Histone H4-related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic activity

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It has been established that regenerating marrow induces an osteogenic response in distant skeletal sites and that this activity is mediated by factors released into the circulation by the healing tissue. In the present study we have characterized one of these factors, a 14 amino acid peptide named osteogenic growth peptide (OGP). Synthetic OGP, identical in structure to the native molecule, stimulates the proliferation and alkaline phosphatase activity of osteoblastic cells in vitro and increases bone mass in rats when injected in vivo. Immunoreactive OGP in high abundance is present physiologically in the serum, mainly in the form of an OGP–OGP binding protein complex. A marked increase in serum bound and unbound OGP accompanies the osteogenic phase of post-ablation marrow regeneration and associated systemic osteogenic response. Authentic OGP is identical to the C-terminus of histone H4 and shares a five residue motif with a T-cell receptor β-chain V-region and the Bacillus subtilis outB locus. Since these latter proteins have not been implicated previously in the control of cell proliferation or differentiation, OGP may belong to a novel, heretofore unrecognized family of regulatory peptides. Perhaps more importantly, OGP appears to represent a new class of molecules involved in the systemic control of osteoblast proliferation and differentiation.
Key words: growth factor/histone H4/bone marrow/osteogenesis/bone remodelling/binding protein

Introduction

Bone and bone marrow are anatomically and functionally related tissues in which cells originating in the marrow serve as precursors for the osteoblasts and osteoclasts required for osteogenesis and bone remodelling (Friedenstein, 1976; Ashton et al., 1980; Schneider et al., 1986; Udagawa et al., 1990). In the adult organism, these processes are ongoing but occur at a relatively slow rate. However, with trauma and in some states of disease, these processes are substantially accelerated. This acceleration is particularly dramatic

following marrow ablation, wherein the marrow regeneration includes an osteogenic phase in which bone trabeculae are formed in the marrow cavity. These trabeculae are transient structures that are subsequently resorbed during the reconstitution of a normal, hemopoietic marrow compartment (Amsel et al., 1969; Patt and Maloney, 1975). In man and experimental animals, the local osteogenic response triggered by marrow injury is accompanied by a stimulation of bone formation at distal skeletal sites (Bab et al., 1985; Foldes et al., 1989; Einhorn et al., 1990; Gazit et al., 1990; Mueller et al., 1991), suggesting that a growth factor(s) responsible for affecting osteogenesis is (are) produced by the regenerating marrow and released into the circulation. The existence of such a growth factor(s) not only follows intuitively from the basic findings, but is also supported by our recent observations that the conditioned medium recovered from cultured regenerating rat bone marrow (HBMCM), but not normal marrow, contains osteogenic cell growth promoting activity. This activity has been partially purified by boiling, size exclusion and heparin – Sepharose chromatography (Bab et al., 1988; Gazit et al., 1989). In the present study we purified to homogeneity the heat stable rat HBMCM-derived activity recovered in the flow-through of the heparin – Sepharose column (Bab et al., 1988; Gazit et al., 1989) and identified a novel osteogenic growth peptide (OGP) identical to the C-terminus of histone H4 (HH4). In vitro the OGP stimulates proliferation and alkaline phosphatase activity in osteogenic cells. In vivo OGP is physiologically present in the serum, mainly as an OGP–OGP binding protein (OGPBP) complex and is highly and transiently increased during the post-ablation local and systemic osteogenic reactions. Exogenously administered OGP markedly enhances bone formation and increases trabecular bone mass.

Results

To characterize the molecules functioning in the post-ablation osteogenic response and stimulation of osteogenic cell DNA synthesis, the heat stable rat HBMCM-derived activity recovered in the flow-through of the heparin – Sepharose column (Bab et al., 1988; Gazit et al., 1989) was further purified by cation exchange and reverse phase chromatography. As previously (Bab et al., 1988; Gazit et al., 1989), the chromatographic fractions were screened for mitogenic activity by measuring [3H]thymidine ([3H]TdR) incorporation into the DNA of the osteogenic rat osteosarcoma cell line, ROS 17/2. Specifically, the fractions of potential interest were recovered from the cation exchanger at 1.0 M NaCl, pH 5.5 and from the reversed phase column at 27% acetonitrile (Figure 1).

An automated Edman's degradation of the protein recovered in the major peak of mitogenic activity from the reverse phase column revealed the presence of a 14 residue peptide of MW 1523. A computerized analysis of the amino

acid sequence of this peptide, designated OGP, revealed an identity with the 14 C-terminal residues of HH4 (Table I). In fact, the stretch of HH4 corresponding to OGP is completely homologous in most plant and animal species, including man, and contains sequences indispensable for cell proliferation and survival (Kayne, 1988). In addition, OGP shares a five amino acid residue motif, QGRTL, with a mouse T-cell receptor β-chain V-region and the *Bacillus subtilis* outB locus (Table I). The similarity of OGP with the T-cell receptor also includes a tyrosine residue at the C-terminus of this motif.

