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EFFECTS OF PROTEIN KINASE INHIBITORS ON THE MITOGENIC ACTIVITY OF HUMAN HEPATOCYTE GROWTH FACTOR ON RAT HEPATOCYTES IN PRIMARY CULTURE

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SUMMARY: To evaluate the role of protein phosphorylation reactions in signal transduction of human hepatocyte growth factor (hHGF), now known to be the same protein as the scatter factor and tumor cytotoxic factor, we examined the effects of various inhibitors of protein kinases on the mitogenic activity of hHGF on rat hepatocytes in primary culture. Genistein, a specific inhibitor of tyrosine kinase, dose-dependently inhibited the effect of hHGF in stimulating DNA synthesis of hepatocytes. By contrast, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), a specific inhibitor of protein kinase C, potentiated the stimulatory effect of hHGF on DNA synthesis of hepatocytes. H7 was effective at over 2 μg/ml and potentiated the effect of hHGF over 2-fold at 20 μg/ml. On the other hand, an inhibitor of Ca^{++}/calmodulin-dependent protein kinase inhibited both the basal and hHGF-stimulated DNA synthesis in the cells, whereas an inhibitor of cyclic nucleotide-dependent protein kinases had little effect on the action of hHGF. These results suggest that tyrosine phosphorylation is required for stimulation of hepatocyte DNA synthesis by hHGF and that the action of hHGF is negatively regulated by protein kinase C activation.

Human hepatocyte growth factor (hHGF) was first identified in the sera and plasma of patients with fulminant hepatic failure as a potent mitogen for rat hepatocytes in primary culture [1, 2] and was purified from the plasma of these patients [3]. hHGF is a heterodimeric glycoprotein consisting of a heavy chain of 55-65 kDa and a light chain of 30-35 kDa linked by a disul-

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The abbreviations used are: HGF, hepatocyte growth factor; hHGF, human HGF; SF, scatter factor; rhHGF, recombinant hHGF; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H8, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

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fide bond(s) [3]. These two chains are encoded by the same mRNA and are produced from a common translation product by proteolytic processing [4, 5]. We have suggested that hHGF is involved in liver regeneration because its plasma and serum levels are higher in patients with liver diseases than in healthy controls [6, 7]. Increase in the level of HGF in the blood of mice and induction of HGF mRNA in the liver and spleen of rats during liver regeneration after partial hepatectomy or administration of hepatotoxins have also been reported [8–10]. Moreover, similar factors have been purified from rat platelets [11] and rabbit and human plasma [12].

Recent studies have shown that HGF has mitogenic effects on various epithelial cells, such as keratinocytes and melanocytes, as well as hepatocytes [13, 14]. Moreover, the identity of hHGF with the scatter factor (SF) [15], originally identified as a chemotactic factor promoting epithelial cell motility and tumor cell invasion [for review, see 16], and a tumor cytotoxic factor, which has cytotoxic activity on some tumor cell lines [17], has been reported. Therefore, HGF is now recognized to have a broadspectrum of biological activities on different target cells. These different biological activities of HGF may be mediated by different intracellular signal cascades or by different cell surface receptors for HGF on these different target cells. Bottaro *et al.* [18] reported that the *c-met* proto-oncogene product, a membrane-spanning tyrosine kinase, is a receptor for HGF/SF, suggesting the possible involvement of tyrosine phosphorylation of the *c-met* protein in the mitogenic activity of HGF. Furthermore, we have recently reported the presence of two classes of high affinity receptors for hHGF on adult rat hepatocytes with dissociation constants of about 4.6 pM and 275 pM and calculated molecular masses of 246 kDa and 146 kDa, the latter being consistent with that of the *c-met* product [19]. The presence on rat hepatocytes of another receptor for HGF with a molecular mass of about 160–220 kDa has also been reported [20, 21]. These receptors may be important for the multifunctional properties of HGF, but to date, little is known about the mechanism(s) of HGF signal transduction.

To determine the role of protein phosphorylation reactions in hHGF signal transduction, we examined the effects of various inhibitors of protein kinases on the stimulation by hHGF of DNA synthesis of rat hepatocytes in primary culture. In this paper, we report that 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), a specific inhibitor of protein kinase C, potentiated the effect of hHGF on DNA synthesis of primary cultured rat hepatocytes. We also show that genistein, a specific inhibitor of tyrosine kinase, inhibited the action of hHGF possibly mediated by the *c-met* receptor.

