Age-Dependent Changes in the Effect of Zinc and Cadmium on Bone Nodule Formation in Rat Calvarial Osteoblasts

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(Received August 25, 2000; Accepted Septeber 7, 2000)

Mechanism of Zn^{2+}-induced stimulation and Cd^{2+}-induced inhibition of bone nodule (BN) formation was studied in calvarial osteoblastic cells isolated from rats of various ages. Zn^{2+} dose–dependently increased BN formation at 10^{-8} M or higher concentrations in the cells from 10-week-old (young) and 90-week-old (aged) rats and the degree of stimulation was much smaller in the aged rat cells. Conversely, Cd^{2+} dose–dependently inhibited BN formation in the young and aged rat cells, with the effect being more prominent in the aged rat cells. The lowest concentrations required to reduce the area of BN in the young and aged rat cells were 10^{-8} and 10^{-9} M, respectively. Insulin-like growth factor (IGF)-I (10^{-6} M) increased the area of BN by 350% and 50% in the young and aged rat cells, respectively. Zn^{2+} (3×10^{-6} M) stimulated IGF-I production by 580% and 100% in the young and aged rat cells, respectively. Anti-IGF-I antibody almost completely inhibited the stimulatory effect of Zn^{2+} on BN formation in the young and aged rat cells. These results indicate that the effect of Zn^{2+} is mediated through the production of IGF-I. The stimulation of IGF-I production by Zn^{2+} (3×10^{-6} M) was blocked by Cd^{2+} (10^{-7} M) in the young and aged rat cells. Cd^{2+} (10^{-7} M) also inhibited the increase in BN formation by IGF-I in the aged, but not in the young rat cells. These results indicate that Cd^{2+} inhibits the induction of IGF-I production by Zn^{2+} and the subsequent action of IGF-I on osteoblasts in the aged rat cells, while only the IGF-I production is affected in the young rat cells.

Key words —— osteoblasts, zinc, cadmium, insulin-like growth factor-I, bone nodule formation, aging

INTRODUCTION

Chronic cadmium (Cd) intake along with other factors such as pregnancy and aging is known to cause "Itai-itai disease" characterized by osteomalacic and osteoporotic lesions. Conflicting results have been reported as to the pathogenesis of these bone lesions. Some researchers suggest that the bone damage is a secondary effect caused through the failure of vitamin D activation due to the renal damage.[1–3] However, it has also been suggested that the direct action of Cd on bone is the major cause of the disease rather than indirect action through kidneys, because osteoporotic changes were observed in bone tissue when no damage was detected in kidneys in some cases.[4,5] In addition, Cd inhibits bone formation and stimulates bone resorption in the organ culture of chick embryo tibia.[6,7]

Zinc (Zn), an essential trace element, is required for the function of many enzymes and other proteins as a cofactor. Zn is essential for the normal bone metabolism, and a chronic Zn deficiency causes bone growth retardation in children[8,9] and experimental animals.[10,11] Furthermore, reduced bone growth is improved with Zn supplementation. Zn has been shown to stimulate bone formation and calcification both *in vivo*[12] and *in vitro*.[13,14] Zn also increases collagen production and ALP activity in rat femora *in vivo*[15] and rat calvaria *in vitro*.[16] In addition, it prevents a decrease in bone growth caused by Cd in femora from chick embryos although the mechanism is not known at present.[17]

A variety of both systemic and local factors are involved in bone metabolism, among which insulin-like growth factor (IGF)-I is a potent stimulator of bone formation. The *in vivo* administration of IGF-I stimulates bone growth in rats.[18–20] Moreover, IGF-I is locally produced by osteoblasts, stimulating their proliferation and synthesis of bone matrix protein such as osteocalcin and type I collagen, a major bone matrix protein, in an autocrine manner.[21–23] IGF-I is also present in blood, which is

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mainly derived from liver cells. Zn deficiencies in children[24] and animals[10,11] result in the reduction of serum IGF-I concentration, which in turn is restored to normal by Zn supplementation.

The purpose of the present study is to clarify the role of IGF-I in Zn^{2+}-induced stimulation and Cd^{2+}-induced inhibition of proliferation and differentiation of osteoblasts. We used the cultures of osteoblast-enriched cells isolated from female rats at various ages to determine the age-related changes in the mechanism of action of Zn^{2+} and Cd^{2+} on osteoblasts.

