Induction of hepatocyte growth factor production in human dermal fibroblasts and their proliferation by the extract of bitter melon pulp

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Abstract

Hepatocyte growth factor (HGF) is useful as a potential therapeutic agent for hepatic and renal fibrosis and cardiovascular diseases through inducing proliferation of epithelial and endothelial cells. HGF inducers may also be useful as therapeutic agents for these diseases. However, there have been no reports on induction of HGF production by plant extracts or juices. An extract of bitter melon (*Momordica charantia* L.) pulp markedly induced HGF production. There was a time lag of 72 h before induction of HGF production after the extract addition. Its stimulatory effect was accompanied by upregulation of HGF gene expression. Increases in mitogen-activated protein kinases (MAPKs) were observed from 72 h after the extract addition. Inhibitors of MAPKs suppressed the extract-induced HGF production. The extract also stimulated cell proliferation. Both activities for induction of HGF production and cell proliferation were eluted together in a single peak with 14 000 Da on gel filtration. The results indicate that bitter melon pulp extract induced HGF production and cell proliferation of human dermal fibroblasts and suggest that activation of MAPKs is involved in the HGF induction. Our findings suggest potential usefulness of the extract for tissue regeneration and provide an insight into the molecular mechanism underlying the wound-healing property of bitter melon.

Keywords: Hepatocyte growth factor; Bitter melon; Extracellular signal-regulated kinase; Cell proliferation; Dermal fibroblast

1. Introduction

Hepatocyte growth factor (HGF), also known as scatter factor, was originally discovered as a mitogenic factor of rat hepatocytes in primary culture [1-5]. HGF is now recognized as a pleiotropic factor that functions as a mitogen, motogen, morphogen, anti-apoptotic factor and angiogenic factor acting on various types of cells [6,7]. Based on these actions, HGF has been shown to play critical roles in developmental and regenerative events of the liver and other tissues. Deficiency of HGF or its receptor, c-Met, causes lethality at embryonic days 13.5-16.5 characterized by failure to develop a normal liver, muscle and placenta [8-10]. In addition, liver regeneration is impaired by pretreatment with an anti-HGF monoclonal antibody or in conditional HGF or c-*met* mutant mice [11,12]. Treatment with HGF stimulates liver growth in normal and partially hepatectomized animals, and injection of HGF is effective for treating animal models of chronic hepatic and renal diseases such as hepatic and renal fibrosis and liver cirrhosis [13-15]. Recent studies have also demonstrated the potential application of HGF for treating cardiovascular diseases such as peripheral vascular disease, myocardial infarction and cerebrovascular disease [16]. Moreover, HGF is capable of stimulating migration and proliferation of keratinocytes and thus has been suggested to be involved in cutaneous physiology and wound healing [17,18]. HGF inducers may also be useful as therapeutic agents for these diseases.

HGF is mainly produced from mesenchymal cells such as fibroblasts and smooth muscle cells [19,20]. HGF production is induced in response to activation of PKA- and PKC-mediated pathways and is also induced by interleukin-1, tumor necrosis factor-α, interferon-γ, oncostatin-M, heparin, norepinephrine, staurosporine, a scatter factor-inducing factor, and growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) [21-30]. To date, however, there have been no reports on the effects of plant extracts or juices on HGF production.

Bitter melon (*Momordica charantia* L.), a member of the Cucurbitaceae family, is a plant that is cultivated throughout the world for use as a vegetable. The unripe fruit has also been used in developing countries as traditional medicine for microbial infections, sluggish digestion and intestinal gas, inflammation, fever reduction, and wound healing [31]. Recent studies on its

pharmacological properties have shown several biological activities of bitter melon, including antidiabetic, antilipidemic, antibacterial, antiviral and anticancer activities [32-36]. Immunosuppressive and immunostimulating activities of bitter melon or its constituents have also been reported [37]. Prompted by the results of studies showing that the bitter melon extract augmentated cytokine secretion, we examined effects of the extract on HGF production. Here we describe that the extract of bitter melon pulp potently promoted HGF production and proliferation of human dermal fibroblasts. Our results suggest that the effect on HGF production is mediated through activation of mitogen-activated protein kinases (MAPKs), especially extracellular signal-regulated kinase (ERK).

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). GF109203X, PD98059 and SB203580 were purchased from Calbiochem (La Jolla, CA, USA). SP600125 and wortmannin were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies against phospho-ERK, phospho-c-Jun N-terminal kinase (JNK) and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA), and antibodies against ERK, JNK and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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 [26].

2.2. Cell culture

Human dermal fibroblasts derived from 200 individual neonatal donors (Cell Systems, Kirkland, WA, USA) and from a 3-day-old male baby (The Riken Cell Bank, Tsukuba, Japan) and the human embryonic lung fibroblast cell line MRC-5 (The American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (DMEM-10) at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air as described previously [21].

2.3. Preparation of bitter melon extract

Unripe bitter melons (Sadowara Shironaga) were cultivated in Miyazaki Agricultural Research Institute (Miyazaki, Japan). The pulp of bitter melons was sliced, freeze-dried and powdered. The pulp powder was mixed with 10 vol of phosphate-buffered saline (PBS) for 1.5 h under continuous stirring at 40ºC followed by centrifugation. The supernatant was collected by decantation, and the resulting precipitate was mixed again with 5 vol of PBS for another 1.5 h under continuous stirring at 40ºC. After centrifugation, the supernatants were combined, filtered through a membrane filter and stored at –80ºC until use. The concentration (w/v) of the extract was calculated by subtracting the weight of lyophilized PBS of the same volume from the weight of the lyophilized PBS extract.

2.4. Gel filtration of bitter melon pulp extract

The extract of bitter melon pulp (2 ml) was applied to a column of Sephadex G-50 (1.5 × 30 cm) equilibrated with PBS and eluted with the same buffer at 4ºC. Fractions of 1 ml were collected.

2.5. Determination of HGF production

The medium of confluent human dermal fibroblasts and MRC-5 cells cultured in 96-well

plates (Nunc, Roskilde, Denmark) was replaced with a fresh medium (DMEM-10) containing various amounts of the bitter melon pulp extract. The conditioned medium was collected after being incubated for various periods and was frozen at –30ºC for a human HGF ELISA. The sandwich ELISA for human HGF was performed at room temperature as described previously [38], with slight modification [39]. HGF levels were expressed as ng/ml or ng/mg of cellular protein as described previously [26].

2.6. MTT assay

Confluent human dermal fibroblasts grown in 96-well plates (Nunc) were incubated for 120 h with the extract of bitter melon pulp as described in the previous section. The medium was then replaced with 100 µl of the fresh medium (DMEM-10), and the cultures were incubated for 1 h. MTT assay was then performed as described previously [40].

2.7. Northern blot analysis

The medium of confluent human dermal fibroblasts grown in 90-mm dishes (Nunc) was replaced with the fresh medium (DMEM-10) containing the extract of bitter melon pulp, and the cells were incubated for 88 h. Total RNA was then isolated from the cells using RNA-Bee (TEL-TEST, Friendswoods, TX, USA). Northern blotting was performed as described previously [26]. The signal intensity of the 6.4-kb HGF mRNA band in the autoradiograms was normalized to the fluorescence intensity of the 28S rRNA band.

2.8. Western blot analysis

The medium of confluent human dermal fibroblasts grown in 24-well plates (Nunc) was replaced with the same fresh medium (DMEM-10), and the cells were incubated for about 18 h. The extract of bitter melon pulp was added without a medium change. After being incubated for

an appropriate period, the cells were washed four times with ice-cold PBS and lysed by adding 40 µl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% Bromophenol Blue). Lysates were boiled for 10 min, briefly sonicated, and centrifuged. Protein in extracts was determined by a modification of the method of Lowry et al. [41]. Equivalent protein aliquots were separated by 10% SDS- polyacrylamide gel electrophoresis (PAGE) and transfered electrophoretically to Immobilon-P membranes (Millipore, Billerica, MA, USA) as reported previously [42]. Blots were probed with various antibodies, incubated with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulins (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and detected with ECL Plus Western blotting detection reagents (GE Healthcare Bio-Sciences Corp.).