A synthetic peptide (sOGP) corresponding to the native OGP was prepared according to the standard solid phase peptide methodology (Barany and Merrifield, 1979). The sOGP reproduces the proliferative effect of the native OGP in the osteogenic ROS cell cultures where cell number is

Fig. 1. Purification of OGP by FPLC. A. Cation exchange separation on Mono S column of HBMCM-derived mitogenic activity. Data are mean of duplicate cultures. B. Reverse phase chromatography of mitogenically active cation exchange fractions on C1/C8 ProRPC column. Data are mean of triplicate cultures; fractions in the peak regions and almost all other fractions had SD < 10% of the mean. Bold dashed line indicates control level of [^{3}H]TdR.

enhanced dose dependently at $10^{-11} - 10^{-8}$ M followed by inhibition at 10^{-7} M. A considerably higher sensitivity to sOGP is expressed by the nontransformed mouse osteoblastic cell line MC3T3 E1 in which the increase in cell number peaks at 10^{-13} M peptide concentration. The peak effect in NIH 3T3 fibroblasts is seen at a sOGP dose of 10^{-10} M (Figure 2). The magnitude of this proliferative activity as well as the stimulation of DNA synthesis rate by native OGP (Figure 1) are smaller than those recorded in ROS 17/2 cells in response to crude HBMCM preparations where several growth factors are likely to act in concert to produce the biological effect (Bab et al., 1988; Gazit et al., 1989).

sOGP has a biphasic effect on osteoblastic cell alkaline phosphatase activity. In MC3T3 E1 cells the enzyme activity is modestly inhibited at 10^{-13} M followed by a marked stimulation at higher peptide concentrations (Figure 2). A similar reciprocal relationship between proliferation and alkaline phosphatase activity is also seen in osteogenic cells allowed to mature in long term cultures or exposed to agents such as transforming growth factor beta and thyroid hormone (Centrella et al., 1987; Noda and Rodan, 1987; Pfeilschifter et al., 1987; Sato et al., 1987; Fedarko et al., 1990). However, it is unlikely that this reciprocal effect on bone cells is a general one inasmuch as factors such as calcitonin and bone morphogenetic protein-2 elicit a conjoint stimulation in proliferation and maturation (Farley et al., 1989; Yamaguchi et al., 1991). Exposure of the cells to a synthetic peptide presenting the reversed sequence of OGP (designated retro-OGP or rOGP) fails to stimulate osteogenic cell proliferation in vitro (Figure 2) or bone formation in vivo (Figure 3) indicating, at least, that the nonpalindromic nature presented by the OGP backbone is essential for its effect on bone. Maintenance of side chain order and the net charge are not sufficient for the retention of OGP-like activity.

To assess the in vivo effect of OGP, either sOGP or rOGP were intravenously administered daily to adult male rats. On day 10 after the onset of treatment, the mandibular condyles were separated and undecalcified sections prepared for light microscope examination. A histomorphometric analysis revealed that sOGP but not rOGP, over the dose range of 0.1 – 100 ng/rat/day, elicits a progressive increase in the trabecular component of the subchondral marrow space (BV/TV) (Figure 3). This increase consists mainly of expanded trabecular volumes and enhanced transition of primary to secondary spongiosa (Figure 4). In the absence of significant alterations in osteoblast and osteoclast surfaces (Table II), the augmentation in trabecular mass must be
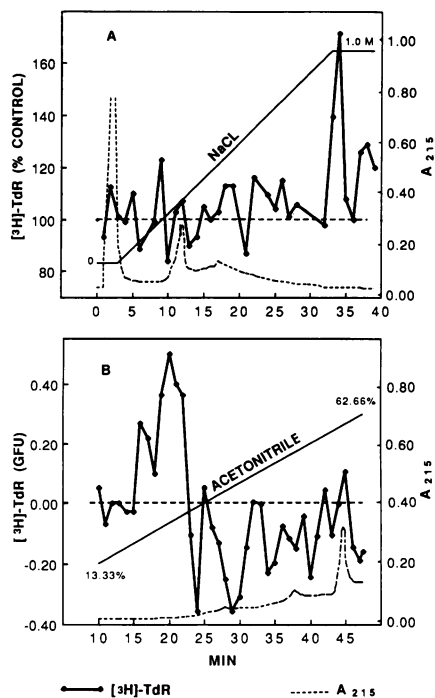
Table I. Amino acid residue alignment of OGP and structurally related sequences[a,b]

