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Kringle 1 of Human Hepatocyte Growth Factor Inhibits Bovine Aortic Endothelial Cell Proliferation Stimulated by Basic Fibroblast Growth Factor and Causes Cell Apoptosis

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Hepatocyte growth factor (HGF), also known as scatter factor, is a mesenchymal or stromal-derived mediator with angiogenic activity. There are four kringle domains in its amino terminus. They display considerable sequence similarity with those of angiostatin, an angiogenesis inhibitor. We now describe that the recombinant kringle1 of HGF (HGFK1) inhibits bovine aortic endothelial (BAE) cell proliferation stimulated by basic fibroblast growth factor in a dose-dependent manner, with an ED_{50} of approximately 0.7 μg/ml, while ED_{50} of angiostatin is 3 μg/ml. Treatment of BAE cell with HGFK1 caused cell apoptosis. This report thus constitutes the first demonstration that kringle1 of HGF is a selective inhibitor for BAE cell proliferation stimulated by bFGF.

Key Words: **kringle; hepatocyte growth factor; angiostatin; angiogenesis inhibitor; apoptosis.**

Kringle domain is a kind of protein module, which usually consists of about 80 amino acids (1). There are two conservative clusters of amino acids and six conservative cysteines in it so as to form certain structure constrained by three pairs of inner disulfide bonds. Kringle domains were found in many proteins from one to about forty copies (2). For example, there are two copies in tissue plasminogen activator, five in plasminogen, and 15 to 30 in apolipoprotein A. Previously, kringle modules in these proteins are thought to function as recognition units for binding of other proteins in solution and on cells (1).

Angiostatin is a circulating angiogenesis inhibitor that has been found to be part of plasminogen (3). It contains the first four kringles of plasminogen. The exact inhibitory mechanism is not completely clear yet. Each kringle of angiostatin and the five kringle of plasminogen were all cloned and proved to be angiogenesis inhibitor, with the fourth kringle to be exceptional (4). Kringle 5 is even more potent in inhibiting the growth of endothelial cell than angiostatin (5). Later, kringle 2 of prothrombin was found to be an angiogenesis inhibitor (6), too, while completely different results were reported as to whether apolipoprotein A has inhibitory effect on endothelial cell growth or not (7, 8).

Hepatocyte growth factor (HGF), also known as scatter factor, is a mesenchymal or stromal-derived mediator with angiogenic activity (2). There are four kringles in its amino terminus, showing a remarkable sequence similarity with those of plasminogen. In order to see whether they exhibit anti-angiogenic activity as kringles of plasminogen or not, kringle 1 of HGF was cloned and expressed in *E. coli*. We demonstrated for the first time that kringle 1 of hepatocyte growth factor was a potent angiogenesis inhibitor and that treatment of BAE cells with kringle 1 of HGF caused cell apoptosis.

MATERIALS AND METHODS

Reagents and materials. DMEM and Trypsin/EDTA were purchased from GIBCO BRL (Rockville, MD). Fetal calf serum were from Hyclone (Logen, UT). Human fresh placenta was from Shanghai No. 1 women and children healthcare hospital. Angiostatin was prepared in our lab as described previously (9).

Abbreviations used: bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; HGFK1, kringle 1 of HGF; BAE cell, bovine aortic endothelial cell; BCE, bovine capillary endothelial cell; HUVEC, human umbilical vein endothelial cell; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle medium; GSH, reduced glutathione; GSSG, oxidized glutathione; IPTG, isopropyl thio-β-D-galactopyranoside; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PI, propidium iodide; PCR, polymerase chain reaction; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-Tetraliumbromide.