MATERIALS AND METHODS

Materials —— Fetal bovine serum (FBS), alpha minimum essential medium (α-MEM) and trypsin were products of Gibco BRL (Lockville, MD, U.S.A.). Ascorbic acid, collagenase and β-glycerophosphate were obtained from Wako Pure Chemical Industries (Osaka). IGF-I from rats, anti-IGF-I and IGF-I radioimmunoasssay kit which does not detect IGF-II were from Gropep Pty Ltd (Adelaide, SA, Australia), Santa Cruz Biotechnology Inc (Santa Cruz, CA, U.S.A.) and Nichols Institute (San Juan Capistrano, CA, U.S.A.), respectively. [Methyl-^{3}H]thymidine and [2,3-^{3}H]proline were from DuPont (Wilmington, DE, U.S.A.). Phenol red-free nutrient mixture Ham's F-12 which contains 3×10^{-6} M $ZnSO_4$ (F-12 medium) and F-12 medium without $ZnSO_4$ (Zn^{2+}-free F-12 medium) were prepared according to the method of Ham *et al.*[25]

Cell Culture —— Cells enriched for osteoblast phenotype were enzymatically isolated from the calvaria of 4- to 110-week-old female Wistar rats (CLEA, Tokyo) according to the method of Bellows *et al.*,[26] as described previously.[27] 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 F-12 medium containing 10% FBS. After reaching confluency, the cells were collected by a trypsin treatment, seeded in 4-well plates at 2×10^3 cells/cm^2 in the same medium and cultured for 4 d (designated as the proliferation period, P1 to 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 d (designated as the mineralization period, M1 to M18).

Determination of Markers for Cell Proliferation and Differentiation —— For the examination of the effects of Zn^{2+} ($ZnSO_4$), Cd^{2+} ($CdCl_2$), IGF-I and/or anti-IGF-I on the proliferation and differentiation of osteoblasts, cells were treated with these reagents for 24 h on day P4. During these treatments, cells were cultured in Zn^{2+}-free F-12 medium and FBS was omitted from the medium. The incubation was continued using agonists-free α-MEM medium described above. An ALP assay was performed at the beginning of day M2 according to the method of Lowry *et al.*[28] using *p*-nitrophenylphosphate as a substrate. For the assay of collagen synthesis, the cells were incubated in serum-free α-MEM medium containing [2,3-^{3}H]proline (1.25 μ Ci/ml) for 5 h at the beginning of day M2. The incorporation of radioactivity into collagen-digestive protein was measured as described by Peterkofsky and Diegelman.[29] The area and number of BN were assessed on day M18 using a colony counter (BMS-400, Toyo Sokki, Tokyo) after visualization by von Kossa's stain.[30] 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 μ Ci/ml) at the beginning of day M1, and the incorporation of radioactivity into DNA was measured.

IGF-I Radioimmunoassay —— Cells were treated for 24 h on day P4 with indicated agonists, and immunoactive IGF-I was quantitated in the serum-free cell culture supernatant using an IGF-I immunoassay kit. Prior to the assay, IGF-I was separated from IGF-I binding proteins by acid-ethanol extraction according to the method of Breier *et al.*[31] Briefly, 10 μl of 5% Tween-20 follwed by 200 μl of 5 M acetic acid was added to 1 ml of cell supernatant and set aside for 10 min. Subsequently, 3 ml of 95% ethanol was added to each tube which was placed at –20°C for 3 h and centrifuged at 13000 *g* at 4°C for 15 min. The supernatant was collected and stored at –80°C until assayed for IGF-I.

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 ± S.D. of 2–3 determinations.

RESULTS

Effect of Zn^{2+} and Cd^{2+} on BN formation by osteoblasts was examined using rat calvarial cells isolated from young (10-week-old) and aged (90-week-old) rats, the results of which are shown in Fig. 1.