OGP	1	A L K R Q G R T L Y *G F G G	14
Histone H4 (human)	85 D V V Y *A L K R Q G R T L Y *G F G G		102
Histone H4 (rat)	86 D V V Y *A L K R Q G R T L Y *G F G G		103
Histone H4 (yeast)	86 D V V Y *S L K R Q G R T I Y *G F G G		103
Histone H4 (Tetrahymena thermophila)	55 D V V Y *A L K R Q G R T		66
T-cell receptor β-chain V-region (mouse)	98 R L Q V A N M S Q G R T L Y *C T C S		115
outB locus (Bacillus subtilis)	181 P L T G L T K R Q G R T L L K E L G		198

[a]Sequence data retrieved from the Genetic Computer Group Sequence Analysis Software Package, Version 6.2, June 1990, NBRF-Protein, Release 27.0, December 1990.
[b]Box indicates sequence identities.
[c]Chymotrypsin cleavage sites are indicated by *.

attributed to the stimulated osteoblastic activity expressed by the dose dependent increases in osteoid thickness (O.Th) and mineral appositional rate (MAR) (Figure 3). The close linear relationship between these parameters and the absence of changes in the mineralization lag time (Table II) indicate that sOGP enhances the formation of normally mineralized bone (Parfitt, 1983).

One plausible explanation for the small dose of sOGP required to evoke the in vivo osteogenic effect is the presence in the serum of endogenous OGP, possibly in a steady state equilibrium with an OGP−OGPBP complex. To test this hypothesis [^{125}I]sOGP was incubated with fresh serum and the relationship between the bound and unbound radio-iodinated peptide was demonstrated using a cathodic native PAGE. The [^{125}I]sOGP is competed out from the [^{125}I]sOGP−OGPBP complex dose dependently by cold sOGP with maximal displacement occurring at sOGP concentrations >300 nmol per ml undiluted serum (Figure 5). The displacement effect is mimicked by OGP-Cys^{15}(NEM)NH$_2$, a synthetic OGP analogue extended at the C-terminal with Cys[S-2(N-ethylsuccinyl)]carboxamide. Ac−Cys^0(NEM)-OGP, a synthetic analogue extended at the N-terminal with acetyl-Cys[S(N-ethylsuccinyl)] as well as rOGP fail to displace the radioiodinated peptide from the [^{125}I]sOGP−OGPBP complex (Figure 5), indicating a role for the OGP N-terminal in the OGP−OGPBP complex formation. The proteinous nature of the OGPBP was demonstrated by inhibition of the complex formation following trypsin or thermolysine digestion prior to the incubation with [^{125}I]sOGP. As in the case of other polypeptide growth regulators such as growth hormone and

insulin-like growth factor (Hintz, 1990), the OGPBP may protect serum OGP against proteolytic degradation thus regulating the level of active OGP. The OGPBP may also participate in the binding of OGP to its putative receptor.

To measure OGP levels in biological fluids, we have designed a competitive ELISA using a polyclonal rabbit anti-OGP antibody preparation that demonstrates specific binding to the OGP C-terminal region; the C-terminal modified analogue OGP-Cys^{15}(NEM)NH$_2$ does not bind to the antibody. To assess the physiological relationship between the bound and steady state levels of serum OGP, the immunoreactive OGP (irOGP) was measured in fresh serum or after incubation of the serum with either OGP-Cys^{15}(NEM)NH$_2$ or sOGP. The results of this experiment are shown in Figure 6. These results indicate that the competitive ELISA does not detect significant amounts of the OGP−OGPBP complex but only the unbound irOGP. The steady state irOGP level in individual rats in the presence of the antibody is 0.27−0.47 nmol/ml. The irOGP is competed out of the complex dose dependently by the OGP-Cys^{15}(NEM)NH$_2$ with maximal displacement occurring at OGP-Cys^{15}(NEM)NH$_2$ concentrations >300 nmol/ml undiluted serum, a value of the same order of magnitude as that obtained by displacing the [^{125}I]sOGP with sOGP (cf. Figures 5 and 6). The irOGP levels measured in different rats at maximal displacement (total irOGP) range between 7.0 and 25.0 nmol/ml (Figures 6 and 7). sOGP added at concentrations <100 nmol/ml induces a maximal 10-fold
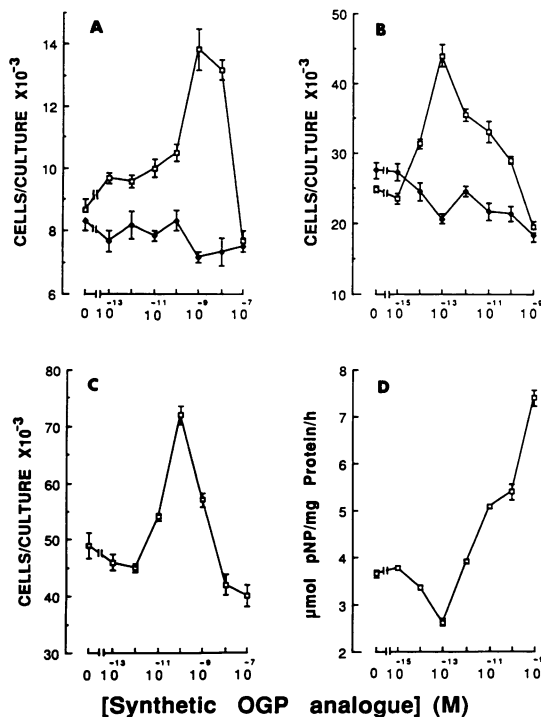