2.9. Determination of DNA synthesis

The medium of confluent human dermal fibroblasts cultured in 96-well plates (Nunc) was replaced with the fresh medium (DMEM-10) containing the extract of bitter melon pulp, and the cells were incubated for 78 h. The cells were then pulse-labeled with [^{3}H]thymidine (0.5 µCi/well, 2.5 Ci/ mmol) for 24 h, trypsinized, and harvested on glass-fiber filters using a cell harvester as described previously [43]. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter.

2.10. Statistical analysis

Results are expressed as means and SD of several independent experiments. Data in two groups were analyzed by Student's *t*-test or Welch's *t*-test. Multiple comparison of the data was done by ANOVA followed by Dunnett's *t*-test, Dunnett's T3 test or Tukey's test. *P* values less than 0.05 were regarded as significant.

3. Results

3.1. Induction of HGF production by the extract of bitter melon pulp

Human dermal fibroblasts derived from 200 neonatal donors were incubated for 5 days with the extract of bitter melon pulp, and HGF secreted from the cells was then determined by an HGF ELISA. The extract dose-dependently enhanced HGF production expressed as either ng/ml (Fig. 1A) or ng/mg of cellular protein (Fig. 1B). The HGF-inducing activity of the extracts prepared from some lots of bitter melon pulp decreased at concentrations of more than 0.85 mg/ml, but the extract exhibited dose-dependent HGF induction similar to that shown in Fig. 1A after being incubated for 10 min at 70ºC (data not shown, see below). HGF production in human dermal fibroblasts derived from a 3-day-old baby and in the human embryonic lung fibroblast cell line MRC-5, which produces a large amount of HGF, was also significantly increased by the extract (Fig. 1C and 1D). The time course of HGF production induced by the extract of bitter melon pulp is shown in Figure 1E. There was a time lag of at least 72 h before upregulation of HGF production after the addition of the extract, whereas the time lag before EGF-induced HGF production was 24 h. These results suggest that the extract increased HGF production through an indirect mechanism.

Fig. 1

Next, we compared the effect of the extract of bitter melon pulp with the effects of other agents known to induce HGF production in human dermal fibroblasts [25,26]. The effect of the extract of bitter melon pulp was stronger than the effects of growth factors such as EGF (3 ng/ml) and PDGF (10 ng/ml) but weaker than the effect of cholera toxin (1 pM) (Fig. 2).

Fig. 2

3.2. Upregulation of HGF mRNA expression by the extract of bitter melon pulp

To investigate whether the extract of bitter melon pulp affects HGF mRNA expression, total RNA was isolated from human dermal fibroblasts incubated for 88 h with or without the extract, and Northern blot analysis was performed. As shown in Figure 3A and 3B, the level of HGF mRNA expression in cells treated with the extract increased by 60% compared to that of the control.

Since induction of HGF production by the extract of bitter melon pulp was slow, we examined the requirement of *de novo* protein synthesis in the extract-induced HGF gene expression using the protein synthesis inhibitor cycloheximide. Confluent cells were incubated for 88 h with or without the extract, and some cultures were also treated with cycloheximide during the last 24 h. The level of HGF mRNA expression was then determined by Northern blot analysis. Treatment with cycloheximide completely inhibited HGF mRNA upregulation induced by the extract (Fig. 3C and 3D).

Fig. 3

3.3. Involvement of MAPKs in HGF production induced by the extract of bitter melon pulp

To reveal what signaling pathway is involved in the extract-induced HGF production, we investigated the effects of selective signal transduction inhibitors on induction of HGF production by the extract. As shown in Figure 4, the ERK kinase inhibitor PD98059, which inhibits phosphorylation of ERK, almost completely suppressed the induction of HGF production. The p38 inhibitor SB203580 and the JNK inhibitor SP600125 also potently inhibited the induction of HGF production. HGF production induced by the extract was moderately suppressed by the PKC inhibitor GF109203X but was not significantly influenced by the phosphatidylinositol 3-kinase inhibitor wortmannin.

Fig. 4

3.4. Effects of the extract of bitter melon pulp on phosphorylation of MAPKs

Since it was suggested that ERK phosphorylation plays a critical role in HGF production induced by the extract of bitter melon pulp, we investigated the effect of the extract on ERK phosphorylation by Western blotting. When the cells were stimulated with growth factors (e.g., EGF) or phorbol 12-myristate 13-acetate, 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 the extract did not increase within 6 h after addition of the extract (data not shown). Thus, we examined whether long-term treatment with the extract of bitter melon pulp could induce ERK phosphorylation. As shown in Figure 5A, ERK phosphorylation gradually increased from 72 h after addition of the extract. Further increase in phosphorylation level was observed at 96 h, whereas the cells cultured with the medium alone showed no change at any times examined. Upregulation of ERK phosphorylation induced by the extract was completely inhibited by PD98059, while neither SB203580 nor SP60012 had an inhibitory effect (Fig. 5B). We also investigated the effects of the extract on phosphorylation of other kinds of MAPK, p38 and JNK. Treatment with the extract significantly increased the phosphorylation levels of p38 and JNK with a time course similar to that of ERK phosphorylation (Fig. 5A).

Fig. 5

3.5. Stimulation of proliferation of human dermal fibroblasts by the extract of bitter melon pulp

Upregulation of MAPKs suggests a potential stimulation of cell proliferation induced by the extract of bitter melon pulp. To investigate an effect of the extract on cell proliferation, confluent human dermal fibroblasts were incubated with or without the extract of bitter melon pulp, and the viable cell number was determined by the MTT assay. The extract significantly increased the cell

number at doses of 0.21 and 0.42 mg/ml (Fig. 6A). Figure 6B shows the effect of the extract on DNA synthesis in the fibroblasts. The extract markedly enhanced [^{3}H]thymidine incorporation. The time course of cell proliferation induced by the extract of bitter melon pulp is shown in Figure 6C. There was a time lag of at least 48 h before enhancement of cell proliferation after the addition of the extract.

Fig. 6

3.6. Properties of an active substance(s)

The extract was subjected to gel filtration to determine the molecular mass of a substance with activity for induction of HGF production and that of a substance with growth-promoting activity. Both activities were eluted in a single peak with almost the same molecular mass (Fig. 7A). The apparent molecular mass was about 14 000 Da, if the active substance(s) is a polypeptide. The HGF production-inducing activity in the extract of bitter melon pulp was stable for 10 min incubation up to 70ºC (data not shown), but the activity was completely inactivated by being incubated for 10 min at 80ºC (Fig. 7B). The activity in the extract was not inactivated by being treated with 0.01% trypsin for 30 min at 37ºC (data not shown). When the extract was precipitated with ethanol, the HGF production-inducing activity was retained in the supernatant of 70% ethanol (data not shown).

Fig. 7

4. Discussion

The data presented in this report show that the extract of bitter melon pulp markedly induced HGF production from human dermal fibroblasts. The extract also stimulated HGF production from the human lung fibroblast line MRC-5, suggesting that the effect is not specific for cell type.

To our knowledge, this is the first report on the stimulatory effect of a vegetable extract on HGF production. HGF is mitogenic to hepatocytes, other epithelial cells and endothelial cells [6,7]. Injection of HGF or HGF gene has been effective for treating animal models of chronic hepatic and renal diseases such as hepatic and renal fibrosis and liver cirrhosis and of cardiovascular diseases [14-16]. Thus, HGF inducers may also be useful as therapeutic agents for these diseases. Studies are under way in our laboratory to determine the effect of the bitter melon pulp extract on HGF production in vivo.

