Journal of Cellular Biochemistry 104:1465–1476 (2008)

Induction of Hepatocyte Growth Factor Expression by Maleic Acid in Human Fibroblasts Through MAPK Activation

Takahiro Motoki,[1] Yoshihiro Sugiura,[1] Yohsuke Matsumoto,[1] Tomoe Tsuji,[1] Satoshi Kubota,[2] Masaharu Takigawa,[2] and Eiichi Gohda[1]*

[1]Department of Immunochemistry, Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima-naka, Okayama 700-8530, Japan
[2]Derpartment of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikata-cho, Okayama 700-8525, Japan

Abstract Carboxylic acids have various biological activities and play critical roles in cellular metabolic pathways such as the tricarboxylic acid (TCA) cycle. It has been shown that some carboxylic acids induce cell proliferation and production of cytokines or growth factors. However, there have been no reports on effects of carboxylic acids on hepatocyte growth factor (HGF) expression. In this study, we found that only maleic acid among various carboxylic acids examined markedly induced HGF production from human dermal fibroblasts. Maleic acid also induced HGF production from human lung fibroblasts and neuroblastoma cells. The stimulatory effect was accompanied by upregulation of HGF gene expression. Increase in phosphorylation of extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) but not in phosphorylation of p38 was observed from 6 h and up to 24 h after maleic acid addition. The ERK kinase inhibitor PD98059 and the JNK inhibitor SP600125 potently inhibited maleic acid-induced HGF production, while the p38 inhibitor SB203580 did not significantly inhibit the production. The protein synthesis inhibitor cycloheximide completely inhibited upregulation of HGF mRNA induced by maleic acid but superinduced HGF mRNA expression upregulated by 12-*O*-tetradecanoylphorbol 13-acetate (TPA). These results suggest that maleic acid indirectly induced HGF expression from human dermal fibroblasts through activation of ERK and JNK and that de novo protein synthesis is required for maleic acid-induced upregulation of HGF mRNA. J. Cell. Biochem. 104: 1465–1476, 2008. © 2008 Wiley-Liss, Inc.

Key words: HGF; maleic acid; carboxylic acids; MAPK

Hepatocyte growth factor (HGF), also known as scatter factor, was originally discovered as a mitogenic factor of rat hepatocytes in primary culture [Michalopoulos et al., 1982; Strain et al., 1982; Nakamura et al., 1987, 1989; Gohda et al., 1988; Miyazawa et al., 1989; Zarnegar and Michalopoulos, 1989]. HGF is now recognized as a pleiotropic factor that functions as a mitogen, motogen, morphogen, and anti-tumorigenic factor acting on various types of cells [Michalopoulos and DeFrances, 1997]. These effects are exhibited through binding to its specific receptor c-Met, a protooncogene product [Bottaro et al., 1991; Naldini et al., 1991]. Many observations support the idea that HGF plays critical roles in developmental and regenerative events of the liver and other tissues. HGF deficiency and c-Met deficiency in mice cause embryonic lethality at embryonic days 13.5–16.5 [Schmidt et al., 1995; Uehara et al., 1995]. The knockout mice fail to develop a normal liver, muscle, and placenta. In rats partially hepatectomized or treated with carbon tetrachloride, plasma concentrations of HGF markedly increase prior to DNA synthesis and liver regeneration. The liver regeneration is impaired by pretreatment with anti-HGF monoclonal antibody or in conditional c-*met* mutant mice [Burr et al., 1998; Huh et al., 2004]. Treatment with HGF stimulates liver growth in normal and partially hepatectomized

*Correspondence to: Eiichi Gohda, Department of Immunochemistry, Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima-naka, Okayama 00-8530, Japan. E-mail: gohda@pheasant.pharm.okayama-u.ac.jp

Received 16 August 2007; Accepted 10 January 2008
DOI 10.1002/jcb.21724

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animals. The livers of HGF-transgenic mice recover in half the time needed for normal mice after partial hepatectomy.

HGF is mainly produced from mesenchymal cells such as fibroblasts and smooth muscle cells [Stoker et al., 1987; Rosen et al., 1989]. Expression of HGF in the liver occurs in Kupffer cells, sinusoidal endothelial cells, and Ito cells. HGF production is induced in response to activation of protein kinase A (PKA)- and protein kinase C (PKC)-mediated pathways and is also induced by interleukin-1 (IL-1), tumor necrosis factor-α, interferon-γ (IFN-γ), oncostatin-M, heparin, norepinephrine, okadaic acid, a scatter factor-inducing factor, and growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [Inaba et al., 1993; Matsumoto et al., 1993; Tamura et al., 1993; Gohda et al., 1994; Matsunaga et al., 1994; Rosen et al., 1994; Broten et al., 1999; Cohen et al., 2006]. Recently, we have shown that staurosporine, a broad-spectrum protein kinases inhibitor, and a combination of IL-1 and IFN-γ significantly induced the production of HGF and that tryptanthrin, an extract from *Polygonum tinctrium*, and ursodeoxycholic acid suppressed HGF induction [Yagi et al., 2003; Motoki et al., 2005; Takami et al., 2005a].

