STIMULATION OF RAT HEPATOCYTE FIBRONECTIN PRODUCTION BY MONOCYTE-CONDITIONED MEDIUM IS DUE TO INTERLEUKIN 6

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Plasma fibronectin (Fn), a 440-kD glycoprotein produced by hepatocytes, plays a role in nonspecific opsonization, "activation" of macrophage complement receptors, and hemostasis (1, 2). The major source of Fn appears to be the liver, since hepatocyte production of Fn is adequate to account for the total circulating Fn pool (3). The plasma concentration of Fn increases three- to fivefold during experimental inflammation, and hepatocytes isolated from animals harboring an inflammatory focus synthesize increased amounts of Fn (4). Thus, Fn appears to be an inducible acute-phase protein (APP) in at least some species.

Like other APPs, hepatocyte synthesis of Fn is stimulated by products contained in conditioned medium (CM) harvested from LPS-activated macrophages (5). Sera from inflamed animals and LPS-stimulated monocyte CM enhance hepatocyte Fn synthesis (5). A hepatocyte-stimulating factor (HSF) has been purified from these sources and found to be identical to IL-6 (6). Since IL-6 has been shown to regulate synthesis of many APPs (6), it seemed possible that IL-6 might also play a role in controlling Fn production.

In this study, the stimulation of rat hepatocyte Fn production by monocyte CM, IL-1, TNF, and IL-6 was investigated. We report that IL-6 accounts for the total Fn-stimulating activity of monocyte CM, and of the three monokines, only IL-6 stimulates hepatocyte Fn production.

Materials and Methods

Reagents. Goat anti-rat fibronectin antiserum was obtained from CalBiochem-Behring Corp., San Diego, CA; rabbit anti-human IgG from Dako Corp., Santa Barbara, CA; rabbit anti-human IL-1α, rabbit anti-human IL-1β, anti-human IL-6, anti-human TNF, recombinant human (rh)IL-1α, rhIL-1β, rhIL-6, and rhTNF from Genzyme, Boston, MA; LPS (*Salmonella minnesota*, strain Re 595), polymyxin B, rat albumin, rat fibrinogen, and rat Fn from Sigma Chemical Co., St. Louis, MO; and all other antibodies from Organon Teknika-Cappel, Malvern, PA.

Isolation and Culture of Rat Hepatocytes. Rat hepatocytes were obtained from adult male CD-1 rats (250–300 g) by collagenase perfusion (7).

This work was supported by National Institutes of Health grant GM-36258. Marc E. Lanser is a recipient of an NIH Research Career Development Award (GM-00511). Addresss correspondence to Marc E. Lanser, Department of Surgery, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.

Monocyte CM. To obtain monocyte CM, human monocytes were isolated by centrifugation over Ficoll-Hypaque and incubated with monocyte media (MEM with 5% FCS) containing 10 μg/ml polymyxin B or 5 μg/ml LPS for 24 h.

Measurement of Secreted Hepatocyte Proteins. Standard ELISA procedures were used to determine the concentrations of fibrinogen, albumin, and fibronectin in the hepatocyte supernatants.

Purity of Monokines and Specificity of Antimonokine Antibodies. IL-6 was assayed by its stimulation of IgG secretion by EBV-transformed B cells (CESS cells) using a modification of the method described (8). IL-1 was assayed on thymocytes from 4–6-wk-old C3H/HeJ mice as described (9). Only anti-IL-1α and anti-IL-1β neutralized the thymocyte-stimulating activity of IL-1α and IL-1β, respectively, while anti-IL-6 and anti-TNF had no effect. TNF was assayed on L929 cells in the presence of 1 μg/ml actinomycin D (10). Only anti-TNF completely neutralized the TNF, while anti-IL-1α, anti-IL-1β, and anti-IL-6 had no effect.

Results

Stimulation of Hepatocyte Production of Fn by Monocyte CM. LPS-induced CM stimulated hepatocyte production of Fn and fibrinogen, and decreased production of albumin (Table I). Fn production increased 80% while fibrinogen production tripled. Albumin production decreased 25%. The addition of polymyxin B to hepatocyte cultures did not decrease the amount of Fn- and fibrinogen-stimulating activity produced by monocyte CM, indicating that LPS-contamination of the control monocyte cultures did not directly affect the hepatocyte cultures. Fn secretion was dose dependent on the amount of CM added (Fig. 1). Fn secretion tripled when monocyte CM was added at a concentration of ~2% (1:60 dilution).

