Synthesis of Osteogenic Growth Peptide and Its Synergetic Effect with Granulocyte-Colony Stimulating Factor

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Abstract Osteogenic growth peptide (OGP) has been synthesized through Fmoc solid phase synthesis procedure. The purity of synthetic OGP (sOGP) is over 98.6% identified by HPLC, the amino acid sequence and electro-spray mass spectroscopy are consistent with theoretical values. The synergetic effect of sOGP with recombinant human granulocyte-colony stimulating factor (rhG-CSF) on the hematopoiesis was investigated in normal mice. To assess the synergy of sOGP with rhG-CSF, two schemes were designed. In one scheme rhG-CSF was used at the last 8 days of a 13-day treatment with sOGP, while in the other one both cytokines were given concurrently for 10 days [sOGP, 0.5 nmol/day (mouse); rhG-CSF, 2 μg/day (mouse)]. Both schemes showed that sOGP remarkably synergized with rhG-CSF on increment of white blood cell number and lymphocyte number in peripheral blood without any change of red blood cell and platelet counts. Quantitative differential analysis of bone marrow and histological examination of the spleen and sternum showed that sOGP plus rhG-CSF did not cause abnormal hyperplasia, so sOGP is a very hopeful new drug to improve the effectiveness of clinical used rhG-CSF.

Key words OGP; G-CSF; synergy; hematopoiesis; solid phase synthesis

Now a newly found peptide, named osteogenic growth peptide (OGP), working like a hematopoietic modulator, increases blood and bone marrow (BM) cellularity [1]. The OGP, a 14-amino acid, highly conserved, H4 histone-related peptide, is characterized in regenerating BM and normal serum. The sequence of OGP is ALKRQGRTLYGFGG. OGP was abundant in human and other mammalian blood, mainly in the form of OGP-OGP-binding protein complex [1-3]. Meanwhile, its C-terminal pentapeptide was also found at a high level in mammalian serum and osteogenic cultures[4]. *In vivo* OGP increased osteogenesis[2, 5, 6] and blood granulocyte count, enhanced engraftment of bone marrow transplants of mice[1]. The C-terminal pentapeptide had also some effects on peripheral neutrophil recovery after cyclophosphamide-induced aplasia, and stem/progenitor cell mobilization from BM into peripheral blood[7]. On human $CD34^+$ enriched cells, the activity of stem cell factor (SCF) and granulocyte-megakaryocyte colony-stimulating factor is increased by the pentapeptide[8].

It is suggested that OGP may enhance some cytokines and/or receptor synthesis, and synergize with some other cytokines, such as rhG-CSF, which is a popular clinically used hematopoietic cytokine, at granulocytosis. The detailed mechanism of its action on hematopoiesis is not clear yet.

In this paper, OGP was synthesized (sOGP, which has the same sequence and the same function of natural OGP [1]) by Fmoc system and the synergetic effect of sOGP with rhG-CSF was studied.

1 Materials and Methods

1.1 Materials

Male BALB/C weighing 16~18 g were purchased from Shanghai Laboratory Animal Center, the Chinese Academy of Sciences. Fmoc-amino acids were purchased from ABI Company. P-alkoxybenzyl alcoholic (HMP)-resin was purchased from Sigma Company. Human recombinant G-CSF (rhG-CSF) was purchased from Kirin Brewery Co., Ltd. (China). Human albumin was purchased from Shanghai RAAS Blood Products Co., Ltd..

1.2 Synthesis of OGP

Fmoc solid phase synthesis (SPS) was performed manually starting from a P-alkoxybenzyl alcoholic (HMP)-resin, N-terminal and side chain protected amino acids were connected step by step as the sequence of natural OGP by Fmoc system. These side-chain protected amino acids are: Fmoc-Arg (Pmc), Fmoc-Lys (Boc), Fmoc-Thr (tBu) and Fmoc-Tyr (tBu). After the final cycle, the resin was cleavaged by TFA containing 5% *p*-cresol and 100 mg phenol for 1 h at room temperature. The other side-chain deprotection

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and resin cleavage conditions were also tried, 2 h reaction, 3 h reaction, or reactive solvent containing 2% water. The crude peptide was desalted on a Sephadex G10 and purified by FPLC (ACTA purifier, Amersham Pharmacia). The column used was SP Sepharose XL (column volume: 1 mL; dimension: 0.7 cm × 2.5 cm). The purity of sOGP was identified by rpHPLC (Hewlett Packard series 1050, HP Company; column: Beckman C8 4.6 mm × 250 mm), the exactness and sequence of sOGP was identified by electro-spray mass spectroscopy (MS) and protein sequence analyzer (ABI 491A protein sequence analyzer).

