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Isolation and Expression of Five-Amino-Acid-Deleted Variant of Feline Hepatocyte Growth Factor (HGF) cDNA

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ABSTRACT. Hepatocyte growth factor (HGF) is a pleiotropic cytokine that stimulates a wide array of cellular targets, including hepatocytes and other epithelial cells, melanocytes, endothelial and hematopoietic cells. We have cloned a different form of cDNA, with a deletion of 15 base pairs predicted to result in the loss of 5 amino acids from the first kringle domain. To investigate the biological activity, original and deleted variant of feline HGF cDNAs were transiently expressed in COS-7 cells. Both recombinant feline HGFs showed almost the same dose-response curves in the stimulation of the growth of BNL CL.2 cells (a mouse hepatocyte cell line) and scatter activity of Madin-Darby canine kidney (MDCK) cells. The findings reported here suggest that the deleted variant of feline HGF has almost the same biological activity as the original in terms of the proliferation and scatter activity.
KEY WORDS: cloning, expression, feline, leukocyte, variant HGF.

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Hepatocyte growth factor (HGF) was first identified and cloned as a mitogen for adult rat hepatocytes in primary culture [10, 16]. It is synthesized as a single chain, 728 amino acid precursor, pre-pro-HGF, with a 31-amino acid signal peptide. The secreted pro-HGF is activated extracellularly by the proteolytic activity of serine proteases [12]. Biologically active HGF is composed of a 69-kDa α-chain and a 34-kDa β-chain, linked by a disulfide bridge and both chains are highly glycosylated. The α-chain contains an N-terminal hairpin domain followed by four kringle domains and the β-chain contains a serine protease-like domain [7].

HGF exerts numerous biological activities on a wide variety of cells, and has mitogenic, motogenic, morphogenic and anti-apoptotic activities. All the effects of this multifunctional cytokine are mediated through its cell surface receptor tyrosine kinase, encoded by the c-MET proto-oncogene [1]. Recent studies have revealed that the HGF/c-Met receptor signaling system plays essential roles in mammalian development, angiogenesis, tumorigenesis and organ regeneration [7]. Physiologically, HGF has an organotrophic role in the regeneration and protection of various organs, including the liver, lung, stomach, pancreas, heart, neurons and kidney [8]. Intravenous injection of recombinant HGF remarkably enhances organic regeneration in various disease models, such as liver cirrhosis, acute and chronic renal failure, and lung fibrosis [3, 6, 13, 24].

There are two known distinct forms of naturally occurring variant HGF. One form has a 5-amino-acid deletion in the first kringle domain [17–20] while the other form consists of only the N-terminal hairpin domain and two kringle domains [2]. The former retains most of the biochemical characteristics of the originally reported HGF whereas the latter form can bind to the HGF receptor with relatively high-affinity and acts as an antagonist. Interestingly, the 5-amino-acid-deleted variant (dHGF) is distinguishable from HGF in its biological activity and tertiary structure: dHGF is more potent than HGF in stimulation of DNA synthesis in epithelial cells, HGF is over 70-fold more soluble than dHGF in PBS, and several monoclonal antibodies raised against dHGF recognized only dHGF and neither HGF nor reduced dHGF, indicating that the deletion caused a tertiary structural change [21].

The potent activity of dHGF might be beneficial in developing HGF-therapy for organ diseases. In fact, dHGF was used to treat an acute and chronic renal failure model mouse, and remarkable therapeutic effects were observed [13, 15, 25]. In this study, we describe the isolation and expression of a 5-amino-acid-deleted variant feline HGF cDNA from leukocytes and characterization of the recombinant protein.