Since the extract of bitter melon pulp upregulated phosphorylation of ERK, p38 and JNK and the extract-induced HGF production was potently inhibited by PD98059, SB203580 and SP600125, which specifically block ERK, p38 and JNK signaling pathways, respectively, it seems likely that MAPKs are important molecules responsible for the HGF production-inducing effect of the extract. There was a time lag of at least 48 h before upregulation of the phosphorylation of MAPKs in the fibroblasts after addition of the extract, whereas upregulation of the phosphorylation of MAPKs occurred within 5 min after addition of EGF (our unpublished data). These results suggest that the extract increases phosphorylation levels of MAPKs indirectly through a mechanism by which an unidentified factor(s) is induced. There was also a time lag of at least 72 h before upregulation of HGF production after addition of the extract, whereas the time lag before EGF-induced HGF production was about 24 h. Thus, induction of HGF production occurs approximately 24 h later than upregulation of the phosphorylation of MAPKs. We have speculated that one of the most likely candidates is a growth factor such as EGF or PDGF. This notion is supported by the fact that the extract stimulated proliferation of the cells and the fact that growth factors such as EGF, PDGF and bFGF induce HGF production in fibroblasts [25]. The notion is also consistent with our results showing that the HGF production-inducing activity and the cell growth-promoting activity of the extract were not separated by gel filtration (Fig. 7A) and that HGF mRNA upregulation induced by the extract was inhibited by treatment with the protein synthesis inhibitor cycloheximide (Fig. 3 C and 3D). Production of EGF and PDGF-AA from human dermal fibroblasts, however, was not induced by the extract of bitter melon pulp (our unpublished data). It has been reported that HGF also has growth-stimulating activity of human

dermal fibroblasts [44]. The extract-induced augmentation of cell proliferation, however, may not be mediated through the induction of HGF production because the former occurred earlier than the latter. Thus, the mediator for induction of HGF production and cell proliferation remains to be determined.

The HGF production-inducing activity in the extract of bitter melon pulp was not reduced by trypsin treatment. Although bitter melon contains protease inhibitors [45], the azocaseinolytic activity of trypsin was only slightly inhibited by the extract (our unpublished results). The results of gel filtration of the extract indicated that the HGF production-inducing activity was eluted in the fractions of high molecular weight. Therefore, the active substance in the extract seems to be a non-proteinous macromolecule. Most of the macromolecular inducers of HGF are proteins such as growth factors and cytokines [24,25,27,28,30]. Non-proteinous macromolecular HGF inducers reported so far is heparin which is effective in MRC-5 cells and others [23]. However, heparin does not upregulate HGF gene expression and does not effectively induce HGF production in human dermal fibroblasts [23]. Thus, it is likely that the active substance in the extract is a hitherto-unidentified inducer of HGF production.

Bitter melon has been shown to possess a wound-healing property [31,46]. Wound healing is a complex process of inflammation, granulation tissue formation, angiogenesis, re-epithelialization and remodeling [47]. The complexity of this process is compounded by the fact that skin is comprised of two distinct compartments, the ectodermally derived epithelial epidermis and the mesodermally derived mesenchymal dermis, which are separated by a basement membrane barrier. To regenerate this organ, numerous cellular, hormonal, matrix, and enzymatic activities in each compartment are required to act in a coordinated manner with those in the other compartment. If re-epithelialization occurs without sufficient underlying granulation tissue formation, which encompasses macrophage accumulation, fibroblast ingrowth, matrix deposition and angiogenesis, then an atrophic scar results. If granulation tissue formation proceeds without the requisite re-epithelialization, then a nonhealed wound with hypergranulation tissue is the outcome. The extract of bitter melon pulp was found to have both HGF production-inducing activity and fibroblast growth-promoting activity. HGF is capable of stimulating migration and

proliferation of keratinocytes and thus has been suggested to be involved in cutaneous physiology and wound healing [17]. The extract is expected to stimulate regeneration of the epithelial epidermis and mesenchymal dermis in a coordinated manner.

In conclusion, this study showed that the extract of bitter melon pulp significantly induced HGF expression in different cell types. The results suggested that the effect is mediated through indirect activation of MAPKs, especially ERK. To the best of our knowledge, this is the first report on the stimulatory effect of a vegetable extract on expression of HGF. This study also showed that the extract markedly stimulated the proliferation of fibroblasts. Our findings suggest potential usefulness of the extract for tissue regeneration and provide an insight into the molecular mechanism underlying the wound-healing property of bitter melon.

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ERK-independent proliferation of murine splenic B cells stimulated by chondroitin sulfate B. Immunol Lett 2005;99:80-4.

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Figure Legends

Fig. 1. Dose-response and time course for HGF production induced by the extract of bitter melon pulp. (A-D) Confluent dermal fibroblasts from neonatal donors (A, B) and a 3-day-old baby (C) and MRC-5 cells (D) were incubated for 120 h, 120 h and 96 h, respectively, with the indicated concentrations of bitter melon pulp extract. (E) Confluent dermal fibroblasts from neonatal donors were incubated for the indicated periods with or without 0.42 mg/ml of bitter melon pulp extract and 3 ng/ml of EGF. The amount of HGF secreted into the medium was measured by an ELISA. The data are means ± SD of three (A, B, D, E) or five (C) independent experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs control (Dunnett's *t*-test); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs control (Dunnett's T3 test).

Fig. 2. Comparison of HGF production induced by the extract of bitter melon pulp with HGF production promoted by other inducers. Confluent dermal fibroblasts from neonatal donors were incubated for 120 h with or without 0.42 mg/ml of bitter melon pulp extract, 3 ng/ml of EGF, 10 ng/ml of PDGF, and 1 pM cholera toxin. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. $^{\#\#\#}p < 0.001$ vs control (Student's *t*-test); $*p < 0.05$ and $**p < 0.01$ vs bitter melon pulp extract (Dunnett's T3 test).

Fig. 3. Up-regulation of HGF gene expression by the extract of bitter melon pulp and inhibition of the extract-induced HGF mRNA expression by cycloheximide. (A, B) Confluent dermal fibroblasts from neonatal donors were incubated for 88 h with or without 0.85 mg/ml of bitter melon pulp extract. (C, D) Confluent dermal fibroblasts were incubated for 88 h with or without 0.85 mg/ml of bitter melon pulp extract. During the last 24 h some cultures were incubated in the presence of cycloheximide (1 μg/ml). Total RNA was isolated and Northern blot analysis was performed using a ^{32}P-labeled cDNA probe for human HGF. The signal intensity of the 6.4-kb HGF mRNA band in the autoradiograms was normalized to the fluorescence intensity of the 28S

rRNA band and expressed as fold-change relative to the control. Autoradiograms and fluorescence photographs (A,C) are representative of three independent experiments with similar results. The data (B, D) are means of three independent experiments. Bars indicate SD. $^{**}p < 0.01$ vs control (Welch's *t*-test); $^{\#\#\#}p < 0.001$ vs bitter melon pulp extract alone (Tukey's test); $^{\ddagger}p < 0.05$ vs medium alone (Tukey's test).

Fig. 4. Suppression of bitter melon-pulp-extract-induced HGF production by inhibitors of MAPKs. Confluent dermal fibroblasts from neonatal donors were preincubated with or without inhibitors of MAPKs and other protein kinases for 2 h and then incubated for 120 h with or without 0.42 mg/ml of bitter melon pulp extract in the presence or absence of the inhibitors. The inhibitors of MAPKs and other protein kinases were dissolved in dimethy sulfoxide, and the final concentration of dimethyl sulfoxide in all cultures, including the control, was 0.1%. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of four independent experiments. Bars indicate SD. $^{\#\#\#}p < 0.001$ vs control (Welch's *t*-test); $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs bitter melon pulp extract alone (Tukey's test).