Carboxylic acids have various biological activities. Butyric acid, a short-chain monocarboxylic acid, is known to have an inhibitory activity against histone deacetylase and to affect cell proliferation and differentiation [Kruh, 1982]. Pyruvic acid upregulates albumin synthesis in hepatocytes [Boot, 1996]. The tricarboxylic acid (TCA) cycle, also known as the citric acid cycle, which acts as the final common pathway for the oxidation of carbohydrates, lipids, and proteins, consists of di- and tri-carboxylic acids. TCA cycle intermediates, especially oxaloacetic acid, stimulate the growth of hepatocytes in primary culture as potently as EGF [Li et al., 1993]. Malic acid and lactic acid induce angiogenesis through upregulation of vascular endothelial growth factor (VEGF) [Murray and Wilson, 2001]. Moreover, diethyl fumarate and dimethyl fumarate, which are converted to fumaric acid by cellular esterase, increase production of VEGF and erythropoietin through stabilization of hypoxia-inducible factor-1α (HIF-1α) [Koivunen et al., 2007]. These findings led us to investigate whether HGF expression is regulated by carboxylic acids. Among carboxylic acids, including members of the TCA cycle, we found that only maleic acid, a geometric isomer of fumaric acid, specifically induced HGF production in human fibroblasts. To the best of our knowledge, this is the first report on the ability of maleic acid to induce cytokine and growth factor production.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Citric, fumaric, L-(−)-malic, oxaloacetic and succinic acids, and sodium salts of maleic, α-ketoglutaric, and pyruvic acids were obtained from Sigma Chemical Co. (St. Louis, MO). GF109203X, PD98059, and SB203580 were purchased from Calbiochem (La Jolla, CA). SP600125 and wortmannin were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Antibodies against phospho-extracellular signal-regulated protein kinase (ERK), phospho-c-Jun N-terminal kinase (JNK), and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA), and antibodies against ERK, JNK, and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human HGF cDNA (*Bam*HI/*Kpn*I fragment, 2.2 kbp) was derived from plasmids obtained from Dr. Naomi Kitamura (Tokyo Institute of Technology, Yokohama, Japan). Other reagents were obtained as described previously [Matsunaga et al., 1994].

Cell Culture

Normal human dermal fibroblasts isolated from 200 individual neonatal donors were purchased from Cell Systems (Kirkland, WA) and used between 7th and 10th passages. The human embryonic lung fibroblast cell line MRC-5 and the human neuroblastoma cell line SK-N-SH were obtained from the American Type Culture Collection (Rockville, MD) and Riken BRC Cell Bank (Tsukuba, Japan), respectively. These cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, as described previously [Matsunaga et al., 1994].

Determination of HGF Level in Conditioned Media

Human dermal fibroblasts and MRC-5 cells were seeded onto 96-well culture plates (Nunc, Roskilde, Denmark) and cultured until they reached confluence. SK-N-SH cells were seeded onto 96-well culture plates at a density of 2.9×10^3 cells/0.17 ml/well and incubated for 24 h. The medium was then replaced with the same fresh medium containing maleic acid, other carboxylic acids and HGF inducers. All carboxylic acids were dissolved in phosphate-buffered saline (PBS) and neutralized with NaOH, if necessary, before addition to the fresh medium. The conditioned medium was collected after incubation for various periods and frozen at $-30°C$ until use in human HGF ELISA. The sandwich ELISA for human HGF was performed at room temperature as described previously [Tsubouchi et al., 1991], with slight modification [Motoki et al., 2005]. HGF levels were expressed as ng/mg cellular protein as described previously [Matsunaga et al., 1994]. Cellular protein was determined using the method of Lowry et al. as described previously [Matsunaga et al., 1994].

Northern Blot Analysis

The medium of confluent human dermal fibroblasts cultured in 9 cm dishes (Nunc) was replaced with the same fresh medium, and the cells were incubated for 24 h. Maleic acid was then added, and the cells were incubated for various periods. Total cellular RNA was isolated by RNA-Bee^{TM} (TEL-TEST, Friendswood, TX) according to the manufacturer's protocol. Northern blotting was carried out as described previously [Matsunaga et al., 1994]. Briefly, total RNA (10 μg) was denatured with 2.2 M formaldehyde and 50% formamide, mixed with ethidium bromide (50 μg/ml), and fractionated on 1% agarose gels containing 2.2 M formaldehyde. The gel was photographed by ultraviolet illumination. The RNA was then transferred from the gel to a Biodyne nylon membrane. The membrane-bound RNA was hybridized to a ^{32}P-labeled human HGF cDNA probe. After being washed, the membrane was exposed to an imaging plate at room temperature, and the plate was analyzed using a Bio-imaging analyzer, BAS-1800II (Fuji Photo Film Co., Tokyo, Japan). The human HGF cDNA fragment was labeled with [α-^{32}P]dCTP by the megaprime DNA labeling system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) according to the manufacturer's instructions.

Real-Time PCR Analysis

The medium of confluent human dermal fibroblasts cultured in 6-well plates (Nunc) was replaced with the same fresh medium, and the cells were incubated for 24 h. After treatment with cycloheximide (1 μg/ml) for 1 h, the cells were incubated for an additional 24 h with or without maleic acid or 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Total cellular RNA was isolated as described above. After treatment with DNase, first-strand cDNA synthesis from 0.5 μg RNA was performed using reverse transcriptase with oligo-dT primer. Real-time PCR was performed with a LightCycler (Roche, Indianapolis, IN) using SYBR® Green Realtime PCR Master Mix (Toyobo Co., Osaka, Japan) according to the manufacturer's protocol. The primers for human HGF were designed on the basis of the nucleotide sequence in a database (NCBI accession number: NM_000601). 28S rRNA was used as an internal control for normalization. The sequences of primers for HGF were as follows: forward, 5′-CAATAGCATGTCAAGTGGAG-3′; reverse, 5′-CTGTGTTCGTGTGGTATCAT-3′ (amplicon size: 180 bp). The sequences of primers for 28S rRNA were: forward, 5′-GTTCACCCACTAATAGGG-AACG-3′; reverse, 5′-GGATTCTGACTTAGA-GGCGTTC-3′ (amplicon size: 213 bp). The PCR conditions were as follows: HGF, 1 cycle of 95°C for 30 s followed by 60 cycles of 95°C for 5 s, 57°C for 0 s, and 72°C for 25 s; 28S rRNA, 1 cycle of 95°C for 30 s followed by 55 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 15 s. Relative cDNA copy numbers were computed based on the data with a serial dilution of a representative sample for each target gene. Melting curve analysis showed a single sharp peak for all samples.

