Journal of Cellular Biochemistry 81:547–556 (2001)

Age-Dependent Decline in Bone Nodule Formation Stimulating Activity in Rat Serum is Mainly Due to the Change in the Corticosterone Level

Masaki Fujieda, Noriaki Takao, Michiaki Kiriu, Shigeki Mizuochi, Hiroyuki Kaneki, and Hayao Ide*

Department of Hygienic Chemistry, School of Pharmaceutical Sciences, Toho University, Japan

Abstract The replacement of fetal bovine serum with rat serum in a culture medium brought about a marked increase in the formation of mineralized bone nodules (BN) in primary cultures of rat calvarial cells. These effects of rat serum were most prominent when added during the early phase of the culture, indicating that the serum factor mainly acts on the cells during the growing phase. A significant increase in BN formation was observable at final rat serum concentration as low as 1%, and the effect was dependent on serum concentration, at least up to 10%. The addition of rat serum also increased alkaline phosphatase (ALP) activity, collagen synthesis, and DNA synthesis in calvarial cells. BN formation stimulating activity was extractable with ethyl acetate. The ethyl acetate extract was purified by TSK-GEL OH-120 column chromatography by monitoring the stimulation of ALP activity in ROS 17/2.8 cells. The chromatographic behavior of the ALP activity was found to be identical to that of corticosterone, the major glucocorticoid in rodents and the preincubation of the purified fraction with anticorticosterone antibody abolished the ALP stimulating activity. These results suggest that BN formation stimulating activity in rat serum is mainly attributable to corticosterone. The concentration of serum corticosterone decreased with age in parallel with BN formation stimulating activity, which suggests that the physiological level of corticosterone may have a regulatory role in the maintenance of osteoblast function. J. Cell. Biochem. 81:547–556, 2001. © 2001 Wiley-Liss, Inc.

Key words: corticosterone; aging; osteoblasts; mineralized bone nodules; serum

Growth factors present in the bone matrix, such as insulin-like growth factors I and II (IGF-I and IGF-II) [Williams et al., 1989; Jones and Clemmons, 1995; Middleton et al., 1995], platelet-derived growth factor [Hauschka et al., 1986, Gowen, 1994], and transforming growth factor-β [Centrella et al., 1987, Bonewald and Mundy, 1990] have been suggested to play key roles in coupling between resorption and formation as autocrine or paracrine regulators. These factors, synthesized and secreted by osteoblasts, are incorporated into the bone matrix during bone formation, and are thought to be released during bone resorption to stimulate osteoblastic cell proliferation and bone matrix synthesis in the local area by autocrine and paracrine mechanisms [Canalis and Lian, 1988; Goldring and Goldring, 1989; Turner et al., 1994]. In contrast, systemic factors present in serum, such as estrogen [Gray et al., 1987; Robinson et al., 2000; Cheng et al., 1999], parathyroid hormone [Hassager et al., 1992] and 1,25-dihydroxyvitamin D_3 [Civitelli et al., 1990; Noda et al., 1990; Matsumoto et al., 1991; Farach-Carson and Ridall, 1998] have also been suggested to play key roles in bone metabolism on direct or indirect mechanisms. These observations suggest that osteoblast functions are under the control of systemic factors as well as local factors.

Cell populations enzymatically isolated from the membranous bone of fetal or newborn rats and mice, containing osteoblasts and their precursor cells, serve as a useful in vitro model for studying the effects of hormones and growth factors on these osteoblastic cells independent of bone resorption by osteoclasts [Bellows et al., 1986; Yokose et al., 1996]. The osteoblastic cells

*Correspondence to: Dr. Hayao Ide, Department of Hygienic Chemistry, School of Pharmaceutical Sciences, Toho University, Chiba 274-8510, Japan.
E-mail: ide@phar.toho-u.ac.jp

Received 7 September 2000; Accepted 2 November 2000

This article published online in Wiley InterScience, February XX, 2001.

form mineralized bone nodules (BN) with a histological, immunohistochemical, and ultrastructural appearance similar to that of woven bone in the presence of ascorbic acid and β-glycerophosphate. Kato et al. [1995], modifying the fetal cell culture technique, recently developed a culture model for BN formation by adult calvarial cells and showed that fibroblast growth factor-2 only marginally enhanced BN formation, although this growth factor has a prominent stimulatory effect on fetal calvarial cells, suggesting the possibility that the responsiveness to regulatory factors differs between adult and fetal cells.

We found in the present study that rat serum contained a factor, which greatly stimulates the proliferation and differentiation of adult rat calvarial cells. Purification of the BN formation stimulating activity revealed that most of it is attributable to corticosterone, the major glucocorticoid in rodents. The age-related changes in the concentration of corticosterone in the serum were also studied.

MATERIALS AND METHODS

Materials

Female Wistar rats (5–90 weeks old) were purchased from Clea Japan, Inc. (Tokyo, Japan). They were allowed free access to Clea CE7 laboratory pellets and water prior to experiments. Rat sera were prepared from blood collected at 2 p.m. Corticosterone was purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), phenol red-free F-12 medium, and α-minimum essential medium (α-MEM), and trypsin were products of Gibco BRL (Rockville, MD). Kanamycin, ascorbic acid, collagenase, and β-glycerophosphate were obtained from Wako Pure Chemical Industries (Tokyo, Japan). ROS 17/2.8 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan).

Cell Culture

Cells enriched for osteoblast phenotype were enzymatically isolated from the calvaria of 25-week-old female Wistar rats according to the method of Bellows et al. [1986], modified by Kato et al. [1995]. Briefly, after five sequential digestions of calvaria with a mixture of collagenase and trypsin, the released cells from the last three digestion intervals were grown in the F-12 medium containing 10% FBS. After reaching confluence, the cells were collected by trypsin treatment, seeded in 4-well dishes at 2×10^3 cells/cm^2 in the same medium and cultured for 4 days (designated as the proliferation period, P1–P4). At the end of day P4, the medium was changed to α-MEM supplemented with 10% FBS, 2 mM β-glycerophosphate, and ascorbic acid (0.1 mg/ml), and the cells were maintained for a further 18 days (designated as the mineralization period, M1–M18).

