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Carboxy-Terminal Fragment of Osteogenic Growth Peptide Regulates Myeloid Differentiation Through RhoA

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Abstract The carboxy-terminal fragment of osteogenic growth peptide, OGP(10–14), is a pentapeptide with bone anabolic effects and hematopoietic activity. The latter activity appears to be largely enhanced by specific growth factors. To study the direct activity of OGP(10–14) on myeloid cells, we tested the pentapeptide proliferating/differentiating effects in HL60 cell line. In this cell line, OGP(10–14) significantly inhibited cell proliferation, and enhanced myeloperoxidase (MPO) activity and nitroblue tetrazolium reducing ability. Moreover, it induced cytoskeleton remodeling and small GTP-binding protein RhoA activation. RhoA, which is known to be involved in HL60 differentiation, mediated these effects as shown by using its specific inhibitor, C3. Treatment with GM-CSF had a comparable OGP(10–14) activity on proliferation, MPO expression, and RhoA activation. Further studies on cell proliferation and RhoA activation proved enhanced activity by association of the two factors. These results strongly suggest that OGP(10–14) acts directly on HL60 cells by activating RhoA signaling although other possibilities cannot be ruled out. J. Cell. Biochem. 93: 1231–1241, 2004.

Key words: OGP(10–14); GM-CSF; RhoA; HL60; cell differentiation

Osteogenic growth peptide (OGP) is a 14-mer peptide, which exerts regulatory effects on bone and bone marrow. This highly-conserved, H4 histone-related peptide was first isolated about 10 years ago in blood during osteogenic remodeling of post-ablation marrow regeneration and it is found in great abundance in the blood, usually conjugated to a binding protein (OGPBP) [Bab et al., 1992].

OGP administration in vivo enhances bone formation and increases trabecular bone mass. In vitro, OGP stimulates proliferation and alkaline phosphatase activity in osteogenic cell lines and exerts mitogenic effects on fibroblasts [Greenberg et al., 1993, 1995]. Moreover, OGP is able to induce in vivo a balanced increase in white blood cell (WBC) count and overall bone marrow cellularity in mice [Gurevitch et al., 1996].

OGP-derived C-terminal pentapeptide, OGP(10–14), is generated by proteolytic cleavage of the full-length OGP upon dissociation from OGPBP [Bab et al., 1999]. Synthetic OGP(10–14) retains the OGP effect on cell proliferation, thus suggesting a specific role of the C-terminal region in binding to the putative OGP receptor [Greenberg et al., 1993]. The mechanism of action of the pentapeptide is not fully known; however, it has been recently shown that OGP(10–14) activates the mitogenic Gi protein MAP kinase-signaling cascade in osteogenic cells. This indirectly suggests the presence of a membrane receptor [Gabarin et al., 2001].

We have previously shown that OGP(10–14) is able to enhance bone marrow recovery after cyclophosphamide administration in mice [Fazzi et al., 2002a]. Moreover, OGP(10–14)

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increases the number of CFU-GM derived from human [Fazzi et al., 2002b] and mouse [Fazzi et al., 2003] blood stem cells cultured in semi-solid medium. This activity appears to be largely mediated by the enhancement of specific growth factors such as GM-CSF [Fazzi et al., 2002b, 2003]. However, it is not clear whether these actions are exerted directly and/or through the induction–modulation of other hemopoietic factors, and whether OGP(10–14) acts exclusively on staminal-progenitor cells or also on precursor cells. In order to study a possible direct activity of OGP(10–14) on myeloid cells and to evaluate its possible role on intracellular signaling, we tested the pentapeptide activity in HL60 cell line grown in serum-free medium with or without GM-CSF. In particular, as RhoA activation appears to be involved in HL60 cell differentiation [Ohguchi et al., 1997], we focused our attention on the involvement of this small GTP-binding protein.

The Rho family belongs to the Ras small-G protein superfamily, and consists of at least 20 members including RhoA, Cdc42, and Rac1 [Ridley, 2001a]. Like Ras, Rho GTPases cycle between a GTP-bound active state and a GDP-bound inactive state. Interestingly, and differently from the other small-G proteins, active and inactive forms of RhoA correspond to their intracellular localization, in the membrane fraction and in the cytosol, respectively [Hall, 1994, 1998; Takaishi et al., 1995]. Rho proteins in the GTP-bound active state can interact with a number of effectors, in order to transduce signals leading to several biological responses, including actin cytoskeletal rearrangements, regulation of gene transcription, cell cycle regulation, control of apoptosis, and membrane trafficking [Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Bishop and Hall, 2000].

Proliferating/differentiating effect of OGP-(10–14) on these cells was assayed, along with its action on membrane RhoA translocation and activation. RhoA activation induced by OGP(10–14) was further evaluated by assaying actin polymerization and by using the specific inhibitor C3 derived from *Clostridium botulinum*.

MATERIALS AND METHODS

Cell Cultures

The human promyelocytic leukemia HL60 cell line, obtained from Interlab Cell Line Collection (Genova, Italy), was grown in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Gibco, Gaithesburg, MD), 2 mM L-Glutamine (Sigma), and 10 μg/ml gentamycin (Sigma). Cells maintained at 37°C in a 5% CO_2 humidified atmosphere were used for the assays in their exponential growth phase, with viability exceeding 90% as determined by trypan-blue exclusion test.

