Regulation of Release of Hepatocyte Growth Factor From Human Promyelocytic Leukemia Cells, HL-60, by 1,25-Dihydroxyvitamin D₃, 12-O-Tetradecanoylphorbol 13-Acetate, and Dibutyryl Cyclic Adenosine Monophosphate

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Hepatocyte growth factor (HGF) secreted from human promyelocytic leukemia cell line, HL-60, is indistinguishable from HGF in human plasma and its release is significantly stimulated by 12-O-tetradecanoylphorbol-13-acetate (TPA), a differentiation-inducer of HL-60 cells into monocytes/macrophages (Nishino T et al: Biochem Biophys Res Commun 181:323, 1991). TPA stimulated HGF release from the cells through an activation of C-kinase, but not through a formation of reactive oxygen species. Furthermore, dibutyryl cAMP (dbcAMP), an activator of A-kinase and granulocyte-inducer, also stimulated HGF release. 1,25-Dihydroxyvitamin D₃, another monocyte/macrophage-inducer, abated either TPA- or dbcAMP-stimulated synthesis and release of HGF in a dose-dependent manner probably via its nuclear receptor as reflected by vitamin D analog study. The effects of these three agents on the steady-state levels of HGF mRNA of 6.0 kb corresponded with their effects on its protein levels. Furthermore, a close correlation between intracellular and extracellular HGF levels strongly suggested that these agents affected HGF release mainly on its synthesis step. Recombinant human HGF significantly stimulated the proliferation and alkaline phosphatase activity of mouse osteoblastic cell line, MC3T3-E1. In summary, HL-60 cells secrete HGF, whose synthesis is specifically regulated by various reagents independent of their differentiation-inducing effects. Because HGF shows a direct effect on osteoblast-like cells, it might be involved in the interaction of bone marrow cells with bone cells.

MATERIALS AND METHODS

Materials. 1,25-(OH)₂D₃, 24,25-dihydroxyvitamin D₃ (24,25-[OH]₂D₃) and 25-hydroxyvitamin D₃ (25-OH-D₃) were gifts from Chugai Pharmaceutical Co (Tokyo, Japan). TPA, dbcAMP, 1-(5-isooquinoline-sulfonyl)-2-methylpiperazine (H-7), N-(2-guanidinoethyl)-5-isooquinolinesulfonamide (HA1004), potassium superoxide, and cumene hydroperoxide were purchased from Sigma (St. Louis, MO). Monoclonal and polyclonal anti-human HGF antibodies, recombinant human HGF, and human HGF cDNA were prepared as described previously. Buffers used were as follows: phosphate-buffered saline (PBS), 15 mmol/L KH₂PO₄, 8.0 mmol/L Na₂HPO₄ (pH 8.0), 137 mmol/L NaCl, 2.7 mmol/L KCl; PBS-T, PBS plus 0.05% Tween-20 (Wako Pure Biochemical, Osaka, Japan); TNMN, 10 mmol/L Tris/HCl (pH 7.5), 0.15 mol/L NaCl, 1.5 mmol/L MgCl₂, 0.65% NP-40; TENUS, 10 mmol/L Tris/HCl (pH 7.5), 10 mmol/L EDTA, 0.35 mol/L NaCl, 7 mol/L urea, 1% sodium dodecyl sulfate (SDS); SSC, 0.15 mol/L NaCl, 0.015 mol/L Na citrate.

Cells and cell culture. HL-60 cells were provided by Otsuka Pharmaceutical Co. MC3T3-E1 cells were kindly provided by Dr Yoshiyuki Hakeda (Meikai University, Saitama, Japan). HL-60 cells were cultured essentially as described. Briefly, cells were cultured in RPMI 1640 (Flow Laboratories, Irvine, Scotland) sup-

plemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), in a humidified air at 37°C. Under these conditions, the doubling time was less than 48 hours. Each flask was usually inoculated at 2.0 × 10^6 cells/mL. Mouse osteoblastic cell line, MC3T3-E1, was cultured in α-Modification of Eagle’s Medium (α-MEM) containing 10% FBS, as previously described. Vitamin D₃ analogs and TPA dissolved in ethanol, were added so that the final concentration of ethanol was less than 0.1%. Cell number was monitored with a hemacytometer and cell viability determined by trypan blue exclusion.

