extensive washing (4 times with PBS + 0.1% (v/v) Tween 20 for 15 min), the membranes were incubated with 1:2,500–1:10,000 dilutions of horseradish peroxidase-conjugated second antibody (Southern Biotechnology, Birmingham, AL) for 1 h at 25°C, and washed 4 times with PBS + 0.1% (v/v) Tween 20. The bands were detected by ECL (Amersham). All antibody dilutions were made in 5% non-fat dry milk.

Statistical Analysis

Statistical significance between samples were calculated using ANOVA and the Tukey-Kramer HSD test employing JMP 3.1 software (SAS Institute, Cary, NC).

RESULTS

Immunohistochemical Evaluation of Bone Sections for Integrin Distribution

The distribution of integrin in bone was analyzed in decalcified sections of growing male rat femurs (4–6-weeks old) by immunohistochemistry, using polyclonal antibodies against a variety of integrins. The results (Fig. 1) demonstrated the presence of α_2, α_3, α_v subunits and $\alpha_5\beta_1$ integrins in the osteoblastic cells of the metaphysis of the bone, but not in hypertrophic chondrocytes of the growth plate. Osteocytes, (cells deeply embedded in bone), also demonstrated the presence of integrins. In the diaphyseal bone marrow, only occasional multinucleated cells (presumably megakaryocytes) stained for α_2, α_3, α_v subunits and $\alpha_5\beta_1$ integrins. No staining was observed in the sections treated with primary antibody alone or secondary antibody alone (data not shown).

In vivo PTH Treatment Results in Altered Integrin mRNA Levels

In order to determine whether an anabolic dose of hPTH (1–34) resulted in alterations in integrin expression by osteoblasts, we next evaluated the steady-state mRNA levels of various integrins. Previous studies have demonstrated the anabolic effects of PTH in young rats (1, 5, and 9). Young rats (75–100 gm) were injected with 80 µg/kg of hPTH (1–34) once daily for 3 days, total cellular RNA was isolated from freshly harvested cells, and the mRNA levels were evaluated by Northern blot analysis using cDNA probes for rat α_v and β_1 integrin subunits. The results (Fig. 2) show that the mRNA levels of both the integrins were greater in metaphyseal-derived osteoblasts from PTH (1–34) treated rats than those from vehicle treated rats (1.9 to 2.6-fold). This increase was not observed in diaphyseal-marrow derived osteoblasts (data not shown).

Effects of In Vitro PTH Treatment on Integrin mRNA Levels

In order to distinguish whether the increase in integrin expression after in vivo administration of PTH was due to a direct or indirect effect, metaphyseal- and diaphyseal marrow-derived osteoblasts and another osteoblast line-ROS17/2. 8 cells were treated in culture with PTH (1–34) for various time intervals and the mRNA levels were evaluated for various integrin subunits. The results (Fig. 3) show that PTH treatment resulted in very little changes in the levels of α_v, α_5, and β_1 integrins in all of these cells and suggest that the changes observed after in vivo PTH treatment were likely to be indirect.

FN Enhances PTH Stimulation of Integrin Expression

We next evaluated whether (a) cells isolated from PTH treated rats retained the increase in integrin levels upon subsequent in vitro culture and (b) whether extracellular matrix proteins influenced PTH effects on integrin levels. Cells from PTH and vehicle treated young rats were grown to near confluency, removed by EDTA,
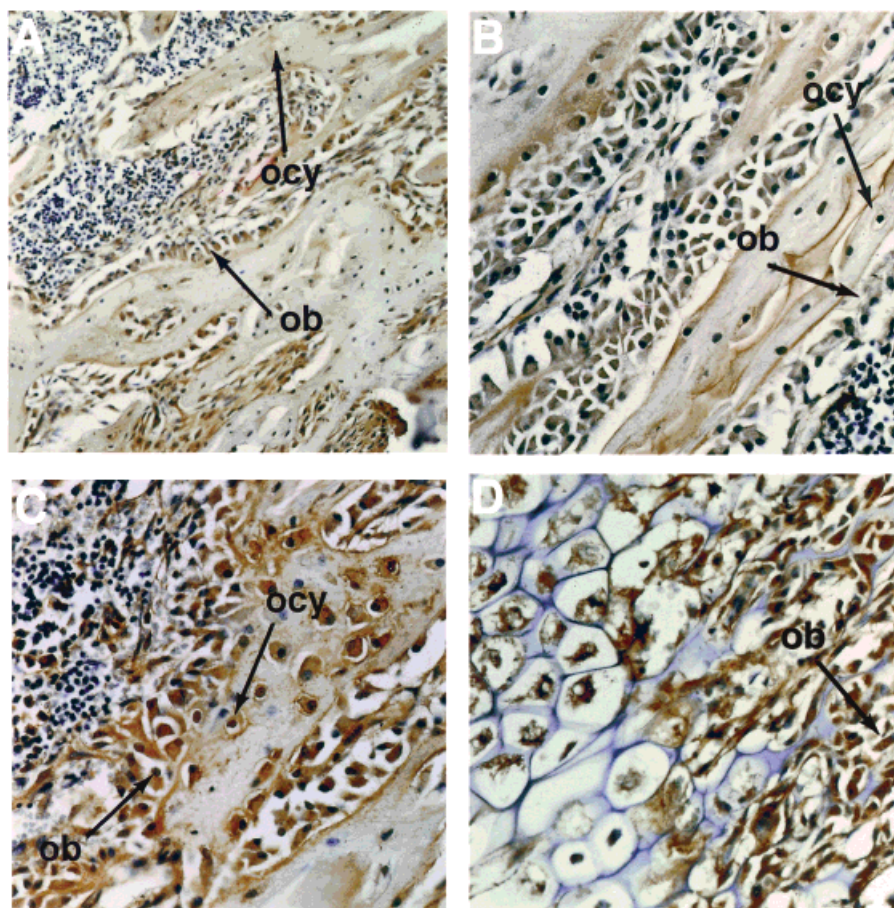
Fig. 1. Immunolocalization of integrins in femur sections of young rats. Decalcified femur sections of young rats (75–100 gm) were immunostained with rabbit antibodies to α_2 (**A**), $\alpha_5\beta_1$ (**B**), α_3 (**C**), and α_v (**D**) integrins, followed by an avidin-biotin-peroxidase detection system. The primary antibodies were used at 1:40 dilution, in goat normal blocking serum. Ob, rosteoblast; ocy, rosteocytes.

