Evidence for modulation of osteocalcin containing γ -carboxyglutamic acid residues synthesis by insulin-like growth factor-I and vitamin K₂ in human osteosarcoma cell line MG-63

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Abstract

The effect of insulin-like growth factor-I (IGF-I) and 2-methyl-3-all-trans-tetraphenyl-1,4-naphthoquinone (vitamin K₂) on the synthesis of osteocalcin containing γ -carboxyglutamic acid (Gla) residues which is the physiologically relevant form in bone metabolism was studied in cultured human osteoblast-like (MG-63) cells. Both IGF-I and vitamin K₂ stimulated 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃)-induced osteocalcin containing Gla secretion in a concentration-dependent manner. This stimulatory effect of IGF-I and vitamin K₂ was additive. Vitamin K₂-enhanced osteocalcin containing Gla secretion was selectively suppressed by 3-(α -acetonil-benzyl)-4-hydroxy-coumarin (warfarin). The stimulatory effect of IGF-I was completely abolished by the presence of cycloheximide; in contrast the effect of vitamin K₂ was still observed in the presence of cycloheximide. Treatment of MG-63 cells with IGF-I caused an approximately 2.2-fold increase in osteocalcin mRNA levels (determined by reverse transcription-polymerase chain reaction). Vitamin K₂ had no effect on either the stimulation of mRNA level by IGF-I or the basal level. IGF-I-stimulated osteocalcin containing Gla secretion was inhibited by one of its binding proteins (insulin-like growth factor binding protein-4) in a concentration-dependent manner. These findings suggest that the modes of action of IGF-I and vitamin K₂ on 1,25(OH)₂D₃-induced osteocalcin containing Gla secretion in MG-63 cells are different.

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Introduction

Bone turnover is regulated by interactions of systemic hormones, locally synthesized growth factors and bone cells (1). Insulin-like growth factor-I (IGF-I) is a predominant growth factor secreted by osteoblasts and stimulates cellular proliferation and functioning via both autocrine and paracrine mechanisms (2–4). At least eight distinct IGF-binding proteins (IGFBPs) have been identified so far, which play important roles in modulating the cellular actions of IGF-I (5–7). IGFBP-4 was originally isolated from the conditioned medium of human bone cells and has been shown to produce potent inhibition of IGF-I-mediated osteoblast function *in vitro* (8).

Osteocalcin is one of the most abundant non-collagenous bone matrix proteins synthesized exclusively by osteoblasts (9). This 49-residue protein contains γ -carboxyglutamic acid (Gla) and has been termed 'bone Gla protein' (10). The Gla residues in osteocalcin bind Ca²⁺ and promote tight binding of the protein to hydroxyapatite. The protein may thereby

function in regulating bone matrix mineralization and bone turnover (11, 12). Gla residues are posttranslationally synthesized from selected glutamic acid residues by the vitamin K-dependent carboxylase in endoplasmic reticulum, where vitamin K is essential as a co-factor for γ -carboxylation (13). Several lines of evidence have suggested that vitamin K has a physiologically significant role in bone metabolism (12).

The regulatory mechanisms responsible for osteocalcin synthesis by 1,25-dihydroxyvitamin D₃, parathyroid hormone and glucocorticoids have been extensively investigated in human primary bone cell cultures, fetal rat calvaria cells, rat osteosarcoma cell lines and human osteosarcoma cell lines (14–18). However, no full report has yet appeared on the regulation of osteocalcin synthesis by IGF-I, nor has its interaction with vitamin K been examined. Therefore, in the present study we have investigated, in parallel, the effects of IGF-I and vitamin K₂ on the synthesis of osteocalcin containing Gla residues using the human osteosarcoma cell line MG-63 in an attempt to delineate further the mechanisms of IGF-I action on stimulating osteoblastic activity

which may be particularly important in bone turnover. The clonal osteosarcoma cell line used for this study secretes osteocalcin into culture medium, possesses receptors for IGF-I and displays a number of features of the osteoblastic phenotype (14, 19, 20).