MATERIALS AND METHODS

Isolation and sequencing of the feline HGF (FeHGF) cDNA: Leukocyte total RNA (American short-hair domestic cat) was isolated using Trizol reagent (Invitrogen Corp., Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. For cDNA synthesis, feline leukocyte total RNA (2 μg) was incubated at 42°C in a reaction mixture (20 μl) containing 200 units of SuperScript II reverse transcriptase (Invitrogen) and an oligo (dT) primer (Invitrogen). The FeHGF cDNA fragment was obtained by nested-reverse transcription (RT)-polymerase chain reaction (PCR), with primers designed based on the sequences conserved between human, mouse, and rat HGF cDNAs (Accession Nos. M29145, D10212 and D90102, respectively). The nested-RT PCR was performed in a reaction

mixture (50 μl) containing an aliquot of the above cDNA solution, 0.05 unit/μl *Taq* DNA polymerase (EX Taq, TaKaRa, Shiga, Japan), and 0.5 pmol/μl each of the sense and antisense primers: 5'-GAACACAGCTTTTTGCCTTC-GAGCTATCG-3' and 5'-GTGTATCCATTTTGCAT-AATATGCTACTC-3' for the first round PCR, and 5'-CCTACAGGAAAACTACTGTCGAAATCCTCG-3', and 5'- GGCACATCCACGACCAGGAACAATGACAC-3' for the nested PCR. Reaction conditions were 2 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. The PCR product (approximately 1.5 kb) was purified from the agarose gel (RECOCHIP, TaKaRa), cloned into the pGEM-T easy vector (Promega, Madison, WI, U.S.A.) and sequenced using the dideoxy-chain termination method (Amersham, Buckinghamshire, UK) and an automated sequencer (Shimadzu, Kyoto, Japan). The final nucleotide sequence was determined by comparing at least three cDNA clones.

To determine the full-length feline cDNA sequence, rapid amplification of cDNA ends (RACE) was performed as described in the SMART RACE amplification kit (Clontech, Palo Alto, CA, U.S.A.) using primers designed based on the sequence data derived above: (F1: 5'-ATGCAGC-CAATACCATCAAGGGAAGGTGAC-3' and F2: 5'-TCAGGACCATGTGAGGGAGATTATGGTGGC-3' for 3' RACE; R1: 5'-CACTGAGGAATGTCACAGACTTCG-TAG-3' and R2: 5'-CCTACAGGAAAACTACTGTC-GAAATCC-3' for 5' RACE). F2 and R2 were used for nested PCR. All PCR amplifications were performed for 25 cycles of 5 sec at 94°C, 10 sec at 68°C, and 2 min at 72°C. The PCR products of 5' and 3' RACE were inserted into the pGEM-T Easy vector and sequenced.

Amplification of the entire coding region and screening for variant clones: To amplify the entire coding region of the FeHGF, RT-PCR was performed using the primers: 5'-GGATCCGCCAGCGCGTCCAGCAGCACC-3', and 5'-TGGGTGCTTCAAATACACTTACATCAG-3' for the first round PCR and 5'-ATGTGGGTGACCAAACTTCT-TCCAGTCCTG-3' and 5' CTATGACTGTGGTATCT-TATATGTTAAT-3' for the nested PCR. PCR amplification was performed for 30 cycles of 30 sec at 94°C, 30 sec at 55°C, and 2 min at 68°C with KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The PCR product was inserted into the *Eco*RV-digested pBluescript (pBS) KS (+) vector (Stratagene, La Jolla, CA, U.S.A.) and sequenced as described above.

The variant HGF clones were screened by PCR using *E. coli* colonies transformed with the above ligation mixture as templates. PCRs were performed using primers (5'-CTAT-CACTAAGAGTGGCATC-3', nucleotides 416–435 and 5'-GGAATGTCACAGACTTCGTAG-3', nucleotides 605–585) designed to amplify an HGF cDNA fragment containing a deleted region in the first kringle domain, previously reported for the human [17, 19, 20], and mouse [18] sequences. Reaction conditions were 9 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster, CA, U.S.A.). The PCR products with an expected size of 190 bp were electrophoresed in a 4% agarose gel, stained with ethidium bromide and photographed under ultraviolet light. The clones that yielded shorter products than expected were selected and their sequences determined.

