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Heparin Protects Heparin-Binding Growth Factor-I From Proteolytic Inactivation In Vitro

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Summary: Heparin inhibits proteolytic digestion of heparin-binding growth factor-I (HBGF-I) by trypsin, plasmin and other proteases. This property is lost after thermal denaturation of HBGF-I, suggesting that a heparin:HBGF-I structural interaction rather than a heparin:trypsin interaction is responsible for the resistance of HBGF-I to digestion with trypsin. Heparin is also able to partially protect HBGF-I from thermal denaturation as demonstrated by the ability of heparin to protect HBGF-I from trypsin digestion. The protective effect of heparin is dependent upon the concentration of heparin as well as temperature and duration of denaturation. Autoradiography of ^{125}I-HBGF-I incubated with human umbilical vein endothelial cells demonstrates near complete protection of HBGF-I from proteolytic modification when the incubation is performed in the presence of heparin. These data suggest that (i) the mechanism of the heparin-induced increase in human endothelial cell number at confluence involves the protection of HBGF-I by heparin against proteolytic inactivation and (ii) heparin provides conformational stability to the polypeptide growth factor which reduces the susceptibility of HBGF-I to denaturation.

Introduction: Endothelial cells are highly specialized, multifunctional cells which play a significant role in such processes as angiogenesis, thrombosis, wound repair and atherogenesis (1). Endothelial cell proliferation is induced by

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a family of acidic and basic polypeptide mitogens collectively termed the heparin-binding growth factor Class I (HBGF-I) and heparin-binding growth factor Class II (2). The HBGF-I (acidic-fibroblast growth factor) and HBGF-II (basic-fibroblast growth factor) families are related structurally with a 55% homology between the classes (3).

The glycosaminoglycan, heparin, enhances but does not promote angiogenesis *in vivo* (5,6), and heparin derived from mast cells stimulates migration of capillary endothelial cells *in vitro* (1,7). In addition, heparin interacts structurally with HBGF-I (8) and HBGF-II (9). Although heparin modulates the biological activity of HBGF-I (10,11,12), the glycosaminoglycan inhibits the biological activity of HBGF-II (13). Further, heparin enhances the binding of HBGF-I to its receptor present on several different cell types (11) and induces the formation of a more compact human endothelial cell monolayer *in vitro* (10).

Although the biochemical basis for the potentiation of HBGF-I activity by heparin is not known, it is thought to involve a structural interaction between the polypeptide growth factor and the glycosaminoglycan (8,11). This has been suggested to affect the stability of HBGF-I (11), and has been reported to prevent inactivation of Class I and II HBGF's (13). We present evidence that suggests the ability of heparin to increase the cell number of HUVEC at confluence is due to the ability of heparin to protect the HBGF-I from proteolytic modification.

MATERIALS AND METHODS

Cells and Reagents: Human umbilical vein (HUVEC) and murine lung capillary (LE II) endothelial cells were cultured *in vitro* as previously described (8,11). HBGF-I was purified as previously described (14) and growth assays performed using 10 ng per ml purified HBGF-I. The polypeptide used for the iodination is identical to the structure reported for alpha-endothelial cell growth factor (14). The polypeptide was labelled with ^{125}I (New England Nuclear) using immobilized lactoperoxidase-glucose oxidase Enzymo-Beads (Bio-Rad) as previously described (15) and was collected in 0.1% bovine serum albumin. The specific activity of ^{125}I-HBGF-I was approximately 60 to 90 mCi per ug. The DNA synthesis assays were performed as previously described (8). Trypsin was purchased from Worthington, immobilized TPCK-trypsin from Pierce, and subtilisin, thermolysin and papain from Boehringer Mannheim. Plasmin was a generous gift of Dr. Dudley Strickland (American Red Cross).

