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Hepatocyte Growth Factor Is a Major Mediator in Heparin-Induced Angiogenesis

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Heparin has a potent angiogenic effect in experimental animals and patients with ischemic diseases; however, the precise mechanism behind this angiogenesis remains to be clarified. The aim of this study was to determine whether the administration of heparin affects the levels of heparin-binding angiogenic factors in human plasma, and to identify the molecule responsible for heparin-induced angiogenesis. Plasma levels of hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) were measured before and after administration of 100 U, 3,000 U or 10,000 U of heparin in patients with coronary artery disease. Administration of 3,000 U or 10,000 U of heparin caused significant increases in plasma HGF (40- and 54-fold, respectively), in absence of obvious increases in bFGF and VEGF levels. Furthermore, compared with the serum collected before heparin administration, the serum collected after heparin administration had more prominent growth-promoting and vascular tube-inducing properties on endothelial cells, and these increased activities were completely inhibited by neutralization of HGF, whereas neutralization of bFGF and VEGF had no effect. These findings suggest that HGF plays a significant role in heparin-induced angiogenesis.

Heparin has long been clinically prescribed as an anticoagulant and its therapeutic efficacy is well recognized. In addition to its anticoagulant property, heparin participates in the regulation of many cellular processes, particularly cell growth and differentiation, tumor metastasis, and lipoprotein metabolism (1). Furthermore, several reports have indicated that heparin has a potent angiogenic effect in experimental animals and in patients with coronary artery disease (CAD) (2–4). On the other hand, several angiogenic factors such as hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF), have a strong affinity for heparin. They are also reported to bind to extracellular matrices such as heparan sulfate proteoglycans (5–9), and to be stored as a complex with such molecules *in vivo* (7, 9–11). The interaction of these growth factors with extracellular matrices has been recently clarified, and the exogenous addition of heparin is considered to modulate their mutual binding *in vitro* (9,12–14). We hypothesized, therefore, that heparin administered *in vivo* may increase the plasma levels of these heparin-binding angiogenic factors, and promote angiogenesis via these molecules.

In this study, we demonstrate that the *in vivo* administration of heparin in patients with CAD causes a significant increase in HGF in the systemic circulation, and that the serum taken from patients receiving heparin has growth-promoting and vascular tube-forming properties on endothelial cells (ECs) *in vitro*, which are completely inhibited by neutralization of HGF. These findings suggest that HGF plays a significant role in heparin-induced angiogenesis.

MATERIALS AND METHODS

Pharmaceutical and biochemical reagents. Heparin sodium salt was purchased from Pharmacia Upjohn Co. Medium 199 and heparin sodium salt (grade I-A from porcine intestinal mucosa) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human bFGF, recombinant human VEGF, anti-human FGF basic neutralizing antibody, and monoclonal anti-human VEGF antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). Fetal calf serum (FCS) was purchased from GIBCO BRL (Grand Island, NY). Matrigel Basement Membrane Matrix was purchased from Collaborative Biomedical Products (Bedford, MA). Recombinant human HGF and polyclonal rabbit anti-human HGF neutralizing antibody were generously provided by Mitsubishi Chemical Co. (Yokohama, Japan).

Study population. After giving their informed consent, 30 patients with CAD undergoing cardiac catheterization for stable angina pectoris (n=18) or old myocardial infarction (n=12) were enrolled in the study. Patients with the diagnosis of unstable angina pectoris, recent and acute myocardial infarction were excluded from

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this study to prevent the effects of recent severe ischemia and thrombotic events on our results (15,16). Clinical data including age, sex, body weight, and prevalence of hypercholesterolemia and diabetes status were collected from the patients' medical records. No patient received heparin in the 24 hours preceding the study. This study was approved by the human research committee of our hospital.

Study protocol. Cardiac catheterization was performed by standard femoral or brachial approaches. After the insertion of arterial and venous sheaths, blood samples were collected just before the randomly assigned, single-blind administration of 10 ml of saline containing 100 U, 3,000 U or 10,000 U of heparin through the venous sheath. Blood samples were collected in the same manner, 15 minutes after the administration of heparin. Serum and plasma were prepared as follows: Serum (for experiments using ECs): The blood taken into a sterile blood collection tube was allowed to clot, and the serum was isolated by centrifugation (1300 g for 15 minutes at room temperature). Plasma (for ELISA): The blood taken into a chilled tube containing sodium ethylenediamine tetra-acetate was centrifuged for 15 minutes at 1300 g at 4°C, and the plasma was isolated. Both serum and plasma were stored at −80°C in multiple aliquots until analysis.