Cell lines and cultures: The MDCK (Madin-Darby canine kidney) and BNL CL.2 (mouse hepatocyte origin) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). COS-7 cells were purchased from the Human Science Research Resources Bank (HSRRB, Osaka, Japan). All were cultured in Eagle's minimum essential medium (EMEM, Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere with 5% CO_2.

Expression of the HGF and the variant HGF cDNAs: To express the FeHGF or the variant FeHGF (deleted FeHGF, dFeHGF), plasmids were constructed by inserting a *Sal* I - *Not* I digested 2.2 kb fragment of pBS-FeHGF or pBS-dFe-HGF into a *Sal* I - *Not* I digested pCI-neo mammalian expression vector (Promega). The plasmid DNAs were purified using Wizard plus SV minipreps (Promega) and adjusted to 1 $\mu g/\mu l$. One day after plating 8×10^5 COS-7 cells in a 60-mm tissue culture plate (Falcon, Franklin Lakes, NJ, U.S.A.), the cells were transfected with 10 μg of either pCI-neo (control), pCI-FeHGF or pCI-dFeHGF using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The conditioned media were collected 48 hr after transfection.

Biological assay: A proliferation assay was performed by the method of Lee *et al.* [5] with some modifications. Briefly, BNL CL.2 cells were seeded in 96-well culture plates (Falcon) at a density of 10^4 cells/100 μl/well. After an overnight culture, the medium was changed to EMEM containing 1% FBS. Ten microliters of EMEM containing 1% FBS (negative control) and 1, 3 or 5 μl of conditioned medium (all diluted to 10 μl with EMEM containing 1% FBS) were added to triplicate wells. The addition of conditioned medium was repeated 2 days later, and viable cell numbers were determined by a colorimetric method (WST cell proliferation assay system, TaKaRa) after incubation for another two days. Absorbance at 450 nm was determined using an ELISA reader (Biorad, Hercules, CA, U.S.A.) 1 hr after addition of the WST solution.

Scatter activity was measured according to the method of Stoker and Perryman [22]. Briefly, MDCK scatter activity was assayed in 96-well plates using serial 2-fold dilutions of conditioned medium in 50 μl of EMEM containing 5% FBS. MDCK cells suspended in EMEM containing 5% FBS were seeded in 96-well culture plates (Falcon) at a density of 3,000 cells/100 μl/well and incubated for 24 hr. After fixation with glutaraldehyde (Wako, Osaka, Japan), the cells were stained with Giemsa's solution (Merck, Darmstadt, Germany), and photographed under a light microscope.

RESULTS AND DISCUSSION

Isolation of the FeHGF cDNA and screening of deleted variants: We cloned the FeHGF cDNA from leukocytes by RT-PCR with the RACE method. The complete nucleotide sequence of the cloned FeHGF cDNA was deposited in GenBank-DDBJ-EMBL, accession no. AB080187. The cloned FeHGF consists of 3,430 nucleotides with 76 bp of a 5' untranslated sequence, followed by 2,187 bp of an open reading frame (ORF) coding for 728 amino acids, and 1,167 bp of a 3' untranslated sequence. The transcription initiation site of the human HGF gene is located 76 bp upstream of the translational start codon [11]. This suggests that we might have cloned the FeHGF cDNA containing the transcription initiation site. The 3' flanking sequence is 1,167 bp, in contrast to the 3.58 kb reported previously for human and rat HGF cDNAs [17, 23]. It is possible that an alternative splice variant of the 3' non-coding region was amplified. Kobayashi *et al.* [4] have reported on the cloning of FeHGF cDNA isolated from the liver. They cloned a FeHGF cDNA fragment whose nucleotide length was 2,257 bp. We have cloned full-length FeHGF cDNA containing a 5' and 3' untranslated region. Our sequence data was identical to the previously reported sequence for the ORF.

