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Effect of Dietary Minerals and Ca-Regulating Hormones on Bone Enzyme, Alkaline Phosphatase Activity

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Abstract

Parathyroid hormone(PTH) is known to stimulate bone resorption and to inhibit bone collagen synthesis. In contrast, as the evidence of stimulation of bone formation by PTH has recently been observed, the study on the role of PTH involved in osteoporosis draws remarkable attention. This study has dealt with the role of alkaline phosphatase(AP), a marker enzyme for bone formation and osteoblast action. Animals(BALB/c mice) were divided into three dietary groups(high and medium Ca and Ca-free) and hormones including PTH, calcitonin(CT), cholecalciferol(vitamin D) were i.p. injected. AP in the serum and liver was measured using Sigma 221 alkaline buffer solutions containing 9mM of *p*-nitrophenyl phosphate. Enzyme was reacted at 37°C for 10 minutes and the reaction was stopped by 1.8ml of 0.1N NaOH and measured at 410nm. We found that serum and liver AP activity was increased by low dietary Ca. Compared to the control, and serum AP activity was enhanced by PTH and vitamin D regardless of the levels of dietary Ca. On the other hand, liver AP activity was inhibited by PTH and vitamin D at all levels of dietary Ca. CT inhibited the action of PTH and vitamin D in the serum. But, the inhibition of PTH and vitamin D action by CT was not observed in the liver, unlike in the case of serum.

Key words: calcium, osteoporosis, alkaline phosphatase, parathyroid hormone, vitamin D

INTRODUCTION

The growth and health of bone is largely dependent on diet and hormonal status. As the life expectancy of people prolongs senile bone diseases, including postmenopausal osteoporosis and senile osteoporosis, in particular, are constantly increasing. These phenomena are mostly due to the lack of Ca intake and increase of bone resorption. Parathyroid hormone (PTH) was known to stimulate bone resorption and to inhibit bone collagen synthesis(1-3). In contrast, morphological studies found the evidence of stimulation of bone formation by this hormone and the bone formation is occuring independent of the action of bone resorption(4). As aging progresses PTH hormone level is increased, consequently stimulating osteoclastic activity and bone resorption. On the other hand, the activity of osteoblast is active in children whose mineral deposition into the bone matrix is necessary for bone growth and development.

Clinical investigations with osteoporotic patients found that their dietary Ca intake is lower than that of the healthy population(5-9). Women whose Ca intake is higher showed 50% less hip fractures(10). Infants with lower calcium intake had a lower serum calcium, higher alkaline phosphatase activity and higher serum PTH level(11).

Alkaline phosphatase is a marker for OBL, which is responsible for bone formation. But, recent studies demonstrate that osteoclastic bone resorption requires the presence of OBL(12). These contrasting observations suggest that PTH might be involved not only in bone resorption but also in bone formation. In fact, several reports(13-17) indicate possible anabolic effects of PTH in bone metabolism.

This study was intendeded to examine the *in vivo* effects of calcitrophic hormones and dietary Ca levels on alkaline phosphatase bone enzyme related to bone growth and osteoporosis.

MATERIALS AND METHODS

Experimental animals and diet compositions

Three months-old BALB/C mice were maintained in a 12 : 12 dark and light cycled room. Before the start of the experiment animals were fed a chow diet, then

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divided into 3 dietary groups of high and medium Ca and Ca-free. Diet groups and the experimental diet composition is shown in Table 1.

Experimental design and hormonal treatment

Animals were divided into 18 groups according to diet and hormones. Hormones including PTH(1-34, bovine, Sigma), calcitonin(CT, from salmon, Sigma), and cholecalciferol(vitamin D, Sigma) were injected intraperitoneally in physiological levels and the initial solution was prepared at 0.83μg/ml, 41.67μg/ml and 83 mg/ml, respectively and the amounts of the hormone and the administration schedule is shown in Fig. 1. The injection of hormone was performed a week after the feeding of experimental diet. Also, the administration of hormone of ALL group was performed in a week interval as the schedule in Fig 1. As cholecalciferol was prepared in ethanol, it was injected twice in half-dose to avoid ethanol overdose.