Measurement of plasma HGF, bFGF, and VEGF levels. Plasma levels of HGF, bFGF, and VEGF were measured by ELISA kits for human HGF (Otsuka Pharmaceutical Co., Tokushima, Japan), for human bFGF (R&D Systems, Inc., Minneapolis, MN), and for human VEGF (Amersham Life Sciences, Arlington Heights, IL), respectively, according to each manufacturer's protocols. With respect to the sensitivity and technical quality of the assay, standard curves were generated for each set of samples assayed. The sensitivities of the assay are 0.1 ng/ml for HGF, 9 pg/ml for bFGF, and 15.6 pg/ml for VEGF. Each plasma sample was tested in duplicate.

Cell culture. ECs were isolated from human umbilical veins as previously described (17) and cultured in Medium 199 supplemented with 20% heat inactivated FCS, 90 μg/ml of heparin sodium salt (grade I-A from porcine intestinal mucosa), and antibiotics (penicillin, 50 U/ml, streptomycin, 50 μg/ml, and amphotericin B, 125 ng/ml) at 37°C in 5% CO2. The cells were characterized by a typical cobblestone appearance and staining for factor VIII antigen by immunofluorescence. Cells at passage level 3 were used for these experiments.

In vitro EC proliferation assay. For measurements of cell proliferation, the colorimetric MTT (3-(4, 5-dimethylthyazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay (18) was performed with CellTiter 96 Non-Radioactive Cell Proliferation Assay kits (Promega Co., Madison, WI) according to the manufacturer's protocol. Briefly, ECs were seeded into gelatin-coated 96-well plates at 2.5×10^3 cells per well in Medium 199 containing 5% FCS. After ECs had adhered to the plates (approximately 3 hours later), the medium was discarded, the cells were washed with PBS, and 100 μl of Medium 199 containing 30% serum collected from patients was added to each well. After incubation of the ECs for 44 hours at 37°C, 10 μl of dye solution (MTT solution) per well were added, incubated for 4 more hours at 37°C, at which point the reaction was interrupted by addition of solubilization/stop solution. After incubation for 1 hour at room temperature, the plates were read on an automatic plate reader with a test wavelength of 570 nm and a reference wavelength of 690 nm. In this assay system, the absorbance (A570nm–A690nm) is directly proportional to the number of cells. All samples were assayed in quadruplicate.

Matrigel endothelial cell tube (capillary-like structure) formation assay. Vascular tube forming-activity was examined by the method described previously (19). Briefly, 300 μl per well (24-well plates) of diluted Matrigel (1:2 with Medium 199) was kept at 4°C overnight for slow polymerization, followed by 37°C for 30 minutes for complete polymerization before use. After polymerization, the remaining Medium 199 was removed from the wells by gentle aspiration. ECs (7.5×10^4 cells per well), suspended in 800 μl of Medium 199 without FCS, were plated on diluted Matrigel, and 200 μl of serums taken from patients were added to each well. After 24 hours of incubation at 37°C in 5% CO2, vascular tube formation was examined by inverted microscopy.

In order to lessen the effect of the serum itself, it was diluted, and a lower concentration (20 or 30%) was chosen for the *in vitro* experiments.

Statistical analyses. Values are presented as means ± SD, and significance was established by one-way ANOVA with multiple comparisons with Fisher's protected least significance difference test. In all analyses, the level of statistical significance was the 95% confidence level.

TABLE I

Clinical Characteristics of Three Patient Groups

	Heparin 100 U (n=10)	Heparin 3,000 U (n=10)	Heparin 10,000 U (n=10)
Age, y	64.1 ± 14.1	68.4 ± 7.9	65.6 ± 5.8
Sex, M/F	6/4	8/2	8/2
Body Weight, kg	56.1 ± 14.2	57.4 ± 6.7	56.8 ± 8.8
Prior MI, %	40	50	30
Diabetes Mellitus, %	10	10	20
Hypercholesterolemia, %	40	40	60

Note. MI: myocardial infarction.

