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Stimulatory Effect of Zinc on Deoxyribonucleic Acid Synthesis in Bone Growth of Newborn Rats: Enhancement with Zinc and Insulin-Like Growth Factor-I

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Abstract. The effect of zinc on *in vitro* deoxyribonucleic acid (DNA) synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats was investigated to determine a role of zinc in bone growth. *In vitro* DNA synthesis was assayed in a reaction mixture containing the 100 *g* centrifugation supernatant, which includes the nucleus of bone cells, of bone issue homogenate with incorporation of [^{3}H]-deoxythymidine 5′-triphosphate (dTTP). DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats was significantly raised with increasing age (1–21 days) after birth. The presence of dipicolinate (10^{-3} M), a chelator of zinc, in the reaction mixture caused a significant decrease in DNA synthesis activity in the diaphyseal and metaphyseal tissues of newborn rats at 7 and 14 days after birth. The addition of zinc sulfate (10^{-6} - 10^{-4} M) resulted in a significant increase in DNA synthesis activity in the diaphyseal and metaphyseal tissues. When the diaphyseal and metaphyseal tissues of newborn rats at 7 days after birth were cultured for 24 hours in a serum-free medium containing either vehicle, zinc sulfate (10^{-4} M), insulin-like growth factor-I (IGF-I; 10^{-8} M) or transforming growth factor-β (TGF-β; 10^{-10} M), bone DNA synthesis activity was significantly elevated. Culture with both zinc and IGF-I enhanced additively bone DNA synthesis activity. Such an effect was not seen in the case of zinc and TGF-β. The effect of zinc, IGF-I, or zinc plus IGF-I in increasing bone DNA synthesis activity was completely prevented by culture with PD98059 (10^{-5} M), an inhibitor of mitogen-activated protein (MAP) kinase. Also, the effect of zinc, TGF-β, or zinc plus TGF-β in elevating bone DNA synthesis activity was significantly inhibited by culture with staurosporine (10^{-6} M), an inhibitor of protein kinase C. The present study demonstrates that zinc, like bone growth factors, has a stimulatory effect on bone DNA synthesis in newborn rats.

Key words: Bone growth — DNA synthesis — Zinc — Insulin-like growth factor-I — Transforming growth factor-β

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Zinc is known to be an essential trace element for the growth of humans and other animals [1, 2]. Zinc deficiency results in a retardation of bone growth [3, 4], suggesting that the element is required for the growth, development, and maintenance of healthy bone. There is growing evidence that zinc plays a role in the regulation of bone metabolism; the metal can stimulate osteoblastic bone formation [5–8] and inhibit osteoclastic bone resorption [8–11], thereby increasing bone mass.

The pathophysiologic role of zinc in osteopenia and osteoporosis has been shown [12–15]. Bone zinc content is reduced with increasing age [12] and skeletal unloading in rats [13]. Osteoporosis patients have been shown to have lower levels of skeletal zinc than normal individuals [14]. Women with osteoporosis excrete a great amount of zinc in urine [15]. Zinc supplementation has been shown to have a preventive and therapeutic effect on bone loss [16–18], suggesting its role as a nutritional and pharmacologic tool in the prevention of osteoporosis with increasing age.

The physiologic mechanism of bone growth has not been fully clarified, although bone growth factor is important. Zinc may play a role as a stimulatory factor in bone growth, since zinc deficiency results in a reduction of serum IGF-I levels and skeletal growth in young rats [4]. The mechanism by which zinc stimulates bone growth, however, is unknown. More recent study has shown that it has a stimulatory effect on bone growth in newborn rats following lactation of maternal animals that have been orally administered zinc [19]. Endogenous zinc in bone tissues has been demonstrated to have an enhancing effect on bone protein synthesis associated with bone growth of newborn rats [20]. Moreover, zinc, like IGF-I and TGF-β, has been shown to increase bone protein components of newborn rats [21]. Zinc may play a special role in bone growth in collaboration with IGF-I.