plated onto FN coated dishes or processed without attachment (suspension), and the mRNA levels were determined by Northern blot analysis with a cDNA probe for rat α_v integrin. The results (Fig. 4) confirm the presence of two different mRNA species as described before [Sheppard et al., 1992]. Metaphyseal osteoblasts from PTH treated rats showed a 50% increase in α_v mRNA level in suspension, and a 300% increase upon adherence to FN. The same cells isolated from placebo-treated controls showed a 200% increase in α_v mRNA levels when plated onto FN coated plates (Fig. 4A and B). The results from three independent experiments (Fig. 4B) confirm that while the adhesion of cells to FN was sufficient to induce α_v integrin expression, a combination of FN matrix in vitro and prior in vivo exposure to PTH resulted in the greatest increase in α_v mRNA levels. In contrast, the levels of α_v integrin in diaphyseal

marrow cells was not significantly affected by FN or previous in vivo PTH-treament. These results demonstrate that (a) cells isolated from PTH treated rats retain the increased in α_v integrin upon subsequent culture, (b) FN increases α_v integrin levels in metaphyseal cells, but the increase was much greater in cells isolated from PTH-treated rats, and (c) neither PTH nor FN had any effect on α_v integrin integrin levels in diaphyseal marrow cells.

In order to determine whether the effects of hPTH (1–34) were specific to α_v integrin, we next evaluated the mRNA levels for α_5 and β_1 integrin subunits from control and hPTH (1–34) -treated rats. The results are shown only for α_5 integrin (Fig. 5). There was a dramatic increase in α_5 integrin levels in both the metaphyseal and diaphyseal-derived cells when plated onto FN. However, prior in vivo PTH treatment resulted in no further change in the
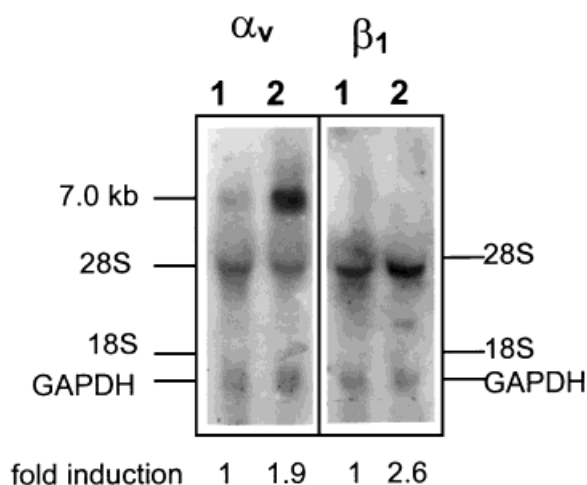
Fig. 2. Effect of in vivo administration of hPTH (1–34) on integrin mRNA levels. Young rats (75–100 gm) were injected once daily with vehicle or 80 μg/kg of hPTH (1–34) for three days. One hour after the last injection, rat femurs (8–12) were harvested, the metaphyseal-derived osteoblasts were isolated, and the total mRNA (cells) was isolated by cesium chloride centrifugation. Equal amounts (15 μg) of total RNA, were subjected to Northern blot analysis using ^{32}P labeled rat cDNA probes specific for α_v and β_1 integrins. For standardization of mRNA applied to the gel, the same blots were also probed with GAPDH. The bands were visualized by autoradiography and the flurorescence intensity was determined by densitometric scanning. The values are expressed as fold induction relative to vehicle-treated control and are normalized relative to GAPDH levels in the corresponding lanes. Lane 1 = cells from vehicle control; Lane 2 = Cells from PTH-treated rats.

levels of α_5. There was a 50–75% increase in β_1 integrin levels in the metaphyseal-derived from PTH-treated rats, but no further changes were seen upon plating in by FN (data not shown).