Neutralization of the Fn-stimulating Activity of Monocyte CM with anti-IL-6. Monocyte CM was preincubated with anti-IL-1α, anti-IL-1β, anti-IL-6, or anti-TNF (Table II). Preincubation of the CM with anti-IL-6 reduced the Fn-stimulating activity of the CM to the same level as no IL-6. Anti-IL-1α and anti-IL-1β had no effect, while anti-TNF partially suppressed Fn production. The suppressive activity of the anti-TNF appeared to be due to its nonspecific cytotoxic effect, since this amount of anti-TNF killed the CESS cells when it was tested against IL-6 in the CESS cell assay (results not shown). In addition, the monocyte CM was tested for TNF activity and none was found (<50 U/ml).

Similar to the case of Fn production, the fibrinogen-stimulating activity of the

TABLE I

Effect of CM on Hepatocyte Protein Production

Added to monocytes	Fibrinogen	Fibronectin	Albumin
No LPS	6.7 ± 3.0 (7)	1.3 ± 0.2 (7)	21.4 ± 2.3 (6)
LPS	21.6 ± 2.7 (7)	2.1 ± 0.5 (7)	16.1 ± 3.9 (6)
Polymyxin B	8.0 ± 5.0 (3)	1.2 ± 0.2 (3)	18.9 ± 3.2 (3)
"Media"	5.0 ± 2.7 (8)	1.2 ± 0.2 (8)	19.4 ± 4.1 (5)

50 μl of CM made by incubating monocytes with the indicated additions was added (in duplicate) to 20-h-old hepatocyte cultures fed with 0.3 ml of hepatocyte media. As an additional control, 50 μl of plain monocyte "media" was also added to some wells of hepatocytes. After an additional 24 h in culture, the concentrations (μg/ml) of Fn, fibrinogen, and albumin were determined in the hepatocyte supernatants. Values represent the mean ± SD, with the number of supernatants or control wells tested shown in parentheses.

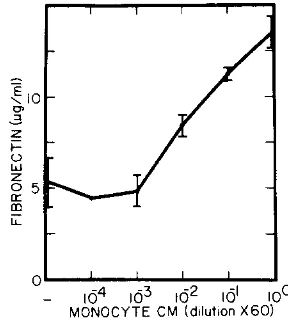

FIGURE 1. Stimulation of hepatocyte Fn production by monocyte CM. Monocyte CM was added in increasing concentrations to hepatocyte cultures and the concentration of Fn in the hepatocyte supernatants was measured after 24 h. Each point represents the mean ± SD of four sets of hepatocytes.

TABLE II

Effect of Antibodies on Hepatocyte Protein Production

Antibodies added	Fibronectin	Fibrinogen
	μg/ml	
Buffer only	6.0 ± 0.2	11.6 ± 2.2
CM only	11.7 ± 1.0	20.7 ± 2.2
Anti-IL-1α IgG	11.6 ± 0.4	20.7 ± 1.8
Anti-IL-1β IgG	10.1 ± 0.4	21.8 ± 3.9
Anti-IL-6 IgG	5.9 ± 1.4	14.8 ± 3.7
Anti-TNF antiserum	8.1 ± 0.7	20.1 ± 3.7

5 μl of CM was incubated with 1 mg/ml of IgG or 40 μl of anti-TNF antiserum overnight at 4°C. 20 μl of the mixtures was then added to 20-h-old hepatocyte cultures (in quadruplicate) freshly fed with 0.3 ml hepatocyte media, and the culture supernatants were harvested after a final 24-h incubation. Values represent the mean ± SD of data obtained from three different monocyte supernatants.

monocyte CM was only neutralized by anti-IL-6, whereas anti-TNF and anti-IL-1 had no effect. None of the antibodies affected albumin production (results not shown).

Effect of Pure Monokines on Hepatocyte Protein Production. Hepatocytes were cultured with rhIL-6, IL-1α, and TNF, and the secretions of Fn and fibrinogen were measured. IL-6 stimulated a dose-dependent increase in Fn and fibrinogen production (Table III). Neither TNF nor IL-1α affected Fn production.