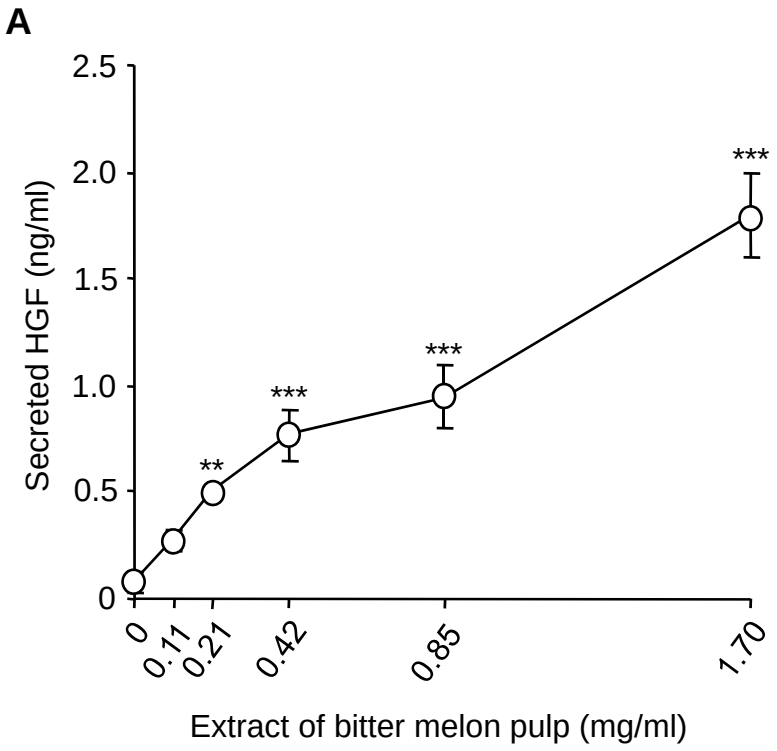
Fig. 5. Up-regulation of phosphorylation of ERK, p38 and JNK by the extract of bitter melon pulp. Confluent dermal fibroblasts from neonatal donors were incubated for the indicated periods with or without 0.85 mg/ml of bitter melon pulp extract in the presence or absence of MAPK inhibitors (25 μM PD98059, 10 μM SB203580 or 10 μM SP600125). Equal amounts of cell extracts (10 μg) were subjected to SDS-PAGE and immunoblotted with specific antibodies against phosphorylated and total ERK, p38 and JNK. Immunoblots are representative of three independent experiments with similar results.

Fig. 6. Stimulation of proliferation of dermal fibroblasts by the extract of bitter melon pulp. Confluent dermal fibroblasts from neonatal donors were incubated for 120 h (A), 102 h (B) and the indicated periods (C) with or without the indicated concentrations (A , B) and 0.21 mg/ml (C) of bitter melon pulp extract. In the time course experiment (C), the medium of confluent cells was

replaced with the same fresh medium (DMEM-10) 24 h before addition of the extract. The extract of bitter melon pulp was then added without a medium change. The number of viable cells and DNA synthesis were then determined by the MTT method (A) and [^{3}H]thymidine incorporation during the last 24 h (B, C), respectively. The data are means ± SD of three (A,B) or four (C) independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control (Dunnett's *t*-test); $^{\#}p < 0.05$ vs control (Welch's *t*-test).

Fig. 7. Gel filtration profile of HGF production-inducing activity and cell growth-promoting activity of bitter melon pulp extract and inactivation of HGF production-inducing activity by heating. (A) Two ml of bitter melon pulp extract was applied to a Sephadex G-50 column, and 1-ml fractions were collected. For the assay of HGF production induction assay, confluent dermal fibroblasts from neonatal donors were incubated for 120 h with or without 5% of each fraction. The amount of HGF secreted into the medium was measured by an ELISA. For the assay of cell growth-promoting activity, confluent dermal fibroblasts from neonatal donors were incubated for 78 h with or without 2.5% of each fraction. The cells were then pulse-labeled with [^{3}H]thymidine for 24 h. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter. Molecular weight markers used are carbonic anhydrase (29 000), myoglobin (17 000) and aprotinin (6 500). The data are representative of three independent experiments with similar results and expressed as means + or ± SD of triplicate cultures. (B) The extract of bitter melon pulp (33.9 mg/ml) was incubated for 10 min at 80ºC and centrifuged. Confluent dermal fibroblasts were incubated for 120 h with 1.25% (v/v) of the supernatant. The amount of HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. $^{***}p < 0.001$ vs untreated extract of bitter melon pulp (Dunnett's *t*-test).

Fig. 1


A
Secreted HGF (ng/ml)
2.5
2.0
1.5
1.0
0.5
0
**
***
***
***
0
0.11
0.21
0.42
0.85
1.70
Extract of bitter melon pulp (mg/ml)

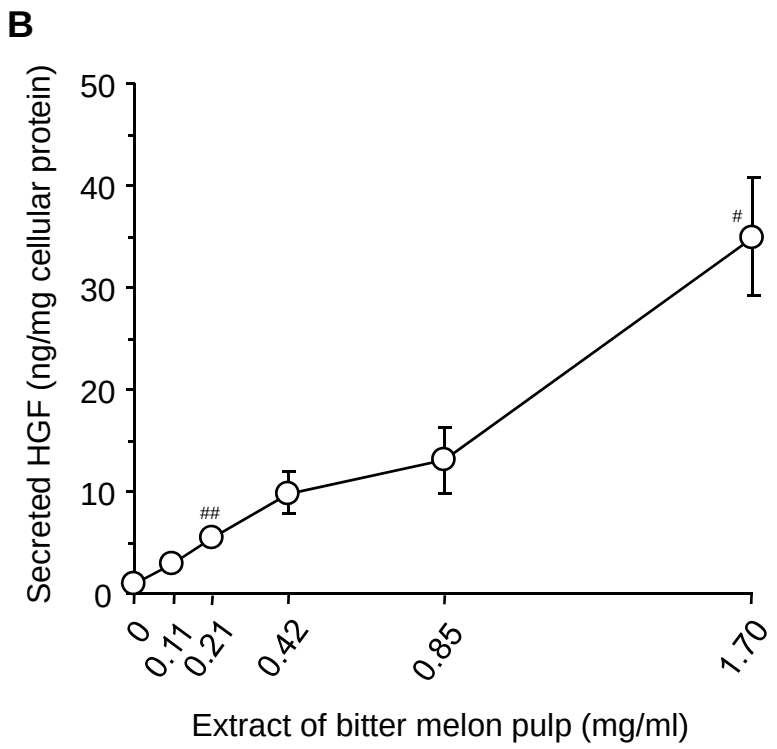
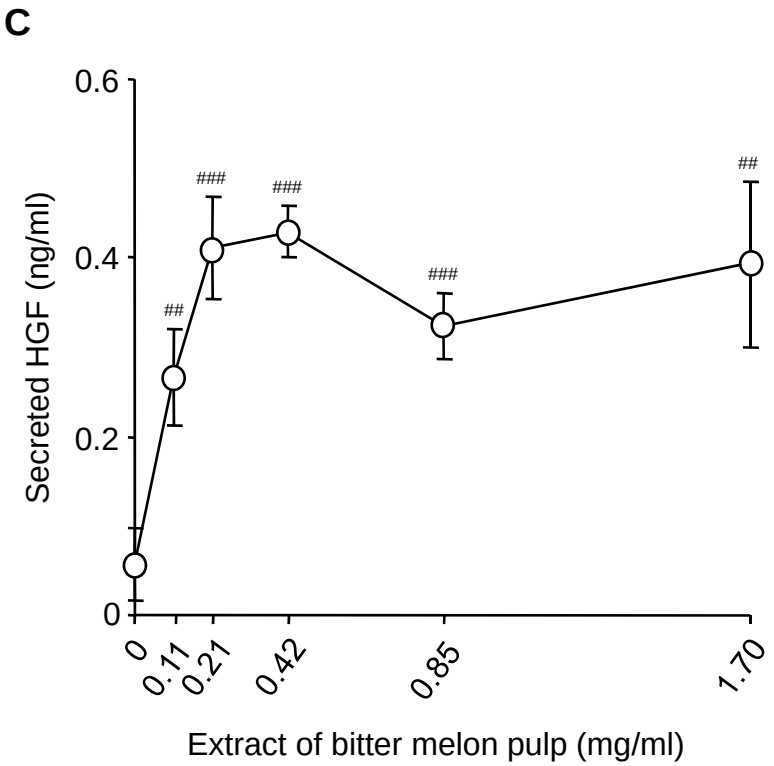

B
Secreted HGF (ng/mg cellular protein)
50
40
30
20
10
0
##
#
0
0.11
0.21
0.42
0.85
1.70
Extract of bitter melon pulp (mg/ml)

Fig. 1


C
0.6
0.4
0.2
0
Secreted HGF (ng/ml)
##
###
###
###
##
0
0.11
0.21
0.42
0.85
1.70
Extract of bitter melon pulp (mg/ml)

