Impairment of Collateral Formation in Lipoprotein(a) Transgenic Mice

Therapeutic Angiogenesis Induced by Human Hepatocyte Growth Factor Gene

Ryuichi Morishita, MD, PhD*; Minako Sakaki*; Kei Yamamoto, MD, PhD; Sota Iguchi; Motokuni Aoki, MD, PhD; Keita Yamasaki, MD; Kunio Matsumoto, PhD; Toshikazu Nakamura, PhD; Richard Lawn, PhD; Toshio Ogihara, MD, PhD; Yasufumi Kaneda, MD, PhD

Background—Although lipoprotein(a) (Lp[a]) is a risk factor for atherosclerosis, no study has documented the effects of Lp(a) on angiogenesis. In this study, we examined collateral formation in peripheral arterial disease (PAD) model in Lp(a) transgenic mice. In addition, we examined the feasibility of gene therapy by using an angiogenic growth factor, hepatocyte growth factor (HGF), to treat PAD in the presence of high Lp(a).

Methods and Results—In Lp(a) transgenic mice, the degree of natural recovery of blood flow after operation was significantly lower than that in nontransgenic mice. Of importance, there was a significant negative correlation between serum Lp(a) concentration and the degree of natural recovery of blood flow ($P<0.05$). In addition, Lp(a) significantly stimulated the growth of vascular smooth muscle, accompanied by the phosphorylation of ERK. These data demonstrated the association of impairment of collateral formation with serum Lp(a) concentration. Thus, we examined the feasibility of therapeutic angiogenesis by using HGF, with the goal of progression to human gene therapy. Intramuscular injection of HGF plasmid resulted in a significant increase in blood flow even in Lp(a) transgenic mice, accompanied by the detection of human HGF protein. A significant increase in capillary density also was detected in Lp(a) transgenic mice transfected with human HGF compared with control ($P<0.01$).

Conclusions—Overall, a high serum Lp(a) concentration impaired collateral formation. Although the delay of angiogenesis in high serum Lp(a) might diminish angiogenesis, intramuscular injection of HGF plasmid induced therapeutic angiogenesis in the Lp(a) transgenic ischemic hindlimb mouse model as potential therapy for PAD. (Circulation. 2002; 105:1491-1496.)

Key Words: arteries ■ lipoproteins ■ angiogenesis ■ gene therapy ■ growth substances

Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million per year.1 In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Thus, the disease frequently follows an inexorable downhill course.2,3 One of the risk factors related to peripheral arterial disease is a high concentration of serum lipoprotein(a) (Lp[a]) because Lp(a) is also a risk factor for atherosclerosis, restenosis after angioplasty, ischemic heart disease, and cerebral stroke.4–9 Lp(a) consists of LDL with an additional protein component, apolipoprotein (apo[a]), a homologue of plasminogen.10 Lp(a) and apo(a) have been thought to enhance proliferation of human vascular smooth muscle cells (VSMCs).11–14 On the other hand, Lp(a) has been postulated to bind to endothelial cells and macrophages and to extracellular components such as fibrin and inhibit cell-associated plasminogen activation.15,16 Moreover, the inhibition of activation of transforming growth factor (TGF)-β by Lp(a) because of its strong homology to plasminogen has been reported.11 Because TGF-β is also known to have proangiogenic properties, Lp(a) might inhibit angiogenesis from this aspect. In contrast, there is less evidence for the stimulation of Lp(a) on angiogenesis, although the proliferation of VSMCs might promote collateral formation in the case that endothelial cells would be stimulated by Lp(a). Nevertheless, no

Received December 6, 2001; revision received January 15, 2002; accepted January 17, 2002.
From the Division of Gene Therapy Science (R.M., M.S., S.I., Y.K.), the Department of Geriatric Medicine (R.M., K. Yamamoto, M.A., K. Yamasaki, T.O.), and the Division of Biochemistry, Department of Oncology, Biomedical Research Center (K.M., T.N.), Osaka University Medical School, Osaka, Japan; and CV Therapeutics (R.L.), Palo Alto, Calif.
*Dr Morishita and Minako Sakaki contributed equally to this work.
Correspondence to Ryuichi Morishita, MD, PhD, Division of Gene Therapy Science, Osaka University Medical School, 2-2 Yamada-oka, Suita 565-0871, Japan. E-mail morishit@geriat.med.osaka-u.ac.jp
© 2002 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000012146.07240.FD

1491
report has documented the effects of a high serum concentration of Lp(a) on the pathogenesis of peripheral arterial disease because no animal model except double transgenic mice expressing human apo(a) and apoB genes is available. Thus, we examined the effects of high serum Lp(a) concentration on collateral formation with the use of an ischemic hindlimb model in Lp(a) transgenic mice. We demonstrated that a high serum concentration of Lp(a) abolished collateral formation after hindlimb ischemia.

