The Effects of Hepatocyte Stimulating Factor on Fibrinogen Biosynthesis in Hepatocyte Monolayers

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ABSTRACT The biosynthesis of fibrinogen increased at least eightfold in primary hepatocytes when incubated in the presence of monocyte/macrophage-derived hepatocyte stimulating factor (HSF). The large increase in fibrinogen production is due to increased availability of the mRNAs for the protein since cytodot analysis of cellular RNA showed a 10-12-fold increase in each of the fibrinogen mRNAs. Pulse-chase experiments showed that the time for fibrinogen synthesis, assembly, and secretion was 40-50 min for both control and stimulating conditions. This indicates that the increased production was due principally to the presence of greater amounts of fibrinogen mRNA rather than translation or secretion-specific events. Three lines of evidence indicate that the increase in fibrinogen production was due to HSF effects on transcription: (a) analysis of cytoplasmic levels of each of the fibrinogen mRNAs showed that all three increased at the same rate and to the same extent, demonstrating that HSF affects the three gene products coordinately; (b) Northern gel analysis of cytoplasmic RNA isolated after very brief exposures to HSF showed increases in a large molecular weight fibrinogen RNA precursor; and (c) actinomycin D blocked the HSF-stimulated increase in fibrinogen mRNA species. Furthermore, experiments in which protein synthesis was inhibited by cycloheximide failed to inhibit the increase in fibrinogen mRNAs, indicating new protein synthesis is not required for the HSF stimulation of fibrinogen mRNA. These results are consistent with our hypothesis that HSF is exerting its control of fibrinogen at the level of gene transcription.

Fibrinogen is a complex glycoprotein whose subunit structure consists of three pairs of nonidentical polypeptide chains. The principle site of synthesis of the protein is in hepatic parenchymal cells (1, 16). The biosynthesis of fibrinogen is under complex coordinated control since its production involves both transcription and processing of three different mRNAs (19, 20) as well as a complicated translational assembly of the three different subunits (35, 36). Under normal circumstances the fibrinogen genes appear to be constitutively regulated. However, during an acute inflammatory reaction the production of fibrinogen and of its mRNAs increases significantly (4, 12, 13). The molecular mechanisms responsible for these changes have not been determined.

We and others have shown that human peripheral blood monocytes/macrophages and murine and human myeloge-
control conditions. Furthermore, studies dealing with the effects of HSF on mRNA levels provided the following information: (a) the addition of actinomycin D to hepatocytes exposed to HSF blocked the HSF-stimulated fibrinogen response, (b) cytoplasmic levels of fibrinogen mRNAs increase 10-12-fold over control by a 12 h exposure to HSF, (c) all three fibrinogen mRNAs increased at the same rate and to the same extent, (d) Northern gel analysis of cytoplasmic RNA showed the presence of a large molecular weight species (presumably fibrinogen mRNA precursors) as early as 30 min after HSF exposure, and (e) the addition of cycloheximide 1 h before adding HSF had no inhibitory effect on increasing cytoplasmic levels of fibrinogen mRNAs, although protein synthesis was completely inhibited. These data imply that the HSF signal affects fibrinogen synthesis primarily at the RNA level. In this regard, the data are consistent with our hypothesis that HSF exerts its control of fibrinogen at the level of gene transcription.

MATERIALS AND METHODS

Preparation of Antigens and Antibodies: Rat fibrinogen was purified to homogeneity from rat plasma (3). Rat fibrinogen antibody was produced in goats and made monospecific by affinity chromatography using fibrinogen covalently linked to Sepharose as described previously (4, 19).

Immunoassays: ELISA: Fibrinogen concentration in the hepatocyte medium was determined using a quantitative enzyme-linked immunoassay as previously described (14, 24). This assay system can detect fibrinogen in the nanogram range.

Immunoabsorption: Fibrinogen was removed from hepatocyte medium by incubating in the presence of Sepharose 4B (Pharmacia Inc., Piscataway, NJ) to which monospecific fibrinogen antibody had been covalently linked (3). Ordinarily 50 ml of the affinity resin was added per milliliter of medium and the slurry gently mixed at 4°C for 16 h. The resin was removed from the medium by centrifugation. 50 ml of the antibody resin could bind 200 ng of fibrinogen/ml.