The present study, therefore, was undertaken to determine whether zinc has a stimulatory effect on bone DNA synthesis associated with bone growth of newborn rats. We found that zinc, like IGF-I and TGF-β, stimulates DNA

synthesis in tissue culture using the femoral-diaphyseal and metaphyseal tissue of newborn rats, and that zinc can enhance the effect of IGF-I.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (high) glucose, (4.5 g/dl) and a penicillin-streptomycin solution (5000 U/mg penicillin, 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, USA). [Methyl-^{3}H]deoxythymidine 5′-triphosphate ([^{3}H]-dTTP; 2.59 TBq/mmol) was obtained from New England Nuclear (Boston, MA). IGF-I (human recombinant), TGF-β (human recombinant), dipicolinate (2, 6-pyridinecarboxylic acid, neutralized with sodium hydroxide prior to use), cycloheximide, PD98059, and staurosporine were obtained from Sigma Chemical (St. Louis, MO, USA). Zinc sulfate and other chemicals were reagent grade from Wako Pure Chemicals Industries (Osaka, Japan). All water used was glass distilled.

Animals and Bone Tissues

Pregnant female Wistar rats, purchased from Japan SLC, Inc. (Hamamatsu, Japan), were fed commercial laboratory chow (solid, Oriental Yeast Co., Ltd., Tokyo, Japan) containing 57.5% carbohydrate, 1.1% calcium, 1.1% phosphorus, and 0.012% zinc, and distilled water, *ad libitum.* The newborn rats were divided into male and female groups, and bred separately. The newborn or weanling male rats were sacrificed by bleeding between 1 and 28 days after birth. The femurs were removed aseptically after bleeding and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. Marrow cells were completely removed by washing diaphyseal and metaphyseal tissues. These bone tissues were homogenized to assay DNA synthesis.

Bone Tissue Culture

The femoral-diaphyseal and metaphyseal tissues were aseptically obtained 7 and 14 days after birth of rats. Bone tissue fragments were cultured for 24 hours in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's modified Eagle's medium (high glucose, 4.5 g/dl) supplemented with antibiotics (100 U penicillin and 100 μg streptomycin/ml of medium) [5]. This medium did not contain serum and did not detect zinc. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air. In another experiment, bone tissues obtained at 7 days after birth were cultured for 24 hours in a medium containing either vehicle, zinc sulfate (10^{-4} M), or dipicolinate (10^{-3} M) in the absence or presence of IGF-I (10^{-8} M) or TGF-β (10^{-10} M). After culture, bone tissues were pooled to assay DNA synthesis.

Assay of Bone DNA Synthesis

Femoral-diaphyseal and metaphyseal tissues were added to ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle, and disrupted for 60 seconds with an ultrasonic device. The supernatant centrifuged at 100 *g* for 5 minutes was used to assay DNA synthesis. DNA synthesis was estimated by the procedure of Lynch et al. [22] with minor modification. DNA synthesis was measured for 30 minutes at 37°C in mixtures (0.5 ml) that contained 0.18 M Tris-HCl buffer (pH 8.2), 4 mM $MgCl_2$, 2 mM ATP, dGTP, dCTP, dATP (each 0.08 mM), 0.06 mM [^{3}H]dTTP, dextran (Type 100 C, 2%), 2.5 mM cadaverine, and the suspension of bone tissue extract containing the nuclei (0.1 ml containing 60– 100 μg DNA). PD 98059 (10^{-5} M) or staurosporine (10^{-6} M) was added as indicated. Reactions were stopped with 0.5 ml of 1 M NaOH, and DNA was precipitated with addition (5 ml) of ice-cold trichloroacetic acid (10% TCA). The DNA was then dissolved (0.5 ml of 1 M NaOH) and precipitated (5 ml of 10% TCA), and the final precipitate, dissolved in 0.5 ml of 1 M NaOH, was heated at 80°C for 15 minutes. Finally, the DNA was precipitated with 10% TCA and the precipitate was washed with acid, ethanol, and ether. The radioactivity in nuclear DNA was measured in a hyamine-toluene liquid scintillation mixture, and all the data were corrected for the incorporation with control mixture that lacked the three unlabeled deoxynucleotides. DNA synthesis was expressed as disintegrations per minute (dpm) per milligram of DNA.

