Pharmacokinetics and Distribution of Heparin-Binding Growth Factor I (Endothelial Cell Growth Factor) in the Rat

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Heparin-binding growth factor I (HBGF I), previously designated as endothelial cell growth factor, is a potent mitogen for endothelial cells in vitro, which may prove useful for promoting endothelial regeneration in vivo. Analysis of the pharmacokinetics and organ distribution of HBGF I is necessary before use of HBGF I as a pharmacological agent. Consequently, pharmacological studies were carried out with [125I]HBGF I in the rat. Intravenous injections of HBGF I were given with or without heparin (2.5 units/ng HBGF I). Blood concentrations of HBGF I decreased by one half 17 seconds after HBGF I bolus. This time was prolonged to 60 seconds when HBGF I was injected with heparin. The elimination half-life of HBGF I was 14 minutes in the presence of heparin. The highest concentrations of HBGF I following intravenous bolus were found in kidney, liver, and spleen, and the lowest in fat and brain. Heparin increased HBGF I concentrations in blood and all organs measured except kidney, which was significantly decreased ($p<0.01$). Intact HBGF I was recoverable from blood 5 minutes following intravenous administration. HBGF I underwent near-complete proteolytic digestion after more prolonged ex vivo incubation with rat plasma, but HBGF I was protected from proteolysis when incubations were conducted in the presence of heparin. Thus, it is feasible that HBGF I can be administered as a pharmacological agent in the presence of heparin. Further studies assessing acceleration of in vivo endothelial growth using HBGF I with heparin appear warranted. (Circulation Research 1989;64:227–234)
Materials and Methods

Reagent Preparation

HBGF I was purified from bovine brain as previously described. Bioactivity was verified by an in vitro human umbilical vein endothelial cell growth assay. Iodination of HBGF I was performed by the immobilized lactoperoxidase glucose oxidase technique using Enzymobeads (Bio-Rad, Cambridge, Massachusetts) and 125I (New England Nuclear, Boston, Massachusetts) as previously described. The specific activity was determined to be approximately 2 x 10^6 cpm/ng protein with greater than 95% protein incorporation as determined by trichloroacetic acid (TCA) precipitation. Heparin (133 USP units/mg, The Upjohn Company, Kalamazoo, Michigan) was coupled to fluoresceinamine and then radioiodinated as described by Smith and Knauer. The [123I]F-heparin maintained its ability to bind HBGF I as demonstrated by ligand blotting (not shown).

Pharmacokinetic Studies

Male Sprague Dawley rats (average weight, 250 g) were anesthetized with 50 mg/kg pentobarbital injected intraperitoneally. Animal care at all times complied with "The Principles of Laboratory Animal Care" and the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 80-23, revised 1978). Eight rats were administered [123I]HBGF I (0.125 ml, approximately 25 ng) diluted to 0.5 ml with phosphate buffered saline and injected as a bolus through the dorsal penile vein. All rats were pretreated with 100 mg NaI injected intraperitoneally 10 minutes before HBGF I injection unless otherwise indicated. HBGF I was injected either with or without heparin at a concentration of 2.5 units/ng HBGF I. Serial blood samples (50 μl) were drawn at specified time intervals into an unheparinized capillary tube from the transected tail and analyzed for gamma emission. Blood samples from four different subjects were counted for 1-minute periods, and a curve was constructed using the average of these counts to represent the blood concentration of HBGF I as a function of time in heparin- and non-heparin-treated rats. An additional eight rats received injections of [125I]HBGF I (25 ng) with or without heparin (2.5 units/ng protein). Five minutes after HBGF I injection, the animals were killed by pentobarbital overdose and the organs rapidly harvested in random sequence. The organs were washed twice in ice-cold phosphate buffered saline and then lyophilized, weighed, and counted for 1-minute periods. Organ concentrations were determined from the average of counts per gram of lyophilized tissue from four different subjects in heparin- and non-heparin-treated rats. In addition, blood was withdrawn directly from the heart immediately before the animals were killed, and the plasma was separated by centrifugation at 7,000 rpm for 5 minutes and saved for further analysis.

Gamma Camera Analysis

Rats received intravenous boluses of [123I]NaI (2.5 x 10^6 cpm) either with or without pretreatment with 100 mg unlabeled NaI, which was injected intraperitoneally 10 minutes before [123I] administration. Other rats were injected with equal counts of [123I]HBGF I after NaI pretreatment. These rats were then viewed with a whole body gamma camera and imaged at 5-minute intervals for 20 minutes.