Western Blot Analysis of Integrin Expression

The effect of PTH on integrin mRNA expression was further confirmed by Western blot analysis. Metaphyseal-derived osteoblasts from PTH- and vehicle- treated rats were allowed to attach to FN for 6 h, the cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the proteins were transferred to nitrocellulose membranes, and were evaluated by polyclonal antibodies against rat α_v and α_2 integrin. As shown in Figure 6, the antibodies recognized 160–168 kD bands that are consistent with the reported molecular weights for the α chains [Lawler and Hynes, 1986; Oldberg et al., 1986; Dedhar et al., 1987; Oldberg et al., 1988; Gehron-Robey et al., 1992; Stein and Lian, 1993; Termine and Robey Gehron, 1996]. Cells

isolated from hPTH (1–34) treated rats exhibited a significant increase in α_v integrin levels, and a decrease in the α_2 levels.

Integrin Expression and Organization in Isolated Bone Cells

In order to determine whether the integrins affected by PTH are involved in osteoblast attachment, we next asked which of the integrins are present in the focal adhesion site. This was evaluated by immunocolocalization studies for integrins and vinculin, since vinculins are components of focal adhesion sites [Singer, 1982; Burridge et al., 1988; Miyamoto et al., 1996]. Osteoblasts were allowed to attach to coverslips coated with FN, or bovine serum albumin (not shown) for 1 h. Vinculin and integrin expression were detected with appropriate primary and secondary antibodies. The results (Fig. 7) show that α_v, α_2, and $\alpha_5\beta_1$ integrins (green fluorescence) colocalize with vinculin (red) at the focal adhesion sites of metaphyseal-derived as well as diaphyseal marrow-derived osteoblasts. The α_3 integrin subunit was restricted to a perinuclear localization, with no apparent correlation to vinculin localization. These results are consistent with the suggestion that PTH regulates only those integrins (α_v, α_2, and β_1) that are integral components of adhesion complex in vitro.

Immunohistochemical Evaluation of Integrin Expression in Ovx Rats Treated with Human PTH (1–34)

We also evaluated whether PTH effects on integrin were can be observed in estrogen deficient rats. Initially, we established that PTH 1–34 was able to restore bone loss associated with ovariectomy, in a dose-dependent manner (Fig. 8). The effect of PTH 1–34 on integrin expression was evaluated by immunohistochemistry on metaphyseal-derived and diaphyseal marrow-derived osteoblasts, plated onto FN coated tissue culture dishes, using various anti-integrin antibodies followed by FITC conjugated second antibody. The fluorescent intensity and the number of cells positive for fluorescence were visually assessed. The results further suggest that in comparison to cells from ovariectomized rats, cells from PTH treated rats showed a dose-dependent increase in α_v expression, and a dose-dependent reduction in α_2 levels, with no discernible changes in α_3, and $\alpha_5\beta_1$ levels (Table II). These changes
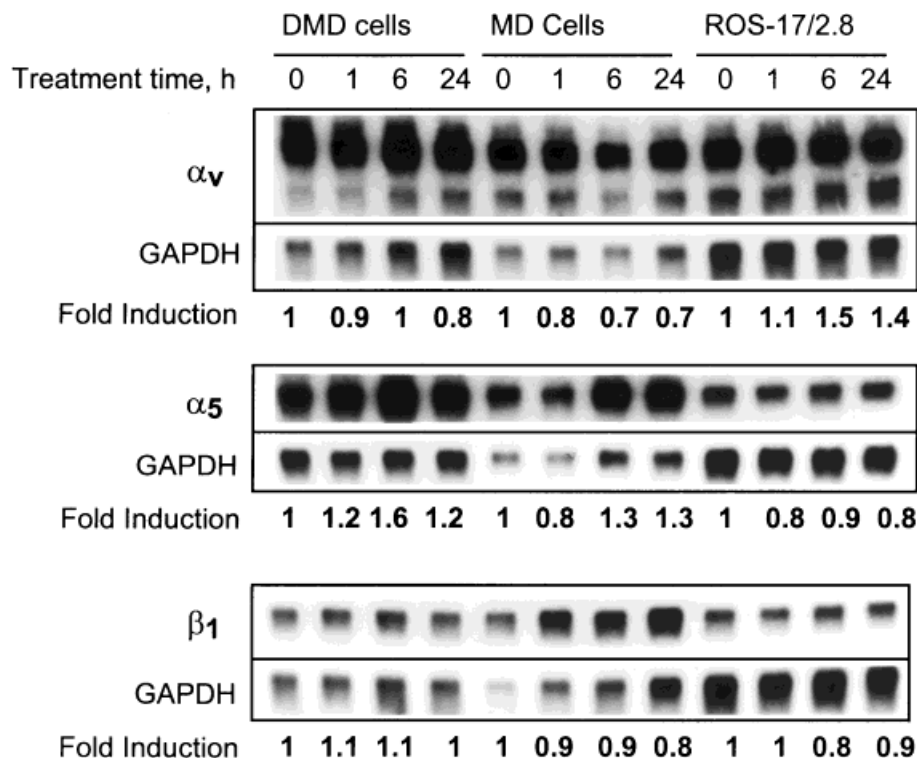
Fig. 3. Effect of hPTH (1–34) on integrin expression in vitro. Metaphyseal-derived osteoblasts (9,000 cells/ cm²), diaphyseal marrow-derived osteoblasts (21,000 cells/ cm²), and ROS 17/ 2.8 cells (12,000 cells/ cm²) were grown to near confluency in 175 cm² tissue culture flasks and were treated with hPTH (1–24) for various time intervals. Equal amounts of total RNA (10 μg/ lane) were subjected to Northern blot analysis using ³²P probes for various integrins. The values normalized to the GAPDH, are expressed as fold induction relative to time ''0''. MM = Metaphyseal marrow-derived; DMD = Diaphyseal marrow derived.