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RT-PCR. Human fresh placenta was homogenized in liquid nitrogen. Total RNA was isolated using TRIZOL reagent (GIBCO BRL). Total RNA was used as the template for cDNA synthesis using Superscript RNase H^- transcriptase (GIBCO BRL) according to manufacturer's instructions. PCR was performed with Ex-Taq DNA polymerase (TaKaRa) according to manufacturer's instructions. The synthetic oligonucleotides were obtained from Shanghai Sangon Co. Ltd. (Shanghai, China). The primers used were as follows: RT primer: 5′ GCAGGTCATGCATTCAAC 3′, primers used for amplifying cDNA encoding HGFK1, sense primer: 5′ GGAATTCCATATGAACTGCATCATTGGTAAAGGA 3′, antisense primer: 5′ ATCGAAGCTTATTAATGGTGGTGATGGTGGTGGCAGGTCATGCATTC, a NdeI site was included in the sense primer, a HindIII site, a stop coden and six histidine coden were incorporated into antisense primer. PCR product of 290 bp was amplified with this primer sets. Reaction were incubated in PE480 thermal cycler (Perkin-Elmers, NJ) for 35 cycles: denaturation 30 s, 94°C; annealing 30 s, 52°C; extension 30 s, 72°C. PCR product was run on 1% agarose gel in TBE (10 mM Tris-Borate, 1 mM EDTA, pH 8.0), and visualized by ethidium bromide staining.

Gene construction and expression. The amplified cDNA fragment was ligated into the NdeI/HindIII sites of *Escherichia coli* expression vector pET24-a (Novagen), resulting in the expression plasmid pETHK1. pETHK1 was transformed into *E. coli* BL21(DE3) and HGFK1 expression was induced by 1 mM IPTG. Cells were harvested by centrifugation for 30 min at 4000 g.

Purification and refolding of recombinant HGFK1. Cells were resuspended in 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 5 mM DTE, and lysozyme was added to the final concentration of 0.5 mg/ml. Cells were incubated at 4°C for 30 min, then were disrupted by sonic homogenizer for 10 s for six times with 30 s interval each time. After centrifugation at 4°C, 12000 g for 30 min, the pellet was collected and resuspended in 8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris–HCl, pH 8.0. Centrifuged again as before, the supernatant was loaded on a Ni^{2+}-nitrilotriacetic acid-agarose column (Qiagen). The recombinant protein was eluted from the column according to manufacturer's instructions. To achieve refolding, the purified protein were adjusted to pH 8.0 and DTT was added to the final concentration of 0.1 M. Following incubation at room temperature for 2 h, the solution was added to refolding buffer (0.1 M Tris–HCl, pH 8.0, 0.5 M arginine, 5 mM EDTA, 1 mM GSSG, 5 mM GSH) with the ratio of 1:200 (v/v). After 24 h incubation at room temperature, the renatured protein was dialyzed against distilled water for 24–48 h and lyophilized.

Bovine aortic endothelial cell proliferation assay. Bovine aortic endothelial cells were isolated as previously described (10) and were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics. Monolayer of BAE cells growing in 60 mm dish were dispersed in 0.05% trypsin solution. Cells were resuspended with DMEM containing 10% FCS. Approximately 3000 cells in 200 μl were added in triplicate to each well of 96-well tissue culture plates and incubated at 37°C (in 10% CO_2). Cells adhere to the plate in about 2–3 h. The medium was replaced with 100 μl of fresh DMEM containing 2% FCS, and samples of HGFK1 or angiostatin were added to each well. After 30 min incubation, another 100 μl DMEM containing 2% FCS and 10 ng/ml bFGF was added to each well. After 72 h incubation, 10 μl MTT (100 mg/ml) was added to each well and incubated for another 4 h at 37°C, 10% CO_2. 180 μl medium was pipetted out from each well and 50 μl DMSO was added, vortex gently to dissolve the pellet. The absorbance of $A_{570\,nm}$, which correlates to the number of cells, was measured with microplate reader (Model 450, Bio-Rad).

Flow cytometry apoptosis analysis by propidium iodide (PI) assay. All the procedures were followed as previously reported (11). Briefly, BAE cells were maintained in DMEM supplemented with 10% FCS till to 60–70% confluence. The medium was changed with DMEM supplemented with 0.5% FCS containing 2 μg/ml HGFK1. An hour later, bFGF was added to the final concentration of 5 ng/ml and cells were cultured overnight at 37°C in 10% CO_2. Cells were trypsinized and washed gently with PBS, and then were fixed with 70% ice cold ethanol for 30 min. Cells were collected by centrifugation. 200 μl 1 mg/ml RNase was added and incubated at 37°C for 30 min, then 400 μl PI (500 μg/ml) was added and incubated dark at 4°C for 30 min. Cells were assessed by FACStar plus flow cytometer (Beckton-Dickinson) for apoptosis and the results were analyzed with CellQuest software.