RESULTS

Effects of heparin administration on the plasma levels of HGF, bFGF, and VEGF in CAD patients. The plasma levels of HGF, bFGF, and VEGF were measured before and 15 minutes after administration of 100 U, 3,000 U, or 10,000 U of heparin in patients with CAD. There was no significant difference, among the three groups, in age, sex, body weight, incidence of myocardial infarction, and prevalence of diabetes mellitus and hypercholesterolemia (Table I).

The plasma HGF levels before and after administration of heparin are shown in Figure 1A. Administration of 3,000 U and 10,000 U of heparin caused significant increases in plasma HGF, to levels 40- and 54-folds higher, respectively, than the baseline levels (3,000 U group: 0.23±0.17 versus 9.32±1.32 ng/ml, $p<0.0001$; 10,000 U group: 0.22±0.10 versus 11.93±2.53 ng/ml, $p<0.0001$). In order to examine the effect of heparin on ELISA, HGF levels in plasma collected before heparin administration were measured in absence of heparin and after the addition of 5 U/ml of heparin. Adding heparin to the blood sample made no significant difference in the assays.

No increase in bFGF and VEGF was observed in most patients, although 3,000 U and 10,000 U of heparin did cause a slight increase in bFGF level in a few patients (Figures 1B and C). In preliminary experiments examining the time course of plasma bFGF and VEGF levels (n=5), no significant rise was seen as late

