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Identification and partial characterization of hepatocyte-stimulating factor from leukemia cell lines: Comparison with interleukin 1

(fibrinogen/thymocyte/acute phase/HPLC/phorbol diester)

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ABSTRACT **Leukemia cell lines of the monocytic series (HL-60, U-937, and P388D$_1$) produce a hepatocyte-stimulating factor (HSF) following induction of differentiation with phorbol diester. In 24–72 hr, these leukemia cells produce 2–30% the amount of HSF as human peripheral blood monocytes. Cells of the series at earlier stages of differentiation produced greater amounts of HSF. Fractionation of the medium from each cell type by HPLC reveals much of the HSF activity in the 25- to 30-kilodalton range. Under the same culture conditions, interleukin 1 is produced; however, its bioactivity is in the 7- to 15-kilodalton range. Neither monokine shows reciprocal bioactivity. Superinducing culture conditions that greatly increase interleukin 1 production completely eliminate HSF production, suggesting that there is different stability of the mRNA coding for each protein or that there are different temporal events important to the induction of synthesis of these proteins.**

A number of well-established physiologic reactions occur during the acute-phase response following inflammation and tissue injury. These include fever, an increase in circulating neutrophil levels, and significant changes in the concentration of many hepatically derived plasma proteins known as acute-phase reactants (1–3). Fibrinogen is one of the plasma proteins whose synthesis is increased severalfold, providing a characteristic marker for the hepatic response to an acute inflammatory challenge (4).

Factors released from leukocytes control much of the acute-phase response (5–10). Medium conditioned by monocytes or Kupffer cells markedly increased cultured hepatocyte synthesis of acute-phase reactants such as fibrinogen (10, 11); keratinocytes also produce a factor with the same activity (12). We have named this factor hepatocyte-stimulating factor (HSF) to describe its direct effects on regulating gene expression in primary hepatocytes (10, 13). Monocytes also produce other regulator proteins or monokines. The best studied of these is interleukin 1 (IL-1), which is an 11- to 16-kDa hormone-like factor that regulates fever and has a mitogenic activity towards T lymphocytes and fibroblasts (6–8, 14). IL-1 has also been implicated as a regulator of hepatic synthesis of the acute-phase reactant serum amyloid A (15) and *in vivo* experiments have suggested that it may play a role in regulating the synthesis of other acute-phase reactants, including fibrinogen (6, 16).

To better understand the relationship between IL-1 and HSF and to develop a cell system to investigate the control of HSF synthesis, we have examined these monokines from three different cell lines of the monocytic series (17, 18). The U-937 and P388D$_1$ cells were of particular interest since they have already been shown to produce IL-1 (19, 20). We report here that all three lines can be induced to produce HSF as well as IL-1. We provide evidence that HSF and IL-1 are not only significantly different in size but also their bioactivities are different and not reciprocal. Furthermore, biosynthesis of IL-1 and HSF differ since there is no HSF production under conditions that cause superinduction of IL-1. These findings allow a clear distinction between IL-1 and the HSF that regulates hepatic synthesis of acute-phase reactants.

MATERIALS AND METHODS

Bioassays. For the bioassay of HSF, normal adult hepatocytes were isolated by a collagenase perfusion technique (21) and cultured as described (9, 10). Fibrinogen levels in hepatocyte media were assayed by using an ELISA (22). IL-1 activity was determined by the thymocyte proliferation assay (23). Ultrapure human IL-1 purified to apparent homogeneity was obtained from Genzyme (Norwalk, CT). Thymocytes from C57BL/J6 mice (The Jackson Laboratory) were plated at 1×10^6 cells per well on 96-well culture plates (Costar); culture medium was RPMI 1640 (KC Biological, Lenexa, KS) containing 10% heat-inactivated fetal calf serum (Flow Laboratories), 0.01 mM 2-mercaptoethanol (Sigma), 2 μg of phytohemagglutinin (PHA) (Wellcome) per ml, and sample, usually at a 1:100 dilution. Thymocytes were cultured for 48 hr at 37°C in 5% CO_2 in air and pulsed for 16 hr with 0.1 μCi of [*methyl*-^{3}H]thymidine (88 Ci/mmol; 1 Ci = 37 GBq; ICN) per well, after which the DNA of cells was precipitated with cold 5% trichloroacetic acid and collected on filter paper and radioactivity was determined by liquid scintillation counting.