Materials and methods

Materials

MG-63 human osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Human recombinant IGF-I (Fujisawa Pharmaceutical Co., Osaka, Japan), vitamin K₂ (2-methyl-3-all-trans-tetraphenyl-1,4-naphthoquinone; menaquinone-4) (Eisai Co., Tokyo, Japan) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) (Chugai Pharmaceutical Co., Tokyo, Japan) were kindly supplied. Recombinant human IGFBP-4 was purchased from AUSTRAL Biologicals (San Ramon, CA, USA). 3-(α -acetylbenzyl)-4-hydroxy-coumarin (warfarin) was from Sigma Chemical Co. (St Louis, MO, USA), tissue culture supplements were from Gibco (Grand Island, NY, USA) and the Gla-type osteocalcin enzyme immunoassay (EIA) kit was from Takara Shuzo Co. (Kyoto, Japan). Moloney murine leukemia virus (M-MLV) reverse transcriptase, deoxynucleotide 5'-triphosphate (dNTP), ribonuclease (RNase) inhibitor and deoxyribonuclease I (DNase I) were purchased from Boehringer Mannheim (Mannheim, Germany) and Taq DNA polymerase was from Takara Shuzo Co. All other chemicals were of the highest purity commercially available.

Cell culture

MG-63 cells were cultured at 37 °C as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air. Cells were subcultured by trypsinizing with 0.05% trypsin in Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffer saline (DPBS) containing 0.02% EDTA, seeded in 35-mm plastic culture dishes and grown for 3–4 days to the stage of confluence. Cells were used between passages 4–10 for experiments. At confluency ((1.45 $\pm$ 0.12) $\times$ 10⁶ cells/dish), cells were washed twice with DPBS and the medium was changed to serum-free DMEM with addition of IGF-I, vitamin K₂, 1,25(OH)₂D₃ or vehicle for the indicated times. Other additions are described in the figure and table legends. At the end of the incubation period, cells were more than 95% viable as assessed by trypan blue dye exclusion, and the number of cells per culture was not affected by each treatment. The conditioned medium was collected and centrifuged at 2000 g, 4 °C for 10 min to remove cellular debris, and stored at –70 °C until used.

Osteocalcin containing Gla secretion

Osteocalcin containing Gla secretion was determined by measuring its concentrations in the conditioned medium by an EIA kit which specifically detects fully carboxylated osteocalcin, containing three Gla residues located at positions 17, 21 and 24. The assay had a detection limit of 1.0 ng/ml with intra- and interassay coefficients of variation of 3.3% and 1.0% respectively in the concentration range of this study.

RNA extraction and reverse transcriptase (RT)-PCR analysis

Total RNA was extracted with RNAzol (Biotecx Laboratories, Houston, TX, USA) according to the manufacturer's protocol. RNA samples were treated with DNase I before RT-PCR to remove any contaminating DNA. The primers used in the subsequent RT-PCR were as follows: osteocalcin (21), forward, 5'-ATGAGAGCCCTCAGAC-TCCCTC-3' and backward, 5'-CGGGCCGTAGAAGCGCC-GATA-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (22), forward, 5'-CGGGAAGCTTGTGATCAA-TGG-3' and backward, 5'-GGCAGTGATGGCATGGAC-TG-3'. The expected sizes of the PCR products were 294 bp for osteocalcin and 345 bp for GAPDH. One microgram RNA was reverse transcribed into cDNA using the backward primer. The reverse transcription reaction, containing 500 μ mol/l dNTP, 0.05 μ mol/l backward primer, 10 unit/ μ l M-MLV reverse transcriptase, 3 mmol/l MgCl₂, 50 mmol/l KCl, 5 mmol/l dithiothreitol and 20 mmol/l Tris-HCl (pH 8.3), was sequentially incubated at 25 °C for 10 min, at 37 °C for 60 min and at 90 °C for 5 min. As a negative control for the absence of exogenous DNA contamination, reactions run without RNA or with RNA in the absence of the reverse transcriptase revealed no amplified product (data not shown). The synthesized cDNA (0.1 μ g equivalent of RNA) was used for PCR amplification in a reaction mixture containing 200 μ mol/l dNTP, 1.1 μ mol/l forward and backward primers, 0.55 unit/ μ l Taq DNA polymerase, 1.5 mmol/l MgCl₂, 50 mmol/l KCl and 20 mmol/l Tris-HCl (pH 8.3). The PCR conditions were: 30 cycles of 94 °C (1 min), 56 °C (1 min), 72 °C (1 min) for osteocalcin and 22 cycles of 93 °C (1 min), 58 °C (1 min), 72 °C (2 min) for GAPDH. The number of cycles was experimentally determined so that quantitative comparison could be made during the exponential phase of the amplification process. PCR products were separated on a 3% agarose gel. Gels were stained with ethidium bromide. The intensity of either the osteocalcin or GAPDH band was quantitated by scanning densitometry and the ratio of the two was used as a normalized expression value of the osteocalcin gene.

