Effect of hepatocyte-stimulating factor and glucocorticoids on plasma fibronectin levels

David L. AMRANI, Debra MAUZY-MELITZ and Michael W. MOSESSON
University of Wisconsin Medical School, Milwaukee Clinical Campus, Department of Medicine, Mount Sinai Medical Center, Milwaukee, WI 53233, U.S.A.

INTRODUCTION

Many studies have shown that a variety of stimuli causing acute inflammation and tissue damage give rise to increased plasma levels of several proteins that are collectively known as 'acute-phase reactants' (Koj, 1974; Feldmann, 1979). These reactants are primarily proteins synthesized by hepatocytes (Courtoy et al., 1981). At least two physiological factors, namely hepatocyte-stimulating factor (HSF) (Ritchie & Fuller, 1983) and glucocorticoids (Miller & Griffin, 1975) have been implicated in the regulation of the acute-phase response. HSF is a protein that is produced by mammalian monocytes and macrophages during an acute inflammatory reaction (Ritchie & Fuller, 1983) as well as in response to fibrogenin degradation products (Ritchie et al., 1982) and lipopolysaccharides (Baumann et al., 1984). It stimulates the production of fibrogenin and certain other acute-phase proteins by 200–400% in rat and mouse hepatocyte cultures (Ritchie & Fuller, 1983; Baumann et al., 1983b). Addition of glucocorticoids to cultured hepatocytes maintained in serum-free medium also induces a 100–300% increase in the levels of secreted fibrogenin and certain other acute-phase proteins (Grieninger et al., 1978; Fouad et al., 1981; Baumann et al., 1983a).

Fibronectin (Fn), which is synthesized by hepatocytes (Tamkun & Hynes, 1983; Amrani et al., 1985), is a part of a family of structurally and immunologically related high-M<sub>r</sub> glycoproteins (Mosesson & Amrani, 1980; Yamada, 1983). These proteins are synthesized by several cells in the body and are active in cellular processes such as cell adhesion, cell migration and phagocytosis (Yamada, 1983; Mosher, 1984). In individuals suffering from severe trauma, burn injury or infection, Fn levels undergo changes similar to those observed for acute-phase proteins (Saba et al., 1978; Richards & Saba, 1983). Owens & Cimino (1982) demonstrated that rat plasma Fn levels rose 100% within 48 h of experimentally inducing acute inflammation, suggesting that plasma Fn is an acute-phase reactant. We previously showed that dexamethasone stimulated plasma Fn production 100–200% in cultured chick hepatocytes (Amrani et al., 1985).

In the present study we have sought to evaluate whether (1) chick plasma Fn is an acute-phase reactant, (2) chick HSF alone stimulates chick hepatocyte Fn production in vitro and (3) there is a specific co-ordinated response in vitro to HSF and glucocorticoids on chick hepatocyte Fn production.

MATERIALS AND METHODS

Experimental induced acute-phase response

Chickens (12 days old) were anaesthetised with diethyl ether (anaesthesia grade; Mallinckrodt, Paris, KY, U.S.A.) and injected subcutaneously on the side of the abdomen with either sterile saline or turpentine (150 μl/100 g body wt.). A blood sample was obtained by venipuncture into a tube containing 3.8% sodium citrate (blood/citrate, 9:1, v/v) and plasma was obtained by centrifugation at 4000 g for 10 min at 4°C.
Blood samples were then taken at the times indicated and the plasma isolated and frozen at −70 °C until assayed.

**Plasma proteins, fibrinogen fragment D₁ and related antisera**

Purified Fn, fibrinogen and albumin were prepared from heparinized adult chicken plasma as previously described (Amrani et al., 1985). Purified fragment D₁ of human and chicken fibrinogen was prepared as previously described for human D₁ (Mosesson et al., 1973). Antisera against chicken Fn, fibrinogen and albumin were produced in rabbits and were made specific as previously described (Amrani et al., 1985; Kalb & Grieninger, 1979).