Preparation of HL-60 cell extract. HL-60 cells were washed twice with PBS. Cell pellets were suspended in PBS-T and sonicated at level 3 in a continuous mode with three 20-second cycles interrupted by 60-second pause by Branson Sonifier (model 200; Branson, Danbury, USA). The sonicate was centrifuged 27,000g for 60 minutes. The supernatant fraction was stored at -30°C until used for the determination of HGF levels by enzyme-linked immunosorbent assay (ELISA).

Determination of HGF concentration. HGF levels in culture medium and cell extract were determined by using ELISA system as we established previously. Briefly, standard human HGF or samples with unknown concentrations of HGF were dispensed into a 96-well microtiter plate coated with a monoclonal antibody (MoAb) against human HGF. After incubation for 1 hour, it was washed three times with PBS-T. After the addition of a 0.1-mL aliquot of a polyclonal antibody against human HGF, the plate was incubated for 1 hour, then washed three times with PBS-T. After the addition of 0.2 mL of diluted goat (antirabbit Ig) IgG-peroxidase conjugate, the plate was incubated for 1 hour and then washed. An aliquot (0.1 mL) of 0.25% 0-phenylenediamine was added and the plate allowed to stand for 10 minutes. After the reaction was stopped by the addition of 0.1 mL of 1.0-N H₂SO₄, the absorbance was read at 492 nm by an automatic plate reader with a reference wavelength of 690 nm.

Northern blot analysis. Total cellular RNA was prepared from HL-60 cells with a minor modification of the method previously described. Briefly, after washed twice with PBS, cells (1.4 × 10⁷ cells) were pelleted in a 1.5-mL Eppendorf tube (Eppendorf, Hamburg, Germany). Then cells were resuspended in 200 μL of ice-cold TNE2MN. After vigorous vortexing, cytoplastmic lysates prepared by centrifugation were transferred to fresh microfuge tubes containing 200 μL of TEFUS. The solution was extracted with phenol/chloroform. RNA (10 μg/lane) was electrophoresed in 1% agarose gel containing formaldehyde, transferred to a nylon filter, and hybridized at 37°C for 40 hours with 3²P-labeled human HGF cDNA as probes in 50 mmol/L Tris/HCl (pH 7.5) 50% formamide, 3 × SSC, 0.1% SDS, 20 μg/mL tRNA, 20 μg/mL boiled salmon sperm DNA, 1 mmol/L EDTA, 0.02% bovine serum albumin (BSA), 0.2% polyvinylpyrrolidone, and 0.02% Ficoll (Pharmacia, Uppsala, Sweden). The filter was washed with 2 × SSC, 15 SDS, 1 × Denhart at 37°C for 1 hour, followed by 0.1 × SSC, 1 × SDS at 50°C for 1 hour.

MC3T3-E1 cell. For proliferation assay, cells were inoculated at 5 × 10⁵ cells per 10 cm² plastic dish. After 24 hours of cultures in MEM containing 10% FBS, cells were washed three times with MEM and then treated for another 48 hours with HGF (150 ng/mL or vehicle in the medium containing 2% FBS). The [³H]-thymidine (6.7 Ci/mmol) was added at a dosage of 1 μCi/well to the medium for the last 6 hours of the culture period. Then the cells were washed three times with PBS to remove exchangeable radioactivity and then treated with 10% trichloroacetic acid (TCA). TCA-insoluble radioactivity was measured. To determine the effect of HGF on cellular alkaline phosphatase activity, cells, inoculated at 5.0 × 10⁴ cells per 10 cm² dish, were cultured for 4 days in the medium supplemented with 10% FBS. After the confluent cells were rested in serum-free medium for 48 hours, they were treated with HGF (150 ng/mL) or vehicle for another 72 hours in MEM containing 2% FBS. Then, the cells were washed three times with PBS and then treated with 1% Triton X-100 (Wako Pure Biochemical) in PBS for 1 hour on ice. Alkaline phosphatase activity in the cellular extract was measured as described. Protein levels were determined as described.