MATERIALS AND METHODS

Materials. N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H8) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) were purchased from Medical and Biological Labs., Nagoya, Japan; 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) was from Biomol. Res. Lab., Plymouth Meeting, PA; genistein was from Funakoshi Co., Tokyo, Japan; [Me-^{3}H]thymidine (3.1 TBq/mmol) was from Amersham Japan Co., Tokyo, Japan. Recombinant hHGF (rhHGF) was prepared from the culture supernatant of Chinese hamster ovary (CHO) cells transfected with cDNA for hHGF as described previously [22, 23]. The structural properties and biological activity of rhHGF were similar to those of native hHGF purified from plasma of patients with fulminant hepatic failure [22, 23]. The concentration of rhHGF was determined by an ELISA for hHGF [7] assuming a molecular weight of 84,000 for this factor. Other reagents for preparation and primary culture of adult rat hepatocytes were as described previously [3].

Preparation of primary culture of adult rat hepatocytes and assay of DNA synthesis. Parenchymal hepatocytes were isolated from adult male Wistar rats weighing about 200 g by the method of Seglen [24] and cultured as monolayers at 37°C in a humidified atmosphere of 5 % CO_2 in air essentially as described by Tanaka *et al.* [25]. The cells were plated into Nunc 24-well plastic dishes (A/S Nunc, Rockiede, Denmark) at a density of 5 x 10^4 cells/0.2 ml/cm^2, and were cultured in Williams medium E supplemented with 5 % fetal calf serum, 5 nM dexamethazone, 100 units/ml of penicillin G and 100 μg/ml of streptomycin (basal medium) as described previously [3]. After 4 hr, the medium was changed to fresh basal medium and test samples were added to the basal medium 20 hr after seeding. After further incubation for 30 hr, [^{3}H]thymidine was added to the medium and culture was continued for 2 hr. Incorporation of [^{3}H]thymidine into DNA was determined as described previously [3].

RESULTS AND DISCUSSION

Inhibition of rhHGF-stimulated DNA synthesis of primary cultured rat hepatocytes by genistein.

Because of the identity of the HGF receptor with the *c-met* proto-oncogene product [18] and reports of stimulation of tyrosine phosphorylation of the *c-met* protein by HGF [18, 26], we first examined the effect of the tyrosine kinase inhibitor genistein on the rhHGF-stimulated DNA synthesis of hepatocytes in primary culture. This flavone compound specifically inhibits tyrosine protein kinase activities such as those of the epidermal growth factor receptor, $pp60^{src}$ and $pp110^{gag-fes}$, whereas it has little effect on the activities of several serine and threonine kinases including protein kinase C [27]. As shown in Fig.1, genistein inhibited the stimulatory effect of rhHGF on DNA synthesis of hepatocytes dose-dependently. The half-maximal effect was observed at about 15 μg/ml of genistein, which was in good agreement with the values reported by others for its inhibition of interleukin-1 stimulated PGE_2 production in mesangial cells [28], and of T cell activation and proliferation [29]. In the concentration range tested, genistein had no cytotoxic effect, as judged by examination of the morphology of control and genistein-treated cells by phase-contrast microscopy (data not shown).

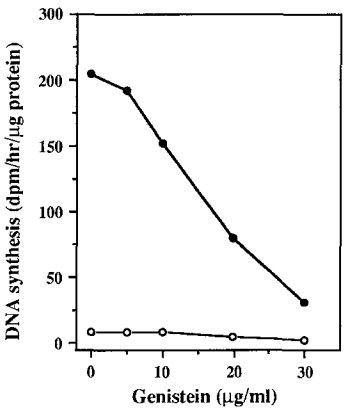

Figure 1. **Inhibition of rhHGF-stimulated DNA synthesis in primary cultured rat hepatocytes by genistein.** Hepatocytes were cultured for 30 hr with (●) or without (○) rhHGF (10 ng/ml) in the presence of the indicated concentrations of genistein. The rate of DNA synthesis was determined as described under "MATERIALS AND METHODS". Data are means for duplicate wells in three separate experiments.

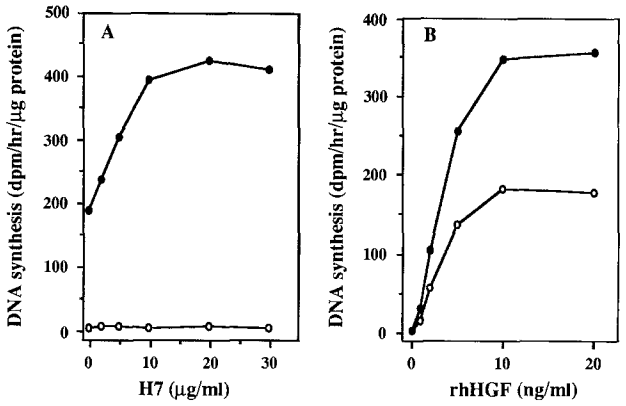

Figure 2. **Potentiation by H7 of the stimulatory effect of rhHGF on hepatocyte DNA synthesis.** *Panel A.* Hepatocytes were were cultured for 30 hr with (●) or without (○) rhHGF (10 ng/ml) in the presence of the indicated concentrations of H7. *Panel B.* Hepatocytes were cultured for 30 hr with (●) or without (○) H7 (20 μg/ml) in the presence of the indicated concentrations of rhHGF. The rate of DNA synthesis was determined as described for Fig. 1. Data are means for duplicate wells in two separate experiments.