The relative responsiveness of hepatocyte production of Fn, fibrinogen, and albumin to increased doses of IL-6 is shown in Fig. 2. In this experiment, Fn production began to increase at a lower dose of IL-6 than did fibrinogen production, though higher doses of IL-6 resulted in a greater maximal increase in fibrinogen production (fourfold) than Fn production (2.2-fold). Suppression of albumin production was the least sensitive to IL-6, occurring only at an IL-6 dose of 1 ng/ml or greater.

The effect of IL-6 on Fn production might have been due to contaminants in the rhIL-6. This possibility was investigated by prior incubation of the IL-6 with anti-IL-6. Anti-IL-6 completely blocked the Fn- and fibrinogen-stimulating effect of the IL-6 (Table IV).

Identity of the Secreted Fn. To determine whether the Fn secreted by the hepatocyte cultures was the plasma or cellular form, the Fn in the hepatocyte supernatants was purified and run in parallel with pure plasma and cellular Fn on an SDS-poly-

TABLE III
Effect of Monokines on Hepatocyte Fibronectin Production

Amount added	Fibronectin produced		
	IL-6	TNF	IL-1
μg/ml		*μg/ml*	
10^{-1}	3.9 ± 1.3	2.5 ± 0.5	ND
10^{-2}	5.5 ± 1.9	2.1 ± 0.6	ND
10^{-3}	4.8 ± 2.2	2.3 ± 0.2	4.1 ± 0.4
10^{-4}	3.6 ± 1.8	2.4 ± 0.3	3.4 ± 0.6
10^{-5}	3.7 ± 1.7	2.4 ± 0.2	3.2 ± 0.2
0	2.5 ± 1.0	2.5 ± 0.3	4.0 ± 0.8

20-h-old hepatocyte cultures were refed with media containing the indicated monokines (in quadruplicate). The concentration of Fn was measured in the hepatocyte supernatants after an additional 24 h. IL-6 = 2×10^4 U/μg; TNF = 2×10^4 U/μg; IL-1 = 10^5 U/μg. Results are the mean ± SD of four sets of hepatocytes.

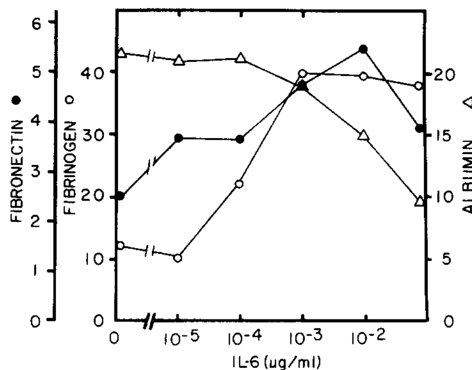

FIGURE 2. Stimulation of hepatocyte production of Fn, fibrinogen, and albumin by IL-6. rhIL-6 was added in increasing concentrations to hepatocyte cultures and the concentrations of Fn, fibrinogen, and albumin in the hepatocyte supernatants were measured after 24 h. Each point is the mean ± SD of four sets of hepatocytes.

TABLE IV
Neutralization of IL-6 Stimulation of Fn and Fibrinogen Production by Anti-IL-6

Additions	Fibronectin	Fibrinogen
	μg/ml	
Buffer	3.1 ± 0.7	5.2 ± 0.8
Buffer + anti-IL-6	3.5 ± 1.5	5.8 ± 1.9
IL-6	6.9 ± 1.6	11.1 ± 1.8
IL-6 + anti-IL-6	3.6 ± 0.9	7.1 ± 2.1

4 μl of IL-6 (2 μg/ml) or of PBS was incubated overnight at 0°C with 80 μl (1 mg/ml) anti-IL-6 or PBS. 19.5 μl of these mixtures was placed into wells of freshly fed (0.3 ml) hepatocytes, and the concentrations of Fn and fibrinogen in the hepatocyte supernatants were measured after 24 h. Each point represents the mean ± SD of four sets of hepatocytes.

acrylamide gel (Fig. 3). The plasma Fn was purified from rat plasma and the cellular form was purified from fibroblast culture supernatants. The secreted Fn and pure plasma Fn ran identically in the gel and slightly slower than the cellular form. Cel-

