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Stimulation of prostaglandin production by hepatocyte growth factor in human gastric carcinoma cells

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Hepatocyte growth factor (HGF), a protein with pleiotropic biological activity affecting cell growth and motility, was found to markedly activate prostaglandin production in human gastric carcinoma TMK-1 cells. HPLC analysis revealed that HGF stimulated the production of prostaglandin E_2 (PGE_2), which is the major prostaglandin produced in these cells. HGF maximally stimulated PGE_2 production at a concentration of 10 ng/ml, and it was a more potent stimulator of PGE_2 production than epidermal growth factor (EGF), which is known to stimulate prostaglandin production in various cell lines. The simultaneous addition of HGF and EGF caused no further stimulation of the PGE_2 production observed in HGF-treated cells. We showed also that HGF increased the arachidonate release from TMK-1 cells, which release was completely suppressed by the addition of phospholipase A_2 (PLA_2) inhibitors. Further studies in vitro showed that HGF enhanced cellular activities of cytosolic PLA_2 and cyclooxygenase 1.5-fold each. These results indicate that HGF stimulates prostaglandin production through increases in both cytosolic PLA_2 and cyclooxygenase activities.

Hepatocyte growth factor; Epidermal growth factor; Prostaglandin; Arachidonic acid; Phospholipase A_2; Human gastric carcinoma cell

1. INTRODUCTION

Hepatocyte growth factor (HGF) was originally identified as a potent mitogen of rat hepatocytes in primary culture [1–3]. HGF was also shown to stimulate the growth of many kinds of cells including melanocytes and endothelial cells as well as a variety of epithelial cells [4], but it was cytotoxic to a variety of tumor cell lines [5]. Recently, Widner et al. [6] reported that HGF is identical to 'scatter factor', which induces the disruption and scattering of epithelial colonies and increases the movement of individual cells. Therefore, HGF induces different growth responses in different cells and is now recognized as a protein with pleiotropic biological activity affecting cell growth and motility.

The HGF receptor is the c-*met* proto-oncogene product, which is rapidly tyrosine phosphorylated in response to HGF [7–8]. Recently, it was suggested that several intracellular molecules are involved in the signaling of HGF. For example, the addition of HGF to rat hepatocytes induced the formation of 1,2-diacylglycerol (DG) via phosphatidylcholine-phospholipase C (PLC) [9]. Furthermore, polyphosphoinositides generated by phosphatidylinositol 3-kinase are suggested to be included in the signaling pathway of HGF [10]. However, details of the signaling pathway following activation of the HGF receptor still remain to be elucidated.

In this study, we examined whether HGF has an activity to stimulate the production of prostaglandins, which have been shown in various studies to play regulatory roles in cell growth and other physiological responses. We found that in TMK-1, a cell line derived from a human gastric carcinoma [11], HGF stimulated PGE_2 production through increases in both cytosolic phospholipase A_2 and cyclooxygenase activity. In addition, HGF was a more effective stimulator than epidermal growth factor, which is known to be a potent stimulator of prostaglandin production in various cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Recombinant human HGF produced in Chinese hamster ovary cells was provided by the Research Center, Mitsubishi Kasei Co. (Yokohama, Japan) [12]. EGF (ultrapure) from mouse submaxillary glands was purchased from Toyobo Co., Ltd. (Osaka, Japan); fetal calf serum (FCS), from Gibco Life Technologies, Inc. (Grand Island, NY); and RPMI1640, from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Prostaglandins were obtained from Sigma Co. (St. Louis, MO). All other reagents were of analytical grade.

2.2. *Cell cultures*

TMK-1 (poorly differentiated adenocarcinoma) isolated from

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human gastric carcinoma [11] was kindly supplied by Dr. E. Tahara of Hiroshima University School of Medicine. The cells (2×10^5) were plated on 35-mm plastic dishes in 2 ml of RPMI1640 supplemented with 5% FCS and 10 μg/ml gentamicin (Sigma) and used for all experiments after 2 days in culture.

2.3. *Assays of prostaglandin production*

Growing TMK-1 cells were incubated with various concentrations of HGF in fresh medium supplemented with 5% FCS. Prostaglandins were extracted from the culture medium by the use of a Sep-Pak C-18 cartridge (Waters; Milford, MA) and were analyzed by HPLC or with a [^{125}I]PGE_2 radioimmunoassay (RIA) kit (New England Nuclear; Boston, MA) as described previously [13].