Trypsin Digestion: In all experiments, 50ul of heparin in varying concentrations was added to Eppendorf tubes as indicated, followed by the addition of 10ul ^{125}I-HBGF-I (approximately 2ng). Solutions were heated in a water bath at specified temperatures for varying periods of time as indicated. After

equilibration at room temperature, trypsin was added to the solution in a 1:1 ratio (w/w) to the BSA present in the ^{125}I-HBGF-I solution (10ug). All dilutions and digestions were carried out in 0.1M ammonium bicarbonate buffer, pH 8.0 except for the papain digestion for which the buffer was adjusted to pH 6.9 with 0.1 N HCl. In separate experiments various other glycosaminoglycans were substituted for heparin. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (16). All samples were lyophilized, resuspended in SDS-sample buffer, and subjected to electrophoresis on 8 to 18% acrylamide slab gradient gels using the Mighty-Small gel apparatus (Hoefer Scientific). The gels were stained as previously described (14), dried and subjected to autoradiography at -70°C using Kodak XAR film and Dupont Lightning Plus intensifying screens.

Conditioned Media: HUVEC and LE-II cells were densely seeded in 12 well cluster dishes and grown to confluence in the presence of purified HBGF-I without heparin. After 24 hrs, media was collected and incubated with ^{125}I-HBGF-I (10ul) overnight at 37°C. In separate experiments, (10ul) ^{125}I-HBGF-I was added directly to the cell culture for overnight incubation at 37°C. One half of all samples were incubated in the presence of 5 units per ml of heparin, and one-half without heparin. Media samples (500ul) were loaded onto C18 Sep-Pak columns (Waters), washed with 2ml of HPLC grade water, followed by 15% acetonitrile, and finally eluted with 1ml of 50% acetonitrile. The 50% elution was collected, lyophilized, resuspended in sample buffer, and subjected to SDS-PAGE and autoradiography as described above. In separate experiments, ^{125}I-HBGF-I was incubated with medium 199 or fetal bovine serum in varying concentrations for varying times and subjected to purification as described above. Some incubations were carried out in the presence of various protease inhibitors as indicated.

RESULTS AND DISCUSSION

Heparin induces an increase in the *in vitro* monolayer number of HUVEC at confluence (Figure 1A). The increase in HUVEC density at confluence occurred within three days after heparin addition at heparin concentrations between 0.1 and 2ug per ml. An identical response was observed with bovine lug heparin, porcine intestinal mucosa heparin or a low molecular weight heparin preparation obtained from Hepar (data not shown).

Higher HUVEC densities could also be achieved in the absence of heparin by the frequent addition of HBGF-I (Figure 1A). The addition of HBGF-I to the culture media every 24 hours resulted in an increase in cell number at confluence that was approximately 75% of the increase observed with a single feeding of HBGF-I and heparin (Figure 1A). Addition of heparin alone caused a decrease in viable cell number and subsequent cell detachment from the monolayer surface. This cell loss was increased with more frequent heparin additions. Since these data suggested that the increase in HUVEC density at confluence was dependent upon the presence of HBGF-I, we examined the