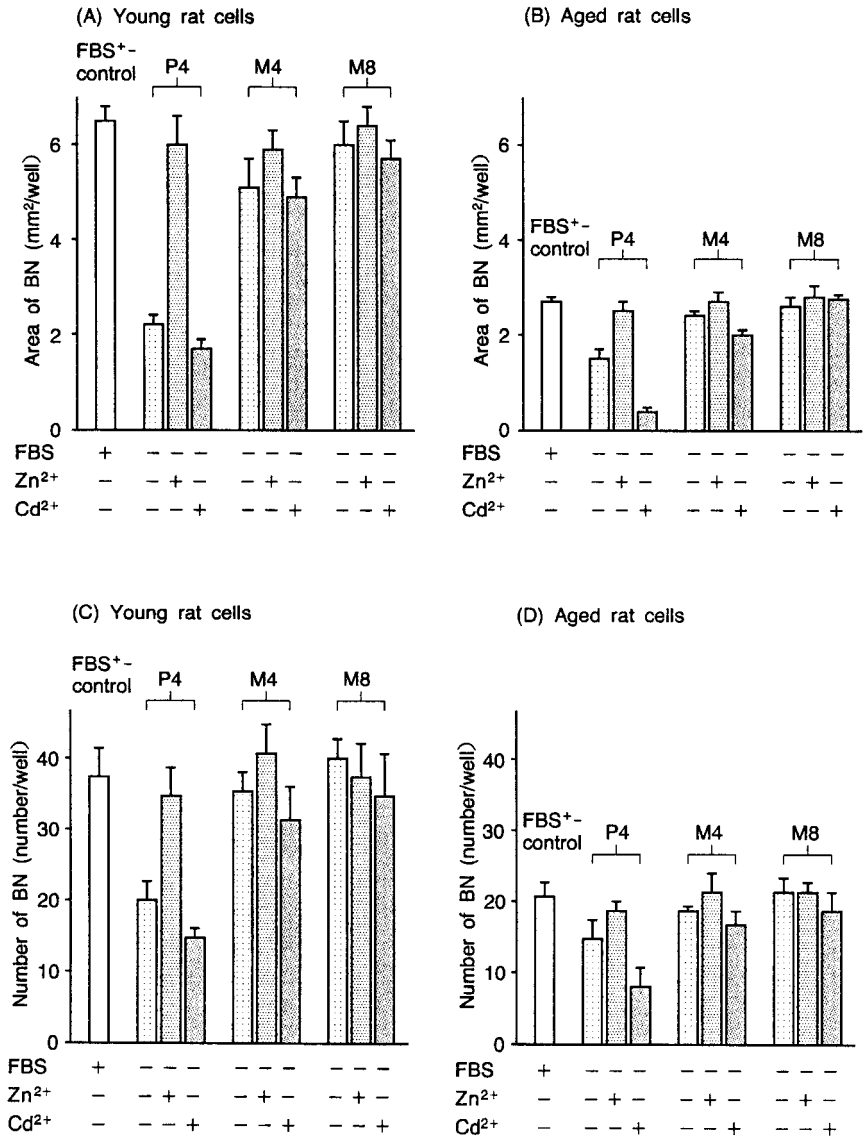

Fig. 1. Effect of Addition of Metal Ions on BN Formation at Various Times in the Culture of Calvarial Osteoblast-Like Cells from 10-Week-Old (Young) and 90-Week-Old (Aged) Rats

Cells were treated with Zn^{2+} (3×10^{-6} M) or Cd^{2+} (10^{-7} M) for 24 h on one of the days of the proliferation period (P4) or the mineralization periods (M4 or M8). The area (A, B) and number (C, D) of BN were measured on day M18. Other conditions were the same as those described in MATERIALS AND METHODS. Each value represents the mean ± S.D. of four cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

The cells were treated with 3×10^{-6} M Zn^{2+} or 10^{-7} M Cd^{2+} for 24 h on day P4, M4 or M8. During these treatments, cells were cultured in Zn^{2+}-free F-12 medium instead of the regular F-12 medium containing 3×10^{-6} M Zn^{2+}, and FBS containing approximately 10^{-5} M Zn^{2+} was omitted from the medium. When cells were incubated in FBS containing medium throughout the culture period (FBS$^+$-control), the area and number of BN formed by the aged rat cells were much smaller than those formed by the young rat cells. Omission of FBS on day P4 reduced the area and number of BN by 65% and 45%, respectively, in the young rat cells, and by 45% and 30%, respectively, in the aged rat cells. These effects of FBS deprivation were not obvious when FBS was omitted on days M4 or M8. BN formation was recovered to the control level by the treatment of cells with Zn^{2+} on day P4. In contrast, treatment of cells with Cd^{2+} on day P4 decreased the area and number of BN by 17% and 24%, respectively, in the young rat cells. The inhibitory effect of Cd^{2+} was more prominent in the aged rat cells in which the decrease in the area and number of BN were 75% and 50%, respectively.