Linear OGP(10–14) (OGP 10–14: thyr-gly-phe-gly-gly; M.W. 499.7) was supplied by Polypeptides Laboratories, Inc. (Torrance, CA, batch no. 9712–006) and provided by Abiogen Pharma SpA (Pisa, Italy).

Twenty-four hours before starting experiments, cells were sub-cultured in serum-free medium supplemented with 5 μg/ml insulin and 5 μg/ml transferrin (IST, Sigma) for 24 h [Breitman et al., 1980; Ohguchi et al., 1997].

OGP(10–14) (10^{-8} M and 10^{-12} M) and/or GM-CSF (0.1 and 1 ng/ml, PrepoTech EC, London, England) were added to the serum-free medium and cultures were stopped at 1 and at 72 h, as reported in the single experiments.

RhoA inhibition tests were performed by adding 2 μg/ml *botulinum* exoenzyme C3 (Upstate Biotechnology, Lake Placid, NY) to cultures 30 min before OGP(10–14) addition. At this concentration, C3 appeared to specifically inactivate RhoA protein [Ridley et al., 1995].

Unless otherwise specified, all experiments were performed in triplicate. To evaluate the statistical significance of the differences between different groups Student's *t*-test was used for parametric results whereas Wilcoxon-*t*-test was employed for non-parametric results such as comparison of percentages.

RhoA Immunocytochemical Detection

After 1 or 72 h, cells cultured with or without OGP(10–14) in the presence or absence of GM-CSF, were harvested, centrifuged on slides, air-dried, and fixed with 1% formalin for 10 min at 4°C. Cytospin preparations were further permeabilized with exposure to 0.2% Triton X-100 solution (Sigma) for 10 min, incubated with 3% H_2O_2 in cold methanol for 5 min to block endogenous peroxidase activity and with 5% swine serum for 20 min to quench non-specific reactivity. Samples were then incubated with the rabbit anti-RhoA polyclonal antibody (1:50 in 0.1% BSA; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The detection

protocol was carried out using biotinylated link antibodies and streptavidin-peroxidase complex (LSAB kit; Dako, Carpinteria, CA). The reaction was developed with DAB-H_2O_2 solution for 5 min in the dark. Finally, after hematoxylin counterstaining, the slides were dehydrated, mounted with DPX mountant (Sigma), and observed with a DMRB Leica microscope by using an 100× oil immersion lens. After each step, slides were washed with phosphate-buffered saline (PBS). All steps were performed at room temperature unless otherwise specified. Negative controls were obtained both by omitting the primary antibody and by using blocked primary antibody as previously described [Mattii et al., 2000].

Activated RhoA Western Blot Analysis

A total of 7.5×10^6 cells, grown with or without OGP(10–14) for 1 h, were harvested, washed twice with tris-buffered saline (TBS), and lysed in Mg^{2+} lysis/wash buffer (MLB: 25 mM Hepes pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol) containing anti-proteases (leupeptin 10 μg/ml, pepstatin 10 μg/ml). Cleared lysates were incubated with Rhotekin RBD (Rho Binding Domain) bound to glutathion-agarose beads (Upstate Biotechnology) for 45 min at 4°C [Ren and Schwartz, 2000]. After three washings with MLB, reduced SDS sample buffer 2X (containing 40 mM dithiothreitol) was added to the beads, which were subsequently heated at 95°C for 5 min. After centrifugation, precipitated GTP-RhoA samples were loaded onto 15% SDS–polyacrylamide gel, subjected to electrophoresis and transferred to nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Upsala, Sweden). The membrane was then incubated for 45 min under constant agitation, in 0.1% Tween 20-TBS solution (T-TBS) containing 5% dry fat milk, washed three times in T-TBS and exposed to rabbit anti-RhoA polyclonal antibody (1:100 in 1% dry fat milk T-TBS; Santa Cruz Biotechnology) overnight at 4°C. Three washings with T-TBS were followed by 30-min incubation with anti-rabbit HRP conjugated antibody (1:10,000 in T-TBS, Sigma), then five washings with T-TBS and one more with bi-distilled water. Detection was performed by enhanced chemiluminescence (ECL, Amersham Biosciences). All steps were performed at room temperature, unless otherwise specified. Negative controls were performed by using blocked primary antibody. A negative control for RhoA pull-down was also performed by loading beads only. Positive controls for total RhoA were carried out both by running non-adsorbed lysates and incubating MLB lysates with GTPγS before precipitation with Rhotekin RBD, so that all (activated and inactivated) cellular RhoA became GTP-bound.

Actin Polymerization

In order to test the OGP(10–14) effect on cellular actin polymerization, cytospins of HL60 cells were exposed to OGP(10–14) for 1 and 72 h, with or without C3. After 1% formalin fixation (10 min at 4°C) and free aldehyde group quenching with 50 mM NH_4Cl (10 min at 4°C), cells were permeabilized with 0.2% Triton X-100 solution for 10 min and subsequently incubated with FITC-phalloidin (1 μg/ml; Sigma) for 30 min. After labeling, samples were washed three times in PBS and mounted with p-phenylenediamine-glycerol solution. F-actin cell distribution was observed with a DMRB Leica fluorescence microscope.