Tissue and sample preparation

Animals were fasted for 12 hours and weighed before being sacrificed by ether.

Blood was taken by cardiac puncture, organs including liver and kidney were taken, weighed and rinsed by physiological saline.

Serum was obtained by centrifugation of blood at 2000rpm(Microspin 24S, Sorvall instruments) for 20 minutes and 1g of liver was homogenized with a sucrose buffer; centrifuged at 2000rpm for 15min, and pellets were discarded and supernatants were obtained and kept frozen at -70°C until assay.

Table 1. Food composition of experimental diet

	High Ca group[1]	Medium Ca group	Ca-free group
Casein[2]	14%	14%	14%
Fat(corn oil)	5%	5%	5%
Corn starch	70.32%	70.82%	70.82%
DL-Methionine	0.18%	0.18%	0.18%
Fiber[3]	5%	5%	5%
Vitamin[4]	1%	1%	1%
Mineral[5]			
Mineral mixture	4%	4%	–
Calcium-free	–	–	4%
$CaCO_3$	0.5%	–	–
Total	100%	100%	100%

[1]Total Ca contents of high, medium, and low Ca diet were 1.09, 0.59, and 0% respectively
[2]Purified high nitrogen casein (ICN)
[3]Alphacel (non nutritive bulk, ICN)
[4]AIN vitamin mixture 76(ICN)
[5]AIN mineral mixture 76(ICN) for high and medium Ca group and calcium-free salt mixture for Ca-free group

Wk 0	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5
Experimental diet Feeding[1]					Sacrificed
	PTH[2] 100μl				
	VD_3[3] 25μl	VD_3 25μl			
			CT[4] 100μl		

Fig. 1. Time table of intraperitoneal(I.P.) hormone injection[5].
[1]Experimental diet was fed throughout the experimental period until sacrifice.
[2]PTH(parathyroid hormone) was injected at the first week of experimental diet feeding.
[3]VD_3(cholecalciferol) was injected at the first and second week of experimental diet feeding.
[4]CT(calcitonin) was injected at the third week of experimental diet feeding.
[5]Hormones were administered at different intervals to avoid possible hormonal and ethanol side effects.

Table 2. Total design of the experiment[1)]

	I.P. Injections[2)]	Diet
Control H	Saline	High calcium
Control M	Saline	Medium calcium
Control L	Saline	Calcium-free
PTH H	PTH	High calcium
PTH M	PTH	Medium calcium
PTH L	PTH	Calcium-free
VD_3 H	Cholecalciferol	High calcium
VD_3 M	Cholecalciferol	Medium calcium
VD_3 L	Cholecalciferol	Calcium-free
PTH+CT H	PTH & Calcitonin	High calcium
PTH+CT M	PTH & Calcitonin	Medium calcium
PTH+CT L	PTH & Calcitonin	Calcium-free
VD_3+CT H	Cholecalciferol & CT	High calcium
VD_3+CT M	Cholecalciferol & CT	Medium calcium
VD_3+CT L	Cholecalciferol & CT	Calcium-free
ALL H	PTH & Cholecalciferol & CT	High calcium
ALL M	PTH & Cholecalciferol & CT	Medium calcium
ALL L	PTH & Cholecalciferol & CT	Calcium-free

[1)]An independent group of calcitonin(CT) was not established in this experimental design as it is a known inhibitor of bone resorption acting coupled with PTH or vitamin D rather than acting independently
[2)]Hormones were I.P. injected during the given experimental period as indicated in Fig. 1.

Alkaline phosphatase(AP) assay

Alkaline phosphatase in 10 to 30μg of liver homogenate and serum protein was measured in Sigma 221 alkaline phosphate buffer solution containing 9mM *p*-nitrophenyl phosphate(*p*-NPP). Reaction was stopped by adding 1.8ml of 0.1N NaOH after incubation at 37°C for 10min. Absorption was measured at 410nm (Kontron Inst., USA).