In addition, recent clinical studies have demonstrated the feasibility of therapeutic angiogenesis through the use of angiogenic growth factors such as vascular endothelial growth factor (VEGF). The efficacy of therapeutic angiogenesis with the use of the VEGF gene has been reported in human patients with critical limb ischemia or myocardial ischemia.17-20 Thus, a strategy for therapeutic angiogenesis with the use of angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia or myocardial infarction. In addition to VEGF, we and others have reported the angiogenic property of hepatocyte growth factor (HGF) in a rabbit ischemia model.21-24 Thus, it is preferable to stimulate angiogenesis by HGF, not only in a normal model but also in other high-risk conditions such as Lp(a) transgenic mice, to consider human disease. Since we have started human gene therapy to treat peripheral arterial disease with the HGF gene from 2001, we chose HGF as the model of therapeutic angiogenesis in this study. We performed preclinical studies that demonstrated that injection of HGF plasmid induced therapeutic angiogenesis to treat peripheral arterial disease in a Lp(a) transgenic mouse hindlimb ischemia model, with the goal of progression to human clinical trials.

Methods

Experiment 1: Lp(a) Transgenic Mouse Hindlimb Ischemia Model

Construction of Plasmids

To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eucaryotic expression plasmid that utilizes the cytomegalovirus (CMV) promoter/enhancer.25 The vector used as a control was the CMV expression vector plasmid, which does not contain HGF cDNA.

In Vivo Gene Transfer With the Direct Injection Approach

Female FVB nontransgenic mice as negative control or Lp(a) transgenic mice (weight, 20 to 30 g; 15 weeks) were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.1 mL/100 mg). Human apo(a) transgenic mice were donated by Dr Lawn (CV Therapeutics, Palo Alto, Calif); apoB transgenic mice were donated by Dr Rubin (UC Berkeley, Calif). Briefly, Lp(a) transgenic mice were created by the mating of human apo(a) transgenic mice and human apoB transgenic mice.13,26-28 Human apo(a) YAC transgenic mice were created by insertion of human apo(a) YAC, including the 110 kb apo(a) gene, 70 kb apo(a)-like gene, and the 270 kb genomic DNA (YAC DNA) containing 5′-prime of plasmid encoding gene. Human apoB transgenic mice were created by insertion of 76 kb genomic DNA (P1 phagemid DNA) containing intact apoB gene. The background of both mice was FVB mice. Lp(a) transgenic mice were created by breeding of both homotransgenic mice and selection of double homotransgenic mice. The procedure to create an ischemic hindlimb model was previously described.19 "Naked" plasmid vector (500 μg/100 μL) containing control vector or human HGF vector was carefully injected directly into the ischemic limb of rats with a 27-gauge needle (Terumo) at 10 days after surgery (day 10). The injection volume of plasmid DNA was 100 μL.

To measure Lp(a) concentration by ELISA, the monoclonal antibody was labeled with horseradish peroxidase (Roche) as ImmunoPure Maleimide Activated Horseradish Peroxidase (Pierce).14 Lp(a) standard serum (Daichi Chemical) was used to calibrate the Lp(a) ELISA. To document successful transfection of HGF vector into the hindlimb, we also measured the production of human immunoreactive HGF.24,29 Four days after transfection, the concentration of HGF in the hindlimb was determined by enzyme-immunoassay with anti-human HGF antibody.29 The antibody against human HGF reacts with only human HGF and not with mouse HGF.29

Measurement of Blood Flow by Laser Doppler Imaging

Measurement of blood flow with a laser Doppler imager (LDI) has been previously described.24 Because laser Doppler flow velocity correlates well with capillary density,24 we measured the blood flow in the ischemic hindlimb by means of a laser Doppler blood flowmeter (Laser Doppler Imager, Moor Instruments). Indeed, we confirmed that the blood flow measured by LDI correlated well with capillary density.24 Low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as white. Perfusion analyses were performed sequentially in (a) the ischemic hindlimb transfected with control vector and (b) the ischemic hindlimb transfected with HGF vector. These laser images were qualitatively converted into histograms that represented the amount of blood flow on the x-axis and the number of pixels on the y-axis in the traced area. The average blood flow in each histogram was calculated for evaluation.