Preparation of Conditioned Medium (C Medium). The human monocytic leukemia cell lines U-937 and HL-60 and the murine histiocytic leukemia cell line P388D$_1$ were obtained from the American Tissue Type Collection; these cell lines have been described in detail by Koeffler (17). Human cells were grown in modified Eagle's minimal essential medium; this contained 25 mM Hepes (Research Organics, Cleveland, OH), 55 mg of sodium pyruvate (GIBCO) per liter, 20 ml of nonessential amino acids (KC Biological) per liter, 125 mg of penicillin per liter, and 63 mg of streptomycin sulfate per liter in Eagle's modified minimal essential medium (GIBCO). The murine cells were cultured in RPMI 1640 medium. Media were supplemented with 5% heat-inactivated fetal calf serum. The lines were subcultured biweekly at a concentration of $1–3 \times 10^5$ cells per ml. Leukemia cell-conditioned media were prepared by washing cells three times in serum-free medium, suspending at 1×10^6 cells per ml in serum-free medium, adding 30 ng of phorbol 12-myristate 13-acetate (PMA) (ITT Research Institute, Chicago) per ml, and incubating at 37°C in a 5% CO_2 in air atmosphere. At appropriate times, cells were removed by centrifugation at $2000 \times g_{av}$ for 20 min. Monocyte-conditioned medium (MC medium) was prepared by incubation of adherent mononuclear cells of

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Abbreviations: HSF, hepatocyte-stimulating factor; PMA, phorbol 12-myristate 13-acetate; IL-1, interleukin 1; PHA, phytohemagglutinin.

whole human blood by previously described procedures (13). In some experiments, P388D$_1$ cells were incubated in a superinduction medium (20). This involved incubation with 10 μg of PMA per ml, 10 μg of cyclohexamide (Sigma) per ml, and 2 mM sodium butyrate (GIBCO) for 5 hr, incubation with 10 μg of actinomycin D (Sigma) per ml for another hour, and a final incubation with 2 mM sodium butyrate for 24 hr. These agents were in RPMI 1640 culture medium with 1% fetal calf serum.

Chromatography. Prior to chromatography of leukemia cell medium, samples were concentrated at 4°C to 0.50 ml and concurrently dialyzed against 20 mM sodium phosphate, pH 7.4/150 mM sodium chloride. HPLC was performed by using a Waters model 510 solvent delivery system, model U6K injector, and model 441 absorbance detector set at 280 nm (Waters Associates) and a MicroPak TSK 2000SW preparative size exclusion column (22 × 300 mm) from Alltech (Deerfield, IL). The solvent used was 20 mM sodium phosphate, pH 7.4/150 mM sodium chloride, which was run at a flow rate of 1 ml/min. Bovine serum albumin, chymotrypsinogen, lysozyme (all from Sigma), ovalbumin (Mann Research Laboratories, New York), and insulin (Eli Lilly) were used as molecular weight markers for the column.

RESULTS

Monokine Bioassays. Medium conditioned by monocytes and leukemia cells contain both HSF and IL-1; the presence of these monokines was assayed in this work by their effects on cultured hepatocytes and thymocytes. The direct effects of supplementation with MC medium on hepatocyte fibrinogen production (HSF bioassay) is shown in Fig. 1*A*, whereas Fig. 1*B* shows the mitogenic response of cultured thymocytes (IL-1 bioassay). Although much of the HSF activity was lost by dilution of 1:100 or greater, IL-1 activity was still present in samples diluted 1:10,000. Although higher levels of IL-1 than HSF may have been present in MC medium, these data suggested that the thymocyte proliferation assay was at least as sensitive a measure of IL-1 activity as the assay of HSF. Also, HPLC-fractionated samples were subsequently assayed by the thymocyte proliferation assay at a dilution of 1:100; IL-1 activity was detected in samples at this dilution and relative activities did not differ significantly from those assayed at dilutions of 1:10 and 1:1000.