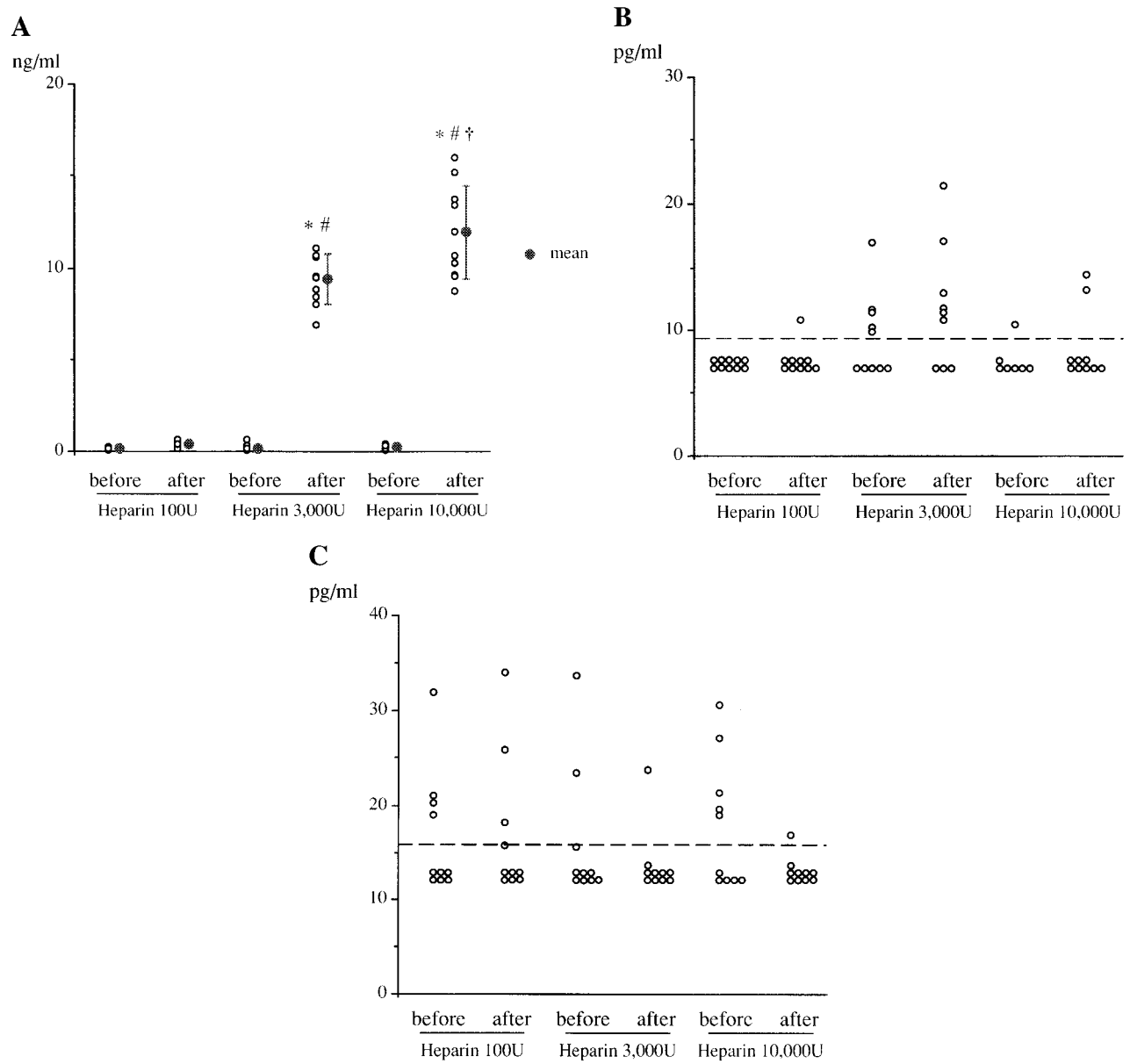

FIG. 1. Effects of heparin administration on the plasma levels of HGF (A), bFGF (B), and VEGF (C). The plasma levels of HGF, bFGF, and VEGF were measured before and 15 minutes after administration of 100 U, 3,000 U, or 10,000 U of heparin in patients with CAD. Error bars represent the standard deviation (SD) of mean levels determined in 10 patients each. *$p<0.0001$ vs the level before administration of heparin, respectively, #$p<0.0001$ vs the level after administration of 100 U of heparin, †$p<0.0001$ vs the level after administration of 3,000 U of heparin.

as 60 minutes after the administration of 10,000 U of heparin (data not shown).

In vitro EC proliferation induced by serum sampled before vs after heparin administration. The growth-promoting activity of the serum collected before and after administration of heparin on EC was examined by cell proliferation assay using MTT. The serum taken from patients 15 minutes after the administration of 10,000 U of heparin exercised more prominent growth-promoting activity than the serum collected before heparin (0.47 ± 0.04 versus 0.39 ± 0.05, $n=6$, $p<0.05$, Figure 2). This increased activity was completely prevented by the neutralization of HGF, while addition of the same amount of unrelated, control antibody did not affect this increased activity (data not shown). In contrast, neutralization of bFGF and VEGF, which inhibits the growth-promoting activity induced by 1 ng/ml of bFGF and 1 ng/ml of VEGF, respectively (data not shown), did not inhibit this increased activity. Serum collected before administration of heparin to

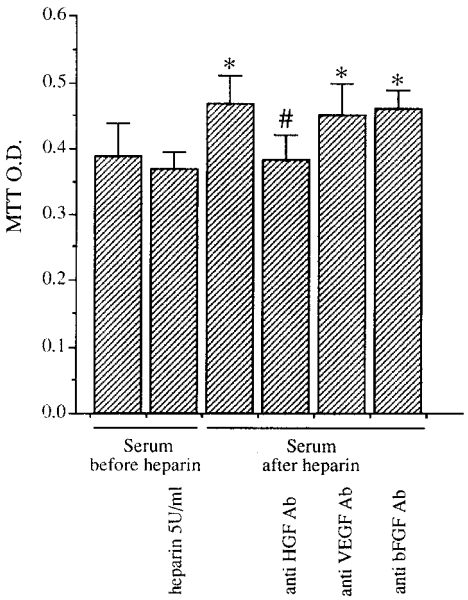

FIG. 2. Effects on EC proliferation of serum collected from patients before and after administration of heparin. The serum sampled after administration of 10,000 U of heparin showed significantly greater growth-promoting activity than before administration of heparin. The effect was completely inhibited by neutralization of HGF. Addition of 5 U/ml of heparin to the serum collected before administration of heparin had no effect. Values are means±SD. *$p<0.05$ vs serum before administration of heparin, #$p<0.05$ vs serum after administration of heparin (untreated control).

which 5 U/ml of heparin was subsequently added did not amplify EC proliferation. These results suggest that HGF induced by heparin, not heparin itself, actively promotes EC proliferation.