Western Blot Analysis

The medium of confluent human dermal fibroblasts cultured in 24-well plates (Nunc) was replaced with the same fresh medium, and the cells were incubated for 24 h. Maleic acid was then added, and the cells were incubated for various periods. Whole cell lysates of human dermal fibroblasts treated with or not treated with maleic acid were prepared using SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% Bromophenol Blue). Lysates were boiled

for 10 min, briefly sonicated, and centrifuged. Western blotting was performed as described previously [Yagi et al., 2003]. Briefly, 10 μg of total protein was subjected to SDS–PAGE and electrophoretically transferred to Immobilon-P membranes (Millipore, Billerica, MA). After blocking with 5% skim milk, membranes were probed with anti-phospho-ERK, anti-ERK, anti-phospho-p38, anti-p38, anti-phospho-JNK, or anti-JNK antibody. Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulins (GE Healthcare Bio-Sciences Corp.) and detected with ECL Plus Western blotting detection reagents (GE Healthcare Bio-Sciences Corp.). Densitometric analysis of the bands was performed using a UMAX PowerLook II flat-bed scanner (UMAX Data Systems, Taipei, Taiwan) and Intelligent Quantifier software (Bio Image Systems, Ann Arbor, MI).

Animal Experiments

Male Wistar rats obtained from Japan SLC (Shizuoka, Japan) were housed in the animal facility of Okayama University at 22°C under a 12-h light–dark cycle and fed a commercial diet with water ad libitum. All experiments were performed according to the guidelines for animal experiments of Okayama University. Rats aged 6 weeks were intraperitoneally administered 100 or 300 mg/kg body weight of maleic acid in PBS or PBS alone for two consecutive days. They were anesthetized with diethyl ether 16 h after the second injection, and blood was collected through the inferior vein using a plastic syringe containing 3.1% sodium citrate solution (1:10 v/v) in order to prepare plasma or a plastic syringe without the citrate solution to prepare sera. After collecting the blood, the liver was perfused with 25 ml of saline through the portal vein. The liver was then homogenized in four volumes of 20 mM Tris-HCl buffer (pH 7.5), containing 2 M NaCl, 1 mM EDTA, and 0.1% Tween 80, with Polytron for 1 min at 0°C. The homogenate was centrifuged for 30 min at 19,000*g* at 4°C, and the resulting supernatant was used for the determination of HGF. Levels of HGF in the plasma and the liver extract of rats were measured by an ELISA kit (Institute of Immunology, Tokyo, Japan) according to the manufacturer's instructions. The activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by a kit (Wako Pure Chemical Industries).

Statistical Analysis

All results were expressed as means and SD of several independent experiments. The data were analyzed by Dunnett's test, Dunnett's T3, Tukey's test, or Student's *t*-test. *P* values less than 0.05 were regarded as significant.

RESULTS

Effect of Carboxylic Acids on HGF Production in Human Fibroblasts

We examined the effects of various carboxylic acids, including members in the TCA cycle, on HGF production from human dermal fibroblasts. Confluent human dermal fibroblasts were incubated in the presence or absence of eight carboxylic acids shown in Figure 1 for 72 h. The HGF concentrations in the conditioned media were determined by an ELISA. Carboxylic acids in the TCA cycle, citric, fumaric, α-ketoglutaric, L-(−)-malic, oxaloacetic, and succinic acids, had no effect on HGF production at 1–10 mM (Fig. 1A). Pyruvic acid did not increase HGF production either, but, interestingly, maleic acid markedly induced HGF production. Its effect peaked at 3 mM as expressed in both ng HGF/ml (data not shown) and in ng HGF/mg cellular protein (Fig. 1A). Maleic acid similarly increased HGF production in the subconfluent fibroblasts incubated for 120 h (data not shown). Cell viability determined by Trypan Blue-exclusion was not affected by maleic acid treatment even at 10 mM (data not shown). Maleic acid significantly promoted HGF production after a time lag of at least 12 h, and HGF levels were increased in a time-dependent manner thereafter (Fig. 1B). The effect of maleic acid on HGF production in other cell types was examined. HGF production in the human embryonic lung fibroblasts cell line MRC-5 and the human neuroblastoma cell line SK-N-SH was also increased in a dose-dependent manner by maleic acid (Fig. 2A,B).

Next, we compared the effect of maleic acid with the effects of the other agents known to induce HGF production in human dermal fibroblasts. The dose of each inducer used was the optimal dose. The effect of 3 mM maleic acid was comparable to the effects of growth factors such as EGF (10 ng/ml) and bFGF (10 ng/ml) and cholera toxin (10 pM) but less than that of 8-bromo-cAMP (1 mM) (Fig. 3). Combined effects