2.4. *Release of [^{3}H]arachidonate*

Growing TMK-1 cells were prelabeled with 0.5 μCi/ml [^{3}H]arachidonic acid (95.1 Ci/ml; NEN) for 24 h. Then the cells were treated with either HGF or EGF in fresh medium. At specified times, the radioactivity released into the medium was measured as described previously [14].

2.5 *Assay of phospholipase A_2 activity*

Growing TMK-1 cells were incubated with or without 10 ng/ml HGF for 4 h. The PLA_2 activitities in TMK-1 cytosol were measured as previously described [15].

2.6. *Assay of cyclooxygenase activity*

The solubilized TMK-1 sonicate was prepared as described by Raz et al. [16], and assayed for cyclooxygenase activity by measuring conversion of the added arachidonic acid to prostaglandin E_2. The assay mixture contained the following in a total volume of 100 μl: 50 mM Tris (pH 8.0), 5 mM tryptophan, 5 mM reduced glutathione, 2 μM hematin, and solubilized cell sonicate (100 μg of protein). The mixture was preincubated for 2 min at 37°C, and the assay was started by the addition of 100 μM arachidonic acid in ethanol. After incubation for 2 min at 37°C, the reaction was stopped by the addition of ice-cold ethanol. Produced prostaglandins were extracted and measured by RIA.

3. RESULTS AND DISCUSSION

Prostaglandin production of TMK-1 cells was analyzed by reverse-phase HPLC. PGE_2 was detected as the major prostaglandin in both the control and the HGF-treated cultures, and its amount was increased by HGF treatment (Fig. 1). The increase in PGE_2 production was confirmed by radioimmunoassay of PGE_2 (Fig. 2). HGF stimulated PGE_2 production in a dose-dependent (Fig. 2A) and time-dependent (Fig. 2B) manner. The maximal effect of HGF was obtained at a concentration of 10 ng/ml and after a 6-h treatment.

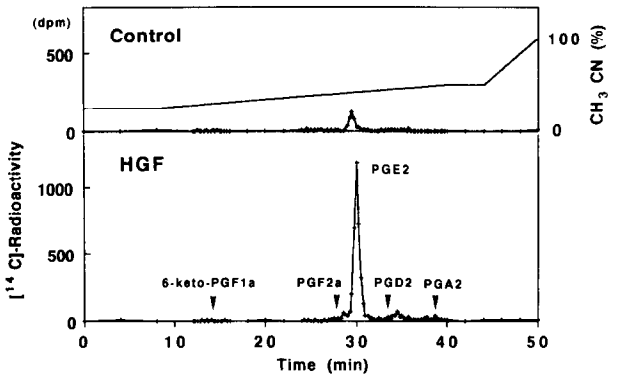

Fig. 1. HPLC analysis of [^{14}C]arachidonic acid metabolites. TMK-1 cells were prelabeled with [^{14}C]arachidonic acid (1 μCi/ml) for 24 h and further incubated with or without 10 ng/ml HGF for 4 h. The supernatants were collected and analyzed by HPLC. Prostaglandins were identified by comparison of their retention times with those of authentic standards.

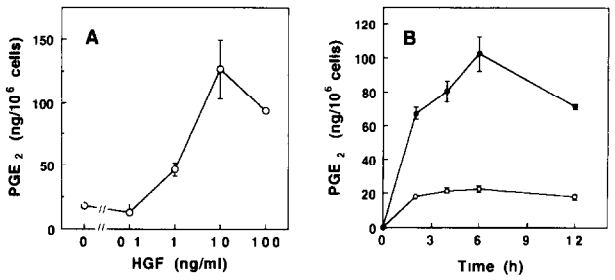

Fig. 2. Dose–response and time course of PGE_2 production by HGF. (A) TMK-1 cells were incubated with indicated concentrations of HGF for 4 h. (B) TMK-1 cells were incubated with (●) or without (○) 10 ng/ml HGF for the indicated times. PGE_2 levels in the culture medium were determined by RIA. Values are means ± S.D. for triplicate cultures. Similar results were obtained in two independent experiments.

We next compared the activity of HGF to stimulate prostaglandin production with that of EGF, whose receptor protein is also known to be a transmembrane protein with tyrosine kinase activity. HGF (10 ng/ml) increased the amount of PGE_2 about 4-fold; whereas EGF (10 ng/ml) increased it about 2-fold (Fig. 3), indicating HGF to be a more potent stimulator of PGE_2 production than EGF. The figure also indicates that treatment of cells with EGF in combination with HGF did not cause any further increase in PGE_2 production by HGF-treated cells.

