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# Isolation of Osteogenic Growth Peptide From Osteoblastic MC3T3 E1 Cell Cultures and Demonstration of Osteogenic Growth Peptide Binding Proteins

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**Abstract** The osteogenic growth peptide (OGP) was recently characterized in regenerating bone marrow. In experimental animals it increases osteogenesis and hemopoiesis. In stromal cell cultures OGP stimulates proliferation, alkaline phosphatase activity, and matrix mineralization. OGP in high abundance is present in normal human and animal serum mainly complexed to OGP binding protein (OGPBP) or proteins. Here we show the presence of two OGPBPs, OGPBP-1, and OGPBP-2, in cultures of osteoblastic MC3T3 E1 cells. Immunoreactive OGP (irOGP) also accumulates in the medium of these cultures and in cultures of NIH 3T3 fibroblasts. A large amount of irOGP was released by heat inactivation of OGPBP-2 and purified by ultrafiltration and hydrophobic HPLC. The purified irOGP was identical to OGP obtained previously from rat regenerating bone marrow and human serum in terms of its amino acid sequence, immunoreactivity, and mitogenicity. Osteoblastic and fibroblastic cell proliferation can be arrested by anti-OGP antibodies and rescued by exogenous OGP, indicating that in the absence of serum or other exogenous growth stimulators the endogenously produced OGP is both necessary and sufficient for baseline proliferation. The OGP production is up- and down-regulated, respectively, by low and high doses of exogenous OGP in a manner consistent with an autoregulated feedback mechanism. The most effective OGP dose in MC3T3 E1 cells is at least two orders of magnitude lower than that in non-osteoblastic cell systems. This differential sensitivity of the osteoblastic cells could result in a preferential anabolic effect of OGP in bone. J. Cell. Biochem. 65:359–367. © 1997 Wiley-Liss, Inc.

**Key words:** osteogenic growth peptide; osteoblasts; fibroblasts; autocrine activity; proliferation

We have recently characterized a positively charged 14-amino acid growth polypeptide identical to the C-terminal region of histone H4 (HH4) [Bab et al., 1992]. The peptide was initially isolated from regenerating bone marrow tracing an osteogenic cell growth promoting activity [Bab et al., 1988; Gazit et al., 1989] and was therefore named osteogenic growth peptide (OGP). OGP in high abundance is also present physiologically in mammalian serum, mainly in the form of an OGP-OGP binding protein (OGPBP) complex [Bab et al., 1992; Greenberg et al., 1995]. A marked increase in the serum bound and unbound OGP accompanies the osteogenic phase of postablation marrow regeneration [Bab et al., 1992]. At this time a sys-

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temic osteogenic response is also evident [reviewed in Bab and Einhorn, 1993, 1994; Bab, 1995]. Synthetic OGP (sOGP) administered to rats increases bone formation and trabecular bone density in a manner reminiscent of the osteogenic response to bone marrow injury [Bab et al., 1992]. sOGP given to mice prior to ablative radiotherapy and bone marrow transplantation markedly enhances the engraftment of the marrow cell inoculum, probably by stimulating the stromal hemopoietic microenvironment [Bab, 1995; Gurevitch et al., 1996]. In vitro, native OGP and sOGP stimulate proliferation of osteoblastic MC3T3 E1 and ROS 17/2.8 cells and of NIH 3T3 fibroblasts [Bab et al., 1992; Greenberg et al., 1995, 1993]. In cultures of stromal cells derived from human and rabbit bone marrow sOGP stimulates proliferation, alkaline phosphatase activity and matrix mineralization [Robinson et al., 1995]. Modifications introduced to the OGP termini suggest a respective role for the N- and C-terminal regions in the OGP-OGPBP complex formation and OGP binding to its putative receptor [Greenberg et al., 1993].