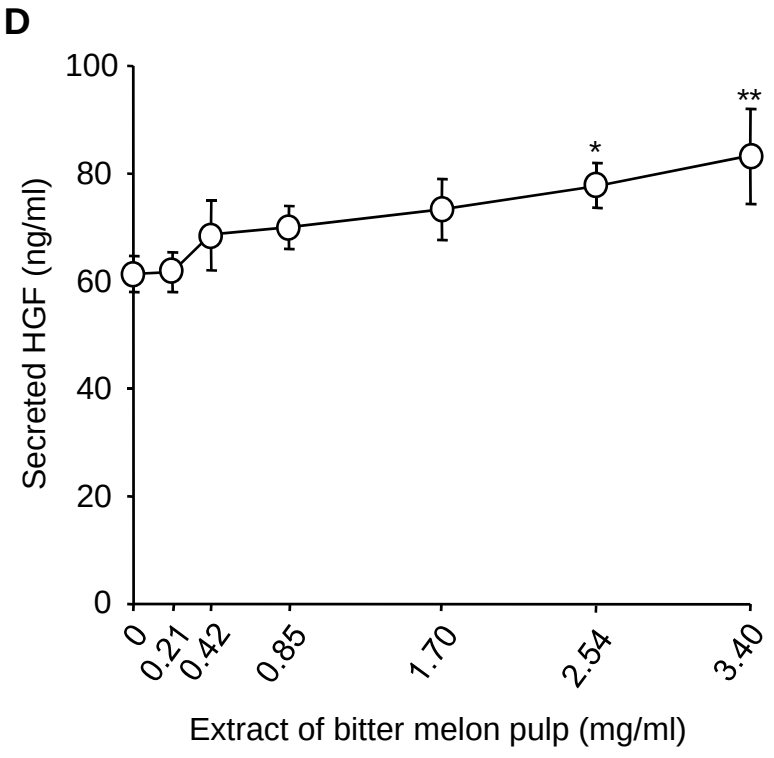

D
100
80
60
40
20
0
Secreted HGF (ng/ml)
*
**
0
0.21
0.42
0.85
1.70
2.54
3.40
Extract of bitter melon pulp (mg/ml)

Fig. 1

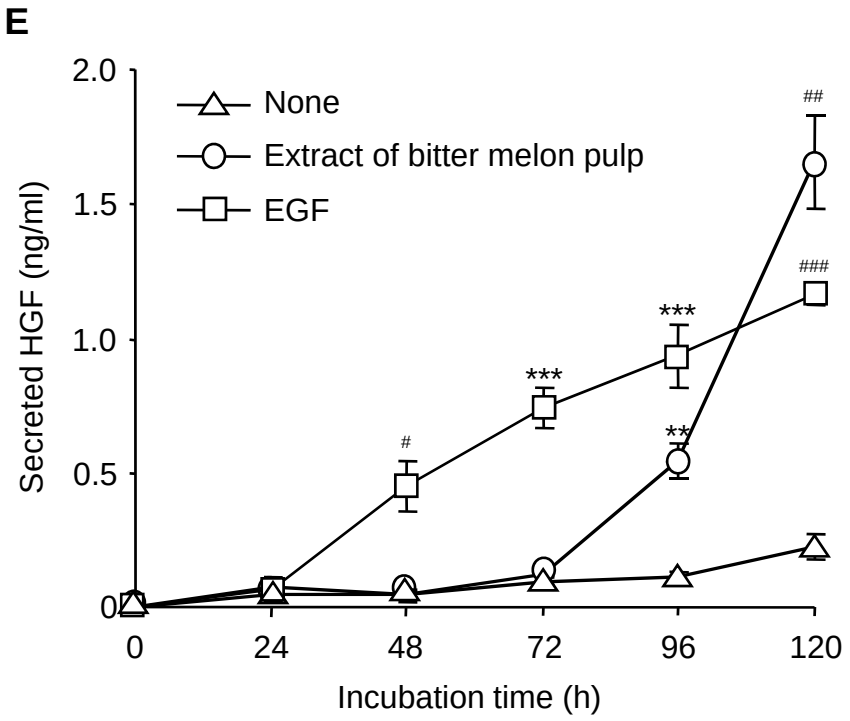

E
2.0
1.5
1.0
0.5
0
Secreted HGF (ng/ml)
None
Extract of bitter melon pulp
EGF
#
***
***
**
##
###
0
24
48
72
96
120
Incubation time (h)

Fig. 2

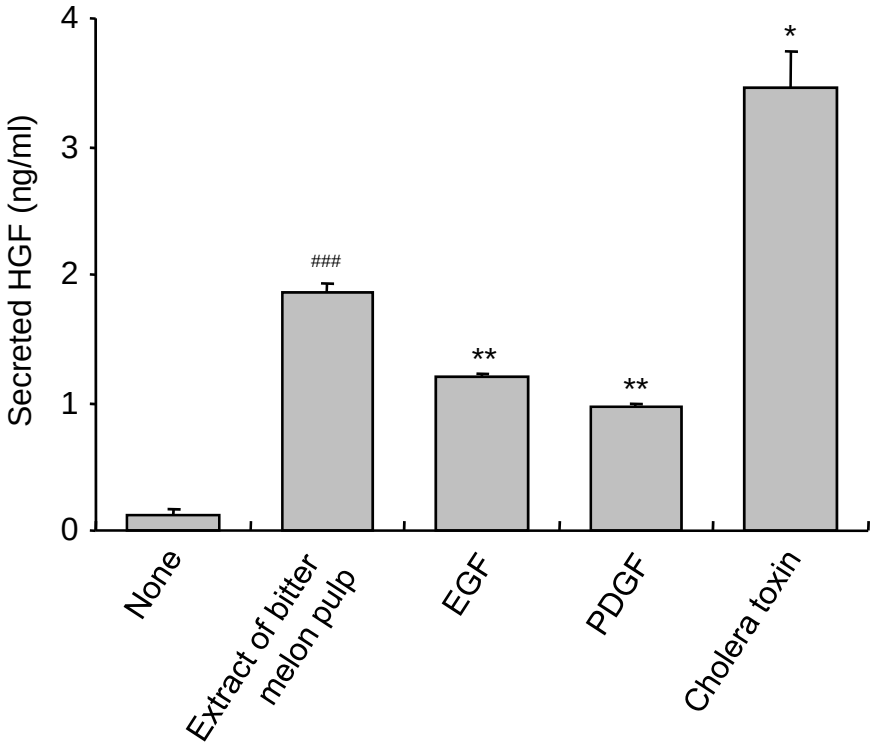

Secreted HGF (ng/ml)
4
3
2
1
0
###
**
**
*
None
Extract of bitter melon pulp
EGF
PDGF
Cholera toxin