1.3 Cytokines and Treatment Schemes

Mice were injected subcutaneously with sOGP, or rhG-CSF, or a combination of these two growth factors. The carrier consists of 10 g/L human albumin in sterile saline. As shown in Table 1, two schemes were applied. Scheme 1, the mice received either sOGP 0.5 nmol /day from day 0 to 12, or rhG-CSF 2 μg/day from day 5 to 12 (from day 0 to day 4, carrier was injected), and a combination group with only sOGP at first 5 days and both at the last 8 days of the 13-day treatment. Scheme 2, the mice received either sOGP 0.5 nmol /day from day 0 to 9, or rhG-CSF 2 μg /day from day 0 to 9, or a combination of sOGP and rhG-CSF concurrently for 10 days. In both schemes, the control mice were injected with carrier only.

Table 1 Normal mice treated with sOGP plus rhG-CSF, sOGP alone and rhG-CSF alone (Days of treatment)

Group		Scheme 1 (day)	Scheme 2 (day)
sOGP plus rhG-CSF	sOGP	0 – 4	0 – 0
	sOGP+rhG-CSF	5 – 12	0 – 9
SOGP		0 – 12	0 – 9
rhG-CSF		5 – 12	0 – 9
Control		0 – 12	0 – 9

1.4 Quantitative differential analysis for peripheral blood (PB) and BM

In scheme 1, PB was collected on days: 0, 4, 7, 9, 11, and 13; while in scheme 2, PB was collected on days: 0, 2, 4, 6, 8 and 10. The mice were bled retro-orbitally, and the samples were analyzed with differential cell count with Coulter counter (MEK-5108-K, Japan). In Scheme 1, BM nucleated cells were also counted with hemocytometer and the stained BM smears were then differentially numerated.

1.5 Histological examination of the spleen and sternum

In scheme 1, histological examination of the spleen and sternum was performed with Bouin's-fixed or formaldehyde-fixed paraffin-embedded sections stained with hematoxylin and eosin and by the Giemsa method, observed under optic microscope.

1.6 Statistical analysis

The statistical analysis of the results was done using the student *t*-test, results are given as mean± SEM.

2 Results

2.1 The purity and exactness of sOGP

The purity of sOGP after purification is over 98.6% identified by HPLC; the molecular weight of sOGP is 1523.5 kD identified by electro-spray mass spectroscopy, which is consistent with the theoretical value (1523.75 kD); the result of whole sequence analysis of sOGP by N-terminal protein sequence analyzer is consistent with the theoretical sequence of OGP. The results of side-chain deprotection and resin cleavage under different conditions indicated that 1 h reaction time without water is the best.

2.2 The synergetic effect between sOGP and rhG-CSF on blood cellularity of mice

In scheme 1 that rhG-CSF was used for the last 8 days of a 13-day treatment with sOGP, there was synergistic increase in circulating WBC as compared with sOGP alone or rhG-CSF alone at day 11 and 13 [Fig.1(A)]. At day 11, a marked increase of circulating WBC after the coinjection of sOGP plus rhG-CSF to $(39.7\pm4.5)\times10^9$ cells/L was observed as compared with $(16.1\pm1.1)\times10^9$ cells/L in rhG-CSF treated mice and $(6.7\pm0.5)\times10^9$ cells/L in sOGP treated mice. At day 13, a marked increase in the number of circulating WBC after the coinjection of sOGP plus rhG-CSF to $(35.1\pm5.67)\times10^9$ cells/L was observed as compared with $(17.6\pm1.6)\times10^9$ cells/L in rhG-CSF treated mice and $(6.6\pm0.4)\times10^9$ cells/L in sOGP treated mice [Fig.1(A)]. A marked increase of circulating lymphocyte could also be seen at day 11 and day 13 [Fig.1(B)]. In scheme 2, coinjection of sOGP and rhG-CSF for 10 days causes a higher synergistic increase in circulating WBC than sOGP alone or rhG-CSF alone at day 10, with increases of $(53.1\pm2.7)\times10^9$ cells/L, $(22.7\pm1.9)\times10^9$ cells/L and $(6.3\pm1.2)\times10^9$ cells/L respectively [Fig.2(A)]. What's more,

a marked increase of the circulating lymphocyte was also caused by the coinjection of these two factors at day 10 [Fig.2(B)].