ROS 17/2.8 cells were maintained in the F-12 medium containing 10% FBS at 37°C in a humidified atmosphere of 5% CO_2/95% air and subcultured every 5 days.

Determination of Markers for Cell Proliferation and Differentiation

For the determination of ALP activity, collagen synthesis, BN formation, and DNA synthesis, cells were cultured in the F-12 medium containing 10% rat serum on days P2–P4. An ALP assay was performed at the beginning of day M2 according to the method of Lowry et al. [1954], using *p*-nitrophenylphosphate as a substrate. For the assay of collagen synthesis, cells were incubated in serum-free α-MEM medium containing [2,3-^{3}H]proline (1.25 μCi/ml, Dupont, Wilmington, DE) for 5 h at the beginning of day M2. The incorporation of radioactivity into collagenase-digestible protein was measured as described by Peterkofsky and Diegelmann [1971]. Quantification of BN was performed by visualization with von Kossa stain [Bhargava et al., 1988], and the total nodule area and the number of nodules were assessed on day M18, using a colony counter (BMS-400; Toyo Sokki, Tokyo, Japan). For the determination of DNA synthesis, the cells were incubated for 3 h in serum-free α-MEM medium containing [^{3}H]thymidine (1.25 μCi/ml, Dupont) at the beginning of day M1, and incorporation of radioactivity into DNA was measured.

Purification of BN Formation Stimulating Activity

Since it takes more than 3 weeks to determine the BN formation stimulating activity using rat calvarial cells, the activity in column chromatography fractions was monitored by the ability to increase ALP activity in ROS 17/2.8 cells for convenience. It takes only 3 days to determine the ALP activity in ROS 17/2.8 cells and the fractions with the BN formation

stimulating activity also increase the ALP activity in ROS 17/2.8 cells so far as we tested. The cells were cultured in 96-well plates at 2×10^3 cells/cm^2 in 200 μl of the F-12 medium/10% FBS for 24 h. The medium was then replaced with 200 μl of the F-12 medium/10% FBS containing an aliquot of a fraction. After incubation for further 48 h, the medium was removed and 25 mM 2-amino-2-methyl-1-propanol, pH 10.5, containing 2 mM $MgCl_2$ and 2 mM *p*-nitrophenylphosphate was added. After 1 h reaction, the *p*-nitrophenol formed from *p*-nitrophenylphosphate was determined by absorbance at 410 nm.

One milliliter of serum from 10-week-old rats was mixed with 5 ml of ethyl acetate in a test tube and the two phases were separated after vigorous shaking. The BN formation stimulating activity was quantitatively recovered in the ethyl acetate phase. The ethyl acetate extract was dried, dissolved in 50 μl of hexane:ethanol (4:1) and applied to a TSK-GEL OH-120 column (4.6×250 mm; TOSOH, Tokyo, Japan). The elution was carried out with 10 ml of hexane:ethanol (4:1) at the rate of 1 ml/min and 1 ml fractions were collected. Each fraction was dried and dissolved in 10 μl ethanol. Aliquots (1 μl) from each fraction were mixed with 1 ml of the F-12 medium/10% FBS and used for the determination of the ability to stimulate ALP activity in ROS 17/2.8 cells. The fractions containing the activity were pooled and re-chromatographed on the same TSK-GEL OH-120 column.

Radioimmunoassay of Serum Corticosterone Concentration

The serum corticosterone was measured by radioimmunoassay using the method of Maldonado et al. [1989] with minor modifications. [^{3}H] Corticosterone (specific activity 100 Ci/mmol; New England Nuclear, Boston, MA), was used with rabbit antiserum to corticosterone (Biogenesis, Poole, UK). Triplicate samples of the serum to be assayed for total corticosterone were extracted with 5 volumes of ethyl acetate at room temperature. Precipitated proteins were removed by centrifugation and samples of the supernatant extract were pipetted into small tubes and evaporated to dryness. After dissolving in absolute ethanol, a part of these was used as a sample. Samples and standards were incubated with antiserum for 30 min at room temperature. Tritiated ligand was added, and incubated at 37°C for 1 h, then cooled on ice at 4°C for 15 min. Unbound ligand was removed with cold dextran-coated charcoal suspension, and residual radioactivity was measured in the supernatant using Beckman scintillation counter. The method had sensitivity in the range 15–2000 pg corticosterone.

Statistical Methods

Data were analyzed by Student's *t*-test or by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. $P < 0.01$ was considered significant. All data are presented as the mean ± SD of four cultures.

RESULTS

We first examined the effect of 10-week-old rat serum on the area and number of BN formation by calvarial cells from 25-week-old rats. When rat serum was added to the culture medium during days 2–4 of the proliferation period (P2–P4), the area and number of BN were increased 4.3- and 2.0-fold, respectively (Fig. 1A, B). Phase-contrast photomicrographs of BN visualized with the von Kossa stain in the culture of osteoblasts from 25-week-old rat are shown in Figure 1C. An almost identical profile was obtained when a medium containing FBS (10%) and rat serum (10%) was added. These results suggest that the rat serum contains a factor(s) which stimulates BN formation.