DNA content in the 100 *g* centrifugation supernatant of the femoral-diaphyseal and metaphyseal tissue homogenate was determined by the method of Ceriotti [23].

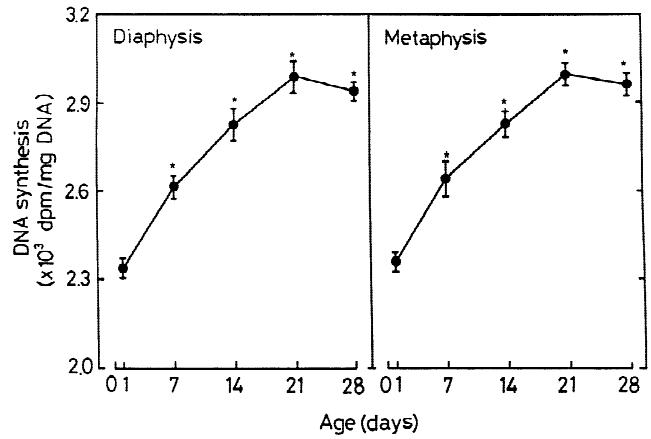

Fig. 1. Changes in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats with increasing age. DNA synthesis was measured by using the 100 *g* centrifugation supernatant of bone homogenate obtained at 1, 7, 14, 21, and 28 days after birth. Data are the mean ± SEM of five rats. * $P < 0.01$, compared with the values obtained at 1 day after birth.

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. *P*-values less than 0.05 were considered to indicate statistically significant differences. Also, we used a multiway ANOVA and Tarky-Kramer multiple comparison test to compare the treatment groups.

Results

Changes in In Vitro *DNA Synthesis Activity in Bone Tissues of Newborn Rats with Increasing Age*

The changes in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats with increasing age after birth is shown in Figure 1. Bone tissues were obtained at 1, 7, 14, 21, and 28 days after birth. *In vitro* DNA synthesis activity was significantly increased by increasing age after birth, as compared with the value obtained at 1 day after birth. An increase in DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues reached a plateau at 21 and 28 days after birth.

The effect of dipicolinate, a chelator of zinc [24, 25], or *in vitro* DNA synthesis activity in the femoral-diaphyseal

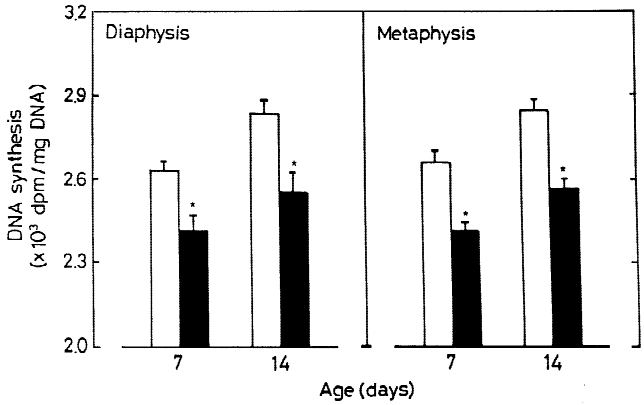

Fig. 2. Effect of dipicolinate, a chelator of zinc, in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats. Bone tissues were obtained at 7 and 14 days after birth. DNA synthesis was measured as described in Figure 1. Data are the mean ± SEM of five rats. * $P < 0.01$, compared with the control (none) values. White bars, control; black bars, dipicolinate.