Protein estimation

Cellular protein was determined by the method of Lowry *et al.* (23) using bovine serum albumin as a standard

after solubilization of cultured MG-63 cells with 0.1% sodium dodecyl sulfate and 0.1 mol/l NaOH solution.

Statistics

Differences between groups were analyzed using two-tailed Student's *t*-test, with results considered statistically significant at $P < 0.05$.

Results

Time course of osteocalcin containing Gla secretion

The secretion of osteocalcin containing Gla by MG-63 cells as a function of incubation time is shown in Fig. 1. The presence of 1 nmol/l $1,25(\text{OH})_2\text{D}_3$ in the incubation medium stimulated osteocalcin containing Gla secretion markedly. A significant stimulatory effect of $1,25(\text{OH})_2\text{D}_3$ on osteocalcin containing Gla secretion was observed at 12 h after the initiation of incubation. The $1,25(\text{OH})_2\text{D}_3$ -induced osteocalcin containing Gla secretion measured under these conditions was a linear function of time for at least the first 36 h of incubation. In the absence of $1,25(\text{OH})_2\text{D}_3$, osteocalcin containing Gla secretion was below 2 ng/mg protein at any period tested. If $1,25(\text{OH})_2\text{D}_3$ was removed after 3 h of treatment and osteocalcin containing Gla secretion was determined 9 h later, there was no stimulation of secretion (control, 1.7 ± 0.5 ; 3 h treatment, 1.9 ± 0.9 ng/mg protein). This suggests that prolonged exposure to $1,25(\text{OH})_2\text{D}_3$ appears to be necessary for stimulation of osteocalcin

Figure 1 Time course of osteocalcin containing Gla secretion. MG-63 cells were cultured in the presence (●) or absence (○) of 1 nmol/l $1,25(\text{OH})_2\text{D}_3$ for the times indicated. Osteocalcin containing Gla (Gla-osteocalcin) concentrations in the conditioned medium were determined as described in Materials and methods. Each point represents the mean $\pm$ s.d. of three separate experiments with triplicate dishes.

containing Gla secretion. Therefore, secretion measured after 24 h of incubation in the presence of 1 nmol/l $1,25(\text{OH})_2\text{D}_3$ was used to estimate the initial rate of osteocalcin containing Gla secretion in the following experiments.

Effect of IGF-I and vitamin K_2 on osteocalcin containing Gla secretion

The effect of different concentrations of IGF-I or vitamin K_2 on $1,25(\text{OH})_2\text{D}_3$ -induced osteocalcin containing Gla secretion by MG-63 cells was determined. As shown in Fig. 2, both IGF-I and vitamin K_2 stimulated osteocalcin containing Gla secretion in a concentration-dependent manner. Significant stimulation was observed at 10^{-10} mol/l for IGF-I and at 10^{-6} mol/l for vitamin K_2 . The results also showed that IGF-I at a concentration of 10^{-8} mol/l could generate a greater increase of osteocalcin containing Gla secretion than vitamin K_2 at saturating doses. In the absence of $1,25(\text{OH})_2\text{D}_3$, no significant stimulation of osteocalcin containing Gla secretion by IGF-I or vitamin K_2 was observed (data not shown).