Protein measurement

Protein was determined by the method of Bradford (18) using Coomassie Brillant Blue G-250 and bovine serum albumin as a standard. Supernatants were diluted with deionized distilled water and 3ml of Coomassie solution was added to each 100μl of sample. Absorption was measured at 595nm(Kontron Instruments, Uvikon Spec.).

Statistical analysis

Data from an individual experiment was expressed as the means±standard error of means(SEM). Statistical analysis was performed by GLM(general linear model) of SAS(statistical analysis system) and Duncan's multivariate analysis.

RESULTS

The effect of PTH hormone

Compared to the control, PTH enhanced AP activity by average 26% in the serum. The overall increase of serum AP at high and medium Ca and calcium-free group showed a constant trend with a similar margin of increment(Fig. 2), whereas liver AP was constantly inhibited by PTH(Fig. 3). T/C ratio(the hormone treated/control) was 0.76. Regardless of the level of dietary calcium, liver AP was inhibited by 24% compared to the control.

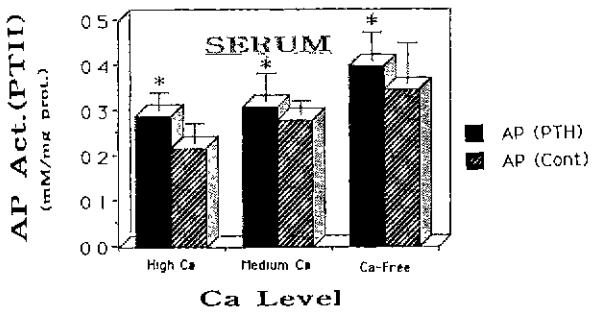

Fig. 2. Stimulation of AP activity in mice serum by PTH at all levels of dietary Ca.
*Indicates that values are significantly different from the control at $p<0.05$. Data are means±SEM.

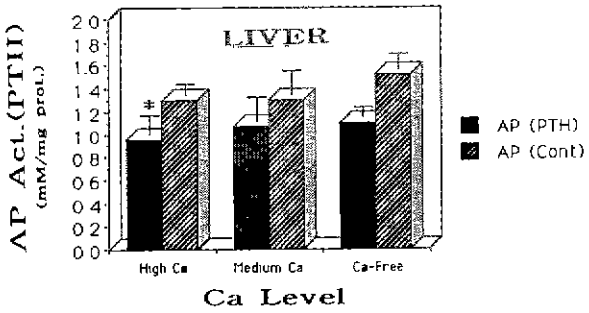

Fig. 3. Inhibition of AP activity in mice liver by PTH at all levels of dietary Ca.
*Indicates that values are significantly different from the control at $p<0.05$. Data are means±SEM.

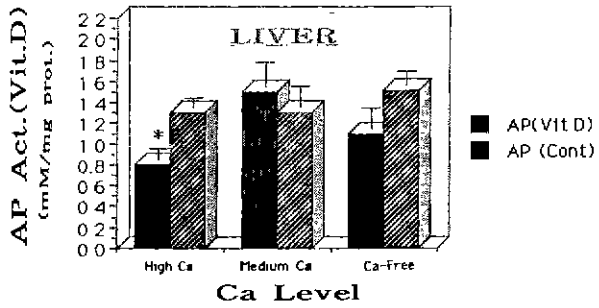

Fig. 5. Overall AP activity in mice liver was inhibited by vitamin D.
*Indicates that values are significantly different from the control at $p<0.05$. Data are means±SEM.

The effect of vitamin D

As shown in Fig. 4, T/C ratio of AP activity in high and medium Ca and calcium-free group was 1.54, 1.09, and 0.98, respectively. These results indicate that when dietary calcium is sufficient enough, vitamin D may increase AP activity in the blood to facilitate osteoblast action and eventually increasing bone formation. Liver AP activity trend was shown in Fig. 5. With a similar phenomenon to the PTH effect, vitamin D inhibited liver AP activity by average 22%.