Vascular tube (capillary-like structure) formation induced by the serum before and after receiving heparin. Vascular tube-forming activity was examined in a diluted Matrigel model. In this model, ECs showed a typical cobblestone appearance in the absence of angiogenic stimuli, and vascular tube formation was induced in the presence of recombinant human HGF, bFGF, and VEGF (data not shown). Serum sampled from patients before administration of heparin did not induce vascular tube formation, and ECs maintained their characteristic monolayer organization (Figure 3A). In contrast, serum collected after the administration of 10,000 U of heparin caused marked, macroscopically evident vascular tube formation (Figure 3C). This induction of vascular tube formation was completely inhibited by neutralization of HGF (Figure 3D), while addition of the same amount of unrelated, control antibody did not affect this phenomenon (data not shown). On the other hand, neutralization of bFGF and VEGF, which inhibits the vascular tube formation induced by 1 ng/ml of bFGF and 1 ng/ml of VEGF, respectively, did not inhibit vascular tube formation induced by the serum collected after heparin administration (Figure 3E, F, respectively). To disprove that heparin itself promotes vascular tube formation, the vascular tube-forming activity of heparin-free serum, to which 5 U/ml of heparin was subsequently added, was measured. No vascular tube-forming activity by heparin-enriched serum was observed (Figure 3B). These results, which were consistent in 5 samples examined, suggest that HGF induced by heparin in the systemic circulation is an actively angiogenic product which exercises not only growth-promoting but also vascular tube-inducing activity on ECs.

DISCUSSION

Several reports have indicated that heparin has potent angiogenic activity (2–4), the mechanism of which has not been precisely defined. This study shows that the *in vivo* administration of heparin causes a significant increase in HGF in the systemic circulation, conferring the serum growth promoting- and vascular tube-inducing activities on ECs which are completely inhibited by neutralization of HGF. Overall, these findings indicate that HGF might play a significant role in heparin-induced angiogenesis.

HGF, also known as scatter factor, was originally described as a potent hepatocytic mitogen (14,20,21). It, subsequently, was shown to act on various cell types, including epithelial cells, melanocytes, endothelial cells, etc., and to elicit a range of cellular responses, including mitogenicity, motility, and morphogenesis (22–24). HGF is now recognized as a pleiotropic factor which participates in various biological processes such as embryogenesis, organ regeneration, wound healing, and angiogenesis (11,22,25,26). Its angiogenic activity was clarified *in vitro* (27,28) and *in vivo* (27,29,30), and we have showed recently that it plays a role in capillary endothelial regeneration in the ischemically injured rat heart (31).

The origin of HGF responsible for angiogenic activity observed in this study remains to be clarified. Given our preliminary finding of a rise in plasma levels within 5 minutes after the administration of heparin, we suspect that HGF is released by heparin from some yet unidentified storage site.

Most cultured cells, whether responsive to HGF or not, appear to possess relatively large numbers of so-called low affinity binding sites for HGF, found to be heparan sulfate and other products (5,6), and HGF is believed to be stored *in vivo* as an inactive complex with extracellular matrices (11). Furthermore, several studies have found that HGF loosely bound to extracellular matrices is released by exposure to heparin *in vitro* (5,12,14). Therefore, one may speculate that endogenous HGF, normally associated with the inner surface of the vessel wall can be mobilized to an active form by heparin.