Effect of warfarin and cycloheximide on osteocalcin containing Gla secretion

Treatment with maximally effective doses of IGF-I (10 nmol/l) induced a 1.7-fold increase of $1,25(\text{OH})_2\text{D}_3$ -induced osteocalcin containing Gla secretion above basal levels. This increase was further stimulated by approximately 40% with 1 $\mu\text{mol/l}$ vitamin K_2 in combination with IGF-I (Table 1). Thus IGF-I and vitamin K_2 have an additive effect on osteocalcin containing Gla secretion. In order to investigate the modes of action of IGF-I and vitamin K_2 on the stimulation of $1,25(\text{OH})_2\text{D}_3$ -induced osteocalcin containing Gla secretion, MG-63 cells were incubated with IGF-I and/or vitamin K_2 in the presence or absence of either warfarin, an inhibitor of γ -carboxylation, or cycloheximide (Table 1). Warfarin had no significant effect on IGF-I-evoked stimulation of osteocalcin containing Gla secretion. The stimulatory effect of vitamin K_2 was significantly suppressed by the presence of warfarin in either the presence or absence of IGF-I. Treatment with cycloheximide decreased osteocalcin containing Gla secretion by 68% in the control culture and the stimulatory effect of IGF-I was completely abolished. However, the significant stimulatory effect of vitamin K_2 was still observed in the presence of cycloheximide.

Effect of IGF-I and vitamin K_2 on osteocalcin mRNA expression

In order to examine the roles of IGF-I and vitamin K_2 as regulators of osteocalcin gene expression in MG-63 cells, osteocalcin mRNA levels after treatment with IGF-I and/or vitamin K_2 were determined by RT-PCR. A single band

Figure 2 Effect of (A) IGF-I and (B) vitamin K₂ (Vit K₂) on osteocalcin containing Gla secretion. MG-63 cells were cultured in the presence of 1 nmol/l 1,25(OH)₂D₃ for 24 h with the indicated concentrations of IGF-I, vitamin K₂ or vehicle (control). Osteocalcin containing Gla (Gla-osteocalcin) concentrations in the conditioned medium were determined as described in Materials and methods. Data represent the means $\pm$ s.d. of three separate experiments with triplicate dishes. *Significantly different from control ($P < 0.05$).

of the expected size for osteocalcin (294 bp) was detected in each treatment (Fig. 3A). IGF-I at 10 nmol/l stimulated osteocalcin mRNA levels after 24 h of treatment, and it increased osteocalcin transcripts after 36 h (data not shown). Densitometry revealed that IGF-I increased osteocalcin mRNA by 2.2-fold after 24 h (Table 2) and by 2.4-fold after 36 h (data not shown). In contrast, vitamin K₂ at 1 μ mol/l affected neither the IGF-I-evoked stimulation of osteocalcin mRNA expression nor the basal level. The density of the RT-PCR product obtained using primers for GAPDH was relatively constant in each treatment (Fig. 3B), which indicated that nearly equal amounts of mRNA from the housekeeping gene were presented in each extracted RNA sample.

Table 1 Effect of warfarin and cycloheximide on IGF-I- and vitamin K₂-evoked stimulation of osteocalcin containing Gla secretion. MG-63 cells were cultured in the presence of 1 nmol/l 1,25(OH)₂D₃ for 24 h with 10 nmol/l IGF-I and/or 1 μ mol/l vitamin K₂ or vehicle (control) in the presence or absence of either 30 μ mol/l warfarin or 50 μ mol/l cycloheximide. Osteocalcin containing Gla (Gla-osteocalcin) concentrations in the conditioned medium were determined as described in Materials and methods. Values are means $\pm$ s.d. of three separate experiments with triplicate dishes.