were not observed diaphyseal marrow-derived osteoblasts. The semi-quantitaive immunohistochemical assessment was also confirmed by slot analyses of mRNA from ovariectomized and PTH treated rats for α_v, α_2 levels, and β_1 levels (Table III).

DISCUSSION

Intermittent administration of PTH induces new bone formation by promoting osteoblast differentiation [Reeve et al., 1980; Tam et al., 1982; Podbesek et al., 1983; Gunness-Hey and Hock, 1984; Tada et al., 1990; Dempster et al., 1993; Onyia et al., 1995, 1997; Sato et al., 1997]. Osteoblast formation is complex and involves recruitment of precursor cells, differentiation of pre-existing cells, and cell–cell and cell–matrix interactions [Gehron-Robey et al., 1992; Stein and Lian, 1993; Onyia et al., 1995; Schmidt et al., 1995; Dobnik et al., 1995; Termine and Robey Gehron, 1996; Onyia et al., 1997]. Previous studies have demonstrated the presence of various integrins on osteoblasts and have

suggested a role for integrins in promoting osteoblast differentiation [Hughes et al., 1993; Hultenby et al., 1993; Castoldi et al., 1997; Ganta et al., 1997; Moursi et al., 1997]. In this report, we provide evidence for the expression of α_2, α_3, α_v and $\alpha_5\beta_1$ integrins in the long bones of aged and young rats. Administration of PTH (1–34) resulted in a significant increase in the levels of α_v mRNA and protein in metaphyseal-derived osteoblasts, a decrease in α_2 levels, and very little changes in α_3 and $\alpha_5\beta_1$. In addition, there was an increase in β_1 mRNA levels. These results are true for both young and aged ovariectomized rats treated with PTH. These results suggest that the PTH action on osteoblast differentiation may at least involve selective modulation of integrin levels.

Integrin expression was evaluated by a variety of techniques: (a) immunohistochemistry of rat bone sections, as well as in isolated metaphyseal-derived and diaphyseal marrow-derived osteoblasts, (b) Northern, and (c) Western blot analyses. In order to evaluate whether the integrin localization correlates with focal
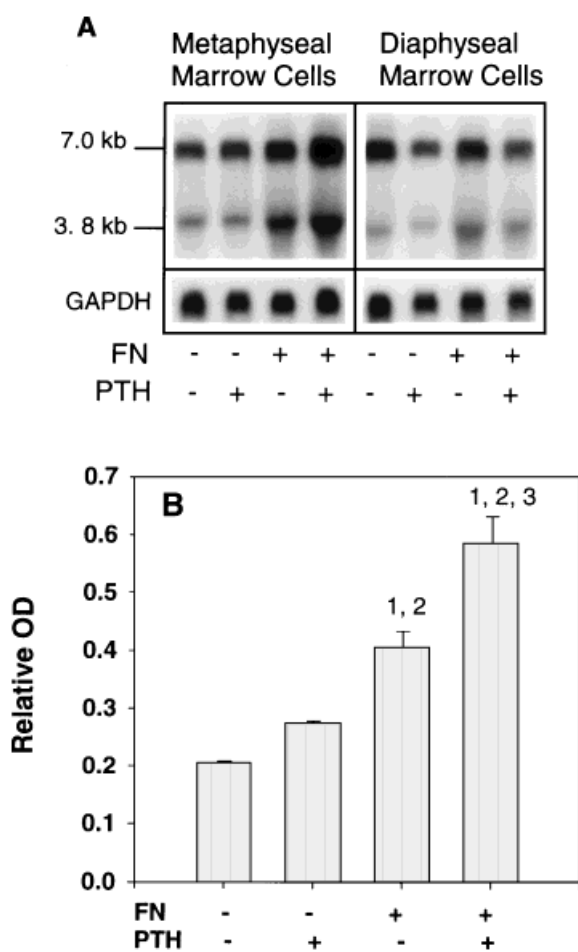
Fig. 4. Osteoblasts from in vivo hPTH(1–34)- treated rats maintain altered integrin expression in vitro. Metaphyseal marrow and diaphyseal marrow derived cells from PTH (80 μg/kg) and vehicle treated young rats, were grown to confluency, released by EDTA, and were then plated onto 175 cm^2 tissue culture flask that were coated with FN (100 μg/plate) or without FN (suspension). After 1 h, equal amounts of (15 μg) of total RNA were analyzed by Northern blot using ^{32}P labeled cDNA probe specific for rat α_v. Figure 4A shows a representative autoradiograph of the northern blot. Figure 4B represents the fold induction average (±SD) of three independent experiments. The values were determined from densitometric scanning of specific hybridized band, normalized relative to that of GAPDH, and are relative to the value of FN-/PTH- sample. The statistical significance ($P < 0.05$) was calculated using ANOVA and Tukey-Kramer HSD test. 1 = Significantly different from cells in suspension of vehicle treated rats; 2 = Significantly different from cells in suspension of PTH treated rats; 3 = Significantly different from all three groups.

adhesion, cells plated onto either FN or BSA were co-stained with antibody for vinculin. At all levels of analysis, hPTH (1–34) effects on increased α_v levels and reduced α_2 levels were confirmed. In addition, the effects of PTH hPTH

(1–34) were observed both in young rats as well in ovariectomized old rats.