**Rocket immunoelectrophoretic analysis**

Quantification of Fn, fibrinogen or albumin levels was accomplished by rocket immunoelectrophoresis with the above specific antisera with a modification (Grieninger et al., 1978) of the Laurell (1966) procedure. Sample values were determined from standard curves (1–40 μg/ml, depending on the protein) of the respective purified proteins that were placed on each assay plate.

**Serum HSF**

Blood was obtained from saline control and turpentine-treated chickens at the times indicated and allowed to clot at 4 °C overnight. Serum samples were then divided into equal portions. One such portion was frozen at −70 °C until assayed for corticosterone. The rest were adjusted to a final (NH₄)₂SO₄ saturation of 30% (w/v) and the resulting precipitate dissolved and dialysed in 0.05 M-sodium phosphate buffer, pH 7.5 containing 0.15 M-NaCl. These serum subfractions (200 μl) were assayed for their ability to stimulate fibrinogen levels in a chick hepatocyte culture system (see below).

**Corticosterone assay**

Plasma corticosterone levels were determined with a radioimmunoassay kit (Radioassay Systems Laboratories, Carson, CA, U.S.A.). The procedure used is as follows. Plasma samples were diluted 1:250 (v/v) with diluent buffer and 0.5 ml was added to 12 mm × 75 mm glass tubes (in duplicate). Corticosterone standard solutions ranging from 0.025 to 1.0 μg/0.5 ml were also added to tubes. Control tubes contained either buffer or [3H]corticosterone (8000–10000 c.p.m./0.1 ml). All tubes were incubated at 98 °C for 10 min and then cooled to room temperature. To all tubes except the buffer control, 0.1 ml of antiserum was added, mixed, and followed by 0.1 ml of [3H]corticosterone. All tubes were mixed and then incubated for 22 h at 4 °C. After incubation, 0.2 ml of cold charcoal/dextran solution was added, mixed for 20 s and incubated for 20 min at 4 °C. All tubes were centrifuged at 2500 rev./min (755 g) for 15 min, then the entire supernatant was decanted into a scintillation vial containing 10 ml of Aquasol (New England Nuclear). The samples were counted for radioactivity in a Packard liquid-scintillation counter (model 2425) with an efficiency for 3H of 56%. The percentage of radioactivity bound was determined by using the following equation:

\[
\text{Percentage bound} = \frac{AC_{\text{sample}} - AC_{\text{blank}}}{AC_{\text{Std.}} - AC_{\text{blank}}} \times 100
\]

where AC is average counts and 0 Std. is the 100% -binding tube. A standard curve was made by plotting the percentage bound, using 100% as the starting point, versus corticosterone standards.

**Hepatocyte cultures**

Primary chick hepatocytes were prepared from 16-day-old chick embryos as previously described (Grieninger et al., 1978; Amrani et al., 1985). Briefly, suspensions of hepatocytes were obtained by liver perfusion with Hank's buffer, followed by treatment of the embryonic liver with purified dissociating enzymes and mechanical disruption. After washing, hepatocyte suspensions (3 × 10⁶ cells/ml) were plated into 35-mm-diameter (2 ml) plastic dishes and maintained in Williams E medium without serum or hormonal supplement. After 24 h, the medium was replaced with fresh arginine-deficient medium supplemented with 0.7 mM-ornithine and 1 mM-carbamyl phosphate. Hepatocyte cultures were used over a 24 h period to assay for stimulation of Fn, fibrinogen and albumin levels. Portions (200 μl) of serum or mononuclear-cell fractions were used for assay of HSF activity.