Treatment	Gla-osteocalcin secretion (ng/mg cell protein)		
	Nil	Warfarin	Cycloheximide
Control	39.6 $\pm$ 3.3	36.7 $\pm$ 4.3	11.7 $\pm$ 2.0
IGF-I	66.9 $\pm$ 5.7*	60.6 $\pm$ 3.6*	14.0 $\pm$ 1.3
Vitamin K ₂	51.3 $\pm$ 3.9*	42.3 $\pm$ 3.9	17.9 $\pm$ 2.6*
IGF-I + vitamin K ₂	83.6 $\pm$ 7.6*	59.3 $\pm$ 5.7*	16.9 $\pm$ 2.2*

* Significantly different from control ($P < 0.05$).

Effect of IGFBP-4 on IGF-I-evoked stimulation of osteocalcin containing Gla secretion

The effect of IGFBP-4 was studied in an attempt to characterize further the action of IGF-I on 1,25(OH)₂D₃-induced osteocalcin containing Gla secretion. As shown in Fig. 4, IGFBP-4 produced a dose-dependent inhibition of IGF-I-stimulated osteocalcin containing Gla secretion. The half-maximal inhibition of IGF-I-stimulated uptake was observed at about

Figure 3 Effect of IGF-I and vitamin K₂ (Vit K₂) on osteocalcin mRNA expression. MG-63 cells were cultured with 10 nmol/l IGF-I and/or 1 μ mol/l vitamin K₂ or vehicle (control) for 24 h. The levels of (A) osteocalcin and (B) GAPDH mRNA were analyzed by RT-PCR as described in Materials and methods. The results shown here are representative of three separate experiments yielding similar results.

Table 2 Relative quantitation of osteocalcin mRNA. The intensity of either the osteocalcin or the GAPDH band was quantitated by scanning densitometry of PCR products in Fig. 3A and the ratio of the two was used as a normalized expression value of the osteocalcin gene. Values are means $\pm$ s.d. of three separate experiments with triplicate dishes, expressed as percentage of control (i.e. values cultured with vehicle alone).

Treatment	Osteocalcin mRNA level (% of control)
Control	100
IGF-I	223.7 $\pm$ 12.7*
Vitamin K ₂	97.3 $\pm$ 6.9
IGF-I + vitamin K ₂	213.6 $\pm$ 9.6*

* Significantly different from control ($P < 0.05$).

1.25 ng/ml and complete inhibition was found at 10 ng/ml IGFBP-4. However, in control cells increasing concentrations of IGFBP-4 had no significant inhibitory effect on osteocalcin containing Gla secretion.

Discussion

This is the first report demonstrating the mechanisms by which IGF-I and vitamin K₂ additively stimulate osteocalcin containing Gla secretion in human osteoblast-like MG-63 cells. That the modes of action of IGF-I and vitamin K₂ on 1,25(OH)₂D₃-induced osteocalcin containing Gla secretion are different is suggested by the

Figure 4 Effect of IGFBP-4 on IGF-I-evoked stimulation of osteocalcin containing Gla secretion. MG-63 cells were cultured in the presence of 1 nmol/l 1,25(OH)₂D₃ for 24 h with 10 nmol/l IGF-I or vehicle (control) in the presence of IGFBP-4 at the concentrations indicated. Osteocalcin containing Gla (Gla-osteocalcin) concentrations in the conditioned medium were determined as described in Materials and methods. Data represent the means $\pm$ s.d. of three separate experiments with triplicate dishes. *Significantly different from values in the absence of IGFBP-4 in IGF-I-evoked culture ($P < 0.05$).