Potentiation by H7 of the stimulatory effect of rhHGF on hepatocyte DNA synthesis.

Due to the selective effect of H7 on protein kinase C, this compound has been widely used in a variety of cellular systems to elucidate the role of protein kinase C in cellular functions, such as serotonin secretion from human platelets [30], and the lytic activity of cytotoxic T lymphocytes produced *in vivo* [31]. Therefore, we next examined the effect of H7 on the action of rhHGF. As shown in Fig. 2A, H7 potentiated the stimulatory effect of rhHGF on hepatocyte DNA synthesis dose-dependently, but itself had little effect on their DNA synthesis. H7 was effective at over 2 μg/ml and caused half-maximal stimulation at about 5 μg/ml (about 13 μM). These concentrations are consistent with those reported by others [30, 31]. At 20 μg/ml, H7 potentiated the effect of rhHGF over 2-fold. As shown in Fig. 2B, H7 increased DNA synthesis to about 2 times the maximum with rhHGF alone, suggesting that it potentiated the responsiveness rather than the sensitivity of hepatocytes to rhHGF.

Effects of inhibitors of cyclic nucleotide- and Ca^{++}/calmodulin-dependent protein kinases on the action of rhHGF.

H7 is a selective but not absolutely specific inhibitor of protein kinase C [32]. Therefore, we tested the effects of H8 and W7: H8 is a more potent inhibitor of cyclic nucleotide-dependent protein kinases (*Ki* = 0.5–1.2 μM) than of protein kinase C (*Ki* = 15 μM) [32], and W7 is a more potent inhibitor of Ca^{++}/calmodulin-dependent protein kinase (*Ki* = 31 μM) [33] than of protein kinase C (*Ki* = ca. 100 μM) [34]. As shown in Table 1, H8 had little effect on the basal or rhHGF-stimulated DNA synthesis of primary cultured hepatocytes when added at 5–20 μg/ml. On the other hand, W7 inhibited the effect of rhHGF on hepatocyte DNA synthesis about 60% at 10 μg/ml, and completely at 20 μg/ml (data not shown). However, it is uncertain whether the effect of W7 on the rhHGF action was specific, because at higher concentrations W7 also inhibited the basal activity for DNA synthesis of hepatocytes (Table 1).

The present results show that, as expected, tyrosine phosphorylation is involved in the stimulatory effect of hHGF on DNA synthesis of hepatocytes, and that the action of hHGF is negatively regulated by protein kinase C activation. These findings are consistent with a recent report by Gandino *et al.* [35] that protein kinase C activation negatively modulates the tyrosine kinase activity of the *c-met* proto-oncogene product.

Table 1. Effects of inhibitors of cyclic nucleotide– and Ca^{++}/calmodulin–dependent protein kinases on the stimulatory effect of rhHGF on hepatocyte DNA synthesis

Inhibitor	Concentration (μg/ml)	DNA synthesis (dpm/hr/μg protein)	
		–rhHGF	+rhHGF
None	–	7.7 ± 4.5	194.6 ± 14.9
H8	5	6.6 ± 0.27	153.0 ± 11.5
	20	7.5 ± 0.44	190.7 ± 10.2
W7	5	6.4 ± 0.3	191.6 ± 11.4
	10	2.5 ± 1.3	85.4 ± 16.0

Hepatocytes were cultured for 30 hr with or without rhHGF (10 ng/ml) in the presence of H8 or W7, and the rate of DNA synthesis was determined. Data are expressed as means ± S.D. for duplicate wells in two separate experiments.

Although the mechanism of negative regulation of the hHGF action by protein kinase C activation is not yet clear, we found that the tumor promoter, 12–O–tetradecanoyl–phorbol–13–acetate (TPA), which is known to be a powerful and specific activator for protein kinase C [36], had little effect on basal or rhHGF–stimulated DNA synthesis of primary cultured hepatocytes (data not shown), although it has been shown to inhibit the tyrosine kinase activity of *c–met* protein [35]. These results suggest that protein kinase C is fully activated in the HGF signal transduction pathway in hepatocytes and thus further activation by TPA does not modulate the action of HGF. In this connection, it is noteworthy that HGF stimulates production of inositol triphosphate and induces a prompt and transient elevation of cytoplasmic free calcium level in rat hepatocytes [37, 38]. It is also possible that the *c–met* receptor is downregulated by protein kinase C or that some novel H7–sensitive enzyme(s) that regulates the action of HGF is present in primary cultured rat hepatocytes. Further investigations are required to elucidate the mechanism of negative control of protein kinase C and determine whether this control is important in regulating the multifunctional effects of HGF.

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