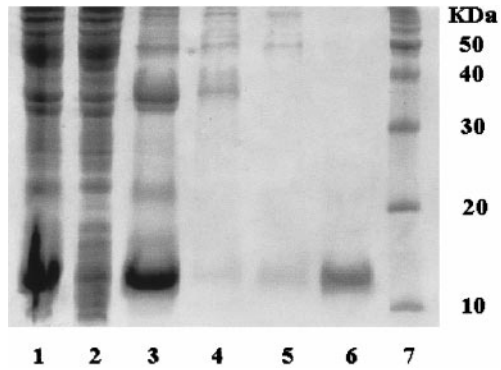

FIG. 1. SDS–PAGE analysis of different fractions of protein. Samples were loaded onto a 15% gel followed by staining with Coomassie Blue. Lane 1, protein of total bacteria; lane 2, soluble protein in *E. coli;* lane 3, insoluble protein in *E. coli;* lane 4, flow through; lane 5, elution by washing buffer, pH 6.3; lane 6, purified HGFK1; lane 7, protein markers.

RESULTS

Purification and characterization of HGFK1. Recombinant protein including amino acids residues from 127–214 of HGF plus six histidines was expressed in *E. coli* and purified to homogeneity using Ni^{2+}-nitrilotriacetic acid-agarose column and was refolded *in vitro* (Fig. 1). Under reducing condition, HGFK1 migrated in PAGE with molecular mass of about 11 kDa, corresponding to the predicted molecular mass.

Inhibition of BAE cell proliferation with recombinant HGFK1 and human angiostatin. HGFK1 and angiostatin were assayed for their inhibitory activities on bovine aortic endothelial cell growth stimulated by bFGF. As shown in Fig. 2, both angiostatin and HGFK1 inhibited BAE cell proliferation in a dose-dependent fashion. The concentration of HGFK1 required to reach 50% inhibition (ED50) was about 0.7 μg/ml, while ED50 of angiostatin is 3 μg/ml, close to data previously reported (4). While both HGFK1 and angiostatin has no inhibitory activities on fibroblast cell line Balb/c3T3 and hepatoma cell line HepG2 (data not shown), which suggests that their inhibitory activity is specific to endothelial cell.

Cell apoptosis detection. As shown in Fig. 3, BAE cells were treated with 2 μg/ml HGFK1 overnight in 0.5% FCS, about 23% cells underwent apoptosis, compared with 3% of untreated cells.

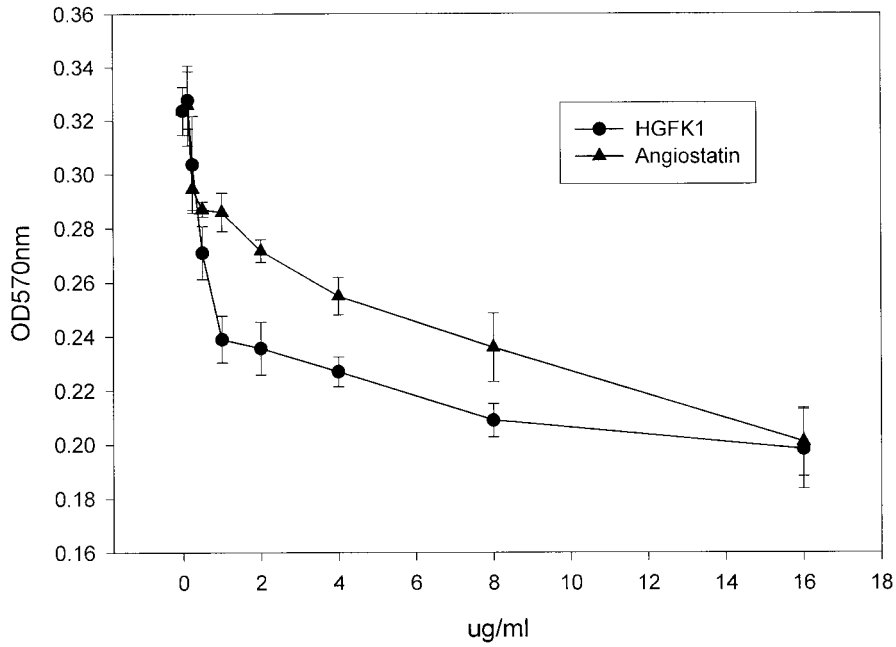

FIG. 2. HGFK1 and angiostatin inhibit the proliferation of BAE cells stimulated by bFGF. Values represent the mean of three determinations (±SE) by MTT assay.