Integrins have been shown to play a role in osteoblast differentiation and function. This has been demonstrated in a variety of cell and organ culture models, including primary or established cells, cells from different regions (calvaria and long bones) and species (rat and humans) and cells displaying varying degrees of osteoblast-related phenotypes, such as alkaline phosphatase and matrix protein expression [Clover et al., 1992; Hughes et al., 1993; Hultenby et al., 1993; Castoldi et al., 1997; Ganta et al., 1997; Moursi et al., 1997]. Since most of the in vitro cell culture models do not consistently display the in vivo anabolic effect of PTH, we have utilized a combination of in vivo/ex-vivo approach in which cells freshly isolated from rats were allowed to complete their differentiation in vitro. Bone cells isolated from metaphyseal spongiosa and diaphyseal marrow, which are repositories of precursor cells that are capable of differentiating towards the osteoblast phenotype [Owen and Friedenstein, 1988; Caplan, 1991; Onyia et al., 1995, 1997]. In young rats, the osteoprogenitor cells that give rise to trabecular osteoblasts in long bone are located in the metaphyseal spongiosa and are the primary target cell for PTH [Onyia et al., 1995, 1997]. In vitro, these cells proliferate and undergo a cascade of phenotypic changes that result in enhanced osteoblast differentiation, including the synthesis of type collagen, osteoclacin, alkaline phosphatase, cAMP production in response to PTH treatment, and ability to form mineralization spontaneously [Onyia et al., 1995, 1997]. Further, cells formed bone nodules when implanted into the peritoneum of rats (Onyia et al., 1999). Previous studies have also demonstrated that the putative precursors from diaphyseal marrow also exhibit its osteogenic potential in vivo, upon implantation in vivo in the diffusion chamber [Bab et al., 1984]. The latter cells also express mRNA for PTH type I receptor, but unlike in the metaphyseal-derived cells, the role of PTH in driving osteogenic potential of the diaphyseal marrow-derived cells is not clear (Kaiser, Chandrasekhar, unpublished data). Our results demonstrate that PTH- mediated integrin changes were observed only in the metaphyseal cells and not in diaphyseal marrow-derived osteoblasts. While we do not know the reason for this apparent difference between the two cell
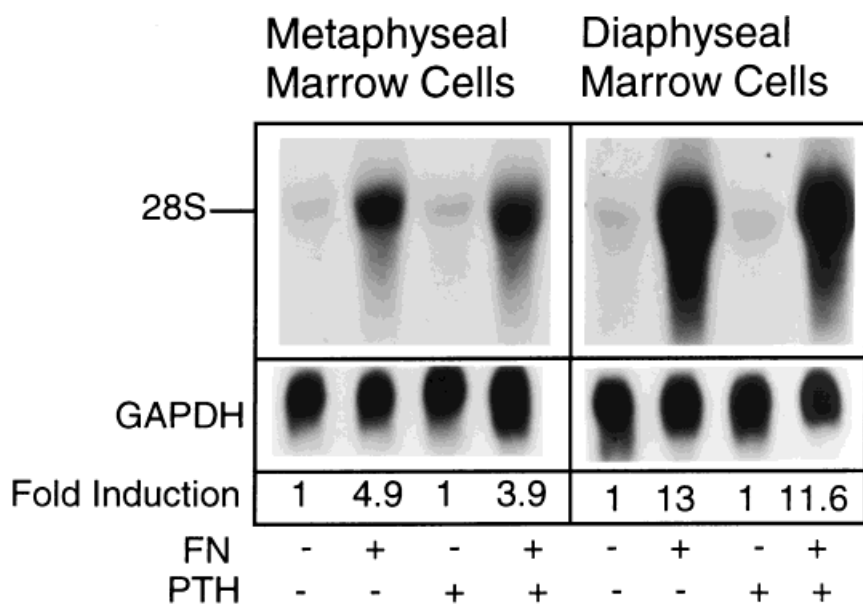
Metaphyseal Marrow Cells   Diaphyseal Marrow Cells

Fig. 5. In vivo PTH treatment does not alter steady-state mRNA levels for α_5 integrin. Metaphyseal marrow and diaphyseal marrow derived cells from PTH (80 μg/kg) and vehicle treated young rats, were grown to confluency, released by EDTA, and were then allowed to attach to 100 cm² tissue culture dish coated with FN or bovine serum albumin (100 μg each). After 1 h, equal amounts of (15 μg) of total RNA were analyzed by Northern blot using ^{32}P labeled cDNA probe specific for rat α_5 integrin and GAPDH. The fold induction was calculated relative to the values for FN-/PTH- samples and was normalized to that of GAPDH expression.

