Hepatocyte Growth Factor Fusion Protein Having Collagen-Binding Activity (CBD-HGF) Accelerates Re-endothelialization and Intimal Hyperplasia in Balloon-injured Rat Carotid Artery

Nana Ohkawara1, Hiroki Ueda1, Shohei Shinozaki1, Takashi Kitajima2, Yoshihiro Ito2,3, Hiroshi Asaoka1, Akio Kawakami1, Eiji Kaneko1, and Kentaro Shimokado1

1Geriatrics and Vascular Medicine, Tokyo Medical and Dental University Graduate School, Tokyo, Japan.
2Kanagawa Academy of Science and Technology, KSP East 309, Kanagawa, Japan.
3The Institute of Physical and Chemical Research, Saitama, Japan.

Aim: Hepatocyte growth factor (HGF) is known to stimulate endothelial cell proliferation. However, re-endothelialization is not enhanced when the native protein is administered to the injured artery, probably due to the short half-life of HGF at the site of injury. Therefore, the effects of an HGF fusion protein having collagen-binding activity (CBD-HGF) on re-endothelialization and neointimal formation was studied in the balloon-injured rat carotid artery.

Methods: The left common carotid artery of male Sprague-Dawley rats was injured with an inflated balloon catheter, and then treated with CBD-HGF (10 μg/mL), HGF (10 μg/mL) or saline (control) for 15 min. After 14 days, the rats were injected with Evans blue and sacrificed.

Results: The re-endothelialized area was significantly greater in the CBD-HGF-treated rats than in the control or HGF-treated rats. Neointimal formation was significantly more pronounced in the CBD-HGF treated rats than in other rat groups. Both HGF and CBD-HGF stimulated proliferation of vascular smooth muscle cells as well as endothelial cells in vitro. Consistent with this, cultured smooth muscle cells were shown to express the HGF receptor (c-Met).

Conclusion: CBD-HGF accelerates re-endothelialization and neointimal formation in vivo. CBD fusion protein is a useful vehicle to deliver vascular growth factors to injured arteries.


Key words: Smooth muscle cells, Endothelial cells, c-Met, Drug delivery system

Introduction

Neointimal hyperplasia occurs in response to various vascular injuries, including those associated with percutaneous transluminal coronary angioplasty. This process involves different steps, including smooth muscle cell (SMC) dedifferentiation, migration, proliferation, and production of extracellular matrix. Among them, smooth muscle proliferation represents a critical step, and inhibition of smooth muscle proliferation with rapamycin has been shown to effectively prevent intimal hyperplasia and re-stenosis. Endothelial cells (ECs) produce substances that suppress smooth muscle proliferation, such as heparin sulfate and cerebral natriuretic peptide. Thus, accelerated re-endothelialization is favorable for reducing intimal hyperplasia. Administration of recombinant vascular endothelial cell growth factor (VEGF) and heparin-binding EGF, or gene transfer of these factors has been shown in several studies to effectively accelerate re-endothelialization and inhibit neointimal formation. On the other hand, contradictory findings have also been reported. Basic fibroblast growth factor (bFGF) stimulates the proliferation of not only ECs, but also SMCs, and therefore, aggravates intimal hyperplasia.

HGF was previously reported as an endothelial cell-specific growth factor with no effects on other vascular cells. Subsequently, it was shown that HGF...
stimulates the migration of SMCs\textsuperscript{10}. Recently, HGF was reported to stimulate the proliferation of a certain population of SMCs expressing c-Met in injured arteries\textsuperscript{10}. Gene transfer of HGF was shown to accelerate re-endothelialization and inhibit neointimal formation in an animal model\textsuperscript{12}. However, recombinant HGF protein showed little or no effect on re-endothelialization \textit{in vivo}, probably due to the short half-life of HGF in the arterial wall\textsuperscript{12}.

Two chimera proteins of growth factors and the collagen-binding domain of fibronectin (FNCBD; hereafter abbreviated as CBD) have been reported to stay bound with the collagen matrix for longer periods of time than native growth factors, and to exert intact growth factor activities. Such a chimera of epidermal growth factor (FNCBD-EGF) was found to bind to collagen with a high binding affinity \textit{in vitro}\textsuperscript{13}, and to accumulate in the intima of injured arteries for longer than 24 hours\textsuperscript{14}. A fusion protein of CBD and VEGF\textsubscript{121} (FNCBD-VEGF), but not VEGF\textsubscript{121}, was also found to remain bound to the collagen matrix and stimulate both ECs and endothelial precursor cells, even after extensive washing of the collagen matrix\textsuperscript{15}. However, it has still not been clarified whether these two chimera proteins might also show any effects on injured arteries \textit{in vivo}.

We prepared a chimera protein of CBD and HGF (CBD-HGF)\textsuperscript{16}. This protein was shown to bind to the collagen matrix and stimulate the proliferation of human umbilical vein endothelial cells as potently as native HGF. In this study, we studied the effects of this protein on EC regeneration and intimal hyperplasia in injured rat carotid arteries, and found that CBD-HGF indeed accelerated re-endothelialization \textit{in vivo}. Unexpectedly, however, it did not suppress intimal hyperplasia. This was partly attributed to the mitogenic activity exerted by HGF on SMCs.