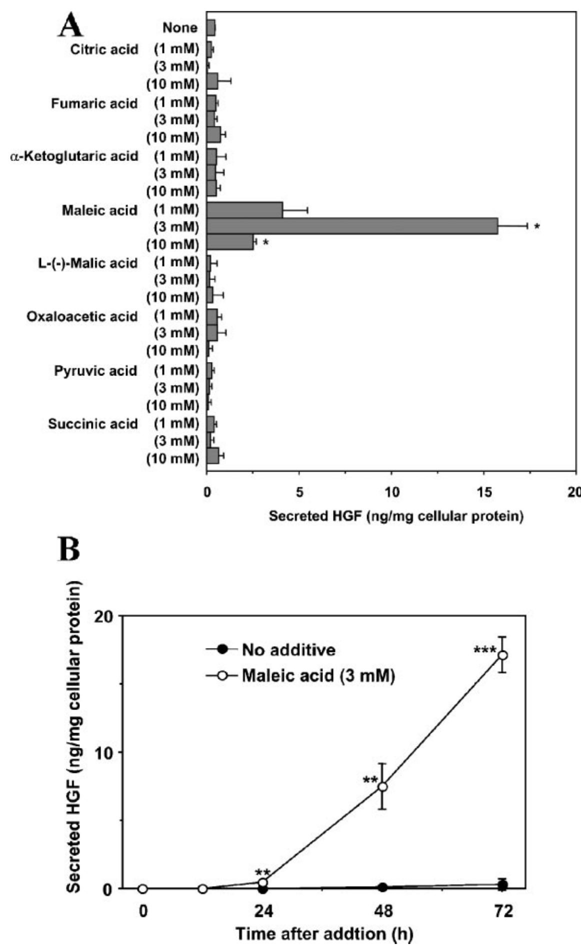

Fig. 1. Maleic acid-induced HGF production in human dermal fibroblasts. **A**: Confluent cells were incubated for 72 h with or without various carboxylic acids at the indicated concentrations. **B**: Confluent cells were incubated for the indicated periods with or without 3 mM maleic acid. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. Values that are significantly different from those of the control are indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Dunnett's T3 (A) or Student's *t*-test (B)).

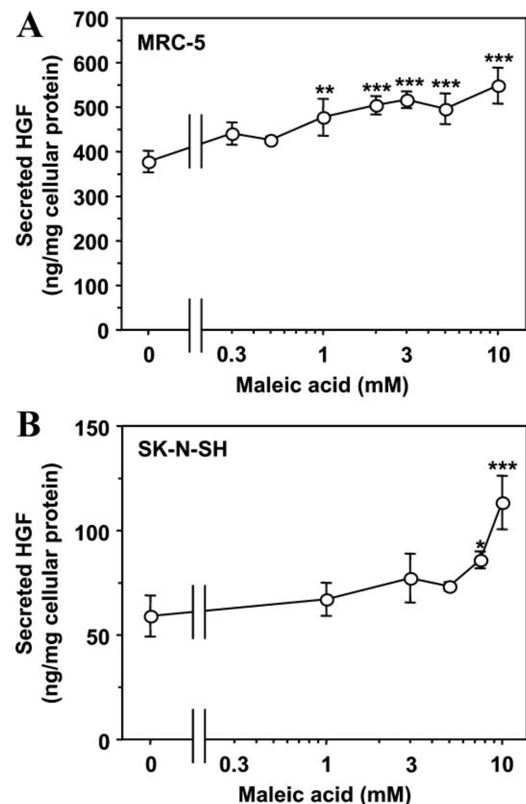

Fig. 2. Maleic acid-induced HGF production in human lung fibroblasts and neuroblastoma cells. **A**: Confluent MRC-5 human fetal lung fibroblasts were incubated for 48 h with or without the indicated concentrations of maleic acid. **B**: Twenty-four hours after plating the neuroblastoma SK-N-SH cells, the medium were changed to a fresh medium containing the indicated concentrations of maleic acid, and the cells were incubated for 48 h. The amount of HGF secreted into the medium was measured by an ELISA. The data are means ± SD of three independent experiments. Values that are significantly different from those of the control are indicated by $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Dunnett's *t*-test).

of maleic acid and growth factors were also examined using various concentrations of maleic acid and circulating or suboptimal concentrations of growth factors. HGF production was synergistically induced by 0.3–3 mM maleic acid and each of EGF (3 ng/ml), PDGF (10 ng/ml), and bFGF (3 ng/ml) (Table I). Synergistic effects of 0.5–3 mM maleic acid and EGF were most marked.

Upregulation of HGF mRNA Expression by Maleic Acid

To investigate whether maleic acid affects HGF mRNA expression, total RNA was isolated from human dermal fibroblasts treated with or not treated with maleic acid, and Northern blot analysis was performed. As shown in Figure 4A and Table II, the levels of HGF mRNA expression markedly increased from 20 h after addition of maleic acid, and an approximately four-fold increase was observed at 40 h compared to that of the control. Maleic acid-induced upregulation of HGF mRNA expression was potently inhibited by the treatment with actinomycin D (Fig. 4B).

Involvement of Mitogen-Activated Protein Kinase (MAPK) in HGF Production Induced by Maleic Acid

Recently, we demonstrated that all-*trans* retinoic acid (ATRA) is a bifunctional modulator

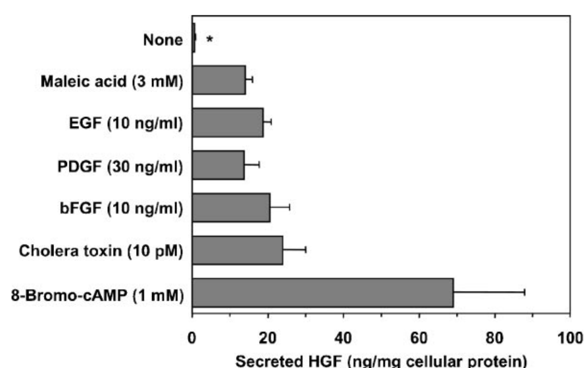

Fig. 3. Comparison of HGF production induced by maleic acid with HGF production promoted by other inducers in human dermal fibroblasts. Confluent cells were incubated for 72 h with or without the indicated concentrations of maleic acid and other HGF inducers. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. Values that are significantly different from those of 3 mM maleic acid are indicated by $^{*}P < 0.05$ (Dunnett's T3).