Prostaglandin production is regulated at either or both of two key enzymatic steps. The first step is the release of arachidonic acid from cellular phospholipids by phospholipase; and the second, conversion of released arachidonate into prostaglandins by cyclooxygenase (PGH synthase). So we first determined the effect of HGF and EGF on arachidonate release from TMK-1 cells. As shown in Fig. 4, both HGF and EGF increased the release in a time-dependent manner for up to 2 h of incubation, and HGF was a more potent stimulator of arachidonate release than EGF. Combined treatment of cells with HGF and EGF did not augment the release over that seen from cells treated with HGF alone. The potency of HGF and EGF for arachidonate release was closely related to that for PGE_2 production; thus the increase in PGE_2 level is thought to reflect the stimulation of arachidonate release. The release of arachidonic acid from the *sn*-2 position of membrane phospholipids has been ascribed either to activity of phospholipase A_2 (PLA_2) or to the sequential action of phospholipase C (PLC) and DG lipase. Recently it was reported that

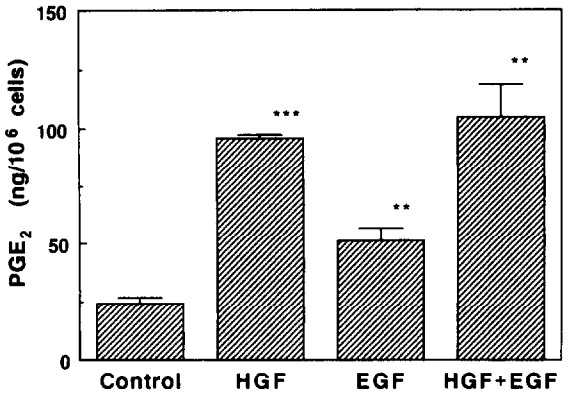

Fig. 3. Stimulation of prostaglandin E_2 production by HGF and EGF. TMK-1 cells were incubated with 10 ng/ml HGF, 10 ng/ml EGF, or their combination for 4 h. PGE_2 levels in the culture medium were determined by RIA. Values are means ± S.D. for triplicate cultures. Similar results were obtained in two independent experiments. Significance of difference was determined by Student's *t*-test. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

HGF stimulated the formation of 1,2-diacylglycerol-(1,2-DG) by PLC, and that the peak of DG formation was observed at 5 min after stimulation [9]. However, in this study the arachidonate release increased up to 2 h after HGF-treatment. This prolonged stimulation may be mediated by PLA_2 rather than PLC. Therefore, we examined effect of PLA_2 inhibitors on the arachidonate release. As shown in the inset of Fig. 4, either *p*-bromophenacylbromide (*p*-BPB) or quinacrine (QC) at a concentration of 30 μM completely inhibited HGF-induced arachidonate release to the control level, whereas neither reagent had a significant effect on arachidonate release from control cultures. These results support our consideration that PLA_2 is involved in the stimulation of arachidonate release in TMK-1 cells.

Furthermore, we measured the enzymatic activity of PLA_2 in vitro. The Ca^{2+} requirement of PLA_2 was examined at pH 7.4 with Ca^{2+}/EDTA buffers [17]. No appreciable activity was detected in the presence of EDTA, while significant activity was observed at micromolar concentrations of free Ca^{2+} (data not shown). HGF caused a 1.5-fold increase in PLA_2 activity at 10^{-5} M free Ca^{2+} (Table I); however, the factor caused no further enhancement of PLA_2 activity even at millimolar concentrations of Ca^{2+}. Next we characterized the substrate specificity of PLA_2. As shown in Table I, TMK-1 PLA_2 hydrolyzed 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine 14-fold more effectively than that it hydrolyzed 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine. These enzymological data indicate that the stimulation by HGF caused the enhancement of cytosolic PLA_2 activity in TMK-1 cells.

Since Lin et al. recently reported the phosphorylation and activation of cytosolic PLA_2 by mitogen-activated protein (MAP) kinase [18], it will be interesting in future studies to elucidate whether MAP kinase plays a role in the signal transduction from HGF and EGF receptors to PLA_2.