28S								
GAPDH								
Fold Induction	1	4.9	1	3.9	1	13	1	11.6
FN	-	+	-	+	-	+	-	+
PTH	-	-	+	+	-	-	+	+

populations, it could be related differences in the differentiation state of these cells, culture conditions, or the time of analysis.

The integrin mRNA levels were evaluated by isolating mRNA from: (a) cells freshly harvested from PTH-treated rats, (b) cells from PTH-
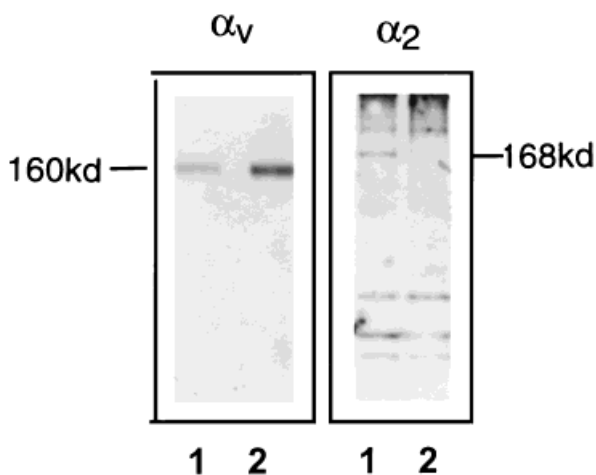

Fig. 6. Immunoblot analysis of α_v and α_2 integrins. Metaphyseal-derived osteoblasts from PTH (80 μg/kg) and vehicle-treated young rats were grown to confluency, serum-deprived for 24 h, released by EDTA and were then allowed to attach to FN for 6 h. Equal amounts of the proteins from cell lysates were separated by SDS–PAGE, transferred to a nitrocellulose filter and probed with polyclonal anti-rat α_v (1:1,000) or α_2 (1:500) integrin antibodies, followed by a peroxidase conjugated secondary antibody. The protein bands were visualized by ECL method. The relative molecular weights were established by protein standards. Lane 1 = cells from vehicle-treated rats; Lane 2 = cells from PTH-treated rats.

treated rats but grown to confluency and subsequently plated onto FN for 4 h, and (c) cells from untreated rats grown to confluency and treated with PTH 1–34 (with or without FN). Cells isolated from PTH (1–34)-treated rats showed an increase in α_v, a decrease in α_2 and very little change in α_5, and β_1 levels in the metaphyseal-derived osteoblasts (Figs. 2 and 4). Similar results were also obtained in ovariectomized old rats that were treated with PTH 1–34 (Tables II and III). Immuno-chemical (Table II) and western blot analysis (Fig. 6) confirmed these observations and further established that in vivo PTH treatment resulted in an increase in α_v levels, a decrease in α_2 with very little change in α_3, and $\alpha_5\beta_1$ levels in the metaphyseal osteoblasts, and not in diaphyseal osteoblasts (Table II). Metaphyseal osteoblasts isolated from in vivo PTH treated rats and subsequently plated onto FN demonstrated a 3–4-fold increase in α_v integrin levels in comparison to cells in suspension (Fig. 3). This increase in α_v integrin was also confirmed by immunoblot analysis of osteoblast cell lysate (Fig. 5). These studies suggest that integrin expression can be regulated by both PTH and by the extracellular matrix onto which cells are plated. While both α_v and α_5 integrins were up-regulated by FN matrix, only α_v integrin was subject to further regulation by PTH.