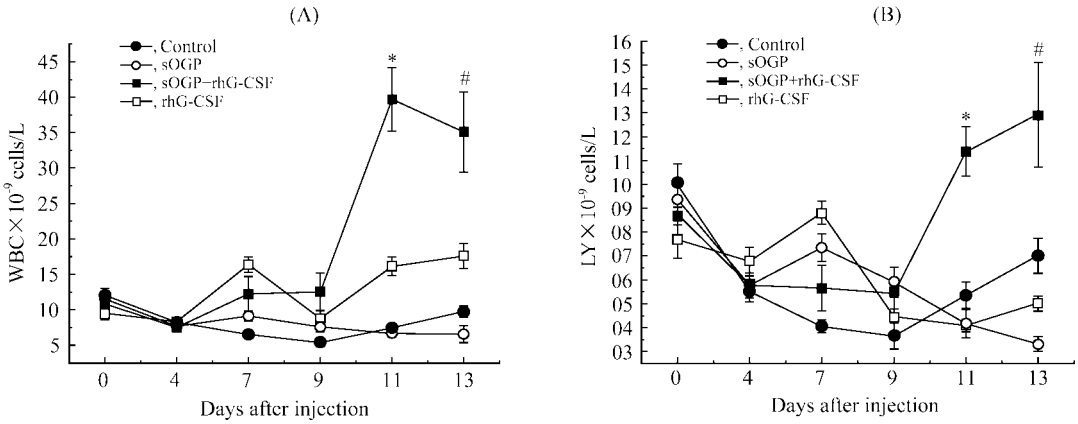

Fig. 1 G-CSF used for the last 8 days of a 13-day treatment with OGP causes a synergistic increase of WBC (A) and a synergistic increase of LY (B)

$n = 7$; $^{*}P < 0.0005$, $^{\#}P < 0.05$ compared with the group that injected with rhG-CSF alone.

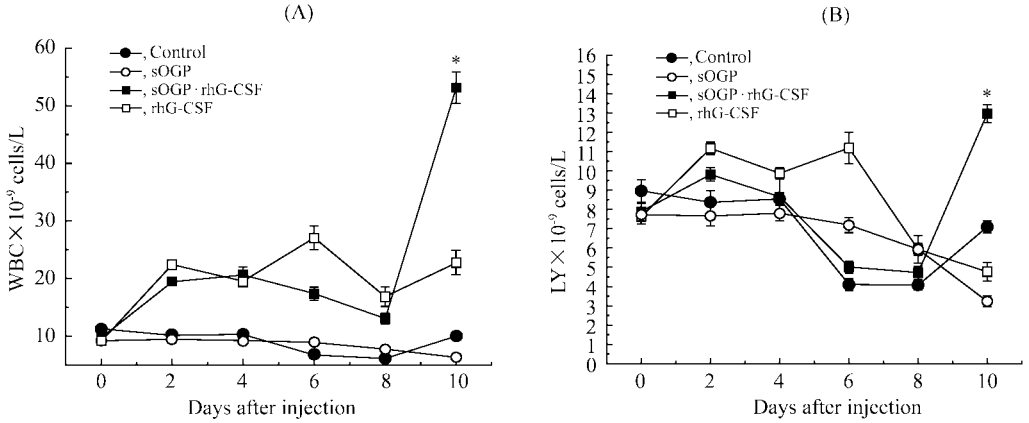

Fig. 2 Daily coinjection of sOGP and rhG-CSF for ten days causes a synergistic increase of WBC (A) and a synergistic increase of LY (B)

$n = 10$; $^{*}P < 0.0005$ compared with the group that injected with rhG-CSF alone.

The proportion of the granulocyte and lymphocyte was altered slightly in sOGP plus rhG-CSF as compared with rhG-CSF or OGP alone. The red blood cells (RBC) and platelets (PLT) counts kept unchanged in different groups (Table 2), showing that the synergistic role mainly acts on neutrophils or perhaps lymphocytes in blood. The BM cellularity was not altered by the treatment of different cytokines. An increase in myelocytes/metamyelocytes was seen in sOGP and sOGP plus rhG-CSF compared with the carrier controls. An increase in neutrophils cells was seen in sOGP, sOGP plus rhG-CSF and rhG-CSF 16 hours later after the cytokine injection (Table 3). No significant increase was noted in sOGP plus rhG-CSF treated mice as compared with sOGP or rhG-CSF alone.

Table 2 Differential counts in blood of normal mice treated with OGP, OGP+G-CSF and G-CSF (G-CSF used for the last 8 days of a 13-days treatment with OGP)

	% WBC presented *		RBC	Platelet
	Granulocyte	Lymphocyte	($\times 10^9$cells/mL)	($\times 10^6$cells/mL)
OGP	48.9±1.8	51.1±1.8	7.0±0.1	500.9±11.2
OGP+G-CSF	63.1±1.2	36.9±1.2	6.7±0.2	495.7±56.6
G-CSF	70.4±1.5	29.6±1.5	6.7±0.1	505.4±335.3
Control	28.3±1.7	71.7±1.7	7.4±0.2	502.3±14.7

*WBC counts are shown in Fig.2(A) mean ± SE ($n = 7$).