Organ Distribution

In separate experiments, an additional eight rats received injections of [123I]HBGF I (25 ng) with or without heparin (2.5 units/ng protein). Five minutes after HBGF I injection, the animals were killed by pentobarbital overdose and the organs rapidly harvested in random sequence. The organs were washed twice in ice-cold phosphate buffered saline and then lyophilized, weighed, and counted for 1-minute periods. Organ concentrations were determined from the average of counts per gram of lyophilized tissue from four different subjects in heparin- and non-heparin-treated rats. In addition, blood was withdrawn directly from the heart immediately before the animals were killed, and the plasma was separated by centrifugation at 7,000 rpm for 5 minutes and saved for further analysis.

HBGF I Characterization

Blood and urine samples were collected at various times up to 24 hours after [125I]HBGF I injection and were subjected to TCA precipitation to ascertain the fate of the labeled protein. Urine or blood samples were mixed with 24% (vol/vol) TCA, spun at 10,000 rpm for 10 seconds, after which the supernatant and pellet were counted. Protein incorporation was determined as the ratio of counts in the pellet versus total counts. Blood samples were further purified with C18 Sep-Pak columns (Waters Instruments, Rochester, Minnesota). One milliliter of plasma was applied to the Sep-Pak column, washed with 2 volumes of high performance liquid chromatography-grade water, then 15% acetonitrile, and finally eluted with 50% acetonitrile. Samples were then lyophilized, resuspended with sample buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide slab gels and then analyzed by autoradiography as described.

Mitogenicity Time Course

Low passage human umbilical vein endothelial cells (HUVEC) were grown to confluence on fibronectin-coated culture plates using Medium 199/10% fetal bovine serum with 10 ng/ml HBGF I as previously described. These were seeded at near confluent density in 24-well dishes and maintained in Medium 199/10% fetal bovine serum without HBGF I for 24 hours. This media was replaced with 1 ml of fresh Medium 199/10% fetal bovine serum plus 10 ng purified HBGF I with or without heparin (5 units) in triplicate wells. After varying time periods, this was aspirated and replaced with fresh Medium 199/10% fetal bovine serum. After 18-hour incubation periods, a standard 4-hour thymidine incorporation assay was performed to evaluate mitogenicity as a function of HBGF I exposure periods.
Pharmacokinetics of Heparin Binding Growth Factor I

The plots of labeled HBGF I blood concentrations versus time (Figures 1 and 2, left) resemble the standard clearance curve of a drug following first-order kinetics. This is confirmed by the linearity of the semilog plot of these data (Figure 2, right). The clearance curve may be divided into an initial distribution phase followed by a terminal elimination phase. The elimination or biological half-life can be determined directly from the semilog plot of these data.

In the distribution phase, HBGF I is rapidly cleared from the circulation after intravenous bolus injection (Figure 1). Blood concentrations are reduced by half in approximately 17 seconds. That time is prolonged to 60 seconds when HBGF I is injected with heparin (2.5 units/ng protein). Blood concentrations of HBGF I after injection without heparin rapidly approach zero, but sustained blood levels were obtained with coadministration of heparin (Figure 1). The HBGF I concentration 5 minutes after injection with heparin was increased approximately fivefold. Following the initial distribution phase, HBGF I injected with heparin was eliminated with a half-life of approximately 14 minutes as determined from the semilog plot of extended blood sampling (Figure 2, right).

Gamma Camera Analysis

Whole body analysis of radioisotope distribution was performed using a gamma camera to ascertain that the tissue distribution of radiolabeled HBGF I was specific to the polypeptide and not the radioisotope. The injection of [123I]NaI demonstrated diffuse activity in the central circulation as well as specific uptake by the stomach (Figure 3, top left). In animals pretreated with unlabeled sodium iodide, rapid elimination of [125I]NaI from the body with specific uptake in the bladder was observed (Figure 3, top right). A different pattern of distribution was observed with [123I]HBGF I in rats pretreated with sodium iodide. Specific uptake of labeled polypeptide was seen in the liver and kidneys (Figure 3, bottom left). These results suggest that the in vivo behavior of [123I]HBGF I reflects the biochemical characteristics of the polypeptide, which is distinct from that of the radioisotope.