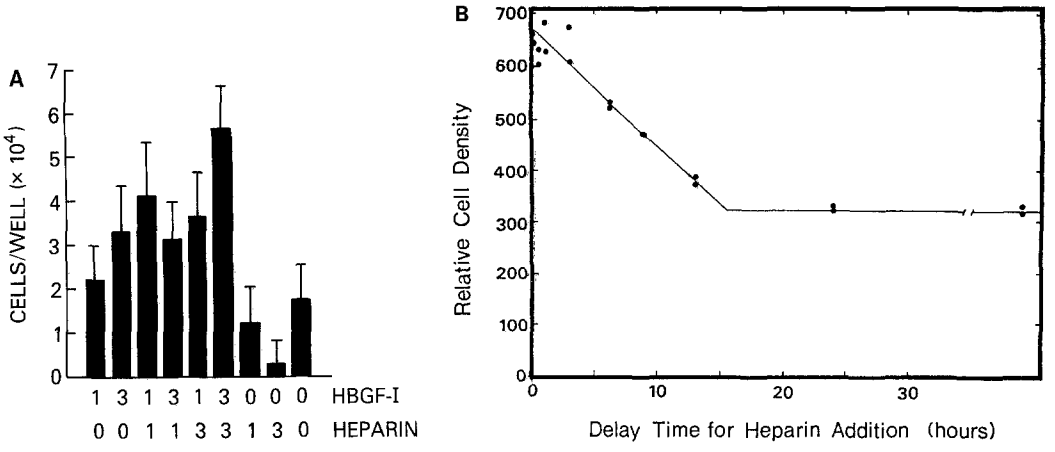

Figure 1. (A) The Heparin-Dependent Increase in HUVEC Density at Confluence Requires HBGF-I and Can Also be Induced by Frequent Additions of HBGF-I. Dishes of HUVEC were obtained as described in Materials and Methods and seeded into 24 well plates at confluent densities. Additions of heparin (5 Units) and purified HBGF-I (10ng) were made every 24 hours as indicated. After three days, viable cells were counted. (B) Delayed Addition of Heparin Diminishes the Density of HUVEC at Confluence. Confluent dishes of HUVEC were grown as described above and seeded into 24 well plates. At the indicate delay time, heparin was added to the cell culture to give a final heparin concentration of 5 units per ml. After three days, viable cells were counted.

importance of the time in which heparin was added to the HUVEC culture medium. We observed that the delayed addition of heparin resulted in a decrease in the final cell number of the HUVEC monolayer at confluence (Figure 1B). Furthermore, heparin does not cause an increase in cell number if added 15 hours after the introduction of HBGF-I to confluent HUVEC compared with the response with HBGF-I addition alone. Since the frequent addition of HBGF-I resulted in an increase in HUVEC number at confluence and the delayed addition of heparin to HBGF-I-supplemented cultures yielded a diminished response, we reasoned that heparin may indeed be stabilizing HBGF-I from inactivation in the HUVEC culture environment.

Autoradiography of ^{125}I-HBGF-I subjected to overnight incubation with HUVEC and LE-II cells *in vitro* showed major HBGF-I digestion fragments (Figure 2). In contrast, a single band corresponding to intact ^{125}I-HBGF-I was observed when incubation was performed with HUVEC and LE-II cells in the presence of heparin. Similar results were also observed after the incubation of ^{125}I-HBGF-I with fetal bovine serum, but did not occur with DMEM or Medium 199 (data not shown). These data suggest the presence of serum-derived

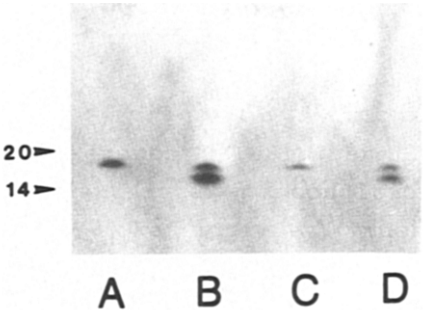

Figure 2. Serum-Derived Proteolytic Activity is Inhibited by Heparin. 125I-HBGF-I, with or without 5 units per ml heparin, was added to confluent cultures of HUVEC and LE-II cells containing Medium 199 and 10% (v/v) FBS. After 18 hours incubation in the presence of cells, the media was aspirated, 25% of total volume was run over a C-18 Sep-Pak column, eluted with acetonitrile (14), lyophilized and subjected to SDS-PAGE and autoradiography as described in Materials and Methods. Heparin (5 units per ml) was added to the sample prior to chromotography to avoid artifacts in purification. Lane A: HUVEC incubated with HBGF-I and heparin. Lane B: HUVEC incubated with HBGF-I without heparin. Lanes C and D: LE-II cells treated respectively with HBGF-I and heparin, and HBGF-I alone. Similar data were obtained after the incubation of ^{125}I-HBGF-I in Medium 199 with 10% FBS in the absence of endothelial cells. In this regard, 5 units per ml of heparin protected ^{125}I-HBGF-I from proteolytic modification by serum-derived proteases and incubation with Medium 199 in the absence of FBS and endothelial cells did not result in the proteolytic modification of ^{125}I-HBGF-I.

proteases which are capable of selective covalent modification of ^{125}I-HBGF-I and are consistent with the observation that heparin may be stabilizing HBGF-I from inactivation by the cell culture environment. Further studies using FBS with various inhibitors demonstrate protease inhibition with 25mM EDTA, but not with EGTA, a specific calcium chelator, suggesting an enzyme of the metallo-protease class. Incubations with 2 mercaptoethanol, soybean trypsin inhibitor, TLCK or iodoacetamide produced no significant inhibition of proteolysis. This protective effect could explain why heparin has little mitogenic potential of its own (10), but markedly potentiates the biological effects induced by HBGF-I (10,11,12).