Several mitogenic regulators such as insulin-like growth factors (IGFs), platelet-derived growth factor, fibroblast growth factors (FGFs), and transforming growth factor beta are expressed by osteoblastic cells [Bab and Einhorn, 1993, 1994; Mohan and Baylink, 1991]. These cells also secrete the corresponding binding proteins, at least in the case of IGF-I [Mohan and Baylink, 1991]. It was therefore of interest to assess whether osteoblastic cells in culture present an OGP-OGPBP system analogues to that shown in serum. To this end we isolated OGP from serum free MC3T3 E1 cell conditioned medium and demonstrated the presence of apparently two OGPBPs.

## MATERIALS AND METHODS

### Preparation of Synthetic OGP

*sOGP* was prepared as previously described [Bab et al., 1992; Greenberg et al., 1993] according to the standard solid phase peptide synthesis methodology [Barany and Merrifield, 1979].

### Cell Cultures

Osteoblastic MC3T3 E1 and fibroblastic NIH 3T3 cell cultures were set as described previously [Bab et al., 1992; Greenberg et al., 1995, 1993]. Cells derived from confluent maintenance cultures grown in alpha minimal essential medium (αMEM) supplemented with 10% FCS were seeded in 16 mm multiwell dishes at $10^4$ cells per $cm^2$ and incubated in the same medium at 37°C in $CO_2$-air. For the initial 46 h the cells were incubated in the same FCS-containing medium. The cultures were than washed and kept for an additional 2 h period under serum-free conditions. Native OGP, sOGP, and/or anti-OGP antibodies were preincubated with 4% fatty acid free BSA (Sigma Chemical Co., St. Louis, MO; cat. no. A-7030). These preparations or BSA alone were then added to the cell cultures for a further 48 h period. Cell counts were carried out using a hemocytometer.

### Binding Assay

OGP binding activity was analyzed in medium supplemented only with BSA, 48 h after its addition to MC3T3 E1 cells, using a competitive binding assay. [3-$^{125}$I(Tyr$^{10}$)]sOGP, $10^4$ cpm, was incubated for 30 min at 37°C with 6 μl of the conditioned medium in the absence or presence of 0.225 or 4.5 nmol unlabeled sOGP. The bound [3-$^{125}$I(Tyr$^{10}$)]sOGP was displayed employing a native anodic 3–12% linear gradient PAGE buffered with 0.041 M tris, 0.040 M borate, pH 8.6 [Van Leuven et al., 1981]. The position of the radiolabeled sOGP was demonstrated by autoradiography. This system exhibits the OGP-OGPBP complexes but not the positively charged free radiolabeled sOGP.

### Immunoreactive OGP Determination

irOGP levels were measured as detailed previously [Greenberg et al., 1995] by competitive ELISA using rabbit anti-OGP antiserum generated against *N*-Ac[(Cys$^0$)]OGP conjugated with maleimide activated Keyhole Limpet Hemocyanin (KLH).

### Purification of OGP

αMEM containing 4% BSA was conditioned with the MC3T3 E1 cells for a 24 h period preceded by a 2 h starvation in medium free of serum and of BSA as above. Further purification steps were carried out as described previously for the isolation of OGP from serum [Greenberg et al., 1995]. Six ml of the conditioned medium were loaded onto microconcentrators with 3,000 MW cutoff (Centricon 3, Amicon, Beverly, MA) and centrifuged at 5,000*g* for 3 h to dispose the majority of small peptides. The retentate, 17.5% of the original volume,

was reconstituted to the original volume with double distilled water and recentrifuged. This step was repeated twice. Then the retentate was diluted with water to a volume 10-fold higher than that of the conditioned medium, boiled for 10 min and centrifuged in the same microconcentrator. The filtrate, 53 ml, was concentrated by evaporation to a 400 μl volume using a SpeedVac apparatus (Savant Instruments Inc., Farmingdale, NY) and subjected to reverse phase HPLC (RP-HPLC) on a 5 μm Lichrocart 125-4 C18 column (Merck, Darmstadt, Germany). Elution was carried out with a 30 min 18–23% linear acetonitrile gradient containing 0.1% TFA at a flow rate of 1 ml/min. Microconcentration and chromatographic fractions were screened for irOGP and total amino acid content using the competitive ELISA and Fmoc amino acid derivatization, respectively.