Fig. 3

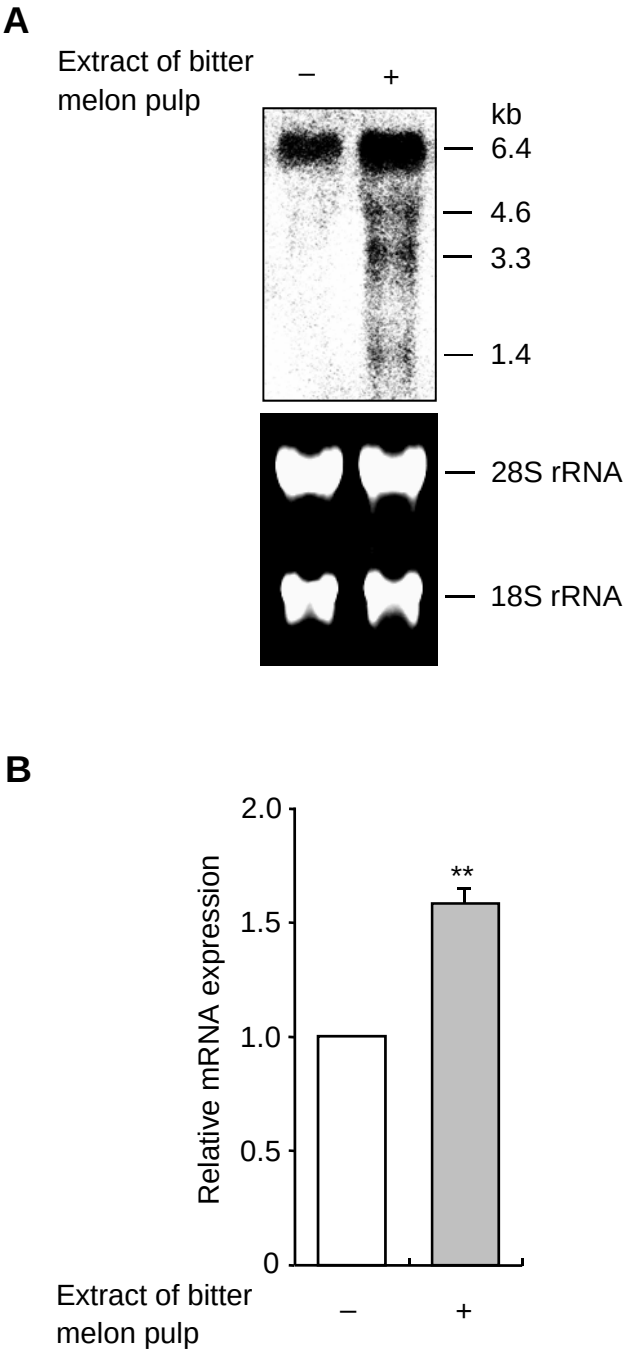

A
Extract of bitter melon pulp
–
+
kb
6.4
4.6
3.3
1.4
28S rRNA
18S rRNA
B
Relative mRNA expression
2.0
1.5
1.0
0.5
0
**
Extract of bitter melon pulp
–
+

Fig. 3

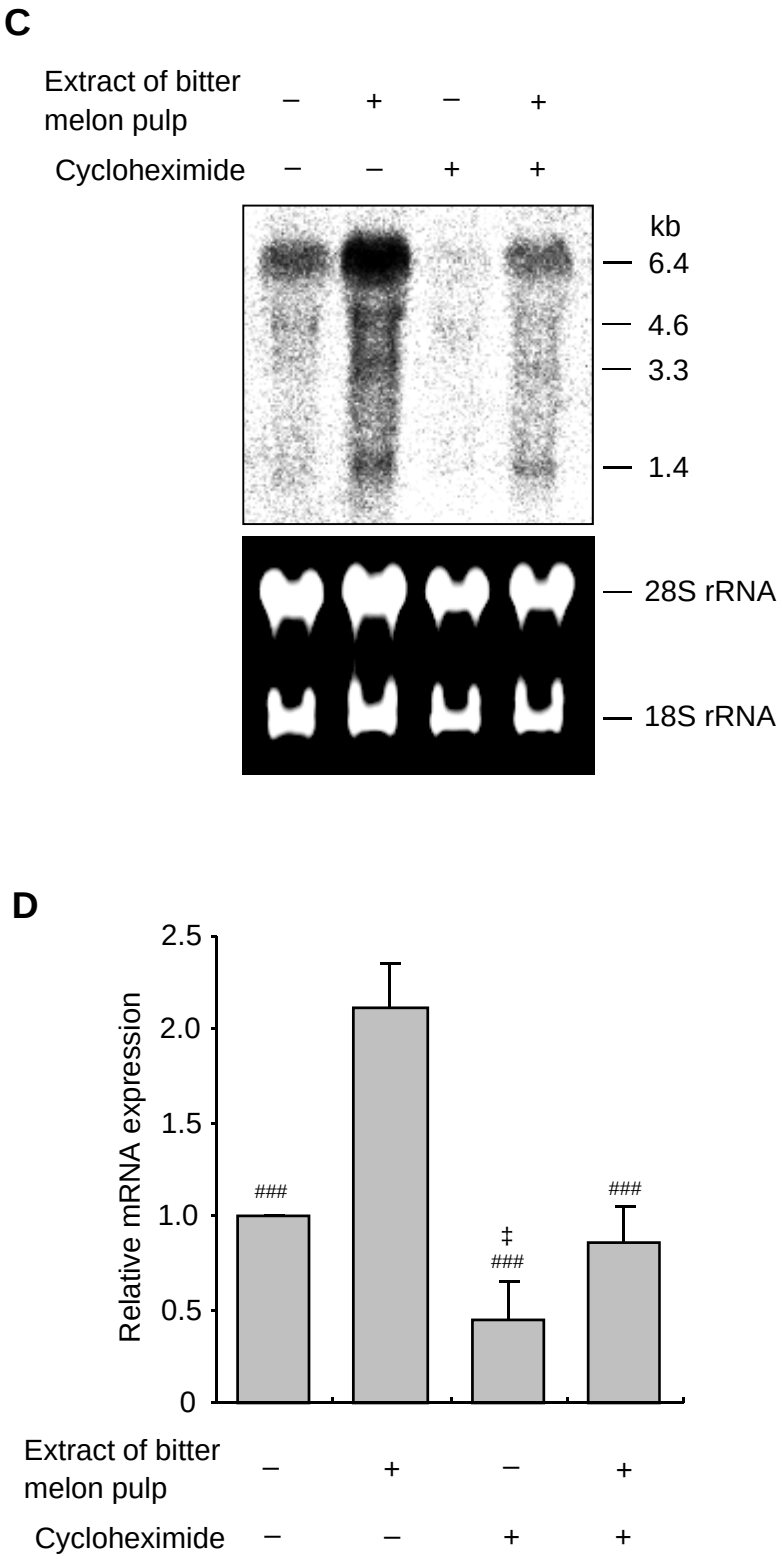

C
Extract of bitter melon pulp
– + – +
Cycloheximide
– – + +
kb
6.4
4.6
3.3
1.4
28S rRNA
18S rRNA
D
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0
###
###
‡
###
Extract of bitter melon pulp
– + – +
Cycloheximide
– – + +

Fig. 4

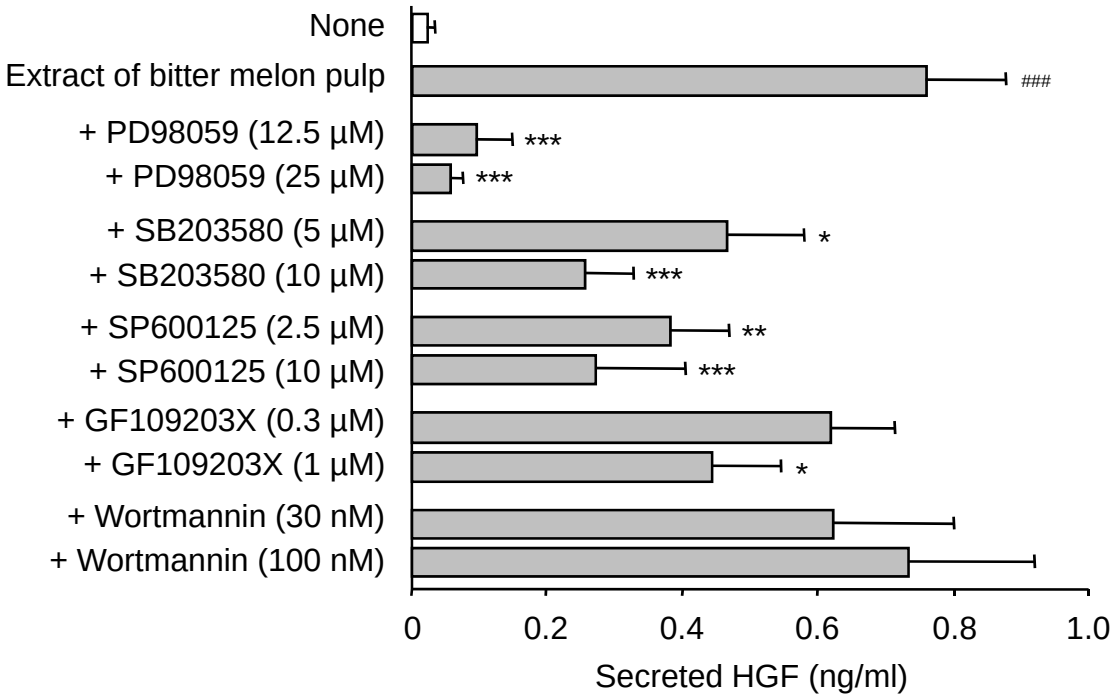

None
Extract of bitter melon pulp
+ PD98059 (12.5 μM)
+ PD98059 (25 μM)
+ SB203580 (5 μM)
+ SB203580 (10 μM)
+ SP600125 (2.5 μM)
+ SP600125 (10 μM)
+ GF109203X (0.3 μM)
+ GF109203X (1 μM)
+ Wortmannin (30 nM)
+ Wortmannin (100 nM)
###
***
***
*
***
**
***
*
0
0.2
0.4
0.6
0.8
1.0
Secreted HGF (ng/ml)