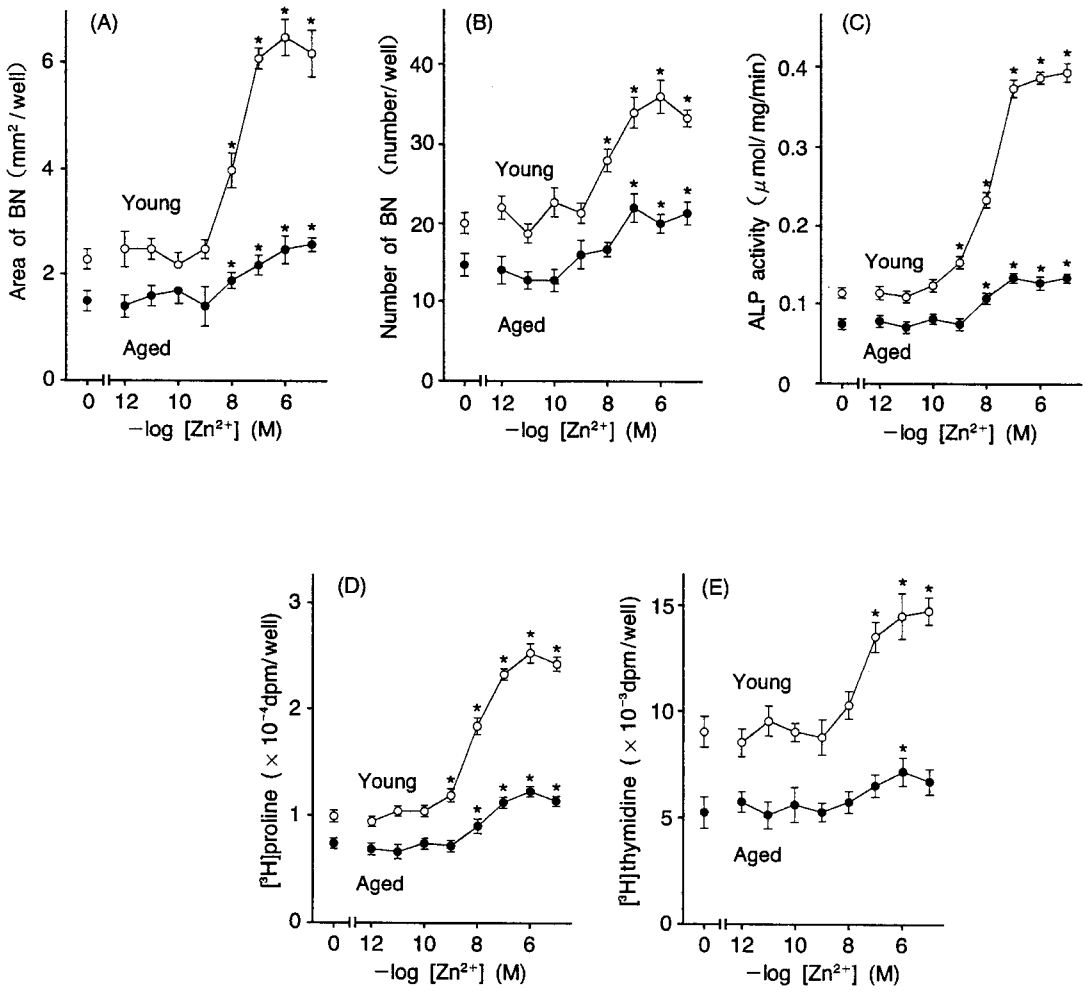

Fig. 2. Effect of Various Concentrations of Zn^{2+} on BN Formation, ALP Activity, Collagen Synthesis and DNA Synthesis in the Culture of Calvarial Osteoblast-Like Cells from 10-Week-Old (Young) and 90-Week-Old (Aged) Rats

The cells were treated with various concentrations of Zn^{2+} for 24 h on day P4. ALP activity (C), the rate of [^{3}H]proline incorporation into collagenase-digestible protein (D) and the rate of [^{3}H]thymidine incorporation into the cells (E) were measured at the beginning of day M2. The area (A) and number (B) of BN were measured on day M18. Other conditions were the same as those described in MATERIALS AND METHODS. Each point represents the mean ± S.D. of four cultures. The asterisk indicates a significant difference ($p < 0.01$) from the control culture. The experiment was repeated twice, and the results were essentially the same as those depicted.

Figure 2 shows dose–dependency of the stimulatory effect of Zn^{2+} treatment on BN formation, ALP activity and the rate of [^{3}H]proline incorporation, which are markers for differentiation of the cells, and the rate of [^{3}H]thymidine incorporation, a marker for cell proliferation. Zn^{2+} enhanced these markers at 10^{-8} M in both the young and aged rat cells, except that significant stimulation of [^{3}H]thymidine incorporation was detectable at 10^{-6} M in aged rat cells. Maximal stimulations of all markers were observed at around 10^{-6} M in both the young and aged rat cells. Increase in the area of BN was 2.8- and 1.7-fold higher in the young and aged rat cells, respectively. Increases in the level of other markers by Zn^{2+} were also higher in the young rat cells.

Figure 3 shows dose–dependency of the inhibitory effect of Cd^{2+} on the differentiation and proliferation markers. In contrast to the case of Zn^{2+}, Cd^{2+} decreased all of these markers at 10^{-8} M, the maximal effect being observed at around 10^{-6} M. The inhibition was more pronounced in the aged rat cells than in the young rat cells and significant inhibition was observed at 10^{-9} M in the case of the area of BN, ALP activity, [^{3}H]proline incorporation and [^{3}H]thymidine incorporation.

To examine the role of IGF-I, which is thought to be an important regulator of osteoblast functions, BN formation and [^{3}H]thymidine incorporation were determined after treatment of the cells with IGF-I or anti-IGF-I in the presence or absence of Zn^{2+} or Cd^{2+}