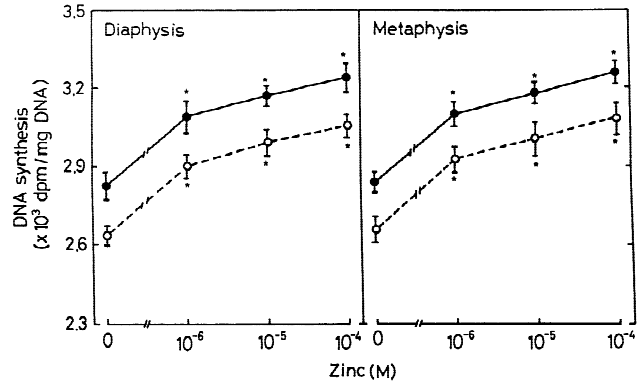

Fig. 3. Effect of zinc on *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats. Bone tissues were obtained at 7 and 14 days after birth. DNA synthesis was measured as described in Figure 1. Zinc sulfate (10^{-6} - 10^{-4} M) was directly added into the reaction mixture for DNA synthesis assay. Data are the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) value. Open circles, 7 days old; closed circles, 14 days old.

tissues of newborn rats at 7 and 14 days after birth is shown in Figure 2. The presence of dipicolinate (10^{-3} M) in the reaction mixture resulted in a significant decrease in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues obtained at 7 and 14 days after birth.

The effect of zinc addition on *in vitro* DNA synthesis in the femoral-diaphyseal and metaphyseal tissues of newborn rats at 7 and 14 days after birth was examined, and the results are shown in Figure 3. Zinc sulfate was directly added into the reaction mixture containing the nuclear fraction from bone tissues for DNA synthesis assay. The addition of zinc sulfate (10^{-6} - 10^{-4} M) in the reaction mixture caused a significant increase in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats at 7 and 14 days after birth.

Changes in In Vitro *DNA Synthesis Activity in Bone Tissues Cultured with Zinc, IGF-I, and TGF-*β

The femoral-diaphyseal and metaphyseal tissues of newborn rats 7 days after birth were cultured for 24 hours in a serum-free medium containing zinc sulfate (10^{-6} - 10^{-4} M). *In vitro* DNA synthesis activity in the diaphyseal and metaphyseal tissues was significantly increased by culture with zinc sulfate (10^{-6} - 10^{-4} M) (Fig. 4). The presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis, in culture medium caused a remarkable inhibition of the zinc (10^{-4} M) - induced increase in *in vitro* DNA synthesis activity in the diaphyseal and metaphyseal tissues of newborn rats at 7 days after birth (Fig. 5).

The effect of IGF-I (10^{-8} M) or TGF-β (10^{-10} M) on *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats at 7 days after birth is shown in Figure 6. Bone tissues were cultured for 24 hours in a medium containing either vehicle, IGF-I (10^{-10} M), or

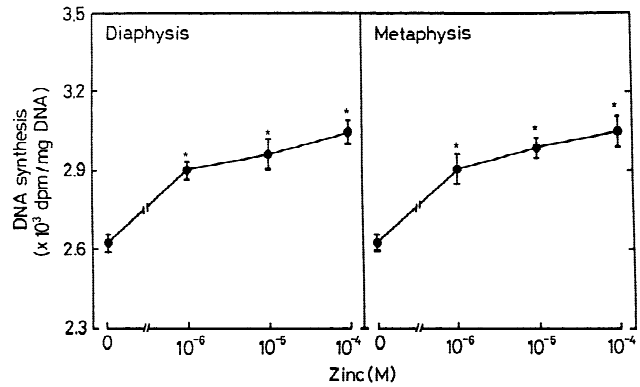

Fig. 4. Changes in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats cultured with zinc. Bone tissues were obtained at 7 days after birth, and were cultured for 24 hours in a medium containing either vehicle or zinc sulfate (10^{-6} - 10^{-4} M). DNA synthesis in the cultured bone tissues was measured as described in Figure 1. Data are the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) values.