RESULTS

The mechanism of TPA for the stimulation of HGF secretion into culture medium. We have previously reported that HGF secreted from HL-60 cells is identical with human HGF in human plasma. TPA significantly stimulates HGF release into culture medium in a dose-dependent manner. We examined whether C-kinase is involved in the stimulation of HGF release from HL-60 cells by TPA, which is known to directly stimulate C-kinase. As shown in Fig 1, 20 μmol/L H7, a C-kinase inhibitor, inhibited TPA-stimulated HGF release, whereas the same concentration of HA 1004, a 10-fold weaker inhibitor of C-kinase, failed to inhibit HGF release. These data suggested that TPA might stimulate HGF release from HL-60 cells through an activation of C-kinase. When exposed to TPA, HL-60 cells undergo a burst of oxygen metabolism with a resultant formation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide. However, 10 μmol/L of potassium superoxide or 50 μmol/L cumene hydroperoxide, enough to stimulate differentiation of Friend erythroleukemia cells, did not affect HGF release from HL-60 cells, suggesting that ROS formation might not play an important role in TPA-stimulated HGF release (data not shown).

The inhibitory effect of 1,25-(OH)₂D₃ on TPA-stimulated HGF release into culture medium from HL-60 cells. If an
Fig 2. Inhibitory effect of 1,25-(OH)2D3 on TPA-stimulated HGF release into culture media. HL-60 cells (2.0 X 10^6 cells/mL) were incubated for 3 days with 1.0 ng/mL of TPA in the presence of varying concentrations of 1,25-(OH)2D3. 1,25-(OH)2D3 inhibited the TPA-induced stimulation in a dose-dependent manner up to 10^{-7} mol/L with the significant inhibition detected by as low as 10^{-10} mol/L. Results are expressed as the mean ± SD of three cultures. *P < .0001 versus control, **P < .005 versus TPA (1.0), ***P < .001 versus TPA (1.0).

increase of HGF release was associated with TPA-induced HL-60 cell differentiation along monocyte/macrophage pathway, 1,25-(OH)2D3, another monocyte/macrophage inducer,25-28 should stimulate HGF release from the cells.

Fig 3. Effects of various vitamin D3 analogs on the TPA-stimulated HGF release into culture media. HL-60 cells (2.0 X 10^6 cells/mL) were incubated for 3 days with TPA in addition to the indicated concentrations of various vitamin D3 analogs. The bioptic potencies of 25-OH-D3 and 24,25(OH)2-D3 were approximately 100-fold less potent than that of 1,25-(OH)2D3, respectively, on the basis of the concentration of each analog required for 50% inhibition of the TPA-induced stimulation. The biologic potency ratio of each vitamin D3 analog was in good agreement with its reported binding affinity for 1,25-(OH)2D3 receptor. Results are expressed as the mean ± SD of three cultures. *P < .005 versus control.