Figure 2 shows the effect of varying the time and duration of serum addition on the area and number of BN by rat calvarial cells. The sera used in this experiment were from 10-week-old (young) and 90-week-old (aged) rats. When young rat serum was present in the culture medium for 24 h on one of the days in the proliferation period or early stage of the mineralization period, a gradual increase in the stimulatory effect of BN formation was observed during the proliferation period with maximal stimulation on day 4 (P4) and the effect gradually decreased from day 1 to day 4 in the mineralization period (M1–M4) (Fig. 2A, C). More marked stimulation was observed when young rat serum was present for 72 h during either the last 3 days during the proliferation period (P2–P4) or the first 3 days during the mineralization period (M1–M3) (Fig. 2B, D). In contrast, aged rat serum

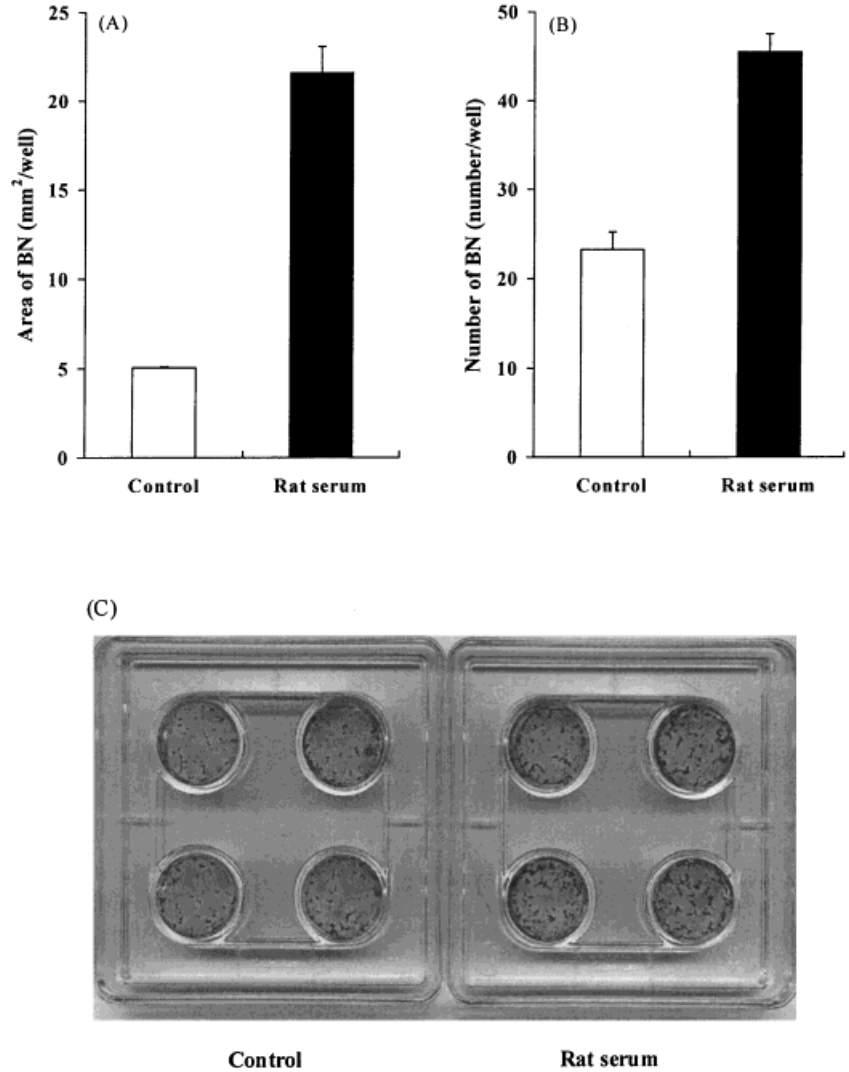

Fig. 1. Effect of 10-week-old rat serum on the formation of BN in the culture of osteoblasts from 25-week-old rats. 10% FBS (□) or 10% 10-week-old rat serum (■) was included in the medium for 72 h during the last 3 days during the proliferation period (P2–P4). Medium for both groups contains 10% FBS during the rest of the culture period. The area (**A**) and number (**B**) of BN were measured on day M18. (**C**) Phase-contrast photomicrographs of BN visualized with von Kossa stain in the culture of osteoblasts from 25-week-old rats. Other conditions were as described in Materials and Methods. Each value represents the mean ± SD of four cultures. The experiments were repeated twice and the results were essentially the same as those depicted.

showed little effect on BN formation when present for 24 h on one of the days during the proliferation period (P1–P4) or early stage of the mineralization period (M1–M4) (Fig. 2A, C). However, aged rat serum significantly stimulated BN formation when it was present for 72 h during either the last 3 days during the proliferation period (P2–P4) or the first 3 days during the mineralization period (M1–M3) (Fig. 2B, D). Since it takes 6–7 days after seeding for the cells to become confluent, the results stated above suggest that rat serum is required to be present continuously when cells are rapidly growing.

Standard media for the control culture of osteoblasts contain 10% FBS. When varying portions of FBS were replaced with young or aged rat serum during P2–P4, a dose-dependent increase in the area and number of BN formation was observed (Fig. 3A, B). Rat serum from either young or aged donors also enhanced the ALP activity, incorporation rate of [^{3}H]thymidine and of [^{3}H]proline in a dose-dependent manner (Fig. 3C–E). The effects of