### Amino Acid Sequence Determination

Material recovered in the reverse phase irOGP peak was subjected to automated peptide sequence analysis in an Applied Biosystems 470A sequencer. Released amino acid derivatives were identified with the aid of an on-line HPLC system.

### Amino Acid Analysis

A sample from the reverse phase irOGP peak was subjected to amino acid analysis in Dionex Amino Acid Analyzer (Dionex, Sun Valley, CA) using a cation exchange column and post-column derivatization by ninhydrin.

### Antibody Purification

Affinity purification of antibodies from the anti-OGP antiserum was carried out using *N*-Ac[(Cys$^0$)]OGP-maleimido conjugated BSA coupled to cyanogen bromide activated Sepharose 4B (CB-Sepharose) (Sigma Chemical Co., St. Louis, MO). The anti-OGP antibodies were released with 100 mM triethylamine, pH 11.5. Anti-KLH antibodies were purified from the antiserum flow-through of the OGP-based affinity column on another affinity column that consisted of KLH conjugated to CB-Sepharose. The anti-KLH antibodies were released with 100 mM glycine, pH 2.5.

## RESULTS

We previously used a native cathodic gel system to demonstrate the presence of a serum-derived OGP-OGPBP complex as well as the free radioligand [Bab et al., 1992; Greenberg et al., 1995, 1993]. This gel system could not be successfully applied for the analysis of conditioned medium-derived OGPBPs. The alternatively employed native anodic PAGE, although failing to show the free [3-$^{125}$I(Tyr$^{10}$)]sOGP, clearly revealed the presence of two [3-$^{125}$I(Tyr$^{10}$)]sOGP-OGPBP complexes in serum free medium collected from 48 h cultures of osteoblastic MC3T3 E1 cells (Fig. 1). The radiolabeled peptide was competed out from either complex dose dependently by cold sOGP. The addition of 300 nmol/ml sOGP resulted in almost complete competition (Fig. 1). The corresponding OGPBPs in the slow and fast electrophoretically migrating complexes were designated OGPBP-1 and OGPBP-2, respectively. Unlike the serum-derived OGP binding activity, which can be completely abolished by boiling, only the complex formed with OGPBP-2 is heat labile. Ten min boiling did not affect the OGP binding properties of OGPBP-1 (Fig. 1).

Competitive ELISA measurements in the osteoblastic MC3T3 E1 and fibroblastic NIH 3T3 cell conditioned medium revealed a continuous accumulation of irOGP mainly during the initial 24 h after changing to serum free, BSA supplemented medium. The levels determined after boiling were approximately 10-fold higher than those found in fresh samples (Fig. 2). Therefore, to conveniently purify substantial amounts of OGP from MC3T3 E1 cell cultures, we employed a strategy based on the release of OGP from its complex with OGPBP-2 by boiling of a 24 h conditioned medium. A similar protocol was recently applied for the isolation and purification of OGP from human serum [Green-

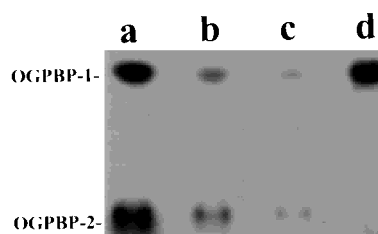


**Fig. 1.** Autoradiograph of anodic native PAGE demonstrating OGP-binding activity in osteoblastic MC3T3 E1 cell culture medium. **Lanes a–c:** [3-$^{125}$I(Tyr$^{10}$)]sOGP preincubated with 1:5 dilution of fresh conditioned medium in the absence of "cold" sOGP **(a)**, or the presence of 15 nmol/ml sOGP **(b)**, or 300 nmol/ml sOGP **(c)**. **Lane d:** Boiled reaction mixture containing [3-$^{125}$I(Tyr$^{10}$)]sOGP preincubated with 1:5 dilution of conditioned medium.