Because heparin protected HBGF-I from proteolytic modification in culture, we examined the susceptibility of HBGF-I to digestion with trypsin in the presence and absence of heparin. As demonstrated in Figure 3, the addition of heparin to the reaction mixture protected HBGF-I from trypsin digestion, as demonstrated by the retention of nearly all radioactivity at the position of intact HBGF-I. This held true even at trypsin to HBGF-I ratios as high as

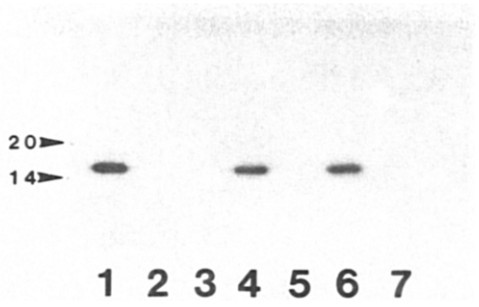

Figure 3. Heparin Protects Native HBGF-I but not Denatured HBGF-I from Digestion by Trypsin. ^{125}I-HBGF-I in 0.1% BSA (2ng HBGF-I) in the presence and absence of heparin (5 units) was treated with 5ug trypsin in 0.1M ammonium bicarbonate buffer, pH 8.0, incubated for 3 hours at 37°C and an additional 5ug trypsin added in order to replenish auto-digested trypsin. The heparin was added prior to trypsin digestion of HBGF-I. After 24 hours incubation, 25% of total volume was removed, lyophilized, electrophoresed and autoradiographed performed as described in Materials and Methods. The independent denaturation of HBGF-I and heparin was performed in water bath for 2 minutes at 90°C. Lane 1: HBGF-I control. Lane 2: HBGF-I plus trypsin. Lane 3: HBGF-I denatured at 90°C prior to the addition of trypsin. Lane 4: HBGF-I treated with trypsin in the presence of heparin (5 units). Lane 5: HBGF-I denatured at 90°C and subsequently treated with heparin and trypsin. Lane 6: Heparin heated at 90°C for 2 min followed by the addition of HBGF-I and trypsin. Lane 7: HBGF-I denatured at 90°C for 2 min in the presence of heparin (5 units) followed by the addition of trypsin.

$5x10^3$:1 (w/w) in the presence of 0.25 units heparin per ng HBGF-I and a similar effect was demonstrated by dextran sulfate but not by hyaluronic acid or chondroitin sulfate (data not shown). In addition, the biological activity of HBGF-I was retained when HBGF-I was treated with immobilized TPCK-trypsin for 30 minutes at 37°C in the presence of heparin (data not shown). In contrast, all mitogenic activity was lost when the proteolytic digestion was performed in the absence of heparin. Subtilisin, thermolysin and papain were also examined in order to determine the specificity of the heparin protective effect. Heparin protected HBGF-I from digestion by thermolysin nearly as completely as against trypsin (Figure 5). Some protection was also extended against HBGF-I digestion by papain and minimal protection against subtilisin (Figure 4) as determined by the relative intensity of intact and high molecular weight fragments observed upon SDS-PAGE analysis of the non-heparin control samples. Heparin also protected HBGF-I from digestion with plasmin (data not shown). In order to determine whether the polypeptide conformation of HBGF-I contributes to the ability of heparin to protect HBGF-I against trypsin digestion, HBGF-I was heat denatured; a process which destroys the biological

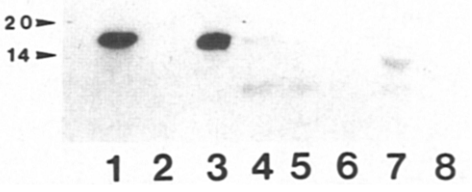

Figure 4. Protective Effects of Heparin Against Various Proteolytic Enzymes. ^{125}I-HBGF-I (2 ng) was premixed with or without 5 units of heparin and exposed to 5 ug of a variety of proteolytic enzymes in 0.1M ammonium bicarbonate, pH 8.0. After incubation for 3 hours at 37°C, 25% of the total mixture volume was aspirated, lyophilized and subjected to SDS-PAGE and autoradiography. Lanes 1 and 2: Trypsin digestion of HBGF-I with or without heparin, respectively. Lanes 3 and 4: Thermolysin digestion with or without heparin, respectively. Lanes 5 and 6: Subtilisin digestion with or without heparin, respectively. Lanes 7 and 8: papain digestion (0.1M) ammonium bicarbonate, adjusted to pH. 6.9 with or without heparin, respectively.