Hepatocytes: Primary hepatocytes were prepared from male 200-300-g Sprague-Dawley rats using the collagenase perfusion procedure originally described by Seglen (29) with several minor modifications (23). Confluent monolayers were prepared by plating 0.5 ml of a washed hepatocyte suspension (3.0 x 10^9) on 35-mm tissue culture plates previously coated with fibronectin (50 mg/ml) in sterile phosphate-buffered saline (PBS). When treated in this way the hepatocytes attach and spread to form a confluent monolayer in 4-8 h. Hepatocytes were cultured in Williams Medium E lacking arginine. This medium was supplemented with ornithine (50 nM), insulin (100 mU), deoxymannojirimycin (50 mg/ml), penicillin G (200 U/ml), streptomycin sulfate (200 mg/ml), and gentamycin (60 mg/ml). Cells incubated in this medium could be maintained without loss of metabolic capability for 48 h. The cells were routinely incubated for only 24 h, after an overnight period of attachment and spreading.

Monocytes: Human peripheral blood monocytes were obtained from the University of Texas Medical School blood bank from anonymous donors. An equal volume of sterile Hank's basic salt solution (HBSS) and heparin (5 U/ml) was added to buffy coat suspensions, and 30 ml of this mixture was layered over 10 ml of Histopaque (Sigma Chemical Co., St. Louis, MO) that had previously been placed in 50-ml sterile centrifuge tubes ( Falcon Labware, Oxnard, CA). The tubes were centrifuged for 40 min at 1,600 x g. Nonmonocuclear leucocytes were collected from the interface and then washed three times using ice-cold 10 mM Tris buffer (pH 7.0) containing 1 mM EDTA and 0.5% Triton X-100. After the three washes with HBSS, the cells were resuspended in McCoy's 5A medium (Gibco Laboratories, Grand Island, NY) containing 5% fetal bovine serum. Cells were counted and plated on 60-mm tissue culture plates at a density of 5 x 10^5 cells/ml followed by an incubation period of 1 h. Nonadherent cells were removed by gently raising the plates twice with medium lacking fetal bovine serum. The purified monocytes which represented the adherent cells were incubated in serum-free McCoy's 5A medium for 24 h in a 5% CO2/95% air tissue culture incubator. The conditioned medium was harvested from the cells after 24 h and served as the starting material for further purification.

Preparation of HSF-III: The monocye conditioned medium was dialyzed against 20 mM Tris/HCl pH 7.2 and chromatographed over a 2.5 x 3 cm DEAE-cellulose column (DEAE 53 from Whatman Inc., Clifton, NJ); dialyzed monocyte conditioned medium (HSF-I) was applied to the column at a flow rate of 20 ml/h, the column was then washed with 150 ml of 40 mM sodium chloride, 20 mM Tris/HCl pH 7.2, and the HSF was eluted from the column with 100 ml of 60 mM sodium chloride, 20 mM Tris/HCl pH 7.2. This pool of monokines (HSF-II) was dialyzed and concentrated using a PD-10 column apparatus. (Bio-Molecular Dynamics, Beaverton, OR). HSF was further purified by gel permeation chromatography using a TSK-3000 column (Altech Associates, Inc., Deerfield, IL) and a Waters high performance liquid chromatography system (Waters Associates, Millipore Corp., Milford, MA) (33), and material of 22-30 kDa was collected. HSF purified in this way was ~500-fold enriched over conditioned medium and contained no interleukin-1 (IL-1) activity when tested by a thymocyte proliferation assay (33).

RNA Extraction and Isolation: Confluent monolayers of hepatocytes (100-mm plates) were incubated with HSF (10-12 U/ml) for varying periods of time. We have previously defined a unit of HSF activity as that amount of HSF required to get a half-maximal fibrinogen synthesis response in primary hepatocytes (23, 24). At the end of each time point the cells were washed with ice-cold PBS twice and then scraped from the plate and RNA extracted by the guanidinium isothiocyanate procedure (6).

Nick Translation: Nick translation of plasmids containing cDNA inserts for each of the fibrinogen genes was done using a commercial translation kit from Bethesda Research Laboratories. These cDNA probes were a generous gift from Dr. Gerald R. Crabtree (Stanford University). 32P-Deoxycytidine triphosphate (3,000 Ci/ mmol, ICN K & K Laboratories, Inc., Plainview, NY) was used to label the cDNAs for each fibrinogen message. The specific radioactivity of each probe was 3.0 x 10^6 cpm/μg of plasmid DNA.