**TABLE I. Purification of OGP***

| Purification step | Total AA (mmol) | Boiled irOGP (nmol) | irOGP recovery (%) | SIR (nmol/mmol) | Purification |
|---|---|---|---|---|---|
| Retentate after initial filtration[a] | 1.25 | 15.4 | 100 | 12.4 | 1 |
| Filtrate after boiling | 0.07 | 12.2 | 79.2 | 184.8 | 15 |
| Retentate after boiling | 1.1 | 2.23 | 14.5 | 2.0 | NA |
| Peak HPLC fractions | 0.00007 | 8.08 | 52.5 | 115428 | 9339 |
| Other HPLC fractions | NA | 0.55 | 3.6 | NA | NA |

*Abbreviations: AA, amino acids; SIR, specific immunoreactivity; NA, not applicable.
[a]Measured after boiling to release irOGP from OGP-OGP binding protein complex.

berg et al., 1995]. The elution profile of the reverse-phase HPLC shows that most of the irOGP loaded on the column recovered in a well defined peak eluted at 21% acetonitrile, a position identical to that of control sOGP (Table I, Fig. 3). The difference in recovery between the filtrate after boiling and the reverse phase eluate reflects mainly loss of material during the pre-HPLC concentration step (data not shown). The amino acid composition and sequence determinations further indicated the purity of the irOGP and its identity with OGP (Table II). An extrapolation based on Table I suggests that at least 93% of the irOGP released by boiling is OGP. This conditioned medium derived OGP shares the full peptide's proliferative activity in the MC 3T3 E1 and NIH 3T3 cell cultures (Fig. 4).

To explore the role of endogenous OGP in the regulation of cell proliferation, affinity purified antibodies raised against the OGP C-terminal region [Bab et al., 1992], the putative OGP bioactive site [Greenberg et al., 1993], were used to sequester the free OGP. In both the MC3T3 E1 and NIH 3T3 cell cultures, antibodies added in the beginning of the 48 h incubation in the defined medium inhibited cell number dose dependently, arresting proliferation completely at 2.5 nM (Fig. 5). Higher antibody concentrations induced a moderate rate of cell death, consistent with the absence of growth factor stimulation [Williams, 1991; Wyllie et al., 1987]. The remaining cells appeared normal. Control cultures treated with anti-KLH antibodies purified from the anti-OGP antiserum did not affect the cell number (Fig. 5). The anti-OGP antibody induced arrest of proliferation was reversed dose dependently by exogenously added sOGP. If added in high enough concentration the exogenous OGP not only restored the baseline proliferation, but triggered the full OGP effect (Fig. 5). To assess whether OGP levels are autoregulated, exogenous sOGP

**TABLE II. Amino Acid Composition and Sequence of irOGP From MC3T3 E1 Cell Culture Medium**

*Amino acid analysis*

| Amino acid residue | Expected no. of residues per molecule | Measured number of residues per molecule: sOGP control | Measured number of residues per molecule: irOGP |
|---|---|---|---|
| Ala | 1 | 1.009 | 0.85 |
| Leu | 2 | 1.88 | 2.75 |
| Lys | 1 | 0.88 | 0.68 |
| Arg | 2 | 2.29 | 2.11 |
| Gln | 1 | 0.998 | 0.99 |
| Gly | 4 | 4.1 | 3.8 |
| Thr | 1 | 1.19 | 0.98 |
| Tyr | 1 | 0.77 | 0.81 |
| Phe | 1 | 0.89 | 0.93 |

*Amino acid sequencing*

| | |
|---|---|
| sOGP: | Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly |
| irOGP: | Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly |

was added to MC3T3 E1 and NIH 3T3 cell cultures and irOGP was measured at different time points thereafter. These levels in the respective cell systems were stimulated dose dependently by $10^{-7} - 10^{-5}$ nmol/ml and $10^{-5} - 10^{-3}$ nmol/ml sOGP added 4 h prior to measurement. Higher doses induced a reversal of this enhancement (Fig. 6). Since the amount of sOGP added was below the detection threshold of our immunoassay, the levels measured accurately represent changes in the production rate of endogenous OGP.