[Synthetic OGP analogue] (M)

Fig. 2. Effects of OGP on cellular activity in osteogenic and fibroblastic cell lines. **A.** Number of osteogenic ROS 17/2.8 cells. **B.** Number of osteogenic MC3T3 E1 cells. **C.** Number of NIH 3T3 fibroblasts. **D.** Alkaline phosphatase activity of osteogenic MC3T3 E1 cells. Open symbols, sOGP; closed symbols, rOGP. Data are mean ± SE of triplicate cultures representing at least three repetitive experiments.
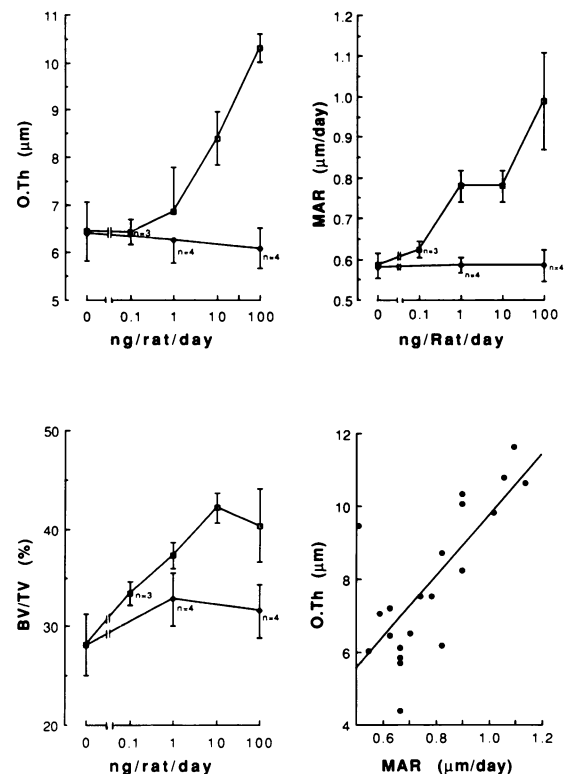

Fig. 3. Effect of OGP on bone remodelling in rat mandibular condyle. Open symbols, sOGP; closed symbols, rOGP. Data are mean ± SE obtained in five condyles (unless otherwise shown), one condyle per animal. The differences in osteoid thickness (O.Th), mineral appositional rate (MAR) and bone volume (BV/TV) between sOGP and rOGP treated animals were significant at $P = 0.0009$ (covariance model), $P = 0.0001$ (covariance model) and $P = 0.0367$ (logit model), respectively. The correlation between O.Th and MAR (lower right panel) shows a close linear relationship ($r = 0.81$; $P = 0.0001$).
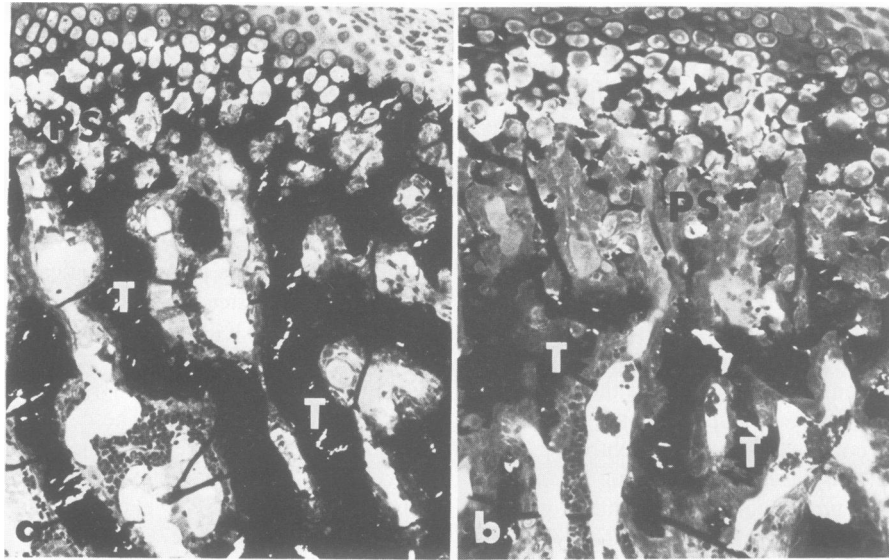
Fig. 4. Photomicrographs of subchondral cancellous bone in mandibular condyle of rat treated with 10 ng/day sOGP (a) and control animal sham injected with PBS (b). Note differences between animals in width of trabeculae (T) and primary spongiosa (PS). Von Kossa and toluidine blue; ×120.