TGF-β (10^{-10} M) in the absence or presence of zinc sulfate (10^{-4} M). The culture with IGF-I or TGF-β caused a significant increase in *in vitro* DNA synthesis activity in the diaphyseal and metaphyseal tissues. *In vitro* DNA synthesis activity in the diaphyseal and metaphyseal tissues was additively enhanced by culture with both IGF-I (10^{-8} M) and zinc (10^{-4} M), as compared with the value obtained from IGF-I or zinc alone. Such an effect was not seen in the case of TGF-β (10^{-10} M) and zinc (10^{-4} M).

Effect of PD98059 or Staurosporine on In Vitro *DNA Synthesis Activity in Bone Tissue Cultured with Zinc, IGF-I, or TGF-*β

The femoral-diaphyseal tissues of newborn rats at 7 days after birth were cultured for 24 hours in a serum-free me-

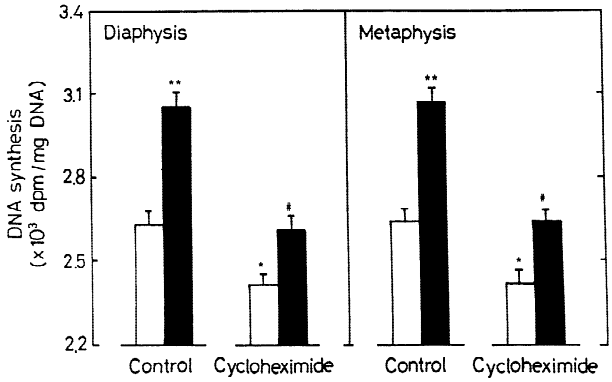

Fig. 5. Changes in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats cultured with zinc in the absence or presence of cycloheximide. Bone tissues were obtained at 7 days after birth, and were cultured for 24 hours in a medium containing either vehicle, zinc sulfate (10^{-4} M), cycloheximide (10^{-6} M), or zinc sulfate (10^{-4} M) plus cycloheximide (10^{-6} M). DNA synthesis was measured as described in Figure 1. Data are the mean ± SEM of five rats. *$P < 0.05$ and **$P < 0.01$, compared with the control (none) value. $P < 0.01$, compared with the value for zinc alone without cycloheximide. White bars, control; black bars, zinc.

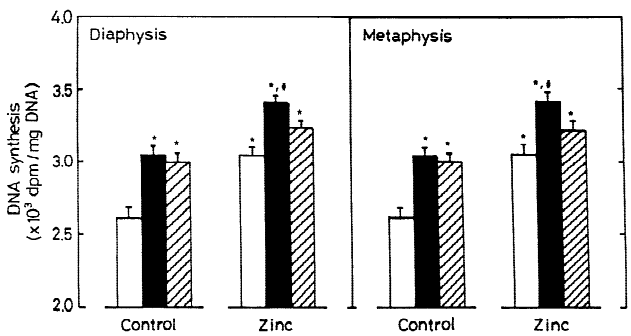

Fig. 6. Changes in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats cultured with IGF-I or TGF-β in the absence or presence of zinc. Bone tissues were obtained at 7 days after birth, and were cultured for 24 hours in a medium containing either vehicle, IFG-I (10^{-8} M), or TGF-β (10^{-10} M) without or with zinc sulfate (10^{-4} M). DNA synthesis was measured as described in Figure 1. Data are the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) values. $P < 0.01$, compared with the value for IGF-I or zinc alone. White bars, control; black bars, IGF-I; hatched bars, TGF-β.

dium containing either vehicle, zinc sulfate (10^{-4} M), IGF-I (10^{-8} M), or zinc sulfate (10^{-4} M) plus IGF-I (10^{-8} M) in the absence or presence of PD98059 (10^{-5} M), an inhibitor of MAP kinase [26] (Fig. 7). The culture with PD98059 did not cause a significant alteration in *in vitro* DNA synthesis activity in the diaphyseal tissues. However, the effect of zinc, IGF-I, and zinc plus IGF-I in elevating *in vitro* DNA synthesis activity in the diaphyseal tissues was completely prevented by culture with PD98059.