Measurement of Capillary Density

Alkaline phosphatase staining was used as a specific marker of endothelial cells in paraffin-embedded sections.24 To analyze the number of vessels in the right ischemic hindlimb transfected with HGF vector or control vector, 3 individual sections from the middle of the transfected muscle were analyzed. The number of vessels was counted under a light microscope (×100) in a blinded manner. The total number of vessels in each section was summed and expressed as number per section. At least 10 individual sections were evaluated in each muscle. The areas in which the number of vessels was quantified were randomly selected in the injected site and around the injected site. The animals were coded so that the analysis was performed without any knowledge of which treatment for each individual animal had received. Intraobserver variability was determined from triplicate measurements performed by one observer for all sections. The mean±SD difference among measurements made by the same observer was 1.8±0.2%. Interobserver variability was determined from measurements of 10 randomly selected sections performed by a second observer in addition to the first. The numeric difference between the measurements made by the two observers was 2.1±0.8%.

Experiment 2: In Vitro Culture Model

Cell Culture

Human aortic VSMCs (passage 3) were obtained from Clonetics Corp (San Diego, Calif) and cultured in modified MCDB131 medium supplemented with 5% fetal calf serum, 50 μg/mL gentamicin sulfate, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor, and 1 mmol/L hydrocortisone.12 An index of cell proliferation was determined with a WST-1 cell-counting kit (Wako). In the preparation of experiments for determination of cell count, the cells were grown to subconfluence. After subconfluence, the medium was changed to DSF (defined serum-free medium) supplemented with insulin (5×10^{-7} mol), transferrin (5 mg/mL), and ascorbate (0.2 mM/L).13 The cells were then incubated overnight. On day 1, the medium was changed to fresh DSF medium with fresh Lp(a). On day 3, an index of cell proliferation was determined with WST-1.
Western Blotting

Western blotting was performed for analysis of ERK with the use of a phosphospecific antibody. After treatment, the cells were extracted with lysis buffer (50 mmol/L Tris-Cl, 2.5 mmol/L EGTA, 1 mmol/L EDTA, 10 mmol/L NaF, 1% DOC, 1% Triton X-100, 1 mmol/L PMSF, 2 mmol/L Na3VO4). Samples containing 20 μg protein were run on 10% SDS-polyacrylamide gels, separated by SDS/PAGE, transferred to nitrocellulose membranes, and incubated with a polyclonal antibody to phosphospecific or total ERK (anti-human IgG, 1:1000, Cell Signaling TECHNOLOGY; Beverly, Mass) at 4°C overnight, respectively. To quantify and compare levels of proteins, the density of each band was measured by densitometry.

Statistical Analysis

All values are expressed as mean±SEM. ANOVA with subsequent Duncan’s test was used to determine the significance of differences in multiple comparisons. Differences with a value of P<0.05 were considered significant.