Table II. Bone cell and mineralization parameters in rats treated with synthetic OGP analogues

Analogue		sOGP				rOGP	
Dose (ng/rat/day)	Control	$0.1^{n=3}$	1.0	10.0	100.0	$1.0^{n=4}$	100.0
Parameter							
Osteoblast surface (Ob.S/BS)	39.3 ± 4.1	41.3 ± 5.8	43.5 ± 3.5	34.9 ± 3.7	39.5 ± 4.9	54.9 ± 11.9	55.1 ± 3.9
Osteoclast surface (Oc.S/BS)	8.3 ± 2.3	16.3 ± 5.2	13.5 ± 3.3	11.0 ± 1.3	13.1 ± 2.0	11.3 ± 2.3	14.2 ± 2.3
Mineralization lag time (days)	13.5 ± 1.1	14.6 ± 1.1	12.4 ± 1.1	15.0 ± 0.7	15.3 ± 0.7	13.9 ± 1.1	14.6 ± 2.2

Data are mean ± SE obtained in five mandibular condyles (unless otherwise shown), one condyle per animal. All differences were not significant statistically.

increase in the steady state irOGP. Measurements done at higher sOGP concentrations are directly proportional to the amount of added peptide (Figure 6). In different rats this competition analysis reveals a serum OGP binding capacity of ~65 nmol/ml.

To further examine the functional relationship of OGP to the systemic post-ablation osteogenic reaction, the serum steady state and total irOGP were measured before ablation of tibial marrow and 1−12 days thereafter. The pattern of response is the same in all rats studied (Figure 7). Both parameters show a highly marked progressive increase, peaking on day five; the maximal elevation in the steady state and total irOGP being 2.5- and 4.5-fold, respectively. While the steady state irOGP remains at its maximum until day 10, the total irOGP is slightly decreased at this time point. From day 10 to day 12 there is a dramatic drop of the total irOGP to normal, with the steady state levels of approximately half the pre-ablation level (Figure 7), suggesting an increase in the serum OGPBP. This temporal pattern of post-ablation serum irOGP corresponds closely to that of the local and systemic osteogenic reactions and associated alterations in growth factor profile (Bab *et al.*, 1985; Gazit *et al.*, 1989, 1990; Einhorn *et al.*, 1990; Mueller *et al.*, 1991; Suva *et al.*, 1991).

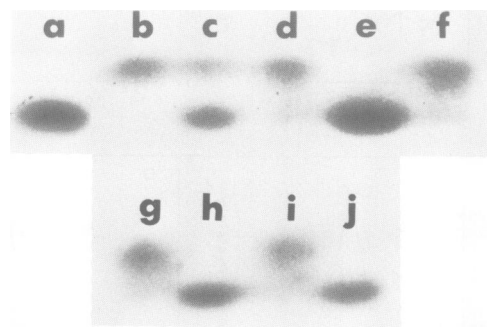The steady state irOGP is measured following prolonged

Fig. 5. Autoradiographs of cathodic native PAGE demonstrating binding of OGP to OGPBP. Lane a, [^{125}I]sOGP alone; lanes b−j, [^{125}I]sOGP preincubated with 1:50 dilution of fresh rat serum in the absence of synthetic OGP analogues (b,g) or presence of 0.625 nmol/ml sOGP (c), 0.625 nmol/ml rOGP (d), 6.25 nmol/ml sOGP (e,h), 6.25 nmol/ml rOGP (f), 6.25 nmol/ml Ac−Cys^0(NEM)-OGP (i) or 6.25 nmol/ml OGP-Cys^{15}(NEM)NH$_2$ (j). Representative of at least three repetitive experiments.

incubation with the anti-OGP antibody and thus provides a marked overestimation of the physiological 'free' or 'active' OGP and indeed, the *in vivo* effective sOGP dose is lower than the irOGP by more than one order of magnitude. The intermittent daily administration of the exogenous sOGP is
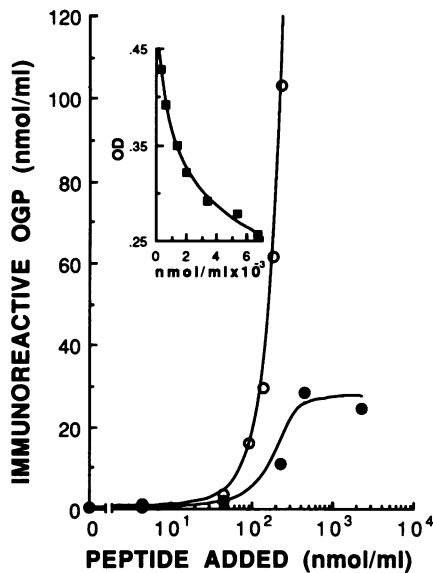
Fig. 6. irOGP in fresh rat serum following preincubation of undiluted specimens for 30 min at 37°C with the indicated concentrations of s at variance to OGP (open symbols) or OGP-Cys^{15}(NEM)NH$_2$ (closed symbols). Insert, standard curve of competitive ELISA for irOGP determination. Representative of at least three repetitive experiments.
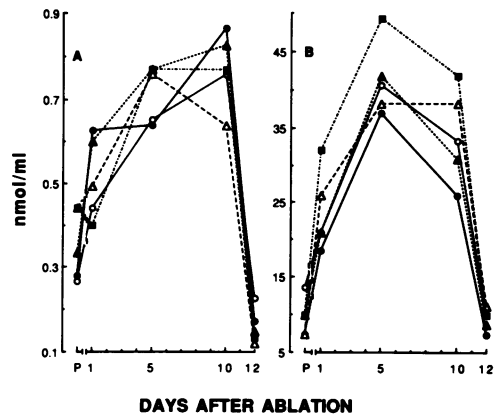

