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Distinct Responses of Different Populations of Bone Cells to Mechanical Stress*

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ABSTRACT

To explore lineage-dependent responses to mechanical stress in bone cells, newborn rat calvarial cells, exhibiting differential characteristics of osteoblastic and osteocytic cells, were compared in their immediate and late responses to stretching. Seven fractions of sequentially prepared cells were cultured on Matrigel to promote their differentiation. By cyclically stretching the flexible bottom of culture plates, cells were exposed to a physiological stress of approximately 4000 microstrain on Matrigel. Cells in fractions IV, V, and VI exhibited striking responses; the levels of cAMP and insulin-like growth factor I, bone Gla protein, and mineral accumulation were significantly elevated in the stretched cells. Also, proliferation was significantly inhibited regardless of the presence of 10^{-6} M indomethacin. In contrast, osteoblasts in fraction III and osteocyte-like cells in fraction VII exhibited no significant response. Thus, these intermediate cells, very mature osteoblasts to young osteocytes, are likely to serve as a mechanosensor in bone, controlling the metabolic aspects of physical stress. We conclude that the responses of these young osteocytes to low level, physiological strain are transmitted in a manner different from the responses of osteoblasts to higher magnitudes of strain in which PGE_2 induces cell proliferation, as reported by others. (*Endocrinology* **137:** 2028–2035, 1996)

BOTH THE REGULATION of bone mass and maintenance of structural integrity are dependent on the mechanical demands placed on bone. This is demonstrated by the effects of deprivation of stress due to weightlessness and immobilization (1–4). It has been shown that the metabolism of bone cells is altered when mechanical loads and physical stress are applied to bones (5–16; see Ref. 15 for review). Before the specific role of osteocytes as a mechanosensor appeared as a subject of discussion (16–19), extensive osteocytic networks attracted the attention of many anatomists in electron microscopic studies. The structure of intercellular junctions, either between osteoblasts and osteocytes or between osteocytes, and their relation to the functions of these junctions have been studied in detail (20–23). Both Ejiri and Ozawa (24) and Baylink and Wergedal (25) showed more interest in the canalicular structure. Emphasized was the fact that the caliber of some canaliculi at the mineralization front are larger than those in more mature bone, and that pericanalicular mineral deposition exists in young osteocytes (24). We and others showed that the network formation or the transition of osteoblasts into osteocytes involves a sequential process of cell differentiation that is reflected in their ability to form and mineralize matrix (25, 26).

Lanyon and his colleagues have made extensive *in vivo* studies on the effects of mechanical loading and have suggested major roles for osteocytes in the response of bone (16–18). They stated that some populations of bone cells must be responsive, directly or indirectly, to the consequences of loading. Their examination of bone cell organization suggests that osteocytes are the sensors and/or transducers that respond to load-induced strain in the bone matrix. In *in vivo* experiments using adult avian bone, they showed that the number of osteocytes expressing glucose 6-phosphate dehydrogenase activity increased transiently immediately after a short period of loading (16). In the same preparation, the number of osteocytes incorporating [^{3}H]uridine increased (17). In a separate study exposing cancellous bone cores to stress, the presence of PGI_2 and/or PGE_2 was suggested in lining cells and osteocytes, and an immediate increase in both PGE_2 and PGI_2 was observed in the perfusing medium of the bone (18). In a more recent study using intact rat tail bone, Lean and colleagues (19) suggested that osteocytes responded to mechanical loading, resulting in the expression of insulin-like growth factor I (IGF-I) messenger RNA within 6 h. Together, these data raised the possibility of specific contribution by osteocytes in response to mechanical stress placed on bone.

The aim of this study was to establish the relationship between responses to mechanical stress, both high and low, in isolated bone cells at different stages of development. As previously reported, fractionated primary cells from newborn rat calvaria exhibit characteristic features of differentiated bone cells on Matrigel (26). Cells in fraction III are round and exhibit the highest alkaline phosphatase (APase) level, whereas the latter fractions (IV–VII) become more osteocytic in both canalicular morphology and biochemical characteristics, *i.e.* low APase level and high bone Gla protein (BGP; osteocalcin) production. By measuring the parameters of bone metabolism in these fractionated cells, we found that the stages of development play a critical role in the response of bone cells to mechanical stress.