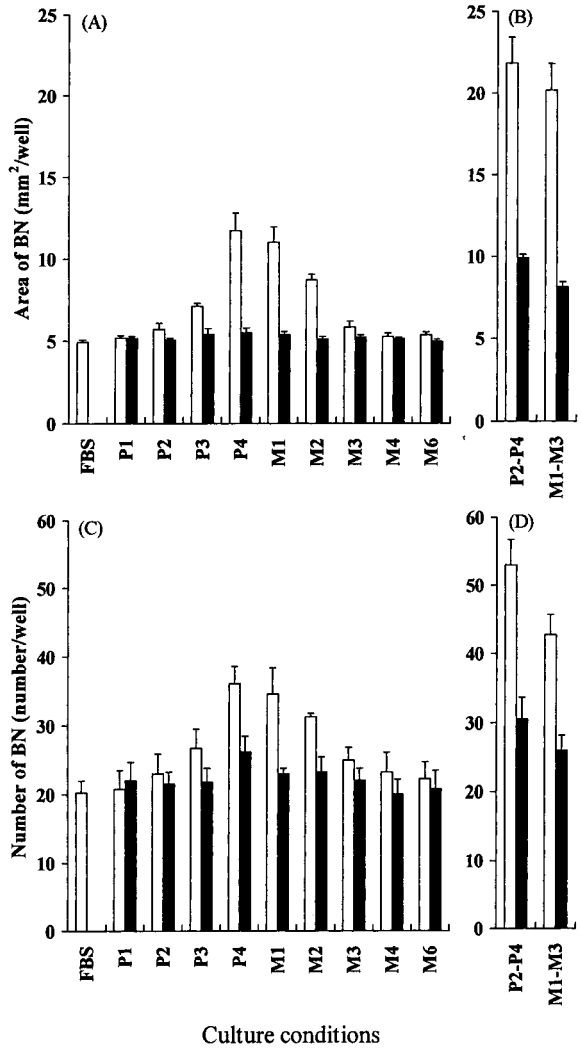

Fig. 2. Effect of varying the time and duration of the addition of rat serum on the formation of BN in the culture of osteoblasts from 25-week-old rats. **A** and **C**: Young (10-week-old, □) and aged (90-week-old, ■) rat sera were included in the medium for 24 h on one of the days during the proliferation period (P1–P4) or during the mineralization period (M1–M6). In a control experiment, FBS was included in the culture medium throughout the culture period. **B** and **D**: Young (□) and aged (■) rat sera were included in the medium for 72 h during the last 3 days during the proliferation period (P2–P4) or the first 3 days during the mineralization period (M1–M3). Rat serum concentration was 10% in all experiments. The area (A and B) and number (C and D) of BN were measured on day M18. Other conditions were as described in Materials and Methods. Each value represents the mean ± SD of four cultures. The experiments were repeated twice and the results were essentially the same as those depicted.

aged rat serum were less potent than those of young rat serum on all of these parameters.

We next tried to purify the BN formation stimulating activity, which was monitored by measuring ALP activity in ROS 17/2.8 cells. After extraction with ethyl acetate, the activity was quantitatively recovered in the organic phase (data not shown). Since the extracted activity was resistant to heat (100°C, 5 min) and trypsin, and filtrated through an Amicon YC-05 membrane (molecular weight cut-off < 500), the BN formation stimulating factor was suggested to be a steroid. When the ethyl acetate-extracted activity was chromatographed on a TSK-GEL OH-120 column, the activity was eluted as a single peak with hexane:ethanol (4:1) solution. The fraction containing the activity was re-chromatographed on a TSK-GEL OH-120 column (Fig. 4A, B), and the retention time of the activity coincided with that of corticosterone (Fig. 4C). The ALP stimulating activity of this fraction was completely blocked by the treatment with neutralizing antibody against corticosterone (Fig. 5).

We next examined the age-dependent changes in the serum corticosterone level and in the effects of the serum on the markers for differentiation and proliferation of osteoblasts. As shown in Figure 6D, the serum corticosterone concentration sharply declined from 10 to 20 weeks, and then gradually decreased up to 90 weeks. The BN formation stimulating activity (Fig. 6A, B) and the rate of [^{3}H]thymidine incorporation into the cells (Fig. 6C) also declined with age in parallel with serum corticosterone concentration (Fig. 6D).

In the next experiment, the ability to stimulate BN formation was compared between rat serum and corticosterone. From the data shown in Figure 6D, the concentration of corticosterone in 10-week-old rat serum was estimated to be approximately 10^{-6} M. When 10-week-old rat serum was added to the culture of calvarial cells from 25-week-old rats on day P4 at a final concentration of 10%, the area and number of BN increased by 130 and 73%, respectively. Corticosterone at a final concentration of 10^{-7} M also increased the area and number of BN both by 112%. These results suggested that corticosterone is as potent as rat serum in the stimulation of BN formation by calvarial cells, as was shown in the ability to stimulate the ALP activity in ROS 17/2.8 cells.

Figure 7 shows a comparison of the degree of stimulation of ALP in ROS 17/2.8 cells among 25-week-old rat serum, the purified fraction from 25-week-old rat serum and corticosterone. Prior to this experiment, the volume of the purified fraction was adjusted to that of