In addition to the HGF cDNA reported originally, a major variant, which lacks 15 nucleotides encoding 5 amino acids in the first kringle domain, has been isolated from the human, mouse, and canine [9, 17–20]. A comparison of HGF and the dHGF revealed that the deletion significantly altered the biological activities; dHGF is 2 to 3 times more potent than HGF in the proliferation activity [21]. Hence, we assumed that dHGF might be more effective than HGF to develop HGF-therapy for organ diseases, and tried to isolate variant clones. The deleted variants were identified by PCR with the amplified fragment of full-length FeHGF cloned into the pGEM-T Easy vector as templates. Figure 1A shows the screening of variant clones; the clones that yielded shorter PCR products were variants lacking 15 bp. We screened 30 clones, three of which yielded shorter PCR products than the original FeHGF. Sequencing of the three variant clones revealed a 15-bp deletion in the first kringle domain and resulted in the deletion of 5 amino acids in the first kringle domain (Fig. 1B); the other sequences were completely identical to the original FeHGF. The deleted sequence is CTTTTTGCCTTCGAG, which is identical to that reported previously for human and mouse variant HGF cDNAs [18–20]. The missing sequence is similar to the conserved 3' acceptor sequence at the splice junction (T/C)n N (T/C)AG [14]. Hence, the spontaneous deletion observed in FeHGF is thought to be generated during RNA splicing.

Biologically active FeHGFs expressed in COS-7 cells: To investigate the functions of the isolated FeHGFs, both FeHGF and dFeHGF cDNAs were subcloned into a mammalian expression vector and transfected into COS-7 cells. The conditioned media were recovered after two days, and used for the HGF activity assay. The proliferation assay was performed using BNL CL.2 cells, differentiated from mouse hepatocytes, which exhibit a proliferative response to HGF [5]. As shown in Fig. 2, the conditioned media from cells transfected with the plasmids (pCI-FeHGF and pCI-dFeHGF) stimulated cell growth in a dose-dependent manner, while conditioned medium from a negative control (pCI-neo) had no such effect. Shima *et al.* [21] have reported that dHGF is 2 to 3 times more potent than the original HGF in stimulating DNA synthesis in rat hepatocytes and epithelial cells, such as LLC-PK1 (pig kidney epithelial cells). However, our results showed that dFeHGF was slightly more potent, but there was no significant difference in the proliferation activity of BNL CL.2 cells (Fig. 2). Biological differences between the two HGFs were observed at higher concentrations (>10 ng/ml) [21]. Another study reported that the specific activities of the two HGFs expressed transiently in COS-1 cells are almost the same [19]. It is likely that the concentrations of transiently expressed FeHGFs in conditioned medium from COS-7 cells were in the range <10 ng/ml, in which the two HGFs showed almost the same activity, or BNL CL.2 cells might be a cell line which does not show potent activity for dHGF. Further studies of recombinant FeHGFs, purification of proteins or high-level expression of recombinant FeHGFs using a baculovirus expression system might reveal the biological differences between the two FeHGFs.

HGF is also known as a scatter factor and we observed scatter activity using MDCK cells. When conditioned media from COS-7 cells transfected with pCI-FeHGF and pCI-dFeHGF were added to the cultures of MDCK cells, the motility of the MDCK cells was markedly enhanced resulting in morphological change and cell dissociation (Fig. 3). On the contrary, most of the cells were tightly packed when the conditioned medium from pCI-neo vector-transfected COS-7 cells was added. Scattering of the cells could be detected even when the conditioned medium was diluted 32-fold.

In this study, we isolated the 5-amino-acid-deleted variant of FeHGF cDNA from leukocytes and successfully expressed biologically active recombinant protein in COS-7 cells. The findings reported here would be a valuable resource for understanding the biological activity of FeHGF and for developing therapeutic applications for cat organ diseases.