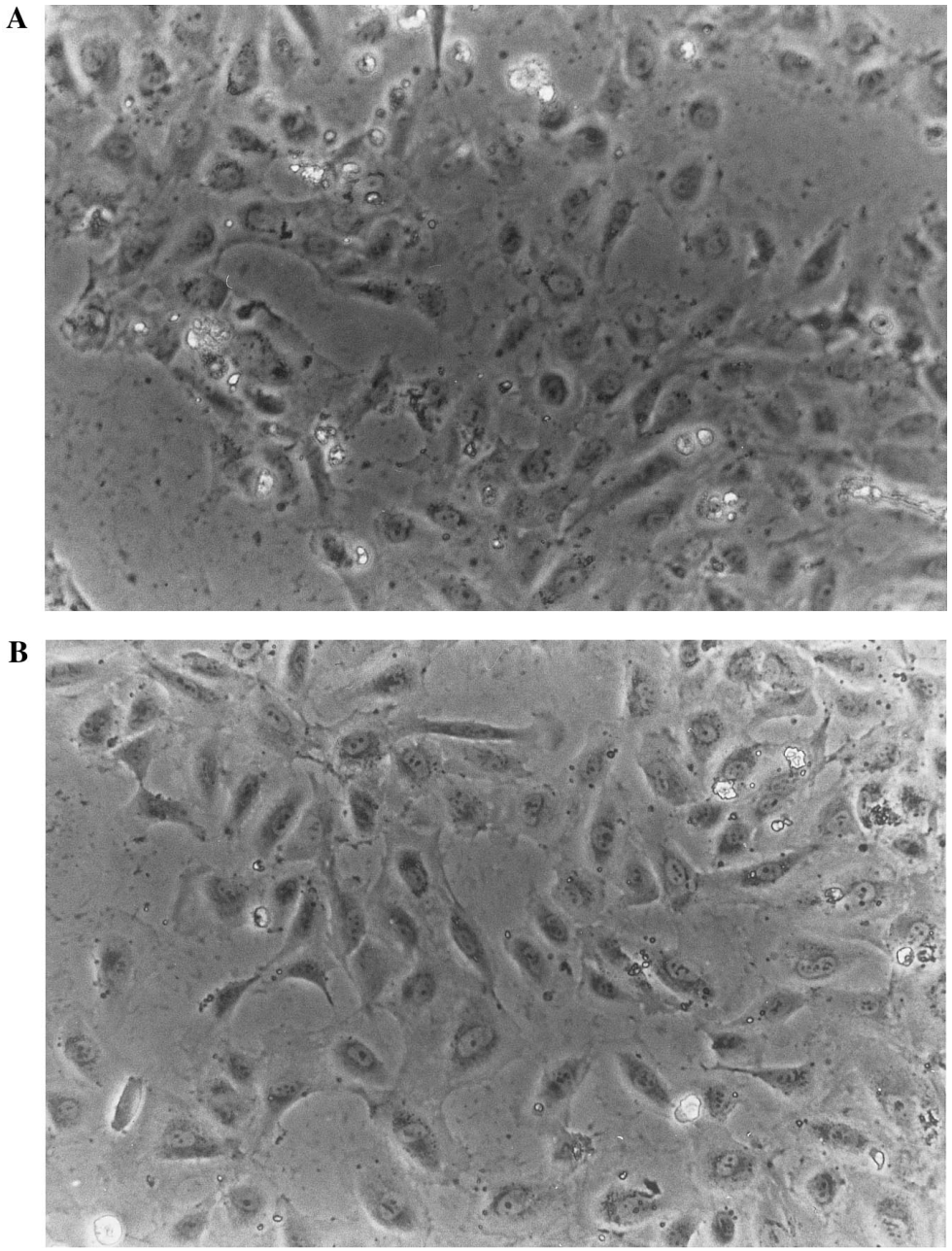

FIG. 3. Vascular tube formation induced by the serum before and after heparin administration. (A) serum sampled from patients before heparin; (B) serum obtained before heparin to which 5 U/ml of heparin was added subsequently; (C) serum sampled after administration of 10,000 U of heparin; (D), (E), (F) serum collected after administration of 10,000 U of heparin, treated with neutralizing antibody against HGF, bFGF, or VEGF, respectively. Consistent results were obtained in 5 samples examined. Representative micrographs are shown.

Although we have shown that HGF induced by heparin in the systemic circulation has growth-promoting and vascular tube-forming activities *in vitro,* it remains to be determined whether circulating HGF can preferentially reach ischemic regions and potentiate angiogenesis *in vivo.* It is well known that HGF exerts its biological effects through binding to, and activation of, a single high affinity tyrosine kinase receptor, the Met receptor protein (14,32–34). In recent experiments from our laboratory, in a rat model of myocardial ischemia-reperfusion, the expression of Met receptor protein was selectively upregulated in the ischemic-reperfused myocardium, although mRNA of HGF was induced not only in the myocardium but also at sites distant from the ischemic stimulus such as