**Chicken mononuclear-phagocytic-cell cultures**

Blood was collected from adult chickens by cardiac puncture into 50 ml polypropylene tubes containing 3.8% sodium citrate (9:1, v/v) and 50 mM-EDTA, pH 7.5. The mononuclear-cell fraction was isolated from blood by centrifugation at 1000 g for 40 min in a Hypaque/Ficoll solution (μ 1.078 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). The mononuclear-cell layer accumulating at the interface of the plasma and Hypaque/Ficoll solution was removed, washed several times by centrifugation at 200 g for 10 min with Hank's balanced salt solution containing 2.5 mM-EDTA, then Hank's solution alone (twice) and resuspended in either pyrogen-free RPMI 1640 or Newman–Tytell medium (GIBCO, Grand Island, NY, U.S.A.) to a final concentration of 10⁶ cells/ml. The cells were plated on to 60 mm-diameter (5 ml) plastic dishes (Corning; Corning, NY, U.S.A.) precoated with gelatin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and human fibronectin as previously described (Bevilacqua et al., 1981). After a 1 h incubation, the medium was removed and lymphocytes were washed off by three washes with RPMI or Newman–Tytell medium. The final concentration of mononuclear cells, which consisted of monocytes and thrombocytes (Sturkie & Griminger, 1976), was (1–3) × 10⁶/plate. Cells were subsequently incubated with LPS (1 μg/ml; Sigma) for 24 h at 37 °C in CO₂/air (1:19). To each dish, 50 μCi of [35S]methionine (sp. radioactivity 1300 Ci:mmol; New England Nuclear) was added.

**RESULTS**

Experimentally induced acute-phase response

Young chickens (12 days old), treated with either saline or turpentine, were examined for changes in plasma Fn, fibrinogen and albumin levels over a 7-day period (Fig. 1). As previously reported (Pindyck et al., 1983), fibrinogen levels were stimulated 200–250% (Fig. 1b), whereas albumin (Fig. 1b) levels decreased 20–40% after turpentine injection. Fibronectin levels also rose 200–250%, rapidly peaking at 48 h (Fig. 1b), whereas the fibrinogen response was maximal at 72 h. The levels of
these proteins returned to control values by day 7 (Fig. 1b). Little or no changes were observed for any of these proteins in control animals over the same period (Fig. 1a).

**Pattern of circulating HSF activity**

Blood serum samples were examined for the levels of circulating HSF activity (Fig. 2) by assessing the ability of serum subfractions to stimulate fibrinogen synthesis in a chick hepatocyte culture system (Amrani et al., 1985). Control samples (0-48 h) produced no significant increases over basal fibrinogen levels (Fig. 2). In contrast, subfractions obtained from sera taken as early as 3 h after turpentine injection produced a 150–200% increase in fibrinogen levels (Fig. 2). This stimulatory activity peaked at approx. 8 h and gradually disappeared from the circulation within the next 24–48 h. To ascertain whether this activity was due to the presence of steroid hormones (e.g., corticosterone), the samples were treated for 20 min at room temperature with charcoal/dextran to bind free steroid hormone (Jones & Bell, 1982). After charcoal treatment, the samples were again assayed for HSF activity, and no changes in stimulatory activity were found when compared with the untreated samples (results not shown). We have found that normal circulating levels of corticosterone (2–5 nM) have only a modest effect in stimulating fibronectin or fibrinogen synthesis compared with dexamethasone in our hepatocyte culture system in vitro (D. L. Amrani, D. Nimmer & G. Bergstrom, unpublished work). Therefore removal of this hormone from serum samples taken at these time points would not be expected to significantly alter the level of stimulatory activity we observed in the serum HSF samples.

To further characterize HSF activity, serum subfractions from the 3, 5 and 8 h samples from the control and turpentine-treated animals were pooled and chromatographed on a G-100 Sephadex column (Fig. 3). The HSF activity from both groups emerged in a fraction corresponding to the molecular-mass range 30–45 kDa, although the activity was much greater in the turpentine-treated group.

The HSF activity from the turpentine-treated sample was pooled and examined for its ability to stimulate Fn, fibrinogen and albumin levels in the presence or absence of dexamethasone (Table 1). With dexamethasone alone, Fn and fibrinogen levels increased 130–150% over basal levels (Amrani et al., 1985). A similar stimulation of Fn and fibrinogen levels was found when a portion of the pooled chromatographic fraction was added to hepatocyte cultures (Table 1). When both HSF and dexamethasone were added together, an additive effect was observed for both Fn and fibrinogen production (Table 1).