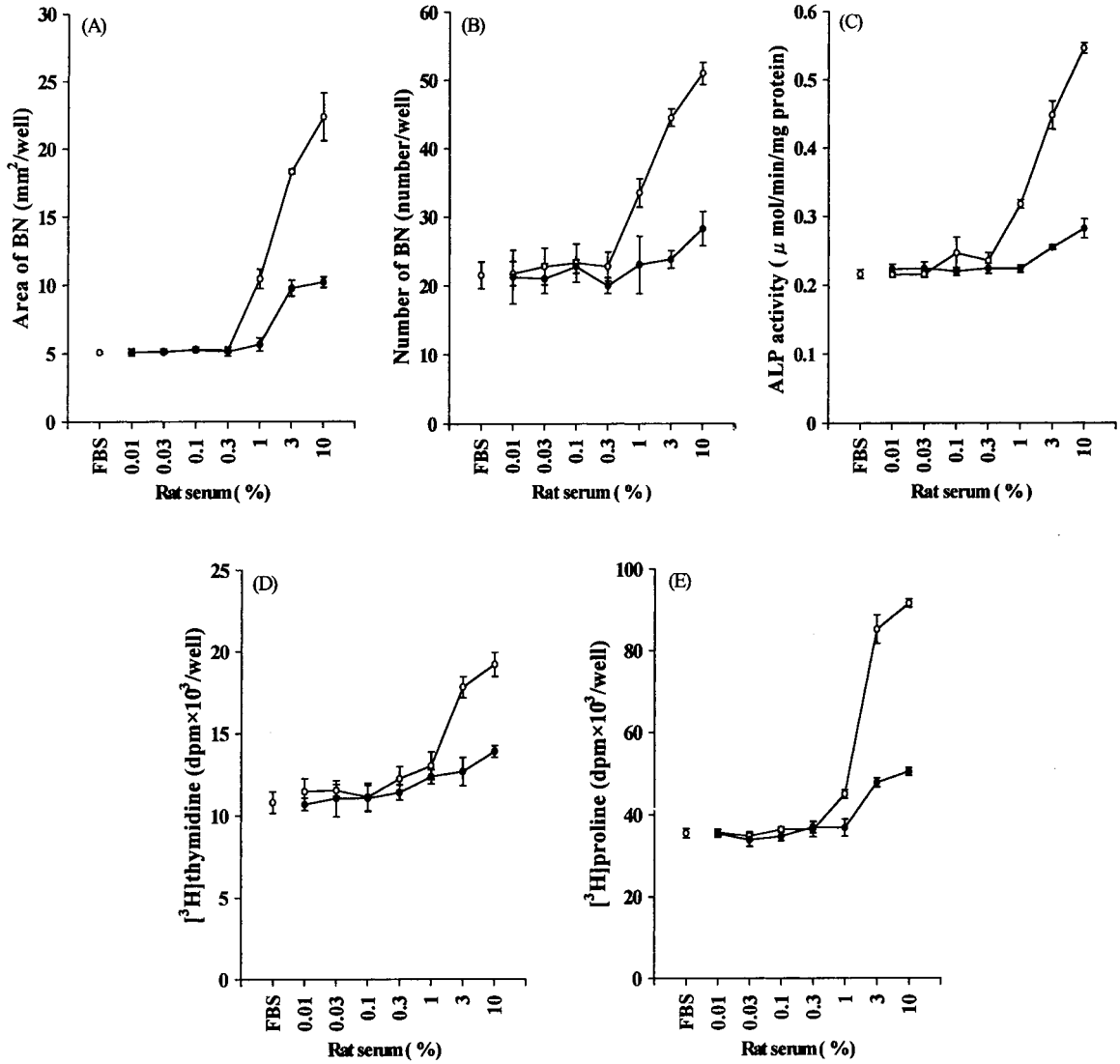

Fig. 3. Dose-dependency of the effect of young and aged rat sera on BN formation, ALP activity, collagen synthesis, and DNA synthesis in the culture of osteoblasts from 25-week-old rats. The cells were treated with various doses of young (○) or aged (●) rat serum for 72 h during the last 3 days during the proliferation period (P2–P4). The area (**A**) and number (**B**) of BN were measured on day M18. ALP activity (**C**) and [^{3}H]proline incorporation into collagenase-digestible protein (**E**) were measured at the beginning of day M2. [^{3}H]Thymidine incorporation into the cells (**D**) was measured at the beginning of day M1. Other conditions were as described in Materials and Methods. Each value represents the mean ± SD of four cultures. The experiments were repeated twice and the results were essentially the same as those depicted.

25-week-old rat serum from which the purified fraction originates. Significant stimulation of the ALP activity was observed when rat serum was added to the culture at final concentration of 10 and 1%, but not at 0.1%. Similar dose dependency was observed with 10, 1 and 0.1% purified fractions. Corticosterone at a final concentration of 10^{-7} M also stimulated the ALP activity to the same level as those obtained by 10% rat serum or 10% purified fraction, indicating that recovery after purification of the serum factor is nearly complete.

From the overall results, it was suggested that the factor responsible for the BN formation stimulating activity in adult rat serum is mainly corticosterone.

DISCUSSION

In the present study, we found that a single pulse exposure of adult calvarial cells to adult rat serum markedly increased BN formation in a dose-dependent manner. The stimulation was observed regardless of the presence of FBS in

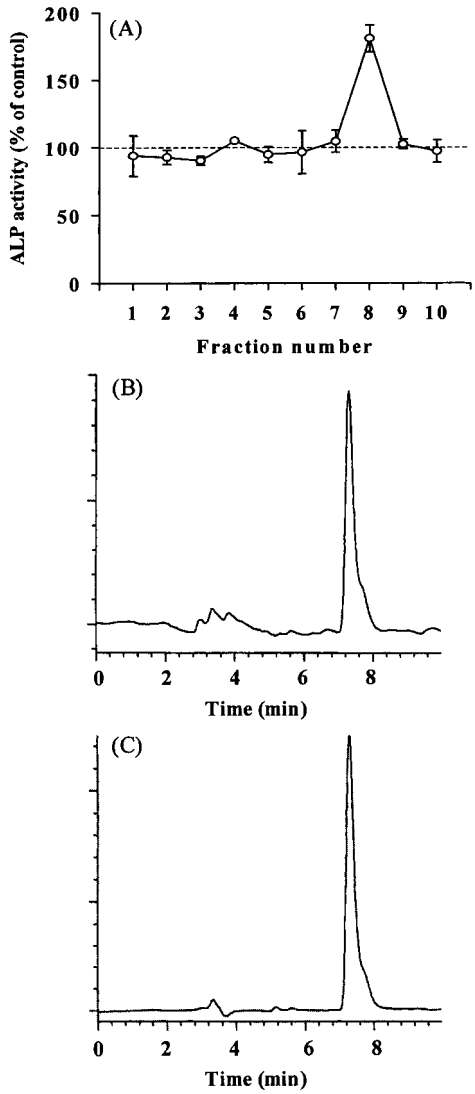

Fig. 4. Re-chromatography on a TSK-GEL OH-120 column of BN formation stimulating activity. Ethyl acetate extract of 1 ml of 10-week-old rat serum was separated on a TSK-GEL OH-120. The fraction containing the activity was re-chromatographed on a TSK-GEL OH-120 column. The eluate was monitored by absorbance at 240 nm (**B**) and elution profile was compared with that of standard corticosterone (**C**). BN formation stimulating activity was monitored by the ability to stimulate the ALP activity in ROS 17/2.8 cells (**A**). Results (mean of triplicates ± SD) were expressed as the percentage of ALP activity in the control culture. Other conditions were as described in Materials and Methods. The experiments were repeated twice and the results were essentially the same as those depicted.