Likewise, diaphyseal tissues were cultured in the presence of either vehicle, zinc sulfate (10^{-4} M), TGF-β (10^{-10} M), or zinc sulfate (10^{-4} M) plus TGF-β (10^{-10} M) without or with staurosporine (10^{-6} M) (Fig. 8). *In vitro* bone DNA

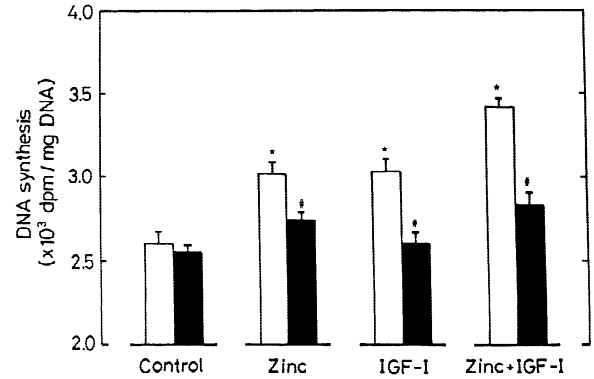

Fig. 7. Changes in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats cultured with zinc and IGF-I in the absence or presence of PD98059, an inhibitor of MAP kinase. Bone tissues were obtained at 7 days after birth, and were cultured for 24 hours in a medium containing either vehicle, zinc sulfate (10^{-4} M), IGF-I (10^{-8} M), or zinc sulfate (10^{-4} M) plus IGF-I (10^{-8} M) without or with PD98059 (10^{-5} M). DNA synthesis was measured as described in Figure 1. Data are the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) value. $P < 0.01$, compared with the control value without PD98059 addition. White bars, control; black bars, PD98059 addition.

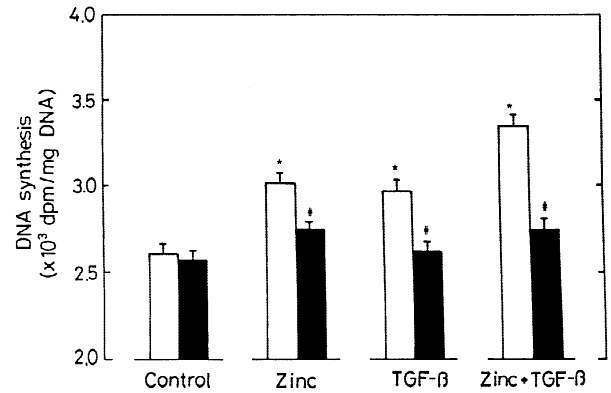

Fig. 8. Changes in *in vitro* DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats cultured with zinc and TGF-β in the absence or presence of staurosporine. Bone tissues were obtained at 7 days after birth, and were cultured for 24 hours in a medium containing either vehicle, zinc sulfate (10^{-4} M), TGF-β (10^{-10} M), or zinc sulfate (10^{-4} M) plus TGF-β (10^{-10} M) without or with staurosporine (10^{-6} M). DNA synthesis was measured as described in Figure 1. Data are the mean ± SEM of five rats. *$P < 0.01$, compared with the control (none) value. $P < 0.01$, compared with the value without staurosporine addition. White bars, control; black bars, staurosporine addition

synthesis activity was not significantly changed by culture with staurosporine in the absence of zinc or growth factors. However, the effect of zinc, TGF-β, or zinc plus TGF-β in elevating *in vitro* DNA synthesis was significantly inhibited by culture with staurosporine, an inhibitor of protein kinase C.