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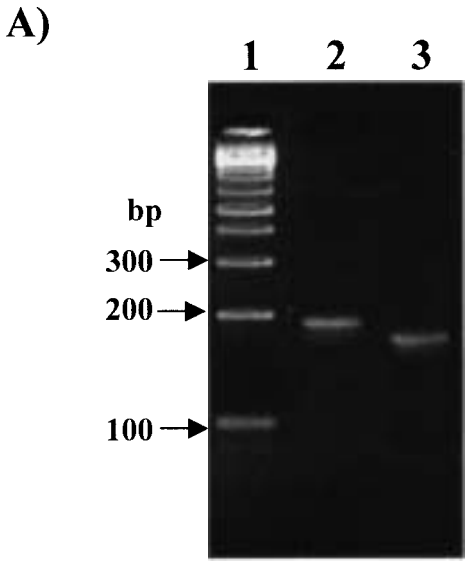

B)

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FeHGF:  457 ATGATACCACATGAACACAGCTTTTTGCCTTCGAGCTATCGGGGTAAAGACCTA 510
dFeHGF: 457 ATGATACCACATGAACACAG----------------CTATCGGGGTAAAGACCTA 495
            MetIleProHisGluHisSerPheLeuProSerSerTyrArgGlyLysAspLeu
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Fig. 1. A) Screening of the 15-bp deleted variant FeHGF cDNA clones. PCR was performed using primers designed to amplify a HGF fragment including the deleted region in the first kringle domain. The PCR products were separated through a 4% agarose gel. Lane 1, 1 kb plus ladder (Invitrogen); lane 2, PCR amplification of FeHGF clone; lane 3, PCR amplification of dFeHGF clone. B) Comparison of the nucleotide and amino acid sequences of FeHGF and dFeHGF in the 5-amino-acid-deleted region. Deleted nucleotide and amino acid sequences are shown by the dashes and bold letters, respectively.

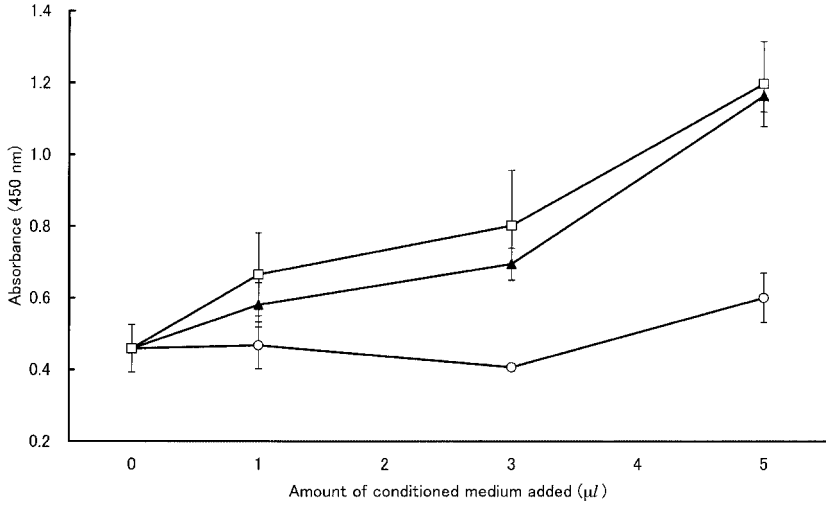

Fig. 2. Dose response effect of FeHGFs, expressed in COS-7 cells, on the proliferation of BNL CL.2 cells (a mouse cell line). Cells were seeded at a density of 10^4 cells/100 μl medium/well in EMEM containing 10% FBS in 96-well culture plates. After changing the medium to EMEM with 1% FBS the next day, 1, 3, and 5μl of the conditioned media from COS-7 cells expressing FeHGF (closed triangles) and dFeHGF (open squares) or pCI-neo vector transformed control medium (open circles) were added and refreshed 2 days later, using triplicate wells. Two days after the last addition, the viable cell numbers were determined by a colorimetric method. Each value represents the mean ± SD of triplicate measurements.