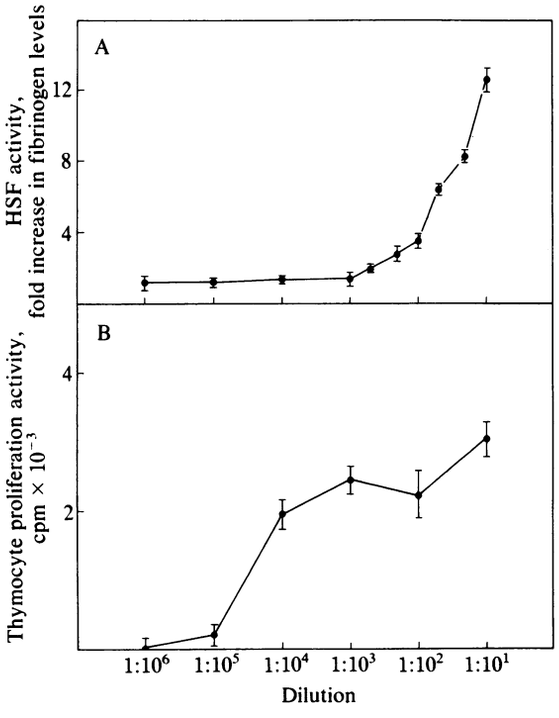

FIG. 1. Comparison of monokine bioassays. MC medium was prepared and used as supplementation in the culture of rat primary hepatocytes and mouse thymocytes. (*A*) Hepatocyte medium levels of fibrinogen; (*B*) incorporation of [*methyl*-^{3}H]thymidine into thymocyte DNA in the presence of 2 μg of PHA per ml. Results shown are the means and variances from the mean of three to five analyses.

Production of HSF. Leukemia cell lines were incubated for 1–3 days in serum-free medium supplemented with PMA and HSF activity in the C medium was tested by the hepatocyte bioassay; the results of these analyses are shown in Fig. 2. The leukemia cell lines released HSF into the culture medium only after PMA had initiated differentiation toward the macrophage lineage since HSF activity was not present in the medium in the absence of PMA. The U-937 cell line produced the highest levels of HSF activity (45 units per 10^6 cells per 24 hr), whereas culture of P388D$_1$ cells produced the lowest levels (4 units per 10^6 cells per 24 hr). These findings contrast with IL-1 production by these cells; there is higher IL-1 production of 3500 units/mg by P388D$_1$ cells (20) than the 2400 units/mg by U-937 cells (24). Moreover, HSF production by HL-60 and P388D$_1$ cells did not persist after 2 days of culture (Fig. 2), whereas IL-1 production continues for at least 5 days in U-937 cells (19).

High-Performance Gel Permeation Chromatography. Commercially available human IL-1 has been tested for ability to stimulate fibrinogen production by hepatocytes and has been found to be without effect at dosages of 0.1–20 units of thymocyte proliferation activity. To make a direct comparison of the sizes of IL-1 and HSF synthesized by sources that produce both of these monokines, high-resolution gel permeation chromatography was performed on C medium and the resulting fractions were assayed for both HSF and IL-1. The results of these analyses are shown in Fig. 3. Most of the HSF activity of MC medium eluted from the gel permeation column with an apparent molecular mass of 25–30 kDa (Fig. 3*A*); this is the same size as determinations of molecular mass by gel filtration of MC medium and Kupffer cell-C medium have indicated (10, 11). The IL-1 of MC medium had an apparent molecular mass of 10–12 kDa (Fig. 3*A*), which agrees with the 11- to 15-kDa size of this molecule that has been reported (8, 14, 19, 20, 25); thymocyte proliferation activity was also present in fractions with apparent molecular

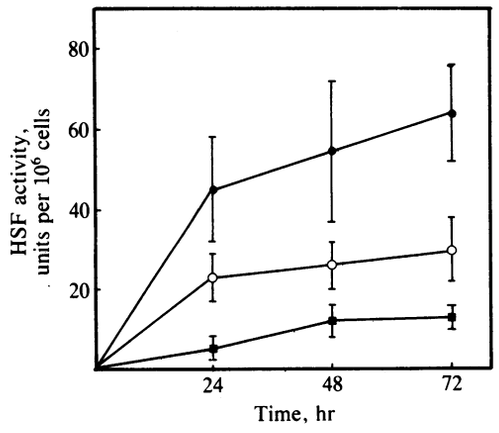

FIG. 2. HSF production by leukemia cell lines. Cells were cultured at 1 × 10^6 cells per ml for the specified times and HSF activity was determined; 1 unit of activity is that amount that elevates fibrinogen levels in hepatocyte medium half-maximally. Cell lines used were U-937 (●), HL-60 (○), and P388D$_1$ (■). Results shown are the means of three to five experiments in which duplicate analyses were performed on medium fibrinogen levels of hepatocyte cultures exposed to doses of 2–200 μl of medium conditioned by leukemia cells for 1–3 days; bars indicate the variances of the results.