## DISCUSSION

The present study in murine osteoblastic cells, which respond to OGP, describes an endog-

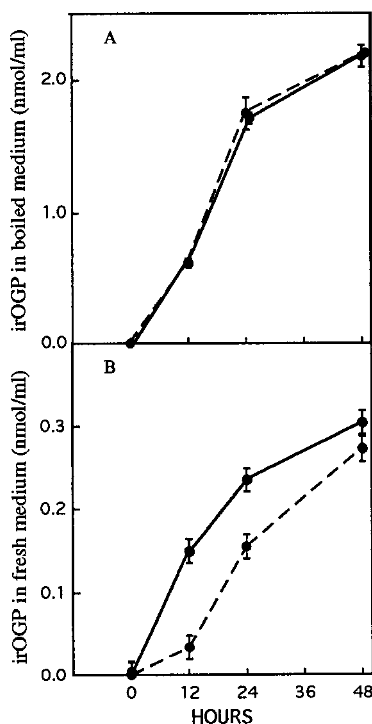


Fig. 2. Accumulation of irOGP in serum free, BSA supplemented cell cultures. A: Boiled medium; B: fresh medium. ——, MC3T3 E1 cells; ----, NIH 3T3 cells. Data are mean ± SE obtained at each time point in triplicate culture wells.

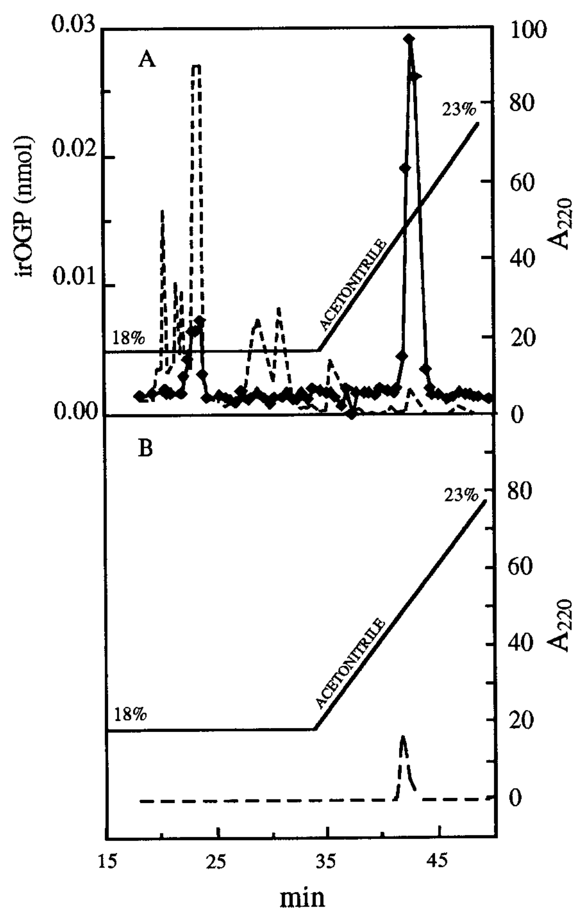


Fig. 3. Purification of OGP from MC3T3 E1 cell culture medium. A: Reverse-phase HPLC of irOGP from post-boiling filtrate. B: Control reverse-phase HPLC of sOGP showing identity of elution position with that of MC3T3 E1 cell derived irOGP. ——, irOGP; ----, light absorbence at 220 nm.

enous OGP-OGPBP system reminiscent of that reported recently in vivo in human and rat serum [Bab et al., 1992; Greenberg et al., 1995]. The presence of such system is supported by two major findings, namely, the demonstration of two radiolabeled OGP-OGPBP complexes in MC3T3 E1 culture medium and the isolation and purification of OGP from the same medium. In addition we show the accumulation of irOGP in serum free medium conditioned with fibroblastic NIH 3T3 cells. In both the osteoblastic and fibroblastic cell cultures the OGP presents a feedback loop. The presence of the endogenously produced OGP in culture medium deprived of serum or other exogenous sources of mitogenic factors is essential for proliferation.