Northern Analysis: Northern procedures were performed as described previously (15). After electrophoresis the RNA was transferred from the agarose gel to nitrocellulose using standard methods (30). After an overnight transfer the nitrocellulose was dried in vacuo and heated to 80°C for 2 h. The denatured RNA was then hybridized with a mixture of the three 32P-labeled fibrinogen cDNA probes. Usually 3-8 x 10^6 cpm/probe was used in the hybridization assay.

Cytodot Analysis: The procedure followed for the cytodot analysis was a modification of that described by White and Bancroft (32). Confluent monolayers of hepatocytes on 35-mm tissue culture plates were scraped into ice cold 10 mM Tris buffer (pH 7.0) containing 1 mM EDTA and 0.5% Nonidet P-40 (Shell Chemical Co., Houston, TX). The cells were homogenized and cell debris removed by centrifugation (10,000 g for 10 min). An equal volume of 20 x SSC/37% formaldehyde (3.2) was added to the supernatant (20 x SSC contains 0.15 M NaCl, 15 mM trisodium citrate) and the mixture incubated at 60°C for 15 min. The RNA was serially diluted and applied to the nitrocellulose using the Schleicher & Schuell minifold (Schleicher & Schuell, Inc., Keene, NH). The dot blots were baked, prehybridized, hybridized, and washed as above except that 3 x 10^6 cpm/probe was used per assay.

RESULTS

Dose-Response Relationship of HSF and Fibrinogen

We have previously shown that the amount of fibrinogen produced by primary hepatocytes is dependent upon the amount of monocyte conditioned medium added to the medium of these cells (25). Fig. 1 shows the relative specific activity of dialyzed monocyte conditioned medium (HSF-I), the DEAE-cellulose fractionated material (HSF-II), and the HPLC-fractionated material (HSF-III). The specific activity of HSF-III is ~500-fold higher than that of HSF-I. HSF-III contained no IL-1 activity when assayed by the thymocyte proliferation assay (data not shown).

Effect of HSF on Fibrinogen Secretion

The time required for fibrinogen synthesis was determined after addition of HSF to confluent monolayers of primary hepatocytes. Medium was harvested at varying times and assayed for the presence of fibrinogen (Fig. 2A). HSF produced an increase in secreted fibrinogen within 3 h. Fibrinogen concentrations increased for the next 15 h and remained elevated thereafter. Fibrinogen production by control cells
also increased but to a lesser extent. The increase in fibrinogen secretion was due to synthesis of new fibrinogen (Fig. 2B). HSF-stimulated and control primary hepatocytes were pulsed with [3H]leucine for 20 min before each time point. The radioactivity of fibrinogen was determined by immunoprecipitation using monospecific antifibrinogen. The quantity of fibrinogen increased eightfold over control during the 24-h period as shown in Fig. 2B.

**Cellular Transit Time for Fibrinogen during HSF Stimulation**

We determined whether HSF affected the time required for the delivery of fibrinogen to the extracellular space. Hepatocytes were pulsed with [35S]methionine for 10 min, washed free of the radioactive label, and fresh warmed nonradioactive medium added. Samples were taken at 10-min intervals to determine the time required for radioactive fibrinogen to appear in the medium. Radiolabeled fibrinogen first appeared at 40 min, indicating that the synthesis, assembly, and transit time through the cell (endoplasmic reticulum to Golgi apparatus to secretory vesicle to plasma membrane) requires 35-45 min (Fig. 3). The time of appearance of radiolabeled fibrinogen was essentially the same for control and the HSF-stimulated cells, indicating that the increased production of fibrinogen caused by HSF does not alter the intracellular transit time of fibrinogen.

**Length of Time Required To Get Maximal Stimulation**

The temporal events involved in HSF induction of fibrinogen synthesis are unknown. To gain some insight, we measured how long the hepatocytes had to be in contact with HSF to get a maximal response. Hepatocytes were exposed to HSF for varying lengths of time (from 2-24 h). The amount of fibrinogen secreted into the medium by hepatocytes ex-
FIGURE 4  Fibrinogen production by hepatocytes after exposure to HSF for varying times. Triplicate 35-mm confluent monolayers of primary hepatocytes were treated with 12 U/ml of HSF. At the end of each time period (2, 6, 9, 12, and 20 h), the HSF-containing medium was removed and chased with fresh medium lacking HSF. For each time point, the two media (HSF and chase) were combined and the amount of fibrinogen determined.

posed to HSF for different times is shown in Fig. 4. The cells began to respond to HSF almost immediately. However, it required a continual presence of HSF for at least 12 h to elicit the maximum response.