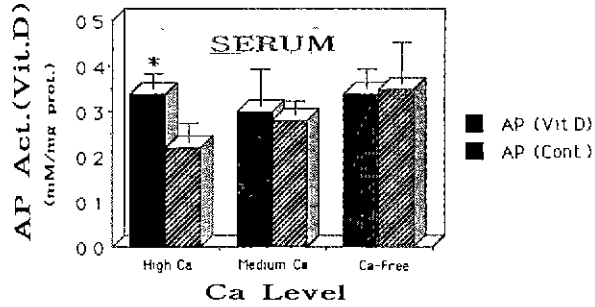

Fig. 4. AP activity in mice serum was enhanced by vitamin D only when dietary Ca supply was adequate.
*Indicates that values are significantly different from the control at $p<0.05$. Data are means±SEM.

The effect of calcitonin

The inhibition of AP by CT was distinctive and evident in the serum(Table 3). CT inhibited the stimulation of AP by PTH even to the lower level of the control. Similarily, CT also inhibited the stimulation of vitamin D to the level of the control. When PTH and vitamin D were administered with CT, serum AP was inhibited to the level of the control.

Although PTH alone inhibited AP in the liver, as shown in Fig. 3, PTH combined with CT showed a 13% increase of AP(Table 4). This explains well the interaction and cooperation between CT and PTH for the control of optimum AP level in the liver. Contrasted to the decrease of AP in the serum, CT increased AP in the liver, further to facilitate bone mineralization.

Table 3. Alkaline phosphatase activity in the mouse serum (unit : mM/mg prot.)

Hormone	High Ca	Medium Ca	Ca-free	Average
Control	$0.218\pm0.035^{1)b)}$	$0.278\pm0.032^{ab)}$	$0.347\pm0.091^{ab)}$	$0.273^{ab)}$
PTH	$0.289\pm0.040^{*ab)}$	$0.312\pm0.063^{a)}$	$0.402\pm0.059^{**a)}$	$0.344^{a)}$
VD_3	$0.335\pm0.027^{a)}$	$0.303\pm0.079^{ab)}$	$0.341\pm0.044^{ab)}$	$0.328^{a)}$
PTH+CT	$0.189\pm0.014^{*b)}$	$0.215\pm0.008^{b)}$	$0.234\pm0.009^{**b)}$	$0.213^{b)}$
VD_3+CT	$0.326\pm0.040^{a)}$	$0.247\pm0.011^{ab)}$	$0.309\pm0.030^{ab)}$	$0.297^{a)}$
PTH+VD_3+CT	$0.196\pm0.018^{*b)}$	$0.292\pm0.045^{*ab)}$	$0.404\pm0.036^{**a)}$	$0.305^{a)}$
Average	0.254*	0.272*	0.337**	

[1)] Mean±S.E.M.(Standard error of mean)
[abc)] Values within the same column with different superscripts were significantly different($p<0.05$) among groups by Duncan's multiple range test
[*,**] Values within the same row with different asterisk were significantly different($p<0.05$) among groups by Duncan's multiple range test
Values with no statistical significance among groups were not indicated

Table 4. Alkaline phosphatase activity in the mouse liver (unit : mM/mg prot.)