Discussion

This study demonstrates that an increase in bone DNA syn-

thesis activity is associated with bone growth of newborn rats with aging. Bone DNA synthesis activity has been shown to be reduced by the presence of dipicolinate, a chelator of zinc [24, 25], in the reaction mixture using the 100 *g* centrifugation supernatant of the femoral-diaphyseal and metaphyseal tissue homogenate of newborn rats at 7 and 14 days after birth. This result suggests that bone endogenous zinc plays a role in the enhancement of bone DNA synthesis associated with bone growth of newborn rats. The addition of zinc in the reaction mixture caused a significant increase in both DNA synthesis activity. Thus, zinc has a direct stimulatory effect on bone DNA synthesis of newborn rats. DNA polymerase, which is related to DNA synthesis, is a zinc enzyme [27] and it is possible that zinc may partly stimulate DNA synthesis due to activating DNA polymerase in osteoblastic cells of bone tissues.

DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats cultured with zinc was also found to be increased, and this effect was significantly blocked in the presence of cycloheximide, an inhibitor of protein synthesis at the translational process. The expression of zinc effect in stimulating bone DNA synthesis may partly require newly synthesized proteins like many other growth factors in the bone tissues of newborn rats. Previous study showed that zinc can stimulate protein synthesis in the bone tissues of newborn rats [20], and that the metal treatment increases many bone protein components [21]. Zinc also has been shown to increase the production of IGF-I and TGF-β in osteoblastic MC3T3-E1 cells [6], and has been reported to enhance IGF-I effect in the cells [28].

The effect of zinc on bone DNA synthesis activity appears to be modulated in the presence of IGF-I or TGF-β, which is bone growth factor. When the femoral-diaphyseal and metaphyseal tissues of newborn rats at 7 days after birth were cultured in a serum-free medium containing zinc, IGF-I, or TGF-β, bone DNA synthesis activity was found to be raised by growth factors. Zinc, like IGF-I or TGF-β, had a stimulatory effect on bone DNA synthesis of newborn rats. The combination of zinc and IGF-I had an additive effect on bone DNA synthesis activity, although such an effect was not seen in the case of zinc and TGF-β. Zinc may especially play a role in bone growth in collaboration with IGF-I. Meanwhile, it is reported that zinc deprivation of murine 3T3 fibroblasts by use of a zinc-chelating chemical impairs DNA synthesis on stimulation with IGF-I [29]. Zinc may be needed to reveal the effect of IGF-I.

The effect of IGF-I in osteoblastic cells is mediated through a MAP kinase pathway which is related to protein tyrosine kinase [26], and the TGF-β effect is mediated through a tyrosine protein serine/threonine kinase pathway [30]. PD98059 is an inhibitor of MAP kinase, and staurosporine is an inhibitor of protein kinase C which is protein serine/threonine kinase. The effect of zinc, IGF-I, or zinc plus IGF-I in increasing bone DNA synthesis activity was significantly inhibited in the presence of PD98059 in culture medium. This finding suggests that the effect of zinc or IGF-I on bone DNA synthesis is partly mediated through a signaling pathway related to MAP kinase. Meanwhile, zinc or TGF-β-induced increase in bone DNA synthesis was significantly prevented by the presence of staurosporine with a 100-fold higher concentration (10^{-6} M) than that used previously in osteoblastic MC3T3-E1 cells [31]. This result suggests that the effect of zinc or TFG-β is partly mediated through a signaling system involved in protein kinase C. Alternatively, the stimulatory effect of zinc on bone DNA synthesis may partly be involved in a signaling pathway which is mediated through MAP kinase and protein kinase C. It is possible that zinc may activate protein kinases. It has been reported that the regulatory domain of protein kinase C coordinates four atoms of zinc [32].

Zinc has been shown to increase DNA content in the femoral-diaphyseal and metaphyseal tissues of newborn rats [19, 21]. This mechanism may be based on the present finding that zinc can elevate DNA synthesis activity in the bone tissues of newborn rats. Zinc has been demonstrated to increase proliferation of osteoblastic MC3T3-E1 cells *in vitro* [31]. Presumably, zinc stimulates proliferation of osteoblastic cells in the bone tissues of newborn rats.

In conclusion, it has been demonstrated that zinc, like IGF-I and TGF-β, can stimulate, with increasing age, DNA synthesis activity in the femoral-diaphyseal and metaphyseal tissues of newborn rats.

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