Fig. 5

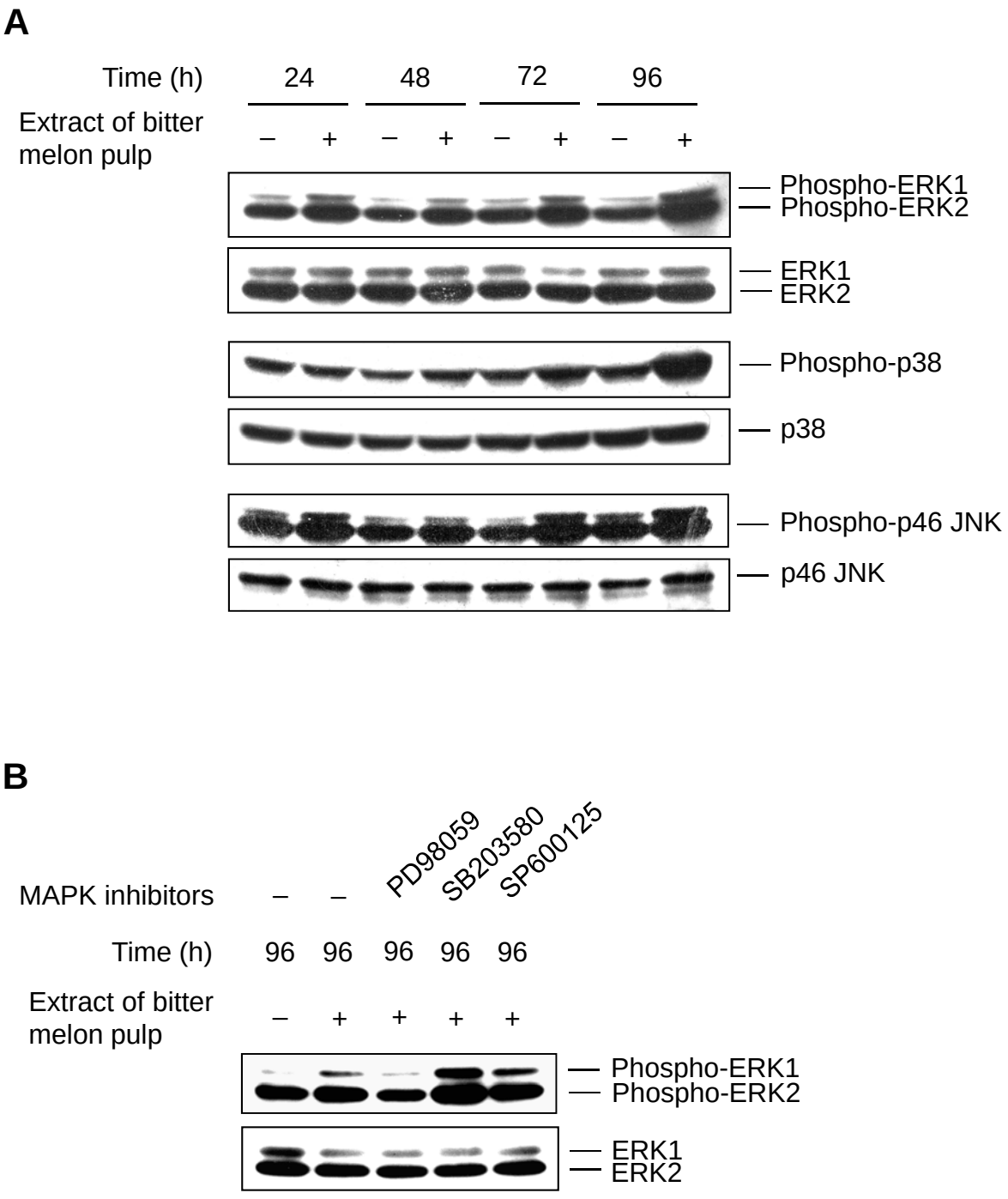

A
Time (h)
24
48
72
96
Extract of bitter melon pulp
– + – + – + – +
Phospho-ERK1
Phospho-ERK2
ERK1
ERK2
Phospho-p38
p38
Phospho-p46 JNK
p46 JNK
B
MAPK inhibitors
– –
PD98059
SB203580
SP600125
Time (h)
96 96 96 96 96
Extract of bitter melon pulp
– + + + +
Phospho-ERK1
Phospho-ERK2
ERK1
ERK2