1,25-(OH)2D3 alone did not affect basal HGF release from HL-60 cells. However, 1,25-(OH)2D3 inhibited TPA-stimulated HGF release in a dose-dependent manner (Fig 2). The inhibitory effect of 1,25-(OH)2D3 was initially detected at 10^{-10} mol/L and the effect increased with concentrations up to 10^{-7} mol/L. Furthermore, to determine whether or not 1,25-(OH)2D3 inhibits HGF secretion via nuclear 1,25-(OH)2D3 receptor, the potencies of various vitamin D3 analogs on this action were examined. The data in Fig 3 clearly show that the relative potencies of the vitamin D3 analogs in inhibiting HGF secretion paralleled their affinities for nuclear 1,25-(OH)2D3 receptor as previously shown.29
together, it was strongly suggested that 1,25-(OH)\textsubscript{2}D\textsubscript{3} exerts its effect on HGF secretion via nuclear 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor. Next, to determine whether TPA and 1,25-(OH)\textsubscript{2}D\textsubscript{3} affect HGF synthesis and/or its secretory process, HGF levels were determined both in culture medium and in cellular extract. TPA- and/or 1,25-(OH)\textsubscript{2}D\textsubscript{3}-induced changes in intracellular HGF levels corresponded very well to those in extracellular levels (Fig 4), suggesting that these reagents affected HGF synthesis but not its secretion. Furthermore, the effects of these reagents on HGF release were preceded by their differentiation-inducing effects (data not shown).

**Stimulatory effect of dbcAMP on the secretion of HGF from HL-60 cells, which was abated by 1,25-(OH)\textsubscript{2}D\textsubscript{3}**. Addition of 10\textsuperscript{-7} mol/L dbcAMP, enough to induce approximately 70% of HL-60 cells to differentiate into granulocytes after 4-day incubation,\textsuperscript{13} caused a significant increase of HGF levels in the culture medium. The stimulation of HGF secretion by dbcAMP was preceded by its differentiation-inducing effect. 1,25-(OH)\textsubscript{2}D\textsubscript{3} (10\textsuperscript{-7} mol/L) attenuated dbcAMP-stimulated HGF release to basal levels as well as TPA-induced stimulation (Fig 5).

**Determination of HGF mRNA levels in HL-60 cells**

![Graph showing the effect of 1,25-(OH)\textsubscript{2}D\textsubscript{3}, TPA, and/or dbcAMP on the steady-state levels of HGF mRNA in HL-60 cells](image)

Fig 6. Effect of 1,25-(OH)\textsubscript{2}D\textsubscript{3}, TPA and/or dbcAMP on the steady-state levels of HGF mRNA in HL-60 cells (A) and its densitometric analysis (B). After treatment of HL-60 cells for 24 hours with 1,25-(OH)\textsubscript{2}D\textsubscript{3} (10\textsuperscript{-7} mol/L), TPA (1.0 ng/mL), and/or dbcAMP (10\textsuperscript{-7}mol/L), total RNA was isolated and electrophoretically size fractionated in 1% agarose/formaldehyde gels. RNAs were transferred to nitrocellulose filter and probed with [\textsuperscript{32}P]-labeled human HGF cDNA as described in Materials and Methods. Lanes: 1, control; 2, TPA; 3, TPA/1,25-(OH)\textsubscript{2}D\textsubscript{3}; 4, 1,25-(OH)\textsubscript{2}D\textsubscript{3}; 5, dbcAMP; 6, dbcAMP/1,25-(OH)\textsubscript{2}D\textsubscript{3}; 7, TPA/dbcAMP. (B) Autoradiogram obtained in (A) was scanned by laser densitometer for the determination of relative levels of HL-60 HGF transcript. Results are expressed as a percentage of control value.

Treated with TPA and/or 1,25-(OH)\textsubscript{2}D\textsubscript{3}. To confirm that TPA, dbcAMP, and 1,25-(OH)\textsubscript{2}D\textsubscript{3} affect HGF synthesis, the effects of those reagents on the steady-state levels of HGF mRNA in the cells were determined. Northern blot analysis of HL-60 cellular RNA using a human HGF cDNA probe showed a single band of 6.0-kb transcript (Fig 6A), as we previously reported.\textsuperscript{1} As semiquantitated by densitometric analysis (Fig 6B), the levels of HGF transcripts increased significantly around 2.6- and 2.1-fold after 24 hours of exposure to either 1.0 ng/mL of TPA or 10\textsuperscript{-7} mol/L dbcAMP, respectively. Treatment of HL-60 cells with a combination of TPA and dbcAMP acted synergistically to increase the levels of HGF transcript around 4.5-fold. The increase induced by either reagent was abated by the simultaneous treatment with 10\textsuperscript{-7} mol/L 1,25-(OH)\textsubscript{2}D\textsubscript{3}, which is in good agreement with the effects of those reagents on the levels of HGF molecule. Of some interest, the level of HL-60 mRNA
in cells treated with 1,25-(OH)2D3 alone were significantly lower than in control cells.