Results

Collateral Formation in Lp(a) Transgenic Mice

First, we used the hindlimb ischemia model to determine the effects of high serum Lp(a) concentration on blood vessel formation. Therefore, we examined the collateral formation after creating the hindlimb ischemia model in nontransgenic mice or Lp(a) transgenic mice. In Lp(a) transgenic mice, serum Lp(a) concentration was much higher in female mice (52.5±6.3 mg/dL, n=10) than in male mice (5.8±1.6 mg/mL, n=10), consistent with previous reports. In contrast, no Lp(a) could be detected in nontransgenic mice. Interestingly, the recovery of blood flow after surgery was significantly impaired in female Lp(a) transgenic mice, which exhibited extremely high serum Lp(a) concentrations as compared with nontransgenic mice lacking Lp(a) (P<0.01, Figure 1A). In addition, serum Lp(a) concentration was significantly negatively correlated with the recovery of blood flow after surgery as assessed by laser Doppler imaging (r=-0.638, P<0.05, Figure 1B). Atherogenic activity of Lp(a) was confirmed by in vitro experiments with human VSMCs. As shown in Figure 2, purified Lp(a) stimulated the growth of human VSMCs in a dose-dependent manner (P<0.01). Interestingly, at an equivalent serum Lp(a) concentration (5 μg/mL), Lp(a) demonstrated potent mitogenic activity on VSMC growth, suggesting that circulating serum Lp(a) acts as a stimulator of atherosclerosis. In addition, we examined the effects of Lp(a) on the ERK pathway, which is activated by growth factors and is involved in mediating cellular proliferation, transformation, and differentiation. As shown in Figure 3A, ERK was phosphorylated by Lp(a) in a dose-dependent manner. More importantly, ERK was phosphorylated from 5 minutes after the addition of Lp(a), and maximal tyrosine phosphorylation was detected at 5 to 10 minutes, whereas total ERK protein was not altered by treatment with Lp(a) (Figure 3B). Lp(a) appears to directly stimulate the growth of human VSMCs. Overall, these data clearly demonstrated that high serum Lp(a) concentration diminished the recovery of blood flow as a marker of collateral formation, probably through stimulation of VSMC growth.

![Figure 1. A. Blood flow in Lp(a) transgenic mice at 4 weeks after operation. Non-TG indicates muscle from ischemic hindlimb of nontransgenic mice, Lp(a) transgenic, muscle from ischemic hindlimb of Lp(a) transgenic mice. Each group contains 8 animals. Values are expressed as percent change of blood flow as compared with Non-TG. B. Correlation of percent change in blood flow and serum Lp(a) concentration in Lp(a) transgenic mice at 4 weeks after operation. Each group contains 8 animals.](Image)

![Figure 2. Stimulatory effect of Lp(a) on growth of human aortic VSMCs at 72 hours (n=8 per group). Control indicates cells treated with serum-free medium; 2.5, 5, and 10, Lp(a) (2.5, 5, and 10 μg/mL) added to VSMCs treated with serum-free medium. *P<0.01 vs control.](Image)
shown in Figure 4. Moreover, transfection of human HGF vector significantly increased capillary density as assessed by alkaline phosphatase (a marker of endothelial cells) staining in the ischemic hindlimb of Lp(a) transgenic mice around the injection site as compared with control vector (Figure 5, \( P<0.01 \)). These results demonstrated that transfection of human HGF vector into the ischemic hindlimb induced therapeutic angiogenesis, which could be applied for the treatment of peripheral arterial disease. Interestingly, the recovery of blood flow was significantly diminished in Lp(a) transgenic mice as compared with nontransgenic mice at 4 weeks after surgery (\( P<0.01 \), Figure 4). Similarly, capillary density was also significantly lower in Lp(a) transgenic mice than in nontransgenic mice at 4 weeks after surgery (\( P<0.01 \), Figure 5). Nevertheless, injection of human HGF vector by the HVJ-liposome method resulted in a significant increase in blood flow and capillary density from 2 weeks after transfection, which continued up to 4 weeks after transfection, as shown in Figures 4 and 5.