Proliferation Assay

Cell proliferation was evaluated by MTT colorimetric assay (Roche, Milan, Italy), which is based on cleavage and reduction of tetrazolium salts. A total of 10^5/well HL60 cells were cultured in 96-well microtitre plates for 72 h with or without OGP(10–14) in presence or absence of GM-CSF.

Peroxidase Activity Test

Cytospin preparations of cells grown for 72 h with or without OGP(10–14) or GM-CSF (1 ng/ml) were fixed in 90% ethanol–10% formalin solution for 1 min, incubated with DAB-H_2O_2 buffer for 10 min and lightly stained with Giemsa. Slides with stained cells were dehydrated and then mounted with cover slips. The 10^{-12} M OGP(10–14) experiments were also repeated after culturing in C3 conditioned medium. Brown staining identified a peroxidase-positive reaction. In all experiments a minimum of 50 cells per sample were evaluated.

The semi-quantitative evaluation scale used to assess the staining for peroxidase was determined by two independent observers and was as follows: no staining (−), light positive staining (+/++), positive staining (+++), and strong positive staining (++++).

Nitroblue Tetrazolium (NBT) Test

The 72-h OGP(10–14) treated and untreated cells were incubated with 1 mg/ml NBT (Sigma) solution for 15 min in a 37°C water-bath. After a further 15 min at room temperature, cells were cyto-centrifuged on the slides and finally slightly stained with Giemsa. The percentage of cells containing cytoplasmic blue–black formazan deposits was scored microscopically on a minimum of 50 cells per sample.

Cytofluorimetric Analysis

After 72 h of OGP(10–14) culture, samples containing approximately 10^6 cells were washed twice with PBS containing 1% FBS and 0.1% sodium azide (washing buffer, WB). Pellets were re-suspended in 100 μl WB at 10^6 cells/ml. CD13(PE), CD14(FITC), and CD45(PerCP) (Becton-Dickinson, Franklin Lakes, NJ) were added to pellets at 4°C for 30 min. Cells were then washed once with WB and analyzed on a FACScan™ by LYSYS software (Becton-Dickinson). In parallel experiments, pellets were incubated with anti-human CD11b (PE) at 4°C for 30 min. After washing with WB, pellets were re-suspended in 1 ml freshly-prepared PLP-buffer (0.2 M sodium monohydrogen phosphate–0.1 M Lysine-HCl–8% paraformaldehyde plus 2.14 mg/ml sodium metaperiodate, 3:1; all reagents from Sigma), and kept at 4°C for 15 min. Cells were washed once with 0.5% Tween-20-PBS, re-suspended in 100 μl WB and incubated with (FITC)-anti-human peroxidase at 4°C for 30 min. Samples were finally washed in WB and analyzed on FACScan™ with LYSYS software.

Six times (2, 6, 7, 26, 48, 72 h) after OGP(10–14) addition, phosphatidylserine externalization was measured by staining cells with FITC-conjugated annexin V (Clontech, PaloAlto, CA) for 5 min at room temperature. Quantification of apoptotic cells was performed on a FACScan™. A simultaneous dye exclusion test with propidium iodide to discriminate necrotic cells was used.

Statistical analysis was performed by Kolmogorov–Smirnov test.

RESULTS

In this study we show that OGP(10–14) inhibits proliferation and enhances myeloperoxidase (MPO) expression of HL60 cell line. Moreover, it induces RhoA activation that may mediate pentapeptide effects. In fact the OGP(10–14)-dependent enhancement of MPO activity and cytoskeleton remodeling are largely prevented by a pre-incubation with C3, a specific RhoA inhibitor. The same biological activities are detected in GM-CSF treated cells, and the inhibition of proliferation and RhoA activation are enhanced after OGP(10–14) addition. Altogether, these results strongly suggest that OGP(10–14) activity on HL60 cell line is largely RhoA-mediated, and this small G protein may be a convergence point for the action of both GM-CSF and pentapeptide.

Immunocytochemical and Western Blot RhoA Detection

Immunocytochemical studies showed that RhoA was mainly localized at the plasma membrane level in HL60 cells treated with OGP(10–14) for both 1 h (Fig. 1) and 72 h. In particular, cells treated for the longest OGP(10–14) exposure time (72 h) displayed the greatest membrane reactivity (data not shown). Conversely, untreated cells (controls) showed reactivity only within their cytoplasm (Fig. 1). Results from experiments performed to evaluate the GM-CSF activity on HL60 were comparable to those obtained by OGP(10–14): both 0.1 and 1 ng/ml GM-CSF induced RhoA translocation at membrane level. This activity was already present after 1 h incubation but it persisted after 72 h culture. The association of OGP(10–14) with GM-CSF resulted in a larger RhoA translocation to the cell membrane (Fig. 1).

Western blotting confirmed OGP(10–14) RhoA activation, demonstrating that a larger amount of RhoA GTP-bound protein was present in pentapeptide treated cells (Fig. 2).

Differences between the two OGP(10–14) concentrations on RhoA behavior were not detectable by these methods.

Effect of OGP(10–14) on Actin Cytoskeleton

Treatment with OGP(10–14) induced a decreased actin polymerization in cytoplasm. Conversely, increased cortical actin polymerization was found. C3 inhibition of RhoA almost totally reversed OGP(10–14) activity on actin re-organization, inducing the disappearance of cortical F-actin (Fig. 3).