Effect of Actinomycin D on HSF Stimulation

We wished to determine if the 12 h of HSF stimulation necessary for maximal increase in fibrinogen production was due to the transcription of new mRNAs. The antibiotic actinomycin D was added to hepatocytes at varying times after the addition of HSF. The results shown in Fig. 5 indicate that the actinomycin D blocking of transcription also blocked the rise in fibrinogen synthesis. These findings suggest that new fibrinogen mRNA synthesis is required for the increased production of fibrinogen under HSF stimulation. When actinomycin D was added to hepatocytes, fibrinogen synthesis was not inhibited, but rather it was maintained at the same levels as that before the addition of the antibiotic. These results indicate that under our experimental conditions actinomycin D does not affect protein synthesis.

Cytodot Analysis for Fibrinogen Messages

Experiments were performed to gain information on the cytoplasmic levels of fibrinogen (messages) at various times after the addition of HSF. Total cytoplasmic RNA containing fibrinogen mRNA was assayed by cytodot analysis. The message for each fibrinogen chain increased during the first 12 h to HSF exposure (Fig. 6). Furthermore, both the response time and the amount of each mRNA species increased in a coordinated fashion, suggesting a highly synchronized control of the synthesis or stabilization of these messages. The fold increase for each fibrinogen mRNA increased to a level 12 times that of the mRNA in control cultures.

HSF Effects on Early Fibrinogen mRNA Synthesis/Stabilization

To examine the effects of HSF on RNA synthesis and/or stabilization, we extracted total cellular RNA at 15, 30, 60, and 90 min after the addition of 12 U of HSF. The specific radioactivity of our cDNA probes was increased by pooling the nick-translated $A_{\alpha}$, $B_{\beta}$, and $\gamma$ probes. A higher molecular weight hybridizing band was detected at the 30-min time point and was even more apparent in the 60- and 90-min samples (Fig. 7). The larger molecular weight RNA species suggests an increase in transcription and/or a stabilization of the fibrinogen mRNA precursors.

FIGURE 5  Effect of actinomycin D on the production of fibrinogen. Confluent monolayers of hepatocytes were incubated in the presence of 12 U/ml of HSF. At the time points indicated, actinomycin D (2.5 $\mu$g/ml) was added and the incubation continued for a total of 24 h. The cell medium was then harvested and the concentration of fibrinogen determined.

FIGURE 6  Cytoplasmic RNA analysis using cDNA probes for $A_{\alpha}$, $B_{\beta}$, and $\gamma$ messages. Primary hepatocytes ($1.5 \times 10^6$) were incubated with HSF (12 U/ml). At each time period shown the cells were washed with cold sterile PBS and the cells scraped and homogenized as described in Materials and Methods. The cell extract was diluted such that the top row contains 1 $\mu$g of RNA, then serially diluted 1:2 for the wells below. The control shown is that taken from a 24-h sample and probed with the $A_{\alpha}$ cDNA. The $B_{\beta}$ and $\gamma$ controls were indistinguishable from the $A_{\alpha}$ control and are not shown in the figure. The fold increase was determined by quantitative scans of the cytodot films.
FIGURE 7 Northern analysis of hepatocyte RNA after incubation with HSF. Twenty 100-mm tissue culture plates containing confluent monolayers of hepatocytes were incubated with 12 U/ml of HSF. At each of the time periods indicated, the RNA was extracted from four plates as described in Materials and Methods. The total cellular RNA was separated by agarose-formaldehyde gel electrophoresis and transferred to nitrocellulose filters. All three cDNA probes were combined and used to identify the larger molecular weight RNA species. Arrows on the left indicate the position of the 28S and 18S ribosomal RNA.