Hormone	High Ca	Medium Ca	Ca-free	Average
Control	1.303±0.087[1)a)]	1.300±0.201	1.505±0.132	1.369[a)]
PTH	0.954±0.173[ab)]	1.070±0.212	1.103±0.084	1.040[b)]
VD_3	0.800±0.093*[b)]	1.490±0.223**	1.078±0.189	1.066[ab)]
PTH+CT	0.780±0.174*[b)]	1.393±0.105**	1.466±0.169**	1.213[ab)]
VD_3+CT	1.404±0.151[a)]	1.183±0.173	1.126±0.172	1.241[ab)]
PTH+VD_3+CT	1.391±0.144[a)]	1.061±0.273	1.445±0.230	1.310[ab)]
Average	1.095	1.248	1.287	

[1)]Mean±S.E.M.(Standard error of mean)
[abc]Values within the same column with different superscripts were significantly different($p<0.05$) among groups by Duncan's multiple range test
[*,**]Values within the same row with different asterisk were significantly different($p<0.05$) among groups by Duncan's multiple range test
Values with no statistical significance among groups were not indicated

Table 5. Protein contents in mouse serum and liver

	Hormone	High Ca	Medium Ca	Ca-free	Average
					(mg/100μl)
Serum	Control	3.972±0.133[1)]	3.860±0.159	4.270±0.589	4.029[NS]
	PTH	3.974±0.064	3.588±0.325	3.763±0.093	3.890
	VD_3	3.840±0.190	4.200±0.471	3.928±0.125	3.974
	PTH+CT	3.710±0.153	4.232±0.379	3.492±0.122	3.811
	VD_3+CT	4.126±0.215	4.878±0.609	4.024±0.173	3.947
	PTH+VD_3+CT	3.550±0.183	4.276±0.376	3.598±0.123	3.848
	Average	3.884[NS]	4.157	3.834	
					(mg/g liver)
Liver	Control	77.76±4.175[1)]	85.92±9.588	88.40±12.318	84.04[NS]
	PTH	85.52±5.145	80.32±8.650	84.96±8.192	83.60
	VD_3	86.40±10.378	84.53±6.468	89.36±4.640	87.12
	PTH+CT	86.32±6.228	86.72±8.805	91.28±10.944	88.12
	VD_3+CT	92.00±7.416	83.10±2.763	95.84±8.370	90.84
	PTH+VD_3+CT	95.00±9.241	83.00±7.846	93.20±8.297	90.60
	Average	86.88[NS]	83.96	90.52	

[1)]Mean±S.E.M.(Standard error of mean)
[NS]Not significant among groups at $p<0.05$ level by Duncan's multiple range test
Values with no statistical significance among groups were not indicated

vitamin D together with CT stimulated AP, while vitamin D alone inhibited AP in the liver. Combination of PTH, vitamin D and CT inhibited AP close to the level of the control.

Protein synthesis

Although a slight increase of protein synthesis was observed in PTH and vitamin D in treated liver tissue, there was no statistical differences between dietary and hormonal groups(Table 5).

DISCUSSION

Dietary calcium and other mineral intake and maintenance of proper calcium-regulating hormone are important in bone growth and development. This study has dealt with the role of PTH, vitamin D, and dietary calcium in terms of AP activity.

The above results of the increment of AP by PTH in the serum corresponds with the observation that serum AP was increased in patients with hyperplasia of parathyroid glands(19). The elevation of both PTH and AP in the serum were also seen in primary hyperparathyroidism(20,21). The ratio of Ca : P may also influence AP activity because calcium can not be incorporated into bone without phosphorus, consequently when phosphorus is limiteds urinary calcium excretion is increased(11).

According to Schapira et al.(22) vertebral mineral contents were maintained during aging with high calcium and vitamin D. The results of this study proved that stimulation of AP by vitamin D and high calcium can lead to increase of bone mass and bone density. Some other studies reported that AP may or may not stimulate AP activity(23,24). This variation may be dependent upon dietary calcium levels. Therefore, the effect of vitamin D *in vivo* may differ with dietary calcium intake levels and body calcium status.

As for the effect of PTH hormone, two phases of the action of PTH proposed-early and late effects. The early phase is mobilization of calcium from bone and the enhanced transfer of this calcium into the extra cellular fluid. The later phase is associated with an increase in the synthesis of bone enzymes, particularly lysosomal enzymes promoting bone resorption and remodeling. As a later effect of PTH, new bone formation through the activation of OBL and bone growth factors is enhanced by PTH(4,25).