OGP(10–14) Activity on Cell Proliferation

Compared to controls (0.459 ± 0.06 nm), OGP(10–14) significantly reduced HL60 prolif-

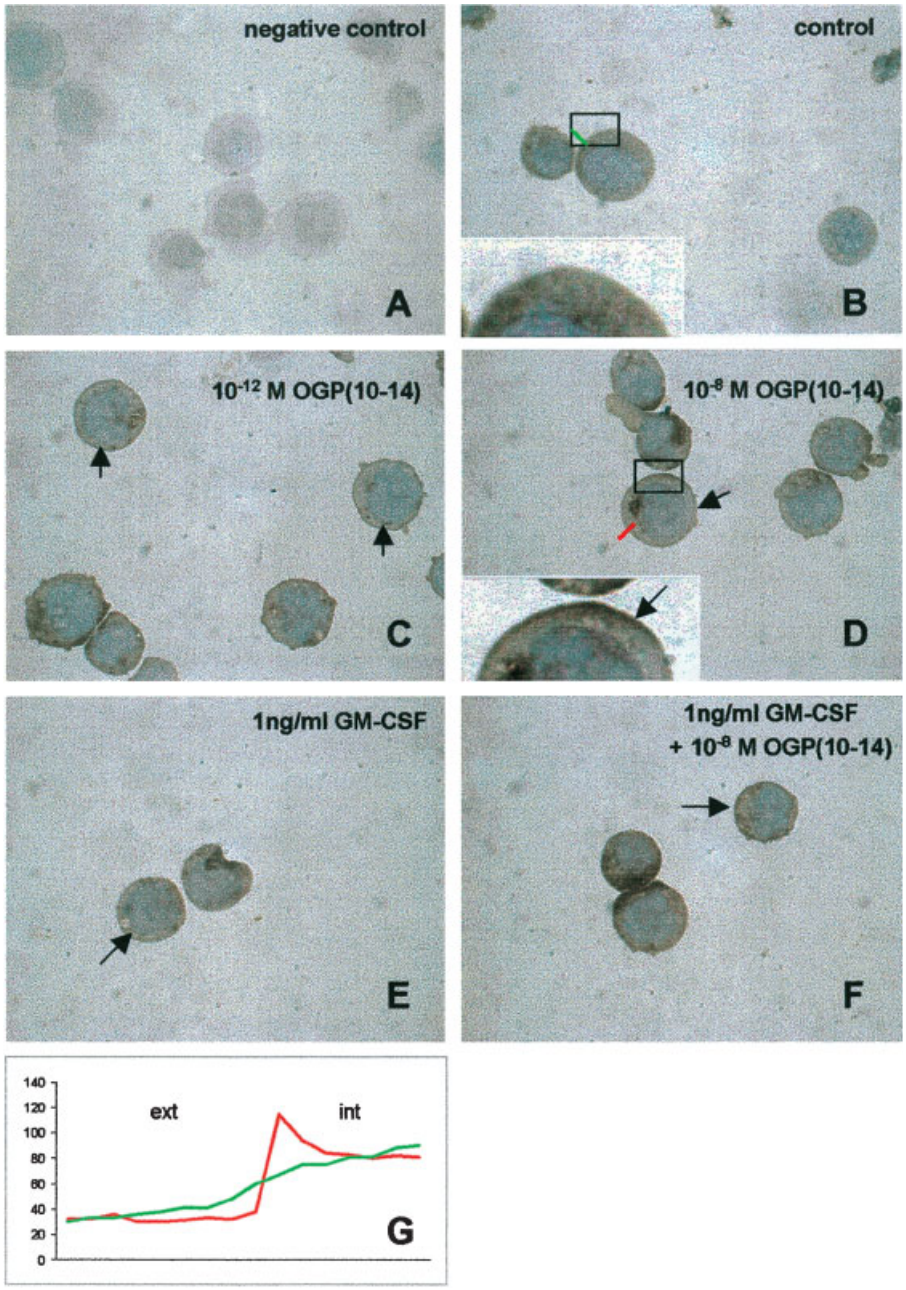

Fig. 1. RhoA immunoperoxidase detection on HL60 cells. **A**: Negative control, obtained by using RhoA blocking peptide (see Materials and Methods), does not react with anti-RhoA antibody. **B**: Control cells, cultured in IST medium, display RhoA presence only within their cytoplasm. **C**, **D**: 1 h OGP(10–14) treatment induces RhoA translocation at the level of the cell membrane (arrows) both at 10^{-12}M and 10^{-8}M. **E**, **F**: Cells treated 1h with 1ng/ml GM-CSF show a membrane RhoA translocation (arrows) that is more evident when this growth factor was associated with OGP(10–14). **G**: Profile of RhoA HRP reactivity levels (obtained by a Leica Quantimet 500+ Image Analysis device) along the lines marked across the plasma membrane in control cell (green line) and in 10^{-8} M OGP(10–14) treated cell (red line); RhoA reactivity is more concentrated at the plasma membrane in treated cell than in control cell. Original magnification 1,000×.

eration, as evaluated by MTT test after 72-h cultures. As shown in Figure 4, both OGP(10–14) 10^{-8} M and 10^{-12} M concentrations were able to decrease cell proliferation (0.232 ± 0.047 nm $P = 6.7\text{E-}07$ and 0.099 ± 0.0030 nm $P = 1.7\text{E-}06$, respectively).