Materials and Methods

DMEM and Mg- and Ca-free PBS [PBS(−)] were purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Pregnant Sprague-Dawley

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rats were purchased from Nippon SLC (Hamamatsu, Japan). FBS was purchased from Whittaker Bioproducts (Walkersville, MD). Bacterial collagenase, ascorbic acid, diaminobenzoic acid dihydrochloride and *n*-2-tosyl-lysine chloromethyl ketone were purchased from Wako Pure Chemical Co. (Osaka, Japan). Penicillin-gentamicin-fungizone antibiotic mixture was purchased from Life Technologies (Grand Island, NY). BSA, APase assay reagents (*p*-nitrophenyl phosphate, *p*-nitrophenol, 2-amino-2-methyl-1-propanol, and β-naphthyl acid phosphate), and Fast blue BB salt for histochemical staining were purchased from Sigma Chemical Co. (St. Louis, MO). The osteocalcin RIA kit was purchased from Biomedical Technologies (Stoughton, MA), the IGF-I and PGE_2 RIA kits were obtained from Amersham International (Aylesbury, UK), and the cAMP RIA kit was purchased from Yamasa Shoyu Co. (Choshi, Japan). ITS Premix and Matrigel (lots 902533 and 921695) were purchased from Collaborative Biomedical Products/Becton Dickinson Labware (Bedford, MA). Proteinase K and bioassay reagent grade PTH-(1–34) (human) were obtained from Stratagene (La Jolla, CA) and Cambridge Research Biochemicals (Wilmington, DE), respectively. Flexible bottomed Flex I plates, coated with a thin film of type I collagen, were purchased from Flexercell Corp. (McKeesport, PA).

Isolation and culture of osteoblastic and osteocytic cells

Calvarial cells were prepared from frontal and parietal bones that were dissected out aseptically from newborn rats. Osteoblastic cells were enriched by collagenase digestion, and the osteocytic cells by treatment with chelator as reported by us previously (26), except that a larger number of rat pups (50 instead of 30) and older animals (4 instead of 2–3 days old) were used to yield more osteocytes. Briefly, pieces of bone were stripped of periosteal soft tissue and sutures, and digested with 25 ml 0.75 mg/ml collagenase. Cells released after the first 30-min and the second through fourth 20-min digestions at 37 C were separately collected (fractions I–IV, respectively), filtered through a nylon screen (40-μm pore size), and cultured at the original density of 5–10 $\times$ 10^4 cells/well in a 48-well plate. Residual bone pieces were additionally treated first with PBS(−) (fraction V), then with 4 mM EDTA in PBS(−) (fraction VI), and finally with collagenase again (fraction VII) for 20 min each. The released cells were cultured similarly to those from the original collagenase digestion. For long term culture, osteoblastic fraction III cells, which expressed the highest level of APase, were maintained separately at 2.5 $\times$ 10^5 cells/well in a flexible bottomed, 6-well plate (Flex I plate). It should be noted that because we used older rats, cells recovered in each fraction were slightly different from those described in our original report (26). Namely, the amount of BGP produced by fraction VII cells, which was the highest by our original method, was lower than that produced by fraction VI cells. Cells were maintained in DMEM containing 10% FBS and insulin, transferrin, and selenium, as reported previously (26).

Substrata for cell culture

One hundred and 400 μl each of Matrigel (a reconstituted basement membrane gel) were used to coat the bottom of wells in 48- and 6-well flexible bottomed plates, respectively. Matrigel was kept in ice, placed in each well, and allowed to solidify at 37 C. The final protein concentration was 11 mg/ml. For the long term culture of osteoblastic fraction III cells, flexible bottomed plates with collagen-coated silicone (Flex I plates) were used without Matrigel.

Stretching procedures

In some experiments, 6-well, flexible bottomed plates were seeded at an original density of 2.5 $\times$ 10^5 fraction III cells/well (5 $\times$ 10^4 cells/cm^2) and stretched at different time points during the 20-day culture period. In other experiments, cells from each fraction were cultured on a Matrigel-coated flexible surface from the beginning of the culture period for about 7 days or were transferred onto the flexible surface before stretching after culture on Matrigel in a 48-well plate. In the latter, the cells together with Matrigel substrate were bound to the flexible plate with a drop of fresh Matrigel placed 5–7 mm from edge. To solidify the Matrigel, the transferred cells were incubated at 37 C for 15 min before stretching. In this case, control cultures were left untouched in the original plate without being exposed to the mechanical stress of transferring them onto another plate. An extra drop of Matrigel was added to the well to equalize their conditions. For stretching at the physiological level, the bottom of the plate was deformed by a computer-operated vacuum system (Flexercell Strain Unit, Flexercell Corp., McKeesport, PA), with a frequency of 1/60 Hz (10 sec of stretching, followed by 50 sec of relaxation). It was stated by Vadiakas and Banes that stretching by applying a negative pressure of 2 kPa (0.3 psi) to the bottom caused 0–30,000 microstrain (μE) depending on the point of growth in the well (27). The actual strain to which most of the cells were exposed was reported to be much less (<10,000 μE) (28). Microstrain is a millionth of the change in length divided by the initial length; therefore, 30,000 μE is equivalent to 3% elongation. When the cells were stressed indirectly on Matrigel that was placed between 5–7 mm from the edge of the well (id, 25 mm), the expected strain was approximately 4,000 μE (0.4% elongation). In the weight-bearing bones of a range of animals, the peak strain during exercise was approximately 3,000 μE (29). That agrees well with the reported level of strain encountered with normal activity of bone (30–32).