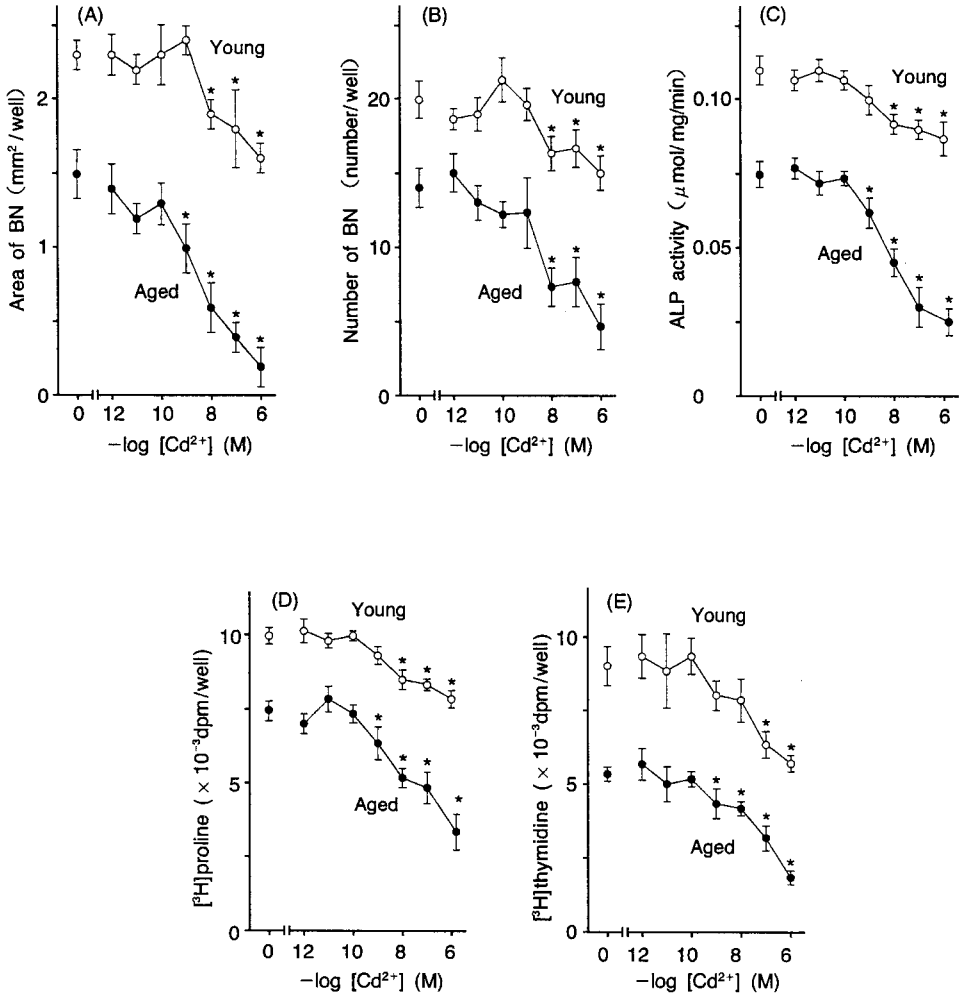

Fig. 3. Effect of Various Concentrations of Cd^{2+} on BN Formation, ALP Activity, Collagen Synthesis and DNA Synthesis in the Culture of Calvarial Osteoblast-Like Cells from 10-Week-Old (Young) and 90-Week-Old (Aged) Rats

The cells were treated with various concentrations of Cd^{2+} for 24 h on day P4. ALP activity (C), the rate of [^{3}H]proline incorporation into collagenase-digestible protein (D) and the rate of [^{3}H]thymidine incorporation into the cells (E) were measured at the beginning of day M2. The area (A) and number (B) of BN were measured on day M18. Other conditions were the same as those described in MATERIALS AND METHODS. Each point represents the mean ± S.D. of four cultures. The asterisk indicates a significant difference ($p < 0.01$) from the control culture. The experiment was repeated twice, and the results were essentially the same as those depicted.

(Fig. 4). IGF-I (10^{-6} M) increased the area and the number of BN and [^{3}H]thymidine incorporation 4.5-, 2.2- and 1.8-fold, respectively, in the young rat cells and the degrees of stimulation were somewhat higher than those by 3×10^{-6} M Zn^{2+} (Fig. 4, A–C) probably because the amount of IGF-I produced in the presence of optimal concentration of Zn^{2+} (3×10^{-6} M) is not sufficient to cause maximal stimulation of these markers. Treatment with IGF-I in the presence of Zn^{2+} increased BN formation and [^{3}H]thymidine incorporation to the levels similar to those obtained with IGF-I alone. Anti-IGF-I almost completely blocked the stimulatory effect of IGF-I and Zn^{2+}, but anti-IGF-I itself showed no effect on BN formation and [^{3}H]thymidine incorporation, raising the possibility that the effect of Zn^{2+} on calvarial cells is mediated through the production of IGF-I. Cd^{2+} (10^{-7} M) inhibited BN formation and [^{3}H]thymidine incorporation and the inhibition was protected by Zn^{2+} in the young rat cells. Interestingly, Cd^{2+} did not block the stimulatory effect of IGF-I, indicating that Cd^{2+} inhibits production of IGF-I, and therefore the effect of the direct addition of IGF-I is not affected by Cd^{2+}.