Both 0.1 and 1 ng/ml GM-CSF showed comparable activity in inhibiting cell proliferation,

Fig. 2. RhoA activation assay on 1 h OGP(10–14) treated HL60 cells. Samples were treated by affinity precipitation assay and precipitated GTP-RhoA were then detected by immunoblot analysis as described in Materials and Methods. The OGP(10–14) treated sample (lane 2) shows a larger amount of activated RhoA than the untreated sample (lane 3). In these positive controls (lane 1,4) all cellular RhoA is shown because total RhoA became bound to GTP by a previous incubation of the lysates with GTPγS; the same results were obtained by running non-adsorbed lysates. Lane 1: Positive control of 10^{-12} M OGP(10–14) treated cells. Lane 2: Activated RhoA present in 10^{-12} M OGP(10–14) treated cells. Lane 3: Activated RhoA present in untreated cells. Lane 4: Positive control of untreated cells.

compared to controls (0.120 ± 0.002 nm and 0.11 ± 0.001 nm, respectively).

In order to evaluate the potential synergy of OGP(10–14) with GM-CSF activity, cells were cultured with OGP(10–14) in the presence or absence of GM-CSF. Both 10^{-8} M and 10^{-12} M OGP(10–14) increased the GM-CSF (mg/ml) inhibition on cell proliferation (0.094 ± 0.015 nm $P = 0.017$; and 0.089 ± 0.002; $P = 0.002$) (Fig. 4). The effect of 0.1 ng/ml GM-CSF was enhanced by the addition of OGP(10–14) 10^{-12} M (0.110 ± 0.008 nm; $P = 0.014$) but not by OGP(10–14) 10^{-8} M. All absorbance values represent the average of three independent hexaplicate experiments.

HL60 Differentiation

Addition of 10^{-12} or 10^{-8} M OGP(10–14) resulted in an increased number of peroxidase-positive cells cultured for 72 h ($98.33 \pm 2\%$ and $97.33 \pm 1.52\%$, respectively versus $51.00 \pm 10.14\%$ of untreated cells) (Fig. 5). Moreover, the peroxidase cellular staining was enhanced, resulting strongly intense (++++) in 10^{-12} M sOGP treated cells, intense (+++) in 10^{-8} M sOGP treated cells and slightly intense (+) in untreated ones (Fig. 6). The OGP(10–14)-induced enhancing effect on peroxidase activity was at least partially RhoA-mediated, since it was largely prevented by C3 pre-treatment; in fact, samples treated by 10^{-12} M OGP(10–14) and C3 resulted in a reduced intensity of peroxidase staining (+) (Fig. 6) and a reduced number of peroxidase-positive cells ($65 \pm 13.50\%$) compared to samples treated with OGP(10–14) alone. Interestingly, 1 ng/ml GM-CSF led to a greater percentage of positive cells ($80.66 \pm 9.71\%$) and a mild increase in peroxidase intensity (+/++).

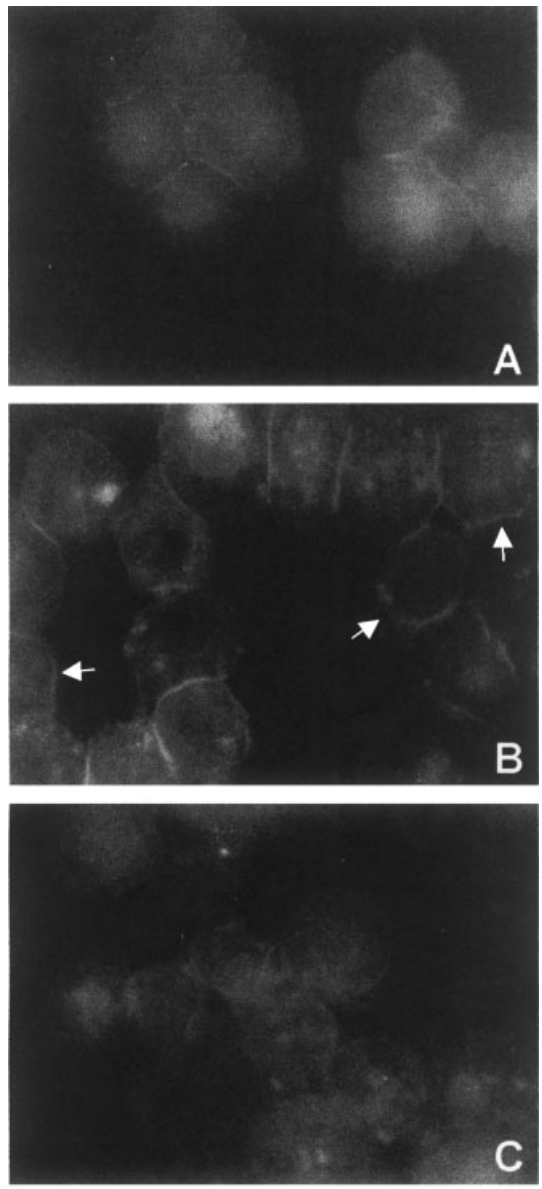

Fig. 3. FITC-phalloidin staining of the cytoskeleton F-actin in HL60 cells. Treatment with OGP(10–14) for 1 h induces an increase in cortical (arrows) and a reduction in cytoplasm F-actin. Addition of C3 to culture medium prevents actin polimerization at the sub-membrane level. **A**: Control cells. **B**: Cells treated with 10^{-12} M OGP(10–14) for 1 h. **C**: Cells treated with 10^{-12} M OGP(10–14) and C3 for 1 h. Original magnification 1,000×.