We show here for the first time the presence of two OGPBPs, OGPBP-1 and OGPBP-2. In spite of the high positive charge of OGP [Greenberg et al., 1993] the corresponding complexes migrate in an anodic PAGE system, indicating that both binding proteins are negatively charged. The binding activity of OGPBP-1 is resistant to boiling, suggesting a high intramolecular content of disulfide bonds [Bare et al., 1994]. The binding of both OGPBPs to OGP is specific inasmuch as the [3-$^{125}$I(Tyr$^{10}$)]sOGP can be competed out from forming the complexes by an excess of unlabeled sOGP. These complexes are rather stable as they resisted the electrophoretic separation. These findings suggest that as in the case of other polypeptide factors, the OGP-OGPBP complexes have a role in maintaining large reservoirs of inactive OGP protected from proteolyitic degradation and clearance, hence providing a mechanism controlling the peptide's availability to its target cells. Such a mechanism may be associated with the regulation of free ligand levels and/or the ligand transport and presentation to its specific tissue or acceptors. Another function attributed to carrier proteins is deactivation of circulating regulators by their removal from the body [LaMarre et al., 1991; Woods et al., 1994].

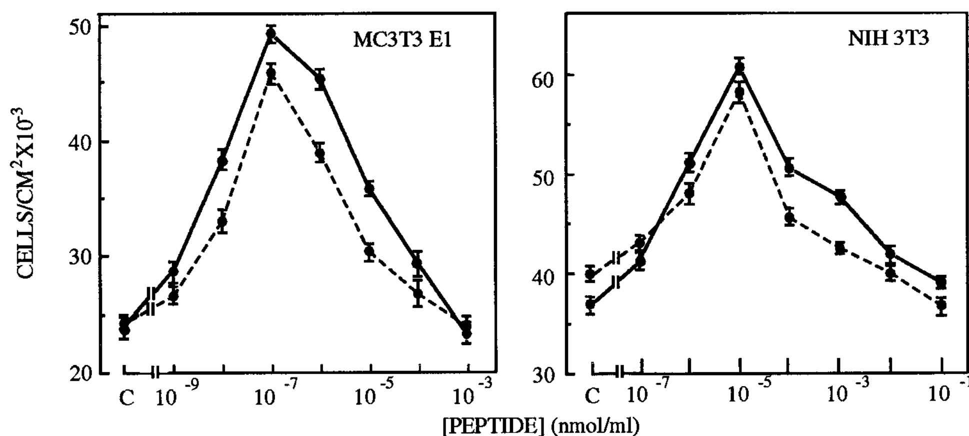


Fig. 4. Proliferative effect of MC3T3 E1 cell derived OGP. ——, peptide recovered in 42–44 min retention time fractions shown in Figure 3; ----, sOGP. Data are mean ± SE obtained in triplicate culture wells per condition 48 h after peptide addition.

The reaction mixture in which the "fresh" irOGP is determined prior to boiling contains both the anti-OGP antibodies and the OGPBPs. OGP binding to the antibodies increases the dissociation of the peptide from the OGP-OGPBP complexes and therefore the corresponding irOGP measurements represent a vast overestimation of the free OGP. Still, the measurements carried out in the serum free culture medium indicate that irOGP in detectable levels is present already several hours after the extensive washes and starvation of the cells for the removal of serum and thus serum-derived OGP. The levels of both "fresh" and boiled irOGP are markedly increased mainly during the first 24 h. During this time the culture is in a rapid proliferation phase [Greenberg et al., 1993] and cell death is minimal. It is therefore unlikely that the OGP originates from HH4 breakdown. More probably, the production of OGP involves alternative translation of HH4 mRNA [B. Frenkel, personal communication].

