Induction of Hepatocyte Growth Factor Production in Human Dermal Fibroblasts by Caffeic Acid Derivatives

Manami Kurisu,[a] Rie Nakasone,[a] Yusaku Miyamae,[a] Daisuke Matsuura,[b] Hirotoshi Kanatani,[b] Shingo Yano,[b] and Hideyuki Shigemori*,[a]

[a] *Graduate School of Life and Environmental Sciences, University of Tsukuba; 1–1–1 Tennodai, Tsukuba, Ibaraki 305–8572, Japan: and* [b] *Products Development Dept., Bathclin Corporation; 29–9 Higashiarai, Tsukuba, Ibaraki 305–0033, Japan.*

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Hepatocyte growth factor (HGF) has mitogenic, motogenic, and morphogenic activities in epithelial cells. Induction of HGF production may be involved in organ regeneration, wound healing and embryogenesis. In this study, we examined the effects of caffeic acid derivatives including 4,5-di-*O*-caffeoylquinic acid (1) and acteoside (2) on HGF production in Neonatal Normal Human Dermal Fibroblasts (NHDF). Both 4,5-di-*O*-caffeoylquinic acid (1) and acteoside (2) significantly induced HGF production dose-dependent manner. To know the important substructure for HGF production activity, we next investigated the effect of the partial structure of these caffeic acid derivatives. From the results, caffeic acid (3) showed strong activity on the promotion of HGF production, while hydroxytyrosol (4) and quinic acid (5) didn't show any activity. Our findings suggest that the caffeoyl moiety of caffeic acid derivatives is essential for accelerated production of HGF. The compound which has the caffeoyl moiety may be useful for the treatment of some intractable organ disease.

Key words caffeic acid derivative; 4,5-di-*O*-caffeoylquinic acid; acteoside; hepatocyte growth factor; Neonatal Normal Human Dermal Fibroblast

Hepatocyte growth factor (HGF) has been shown to play important role in the liver regeneration.[1)] It is generated when the tissue is harmed from some damages, so HGF is believed to play important role for supporting our natural healing ability. Human HGF was first purified from the plasma of patients with fulminant hepatic failure in 1986.[2)] HGF is a heterodimer which consists of a heavy chain of about 60000 Da and a light chain of about 35000 Da, linked together, by a disulfide bond.[2)] HGF has been proved to have various activities such as mitogenic, motogenic, morphogenic, and antiapoptotic activities through binding to its receptor, c-met.[3,4)] Thereafter, it was shown that HGF affects to various kinds of cells from various organs.[5–7)] In our previous study of naturally occurring compounds possessed HGF promoting activity, we isolated and identified daphnane diterpenoids from *Daphne odora*.[8)]

Recently, there are some reports suggesting that HGF regulates neuronal survival. For example, HGF is thought to be a potential therapeutic agent for the treatment of amyotrophic lateral sclerosis (ALS) patients. Since HGF has neurotrophic activity on motoneurons and suppress the gliosis, it may retard disease progression.[9)] HGF is also thought to be useful for Alzheimer's disease (AD) therapy according to the recent study showing that gene transfer of HGF in a mouse model alleviate amyloid β induced cognitive impairment.[10)]

Thus, HGF may be useful for curing for intractable diseases including neurodegenerative diseases, and its inducers may also be useful as therapeutic agent. For example, interleukin-1, one of growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) are known to induce HGF production.[11,12)] Chicoric acid, a caffeic acid derivative, isolated from *Taraxacum* which has been utilized as a traditional medicine for dyspepsia, heartburn, spleen and liver complaints, hepatitis, also proved to have accelerated ability on the production of HGF in normal human dermal fibroblasts.[13,14)] Although these bioactive caffeic acid derivatives may have possibility to promote HGF production, there are few reports about their promoting activity.

In our previous study, we reported that caffeoylquinic acids (CQAs) found in natural resources such as coffee beans, sweetpotatos, propolis, and other plants,[15–18)] inhibited 42-mer amyloid β-protein (Aβ42) induced cytotoxicity on human neuroblastoma SH-SY5Y cells by enhancing the expression of mRNA of glycolytic enzymes and intracellular ATP.[19)] We also reported that CQAs treatment improved spatial learning memory on senescence accelerated-prone mice 8 through increasing the mRNA expression of phosphoglycerate kinase 1.[20,21)] Furthermore, acteoside (**2**) found in natural resources of *Orobanche minor*, olive, and other plants,[22)] is reported that it protects human neuroblastoma SH-SY5Y cells against amyloid β cell injury by protecting reactive oxygen species (ROS) production and modulating apoptotic signal pathway.[23)] We also reported that CQAs and acteoside (**2**) possessed anti-amyloidogenic effect.[24,25)]