NBT Test

When HL60 cells were cultured with OGP(10–14), they acquired a clear ability to reduce NBT. In fact, the percentages of positive

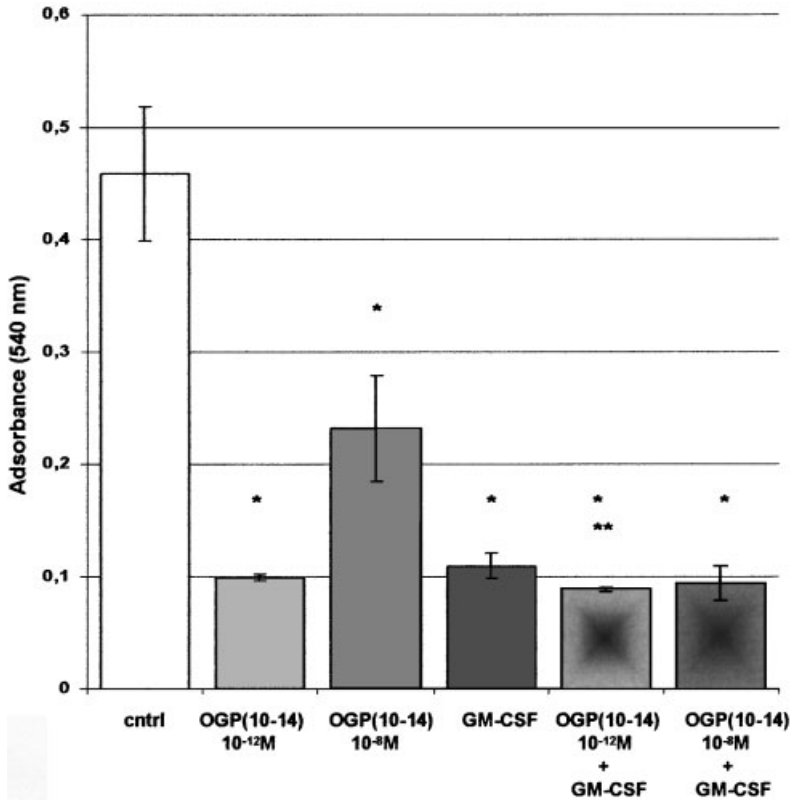

Fig. 4. OGP(10–14) and GM-CSF (1 ng/ml) effect on HL60 cells proliferation. OGP(10–14) induced a significant decrease in cell growth when added to culture medium both at 10^{-12} and 10^{-8} M. Moreover, both OGP(10–14) concentrations are able to significantly increase the GM-CSF (1 ng/ml) inhibitory effect on cell proliferation. $^{*}P < 0.0005$ versus control; $^{**}P < 0.05$ versus GM-CSF 1 ng/ml.

cells were 48.50 ± 4.94 and 44.00 ± 4.24 in 10^{-12} M and 10^{-8} M OGP(10–14) treated samples respectively, versus the $1.50 \pm 0.70\%$ of positive cells found in untreated samples (Fig. 5).

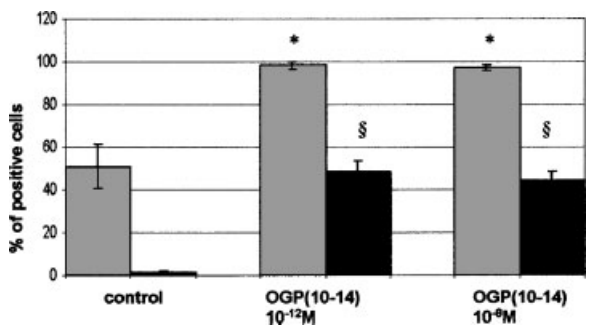

Fig. 5. Seventy-two hours OGP(10–14) treatment of HL60 cells induced an increase in both peroxidase activity and ability of NBT reduction; $^{*}P < 0.005$ versus peroxidase positive cells of control. $^{\S}P < 0.005$ versus NBT positive cells of control. ▒: Percent of cells expressing peroxidase activity, ■: Percent of cells able to reduce NBT.

Cytofluorimetric Analysis

OGP(10–14) treatment induced increased side scatter (SSC) (D/s(n) = 29.15) and MPO (D/s(n) = 35.95) (Fig. 7) without modification of CD45 and CD11b expression. No significant differences were found in CD13 and CD14 expression between control and OGP(10–14) treated samples.

OGP(10–14) (10^{-8} M and 10^{-12} M) treatments did not induce apoptosis in HL60 cells. In fact the Annexin V binding assay revealed less than 5% apoptotic cells in all the samples, independent of the treatment.