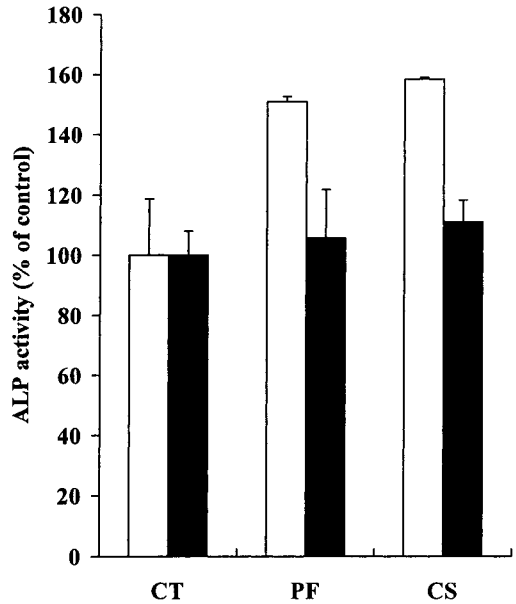

Fig. 5. Effect of antibody against corticosterone on ALP activity induced by the purified fraction obtained from the second TSK-GEL OH-120 column. Purified fraction (1 ml) from second TSK-GEL OH-120 column was dried and dissolved in 10 μl of ethanol and F-12 medium was added to give final volume of 1 ml (designated as PF). ROS 17/2.8 cells were treated with PF (final concentration of 10%) or corticosterone (CS, 10^{-7} M) in the presence (■) or absence (□) of an antibody against corticosterone. Results (mean of triplicates ± SD) were expressed as the percentage of the ALP activity in the control culture (CT). Other conditions were as described in Materials and Methods. The experiments were repeated twice and the results were essentially the same as those depicted.

the medium, indicating that a factor(s) responsible for the stimulation seemed to be either not or poorly included in FBS. Most of the BN formation stimulating activity in rat serum seems to be attributable to corticosterone, because the chromatographic behavior of the activity monitored by the ALP activity in ROS 17/2.8 cells was identical to that of standard corticosterone and the stimulation of the ALP activity by the purified fraction was completely blocked by the treatment with anticorticosterone antibody.

Bellows et al. [1987, 1990] and Turksen and Aubin [1991] have shown that dexamethasone, a synthetic glucocorticoid, stimulates BN formation in fetal rat calvarial cells. Upon the addition of dexamethasone, BN formation is enhanced in both the number and size, suggesting that the glucocorticoid stimulates a population of cells, which does not become BN forming cells in its absence, in addition to a population of cells, which undergoes osteogenic differentiation under the standard culture condition. Turksen and Aubin [1991] suggested that glucocorticoid-dependent osteoprogenitor cells are less matured than glucocorticoid-independent osteoprogenitor cells, and do not express ALP on their surface. In the present

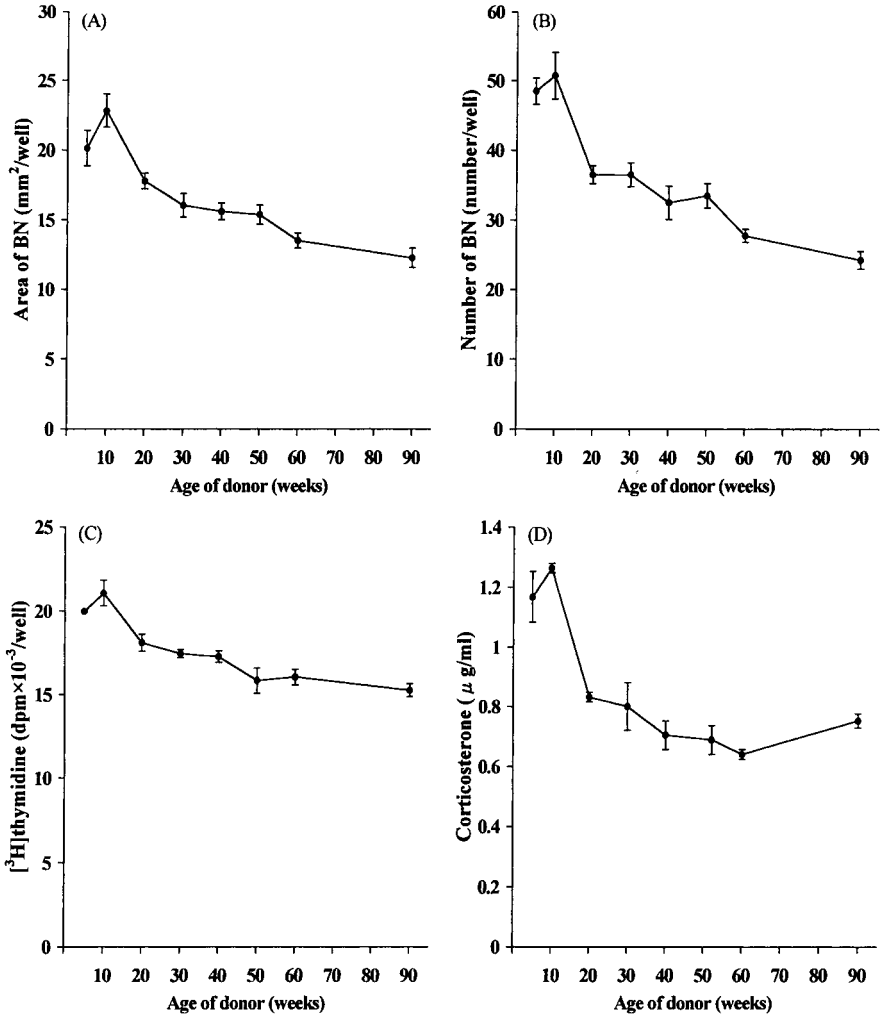

Fig. 6. Donor age-dependent change in the effect of serum on BN formation and DNA synthesis by calvarial cells from 25-week-old rats and in serum corticosterone concentration. The culture medium contains 10% serum from rats of the indicated ages for 72 h during the last 3 days during the proliferation period (P2–P4) and 10% FBS during the rest of the culture period. The area (**A**) and number (**B**) of BN were determined on day M18 and the incorporation of [^{3}H]thymidine into the cells (**C**) was measured at the beginning of day M1. Serum corticosterone concentration (**D**) was determined by radioimmunoassay (mean ± SD of three determinations). Other conditions were as described in Materials and Methods. Each value in (A), (B), and (C) represents the mean ± SD of four cultures. The experiments were repeated at least twice and the results were essentially the same as those depicted.