**Changes in circulating corticosterone levels**

To assess changes in glucocorticoid levels after turpentine injection, we determined the circulating levels of corticosterone, the major glucocorticoid in chickens (Beuving & Yonder, 1977). Control corticosterone levels varied between 1.8 and 5 nM (mean values) over the entire 48 h period examined, a result consistent with previous findings (Beuving & Yonder, 1977). A comparison of plasma corticosterone levels from saline-treated chicks (4.5 ± 2.3 nM, P < 0.05) and turpentine-treated chicks (10.1 ± 3.1 nM, P < 0.05) at 24 h indicated a significant
Fig. 3. Sephadex G-100 column chromatography of serum subfractions from saline-treated and turpentine-treated chickens

Serum subfractions (5 ml) from saline-treated (a) and turpentine-treated chickens (b) were applied to a 2.6 cm x 60 cm column in 20 mM-Hepes buffer, pH 7.6, containing 0.15 mM-NaCl. Fractions were assayed for protein (——) and their ability to stimulate secreted fibrinogen (φ) (--●--) in a serum-free chick hepatocyte culture system (Amrani et al., 1985). The column was pre-run with markers to determine the approximate molecular exclusion of the HSF activity. Stimulation is presented as the increase over basal immunoassayable fibrinogen levels. In both serum samples, there was less than 1 μg of detectable intact fibrinogen, fragment D or E/ml as assessed by immunoassay or by immunoprecipitation and gel analysis of serum fractions (results not shown).

Table 1. Effect of dexamethasone and serum HSF on fibronectin, fibrinogen and albumin levels

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fn (μg/24 h per dish (n = 6))</th>
<th>Fibrinogen</th>
<th>Albumin</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.6±0.3</td>
<td>3.9±0.3</td>
<td>6.0±0.1</td>
<td>0</td>
</tr>
<tr>
<td>Dexamethasone (2 nM)</td>
<td>8.3±0.4</td>
<td>9.8±0.3</td>
<td>6.1±0.2</td>
<td>131</td>
</tr>
<tr>
<td>HSF</td>
<td>8.4±0.3</td>
<td>9.6±0.3</td>
<td>5.8±0.2</td>
<td>133</td>
</tr>
<tr>
<td>Dexamethasone + HSF</td>
<td>16.8±0.3</td>
<td>18.7±0.4</td>
<td>6.2±0.2</td>
<td>367</td>
</tr>
</tbody>
</table>

Abbreviation: NC, no significant change.

rise in the level of this hormone in serum samples from turpentine-treated chickens. At 48 h, the maximum serum corticosterone level had decreased to some extent, but still remained elevated compared with control values (4.1±1.8 nM as against 7.9±2.4 nM). The rise in corticosterone levels occurred later than that seen for circulating HSF (Fig. 2).

Production of HSF by chicken mononuclear phagocytes

We examined whether chicken mononuclear cells were a source of HSF in chickens. Previous studies on human monocytes (Ritchie & Fuller, 1983), rat and mouse macrophages (Ritchie et al., 1982; Ritchie & Fuller, 1983; Baumann et al., 1984) demonstrated that production of HSF could be induced with either fibrinogen fragment D1 or LPS.

Medium for mononuclear cells, incubated with or without LPS for 24 h, was subjected to Sephadex G-150 column chromatography to determine whether HSF activity made by these cells was eluted in a molecular-mass range corresponding to serum HSF activity. Medium from LPS-treated cells demonstrated two peaks of fibrinogen-stimulatory activity (Fig. 4b). The first peak was small and emerged at about 70 kDa. The second peak contained most of the activity from the stimulated group and all of the activity from the control group. This activity was eluted in the 25-40 kDa range (Fig. 4b). Medium from untreated cells showed much less activity than in the LPS-stimulated cells (Fig. 4a), suggesting that mononuclear cells may produce low levels of HSF activity in the absence of any specific inducer.