of HGF production; HGF production via PKC or MAPK is inhibited, whereas HGF production via PKA is increased [Takami et al., 2005b]. Therefore, to reveal what signaling pathway is involved in maleic acid-induced HGF production, we investigated the effect of ATRA on maleic acid-induced HGF production. ATRA reduced HGF production by 50% at 1 μM and by 80–90% at 10 μM compared with that induced by maleic acid alone (data not shown). Then we examined effects of selective signal transduction inhibitors on HGF induction by maleic acid. As shown in Figure 5, the MEK1/2 inhibitor PD98059, which inhibits phosphorylation of ERK, almost completely suppressed the HGF induction. Moreover, the JNK inhibitor SP600125 also potently inhibited the induction. Maleic acid-induced HGF production was moderately suppressed by the PKC inhibitor GF109203X but was not significantly suppressed by the p38 inhibitor SB203580 or the phosphatidylinositol 3-kinase inhibitor wortmannin.

Effects of Maleic Acid on Phosphorylation of MAPKs

Since it was suggested that ERK phosphorylation plays a critical role in HGF production induced by maleic acid, we investigated the effect of maleic acid on ERK phosphorylation by Western blotting. When the cells were stimulated with growth factors (e.g., EGF) or TPA, a PKC activator, ERK phosphorylation transiently occurred within a few minutes and thereafter rapidly declined to the control level (data not shown). In contrast, the levels of ERK phosphorylation in cells treated with maleic acid did not increase within 2 h after addition of maleic acid (data not shown). Thus, we examined whether long-term treatment with maleic acid could induce ERK phosphorylation. As shown in Figure 6 and Table III, ERK phosphorylation gradually increased from 6 h after addition of maleic acid. The increase in phosphorylation level was still observed at 24 h, whereas the cells cultured with the medium alone showed no change at any times examined. We also investigated the effects of maleic acid on phosphorylation of the other kinds of MAPK, JNK, and p38. Maleic acid treatment significantly increased the phosphorylation level of JNK with a time course similar to that of ERK phosphorylation but not phosphorylation of p38 (Fig. 6 and Table III).

TABLE I. Combined Effects of Maleic Acid and Growth Factors on HGF Production in Human Dermal Fibroblasts

	Secreted HGF (ng/mg cellular protein)			
Maleic acid (mM)	None	EGF (3 ng/ml)	PDGF (10 ng/ml)	bFGF (3 ng/ml)
0	0.5 ± 0.2	10.1 ± 0.5	11.7 ± 2.2	13.2 ± 1.2
0.3	1.0 ± 0.2	$20.8 \pm 1.7^{###}$	$18.9 \pm 2.1^{##}$	$16.4 \pm 1.2^{#}$
0.5	$1.6 \pm 0.1^{*}$	$32.9 \pm 1.9^{***,###}$	$25.0 \pm 5.0^{##}$	$20.5 \pm 1.9^{*,##}$
1	3.2 ± 0.9	$53.6 \pm 5.7^{***,###}$	$34.6 \pm 3.1^{**,###}$	$34.1 \pm 3.6^{***,###}$
2	$9.2 \pm 1.3^{*}$	$64.7 \pm 5.5^{***,###}$	$38.4 \pm 0.1^{*,###}$	$50.7 \pm 5.0^{***,###}$
3	$12.9 \pm 0.5^{***}$	$55.5 \pm 6.0^{***,###}$	$32.1 \pm 2.5^{**,##}$	$48.4 \pm 1.7^{***,###}$
5	$9.8 \pm 1.0^{*}$	$30.4 \pm 8.9^{***}$	20.5 ± 2.3	$27.2 \pm 1.7^{***,##}$

Confluent cells were incubated for 72 h with or without the indicated concentrations of maleic acid plus each of EGF, PDGF, and bFGF. The amount of HGF secreted into the medium was measured by an ELISA. The data are means ± SD of three independent experiments. Values that are significantly different from those of untreated control or growth factor alone are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Dunnett's *t*-test or Dunnett's T3). Synergistic induction of HGF production is indicated by $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ (two-way ANOVA).