It is however interesting to note that the integrin sub-unit alterations were observed only in cells isolated from rats previously
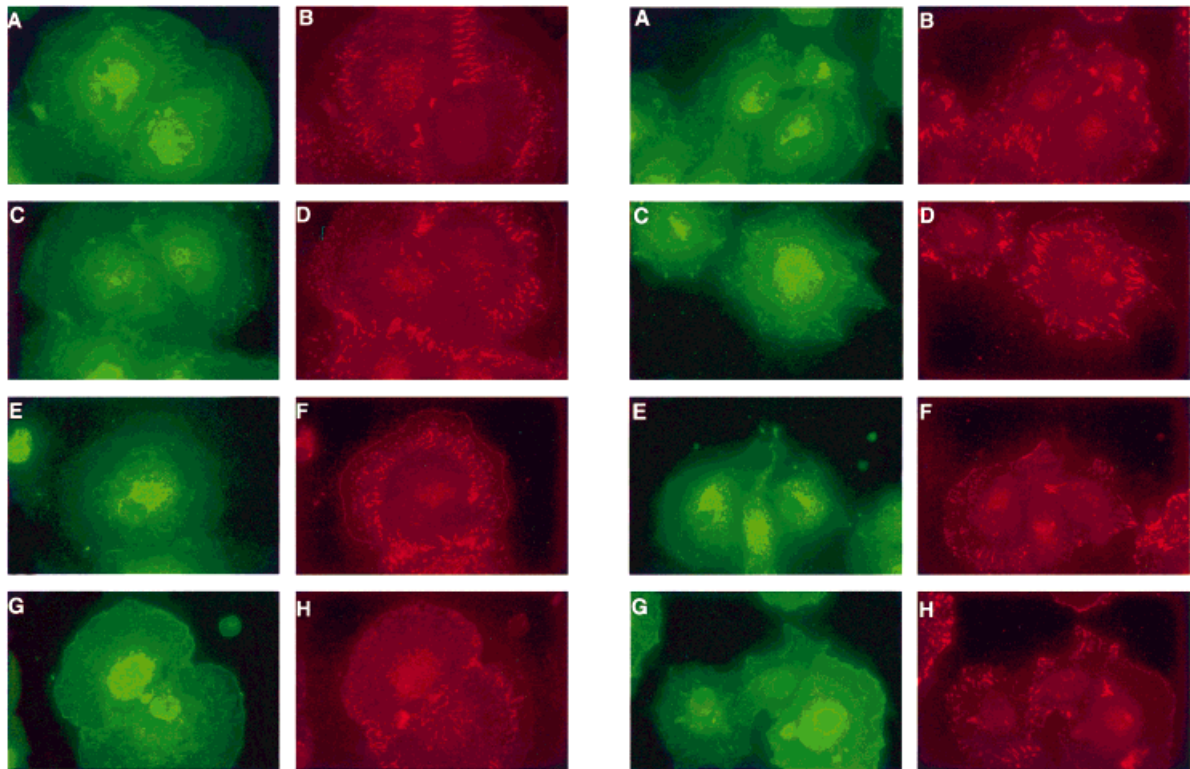
Fig. 7. Immunolocalization of integrins in isolated osteoblasts plated on FN. Metaphyseal and diaphyseal derived osteoblasts from young (4–6 weeks old) rats were grown to confluency, serum deprived for 24 h, released by EDTA, plated onto FN for 1 h, fixed, permeabilized, and incubated for 1 h with 1:20 anti-vinculin antibodies, followed by a rhodamine conjugated secondary antibody. Subsequently, cells were also incubated with 1:40 dilutions of rabbit antibodies to α_v, α_2, α_3, and $\alpha_5\beta_1$ integrins, followed by a 1 h incubation with fluorescein isothiocyante conjugated anti-rabbit secondary antibody. Cells were photographed using appropriate filters for capturing green or red fluorescence. A, C, E, and G = α_v, α_2, α_3, and $\alpha_5\beta_1$ integrins respectively; **B**, **D**, **F**, and **H** represent corresponding cells stained for vinculin.

treated with PTH for at least three days. Integrin levels were not significantly changed after one injection of PTH (1–34) (data not shown). More significantly, PTH treatment of isolated osteoblasts in vitro revealed no such changes. This was true for metaphyseal or diaphyseal marrow cells or for an established osteoblast cell line (ROS 17/2.8) that undergoes in vivo mineralization (Fig. 7). The reason for the discrepancy between in vitro and in vivo experiments are not clear, but are consistent with various reports on other anabolic activity [Gehron-Robey et al., 1992; Stein and Lian, 1993; Onyia et al., 1995, 1997; Termine and Robey Gehron, 1996]. It is possible that PTH may trigger other factors that are responsible for altered integrin expression. It is also likely that the primary effect of PTH may be on osteoblast differentiation and that the altered integrin expression is a reflection, rather than, the cause of osteoblast differentiation.

Although osteoblasts express several integrins, not all integrins are likely to be utilized for all functions. For example, although $\alpha_5\beta_1$ and $\alpha_8\beta_1$ integrins may be utilized for attachment, only $\alpha_5\beta_1$ may be involved in rat calvarial osteoblast differentiation [Hughes et al., 1993; Hultenby et al., 1993; Castoldi et al., 1997; Ganta et al., 1997; Moursi et al., 1997]. The immuno-colocalization studies suggested that α_v, α_2 and $\alpha_5\beta_1$ were components of focal adhesion complex, and presumably play a role in cell attachment to matrix. In contrast, α_3 integrin was present primarily in the perinuclear region of the cell and not in the focal adhesion sites. Previous studies appear to suggest that $\alpha_3\beta_1$ does not play a role in adhesion [Elices et al., 1991]. The perinuclear distribution pattern of α_3 integrin may be reflect an association with endoplasmic reticulum and related functions such as protein sorting and secretion. It could also be associated with calreticulin, a protein
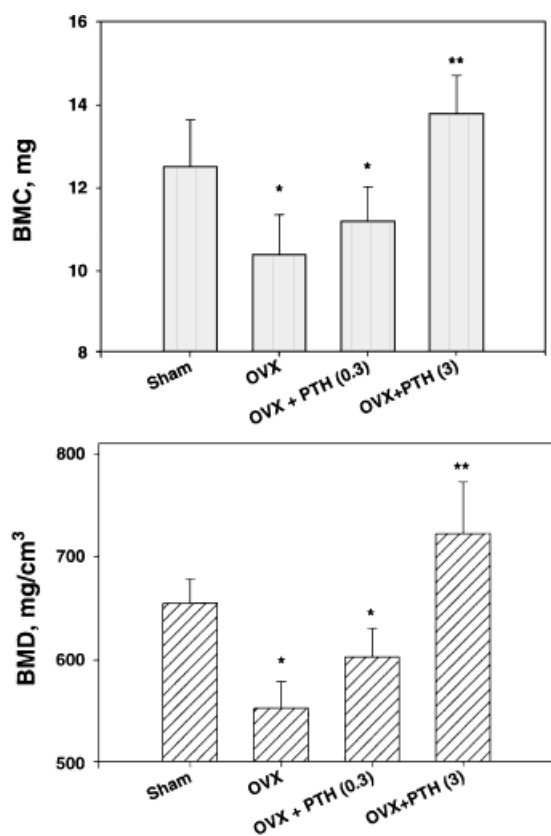
Fig. 8. QCT analysis of PTH (1–34) effects on the proximal tibiae of ovariectomized rats. Six month old rats (n = 8/group) were ovariectomized and allowed to lose bone for 1 month before treatment with PTH (1–34) at 0.3 or 3 µg/kg/day for the following 3 months. At termination, the bone mineral content and volumetric bone mineral density of the proximal tibial metaphysis were analyzed, as described. The values represent the average ±SD. The statistical significance was determined using ANOVA and the Tukey-Kramer HSD test. * = Significantly different from sham (<0.0001); ** = Significantly different from OVX (<0.0001).