Effects of Cycloheximide on HSF-stimulated mRNA Increases

To determine whether the increase in fibrinogen mRNAs after the addition of HSF requires the synthesis of a protein, we performed the following experiment. Hepatocyte cultures were treated with HSF in the presence and absence of cycloheximide (2 μg/ml). After a 20-h incubation, RNA was extracted and dot analysis performed. The results of the experiment shown in Fig. 8 indicate that the presence of cycloheximide in the medium did not diminish the stimulatory signal of HSF for fibrinogen mRNAs. Medium taken from cell plates treated with cycloheximide contained no detectable fibrinogen, indicating the synthesis of this protein had been blocked (data not shown). The results of these experiments indicate that the stimulatory action of HSF is not dependent upon protein synthesis for increasing the three fibrinogen mRNAs and strongly suggests that HSF is acting through a direct pathway.

DISCUSSION

In these studies we have examined the regulation of fibrinogen biosynthesis by HSF in isolated hepatocytes and have shown that HSF exerts rapid and specific effects on the concentration of mRNA for each of the three different fibrinogen genes. Within 15–30 min after HSF exposure, larger molecular weight RNA species that hybridize with cDNAs made from fully processed fibrinogen mRNAs are present. This demonstrates that these species are fibrinogen mRNA precursors. The results presented here also show that there is a time-dependent increase in the cytoplasmic mRNA of fibrinogen that correlates precisely with an increase in the synthesis, assembly, and secretion of fully functional fibrinogen.

The effects of HSF on fibrinogen translation occur concomitantly with increased fibrinogen mRNAs; however, when actinomycin D is added, the rise in fibrinogen synthesis ceases. Since it is well established that actinomycin D inhibits gene transcription (9), our findings are consistent with the hypothesis that the principal site of HSF action is at the transcriptional level and that the increases in fibrinogen concentrations are due to increased amounts of fibrinogen mRNAs. It is unlikely that HSF is exerting its stimulatory influence on protein translation in general since (a) the synthesis of many of the hepatocyte-derived proteins are unaltered as a result of exposure to HSF (data not shown) and (b) hepatocyte albumin synthesis decreases in the presence of HSF (27, 33).

It is well recognized that monocytes/macrophages and a variety of established cells lines of the myelogenous series produce IL-1, a potent monokine with thymocyte proliferation activity. Although several reports have indicated that IL-1 stimulates fibrinogen synthesis (10, 11, 21), we could not duplicate these results. In experiments using IL-1 from two sources and in concentrations as high as 10^4 U, we observed no increase in fibrinogen production over control (33). Furthermore, the HSF-III used in our studies had no IL-1 activity. Thus, the results shown are directly attributable to 25–30-kD proteins that we have previously described and have named HSF (22).

Although hepatocytes required exposure to HSF for 12 h before reaching a maximal response, a clearly detectable increase in mRNA precursors occurred as early as 15 min. This rapid response in mRNA precursors is similar to that

\footnote{Human IL-1 was purchased from Genzyme Corp., Boston, MA. Cloned mouse IL-1 was a generous gift from Dr. Peter Lomedico of Hoffmann-La Roche, Inc., Nutley, NJ.}
reported for the effect of thyrotropin-releasing hormone on transcriptional activity of the prolactin gene (17). The HSF-induced increase in fibrinogen mRNA levels does not require protein synthesis and differs from that of another heptatically derived protein induced during inflammation (α1 acid glycoprotein) (31). This finding suggests that there may be several pathways involved in the induction of different plasma proteins during an acute inflammatory response.

We have shown here that a hormone-like polypeptide, HSF, made by monocytes acts directly on hepatocytes to increase fibrinogen synthesis. This marked increase in fibrinogen is directly related to the dramatic increase in each of the messages coding for the protein. There are several lines of evidence presented in our study strongly implying that HSF is exerting its control of fibrinogen by regulating transcription of the three genes in an elegantly coordinated manner. Several different molecular processes could be envisioned for such a tightly coordinated system, the simplest being the presence of common regulatory sites 5’ to the coding region. Crabtree and Kant suggested (5) that the highly coordinated regulation of mRNAs for the chains of fibrinogen may be under the control of a single regulatory molecule that would be interacting with sites in or around each gene. Our evidence that HSF brings about a coordinated increase in all three primary gene products in both time and amount provides a strong argument that HSF functions as such a molecule. Moreover, Fowlkes et al. (7) reported an extensive nucleotide homology in the 5’ flanking region of all three fibrinogen genes. This finding is consistent with the hypothesis predicting a common regulatory site through which HSF would be exerting its control.

Our data show that HSF is a critically important regulatory protein for fibrinogen biosynthesis. We are now investigating the precise molecular mechanism involved in this type of coordinated gene regulation.

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