following pieces of evidence shown in Tables 1 and 2. IGF-I and vitamin K₂ have an additive effect on 1,25(OH)₂D₃-induced osteocalcin containing Gla secretion. The stimulatory effect of IGF-I was completely abolished by the presence of cycloheximide; in contrast, the effect of vitamin K₂ was still observed in the presence of cycloheximide. Warfarin, an inhibitor of γ -carboxylation, had no significant effect on IGF-I-evoked stimulation of osteocalcin containing Gla secretion. The stimulatory effect of vitamin K₂ was significantly suppressed by the presence of warfarin in either the presence or absence of IGF-I. Treatment of cells with IGF-I stimulated osteocalcin mRNA expression, while vitamin K₂ had no effect on its expression. The vitamin K₂-dependent stimulation of osteocalcin containing Gla secretion seems to be related to enhanced γ -carboxylation of glutamic acid residues of osteocalcin since warfarin produced marked inhibition of vitamin K₂-stimulated osteocalcin containing Gla secretion. The regulation of osteocalcin containing Gla secretion by IGF-I may involve changes in the efficiency of either the transcriptional or the translational step of osteocalcin *de novo* synthesis. Thus the stimulatory effect of IGF-I on osteocalcin containing Gla secretion by MG-63 cells could be attributed to the enhanced synthesis of osteocalcin acting as a substrate of vitamin K₂-dependent γ -carboxylation of glutamic acid residues; in this way IGF-I and vitamin K₂ have an additive stimulatory effect on osteocalcin containing Gla secretion. A dose-dependent inhibition of IGF-I-stimulated osteocalcin containing Gla secretion was observed with increasing concentrations of IGFBP-4 (Fig. 4). These data suggest that IGFBP-4 is one of the IGFBPs which plays an important role in modulating the effect of IGF-I on bone osteocalcin biosynthesis via autocrine or paracrine mechanisms and, further, that 17 β -estradiol and parathyroid hormone may modify osteocalcin biosynthesis by regulating the binding activity of IGFBP-4 since previous studies have shown that osteoblasts secrete various IGFBPs and the binding activity of IGFBP-4 in the conditioned medium is controlled by 17 β -estradiol and parathyroid hormone (24–26).

The precise physiological roles of osteocalcin containing Gla have not been clearly defined. Some of the properties of osteocalcin containing Gla, such as its binding to hydroxyapatite, its concomitant appearance with bone formation and the constant molar ratio of osteocalcin to collagen and to calcium in bone, indicate that osteocalcin containing Gla synthesis is closely related to bone formation and mineralization. Previous observations have indicated that IGF-I mediates the effects of 17 β -estradiol in a variety of tissues including bone cells (27, 28). Therefore, it seems that the effect of estrogen replacement therapy on increasing bone mineral density is, in part, mediated through IGF-I-evoked stimulation of osteocalcin containing Gla synthesis by osteoblasts. Vitamin K₂ administered orally significantly increased bone mineral density of

patients with osteoporosis (29). Since therapeutic concentrations of vitamin K₂ in plasma are several times lower than those used in this study, we cannot clearly define the pharmacological significance of our observation. However, on the basis of the work presented here it is reasonable to speculate that estrogen replacement therapy may be more effective in the treatment of osteoporosis if used in combination with vitamin K₂.

In the series of experiments designed to delineate the role of IGF-I in osteoblastic activity we have investigated, in parallel, the effects of IGF-I and vitamin K₂ on 1,25(OH)₂D₃-induced osteocalcin containing Gla secretion by human osteoblast-like MG-63 cells. The results obtained show that IGF-I and vitamin K₂ have an additive stimulatory effect on osteocalcin containing Gla secretion. Thus the stimulatory effect of IGF-I and vitamin K₂ on osteocalcin containing Gla secretion appears to be the consequence of at least two regulatory steps: the first is an increase in osteocalcin synthesis by IGF-I to act as a precursor of γ -carboxylation, while the second is a direct effect of vitamin K₂ on γ -carboxylation of glutamic acid residues. Since the present study was performed in an osteoblast-like osteosarcoma cell line, it remains to be investigated whether the same phenomenon is found in osteoblasts *in vivo*.

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