**Materials and Methods**

**Materials**

CBD-HGF was produced in the insect cell Sf9 using the baculovirus expression system and purified by heparin affinity\textsuperscript{16}. Recombinant human HGF and human bFGF were purchased from PeproTech Inc. (NJ, USA). CBD polypeptide (FNCBD) was prepared as described previously\textsuperscript{13}.

**Materials**

Dulbecco’s Modified Eagle medium (DMEM), L-glutamine, penicillin, streptomycin and PBS (−) were purchased from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Gibco (Auckland, New Zealand).

**Animal Experiments**

A total of 32 male Sprague-Dawley rats (300–400 g; Sankyo Labo Service Corporation, Tokyo, Japan) were anesthetized by intraperitoneal injection of pentobarbital sodium. The entire carotid artery was injured with a 2F Fogarty catheter (Baxter, IL, USA)\textsuperscript{17}. The catheter was replaced with an infusion cannula, and an approximately 1 cm length of the artery from the bifurcation was incubated with CBD-HGF (13 rats), HGF (6 rats) or saline (control, 13 rats) for 15 min. During this incubation, this segment of the carotid artery was isolated from the blood circulation by temporary ligation.

Two weeks after the balloon injury, the rats were intravenously injected with Evans blue dye (50 mg/kg) and perfusion-fixed with 10% neutralized formalin under a pressure of 110 mmHg. The artery was then dissected longitudinally, and the distance between the distal edge of the denuded area, which was stained blue, and the bifurcation was determined as the length of the re-endothelialized area.

All procedures were in accordance with the guidelines for the proper care and use of laboratory animals proposed by the Committee for Animal Welfare at Tokyo Medical and Dental University.

**Intimal Hyperplasia**

Thick sections of the artery were stained with hematoxylin–eosin and elastica Van Gieson. The ratio of the intima/media area (I/M) was determined by a computer sketching program (NIH image, ver. 1.62).

**Cell Culture**

Rat aortic endothelial cells were purchased from Cell Applications, Inc. (RAOEC, R304-05, CA, USA) and grown in DMEM supplemented with 20% FCS, 10 ng/mL bFGF, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO\textsubscript{2}/95% air at 37°C.

Rat SMCs were isolated from the thoracic aorta of Sprague-Dawley rats as reported before and maintained in Dulbecco’s Modified Eagle medium supplemented with 10% FCS, 100 U/mL penicillin and 10 μg/mL streptomycin\textsuperscript{18}. Cells were trypsinized and the cell number was determined in triplicate for each sample with a hematocytometer.

**Real-time PCR**

Real-time PCR was carried out using the Light Cycler (Roche Diagnostic GmbH, Mannheim, Germany) and the SYBR green PCR kit (Qiagen Inc., CA, USA), as described previously\textsuperscript{19}. The gene-specific oligonucleotide sequences were as follows. c-Met: for-
ward: 5'-CAG AGG CCT TGT ATG AAG T-3' reverse: 5'-CAT AAG TAG CGT TCA CAT GG-3'; GAPDH: forward: 5'-CTC ATG ACC ACA GTC CAT GC-3' reverse: 5'-CAG TGA GCT TCC CGT TCA G-3'.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed by analysis of variance followed by Fisher’s LSD.

Results

CBD-HGF Stimulated Endothelial Cell Proliferation in vitro

To study whether the biological activity of HGF is retained in CBD-HGF, the effects of HGF and CBD-HGF on cultured rat aortic endothelial cells were examined. CBD-HGF stimulated proliferation of the EC in a time-dependent manner, while FNCBD itself did not stimulate EC proliferation (Fig. 1A). CBD-HGF stimulated EC proliferation in a dose-dependent manner (Fig. 1B). The highest dose tested (100 ng/mL) yielded an EC cell number equivalent to that obtained with 50 ng/mL of native HGF.

CBD-HGF Accelerated Both Re-endothelialization and Intimal Hyperplasia of the Injured Artery

As reported before, the injured arterial segments were re-endothelialized from the bifurcation [7]. At 2 weeks after the injury induction, a slight but significant increase in the re-endothelialized area was observed in the CBD-HGF treated group as compared with that in the saline-treated group (Fig. 2). On the other hand, neither native HGF nor saline promoted re-endothelialization.

The re-endothelialized area of the carotid artery in the CBD-HGF-treated rats showed a significantly increased intimal thickness (Fig. 3). The intimal hyperplasia was more pronounced in the CBD-HGF-treated group at a point 3 mm from the bifurcation, which was covered by ECs only in this group, than in the other groups. Similarly, pronounced intimal hyperplasia was observed in the CBD-HGF-treated group at 5 mm from the bifurcation, where no evidence of re-endothelialization was observed in any groups. There was no difference in I/M ratio between these two points (data not shown).