DAYS AFTER ABLATION

Fig. 7. Post-ablation steady-state (A) and total (B) serum irOGP in individual rats. Respective symbols in both panels indicate measurements in aliquots from same serum sample. P, pre-ablation levels.

at variance to the continuously elevated post-ablation serum OGP levels. This difference and the involvement of additional factors in the systemic post-ablation osteogenic reaction (Bab et al., 1988; Gazit et al., 1989; Suva et al., 1991) are presumably responsible for some variations in the overall pattern of the osteogenic response to sOGP (Figure 3, Table II) as compared with the events triggered by marrow ablation and regeneration where BV/TV is affected only marginally and an increase in osteoblast number is observed (Bab et al., 1985; Gazit et al., 1990). Such additional factors, which would not be present in purified synthetic or native OGP, may also be related to the above noted differences in the in vitro experiments.

Discussion

The structural identity of OGP with a portion of HH4 is very surprising. Histones are highly conserved proteins whose synthesis is characteristically linked to DNA replication and whose cellular location is typically within the chromatin structure of the nucleus (Spalding et al., 1966; Robbins and Borun, 1967; Stein and Borun, 1972; McGhee and Felsenfeld, 1980; Wu and Bonner, 1981; Weisbrod, 1982). Thus, at first glance, such proteins or protein fragments thereof would seem to be improbable candidates as cytokines affecting neighbouring or distant cells. However, HH4 or related peptides might become available for such a role following injury-induced cell death and degeneration. That this is unlikely to be the case follows from (i) the precise, closely repetitive, temporal up- and then keen-edged down-regulation of serum irOGP following marrow ablation and (ii) the observation that other types of injury, which also result in cell death (e.g. partial hepatectomy) do not influence skeletal growth (Gazit et al., 1990). A more likely alternative to account for the extracellular availability of OGP would be its production in association with some level of histone expression which is cell cycle independent. This may be pertinent particularly to HH4 for which there are several genes with identical coding but different regulatory sequences

(Lichtler et al., 1982 Grimes et al., 1987; Wolfe et al., 1989). In HH4 the sequence preceding the N-terminal of OGP comprises a potential chymotrypsin cleavage site (Table I) and thus a chymotrypsin-like activity may constitute a major step in the post-translational processing leading to the OGP production.

The large physiological OGP serum reservoir is consistent with the occurrence of HH4 and/or HH4-derived antigenically recognizable fragments on the surfaces of intact cells and in serum and milk (Holers and Kotzin, 1985; Waga et al., 1987). Also consistent with a possible regulatory function of OGP, is the finding that a pentapeptide homologous to the C-terminal pentapeptide of HH4 (10 − 14 OGP residues) has chronotropic and opiate-like effects in heart muscle and intestine (Kharchenko, 1987). Although the in vivo effects of OGP on tissues other than bone have not yet been investigated, the in vitro data on osteogenic and fibroblastic cell lines indicate that distinct cell types may exhibit a range of concentration dependent responses. One relevant mechanism for regulating the OGP concentration at the cellular level would be the OGP−OGPBP−OGP receptor interaction.

Aside from the distinctive biochemical nature of OGP, it differs in its mode of activity, particularly in vivo, from most other osteoblast affector substances. For example, while sOGP induces an acute enhancement in bone formation, factors such as prostaglandin E$_2$ and parathyroid hormone (PTH), when administered in vivo, initially stimulate osteogenic cell proliferation with a resulting delay in the promotion of bone formation (Jee et al., 1985; Liu and Kalu, 1990). Finally, the marked temporal elevation in serum OGP induced by marrow ablation, the in vivo effect of exogenously administered OGP and the in vitro data together indicate that OGP is a key factor in the mechanism of the systemic osteogenic reaction to marrow injury. This reaction, reported also in man (Foldes et al., 1989), suggests a potential role for OGP in local and systemic skeletal defects including fracture nonunion, periodontal disease and osteoporosis.