Effect of HGF on mouse osteoblastic cell line, MC3T3-E1. It has been recently reported that HGF exerts its effect on various kinds of cells besides hepatocyte, although hepatocyte was originally considered as an only and specific target cell for HGF. Furthermore, because HGF release is specifically regulated by 1,25-(OH)2D3, which is known to have its major effect on bone, we examined the possible involvement of HGF in the interaction of BM cells with bone cells. As shown in Fig 7, treatment of MC3T3-E1 cells, which have osteoblast phenotype, with 150 ng/mL of HGF for 48 hours, significantly stimulated [3H]thymidine incorporation. The increase in [3H]thymidine incorporation by the cells was in a dose-dependent fashion. HGF increased the number of MC-3T3-E1 cells when they were treated with HGF in the logarithmic phase of growth (data not shown). Furthermore, treatment of confluent cells for 72 hours with the same concentration of HGF resulted in a significant increase in alkaline phosphatase activity.

Statistical significance. Results were expressed as means ± SD. Difference between groups were determined by Student's t-test.

DISCUSSION

In the present study, we showed that TPA stimulated HGF release from HL-60 cells through an activation of C-kinase and that 1,25-(OH)2D3, another differentiation inducer along monocyte/macrophage pathway, did not affect HGF release alone but attenuated TPA-stimulated HGF release from the cells, indicating the specific effect of TPA or 1,25-(OH)2D3, but not the effect associated with cell differentiation. The data supportive of this hypothesis are that dbcAMP, a granulocyte inducer, also stimulated HGF release from the cells that preceded dbcAMP-induced cell differentiation. This stimulation was also attenuated by 1,25-(OH)2D3. It was very likely that TPA, dbcAMP, and 1,25-(OH)2D3 modulated HGF release from the cells by affecting its synthesis as reflected by both Northern blotting experiment and by a close correlation between extracellular and intracellular HGF levels. Very recently, it was reported that a 28-Kd HGF-immunoreactive peptide is synthesized by various cell lines, human placenta, and fibroblasts from different tissues, which is derived from an alternative human HGF transcript and that this smaller HGF variant, whose transcript is detected as 1.3-kb message, specifically inhibits HGF-induced mitogenesis of B5/589 human mammary epithelial cells. However, in HL-60 cells, neither a 28-Kd-immunoreactive peptide nor 1.3-kb HGF RNA species was detected, thus validating our ELISA system to determine HGF levels in HL-60 cell system. TPA is known to interact with its specific receptor on the plasma membrane and recent investigations point to C-kinase as the receptor for TPA. With regards to stimulatory action on HGF release, TPA seemed to work through an activation of C-kinase because 20 μmol/L H7 but not HA1004 could completely block TPA-stimulated HGF release from the cells. Injury of the liver, which is known to cause ROS formation, leads to the stimulation of HGF synthesis, suggesting ROS formation as a possible stimulus. Because TPA is also known as a stimulator of ROS formation, we studied the involvement of ROS in TPA-induced HGF synthesis in HL-60 cells. However, TPA seemed not to stimulate HGF release via ROS formation as reflected by the absence of the effect of potassium superoxide or cumene hydroperoxide.