Like the serum derived OGP binding activity [Greenberg et al., 1995], that of OGPBP-2 is heat labile as the integrity of its tertiary structure and function can be damaged by thermal denaturation and unfolding [Tanford, 1968, 1970; Schellman, 1987]. A small flexible peptide like the OGP is resistant to heat inactivation. Therefore, large amounts of irOGP could be released by boiling and then conveniently purified. The hydrophobic properties, amino acid sequence and mitogenic activity of the purified MC3T3 E1 derived OGP are identical to those of the human and rat OGP [Bab et al., 1992; Greenberg et al., 1995]. This evolutionary conservation, the high levels of serum OGP [Bab et al., 1992; Greenberg et al., 1995] and abundant production of endogenous OGP in the cultures implicate the potential biological importance of this peptide. This is especially emphasized by the comparison with other regulatory polypeptides, such as IGF-I and FGF, which show only low levels of expression in cultures of non-transformed osteoblastic and fibroblastic cells [Underwood et al., 1986; Di Francesco et al., 1989; Mohan et al., 1990; Yayon and Klagsbrun, 1990; Canalis et al., 1991]. The comparison with IGF-I is of particular relevance because, like the OGP, it is an important regulator of osteoblastic and other stromal cells and has binding proteins produced by its cells of origin [Mohan and Baylink, 1991].

The inhibition of cell number induced by the anti-OGP antibodies and its reversal by the exogenously added sOGP indicate that in the absence of serum or other exogenous growth stimulators the endogenously produced OGP is both necessary and sufficient for baseline proliferation. That this inhibition is the consequence of OGP removal by its specific antibodies and not an antibody stimulated non-specific cytotoxicity is shown by the uninterrupted proliferation in the presence of anti-KLH antibodies purified from the anti-OGP antiserum.

The stimulation of the endogenous OGP production by the exogenous sOGP is consistent with an autoregulated feedback mechanism. In fact, an OGP autocrine feedback loop has been