Comparison of serum and mononuclear-cell HSF activity

We compared the ability of the HSF activity from chromatographic fractions of serum or cell medium to
stimulate basal levels of Fn and fibrinogen. Basal Fn and fibrinogen levels produced by chick hepatocytes over a 24 h period were 1.6 and 2.1 μg/ml respectively. The addition of LPS to hepatocyte cultures had little or no effect on Fn or fibrinogen levels (Table 2), whereas fragment D₁ from either human or chicken fibronogen had a modest stimulatory effect. When dexamethasone (2 nm) was added alone or with either of these components, a 130–140% increase in the levels of the proteins was found, indicating that, in the presence of dexamethasone, no additional effect beyond that for the hormone alone was observed. The mononuclear-cell HSF fraction produced an approx. 100% greater increase in both Fn and fibrinogen levels than did serum HSF (Table 2). When either HSF fraction was added to hepatocytes in the presence of dexamethasone, the levels of both proteins rose to values suggesting an additive effect of the hormone and HSF fractions (Table 2). Additional analysis of the HSF activity from both serum and cell medium indicated that the activity from either source was heat-stable (55 °C, 30 min), but completely destroyed by trypsin (1 μg/ml, 37 °C, 15 min) (Table 2).

**DISCUSSION**

Acute inflammation brought about by trauma, physical injury or infection produces relatively dramatic changes in plasma fibronectin levels in humans (Saba

---

Table 2. Effect of fragment D₁, LPS and HSF on fibronectin and fibrinogen synthesis

<table>
<thead>
<tr>
<th>Addition</th>
<th>Fn</th>
<th>Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LPS</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Fragment D₁ (0.5 mg/ml)</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Dexamethasone (2 nm)</td>
<td>131</td>
<td>251</td>
</tr>
<tr>
<td>Fragment D₁ + dexamethasone</td>
<td>139</td>
<td>139</td>
</tr>
<tr>
<td>Serum HSF fraction</td>
<td>130</td>
<td>168</td>
</tr>
<tr>
<td>Serum HSF fraction + dexamethasone</td>
<td>359</td>
<td>489</td>
</tr>
<tr>
<td>Mononuclear-cell medium HSF</td>
<td>237</td>
<td>278</td>
</tr>
<tr>
<td>Mononuclear-cell medium HSF + dexamethasone</td>
<td>357</td>
<td>429</td>
</tr>
<tr>
<td>Serum HSF, heated (55 °C, 30 min)</td>
<td>119</td>
<td>167</td>
</tr>
<tr>
<td>Serum HSF, trypsin (1 μg/ml, 37 °C, 15 min)</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Mononuclear-cell medium HSF, heated</td>
<td>348</td>
<td>349</td>
</tr>
<tr>
<td>Mononuclear-cell medium, trypsin</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

---

Fig. 4. Sephadex G-150 column chromatography of medium from metabolically labelled chicken mononuclear phagocytes treated (b) or not treated (a) with LPS

Serum-free medium (5 ml) from metabolically labelled ([³⁵S]methionine, 50 μCi/100 mm-diameter dish) cultures of chicken mononuclear phagocytes treated (b) or not treated (a) with LPS (1 μg/ml) for 24 h were applied to a 2.5 cm x 83 cm column in 20 mM-Hepes buffer, pH 7.6, containing 0.15 M-NaCl. Protein fractions (4 ml) were assayed for incorporated radioactivity (——) and fibrinogen-stimulating activity (●●●).
et al., 1978) and experimental animals (Richards & Saba, 1983, 1985; Richards et al., 1983). In studies of patients after surgery, acute depletion of plasma Fn is observed, followed by an acute rise and a subsequent return to normal levels within 7–14 days (Rubli et al., 1983). However, in surgical patients who developed septicemia, Fn levels were either below, or at low, normal levels (Rubli et al., 1983). Owens & Cimino (1982) reported that rat plasma Fn levels were elevated at 48 h after turpentine treatment. Our results clearly demonstrate that plasma Fn responds to experimentally induced inflammation by exhibiting an acute rise in the circulating level between 24 and 72 h (Fig. 1b) as do other known acute-phase reactants such as fibrinogen (Koj, 1974; Pindyck et al., 1983). Thus the rise observed in plasma Fn levels in patients after trauma, surgery or burn injury (Saba et al., 1978; Richards et al., 1983; Rubli et al., 1983) without complicating septicemia or in experimentally induced inflammation may be regulated by HSF and glucocorticoids, substances that also control the production of other liver derived acute-phase proteins.