DISCUSSION

The OGP was initially characterized only as a bone growth factor due to its mitogenic effect on fibroblast cells and mitogenic/differentiating-effect on osteogenic cells, as well as, an in vivo bone formation enhancer [Bab et al., 1988; Greenberg et al., 1993, 1995]; subsequently,

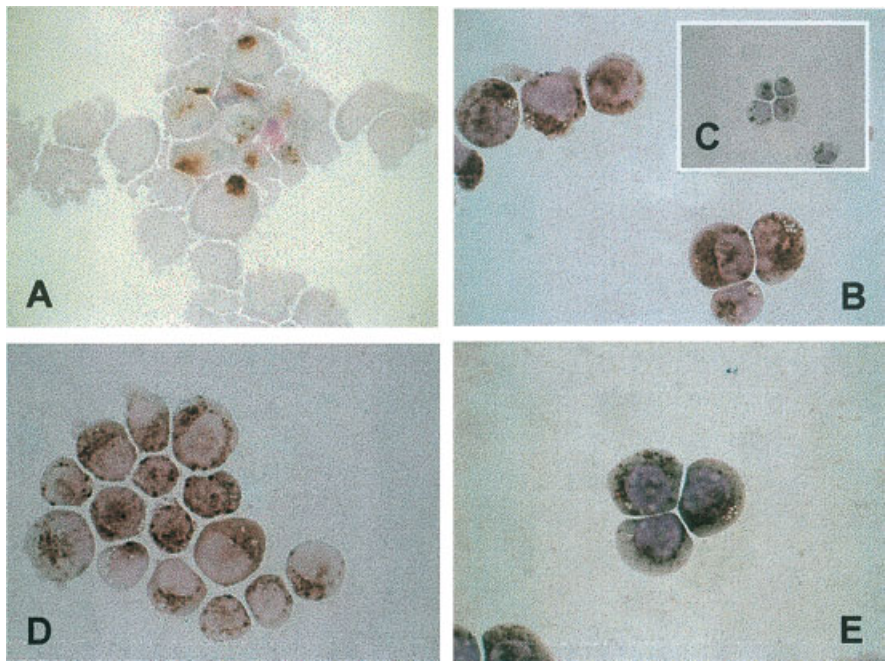

Fig. 6. Peroxidase test. **A**: Control HL60: Cells showed slight (+) brown staining. **B**: 10^{-12} M OGP(10–14) treated HL60: Cells showed intense (++++) brown staining. **C**: 10^{-12} M OGP(10–14)–C3 treated HL60: Cells showed (+) brown staining. **D**: 10^{-8} M OGP(10–14) treated HL60: Cells showed a strong (+++) brown staining. **E**: 1 ng/mlGM-CSF treated HL60: Cells showed (+/++) brown staining. Original magnification 1,000×.

the full-length peptide and the carboxy-terminal fragment of osteoblast growth peptide (OGP(10–14)) have been indicated as pleiotropic factors, since they also show important hematological activity [Gurevitch et al., 1996; Fazzi et al., 2002a, 2002b].

The greatest effects of OGP(10–14) on hematopoietic cells were shown in the presence of specific growth factors and on the staminal-progenitor stage of cell differentiation [Fazzi et al., 2002b, 2003].

In order to study a possible direct activity of OGP(10–14) on myeloid cells and to evaluate its possible role on intracellular signaling, we tested its activity in a HL60 cell line grown in serum-free medium. In fact, the strictly-

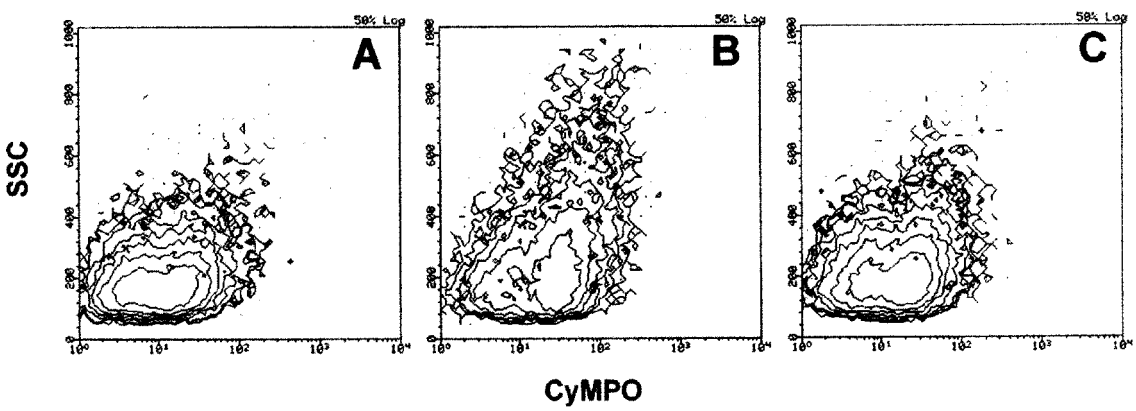

Fig. 7. Contour plot of MPO versus SSC expression in OGP(10–14) treated HL60 cells. The 72-h treatment with 10^{-12} M OGP(10–14) induced a significant increase in side scatter (SSC) and myeloperoxidase (MPO). **A**: Control cells, (**B**) 10^{-12} M OGP(10–14) treated cells, (**C**) 10^{-8} M OGP(10–14) treated cells.

controlled culture conditions used in these experiments decreased the risk of possible interactions between pentapeptide and binding proteins, and/or the unspecific activities on cell proliferation.