Discussion

Epidemiological studies have indicated Lp(a) to be an independent risk factor for cardiovascular disease.\(^4\)–\(^9\) Lp(a) and apo(a) have been thought to enhance proliferation of human VSMCs in culture by inhibiting activation of plasminogen to plasmin, thus blocking the proteolytic activation of TGF-\( \beta \).\(^1\)–\(^4\) In addition, Lp(a) has been postulated to bind to endothelial cells and macrophages and to extracellular components such as fibrin and inhibit cell-associated plasminogen activation.\(^15\)–\(^16\) Because the apo(a) gene is absent in rodents and nearly all subprimate species, there are limited available animal models. Currently, transgenic technology is an attractive system because it provides the means to isolate the effects of a single gene product on the complex pathophysiological processes that underlie thrombosis and atherosclerosis. In apo(a) transgenic mice, accumulation of apo(a) near the luminal surface of the aortic wall and the presence of apo(a) throughout the media of the vessel were observed.\(^2\) Activation of TGF-\( \beta \) is inhibited in the aortic wall and serum of mice expressing apo(a), possibly resulting in the development of atherosclerosis.\(^3\) In addition to the action of apo(a) on TGF-\( \beta \) activation, our previous experiments revealed the potential TGF-\( \beta \)-independent mitogenic mechanisms of Lp(a).\(^2\) Moreover, Lp(a) is more highly concentrated in the arterial wall than in plasma. Plasma-derived Lp(a) is known to penetrate human arteries\(^3\) and the arteries of mice.\(^2\) Therefore, a high serum Lp(a) concentration might affect blood vessel formation, such as collateral formation in
ischemic disease. Nevertheless, none of the studies has documented the effects of high serum Lp(a) concentration on collateral formation because of the lack of adequate animal models. Thus, in this study, we focused on the effects of high serum Lp(a) concentration on collateral formation in the Lp(a) transgenic mouse hindlimb ischemia model. As we expected, the present study demonstrated that a high serum Lp(a) concentration abolished collateral formation after surgery. Proliferation of VSMCs caused by the phosphorylation of ERK might be linked to the impairment of collateral formation. The present study demonstrated that Lp(a) inhibited angiogenesis, as Lp(a) stimulated the proliferation of VSMCs, in addition to the inhibition of activation of TGF-β.

This finding is supported by the previous observation that removal of Lp(a) from plasma may reduce coronary events such as restenosis.33,34

In patients with critical limb ischemia, amputation, despite its associated morbidity, mortality, and functional implications, is often recommended as a solution to the disabling symptoms. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling. From this viewpoint, a novel therapeutic strategy that uses angiogenic growth factors to expedite and/or augment collateral artery development has recently entered the realm of treatment of ischemic diseases. The clinical utility of gene therapy with the VEGF gene has been reported for the treatment of critical limb ischemia.17,18 In addition, recent studies raise the possibility of a new strategy, therapeutic angiogenesis with HGF instead of VEGF, for the treatment of patients with critical limb ischemia. HGF is a mesenchymederived pleiotropic factor that regulates cell growth, cell motility, and morphogenesis of various types of cells and is thus considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis. Recently, direct in vivo evidence of angiogenesis induced by recombinant HGF or HGF gene transfer was reported.21–24 Nevertheless, no report has examined the potential utility of HGF gene transfer to stimulate angiogenesis in a high serum Lp(a) concentration model that attenuated collateral formation, as presented in this study. Therefore, we examined the feasibility of therapeutic angiogenesis by using the HGF gene in an Lp(a) transgenic mouse limb ischemia model because a common complication of high serum Lp(a) concentration is peripheral vascular disease. Although in Lp(a) transgenic mice, measurement of the Doppler flow ratio between the ischemic and normal limb indicated that restoration of perfusion in the ischemic hindlimb was significantly impaired, a single intramuscular injection of HGF plasmid was sufficient to induce therapeutic angiogenesis even in the Lp(a) transgenic mouse hindlimb ischemia model.

Overall, high serum Lp(a) concentration impaired collateral formation in Lp(a) transgenic mice. Although the delay of angiogenesis in the presence of a high serum Lp(a) concentration might diminish angiogenic activity of transfection of an angiogenic growth factor gene, intramuscular injection of human HGF plasmid induced therapeutic angiogenesis in a hindlimb ischemia model in the Lp(a) transgenic mouse, as potential therapy for peripheral arterial disease.

Acknowledgments

This work was partially supported by a grant from the Japan Health Sciences Foundation, a Grant-in-Aid from the Ministry of Public Health and Welfare, a Grant-in-Aid for the Development of Innovative Technology, and a Grant-in-Aid from Japan Promotion of Science and through Special Coordination Funds of the Ministry of Education, Culture, Sports, Science, and Technology of the Japanese Government.

References


Impairment of Collateral Formation in Lipoprotein(a) Transgenic Mice: Therapeutic Angiogenesis Induced by Human Hepatocyte Growth Factor Gene
Ryuichi Morishita, Minako Sakaki, Kei Yamamoto, Sota Iguchi, Motokuni Aoki, Keita Yamasaki, Kunio Matsumoto, Toshikazu Nakamura, Richard Lawn, Toshio Oghara and Yasufumi Kaneda

Circulation. 2002;105:1491-1496; originally published online February 25, 2002; doi: 10.1161/01.CIR.0000121460.7240.FD
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/12/1491

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/