Studies by Rupp & Fuller (1979) provided the first evidence that a circulating factor(s), now termed HSF, was present in sera from traumatized animals or in crude leucocyte supernatants from the same animal, and could stimulate increased fibrinogen production in rat hepatocyte cultures. Our present results (Fig. 2) indicate that HSF release into the circulation occurs within 5 h after turpentine treatment (Fig. 2). Furthermore, monocyte cells from different species respond quickly after an acute inflammatory stimulus to produce and/or secrete HSF (Ritchie et al., 1982; Ritchie & Fuller, 1983; Baumann et al., 1983b, 1984; Woloski & Fuller, 1985a; Koj et al., 1984; the present study). Recent studies by Woloski & Fuller (1985a) demonstrated HSF size heterogeneity in LPS-stimulated human leukaemic-cell lines. HSF activity can be separated by h.p.l.c. chromatography from that of interleukin I, and only HSF (peaks at ~70 kDa and ~30 kDa) stimulates fibrinogen production by cultured rat hepatocytes (Woloski & Fuller, 1985a). Our studies showed that, like mammalian mononuclear phagocytic cells, chicken mononuclear phagocytic cells (i.e., monocytes and thrombocytes) produce an HSF-like activity (Fig. 4, Table 2). Chicken HSF apparently exhibits the same size heterogeneity (Figs. 3 and 4) as has been found for mammalian HSF preparations (Baumann et al., 1984; Woloski & Fuller, 1985a).

Several studies, including our own, suggest that effective stimulation of acute-phase proteins by HSF differs from species to species and may be modulated by glucocorticoids. Fn synthesis by human fibroblasts (Oliver et al., 1983), endothelial cells (Piovella et al., 1982), rat hepatoma cells (Baumann & Eldridge, 1982) and human fibrosarcoma cells (Oliver et al., 1983) is stimulated by dexamethasone. In the chick hepatocyte system, hepatocyte Fn production is stimulated by HSF or dexamethasone and together their effect is additive (Tables 1 and 2). In the case of rat hepatocytes, however, HSF stimulation of acute phase protein synthesis occurs only in the presence of dexamethasone (Baumann et al., 1984; Koj et al., 1984). More recently, Fuller and Otto (1985) reported that a synergism exists between HSF and dexamethasone effects on rat hepatocyte fibrinogen synthesis. A specific role for glucocorticoids in the production of acute phase proteins is currently undefined. Even though they can stimulate production of fibrinogen and fibronecint in cultured hepatocytes (Baumann et al., 1983a, 1984; Grienger et al., 1978; Fouad et al., 1981; Amrani et al., 1985), it is still uncertain from glucocorticoid infusion studies whether these hormones alone can stimulate the levels of these proteins in vivo (Koj, 1974; Miller & Griffin, 1975). Woloski et al. (1985b) recently reported that HSF induced cultured pituitary cells to release ACTH, suggesting that HSF has a role in stimulating glucocorticoid levels in vivo. This observation would be consistent with the appearance of circulating glucocorticoids following the appearance of HSF after turpentine treatment. One might speculate from the same data that glucocorticoid increase would have a negative feedback effect on monocyte/macrophage production of HSF. One possible effect of circulating glucocorticoids on the liver during inflammation may involve modulation of the liver cell response to HSF or to the products of the HSF reponse, i.e., stabilization of mRNAs for acute-phase proteins.

Stimulation of fibrinogen mRNA levels by HSF in rat hepatocytes has been reported (Fuller & Otto, 1985), suggesting that the effects of HSF and dexamethasone are both due to increases in the number of mRNAs for acute phase proteins (Baumann & Eldridge, 1982; Oliver et al., 1983). Studies designed to address the molecular mechanism(s) by which HSF and dexamethasone stimulate chicken plasma Fn production are needed.

We thank Ms. Julie Erickson for secretarial assistance in typing this manuscript and Ms. Doreen Diekfuss for preparing the Figures. This research was supported by a Wisconsin Heart Association grant-in-aid, NIH Program Project Grant HL 28444, and by an NIH New Investigator Award (NIH R23-AM32762) to D. L. A.

REFERENCES


1986

Received 30 December 1985/14 April 1986; accepted 1 May 1986