As an important mediator of growth hormone, IGF-I is involved in bone growth and development. IGF-I is synthesized in the liver and stimulates the growth and differentiation of its target tissue, collagen and muscle(26). Many growth factors are identified in many different cell lines(27-30), which might play important roles in bone mineralization and growth.

According to McCarthy et al.(31) osteoblastic cAMP stimulates the synthesis of IGF-I. In addition, this kind of action is also stimulated by PTH in rat OBL cells(4).

The use of calcium-regulating hormone for the inhibition of bone turnover in Paget's bone disease(24) and osteoporosis(32,33) is clinical in application. Further, to prevent osteoporosis the utilization of calcitrophic hormones including PTH, CT and vitamin D can be proposed in addition to the use of diet therapy.

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REFERENCES

1. Chambers, T. J. : The cellular basis of bone resorption. *Clin. Orthop.*. **151**, 283(1980)
2. Raisz, L. G. · Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. *J. Clin. Invest.*, **44**, 103(1965)
3. Dietrich, J. W., Canalis, E. M.. Maina, D. M. and Raisz, L. G. : Hormonal control of collagen synthesis *in vitro* : effect of parathyroid hormone and calcification. *Endocrinology*, **98**, 943(1976)
4. Tam, C. S., Heersche, N. M., Murray, T. M. and Parsons, J. A. : Parathyroid hormone stimulates the bone apposition rate independently of its resorptive action : Differential effects of intermittent and continuous administration. *Endocrinology*, **110**. 506(1982)
5. Paulsen, N. V. : Calcium and phosphorus intake in senile osteoporosis. *Geriatrics*, **8**, 76(1953)
6. Wardlaw, G. : The effect of diet and life style on bone mass in women. *JADA*, **88**, 17(1988)
7. Alan, D. and Martin, C. : Osteoporosis ; calcium and physical activity. *CMAJ*. **136**, 587(1987)
8. Kim, H. K. and Yoon, J. S. : Factors influencing the bone status of Korean elderly women. *Kor. J. Nutr.*, **24**, 30(1991)
9. Hong, H. O. and Yu, C H. : The effect of Ca and vitamin D supplementation on bone metabolism in postmenopausal women. *Kor. J. Nutr.*, **27**, 1025(1994)
10. Matokovic, V.. Kostial, K.. Simonovic, I., Buzin, R.. Brodarec, A. and Nordin, B. E. C. : Bone status and fracture rates in two regions of Yugoslavia. *Am. J. Clin. Nutr.*, **32**, 540(1979)
11. Hillman, L. S. : Nutritional factors affecting mineral homeostasis and mineralization in the term and preterm infant. In "*Nutrition and bone development*" Simmons, D J.(ed.), Oxford Univ. Press, New York, p.55(1990)
12. McSheehy, P. M. J. and Chembers, T J. : Osteoblastic cell mediate osteoclastic responsiveness to parathyroid hormone. *Endocrinology*, **118**, 824(1986)
13. Gaillard, P. J., Wassenaar, A. M. and Wijhe Wheeler, M. Z. : Effects of parathyroid hormone and a synthetic fragment(PTH 1-34) in bone *in vitro*. *Proc. K. Ned. Akad. Wet.(Biol Med)*, **C80**, 267(1977)
14. Chung, C. K. : Parathyroid hormonal effect on bone formation and resorption in culture mouse calvarial tissues. *Kor. J. Endocrinology*, **4**, 126(1989)
15. Chung, C. K. : *In vitro* stimulation of bone formation and protein synthesis by parathyroid hormone in cultured neonatal mouse calvarial tissue. *Hallym Univ. J.(Natural Sciences & Medicine)*, **5**, 115(1987)
16. Chung, C. K. and Ha, K. S. : Effect of parathyroid hormone and calcitonin on the enzyme and mineral metabolism of bone cells and phosphorylation. *Kor. J. Nutr.*, **28**, 737(1995)
17. Raisz, L. G., Canalis, E. M.. Dietrich, J. M., Kream, B. E. and Gworek, S C. · Hormonal stimulation of bone formation. In "*Recent progress in hormone research*" Academic Press Ins., New York, p.335(1978)
18. Bradford, M. M. · A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochm.*, **72**, 248(1976)
19. Huang, Y. Y., Hsu, B. R., Huang, B. Y., Huang, H. S. and Huang, M. J. : Values of serum alkaline phosphatase evaluating hyperplasia of parathyroid glands in chronic