Biochemical measurements

After 12-h stretching and the additional 24-h incubation, cultures were harvested, washed with Tris-buffered saline (TBS), and subjected to fluorometrical DNA measurements with diaminobenzoic acid dihydrochloride. Before the DNA assay, proteinatious materials in the culture wells were digested with proteinase K, and DNA was precipitated with 70% ethanol containing 0.15 M NaCl (26). Data were standardized against salmon sperm DNA. A separate set of samples was subjected to the APase measurement after two cycles of freeze-thawing, using *p*-nitrophenyl phosphate as a substrate. The enzyme activity in 25 μl medium was also measured for the APase released. To measure mineral and the associated matrix proteins, cultures were harvested, washed with phosphate-free TBS extensively, and subjected to acid extraction with 0.1 N HCl. With the extract, BGP (osteocalcin) was assayed using the rat osteocalcin ^{125}I RIA kit, and inorganic phosphate by a colorimetric microassay method (33). In some cases, residues of the acid extraction were subjected to DNA assay after proteinase K digestion and ethanol precipitation. RIAs for ligands such as cAMP, IGF-I, and PGE_2 were performed according to the procedures provided by suppliers. cAMP was extracted from the cells with 1 ml/well ethanol containing 0.01 vol 1 N HCl. After 2-h incubation at 4 C, the ethanol extract was transferred into a glass tube, dried at 90 C, and dissolved in 200 μl 3 mM HCl for further analysis. IGF-I in the medium was extracted with 1.5 vol acidic ethanol, which contains 85% ethanol and 15% 2 N HCl. After 30-min incubation on a shaker, samples were centrifuged, and aliquots of supernatant solution were subjected to RIA after neutralization with Tris base. Sample numbers for each experiment were specified in a figure legend, and statistical significance was analyzed by Student's t test.

Results

Effects of stretching on matrix mineralization in long term cultures of rat calvarial osteoblasts

In Fig. 1, calvarial cells recovered in fractions III, V, and VII were compared in their morphology on both Matrigel (Fig. 1, A, B, and C) and tissue culture plastic (Fig. 1, each *inset*). Fraction III cells, which exhibit the highest APase level on Matrigel (26), are of a typical polygonal shape on either tissue culture plastic or a flexible bottomed plate (Fig. 1A, *inset*). It has been reported that primary calvarial cells, such as fraction III osteoblasts, mature in culture to some extent, especially on collagenous substrate (34). Therefore, we first examined the changes in response to mechanical stress during their 20-day culture period. On days 5, 9, 13, 15, and 20, respectively, the plates were placed in a Flexercell Strain Unit, and the cells were stretched at a physiological level by deforming the bottom of the plate at −2 kPa (see *Materials and*

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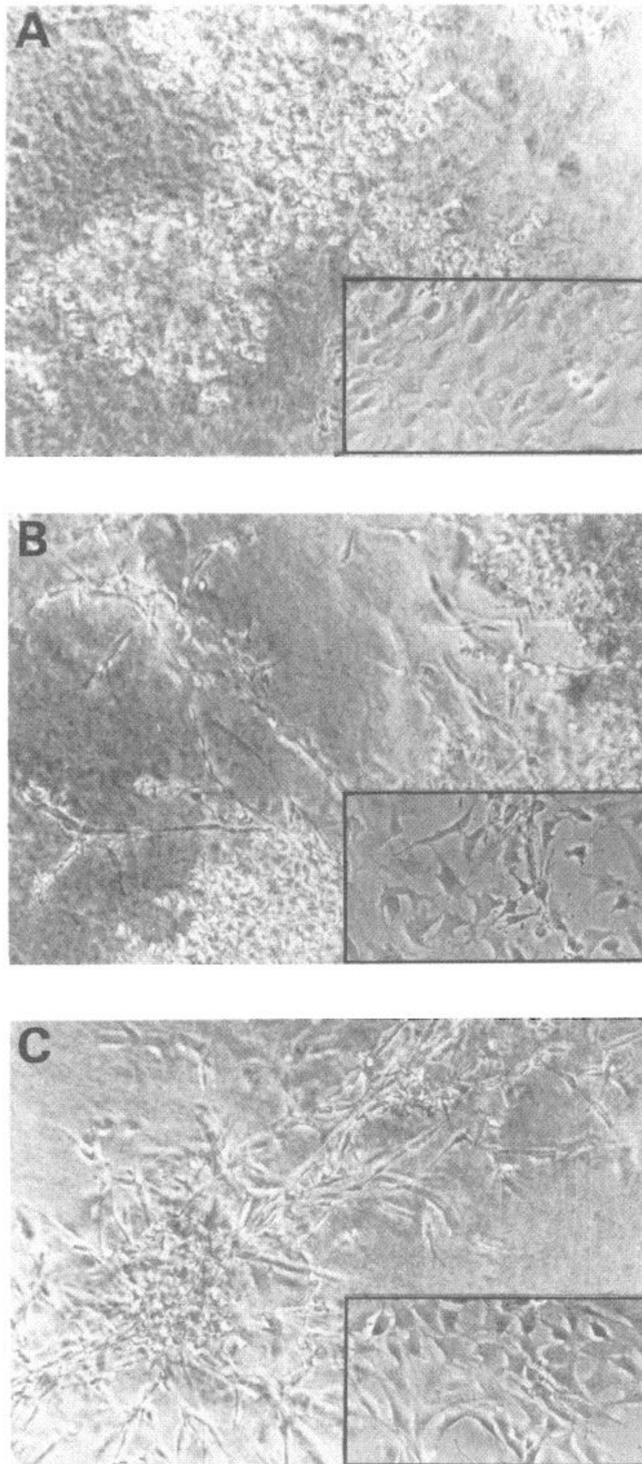