Organ Distribution

Highest concentrations of HBGF I following intravenous injection of HBGF I were found in organs with the greatest “filtering” function, such as kidney, liver, and spleen (Table 1). HBGF I concentrations in these organs were approximately 10 times greater than equivalent blood concentrations, assuming 1 g tissue equals 1 ml blood. It is unlikely...
that tissue concentrations of [125I]HBGF I represent residual blood trapped in these organs following washings. Organs with "tight" capillary junctions (brain) or relatively low vascularity (fat) demonstrated the lowest uptake of labeled protein, being less than 1% of that found in organs with the highest HBGF I concentrations. Organs such as heart, lung, and aorta demonstrated intermediate uptake of HBGF I (Table 1).

Heparin increased blood levels of HBGF I as well as uptake of HBGF I by all organs measured except the kidney (Table 2). The increase was significant in all organs measured except the liver. The kidney actually demonstrated a significant decrease in HBGF I binding in the presence of heparin (p<0.01).

Whole-organ distribution patterns of HBGF I were calculated from whole-organ weights and total blood volumes (Figure 4). Heparin significantly increased the portion of HBGF I remaining in the blood compartment 5 minutes after intravenous bolus compared with injection without heparin (15% and 5%, respectively). Liver and kidney accumulated approximately 75% of total injected HBGF I dose in both the heparin- and non-heparin-treated animals. Heparin, however, appeared to shift the uptake of HBGF I from kidney to liver. Uptake by other organs of potential interest (e.g., heart and vasculature) appeared to account for less than 1% of the total injected dose. Total uptake at the site of injection (penis) represented less than 1% of total injected dose in heparin- and non-heparin-treated animals and thus was an insignificant source of error.

In separate experiments, [125I]F-heparin was injected in three rats each either with or without 25 unit/kg heparin. Autoradiographic analysis of serial urine samples demonstrated that all radioisotope activity found in urine represented free label and not polypeptide. Autoradiography after SDS-PAGE analysis demonstrated that all radioisotope activity found in urine represented free label and not polypeptide. Autoradiography after SDS-PAGE analysis demonstrated that all radioisotope activity found in urine represented free label and not polypeptide.
FIGURE 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of heparin-binding growth factor I (HBGF I) after intravenous bolus. Blood was collected 5 minutes after intravenous injection of HBGF I with heparin, and the plasma purified and subjected to SDS-PAGE analysis and autoradiography as described in "Materials and Methods." Lane 1: HBGF I in 1 ml phosphate-buffered saline subjected to purification process without intravenous injection. Lane 2: HBGF I recovered from 1 ml plasma.

injection also demonstrated a single band corresponding to intact HBGF I (not shown).

The apparent molecular weight of HBGF I is higher in plasma samples because of gel artifact. [125I]HBGF I added to plasma immediately before C18 purification without intravenous injection demonstrated a similar shift. This shift is increased with increasing plasma concentrations (not shown).

It is thus unlikely that this shift represents HBGF I digestion or modification in vivo. It was not practical to obtain sufficiently concentrated blood samples of HBGF I at later time points following intravenous administration to perform SDS-PAGE analysis due to the relatively rapid elimination half-life of HBGF I. However, 18-hour incubations of HBGF I in freshly obtained rat plasma at 37° C with or without heparin were performed in vitro. Analysis of the samples containing heparin (2.5 units/ng protein) demonstrated intact HBGF I. In contrast, the samples that did not contain heparin revealed significant proteolytic digestion of HBGF I (Figure 6).

Mitogenicity Time Course

The mitogenic effect of HBGF I with and without heparin on endothelial cell (HUVEC) cultures as a function of exposure time is depicted in Figure 7. This in vitro determination is prerequisite to subsequent in vivo endothelial growth experiments. These data indicate that HBGF I exposure times as short as 15 minutes in the presence of heparin increase the thymidine incorporation rate in HUVEC. Thymidine incorporation has been shown to correspond to cell replication rates in HUVEC.10 This mitogenic effect reaches maximal levels with exposure times of approximately 4 hours. HBGF I produces significantly less increased growth compared to controls even at 4 hours, when incubation is carried out without heparin. Similar results were also obtained with murine lung capillary endothelial cells.