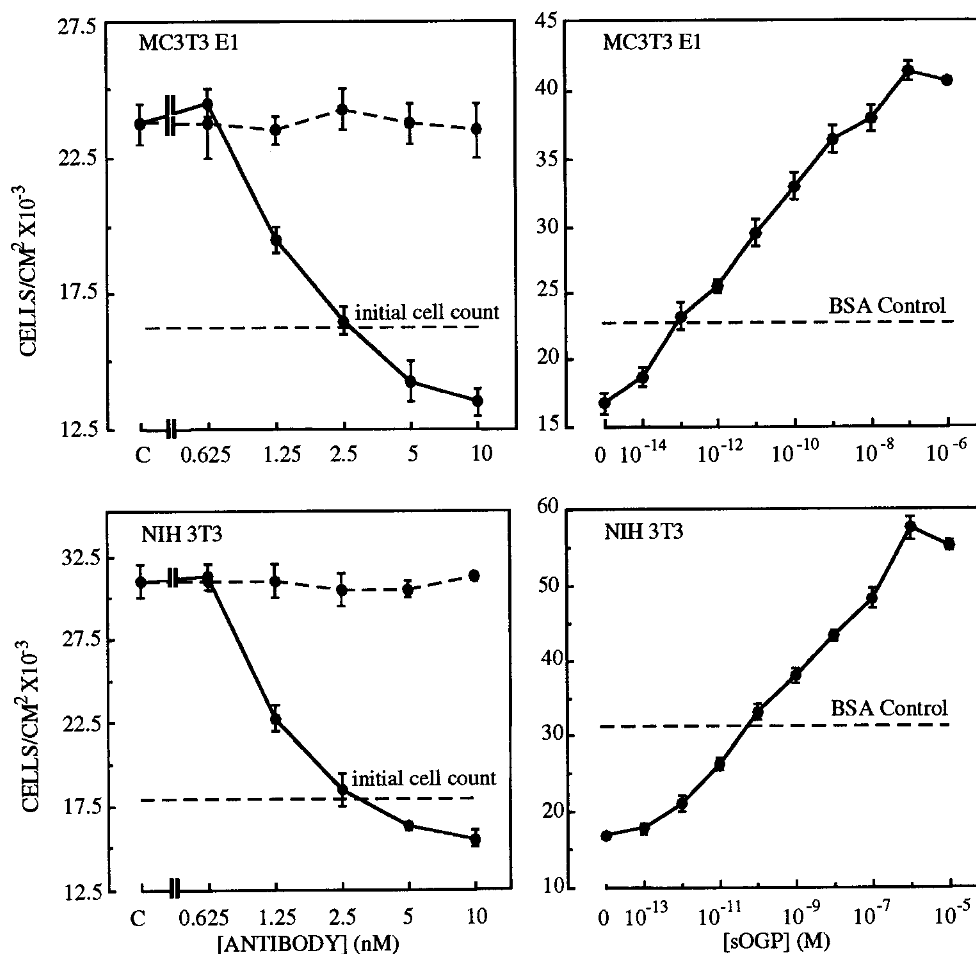


**Fig. 5.** Inhibition of cell proliferation following sequestration of endogenous OGP. **Left:** ——, inhibition of cell number by affinity purified polyclonal anti-OGP antibodies; ----, control cultures treated with anti-KLH antibodies purified from same antiserum. **Right:** sOGP rescue of cell proliferation from quiescence induced by 5 nM anti-OGP antibodies. Data are mean ± SE obtained in triplicate culture wells per condition 48 h after antibody and/or sOGP addition. BSA controls are cultures treated with medium supplemented with BSA only.

recently demonstrated in vivo in mice [Gurevitch et al., 1996]. The levels of endogenous OGP were undetectable earlier than 4 h after changing the serum free to BSA supplemented medium. Moreover, it is this time period during which the endogenous production is particularly sensitive to the exogenously added sOGP. It is therefore suggested that the direct effect of sOGP is limited to a rather short period of time and that its proliferative effect is mediated by the induced increase in the endogenous OGP, and possibly OGPBP, levels. It further appears that an initial small increase in the level of endogenous OGP triggers a chain response which results in amplification of the mitogenically active OGP. Likewise, it is implicated that the reversal of the OGP proliferative effect is mediated by the reversal of the increase in endogenous OGP induced by supraoptimal sOGP doses. The availability of this OGP to the cells may be finely tuned by the OGPBPs.

So far all stromal cells tested, i.e., osteoblastic, fibroblastic, and cartilaginous [M. Pines, personal communication] from human, mouse, rat, rabbit, and chicken responded mitogenically to OGP [Bab et al., 1992; Bab, 1995; Greenberg et al., 1993; Robinson et al., 1995]. OGP does not affect COS (data not shown) and hematopoietic cells [Gurevitch, 1996]. The most effective OGP dose in MC3T3 E1 cells is at least two orders of magnitude lower than that in non-osteoblastic cell systems. This differential sen-

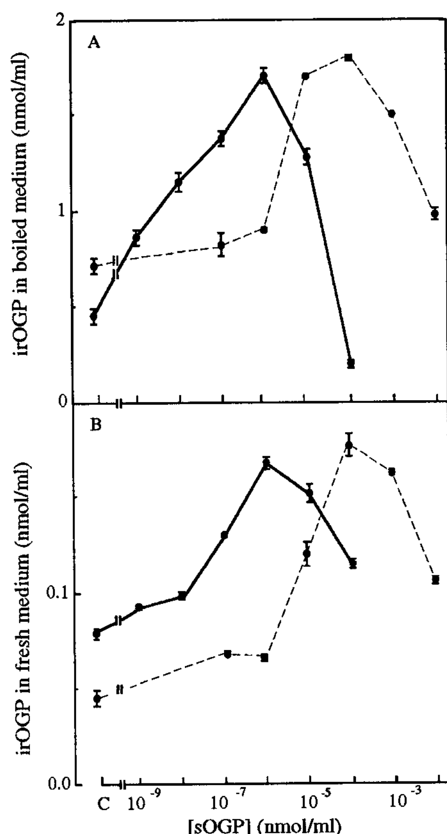


**Fig. 6.** Regulation of endogenous OGP by exogenous sOGP. **A:** Boiled medium; **B:** fresh medium. ——, MC3T3 E1 cells; ----, NIH 3T3 cells. Data are mean ± SE obtained in triplicate culture wells per condition 4 h after challenge with sOGP.

sitivity of osteoblastic cells could result in a preferential anabolic effect of OGP in bone.

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