FIG. 1. Morphology of the cells recovered in fractions III, V, and VII. Cells in three fractions were compared on Matrigel (A, fraction III; B, fraction V; C, fraction VII) and on tissue culture plastic (each *inset*) after 6-day incubation. Original magnification, ×100.

Methods). After 12-h stretching and an additional 24-h incubation period, DNA contents and APase levels were determined. The DNA content in each well showed that cells increased their number continuously throughout the culture period (Fig. 2A). Although cellular APase showed a steady decrease from day 5 (Fig. 2B), there seemed to be a shift, by stretching, from a positive early effect to a negative effect toward the end of the culture period. Stretching, however, did not have a significant effect throughout the incubation period. One the other hand, the matrix contents of mineral (Fig. 3A) and BGP (Fig. 3B) and the amount of BGP secreted into the culture medium (Fig. 3C) showed dramatic changes. After day 9, cells started to respond to the stress significantly. A significant increase in BGP production was detected on day 9 in stretched cells. The effect on mineral deposition was apparent by day 13 ($P < 0.01$), which peaked on day 20 in control cultures. Stretching accelerated the accumulation of BGP and mineral, such that higher levels were reached at an earlier time point.

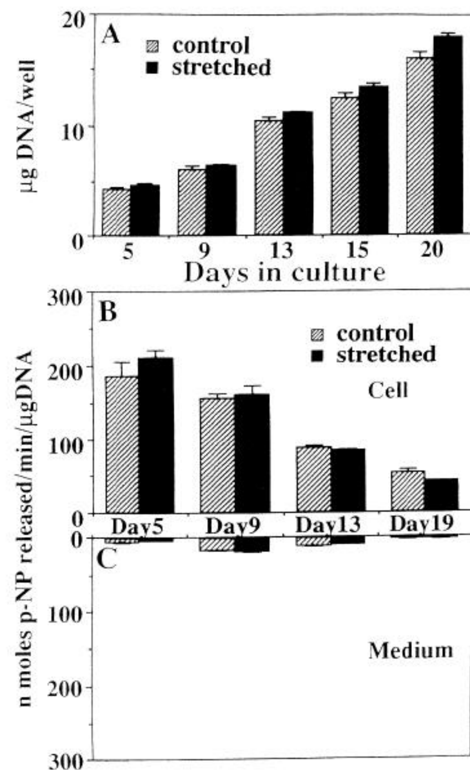

FIG. 2. Effects of physiological strain on proliferation and cell and medium APase levels and in long term cultures of rat calvarial osteoblasts (C). Fraction III osteoblasts were cultured on a six-well, flexible bottomed plate coated with type I collagen at the original density of 2.5×10^5 cells/well. On days 5, 9, 13, 15, and 20 of culture, negative pressure of 2 kPa was applied cyclically to the bottom of plates. After 12-h stretching and the additional 24-h incubation, cultures were harvested, washed with TBS, and frozen. DNA contents (A) and APase levels (B) in the stretched cultures (■) and in the controls (▨) were determined as described in *Materials and Methods*. In C, APase activity in 25-μl medium was also measured to calculate the released enzyme into medium during the incubation period. n = 6 in each experiment. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$.