Discussion

These studies were undertaken to assess the pharmacokinetics and distribution of HBGF I following intravenous administration in an animal. This is a preliminary step that is necessary to determine appropriate HBGF I dosages before use...
of HBGF I as an infusible agent to accelerate endothelial cell growth in vivo. Pharmacologic use of HBGF I presents a unique problem because the target organ of this polypeptide is the vascular endothelium itself. Further, the polypeptide avidly binds to heparin,1-4,6 and heparan sulfate is found in abundant quantities on the luminal surface of the vasculature.19 Consequently, it was unclear at the start of these studies whether it would be possible to administer HBGF I in quantities sufficient to overcome uptake by endothelium in the immediate vicinity of the injection site.

In vitro studies have demonstrated a high-affinity, low-capacity binding site for HBGF I on the endothelial cell surface.20 The high-affinity site appears to represent a specific receptor site on the endothelial cell.6-7 Binding to this site appears to be enhanced by heparin.6-7 A low-affinity, high-capacity HBGF I binding site also appears to be present on endothelial cells in vitro and may represent HBGF I binding to extracellular matrix components (T. Maciag and T. Rosengart, unpublished observations). This cell-associated binding has been reported to be decreased by heparin and eliminated by pretreatment with heparinase.20

These in vitro observations may account for some of the observations made in vivo. Specifically, it may explain the decreased uptake of HBGF I by the kidney and the increased uptake seen in other organs when HBGF I is administered with heparin compared with uptake following HBGF I injection alone. HBGF I conceivably is filtered past the endothelium and through the heparan sulfate-rich basement membrane in the renal glomeruli19 and undergoes a significant amount of binding to the basement membrane in the kidney. In vitro observations suggest that this binding would be inhibited by coadministration of heparin as was demonstrated.

The heparin-enhanced binding of HBGF I to endothelial cell receptors would predominate in other organs with a continuous endothelium in which there would be less extensive exposure to subendothelial matrix components. Our data are consistent with this suggestion because coadministration of heparin leads to an increase in total binding in these organs. The elevated blood levels of HBGF I found with heparin administration may contribute to the enhanced binding in these cases. In vitro studies, however, have demonstrated that heparin increased HBGF I binding in a manner independent of HBGF I concentrations.6,7

The highest concentrations of radiolabeled heparin administered in separate experiments were found in kidney, while heparin was found to cause a decrease in renal uptake of HBGF I. It is therefore unlikely that enhanced tissue uptake of HBGF I by heparin could be ascribed simply to a heparin "trapping" phenomenon.

Heparin markedly decreases the time endothelial cells must be exposed to HUVEC to produce a mitogenic effect. This may in some way be related to conformational changes in the HBGF I molecule induced by heparin and in the way HBGF I is presented to the cell in the presence of heparin.1,3-6 HBGF I alone does produce an equivalent mitogenic response with longer exposure periods, suggesting that this effect is different from the ability of heparin to protect HBGF I from proteolytic inactivation.10

Data from previous work indicate that the maximal mitogenic effect of HBGF I occurs at media concentrations of approximately 10 ng/ml.3 This corresponds to the concentration required for in vitro receptor saturation.6 Previously published in vivo studies in the rat suggest that saturation similarly occurs at blood concentrations of approximately 10 ng/ml.21 HUVEC in culture require exposure to HBGF I and heparin for at least 15 minutes before they exhibit an increase in thymidine incorporation, which is also approximately the elimination half-life of HBGF I in vivo. Consequently, in vivo endothelial growth experiments are probably best designed with an intravenous infusion of HBGF I or some other means to provide prolonged exposure of endothelial cells to HBGF I. Half-life data presented in this paper can be used to calculate infusion rates necessary to provide an adequate level of circulating HBGF I for in vivo experiments.

The data accumulated in this study suggests that HBGF I can be given intravascularly as a pharmacologic agent. Administration of heparin with HBGF I seems to be important in that heparin 1) significantly increases the blood levels of HBGF I, 2) enhances binding of the polypeptide to most organs including the vasculature, and 3) protects HBGF I from proteolytic digestion by plasma-derived proteins. The high degree of HBGF I accumulation in "filtering" organs such as liver and kidney, as compared with the binding to other potential target organs such as heart or aorta, suggests that future infusion studies might best use selective growth factor administration upstream from the target site in these organs rather than general intravenous infusion.

HBGF I has a relatively rapid elimination half-life in vivo but demonstrates binding properties and interactions with heparin similar to those seen in vitro. HBGF I may well be useful as a pharmacologic agent in vivo and further studies examining its effectiveness in accelerating endothelial regeneration appear warranted.

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