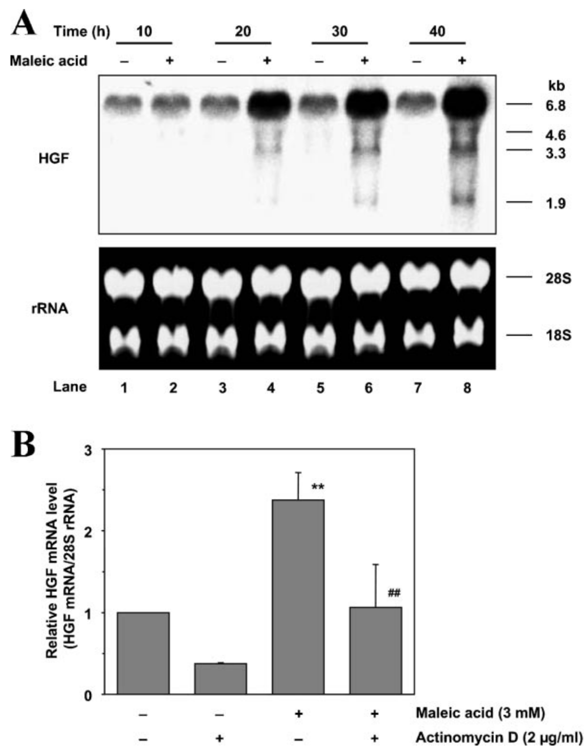

Fig. 4. Upregulation of HGF gene expression by maleic acid and its inhibition by actinomycin D in human dermal fibroblasts. **A**: Confluent cells were incubated for the indicated periods with or without 3 mM maleic acid. Total RNA was then isolated and Northern blotted using ^{32}P-labeled cDNA probes for human HGF. Autoradiograms and fluorescence photographs are representatives of three independent experiments. **B**: Confluent cells were incubated for 17 h with or without 3 mM maleic acid followed by incubation for an additional 7 h with or without 2 µg/ml of actinomycin D. Total RNA was then isolated and reverse-transcribed. The expression levels of HGF mRNA and 28S rRNA were measured by real-time PCR. Results are expressed as relative levels to the value of untreated cells after being normalized to 28S rRNA levels. The data are means of three independent experiments. Bars indicate SD. Values that are significantly different from those of untreated cells are indicated by $^{**}P < 0.01$ (Tukey's test). $^{\#\#}P < 0.01$, as compared with the values of maleic acid alone (Tukey's test).

Requirement of De Novo Protein Synthesis for Maleic Acid-Induced HGF Expression

Since the phosphorylation of ERK and JNK was slow and sustained even at 24 h and since HGF mRNA expression markedly increased from 20 h after maleic acid stimulation, we examined the requirement of de novo protein synthesis in maleic acid-induced HGF gene expression using the protein synthesis inhibitor cycloheximide. Confluent cells were treated with or not treated with cycloheximide prior to addition of maleic acid. The level of HGF mRNA expression was then determined by quantitative real-time PCR. Treatment with cycloheximide completely inhibited HGF mRNA upregulation induced by maleic acid, whereas it markedly augmented HGF mRNA expression induced by TPA (Fig. 7).

Increase in Plasma and Hepatic HGF Levels in Rats Treated With Maleic Acid

In order to test the in vivo effect of maleic acid on HGF production rats were intraperitoneally administered maleic acid (100 or 300 mg/kg) or a vehicle (PBS) for consecutive 2 days. Both the plasma and hepatic HGF levels were significantly increased by the treatment with maleic acid (Fig. 8). Levels of serum ALT and AST did not change by the treatment (data not shown).

DISCUSSION

The data presented in this report show that among eight carboxylic acids only maleic acid markedly induced HGF production from human dermal fibroblasts. Although maleic acid alone was effective at millimolar concentrations, in combination of growth factors it induced HGF production at submillimolar concentrations. Moreover, the injection of maleic acid to rats increased plasma and hepatic HGF levels. The reason why HGF production is specifically responsive to maleic acid is unknown. Since fumaric acid, a geometrical isomer of maleic

TABLE II. HGF Gene Expression Upregulated by Maleic Acid in Human Dermal Fibroblasts

	Relative HGF mRNA level (HGF mRNA/28S rRNA)			
	10 h	20 h	30 h	40 h
None	1.00 ± 0.00	1.19 ± 0.20	1.06 ± 0.38	1.38 ± 0.14
Maleic acid (3 mM)	1.14 ± 0.07*	2.71 ± 0.71*	4.09 ± 0.65**	5.31 ± 1.39**

Confluent cells were incubated for the indicated periods with or without maleic acid, and Northern blotting was performed. The signal intensity of the 6.8-kb HGF mRNA was normalized to the fluorescence intensity of the 28S rRNA and expressed as fold-change relative to the value of cells incubated in the medium alone for 10 h. The data are means ± SD of three independent experiments. Values that are significantly different from those of the respective control are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's t-test).

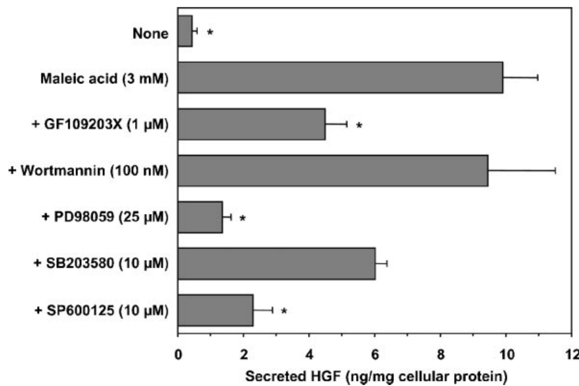

Fig. 5. Effects of signal transduction inhibitors on maleic acid-induced HGF production from human dermal fibroblasts. Confluent cells were preincubated for 2 h with or without the indicated inhibitors of signal transduction followed by incubation for an additional 72 h with or without 3 mM maleic acid in the presence or absence of the signal transduction inhibitors. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. Values that are significantly different from those of the maleic acid alone are indicated by $*P < 0.05$ (Dunnett's T3).

acid, had no effect on HGF production, the configuration of two carboxyl groups may be important for this effect. Moreover, maleic acid also stimulated HGF production from the human lung fibroblast line MRC-5 and the neuroblastoma cell line SK-N-SH, suggesting that the effect is not specific for cell type. It is assumed that maleic acid is devoid of pleiotropic effects, and the acid is therefore occasionally used to obtain the corresponding salts of basic pharmaceuticals such as anti-histamines. To our knowledge, this is the first report on the stimulatory effect of maleic acid on cytokine and growth factor production. HGF is mitogenic to hepatocytes, other epithelial cells and endothelial cells. Injection of HGF is effective for treating animal models of chronic hepatic and renal diseases such as hepatic and renal fibrosis and liver cirrhosis [Yasuda et al., 1996]. Thus, HGF inducers, especially low-molecular-weight ones, may also be useful as therapeutic agents for these diseases. Studies are under way to determine the in vivo beneficial effect of maleic acid on hepatic fibrosis through the stimulation of HGF production.