SMCs Express the HGF Receptor and Show Mitogenic Response to Both CBD-HGF and HGF

It was reported previously that HGF does not
stimulate SMC proliferation. Recently, however, it was reported that it stimulated not only migration, but also proliferation of a subgroup of SMCs. We tested the mitogenic response of cultured SMCs to HGF and CBD-HGF. Both enhanced SMC proliferation in a dose- and time-dependent manner (Fig. 4). CBD-HGF was less potent than native HGF. HGF 50 ng/mL gave a significantly higher cell number than CBD-HGF 100 ng/mL ($p<0.01$), and HGF 5 ng/mL gave a higher cell number than CBD-HGF 10 ng/mL ($p=0.05$). FNCBD by itself did not stimulate SMC proliferation. Consistent with the mitogenic response of the SMCs to HGF, the cells also expressed c-Met mRNA (Fig. 5).
Discussion

In the present study, we showed for the first time that CBD-HGF enhances EC regeneration in injured arteries under conditions where native HGF does not accelerate re-endothelialization. Contrary to some previous reports that endothelial growth factors suppress intimal hyperplasia in injured arteries, CBD-HGF does not suppress intimal hyperplasia and rather aggravate it. Consistent with the in vivo findings, cultured SMCs express c-Met and show mitogenic response to both CBD-HGF and HGF.

CBD-HGF is the third fusion protein prepared using FNCBD, after FNCBD-EGF and FNCBD-VEGF, that has been shown to exhibit biological activity equivalent to that of the native growth factor. These chimeric growth factors show more prolonged binding to the collagen matrix than the corresponding native growth factors. FNCBD-EGF has been shown to persist at the site of de-endothelialization in arteries for 24 hours or longer. While CBD-HGF was less potent than native HGF on a molar basis, it accelerated re-endothelialization, even though native HGF did not affect wound healing in injured arteries in vivo. FNCBD by itself does not have any effect on endothelial cell proliferation, and this domain likely prolongs the retention of the growth factor at the site of injury, thereby enhancing its activity.

One of the unexpected findings of this study was that CBD-HGF did not suppress intimal hyperplasia, and
and in fact, significantly aggravated it. Hayashi and colleagues reported based on experiments on the same model, that local gene transfer of HGF completely suppressed intimal hyperplasia \(^1\). The precise reasons for the discrepant results are not clear. The potential reasons include differences in the procedures employed, local concentrations of HGF, and the duration of HGF exposure. In their report of HGF gene therapy, endothelial cells covered more than 80% of the denuded area in treated rats and 40% of the denuded area in control rats at 2 weeks after the injury \(^2\). In contrast, only mild recovery was observed in our experiments. Since the injury to the vascular wall caused by balloon catheters can vary in severity from simple deendothelialization to injury of the entire wall \(^3\), it is possible that the different re-endothelialization responses were related to different extents of injury of the vessels. In their report, the sum of the tissue concentrations of native rat HGF and transfected human HGF in the injured arteries was almost twice as much as that in uninjured arteries \(^2\). Although we did not estimate the tissue concentration of CBD-HGF, previous data obtained using FNCBD-EGF suggest that only a small portion of administered CBD-HGF remains at the site after 24 hours \(^4\). Although there is no evidence that differences in the concentrations of HGF affect the degree of intimal hyperplasia, concentration-dependent bidirectional effects of bFGF have been reported. At low concentrations, bFGF accelerates endothelial cell regeneration and suppresses intimal hyperplasia, while at higher concentrations, it accelerates intimal hyperplasia \(^5\). Gene transfer of HGF results in more prolonged HGF action than that following local administration of CBD-HGF. The timing of exposure of the SMCs to HGF might be important, because SMCs gradually change their phenotype and express c-Met after injury. However, it remains unclear how difference in the timing of exposure might yield the opposite effect on intimal hyperplasia.

Our \textit{in vitro} findings, taken together with other reports \(^6\), \(^7\), suggest that CBD-HGF might aggravate intimal hyperplasia, at least partly, by stimulating SMC proliferation. Some other mechanisms may also be involved in CBD-HGF-induced intimal hyperplasia. HGF has been shown to stimulate SMC locomotion \(^8\), which can contribute to intimal hyperplasia \(^9\). VEGF has been shown to aggravate intimal hyperplasia by inducing perivascular angiogenesis in a silicon-color injury model \(^10\), and HGF, a strong angiogenic factor, may aggravate intimal hyperplasia by the same mechanism. The precise contributions of the mitogenic effect exerted by HGF and the other mechanisms remain to be determined.

From a clinical point of view, proliferation of SMCs is an important part of wound healing of the artery. Inadequate SMC proliferation causes rupture of atheroma \(^11\). Therefore, CBD-HGF may be a useful therapeutic agent for accomplishing re-endothelialization with a solid neointima of SMCs and extracellular matrix in vascular diseases.

In summary, we report that the biological activity of HGF is retained in CBD-HGF, as demonstrated both \textit{in vitro} and \textit{vivo}. FNCBD polypeptide thus appears to be a useful vehicle to deliver vascular growth factors to injured arteries.

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\section*{References}

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