The increases in BN formation and [^{3}H]thymidine incorporation by IGF-I and Zn^{2+} were also observed in the aged rat cells, but the degree of the stimulation was much lower compared to that in the young rat cells (Fig. 4, D–F). The effect of IGF-I and Zn^{2+} were completely blocked by anti-IGF-I

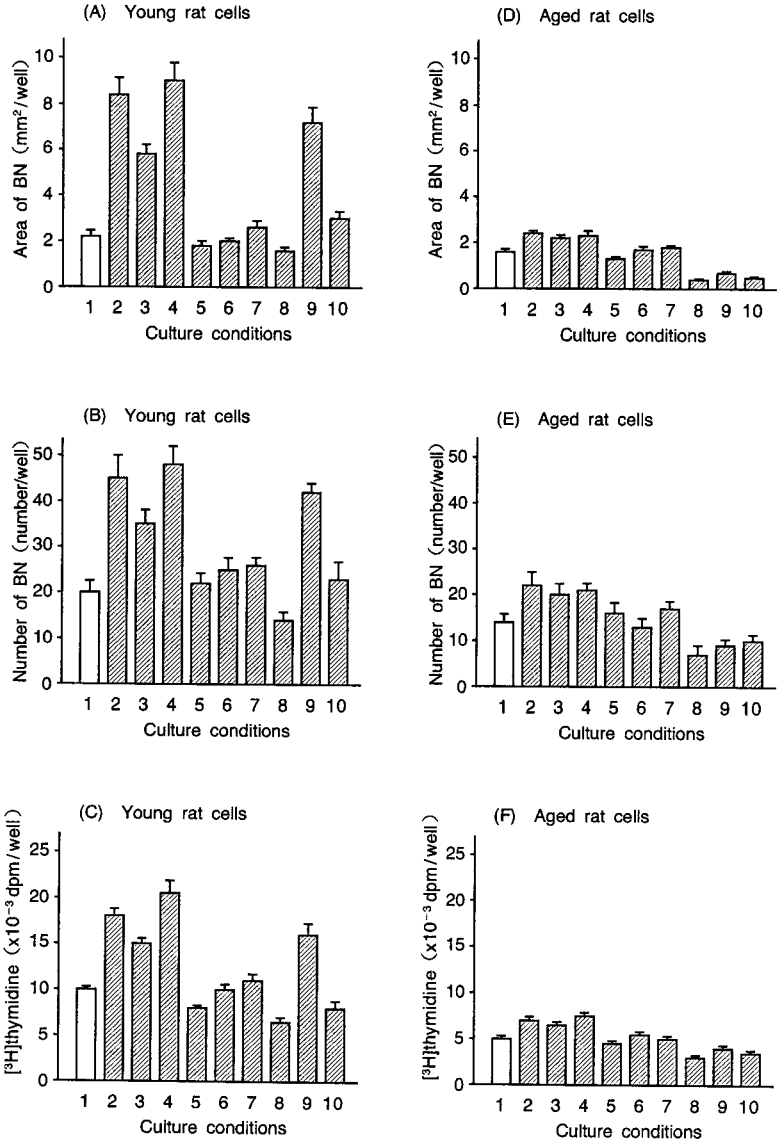

Fig. 4. Effect of Metals and Anti-IGF-I on IGF-I-Induced BN Formation and DNA Synthesis in the Culture of Calvarial Osteoblast-Like Cells from 10-Week-Old (Young) and 90-Week-Old (Aged) Rats

The cells were treated with IGF-I (10^{-6} M), Zn^{2+} (3×10^{-6} M) and/or anti-IGF-I antibody (30 μg) in the presence or absence of Cd^{2+} (10^{-7} M). Culture conditions on day P4 were as follows: 1, control; 2, IGF-I; 3, Zn^{2+}; 4, IGF-I and Zn^{2+}; 5, anti-IGF-I; 6, IGF-I and anti-IGF-I; 7, Zn^{2+} and anti-IGF-I; 8, Cd^{2+}; 9, Cd^{2+} and IGF-I; 10, Zn^{2+} and Cd^{2+}. The rate of [^{3}H]thymidine incorporation into the cells (C, F) was measured at the beginning of day M2. The area (A, D) and number (B, E) of BN were measured on day M18. Other conditions were the same as those described in MATERIALS AND METHODS. Each point represents the mean ± S.D. of four cultures. The experiment was repeated twice, and the results were essentially the same as those depicted.

in the aged rat cells. Cd^{2+} strongly inhibited BN formation and [^{3}H]thymidine incorporation, and Zn^{2+} partially blocked the inhibitory effect of Cd^{2+}. In contrast to the results obtained with the young rat cells, the stimulatory effect of IGF-I on BN formation and [^{3}H]thymidine incorporation was completely blocked in the presence of Cd^{2+}, indicating that Cd^{2+} exerts its inhibitory effect on both production of IGF-I and response of the cells to IGF-I.