activity of the polypeptide. Incubation of ^{125}I-HBGF-I at 90°C for 2 minutes resulted in the complete loss of the ability of heparin to protect HBGF-I from subsequent trypsin digestion (Figure 3). However, if heat treatment is conducted in the presence of heparin, subsequent trypsin digestion can be reduced (Figure 5). This effect was observed at 90°C with heating for 40 seconds in the presence of 2.5 units heparin per ng ^{125}I-HBGF-I, but was lost

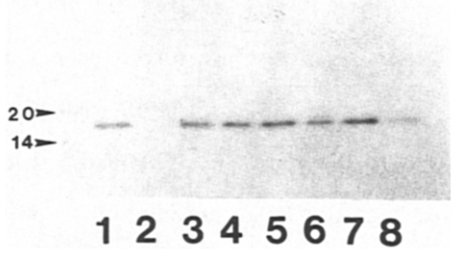

Figure 5. Heparin Stabilization of HBGF-I During Thermal Denaturation is Time Dependent: ^{125}I-HBGF-I (2 ng) was heated at 55°C in a water bath for varying amounts of time with or without 5 units of heparin. Heparin (5 units) was then added to tubes not pre-treated with heparin, followed by the addition of 5ug trypsin. After 3 hours incubation at 37°C, 25% of the total volume was removed, lyophilized and subjected to SDS-PAGE and autoradiography as indicated in Materials and Methods. Lane 1: HBGF-I treated with 5 units of heparin prior to trypsin addition. Lane 2: HBGF-I and trypsin at room temperature in the absence of heparin. Lanes 3 and 4: HBGF-I denatured for 30 seconds at 55°C with and without heparin, respectively, prior to trypsin digestion in the presence of heparin. Lanes 5 and 6: HBGF-I with and without heparin, respectively, prior to denaturation at 55°C for 1 minute followed by trypsin digest in the presence of heparin. Lanes 7 and 8: HBGF-I heated at 55°C for 2 minutes with and without heparin, respectively, and then treated as above.

with longer incubations at the same temperature. HBGF-I stabilization by heparin also occurred with heating at 55°C for 2 minutes, but no difference could be detected at temperatures below 55°C or with shorter time periods of incubation, suggesting that limited thermal denaturation of HBGF-I occurs at these lower temperatures (Figure 5). The heparin concentration could be lowered to 0.25 units per ng HBGF-I before the loss of the heparin stabilizing effect was observed.

The ability of heparin to protect HBGF-I against digestion with trypsin argues that the arg and lys residues present within the primary structure of HBGF-I may be involved in the formation of the heparin:HBGF-I complex and, as a result, are not susceptible to covalent modification by trypsin. The data obtained from thermal denaturation studies with the polypeptide mitogen are consistent with this suggestion and support our initial observation that heparin provides conformational stability to the angiogenic polypeptide (11). Further, these data suggest that heparin inhibits HBGF-I proteolysis by a direct and specific interaction with HBGF-I and is consistent with the observation that the esterolytic activity of trypsin is not altered in the presence of HBGF-I and heparin (data not shown). It is of interest that heparin also protects the biological activity of basic fibroblast growth factor (Class II HBGF) from denaturation but inhibits the biological activity of the polypeptide (13).

The mechanism of release and extracellular fate of HBGF-I is not known. The absence of an apparent signal peptide sequence within the primary structure of the HBGF-I precursor (17) suggests an alternative mechanism of release from cells which synthesize the angiogenic polypeptide. Since such a mechanism may include cell death as an important component, it is reasonable to suggest that the polypeptide should be protected from enzymatic modifications by hydrolyases which may be released concurrently with the growth factor. The resistance of HBGF-I to proteolytic modification in the presence of heparin is consistent with such a mechanism.

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