Effects of stretching on osteoblastic and osteocytic calvarial cells cultured on Matrigel

Instead of comparing osteoblastic cells at different points in the 20-day culture period, sequentially prepared cells at

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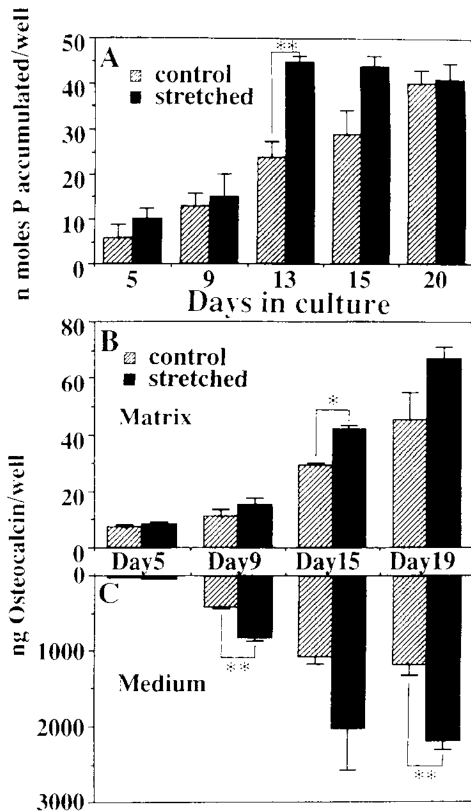

FIG. 3. Effects of physiological strain on matrix mineralization in long term cultures of rat calvarial osteoblasts. Fraction III osteoblasts were cultured, stretched as described in Fig. 2, harvested, washed with phosphate-free TBS, and subjected to acid extraction to recover mineral and the associated matrix proteins. Methods for the determination of BGP and phosphorus in the stretched cultures (■) and in the controls (▨) were described in *Materials and Methods*. n = 6 in each experiment. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$.

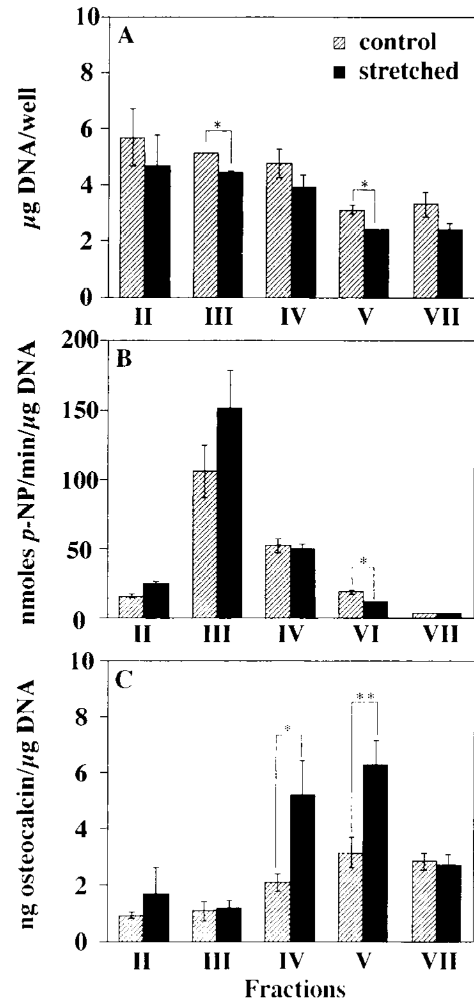

FIG. 4. Lineage-dependent effects of physiological strain on proliferation and levels of APase and BGP in differentiated bone cells. Isolated cells from fractions II–VII (2.5×10^5/well) were cultured for 6 days on Matrigel and then exposed to the strain of about 4000 μE, as described in *Materials and Methods*. After 12-h stretching and another 24-h incubation, cells were harvested together with Matrigel. DNA (A) and APase (B) levels and matrix contents of BGP (C) were determined in stretched cells (■) and in the controls (▨). Because of the limited recovery of cells, either fraction V or VI was included in each set of experiments to make n = 4. *, $P < 0.05$; **, $P < 0.01$.

different stages in the bone cell lineage were compared on Matrigel, which is a reconstituted basement membrane matrix (26). As described in *Materials and Methods*, cells in fractions II–VII were subjected to a strain of approximately 4000 μE on Matrigel. In a six-well plate, cells resided on Matrigel, which was placed 5–7 mm from the edge of the cyclically stretched flexible substrate. The effects of the stretching on DNA (A), APase (B), and matrix BGP (C) levels are depicted in Fig. 4. The results show a trend toward decreased cell proliferation based on the DNA contents in response to stain. Proliferation seems to be generally suppressed judging from the DNA contents (see Fig. 4A; fractions III and V for significant results). In addition, osteocytic fraction VI cells exhibited a lower APase level than the control cells (Fig. 4B). Similarly, cells in fractions IV and V were responsive to the strain, accumulating a significantly greater amount of BGP, as shown in Fig. 4C. The results of other fractions in Fig. 4, B and C, did not show any significant difference between the experimental and the unstrained control samples. Results from further analysis of DNA contents in the presence and absence of 1×10^{-6} M indomethacin are shown in Fig. 5. Proliferation of cells was unaffected by stretching in fractions II, III, and IV. It was suppressed in fractions V and VII (Fig. 5A) and fraction VI (Fig. 5B). Apparently, changes in the presence of indomethacin (Fig. 5B) were almost identical to those in its absence (Fig. 5A). We also tested the effectiveness of indomethacin in BGP synthesis between 1×10^{-8} and 1×10^{-6} M. The results showed that indomethacin by itself induced the synthesis of BGP in a dose-dependent manner, and there was no inhibition of stretch-induced BGP accumulation by indomethacin (data not shown). Further analysis showed that inhibition by indomethacin of stretch-induced PGE_2 release was observed only in fractions II and III (Table 1).