2.3 The influence of sOGP plus rhG-CSF at sternum BM and spleen of mice

Histological examination of sternum BM shows that the percentages of erythroid line, myeloid line and megakaryocyte were normal in sOGP, sOGP plus rhG-CSF and rhG-CSF treated mice. The spleens of sOGP plus rhG-CSF treated mice and rhG-CSF treated mice show increased myelopoiesis, erythropoiesis, and megakaryocytopoiesis as compared with the spleens of the carrier control and sOGP treated mice. Although the average weight of spleen of the sOGP plus rhG-CSF treated mice was larger than that in the rhG-CSF treated mice, yet there was no significant difference.

Table 3 The influence of OGP and G-CSF on BM total nucleated cell number and the percent of differential cells when G-CSF was used for the last 8 days of a 13-days treatment together with OGP

	sOGP	SOGP +rhG-CSF	rhG-CSF	Control
Erythroid				
Pronormoblasts	1.1±0.3	1.4±0.4	1.6±0.3	1.6±0.4
Early normoblasts	3.4±0.4	3.3±0.5	2.5±0.3	4.6±0.5
Intermediate normoblasts	13.8±1.6	11.4±0.7	12.1±1.9	12.1±0.9
Late normoblasts	19.6±2.2	19.6±1.8	16.0±2.1	22.1±1.5
Myeloid				
Myeloblasts	0.8±0.3	0.6±0.2	0.6±0.4	0.9±0.3
Promyelocytes	1.5±0.3	1.8±0.4	1.9±0.4	2.3±0.4
Myelocytes/Metamyelocytes	13.8±1.4	14.3±1.5	11.0±1.0	11.4±0.9
Neutrophils	24.3±1.6	24.8±2.4	30.8±2.8	21.4±1.8
Eosinophils/Basophils	1.6±0.9	1.5±0.3	1.6±0.4	1.7±0.3
Megakaryocyte	1.0±0.23	0.8±0.3	1.1±0.2	1.3±0.2
Lymphoid				
Lymphocytes and Plasma cells	19.3±1.67	20.8±1.4	19.4±2.0	20.6±1.5
Total nucleated cells/femur ($\times 10^7$)	1.8±0.1	2.0±0.1	1.9±0.1	2.0±0.1

mean±SE(n = 7)

3 Discussion

Most hematopoietic cytokines are synergistic with others. SCF synergizes with G-CSF when administered *in vivo* to mice [9]. Flt-3 ligand (FL) is synergetic with combinations of GM-CSF, IL-3, erythropoietin (EPO) and SCF to promote growth of CFU-GEMM, HPP-CFC or CFU-Blast[10-13]. Here we found that OGP synergizes with rhG-CSF.

Our results show that the coinjection of sOGP and rhG-CSF causes a synergistic increase of the circulating WBC and lymphocytes. The proportion of the granulocyte and lymphocyte changed by coinjection of sOGP and rhG-CSF was different from sOGP or rhG-CSF alone, that could be ascribed to the synergistic function of sOGP and G-CSF. In BM smears and histological study of the spleen and sternum, there were no significant difference between the mice treated with sOGP plus G-CSF and rhG-CSF alone, which was significantly different from the mice treated with sOGP or carrier alone. The BM cellularity was not altered by the treatment of different cytokines showing that coinjection of sOGP and rhG-CSF did not cause abnormal hyperplasia in BM.

Previous experiment proved that sOGP increased blood and BM cellularity when injected subcutaneously every other day to normal C57 mice for two weeks[1]. Our results showed that sOGP significantly increased the circulating WBC and lymphocyte number as compared with the carrier control at day 6-9. The difference from the previous experiment may be ascribed to the dose of sOGP, test time after the injection of sOGP and the consecutive injection of the peptide.

In *in vitro* study, we didn't find the synergetic effect between sOGP and rhG-CSF at BM colony conformation of mice (data not shown). Considering the *in vivo* and *in vitro* results together, we think that the synergetic effects *in vivo* between sOGP and rhG-CSF depend upon hematopoietic microenvironment and/or some other growth factors. OGP may improve the stromal hematopoietic microenvironment by regulating the stromal cell number and/or expression of stromal cell derived factors, such as GM-CSF and SCF that are all synergetic with rhG-CSF. It has been proved that sOGP enhances the mRNA level of TGFβ and IGF involved in hematopoiesis[14]. Perhaps, sOGP enhances the expression of rhG-CSF receptors on granulocytic progenitor cell surface, which manifolds the targets of rhG-CSF. Whether or not sOGP increases the expression of rhG-CSF receptors on granulocytic progenitors is an unsolved but critical

question. The next step of our program is to study the mechanism of the synergy between sOGP and rhG-CSF and confirm the synergy effects of sOGP with rhG-CSF on the mobilization of hematopoietic stem/progenitor cells from BM into peripheral blood to improve the collection of stem/progenitor cell for clinical transplantation.

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