Materials and methods

Cation exchange chromatography

Cation exchange separation of HBMCM derived mitogenic activity in osteogenic cell cultures (ROS 17/2) was carried out by FPLC on Waters 650 Advanced Protein Purification System (Millipore Corporation, Milford,

MA). Material recovered from the heparin−Sepharose column at 0.15 M NaCl was desalted by dialysis against 5 mM ammonium acetate and freeze dried. Sodium acetate buffer (SAB), 50 mM, pH 5.0, was added to the dried material, 1.0 mmole SAB/1.65 mg protein. The mixture was centrifuged at 10 000 g for 15 min and the pellet, which contained ~85% of protein in the mixture, was discarded. The supernatant, containing 0.4−0.7 mg protein, was loaded on Mono-S HR 5/5 column (Pharmacia, Uppsala, Sweden) pumped at a flow rate of 1.0 ml/min with a linear 0.0−1.0 M NaCl gradient in SAB. Fractions were screened in the ROS 17/2 cell-[^{3}H]TdR assay (Bab et al., 1988; Gazit et al., 1989) with individual controls prepared by running an identical protein free gradient.

Reverse phase chromatography
Reverse phase chromatography of active fractions pooled for several cation exchange runs was performed using the same FPLC system. Approximately 18.0 µg protein were loaded on C1/C8 ProRPC HR 5/2 column (Pharmacia, Uppsala, Sweden) and eluted at a flow rate of 0.5 ml/min with a linear gradient of 0−100% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Fractions were screened as above. Activity growth factor units (GFU) were defined as half the effect of 10% fetal calf serum (Bab et al., 1988; Gazit et al., 1989).

Amino acid sequence determination
Protein in the main peak of mitogenic activity that recovered at 27% acetonitrile was subjected to automated peptide sequence analysis in an Applied Biosystems 470A Sequencer using the program and reagents supplied by the manufacturer. Released amino acid derivatives were identified with the aid of an on-line HPLC system.

Peptide synthesis
Synthetic peptides were prepared according to the standard solid phase peptide methodology (Barany and Merrifield, 1979). Side chains were deprotected and the peptide was cleaved from the resin using the HF procedure. The crude synthetic peptide was initially purified on a Sephadex G15F 3 × 35 cm column eluted with 50% (v/v) aqueous acetic acid. Further purification was accomplished on a Waters DeltaPrep 3000 HPLC instrument equipped with a PrePak 1000 module (Millipore Corporation, Milford, MA). The cartridge was pumped with 2 l of 5−33.5% acetonitrile gradient at a flow rate of 100 ml/min. Peptide composition was confirmed by amino acid sequence determination. The identity between the synthetic and native OGP was further established by mass spectroscopy and retention time contiguity on reversed phase chromatography.

Ac−Cys^0(NEM)-OGP and OGP-Cys^{15}(NEM)NH$_2$ were prepared by incubating Ac−Cys^0-OGP or OGP-Cys^{15}-NH$_2$, respectively, with a 10-fold molar excess of N-ethylmaleimide (NEM) in 0.5 M PBS, pH 7.0, for 1 h at room temperature. The NEM analogues were purified on HPLC Vydac Protein C18 reversed phase column (The Separation Group, Hesparia, CA) eluted with 2 l of 0−40% acetonitrile gradient at a flow rate of 100 ml/min.

Cell cultures
Tissue culture ingredients were purchased from Biological Industries, Beit Haemek, Israel. Cells in the test cultures were seeded in 16 mm multiwell dishes (Nunc, Roskilde, Denmark) at 2 × 10^4 cells/well and incubated at 37°C in CO$_2$−air. Osteogenic ROS 17/2.8 were maintained in F12 medium supplemented with 10% fetal calf serum (FCS) and subcultured once a week. Cells for experiments were derived from maintenance cultures containing 2.4 × 10^4−2.8 × 10^4 cells/cm^2. Test cultures were prepared in the same medium. For the initial 24 h, the medium was supplemented with 2% FCS followed by an additional 24 h period under serum-free conditions. sOGP or rOGP, preincubated with 4% BSA (Sigma Chemical Co., St Louis, MO; cat no. A-7030) for 30 min at 37°C were then added to the cell cultures. Cell counts were carried out after 48 h, using a hemocytometer.

Osteogenic MC3T3 E1 (the gift of Dr H.Kodama, Tohoku Dental University, Fukushima, Japan) and fibroblastic NIH 3T3 cells were maintained in αMEM supplemented with 10% FCS and subcultured twice a week. Cells for experiments were derived from maintenance cultures at confluency. Test cultures were prepared in the same medium. For the initial 46 h the medium was supplemented with 10% FCS and 0.2% nucleotide/ribonucleoside. The addition of sOGP or rOGP, preincubated with BSA as above, was preceded by washing of the cells (× 1) and 2 h incubation in serum-free medium. Determination of cell number, protein content and alkaline phosphatase activity was done after 48 h.