Table I

Cytosolic phospholipase A_2 activity of TMK-1 cells stimulated by HGF

Substrate	PLA_2 activity (pmol/min/mg protein)	
	Control	HGF
2-Arachidonoyl-PC	142 ± 16	212 ± 13***
2-Linoleoyl-PC	10 ± 2	16 ± 2***

TMK-1 cells were incubated with or without 10 ng/ml HGF for 4 h. PLA_2 activities in TMK-1 cytosol were assayed with 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (2-Arachidonoyl-PC) or 1-palmitoyl-2-[^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine (2-Linoleoyl-PC) as described previously [15]. Values are the means ± S.D. of ten determinations. Similar results were obtained in two independent experiments. Significance of difference was determined by Student's *t*-test.

$^{***}P < 0.001$ vs. control.

Next, we measured the activity of cyclooxygenase in TMK-1 cells that had been preincubated with either HGF or EGF. Solubilized fractions prepared from these cells were incubated with arachidonic acid, and the PGE_2 produced was measured by the radioimmunoassay for PGE_2. HGF caused about a 1.5-fold increase in PGE_2 production over the level in control cells, whereas EGF did not enhance cyclooxygenase activity (Table II). The combination of HGF and EGF also

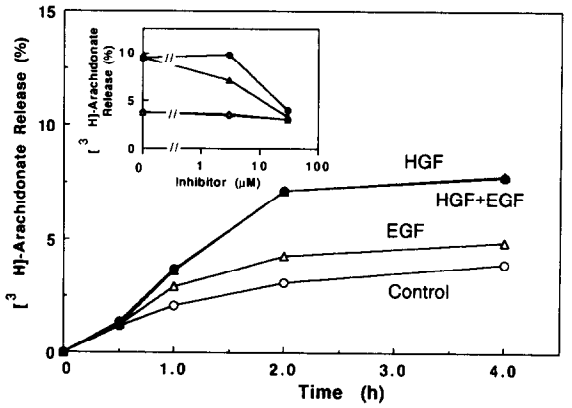

Fig. 4. Arachidonate release stimulated by HGF and EGF. Arachidonate release was determined at various times after the addition of the growth factors. TMK-1 cells prelabeled with [^{3}H]arachidonic acid (0.5 μCi/ml) were incubated in 5% FCS/RPMI containing 10 ng/ml HGF (●), 10 ng/ml EGF (△), combination of HGF and EGF (▲), or no growth factor (○). Amount of the released [^{3}H]arachidonate was expressed as a percentage of total counts incorporated into the cells. Inset: the effect of PLA_2 inhibitors on the arachidonate release. The cells were incubated with PLA_2 inhibitors, *p*-bromophenacylbromide(*p*-BPB; ○, ●) or quinacrine (QC; △, ▲), in the presence (closed symbols) or absence (open symbols) of 10 ng/ml HGF. Released arachidonate after a 2-h incubation is indicated as a percentage. Values are means for triplicate cultures. Similar results were obtained in two independent experiments.

Table II

Stimulation of the cyclooxygenase activity in TMK-1 cells

	Enzyme activity (PGE_2 ng/min/mg protein)
Control	32.1 ± 3.4
HGF	49.2 ± 2.3***
EGF	37.0 ± 2.6
HGF + EGF	49.6 ± 6.1*

Cells were preincubated with 10 ng/ml HGF and/or 10 ng/ml EGF for 4 h. Cell lysates were prepared from these cells and incubated with 100 μM arachidonic acid for 2 min at 37°C. PGE_2 produced was determined by RIA. Each value is the mean ± S.D. of four determinations. Similar results were obtained in two independent experiments. Significance of difference was determined by Student's t-test.
*$P < 0.05$, ***$P < 0.001$ vs. control.

resulted in increased cellular cyclooxygenase activity; however, this activity was not greater than that induced by HGF alone. These data suggest that HGF stimulated the production of PGE_2 via not only increasing arachidonate as a substrate of cyclooxygenase but also inducing the activity of cyclooxygenase in human gastric carcinoma cells.

Prostaglandins induced by a variety of growth factors are known to modulate mitogenic activity of these growth factors [19–22]. In TMK-1 cells, HGF did not affect the cell growth evaluated by [^{3}H]thymidine incorporation or cell number (data not shown). Furthermore, the addition of indomethacin to HGF-treated cultures caused no change in the cell growth (data not shown). These data suggest that prostaglandins are not crucial in the regulation of the growth of TMK-1 cells. We recently reported that HGF induced scattering and cell migration of human gastric carcinoma MKN-74 [23]. TMK-1 cells, as well as MKN-74 cells, were stimulated to migrate in response to HGF (unpublished data). Further study will be required to evaluate the involvement of prostaglandin in the regulation of the cell migration.

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