that interacts with the cytoplasmic domain of all known α subunits and may play a role in "inside-out" signal transduction [Dedhar, 1994; Leung-Hagesteijn et al., 1994]. Irrespective of the potential function of α_3 integrin, PTH has no effect on the levels of this integrin. It is however interesting that PTH upregulates the expression of α_v and down regulates α_2 integrins. Previous studies have established that $\alpha_v\beta_1$ integrin utilizes vitronectin and to a lesser extent FN for attachment, while $\alpha_2\beta_1$ integrin is the receptor for native type I collagen [Pierschbacher and Ruoslahti, 1984; Ruoslahti, 1991; Hynes, 1992; Clark and Brugge, 1995; Meredith et al., 1996]. It is possible that the initial action of PTH is to facilitate osteoblast progenitor adherence to vitronectin and/or FN, while reducing interaction with type I collagen. Once the cells begin to establish an osteoblast phenotype that includes the production of type I collagen [Stein and Lian, 1993; Termine and Robey Gehron, 1996], it is possible that the cells may begin to utilize $\alpha_v\beta_1$ integrin. It is important to note that in our studies, we have not identified the hetero-dimeric integrin complexes associated with α_v and α_2 chains and have only identified the individual chains. However, previous studies have established that α_v can associate with other β chains (β_1, β_3, and β_5). We have demonstrated the presence of β_1 chain (Fig. 4) and β_3 chain (Kaiser and Chandrasekhar, unpublished PCR data) and therefore an opportunity exists for such an assembly. However, these suggestions are speculative and need to be established.

In summary, the results presented in our studies suggest that metaphyseal and diaphy-

TABLE II. Effect of PTH 1-34 Treatment on Integrin Expression

Treatments	α_2	α_3	α_v	$\alpha_5\beta_1$
Metaphyseal Marrow cells				
Ovx	NC	NC	NC	NC
PTH (0.3 µg/kg)	−	NC	+	NC
PTH (3.0 µg/kg)	− − −	−	+++	NC
Diaphyseal Marrow cells				
Ovx	NC	NC	+	NC
PTH (0.3 µg/kg)	NC	NC	NC	NC
PTH (3.0 µg/kg)	−	−	NC	NC

Ovariectomized rats were injected once daily with either PTH 1−34 (0.3 and 3 µg/kg) or vehicle for 12 weeks, the metaphyseal- and diaphyseal derived cells were isolated as described, and plated onto FN coated chamber slides. After 1 h, integrin expression was evaluated using rabbit polyclonal antibodies to rat (α_v, α_2, α_3) and human ($\alpha_5\beta_1$) integrins, followed by FITC conjugated anti-rabbit second antibody. For each treatment, cells were plated in triplicte and in each slide at least three fields (10 cells/field) were evaluated for both the intensity and number of cells stained for the label. In order to avoid subconscious observer bias, slides were evaluated in a blinded fashion. The results are expressed relative to sham control. The experiment done two times with similar results.
NC = No change from sham control.
− or + = 20% of cells show reduced or increased intensity.
− − − or +++ = 60% of cells show reduced or increased intensity.

TABLE III. Effect of hPTH 1-34 on Integrin mRNA Levels in Osteoblasts from Ovariectomized Rats

	Relative mRNA levels, fold inductio (metaphyseal-derived osteoblasts)	
Integrins	Ovariectomized	PTH-treated
α_v	1	5.2±0.3
α_2	1	0.48±0.08
β_1	1	4.3±0.2

Metaphyseal-derived osteoblasts were isolated from ovariectomized rats treated with 30 µg/kg of hPTH (1−34) as in Table II, cultured for 1 week and the total mRNA was evaluated by northern blot, using a slot apparatus. The results represent the average ± SE of three determinations.

seal marrow display a variety of integrin subunits. PTH, an anabolic agent that promotes osteoprogenitor differentiation in vivo, also modulates select integrins indirectly. The changes are cell-type and integrin-type specific. Thus, by selective modulation of integrins on the target cells, PTH may promote appositional bone formation in metaphyseal spongiosa.

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