study, rat serum increased the incorporation rate of [^{3}H]thymidine, ALP activity and both number and size of BN in the culture of adult rat calvarial cells. These results suggest that, similar to the effects of dexamethasone, the serum factor stimulates glucocorticoid-dependent cells to become BN forming cells.

Glucocorticoids are widely used for anti-inflammatory therapy, but excess circulating glucocorticoids due to such prolonged therapeutic use of the steroid or Cushing's syndrome cause imbalance of bone remodeling, leading to osteoporosis [Canalis, 1996]. The processes underlying these unfavorable effects of glucocorticoids are poorly understood. Glucocorticoids affect intestinal calcium absorption and urinary excretion of calcium, leading to secondary hyperparathyroidism [Ritz et al., 1984; Canalis, 1996]. However, the most interesting point to investigate seems to be the mechanism of the direct action of glucocorticoids on osteoblasts and their progenitor cells. Glucocorticoids have been known to decrease the synthesis of type I collagen and osteocalcin [Lukert and Raisz, 1990; Delany et al., 1995]. In contrast, in vitro studies showed that glucocorticoid stimulates BN formation by isolated calvarial cells as described above. Bellows

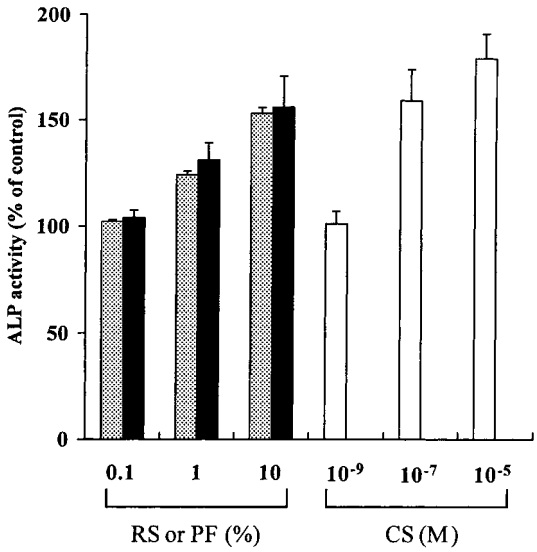

Fig. 7. Comparison of the degree of stimulation of ALP in ROS 17/2.8 cells among 25-week-old rat serum, the purified fraction from 25-week-old rat serum and corticosterone. ROS 17/2.8 cells were treated with 25-week-old rat serum (RS, ■), PF from 25-week-old rat serum (▨, see legend for Fig. 5), or corticosterone (CS, □) at the indicated concentration. Results (mean of triplicates ± SD) were expressed as a percentage of ALP activity in the control culture. Other conditions were as described in Materials and Methods. The experiments were repeated twice and the results were essentially the same as those depicted.

et al. [1987] reported that the peak effect of dexamethasone and hydrocortisone in increasing the number and area of BN was observed at 10–50 nM in the culture of fetal calvarial cells. Since higher doses showed little or no effect, they concluded that corticosteroids regulate the differentiation of osteoblasts and the homeostasis of bone within the physiological concentration range.

The most interesting finding in the present study was that the serum corticosterone concentration showed an age-dependent decline, which was paralleled with the reduction of BN formation stimulating activity in the serum (Fig. 6). These results are compatible with the idea that corticosterone is the major factor responsible for the BN formation stimulating activity in rat serum. The correlation between the age-dependent decline of the serum corticosterone level and that of BN formation stimulating activity suggests that the glucocorticoid may have an important role in the regulation of bone metabolism within the range of the circulating level. In earlier studies on age-dependent change in plasma corticosterone concentration in rats, controversial results were obtained; increased, unchanged, or decreased [DeKosky et al., 1984, Meaney et al., 1988, Goya et al., 1989]. However, more extensive studies revealed that the plasma corticosterone level is consistently lower in old rats [Ait-Chaoui et al., 1995]. The discrepancy has been attributed to difference in sex, the time point of blood collection in the 24 h circadian concentration pattern, a limited number of age groups were examined and analytical methods utilized, but the most important factor seems to be the timing of blood collection. In the present study, serum samples were collected from female rats of eight different age groups at 2 p.m. when near-maximal concentration of corticosterone has been reported to occur to minimize variation by circadian rhythm.

To the best of our knowledge, this is the first study to show the serum activity to stimulate BN formation and its age-related change. The precise mechanism of BN stimulation by corticosterone is now under investigation in our laboratory.

REFERENCES

Ait-Chaoui A, Rakotondrazafy J, Brudieux R. 1995. Age-related changes in plasma corticosterone and aldosterone responses to exogenous ACTH in the rat. Horm Res 43:181–187.

Bellows CG, Aubin JE, Heersche JNM. 1987. Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro. Endocrinology 121:1985–1992.

Bellows CG, Aubin JE, Heersche JNM, Antosz ME. 1986. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38:143–154.

Bellows CG, Heersche JNM, Aubin JE. 1990. Determination of the capacity for proliferation and differentiation of osteoprogenitor cells in the presence and absence of dexamethasone. Dev Biol 140:132–138.

Bhargava U, Bar-Lev M, Bellows CG, Aubin JE. 1988. Ultrastructural analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells. Bone 9:155–163.