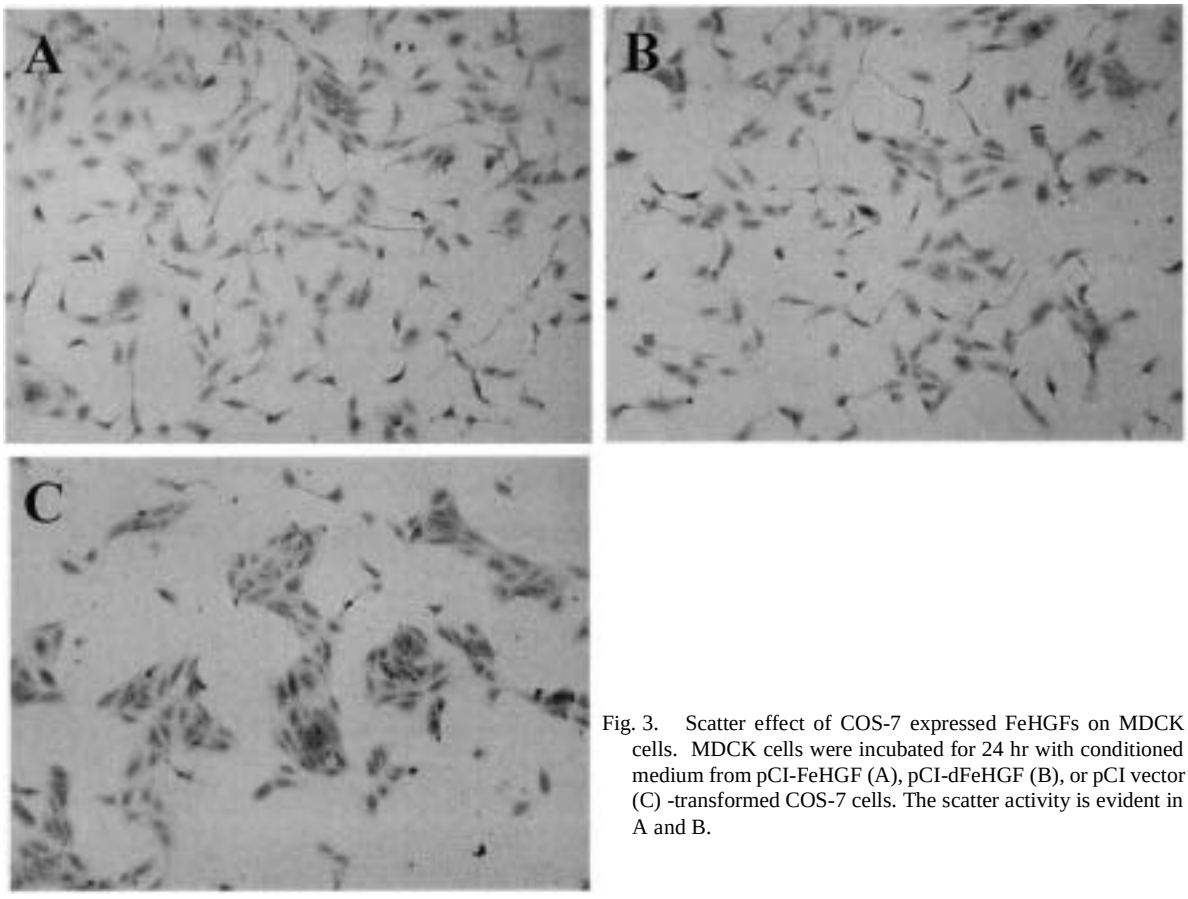

Fig. 3. Scatter effect of COS-7 expressed FeHGFs on MDCK cells. MDCK cells were incubated for 24 hr with conditioned medium from pCI-FeHGF (A), pCI-dFeHGF (B), or pCI vector (C) -transformed COS-7 cells. The scatter activity is evident in A and B.

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