DISCUSSION

Kringle domains were found in many proteins, most of which are important molecules mediating coagulation and fibrinolysis or lipid transportation. Therefore, kringle modules were thought to function as recognition unit for protein and protein or protein and cell surface (1). However, in recent years, several proteins containing kringle module were found to inhibit specifically the proliferation of endothelial cell. Angiostatin, which is composed by the first four kringles of plasminogen was found to inhibit endothelial cell proliferation stimulated by bFGF (9) and except kringle 4, each kringle of plasminogen had more or less anti-angiogenesis activity (5). Lee *et al.* (6) reported that

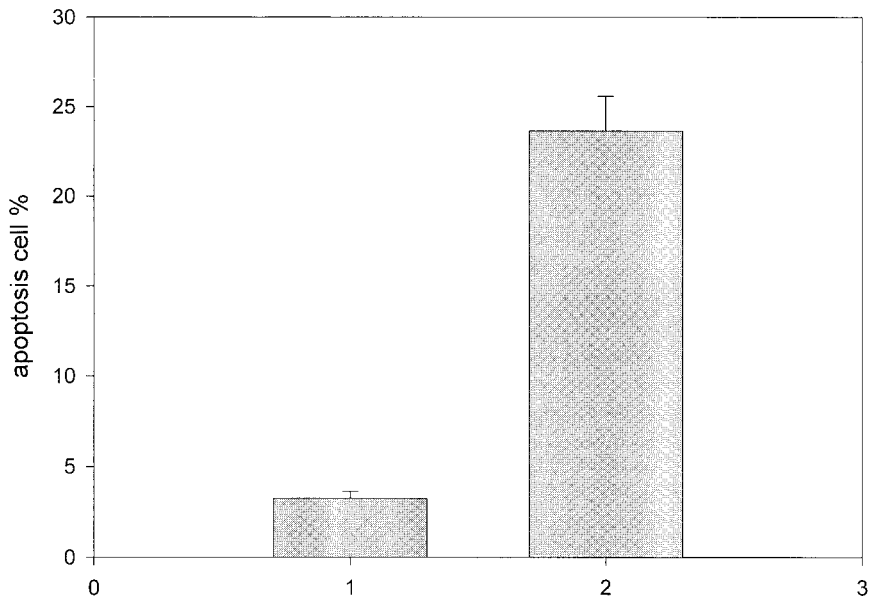

FIG. 3. HGFK1 induces apoptosis in BAE cells. BAE cell monolayers were exposed overnight to HGFK1 (2 μg/ml) in 0.5% serum, and apoptosis was assessed by propidium iodide. Data were pooled from two experiments and results were expressed as the mean percentage (±SEM) of cells with evidence of apoptosis. Column 1, control; column 2, cells treated with HGFK1 (2 μg/ml).