hemodialysis patients. *J. Clin. Ultrasound,* **22**, 193(1994)
20. Pfeilschifter, J., Siegrist, E., Wuster, C., Blind, E. and Ziegler, R. : Serum levels of intact parathyroid hormone and alkaline phosphatase correlate with cortical and trabecular bone loss in primary hyperparathyroidism. *Acta Endocrinol. Copenh.*, **127**, 319(1992)
21. Slovik, D. M., Gundberg, C. M., Neer, R. M. and Lian, J. B. : Clinical evaluation of bone turnover by serum osteocalcin measurements in a hospital setting. *J. Clin. Endcrinol. Metab.*, **59**, 228(1984)
22. Schapira, D., Linn, S., Sarid, M., Mokadi, S., Kabala, A. and Sibermann, M. : Calcium and vitamin D enriched diets increase and preserve vertebral mineral content in aging laboratory rats. *Bone,* **16**, 575(1995)
23. Shigematsu, T., Kawaguchi, Y., Unemura, S., Yamamoto, H., Momose, M., Yokoyama, K., Wakabayashi, Y., Ikeda, M., Hasegawa, T. and Sakai, O. : Suppression of secondary hyperparathyroidism in chronic dialysis patients by single oral weekly dose of 1,25-dihydroxycholecalciferol. *Intern. Med.*, **32**, 695(1993)
24. Nunziata, V., Giannattasio, R., di Giovanni, G., Lettera, A. M. and Nunziata, C. A. : Vitamin D status in Paget's bone disease. Effect of calcitonin therapy. *Clin. Orthop.*, **293**, 366(1993)
25. Canalis, E. : Effect of growth factors on bone cell replication and differentiation. *Clin. Orthop.*, **193**, 246(1985)
26. Daughaday, W. H. : Growth hormone and the somatomedins. In "*Endocrine control of growth*" Daughaday, W. H.(ed.), Elsevier, New York, p.1(1981)
27. Oger, J., Arnason, B. G. W., Pantozis, N., Lehrich, J. and Young, M. : Synthesis of nerve growth factor by L and 3T3 cells in culture. *Proc. Natl Acc. Sci. USA,* **71**, 1554 (1974)
28. Klagsburn, M. and Smith, S. : Purification of a cartilage-derived growth factor. *J. Biol. Chem.*, **255**, 10859(1980)
29. De Larco, J. E. and Todaro, G. J. : A human fibrosarcoma cell line producing multiplication stimulating activity (MSA) related peptides. *Nature,* **272**, 356(1978)
30. De Larco, J. E. and Todaro, G. J. : Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA,* **75**, 4001(1978)
31. McCarthy, T. L., Centrella, M. and Canalis, E. : Cyclic AMP induces insulin-like growth factor I synthesis in osteoblast-enriched cultures. *JBC,* **265**, 15353(1990)
32. Staffan, A. V. and Lindgren, J. U. : Combined treatment with calcitonin and 1,25$(OH)_2D_3$ for osteoporosis in women. *Calcified Tissue Int.*, **53**, 26(1993)
33. Shiraki, M. and Orimo, H. : The effect of estrogen and sex-steroids and thyroid hormone preparation on bone mineral density in senile osteoporosis-a comparative study of the effect of 1 alpha-hydroxycholecalciferol in senile osteoporosis. *Nippon Naibupi Gakkai Zasshi,* **67**, 84(1991)

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