Distinct responses of young osteocytes to stretching

Notable responses to the low strain among the latter fractions were further explored in the production of potential transducers, such as IGF-I and cellular cAMP. In Fig. 6, effects of stretching and PTH administration on cAMP levels

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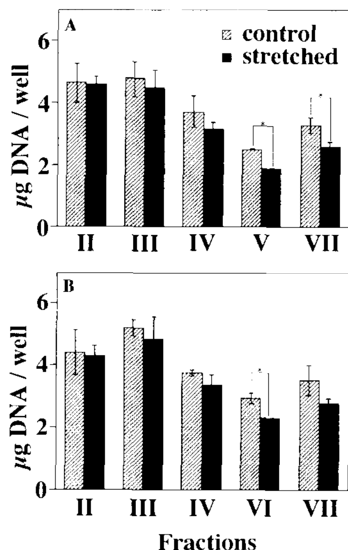

FIG. 5. Effects of strain on proliferation in the presence and absence of 1×10^{-6} M indomethacin. As in Fig. 4, 2.5×10^5 cells were cultured for 6 days on Matrigel, preincubated with and without 1×10^{-6} M indomethacin for 2 h, and then exposed to the strain of about 4000 μE, as described in *Materials and Methods*. After 12-h stretching and another 24-h incubation, cells were harvested for DNA assay. DNA in stretched cells (■) and in the control cells (▨) are plotted in the absence (A) and presence (B) of indomethacin. Either fraction V or VI was included in each set of experiments to make n = 4. *, $P < 0.05$; **, $P < 0.01$.

TABLE 1. Changes in the amount of PGE_2 released from stretched bone cells by the presence of 1×10^{-6} M indomethacin

	Fractions				
	II	III	IV	V	VII
Difference in PGE_2 (pg/ml)	−210 ± 107	−74 ± 43	22 ± 26	28 ± 19	9 ± 20

Values are the mean and SEM (n = 3). The figures represent the difference caused by the inclusion of 1×10^{-6} M indomethacin 2 h before stretching. Cells were stretched for 15 min, and the medium was collected for PGE_2 assay.

were compared among fractions II–VII. Stretching affected fraction IV and VI cells significantly, as shown in Fig. 6A. Osteoblastic fraction III cells, however, did not show any significant response to the stretching. When PTH was tested, not only fractions IV and V, but all other fractions as well responded to PTH in a distinct manner (Fig. 6B). In another experiment, we measured IGF-I secretion in stretched and control cells and compared the results among the fractions. Similar to the results presented in Fig. 6A, cells in fractions IV and VI responded the most to stretching, resulting in the secretion of 2–3 times more IGF-I into the medium. Fraction III osteoblasts did not respond to the stress in any measurable way (Fig. 7). The IGF-I content in the cell layer including Matrigel was approximately 20 ng/well in every fraction regardless of exposure to stretching.

Discussion

More than 90% of bone cells are osteocytes (35). Young osteocytes have been reported to respond to modulators such

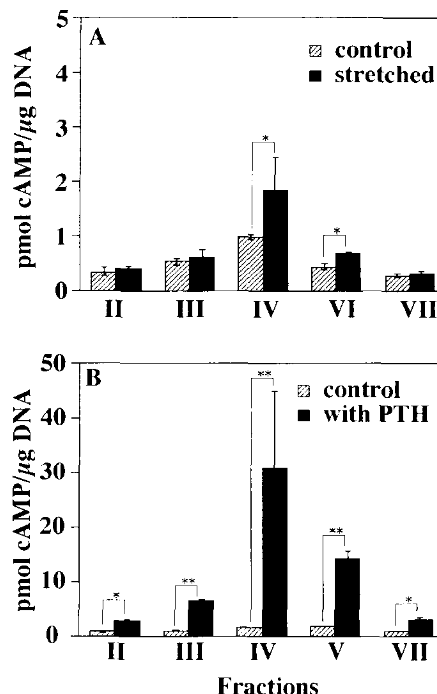

FIG. 6. Effects of stretching (A) and PTH administration (B) on cAMP levels in the cultures of fractions II–VII. cAMP was extracted from the cells after 15-min stretching with acidic ethanol, as described in *Materials and Methods*. Before stretching, cells were incubated with 5 mM isobutylmethylxanthine for 20 min. In Fig. 4B, 200 ng/ml PTH-(1–34) were administered instead of stretching the cells after the 20-min incubation with isobutylmethylxanthine. After an 8-min incubation of the cells at 37 C, cAMP was extracted with acidic ethanol. An RIA kit was used to determine the levels of cAMP, as described in *Materials and Methods*. Either fraction V or VI was included in each set of experiments to make n = 4. *, $P < 0.05$; **, $P < 0.01$.