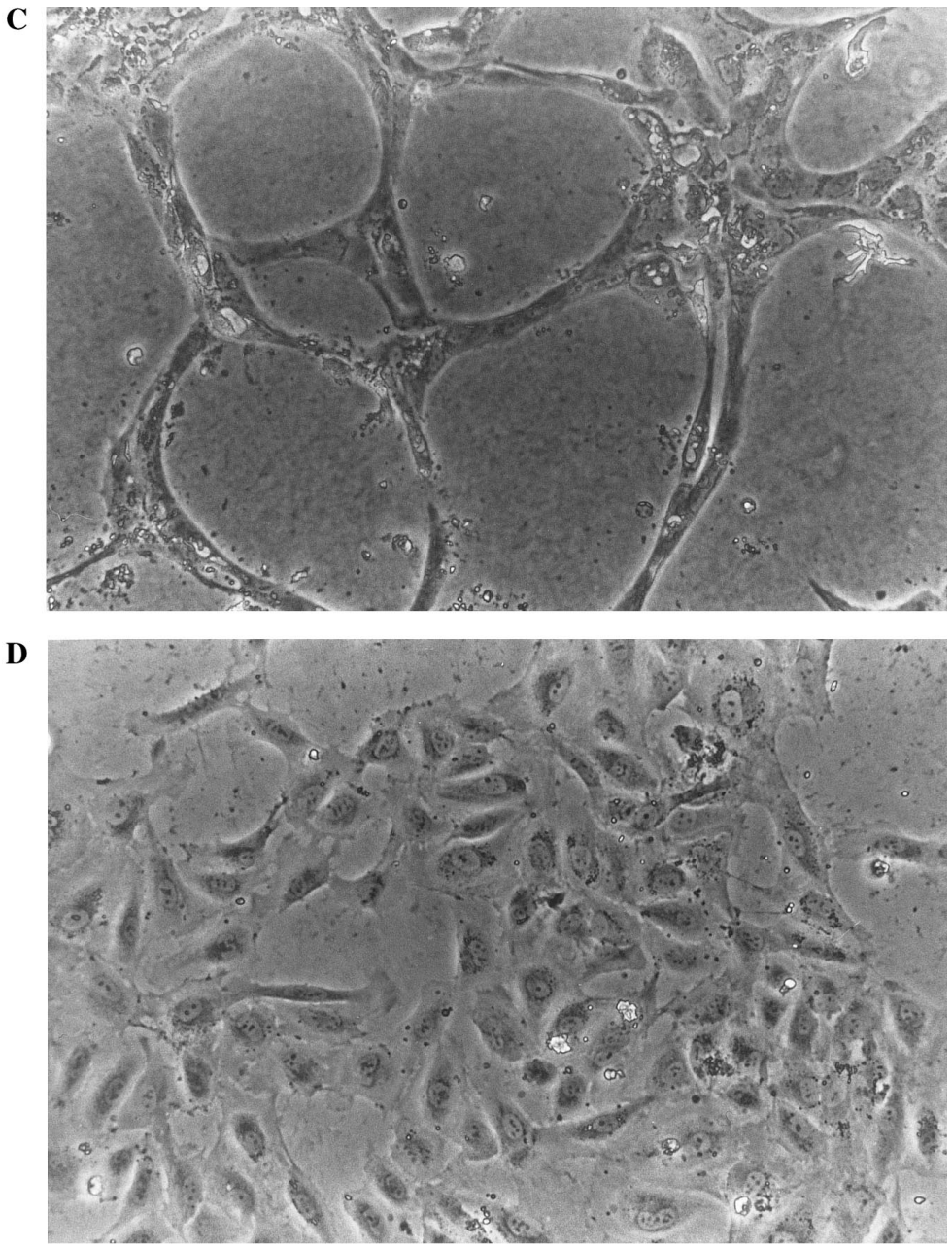

FIG. 3—*Continued*

liver, kidney, spleen, and lung (31). From this observation, HGF induced in the systemic circulation is expected to act preferentially at sites exposed to ischemia.

VEGF and bFGF, as well as HGF, have an affinity for the extracellular matrix components, heparan sulfate proteoglycans, and are believed to be stored with such molecules as a complex *in vivo* (7–10). However, in comparison with HGF, plasma levels of VEGF and bFGF did not change significantly with the administration of heparin, and neither growth-promoting nor vascular tube-inducing activities on ECs were influenced by the neutralization of VEGF and bFGF, suggesting that the contribution of these molecules to heparin-induced angiogenesis is low. It is, nevertheless, conceivable that heparin causes the local release of VEGF and bFGF, promoting their paracrine or autocrine functions, and that it enhances their transcription and later up regulates their plasma levels. Medalion et al. have recently reported that heparinization during open heart surgery and cardiopulmonary bypass causes an approx-