TABLE 1

Amino Acids Sequence Alignment of Kringle Domains of Plasminogen and HGF

```
PK1          CKTGNGKNYRGTMSKTKNGITCQKWSSTSPHRP-RFSPATHPSEGLEENYCRNPDNDPQG
PK5          CMFGNGKGYRGKRATTVTGTPCQDWAAQEPHRHSIFTPETNPRAGLEKNYCRNPDGDVGG
PK4          CYHGDGQSYRGTSSTTTTGKKCQSWSSMTPHRH-QKTPENYPNAGLTMNCRNPDAK-G
HK1          CIIGKGRSYKGTSITKSGIKCQPWSSMIPHEH-SFLPSSYRGKDLQENYCRNPRGEEGG
PK2          CMHCSGENYDGKISKTMSGLECQAWDSQSPHAH-GYIPSKFPNKLKKNYCRNPDREL-R
PK3          CLKGTGENYRGNVAVTVSGHTCQHWSAQTPHTH-NRTPENFPCKNLDENYCREPDGKR-A
             *  : *..* *. : * .*  ** * :  **.:    *.  : ..*  ****:*:.   :

PK1          PWCYTTDPEKRYDYCDILEC
PK5          PWCYTTNPRKLYDYCDVPQC
PK4          PWCFTTDPSVRWEYCNLKKC
HK1          PWCFTSNPEVRYEVCDIPQC
PK2          PWCFTTDPNKRWELCDIPRC
PK3          PWCHTTNSQVRWEYCKIPSC
             ***.*::. .:::.*::: *
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Note. The sequences of five kringle domains were aligned according to their conserved cysteines and amino acids clusters. PK represents the kringle of plasminogen, while HK represent the kringle of HGF. Conserved amino acid residues are marked with "*"; highly conserved residues are marked with ":"; less conserved residues are marked with ".".

kringle2 of prothrombin had inhibitory activity against BCE proliferation. Reports about apolipoprotein A were contradictory. Lou *et al.* (7) found that it displayed no such kind of activity despite that its kringle showed great sequence similarity with kringle 4 of plasminogen; while Trieu *et al.* (8) reported that recombinant apolipoprotein A with 18 kringle4 repeats impaired angiogenesis in animal model experiment. These facts, combined together, implied that some kringle domains manifested anti-angiogenic activity. Amino acid sequence alignment of the kringle domains of plasminogen and HGFK1 showed that they displayed considerable similarity (40–50% identity, Table 1). We thus wondered whether HGFK1 was also an angiogenesis inhibitor. Our result in this paper demonstrated for the first time that HGFK1 is indeed an angiogenesis inhibitor.

In the examples above, the "mother" proteins display no inhibitory activity on angiogenesis and HGF is even a growth factor with angiogenic activity. Therefore, we proposed that in "mother" proteins the functional elements of kringle were shielded and could not interact with endothelial cell effectively. While after kringle domains were set free from "mother" molecules, they were more freely to access the binding sites on endothelial cell membrane and manifested anti-angiogenic function. This phenomenon might be regarded as the result of the diversity of protein function in molecule evolution.

Then, why different kringles inhibited the proliferation of endothelial cell in different degree? We contributed it to their subtle differences in amino acids sequence, as well as in their second structure. It has been reported that lysine-binding site in kringle was not related with inhibitory activity (4); the treatment of kringle structure by reductive reagent may compromise its inhibitory activity (4); kringle 4 is distinctive among kringles of plasminogen for not having such inhibitory activity. Cao *et al.* (5) contributes it to two sets of consecutive lysine residues in its amino acids sequence. Therefore, much effort is needed to locate the exact amino acids clusters that count most.

As was seen in Fig. 2, HGFK1 was 3 times stronger than angiostatin in inhibiting the proliferation of endothelial cell. We believe that HGFK1 might share the same receptor with angiostatin on cell membrane with different Kd, which led to the different degree of their inhibitory activity. Moser *et al.* reported that ATP synthase on the membrane of HUVEC cell line was the receptor of angiostatin (12). They suggested that angiostatin bound to ATP synthase and rendered endothelial cells more vulnerable to hypoxic challenge and eventual irreversible cell damage (12). However, direct evidence has not been reported as to whether binding of ATP synthase to angiostatin could block its enzymatic activity or not.

Angiostatin was reported to induce endothelial cell apoptosis (13). We here demonstrated that HGFK1 could induce BAE cell apoptosis, too. Little is known about their mechanism. Liu *et al.* reported that angiostatin treatment of ECV304, a well characterized human umbilical endothelial cell line, could block VEGF or bFGF induced down regulation of caveolin-1 (14). Caveolin-1 is the marker protein of caveolae, whose function was regarded to be transportation of materials from outside to inside cell membrane (15). ATP synthase was found in caveolae (16), which made us suspect that kringles in angiostatin and HGFK1 may inhibit cell proliferation stimulated by bFGF and induce cell apoptosis at least partly by disturbing material transportation through caveolae on cell membrane.

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