Fig. 6

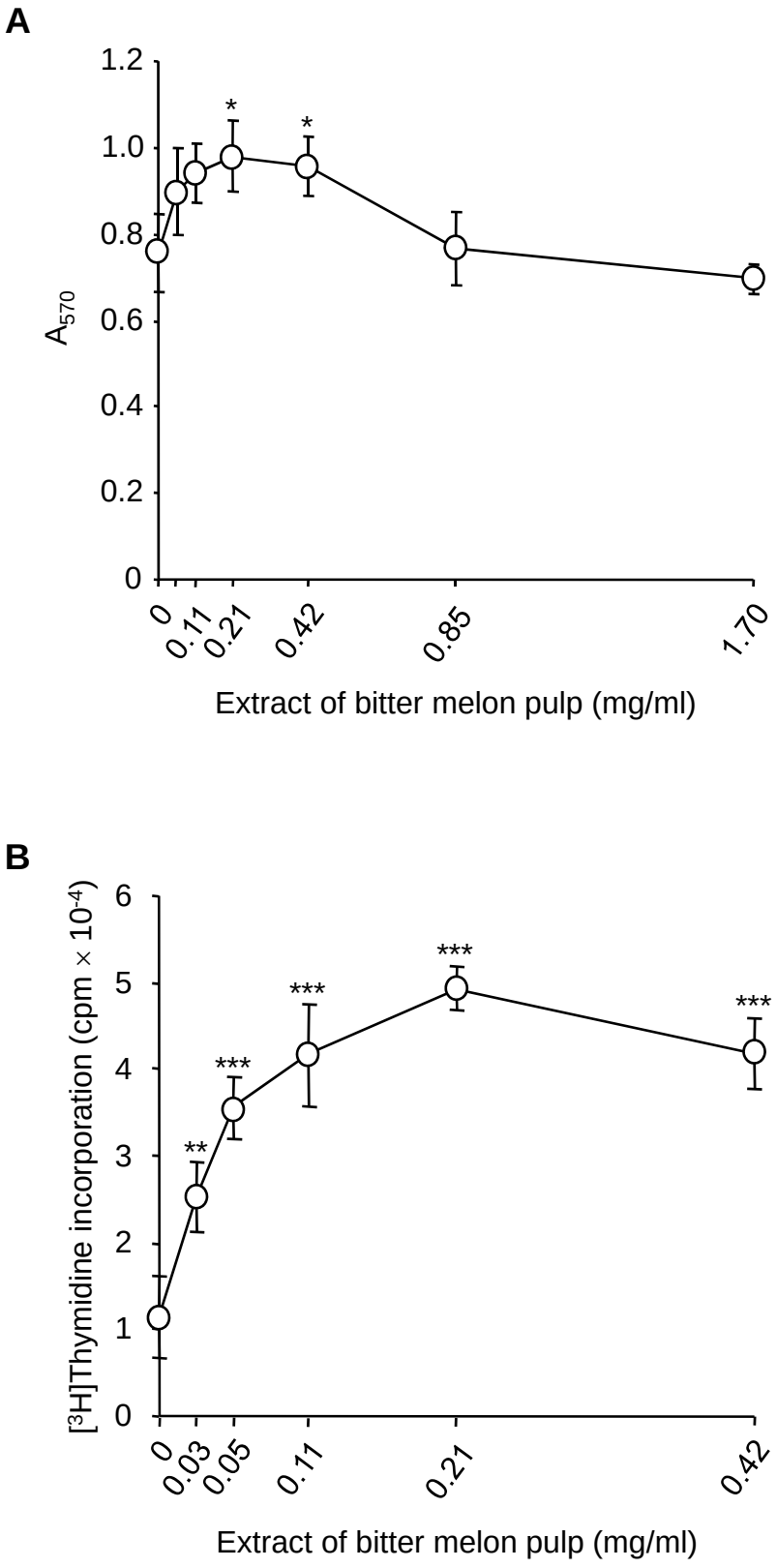

A
1.2
1.0
0.8
0.6
0.4
0.2
0
A_{570}
*
*
0
0.11
0.21
0.42
0.85
1.70
Extract of bitter melon pulp (mg/ml)
B
6
5
4
3
2
1
0
[³H]Thymidine incorporation (cpm × 10⁻⁴)
**
***
***
***
***
0
0.03
0.05
0.11
0.21
0.42
Extract of bitter melon pulp (mg/ml)

Fig. 6

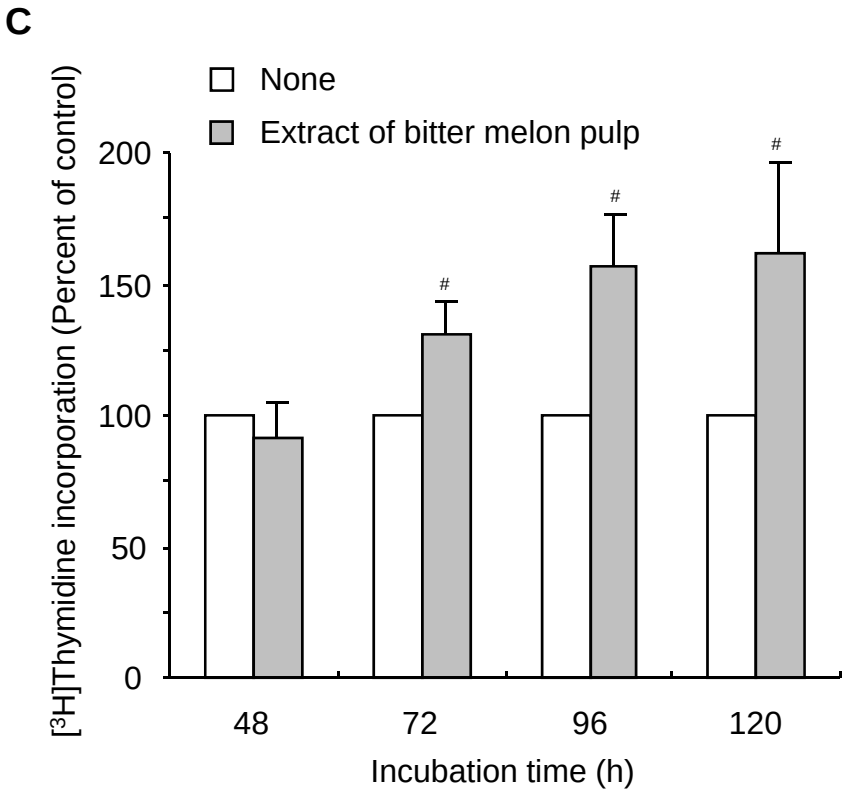
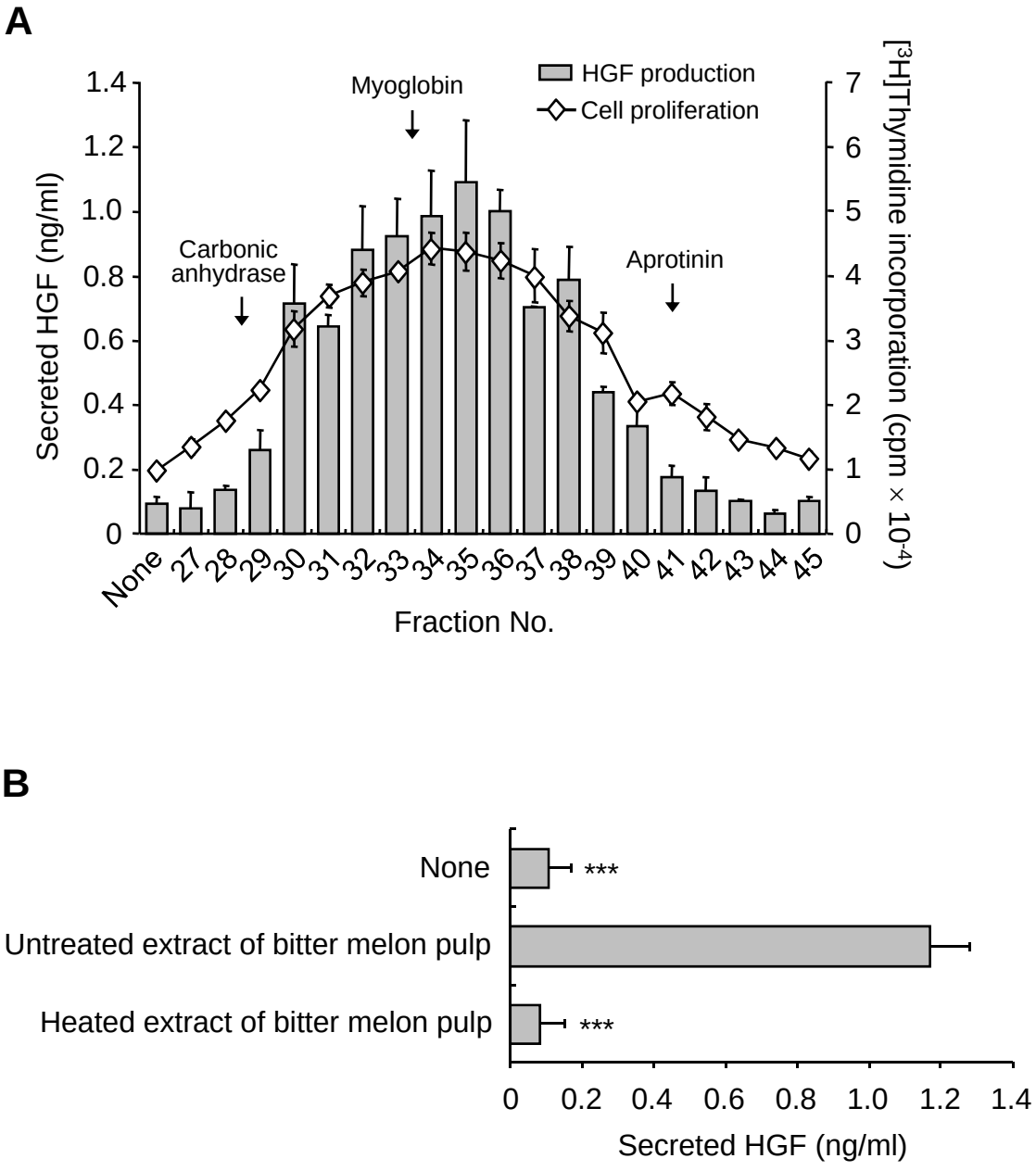

C
None
Extract of bitter melon pulp
[3H]Thymidine incorporation (Percent of control)
200
150
100
50
0
#
#
#
48
72
96
120
Incubation time (h)

Fig. 7


A
HGF production
Cell proliferation
Myoglobin
Carbonic anhydrase
Aprotinin
Secreted HGF (ng/ml)
[3H]Thymidine incorporation (cpm × 10-4)
Fraction No.
None
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
B
None
Untreated extract of bitter melon pulp
Heated extract of bitter melon pulp
***
***
Secreted HGF (ng/ml)