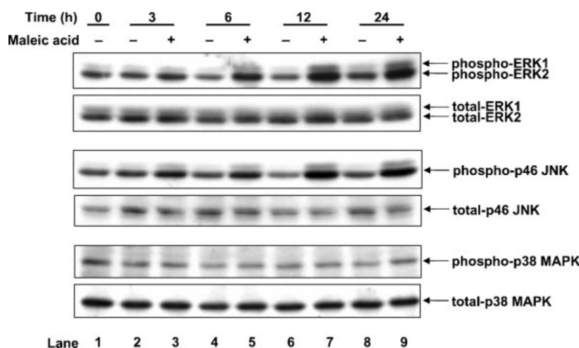

Fig. 6. Upregulation of phosphorylation of ERK and JNK by maleic acid in human dermal fibroblasts. Confluent cells were incubated for the indicated periods with or without 3 mM maleic acid. Equal amounts of cell extracts (10 μg) were subjected to SDS–PAGE and immunoblotted with specific antibodies against phosphorylated and total ERK, JNK, and p38. Immunoblots are representative of at least three independent experiments.

Administration of high doses of maleic acid to experimental animals induces a rapid, reversible and complex dysfunction of proximal renal tubules resembling Fanconi syndrome, which is characterized by various combinations of renal tubular transport dysfunction involving amino acids, glucose, protein, and other substances [Verani et al., 1982]. The biochemical mechanism responsible for maleic acid-induced alterations in renal tubular function is not completely understood, but it has been suggested that a decrease in Na^+, K^+-ATPase in proximal renal tubules underlies the many consequences of this model of Fanconi syndrome [Eiam-ong et al., 1995]. Under our experimental conditions, however, maleic acid did not show any effect on cellular viability.

To date no information on the effect of maleic acid on intracellular signaling pathways has been provided. Since Roth et al. [1978] demonstrated that maleic acid penetrated into renal tubules by simple diffusion, maleic acid might get into the cells. Our results showed that maleic acid markedly upregulated phosphorylation of ERK1/2 and JNK and that maleic acid-induced HGF production was potently inhibited by PD98059 and SP600125, which specifically block ERK1/2 and JNK signaling pathways, respectively. On the other hand, the p38 specific inhibitor SB203580 was less effective, and phosphorylation of p38 was not significantly influenced by maleic acid. These results suggest that ERK1/2 and JNK are important molecules involved in the inducing effect of maleic acid. Since there was a time lag of at least 2 h before upregulation of ERK and JNK phosphorylation after maleic acid addition, maleic acid probably increased both phosphorylation levels indirectly through a mechanism by which an unidentified factor(s) was induced or activated. This hypothesis is supported by results showing that maleic acid-induced HGF mRNA expres-

TABLE III. Phosphorylation of ERK and JNK Upregulated by Maleic Acid in Human Dermal Fibroblasts

	Relative phosphorylation level					
	ERK1/2		JNK		p38	
Time (h)	None	Maleic acid	None	Maleic acid	None	Maleic acid
3	0.90 ± 0.37	1.62 ± 0.56	0.80 ± 0.16	1.07 ± 0.48	0.63 ± 0.19	0.88 ± 0.29
6	0.64 ± 0.25	1.77 ± 0.35**	0.54 ± 0.18	1.11 ± 0.10**	0.48 ± 0.18	0.64 ± 0.07
12	0.64 ± 0.32	2.44 ± 0.64**	0.55 ± 0.07	1.88 ± 0.36**	0.80 ± 0.12	0.84 ± 0.14
24	0.81 ± 0.50	2.73 ± 0.71**	0.63 ± 0.16	2.10 ± 0.53*	1.19 ± 0.68	1.44 ± 0.52

Confluent cells were incubated for the indicated periods with or without 3 mM maleic acid, and Western blotting was performed. The signal intensity of the phosphorylated bands was normalized to that of the total bands and expressed as fold-change relative to the value of 0-h cells. The data are means ± SD of at least three independent experiments. Values that are significantly different from those of the respective control are indicated by $*P < 0.05$, $**P < 0.01$ (Student's *t*-test).

sion was almost completely inhibited by the de novo protein synthesis inhibitor cycloheximide. In contrast to maleic acid-induced HGF mRNA expression, HGF mRNA expression upregulated by TPA was superinduced in the presence of cycloheximide. This suggests that de novo protein synthesis is not required for the induction of HGF mRNA by TPA and that a labile protein might negatively regulate HGF gene expression at either the level of transcription or mRNA degradation. Superinduction of mRNA expression of certain cytokines and protooncogenes by the combination of TPA and cycloheximide has been shown as well [Mitchell et al., 1986].