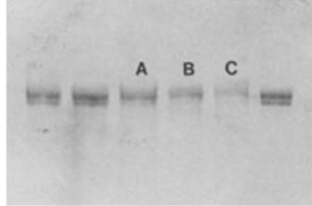

FIGURE 3. SDS-PAGE of hepatocyte fibronectin. Rat serum and culture supernatants from F2408 rat fibroblasts and monocyte CM-stimulated rat hepatocytes were adsorbed onto gelatin-agarose. The complexes were washed with PBS and bound material eluted with 6 M urea. The eluted material was dialyzed for 24 h against PBS. These fibronectin samples were diluted into sample buffer, subjected to electrophoresis into a 7.5% SDS-PAGE plate, and stained with Coomassie blue. (Lane *A*) F2408 culture supernatant; (lane *B*) hepatocyte culture supernatant; (lane *C*) rat serum.

lular Fn was not apparent in the hepatocyte supernatants. Thus, IL-6 appeared to specifically stimulate the production of the plasma form of Fn.

Discussion

We have shown that stimulation of in vitro hepatocyte production of Fn by monocyte CM is due to the monokine IL-6. Amrani et al. (11) stimulated hepatocyte Fn production by exposing them to either monocyte CM or sera fractions containing HSF activity from inflamed animals. These sera fractions presumably contained IL-6, since IL-6 has been shown to be the major source of HSF activity (6). However, since both CM and these sera fractions contain other monokines, the factor responsible for the Fn-stimulating activity could not be identified from these studies. By neutralizing the Fn-stimulating activity of CM with anti-IL-6 and by showing that rhIL-6 possesses strong Fn-stimulating activity, we have demonstrated that the source of the Fn-stimulating activity in monocyte CM is IL-6.

Neither IL-1 nor TNF stimulated Fn production when added to hepatocytes in doses ranging from 10^{-5} to 10^{-1} μg/ml. Rat hepatocyte Fn production is thus similar to that of fibrinogen, in that IL-6 appears to be the only one of these three monokines that significantly stimulates production of these APPs.

Our results are in contrast to those of Castell et al. (12), who found that IL-6 at 100 U/ml suppressed human hepatocyte synthesis of Fn ~50%. These contrasting results are most likely due to the different species (human vs. rat) used as sources of hepatocytes. The large increases in plasma Fn levels seen in experimental inflammation do not occur in clinical infection. These conflicting results suggest that Fn may not be an APP in humans.

In our study, the Fn-stimulating activity of CM was completely neutralized only with anti-IL-6. Crossreactivity with other monokines was ruled out by demonstrating that the anti-IL-6 did not neutralize either IL-1 or TNF activity. IL-6 itself was found to have a small amount of IL-1 (thymocyte-stimulating) activity, but we did not attribute the Fn-stimulating activity of IL-6 to contamination with IL-1, since rhIL-1 itself had no effect on Fn production.

IL-6 increased secretion of the plasma form of Fn, rather than the cellular form. Hepatocytes have been reported to possess only plasma Fn mRNA and to produce only the soluble plasma form of the molecule (3). In addition, production of only the plasma form of Fn increases during inflammation (4).

Fn production was at least as sensitive to IL-6 as fibrinogen production. It is not clear whether this enhanced production of Fn is due to effects on transcription or on secretion, since IL-6 increases secretion as well as transcription of some APPs.

Summary

In this report, conditioned media from LPS-stimulated monocytes increased rat hepatocyte production of fibronectin (Fn) in a dose-dependent manner. Preincubation of the conditioned media with anti-IL-6, but not with anti-IL-1α, anti-IL-1β, or anti-TNF, completely neutralized the Fn-stimulating activity. 10–100 pg/ml of rIL-6 was sufficient to increase Fn production. Neither IL-1 nor TNF had an effect on Fn production. The Fn-stimulating activity of IL-6 could be specifically neutralized only with anti-IL-6, but not with anti-IL-1 or anti-TNF. The increased Fn produced was shown to be of the plasma rather than the cellular form. These results demonstrate that IL-6 is the factor in monocyte-conditioned media that stimulates Fn production, and that IL-6 is the monokine tested with such activity.

We thank Ms. Laura Praddaude and Ms. Beth Reed for technical assistance. We also thank Dr. Brian Cohen for his help in isolation of the hepatocytes.

Received for publication 26 June 1989 and in revised form 28 August 1989.

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