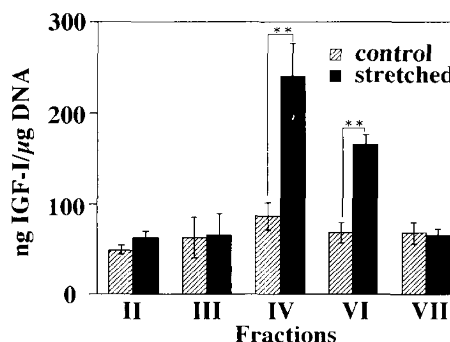

FIG. 7. Effects of stretching on IGF-I secretion in the cultures of fractions II–VII. IGF-I was extracted from the medium after 12-h stretching, as described in *Materials and Methods*. An RIA kit was used to determine the levels of IGF-I. *, $P < 0.05$; **, $P < 0.01$.

as PTH (36) and vitamin D_3 (37). In our previous study, we proposed the involvement of young osteocytes in the extracellular processing of matrix proteins, leading matrix to mineralization (26). By sequentially preparing calvarial cells and culturing them on Matrigel to promote their differentiation, it became possible to demonstrate the consequences of osteocytic development in biochemical terms (26). The study reported in this article was specifically designed to analyze the lineage-dependent responses of bone cells to mechanical stress. Our observations of differential responses in isolated

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bone cell populations demonstrate a specific role of osteocytes in mechanical responses and are consistent with recent results of *in situ* experiments (18, 19). Additionally, we found that the most responsive populations are of the intermediate cells, which we recovered in low abundance between mature osteoblasts and osteocyte-like cells. We believe that they correspond to transitional young osteocytes with characteristic morphology and biochemical features (26). Because of the large osteocyte population in bone and their ability to direct the entire bone through an extensive network of cell processes, one would expect the aforementioned transient young osteocytes to play a major role in bone metabolism. We have clearly shown that these cells are much more sensitive to low level mechanical stress of a physiological magnitude than cells in other phases of development.

The general effect of mechanical stress on bone has been a subject of continued interest over 3 decades (2–4). In a previous study from this laboratory, increased proliferation of primary rat osteoblasts was demonstrated (9). Since then, diverse effects of mechanical stress have been reported in a variety of systems: osteoblasts, primary or after a few passages (6–11), and the cells of osteoblast-like cell lines (11–14). Generalization of the effects was difficult due to additional variation in the magnitude and duration of the stress applied to these cells, the devices used to induce stress, and the parameters chosen in the studies. In a more recent review, however, Burger and Veldhuijzen (15) pointed out that the confusion stems from the fact that osteoblastic cells respond differently to high and low stress. To high stress, the cells respond with increased PGE_2 synthesis followed by increased proliferation. The release of resorption-stimulating factors is also induced, whereas APase activity, collagen synthesis, and matrix calcification are reduced. On the other hand, with low cyclic stress of physiological magnitude, cell proliferation is slightly inhibited, whereas APase activity and protein synthesis are increased. In organ cultures, matrix mineralization is also stimulated. These two differing responses correspond to the two phases of bone formation, *i.e.* growth of trabecular bone and mineral apposition, respectively (15). In this report, we reveal a new factor of complication, the stages of differentiation of cultures subjected to the stress. We know of no studies that analyze the response of isolated bone cells to mechanical stress with respect to the lineage of bone cells. A few studies have indicated a difference in response among cell populations (28, 38, 39).