Bonewald LF, Mundy GR. 1990. Role of transforming growth factor-beta in bone remodeling. Clin Orthop 250: 261–276.

Canalis E. 1996. Mechanisms of glucocorticoid action in bone: implications to glucocorticoid-induced osteoporosis. J Clin Endocrinol Metab 81:3441–3447.

Canalis E, Lian JB. 1988. Effects of bone associated growth factors on DNA, collagen and osteocalcin synthesis in cultured fetal rat calvariae. Bone 9:243–246.

Centrella M, McCarthy TL, Canalis E. 1987. Transforming growth factor β is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. J Biol Chem 262:2869–2874.

Cheng MZ, Zaman G, Rawlinson SC, Mohan S, Baylink DJ, Lanyon LE. 1999. Mechanical strain stimulates ROS cell proliferation through IGF-II and estrogen through IGF-I. J Bone Miner Res 14:1742–1750.

Civitelli R, Kim YS, Gunsten SL, Fujimori A, Huskey M, Avioli LV, Hruska KA. 1990. Nongenomic activation of the calcium message system by vitamin D metabolites in osteoblast-like cells. Endocrinology 127:2253–2262.

DeKosky ST, Scheff SW, Cotman CW. 1984. Elevated corticosterone levels. A possible cause for reduced axon sprouting in aged animals. Neuroendocrinology 38:33–38.

Delany A, Gabbitas B, Canalis E. 1995. Cortisol downregulates osteoblast α1 (I) procollagen mRNA by transcriptional and post-transcriptional mechanisms. J Cell Biochem 57:488–494.

Farach-Carson MC, Ridall AL. 1998. Dual 1,25-dihydroxyvitamin D_3 signal response pathways in osteoblasts: cross-talk between genomic and membrane-initiated pathways. Am J Kidney Dis 31:729–742.

Goldring MB, Goldring SR. 1989. Skeltal tissue response to cytokine. Clin Orthop 258:245–278.

Gowen M. 1994. Cytokines and cellular interactions in the control of bone remodeling. In: Heersche JNM, Kanis JA, editors. Bone and Mineral Research. Vol. 8, Amsterdam: Elsevier Science, p. 77–114.

Goya RG, Castro MG, Sosa YE. 1989. Diminished diurnal secretion of corticosterone in aging female but not male rats. Gerontology 35:181–187.

Gray TK, Flynn TC, Gray KM, Nabell LM. 1987. 17 beta-estradiol acts directly on the clonal osteoblastic cell line UMR106. Proc Natl Acad Sci USA 84:6267–6271.

Hassager C, Fitzpatrick LA, Spencer EM, Riggs BL, Conover CA. 1992. Basal and regulated secretion of insulin-like growth factor binding proteins in osteoblast-like cells is cell line specific. J Clin Endocrinol Metab 75:228–233.

Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M. 1986. Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. J Biol Chem 261:12665–12674.

Jones JI, Clemmons DR. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3–34.

Kato H, Matsuo R, Komiyama O, Tanaka T, Inazu M, Kitagawa H, Yoneda T. 1995. Decreased mitogenic and osteogenic responsiveness of calvarial osteoblasts isolated from aged rats to basic fibroblast growth factor. Gerontology 41:20–27.

Lowry OH, Roberts NR, Wu ML, Hixton WS, Crawford EJ. 1954. The quantitative histochemistry of brain II: enzyme measurements. J Biol Chem 207:19–37.

Lukert BP, Raisz LG. 1990. Glucocorticoid-induced osteoporosis: pathogenesis and management. Ann Int Med 112:352–364.

Matsumoto T, Igarashi C, Takeuchi Y, Harada S, Kikuchi T, Yamato H, Ogata E. 1991. Stimulation by 1,25-dihydroxyvitamin D_3 of in vitro mineralization induced by osteoblast-like MC3T3-E1 cells. Bone 12:27–32.

Meaney MJ, Aitken DH, Van Berkel C, Bhatnagar S, Sapolsky RM. 1988. Effect of neonatal handling on age-related impairments associated with the hippocampus. Science 239:766–768.

Middleton J, Arnott N, Walsh S, Beresford J. 1995. Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type 1 IGF receptor. Bone 16:287–293.

Maldonado R, Dauge V, Callebert J, Villette JM, Fournie-Zaluski MC, Feger J, Roques BP. 1989. Comparison of selective and complete inhibitors of enkephalin-degrading enzymes on morphine withdrawal syndrome. Eur J Pharmacol 165:199–207.

Noda M, Vogel RL, Craig AM, Prahl J, DeLuca HF, Denhardt DT. 1990. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D_3 receptor and 1,25-dihydroxyvitamin D_3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression. Proc Natl Acad Sci USA 87:9995–9999.

Peterkofsky B, Diegelmann R. 1971. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10:988–994.

Ritz E, Kreusser W, Rambausek M. 1984. Effects of glucocorticoids on calcium and phosphate excretion. Adv Exp Biol Med 171:381–397.

Robinson JA, Waters KM, Turner RT, Spelsberg TC. 2000. Direct action of naturally occurring estrogen metabolites on human osteoblastic cells. J Bone Miner Res 15:499–506.

Turksen K, Aubin JE. 1991. Positive and negative immunoselection for enrichment of two classes of osteoprogenitor cells. J Cell Biol 114:373–384.

Turner RT, Riggs BL, Spelsberg TC. 1994. Skeletal effects of estrogen. Endocr Rev 15:275–300.

Williams DW, Williams ED, Wynford-Thomas D. 1989. Evidence for autocrine production of IGF-1. Mol Cell Endocrinol 61:139–143.

Yokose S, Ishizuya T, Ikeda T, Nakamura T, Tsurukami H, Kawasaki K, Suda T, Yoshiki S, Yamaguchi A. 1996. An estrogen deficiency caused by ovariectomy increases plasma levels of systemic factors that stimulate proliferation and differentiation of osteoblasts in rats. Endocrinology 137:469–478.