Effects of Zn^{2+} and/or Cd^{2+} on IGF-I production in the young and aged rat cells are shown in Fig. 5. IGF-I production was increased in the presence of Zn^{2+} (3×10^{-6} M) by 580% and inhibited by Cd^{2+} (5×10^{-7} M) by 60% as compared to the control in the young rat cells. The Zn^{2+}-induced production of IGF-I was blocked by Cd^{2+}. In the aged rat cells, the increase in IGF-I production by Zn^{2+} was only 100%, and Cd^{2+} completely blocked basal and Zn^{2+}-induced IGF-I production.

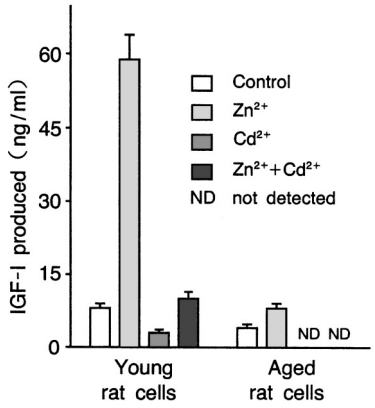

Fig. 5. Effect of Zn^{2+} and/or Cd^{2+} on IGF-I Production in the Culture of Calvarial Osteoblast-Like Cells from 10-Week-Old (Young) and 90-Week-Old (Aged) Rats

The cells were treated with Zn^{2+} (3×10^{-6} M) and/or Cd^{2+} (10^{-7} M) for 24 h on day P4. The amount of IGF-I produced was measured at the beginning of day M1 in the cell culture supernatant using an IGF-I immunoassay kit as described in MATERIALS AND METHODS.

Age-related changes in the effects of Zn^{2+} and Cd^{2+} on BN formation and the rate of [^{3}H]thymidine incorporation are shown in Fig. 6. The stimulatory effects of Zn^{2+} on both BN formation and [^{3}H]thymidine incorporation sharply decreased from 10 to 50 weeks of the cell donor age, and kept relatively constant from 50 to 110 weeks. In contrast, the inhibitory effects of Cd^{2+} on both BN formation and [^{3}H]thymidine incorporation gradually increased from 10 to 110 weeks of the cell donor age.

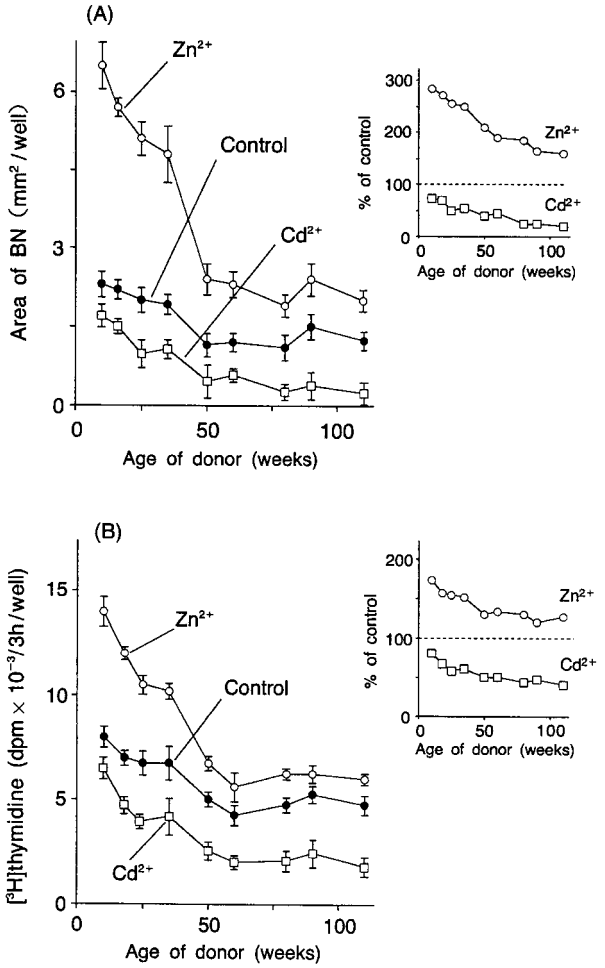

Fig. 6. Effect of Zn^{2+} or Cd2+ on BN Formation and DNA Synthesis in the Culture of Calvarial Osteoblast-Like Cells from Rats of Various Ages

The cells were treated with Zn^{2+} (3×10^{-6} M) or Cd^{2+} (10^{-7} M) for 24 h on day P4. The rate of [^{3}H]thymidine incorporation into the cells (B) was measured at the beginning of day M2 and the area of BN (A) was measured on day M18. Inset compares the effect of metals on the ratio of BN formation and DNA synthesis in metal-treated cultures with that in the untreated cultures. Other conditions were the same as those described in MATERIALS AND METHODS. Each point represents the mean ± S.D. of four cultures. The experiments with rats aged < 100 weeks were repeated at least three times, while those with 100- and 110-week-old rats were repeated twice, and the results were essentially the same as those depicted.