Protein and alkaline phosphatase determination
Prior to protein and alkaline phosphatase determination the cultures were thoroughly washed (× 5), the cells scraped into distilled water and the assays carried out as previously (Bab et al., 1988; Gazit et al., 1989).

In vivo bone remodelling
sOGP or rOGP were administered to male, 230−250 g, rats of the Hebrew University-Sabra strain. The administration consisted of nine consecutive daily injections into the tail vein of 100 µl of the peptides as phosphate buffered saline (PBS) solution. Control animals received peptide-free PBS. Tetracycline (Ledermycin, Lederle, FRG; 25 mg/kg BW) was injected intraperitoneally twice, on days 2 and 9. The animals were killed on day 10. The mandibular condyles were removed and processed undecalcified for light microscopy. The subchondal trabecular bone was analysed histomorphometrically in sections stained with Von Kossa−toluidine blue and unstained sections (for fluorescent microscopy of the tetracycline labels) as described (Bab et al., 1985; Gazit et al., 1990). The analysis comprised three sections per condyle, each a distance of 0.5 mm from the other. The bone histomorphometric parameters are those adopted by the American Society for Bone and Mineral Research (Parfitt et al., 1987).

Radioiodination
Radioiodination of sOGP was carried out in a reaction mixture containing sOGP, 40 mCi/ml [^{125}I]Na (Nuclear Research Center, Negev, Israel) and 100 µg/ml Iodo-gen (Pierce Chemical Company, Rockford, IL). The unreacted iodide was removed using a reversed phase SEP-PAK C18 cartridge (Waters Associates, Milford, MA). The [^{125}I]Na was washed out with 100 mM phosphate buffer, pH 7.5, followed by elution of the radioiodinated peptide with 50% acetonitrile/0.1% TFA solution. The monoiodinated sOGP was purified on a reversed phase HPLC Vydac Protein C4 column (The Separation Group, Hesparia, CA) pumped at a flow rate of 1.0 ml/min with 30 ml linear gradient of 15−25% acetonitrile containing 0.1% TFA.

Binding assay
[^{125}I]sOGP, 10^5 c.p.m./ml, was incubated for 30 min at 37°C with 1:50 dilution in PBS of fresh serum obtained from 300 g male Sabra rats. The incubation was carried out in the absence of unlabelled peptides or in the presence of 0.0625−6.25 nmol/ml sOGP, rOGP, Ac−Cys^0(NEM)-OGP or OGP-Cys^{15}(NEM)NH$_2$. The bound and unbound [^{125}I]sOGP were separated using a cathodic native PAGE system consisting of 15% polyacrylamide gel and 1 mM phosphate buffer pH 6.0, as the electrophoresis buffer. The gels were dried and autoradiographed by exposure to AGFA CURIX RP2 film.

Antibody generation
Anti-OGP antiserum was generated in rabbits in the standard manner using maleimido modified KLH which was conjugated with either Ac−Cys^0-OGP or OGP-Cys^{15}-NH$_2$ as before (Lerner et al., 1981). The IgG fraction was collected from the antiserum by ammonium sulphate fractionation. In ELISA this antibody preparation reacted specifically with the OGP C-terminal region as revealed from its binding to Ac−Cys^0(NEM)-OGP and failure to bind to OGP-Cys^{15}(NEM)NH$_2$, iodine labelled OGP at Tyr^{10} and rOGP. In addition, the anti-OGP IgG failed to react with HH4, PTH (1-34), bradykinin and substance P.

Competitive ELISA
Dynathec microtiter plates were coated with 100 µl of 10 µg/ml sOGP in 0.5 M sodium carbonate buffer, pH 9.5, overnight at 4°C, blocked with 1% gelatin in PBS at 37°C for 1 h and incubated with 100 µl anti-OGP IgG for 2 h at room temperature. Antibody binding was detected by the binding of anti-rabbit IgG-alkaline phosphatase conjugate and visualized with p-nitrophenyl phosphate substrate in diethanolamine buffer, pH 8.4. In competitive ELISA the anti-OGP IgG was preincubated for 30 min at 37°C with the respective test sample or samples of known sOGP content for calibration (Figure 6).

Post-ablation irOGP measurements
Ablation of tibial marrow was carried out in one limb of male, 230−250 g, rats of the Hebrew University-Sabra strain as before (Bab et al., 1985). Blood was drained from the tail immediately prior to ablation and 1−12 days thereafter. The resultant serum samples were collected frozen at −70°C. The samples were thawed simultaneously and assayed by the competitive ELISA. Aliquots allocated for total irOGP measurement were preincubated with 450 nmol/ml OGP-Cys^{15}(NEM)NH$_2$ for 30 min at 37°C.

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