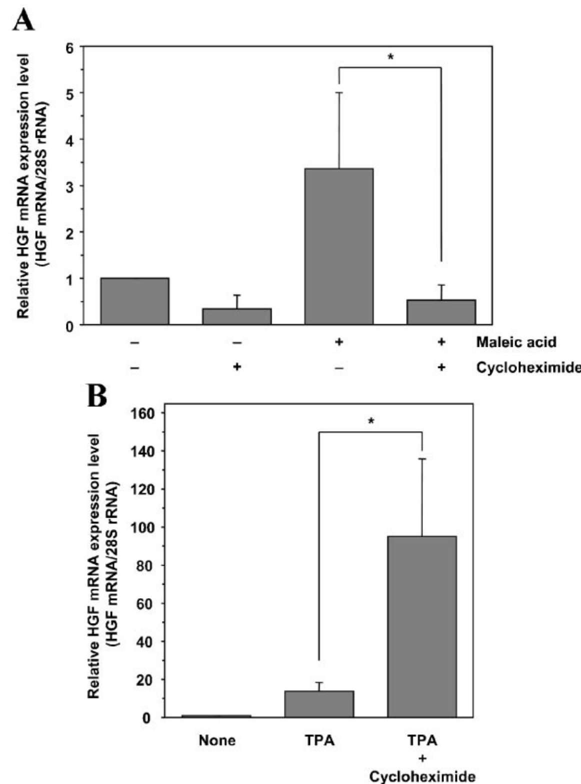

Fig. 7. Inhibition of maleic acid-induced HGF mRNA expression by cycloheximide in human dermal fibroblasts. Confluent cells were preincubated for 1 h with or without cycloheximide (1 μg/ml) followed by incubation for an additional 24 h with or without 3 mM maleic acid or 30 nM TPA. Total RNA was then isolated and reverse-transcribed. The expression levels of HGF mRNA and 28S rRNA were measured by real-time PCR. Results are expressed as relative levels to the value of untreated cells after being normalized to the 28S rRNA levels. The data are means of three independent experiments. Bars indicate SD. Values that are significantly different from those of cycloheximide-untreated cells are indicated by $*P < 0.05$ (Student's *t*-test).

ERK1/2 and JNK activate various transcription factors such as AP-1 (Fos/Jun heterodimers or Jun/Jun homodimers), Elk-1, and c-Myc. Activated transcription factors bind to their elements on target gene promoters and regulate transcription of the genes. The nucleotide sequences of mouse, rat, and human HGF promoter regions have been demonstrated [Miyazawa et al., 1991; Liu et al., 1994]. In the mouse HGF promoter region, previous studies have revealed the presence of *cis*-acting regulatory elements that respond to various transcription factors, including signal transducer and activator of transcription (Stat)-3, Sp-1, and CCAAT/enhancer binding protein (C/EBP)-β and -δ [Jiang and Zarnegar, 1997; Jiang et al., 1997; Wojcik et al., 2006]. Although these transcription factors have been shown to regulate mouse HGF promoter activity, some regulatory elements are not conserved in the human HGF promoter region, and it remains to be elucidated whether those factors also regulate human HGF promoter activity. In this regard, Tomida and Saito [2004] have recently shown that leukemia inhibitory factor (LIF) stimulates HGF gene expression in human embryonic kidney 293 cells and melanoma SEKI cells by activation of the HGF promoter through Stat3 activation. However, they showed that LIF had

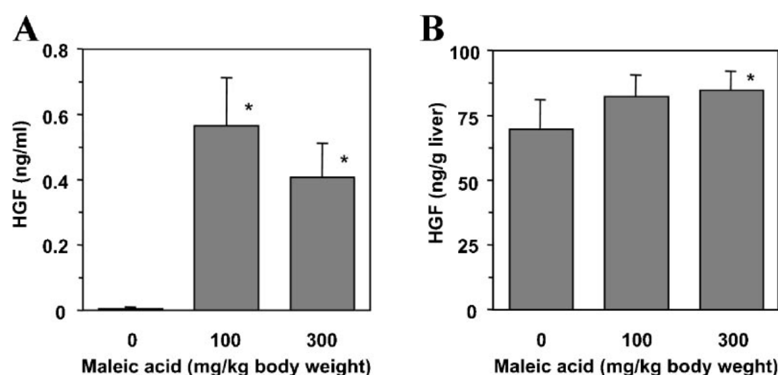

Fig. 8. Increase in hepatic and circulating HGF levels in rats by the treatment with maleic acid. Rats were intraperitoneally administered 100 or 300 mg/kg body weight of maleic acid in PBS or PBS alone for two consecutive days. Sixteen hours later, plasma (**A**) and hepatic (**B**) HGF levels were measured. The data are means ± SD of 3 (A) or 6 (B) rats. Values that are significantly different from those of control are indicated by $*P < 0.05$ (Dunnett's T3 (A) or Dunnett's *t*-test (B)).

no effect on HGF expression both at the mRNA level and protein level in MRC-5 cells [Tomida and Saito, 2004], suggesting that the effects of cytokines or growth factors on HGF expression are cell type-specific. Our results of in silico analysis using AliBaba 2.1 (http://darwin.nmsu.edu/~molb470/fall2003/projects/solorz/alibaba_2_1.htm) and TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/tfsearch.html) predict that there are binding elements of C/EBPβ, NF-κB, or AP-1 in the human HGF promoter region. Since recent studies have indicated involvement of MAPK pathways in C/EBPβ, NF-κB, or Stat3 activation [Wierenga et al., 2003; Larsen et al., 2005; Marcinkowska et al., 2006], it is possible that maleic acid-induced HGF expression is mediated through activation of these transcription factors via MAPK signaling. In fact, phosphorylation and expression of c-Jun and expression of ~50 kDa phosphorylated active isoform of C/EBPβ were increased by maleic acid treatment with the similar time-course to ERK and JNK phosphorylation (our unpublished data). However, the possibility that maleic acid-induced HGF expression is mediated by post-transcriptional mRNA stabilization cannot be ruled out. In fact, TGF-β1 has been shown to regulate HGF gene expression as a consequence of affecting HGF mRNA stability [Harrison et al., 2000]. Further studies are necessary to elucidate the mechanism by which human HGF gene expression is induced by maleic acid.

In conclusion, this study showed that the dicarboxylic acid maleic acid significantly induced HGF expression in different cell types. The results suggested that the effect is mediated through activation of MAPKs, especially ERK and JNK, and that de novo protein synthesis is required for maleic acid-induced upregulation of HGF mRNA expression: the carboxylic acid indirectly upregulated HGF mRNA expression. To the best of our knowledge, this is the first report on a stimulatory effect of maleic acid on expression of growth factors and cytokines.

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