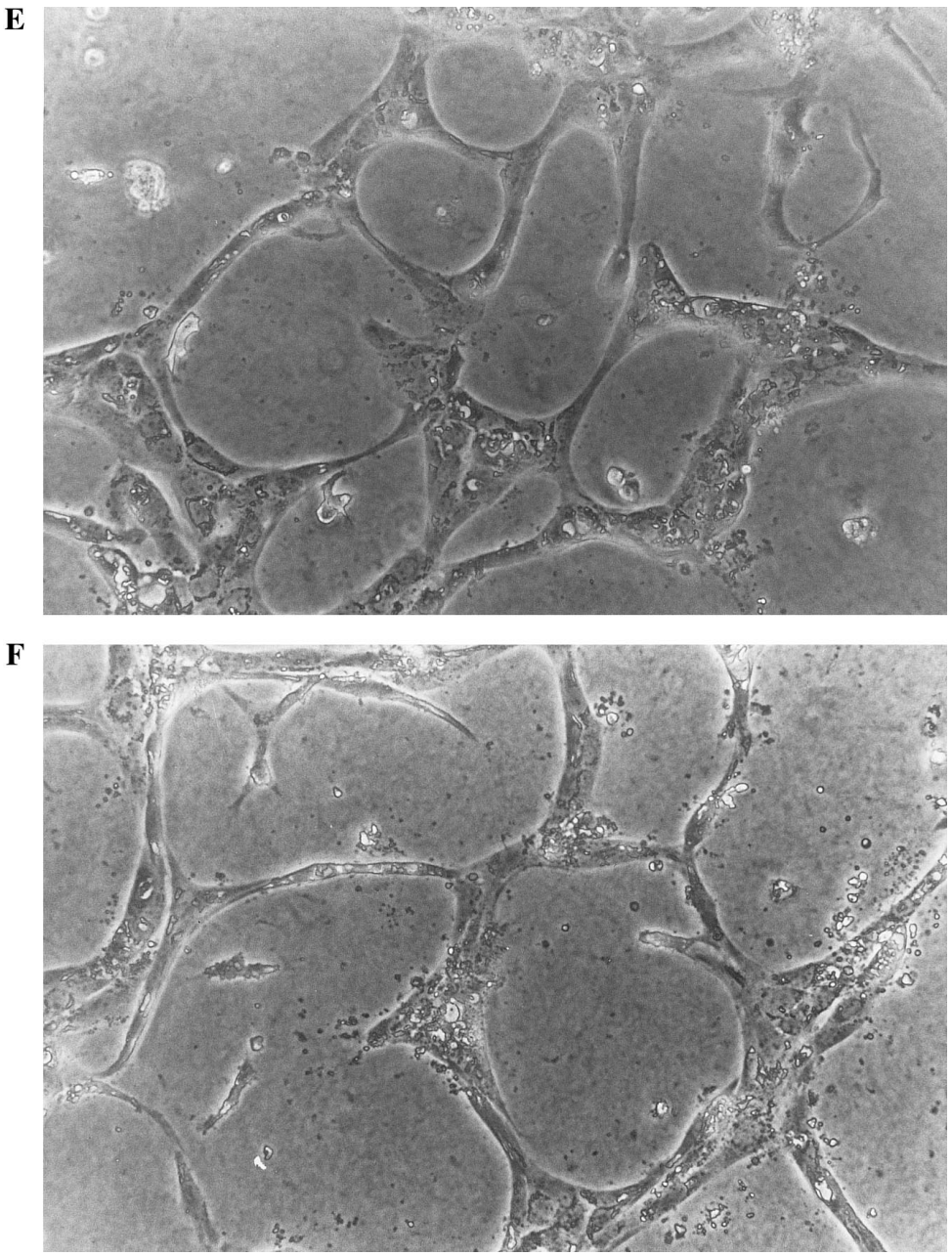

FIG. 3—*Continued*

imately 4-fold increase in plasma bFGF levels (35). This suggests that, in higher doses, heparin does affect plasma bFGF levels. Therefore, a participation of these molecules in heparin-induced angiogenesis cannot be completely excluded. However, within the range of doses administered in usual clinical practice, HGF is thought to play a greater role in heparin-induced angiogenesis than bFGF or VEGF.

In summary, heparin administration caused a marked increase in HGF which has angiogenesis-promoting activity. Since angiogenesis is desirable in ischemic diseases, our observations offer another therapeutic approach to ischemia.

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