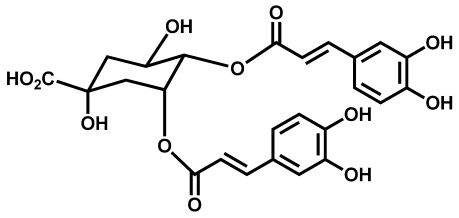
With the raising interest studies these caffeic acid derivatives have a potential to promote production of HGF that is also suggested to regulate neuronal survival,[9,10)] we performed HGF enzyme-linked immunosorbent assay (ELISA). Here, we describe caffeic acid derivatives like 4,5-di-*O*-caffeoylquinic acid (**1**) or acteoside (**2**) potently promoted HGF production in Neonatal Normal Human Dermal Fibroblasts (NHDF).

MATERIALS AND METHODS

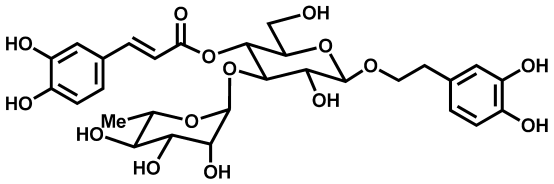
Materials 4,5-Di-*O*-caffeoylquinic acid (**1**) was synthesized as previously described,[19)] while caffeic acid (**3**), hydroxytyrosol (**4**), and quinic acid (**5**) were purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, U.S.A.). On the other hand, acteoside (**2**) was isolated from the roots of *O. minor* in

The authors declare no conflict of interest.

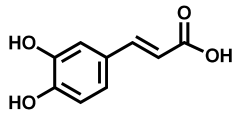
* To whom correspondence should be addressed. e-mail: shigemori.hideyuk.fn@u.tsukuba.ac.jp



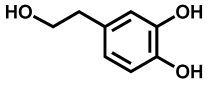
4,5-Di-*O*-caffeoylquinic acid (**1**)



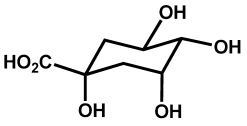
Acteoside (**2**)



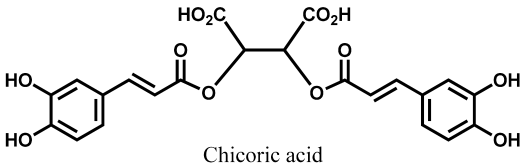
Caffeic acid (**3**)



Hydroxytyrosol (**4**)



Quinic acid (**5**)



Chicoric acid

Fig. 1. Structures of the Tested Compounds **1**–**5** and Chicoric Acid

our previous report.[25)]

Determination of HGF Levels in Conditioned Media Normal human dermal fibroblasts (NHDF) were purchased from Kurabo Co. (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Co., LLC, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Briefly, NHDF were trypsinized and suspended in 100 μL of medium described in the previous section, and were seeded in 96 well-plates (Nunc, Roskilde, Denmark) at a density of 5000 cells/well. After culturing overnight, cells were treated with each compound dissolved in 100 μL of DMEM supplemented with 0.5% FBS. After 24 h incubation, the conditioned medium was collected. Cell layers were then washed 1 time with phosphate buffered saline (PBS) and disrupted in PBS containing 0.5% Triton X-100. Then, the amount of cellular protein was quantified using *DC* protein assay (BIO-RAD Laboratories, Hercules, CA, U.S.A.).

The sandwich human HGF ELISA was performed at room temperature. Briefly, 96 well plates were prepared with an anti-human HGF monoclonal antibody (R&D Systems, Minneapolis, MN, U.S.A.), and incubated overnight. Then wells were washed with 0.05% Tween 20 in PBS and blocking the wells with PBS containing 1% BSA for 1 h. Then the wells were washed again and the conditioned medium was added to the plate. At the same time, human HGF for standard curve was also added within the range of 0 to 5 ng human HGF/mL. After 2 h incubation, the wells were washed and biotinylated goat anti-human HGF antibody (R&D Systems, Minneapolis, MN, U.S.A.) was added. After another 2 h incubation, the wells were washed and streptavidine HRP conjugate (R&D Systems, Minneapolis, MN, U.S.A.) was added. After 30 min incubation, the wells were washed and the substrate solution was added, and then, incubated for another 30 min. Stop solution was added, and the optical density of each well was determined at 450 nm. The HGF levels were expressed as ng per mg of cellular protein.

RESULTS AND DISCUSSION

To investigate the ability of caffeic acid derivatives (Fig. 1) to promote HGF production in NHDF, we first checked the cytotoxicity of the tested compounds. As far as we tested, all the compounds didn't show cytotoxicity (cell viability: >90% at 100 μM) using the WST assay,[26)] so we decided the concentration of the compounds from 0 to 100 μM to investigate the ability to promote HGF production.