In HL60 cell line cultured in a serum-free medium, OGP(10–14) significantly inhibits cell proliferation. In particular, this effect is more evident at the lowest concentration employed. This phenomenon has been previously reported and explained, in different cellular types, by the occurrence of a negative feedback circuit at the higher concentrations [Bab and Chorev, 2002]. OGP(10–14) is also able to enhance MPO expression and activity, and considering that our sub-clone of HL60 basally expresses a very low peroxidase level, both the increased percentage of positive cells and the enhanced cellular expression suggest a differentiating activity of the peptide. Moreover, the increased presence of intracellular granules, as evaluated by SSC analysis and the ability to reduce NBT, confirmed and sustained the pentapeptide-induced cell differentiation. However, terminal differentiation did not take place and apoptosis was not induced.

It has been suggested that during the differentiation process in HL60 cell lines, RhoA protein expression increases in the membrane [Ohguchi et al., 1997], as an activated form of GTP-binding protein [Hall, 1994, 1998; Takaishi et al., 1995]. Thus, we evaluated whether the OGP(10–14) was able to activate RhoA and whether pentapeptide-dependent cellular modifications could therefore be explained through RhoA activation. Our immunocytochemical studies showed that OGP(10–14) induces cell membrane RhoA translocation. RhoA activation was confirmed by immunoblotting; a larger amount of RhoA was GTP-bound in pentapeptide-cultured cells with respect to control cells. Moreover the presence of activated RhoA was provided by OGP(10–14) capability in cellular actin remodeling, as expected by the well-known involvement of RhoA on cytoskeleton rearrangement [Hall, 1998; Ridley, 2001b; Wang et al., 2003; Aspenstrom et al., 2004].

Altogether, these results show that OGP(10–14) is active on HL60 cell line and its action may be mediated by RhoA activation; in fact, the cytoskeleton remodeling as well as MPO expression was largely abolished by pre-incubating cells with the RhoA inhibitor, C3.

RhoA intracellular signaling may be modulated by some growth factors [Moon and Zheng, 2003]. Interestingly, treatment of HL60 cells with GM-CSF appeared to be comparable to treatment with OGP(10–14) regarding cell proliferation and MPO expression, again inducing RhoA activation. It is noteworthy that GM-CSF and OGP(10–14) association enhanced the evaluated activities on HL60 cell line, showing their synergic/additive activity and confirming our previous results obtained in different experimental models [Fazzi et al., 2002b].

A recent report shows that the intracellular signaling cascade elicited by OGP(10–14) involves a G protein-coupled receptor-dependent activation of MAP kinases [Gabarin et al., 2001] and studies carried out on HL60 cells demonstrate that heterotrimeric G proteins trigger various signals, including Rho protein activation [Xu et al., 2003]. In this study we showed that GM-CSF is also able to activate RhoA, in spite of the fact that the interaction of GM-CSF with its specific tyrosine kinase receptor does not activate the G protein pathway [Al-Shami and Naccache, 1999], but instead activates the intracytoplasmatic tyrosine kinase cascade. This picture of complex interactions between several intracellular signaling mechanisms may find its convergence point in RhoA activation; GM-CSF and OGP(10–14) are able to interact with GTP-binding proteins, probably by two independent pathways. This mechanism could also explain the synergic/additive effects of GM-CSF and OGP(10–14) shown in this and in previous studies [Fazzi, 2002b].

In conclusion, our study shows one possible biochemical way by which the pentapeptide acts on hematological cell proliferation/differentiation. Previous results showed that OGP(10–14) acts on hematopoietic cells by enhancing the activities of specific growth factors and/or through the activity of stromal cells as supported by previous studies on hematopoiesis [Fazzi et al., 2002b] and advocated by Gurevitch et al. [1996]. Our preliminary results suggest the possible direct action of OGP(10–14) on HL60 cells, but other possibilities cannot be ruled out. In fact, the documented positive interaction with GM-CSF and previous reports showing that on hematological progenitors pentapeptide action depends upon specific growth factors, could suggest that, in this model, pentapeptide acts by enhancing some

autocrine or paracrine signals able to sustain HL60 cell growth in the absence of exogenous growth factors. In particular, RhoA activation may be related to an enhancing activity of OGP(10–14) on (undetected) growth factor interaction with the specific receptor and/or on intracellular signal transduction after receptor–ligand interaction. However RhoA pathway may not be the unique intracellular signaling triggered by OGP(10–14); studies are in progress to further investigate the role of either other signal molecules or OGP(10–14)-growth factors interaction in HL60 cell differentiation.

In conclusion, this study clearly demonstrates a different mechanism of action of OGP(10–14) on myeloid precursor cells compared to staminal or myeloid progenitors cells; in fact, our previous study focusing on the OGP(10–14) activity on staminal and progenitor hematopoietic cells demonstrated an enhanced clonogenic activity [Fazzi et al., 2002b]; conversely OGP(10–14) seems to play a direct differentiating activity on more mature precursor myeloid cells.

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