1,25-(OH)2D3 induces HL-60 cells to differentiate into monocytes/macrophages. Furthermore, it induces up-regulation of 1,25-(OH)2D3 receptor and 24-hydroxylase activity in HL-60 cell mitochondria via nuclear 1,25-(OH)2D3 receptor. It was hypothesized that C-kinase is intimately involved in the effect of 1,25-(OH)2D3 on HL-60 cells. Supportive of this hypothesis are the recent reports that an early event occurring during 1,25-(OH)2D3-induced HL-60 cell differentiation is associated with a specific increase in calcium and phospholipid-dependent phosphorylation and in protein kinase C activity. 1,25-(OH)2D3 increased phorbol ester receptors in HL-60 cells as reflected by an increase (70%) in [3H]phorbol-12,13-dibutyrate ([3H]PDBu) binding to HL-60 cells, suggesting 1,25-(OH)2D3-induced augmentation of the cell's sensitivity to TPA. Lastly, a specific C-kinase inhibitor, H7, partially blocked 1,25-(OH)2D3-induced HL-60 cell differentiation. The IC50 for 1,25-(OH)2D3-induced HL-60 cell differentiation was approximately 15 μmol/L for H7 and 170 μmol/L for HA1004, respectively. Previous reports of ours showed that 1,25-(OH)2D3 shares common phenomena with TPA, such as the induction of ornithine decarboxylase activity and of upregulation of 1,25-(OH)2D3 receptor (unpublished observation). However, 1,25-(OH)2D3 still retains its specific effect on HL-60 cells besides stimulation of C-kinase, such as the induction of 1,25-(OH)2D3 and 25-OH-D3-24-hydroxylase activity in HL-60 cell mitochondria. With regards to modulatory action on HGF synthesis by HL-60...
cells, 1,25-(OH)₂D₃ abated TPA-stimulated HGF release from the cells, thus strongly suggesting that 1,25-(OH)₂D₃ exerts its effect through a 1,25-(OH)₂D₃-specific pathway but not by C-kinase.

HGF, also known as hepatopoietin A, is a heparin-binding heterodimeric protein of 105 Kd, consisting of a heavy and a light chain with an apparent molecular weight of 54 to 65 and 31.5 to 34.5 Kd, respectively.⁶ It was originally identified as a complete mitogen for hepatocytes in primary cultures and plays a physiologic role in liver regeneration.² However, it has no homology with the various known growth factors. It has high homology with plasminogen (38%) and proteins of the blood coagulation cascade provided with 'krisp' motifs, such as tissue-plasminogen activator, urokinase, factor XII, and prothrombin, but deficient of the proteolytic activity.³ Cross-linking experiments showed that HGF binds a protein of the apparent molecular weight of 150 Kd, a size consistent with the molecular weight of the β subunit of the c-MET receptor.⁴ Further, HGF stimulates the phosphorylation on tyrosine of p190ⁿ’ kinase.⁵ Northern blot analysis showed that HGF mRNA is expressed in various rat tissues, including liver, kidney, lung, thymus, and brain.³ It has been reported recently that HGF exerts its effect on various kinds of cells such as endothelial cells and melanocytes.³ Furthermore, it was shown that HGF is identical with scatter factor, a fibroblast-secreted protein that promotes motility and matrix invasion of epithelial cells.¹¹ These data strongly suggested an important physiologic role of HGF in the proliferation of a much broader spectrum of cell types than originally considered. Because 1,25-(OH)₂D₃ is known to stimulate bone resorption by increasing osteoclast numbers in bone⁴¹ and it affects HGF synthesis in the HL-60 cell system, a relevant system for the investigation of molecular mechanism of 1,25-(OH)₂D₃ on bone,³²³⁴ we checked the effect of HGF on bone cells. The activities and numbers of osteoclast are known to be intimately regulated by osteoblast, which is responsive to 1,25-(OH)₂D₃,⁴³ as reflected by the presence of 1,25-(OH)₂D₃ receptor.⁴⁴ As shown in Fig 7, HGF stimulated mouse bone cells with osteoblastic phenotype, MC3T3-E1. Taken collectively, these data raised the possibility that 1,25-(OH)₂D₃ might exert its effects on bone at least partly by affecting HGF synthesis.

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