NHDF was incubated for 24 h with 4,5-di-*O*-caffeoylquinic acid (**1**) or acteoside (**2**), and the level of HGF produced from the cells was determined by HGF ELISA. Chicoric acid was used as a positive control. From the results, 4,5-di-*O*-caf-

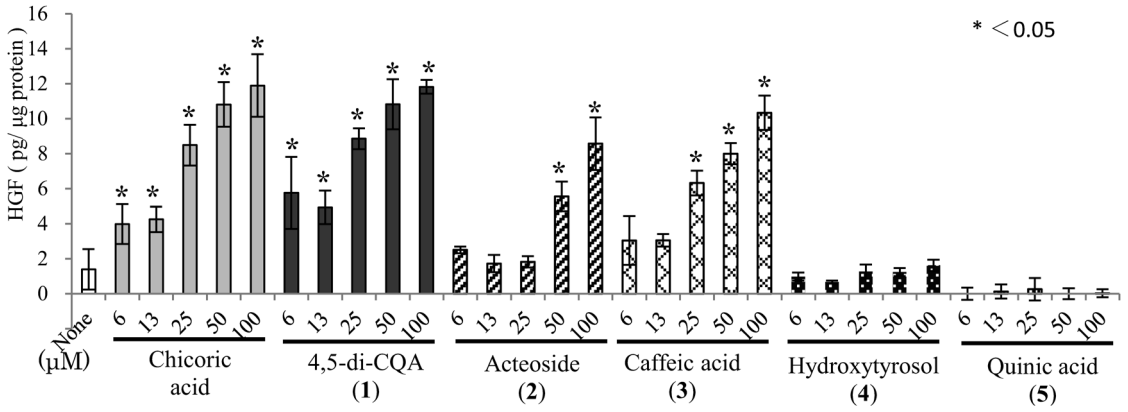


Fig. 2. Comparison of HGF Production Induced by the Caffeic Acid Derivatives **1**–**5** and Chicoric Acid

Normal human dermal fibroblasts were incubated with each compound (6, 13, 25, 50, and 100 μM) at 37°C for 24 h. The amount of HGF secreted into the medium was measured by an ELISA. Each bar represents the mean±S.D. (n=4). *p<0.05.

feoylquinic acid (**1**) and acteoside (**2**), both promoted the HGF production dose-dependent manner. Best of all, 4,5-di-*O*-caffeoylquinic acid (**1**) showed equivalent efficacy to chicoric acid (Fig. 2).

To know the important substructure for HGF production activity, we next investigated the effect of the partial structure of these caffeic acid derivatives (Fig. 1). In particular, caffeic acid (**3**), hydroxytyrosol (**4**), and quinic acid (**5**) were used for the assay. From the results, caffeic acid (**3**) showed strong activity on the promotion of HGF production, while hydroxytyrosol (**4**) and quinic acid (**5**) did not show any activity. These results suggest that the caffeoyl moiety in both 4,5-di-*O*-caffeoylquinic acid (**1**) and acteoside (**2**) is important to promote HGF production.

HGF is known as a multifunctional growth factor and play important roles for developmental and regenerative events in many organs.[3–7] Moreover, it is suggested to regulate neuronal survival and seemed to be useful for AD treatment, considering the studies like showing gene transfer of HGF in a mouse model alleviate amyloid β induced cognitive impairment.[10] So, pharmacological approach including gene therapy to increase the HGF level in the brain is going on. Thus, HGF inducers may also be useful as therapeutic agent for AD treatment.

In our previous study, we reported that neuroprotective effect from Aβ42-induced cytotoxicity of CQAs,[19–21] and the inhibition of the amyloid β aggregation by CQAs and acteoside (**2**).[24,25] Combined with these previous studies and this study about HGF promoting activity of CQAs such as 4,5-di-*O*-caffeoylquinic acid (**1**) and acteoside (**2**), they have a potential to cure neurodegenerative diseases like AD, the evidence that having not only the direct protect activity against amyloid β toxicity but also the promoting activity of HGF. There are some reports on the mechanism of founded inducers to promote HGF production. It is said that inducers may promote HGF production through affecting to HGF gene expression.[5] However, the inducers are wide-ranging substances, the promoting mechanism may varies in quality.

In conclusion, this research showed 4,5-di-*O*-caffeoylquinic acid (**1**), acteoside (**2**), and caffeic acid (**3**) have activity to promote HGF production in NHDF. From the structure–activity relationship studies of these caffeic acid derivatives, caffeoyl moiety is important to promote HGF production. Further research is needed to clear the mechanism that caffeic acid derivatives promote HGF production.

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