Development of the osteocytic canalicular network has distinct advantages in mechanosensory transduction. It has been postulated that osteocyte processes not only sense the deformation of bone directly through the strained cell processes, but also sense small fluid shear stress acting on the membranes of extensive cell processes (40, 41). Osteocytes are well equipped to transmit local mechanical stimuli to other resident osteogenic cells throughout the bone via the canaliculi and cell-cell junctions (20–23; see Ref. 42 for a review). We have shown through two experimental approaches that the responses of calvaria-derived primary cells to stretching are dependent on their maturation. In our first approach, the osteoblastic fraction III cell population, which expressed the highest level of APase, was exposed to strain at different time points during the 20-day culture period. In this system, cells underwent differentiation to a limited extent from mature osteoblasts to osteocytic cells. Mineralization was apparently accelerated by stretching judging from both the decline of APase and the accumulation of BGP/mineral in the matrix. This phenomenon was especially significant in the later phase of the 20-day culture period. Our findings are consistent with stage-specific responses to other modulators that have been observed by other researchers in a similar culture of rat calvarial cells (34, 43). In our second approach, differentiated osteocytic cells derived from the interior of calvarial bone chips (fraction IV–VII) were compared to osteoblastic cells from the surface (fractions II and III) on Matrigel (26). In addition to promoting differentiation of osteocytic cells, the extended use of Matrigel in stress-related experiments has a definitive advantage over the use of tissue culture plastic. The biological characteristics, such as platelet-derived growth factor-stimulated receptor autophosphorylation, are of stressed cells in monolayer culture on plastic and not of relaxed cells on matrix such as collagen gel (44). In other words, artificially stretched cells adhering to the plastic support cannot serve as a control to the mechanically stretched cells. On Matrigel, we showed the magnitude of response to stretching was most significant in cells recovered between the osteoblastic fraction III and osteocytic fraction VII cells. The response of osteoblastic fraction III cells was not significant. A similar response was observed among the fractions in the levels of IGF-I and cAMP as well. These responsive cells, which, we believe, correspond to young osteocytes or very mature osteoblasts, can react better to low level physiological strain than cells at any other stage. It should be noted that the expression of PGE_2 or PTH receptors may not necessarily be proportional to the response measured by cAMP; for example, in rat bone marrow cells. The response is higher at much later stages of development compared to the stages of expression of receptors (Kasugai, S., personal communication). In this context, we have to reserve the possibility that young osteocytes are somehow extremely sensitive to a low level of PGE_2 or PTH than any other type of bone cell.

Generally, PGE_2 is accepted as the mediator of strain-induced bone remodeling, because strained bone cells release PGE_2, increasing cellular cAMP and DNA synthesis (6–8, 12). An increased bone mass is reported *in vivo* as a result of prostanoid administration (45). Elevated biosynthesis of both DNA and collagen by 0.1 μM PGE_2 was reported in fetal rat calvaria and osteoblast-enriched cell populations. Therefore, stretch-induced PGE_2 release has been suggested as the cause of increased DNA and matrix synthesis in bone-derived cells, an effect that could be blocked by indomethacin. Indomethacin, an inhibitor of cyclooxygenase (now called prostaglandin G/H synthetase), in both constitutive and inductive forms, has been said to abolish these effects of strain, supporting the idea that prostanoids act as a mediator (45). In these experiments, however, mechanical stress applied to the cells was relatively high compared to the physiological intensity experienced by the bone cells. Murray and Rushton (30) pointed out that the magnitude of strains used for these studies was between 10–100 millistrain, which is out of the physiological range and should, instead, be associated with microfracture. They postulated that PGE_2 stim-

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ulates the osteogenesis caused by increased functional demands and initiates bone remodeling (30). By using the Flexercell Strain Unit, a number of groups reported that osteoblastic cells responded to 17–24% elongation by aligning themselves perpendicularly to the strain field (10, 14, 28). In our study with calvarial fraction III osteoblasts, a maximum 6% elongation did not cause any such morphological changes (data not shown), which is consistent with the report by Murray and Rushton (30). We showed that indomethacin decreased the level of secreted PGE_2 only in fractions II and III, not in fractions IV–VII. Neither the effect of stretching on young osteocytes, the inhibition of cell proliferation, or the stimulated accumulation of BGP was reversed by indomethacin treatment. In our preliminary experiment, however, indomethacin was effective on the responses of osteoblasts to the higher strain of a maximum 18% elongation, among fraction I–IV cells, PGE_2 secreted by the stretched cells was more significant in the less differentiated fractions, and this effect of strenuous 18% stretching was blocked by indomethacin (Suzuki, Y., and Y. Mikuni-Takagaki, unpublished results). This agrees well with the report that the PGE_2 receptor EP_2, which is responsible for cAMP production, appears in osteoblast progenitor cells in rat bone marrow cell culture (46). Therefore, it is likely that strenuous strain affects preosteoblasts in an indomethacin-dependent manner, and the physiological strain affects young osteocytes in an indomethacin-independent manner. Carvalho *et al.* (28) also reported that an effect of stretching on both cAMP and inositol trisphosphate levels, measured in the presence of PTH, was not sensitive to indomethacin. In our system of isolated osteocytic cells, prostanoid does not seem to be involved directly in the anabolic effect of low level physiological stress on young osteocytes. The indirect effect of prostanoids, however, may explain the effectiveness of indomethacin in *in vivo* systems, for example, probably due to a mixed cell population. On the other hand, the response of osteocytic cells to stress at the physiological level was significantly affected by cation channel blockers such as gadolinium or amiloride (Mikuni-Takagaki, Y., unpublished observation). The involvement of stretch-activated cation channels that are activated by PTH (47) may explain the complex nature of signal transduction pathway(s) in mechanically stressed cells. The transducers are very likely to cross-talk to other transducers, which are already known to influence each other in other signal transduction pathways (28, 48–56). In conclusion, we state that the young osteocyte is the mechanosensor in bone responding to physiological, low level strain. Further studies to characterize the underlying transduction mechanisms in the responsive population will shed light on the regulation of bone mass in health and diseases.

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