DISCUSSION

In this study, we used osteoblasts-enriched cells isolated from rat calvaria to study changes in the effect of Zn^{2+} and Cd^{2+} on the proliferation and differentiation of osteoblasts. These cells have been considered to be composed of mixed populations at different stage of osteoblastic differentiation, including those that differentiate spontaneously under standard culture conditions, and those that respond to a specific stimulus and differentiate into BN forming cells.[26,32] The increase in the number of BN indicates the spontaneously differentiating cells, while the increase in the area of each BN indicates the increase in the rate of cellular replication in each colony. This system enables us to study the age-dependent changes in the characteristics of osteoblasts. In addition, we have shown in our previous study that using this system, the signal transduction pathway for PGE_2 through EP_1 subtype of PGE_2 receptor becomes inactive with age.[33]

From the results obtained in the present study, we propose a model for the mechanism of actions of Zn^{2+} and Cd^{2+} on rat calvarial osteoblasts, which is shown in Fig. 7. Basal level of BN formation under the standard culture condition in the young rat cells is higher than that in the aged rat cells (Fig. 1). The major part of basal BN formation seems to be independent of IGF-I production, because it is insensitive to the treatment with anti-IGF-I antibody (Fig. 4). Zn^{2+} stimulates IGF-I production in both

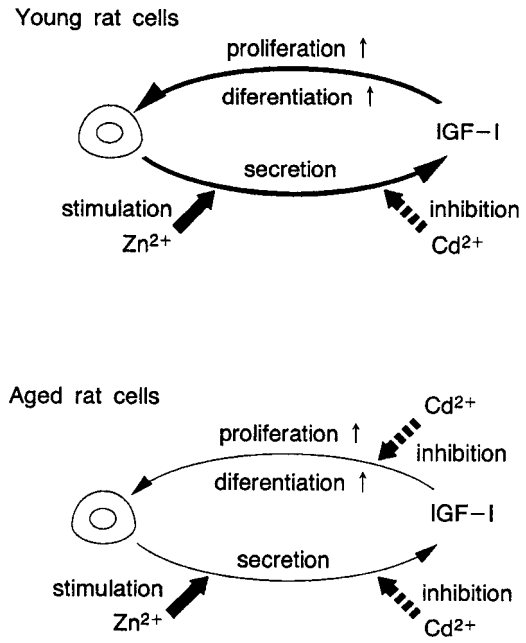

Fig. 7. A Proposed Model for Mechanism of Action of Metals in the Culture of Calvarial Osteoblast-Like Cells from 10-Week-Old (young) and 90-Week-Old (Aged) Rats

In young rat cells, Zn^{2+} stimulates production of IGF-I through which proliferation and differentiation of osteoblasts are accelerated and Cd^{2+} blocks the process of the induction of IGF-I production by Zn^{2+}. In contrast, Cd^{2+} is likely to block both IGF-I production and action of IGF-I on osteoblasts in aged rat cells.

the young and aged rat cells, the degree of stimulation being much greater in the former cells (Fig. 5). The secreted IGF-I mediates the Zn^{2+}-induced increase in BN formation, because the induction is blocked by the treatment with anti-IGF-I antibody, and hence IGF-I stimulates BN formation (Fig. 4). The degree of Zn^{2+}-induced increase in BN formation is also much greater in the young rat cells. Cd^{2+} inhibits the BN formation in the absence or presence of Zn^{2+}, the degree of the inhibition being much greater in the aged rat cells (Fig. 4). Cd^{2+} also inhibits production of IGF-I, the inhibition being partial in the young rat cells and complete in the aged rat cells (Fig. 4). It is interesting to note that Cd^{2+} inhibits the IGF-I-induced increase in BN formation in the aged but not in the young rat cells (Fig. 5). It is not known at present why Cd^{2+} inhibits IGF-I-induced BN formation only in the aged rat cells. Cd^{2+} may affect the process after the receptor binding because the number of IGF-I receptors does not decrease with age.[34]

Potent inhibitory activity of Cd^{2+} on BN formation found in this study strongly suggests a possible involvement of direct action of Cd on bone in the pathogenesis of Itai-itai disease. The increase in the inhibitory activity with cell donor age may partly explain the high incidence of occurrence in aged people. Reduction in the inhibitory activity of Cd^{2+} on BN formation in the presence of Zn^{2+} (Fig. 4) suggests a protective role of Zn against Cd-induced toxicity in bone tissue. Zn deficiency due to inadequate food intake in aged people is associated with low serum IGF-I concentration, which may increase sensitivity of bone to Cd.[35] Reduction of the Zn^{2+} concentration in the culture medium caused reduction in the production of IGF-I by calvarial cells (Fig. 5), suggesting that Zn deficiency may also affect IGF-I production by osteoblasts especially in the liver.

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