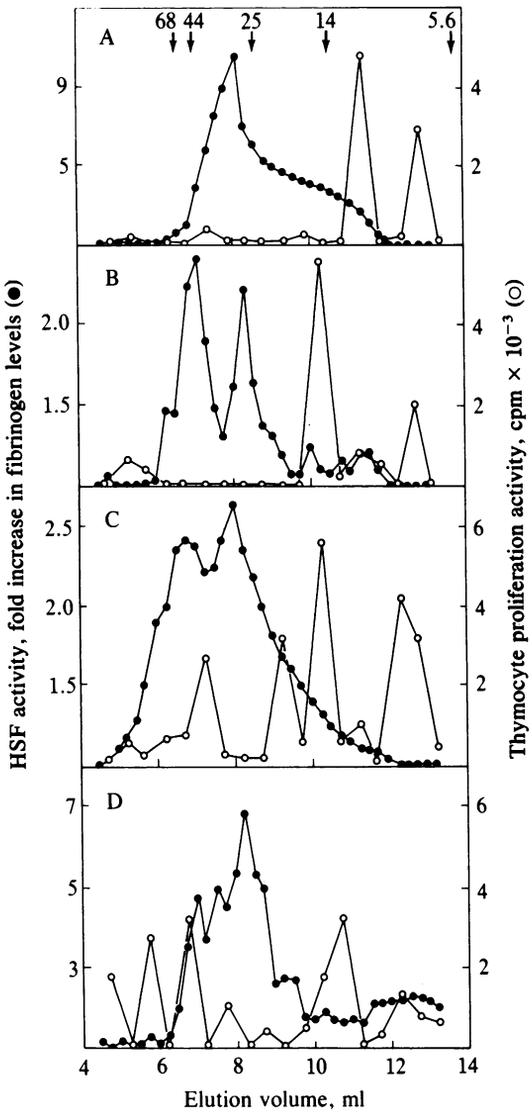

FIG. 3. HSF and IL-1 activity in gel permeation fractions of C media. Shown are column profiles of material produced by 3×10^7 monocytes cultured for 24 hr (*A*) and medium conditioned for 48 hr by 6×10^6 P388D$_1$ cells (*B*), U-937 cells (*C*), and HL-60 cells (*D*). HSF activity is shown in units of fold elevation of hepatocyte medium fibrinogen levels elucidated by 50 μl of MC medium or 200 μl of leukemia cell-C medium. Elution positions of marker proteins are indicated by their molecular masses in kDa.

mass of 7–8 kDa (Fig. 3*A*). The C media of the leukemia cells all contained the 25- to 30-kDa HSF (Fig. 3 *B–D*) that was seen in MC media; the leukemia cells also produced HSF activity in fractions of apparent molecular mass greater than 40 kDa. Leukemia cell IL-1 had an apparent molecular mass of about 15 kDa (Fig. 3 *B–D*); thymocyte proliferation activity of the 7- to 8-kDa size was also present. In addition, human U-937 and HL-60 cells produced thymocyte proliferation activity with higher molecular masses but clearly not in the 25- to 30-kDa fractions. Different sizes exist for both HSF and IL-1; however, these monokines do not copurify.

Effect of the Superinduction Method on P388D$_1$ Monokine Production. Mizel and Mizel (20) have reported a superinduction procedure that stimulated the production of IL-1 by P388D$_1$ 1400-fold. This method capitalized on IL-1 mRNA synthesis within 4 hr of PMA treatment and IL-1 mRNA stability over 24 hr of culture. Treatment with PMA stimulates the production of both IL-1 and HSF by P388D$_1$ cells, which suggests that the production of these monokines may be concomitantly induced. The comparative effects on HSF and IL-1 production of the superinduction procedure, which uses inhibitors of protein and RNA synthesis at early times of treatment, were examined to determine if mRNAs for HSF and IL-1 were similar in their stabilities and timing of appearance after PMA treatment. Fig. 4 shows the results of both PMA treatment and the superinduction procedure on HSF and IL-1 production by P388D$_1$ cells. Although C medium from superinduced cells had levels of IL-1 about 5000-fold higher than control C medium, as determined by the dilution necessary to eliminate thymocyte proliferation activity (Fig. 4*B*), C medium from superinduced cells had no more HSF activity than C medium from untreated cells (Fig. 4*A*). These effects were not due to components of the superinducing medium as the same results were obtained with dialyzed samples of the C medium. Lowered responses that were seen with higher levels of superinduced C medium may be due to the presence of other leukemia cell products. The effect of the superinduction procedure on the production of monokines contrasts with the effect of PMA treatment alone. Phorbol activation of P388D$_1$ cells results in a 1000-fold stimulation of IL-1 production, as determined by the concentration-dependent effects on thymocyte proliferation (Fig. 4*B*), that is similar to that already reported for this treatment (26) and also results in a dramatic increase in HSF production (Fig. 4*A*). These data may result from different timing of transcription of HSF and IL-1 mRNAs at early times after PMA treatment or from different stabilities of the mRNAs for the two molecules; the monokines themselves have been shown to have similar stabilities (10).

DISCUSSION

Following tissue injury and during infections, blood monocytes migrate to the site of injury or infection and differenti-

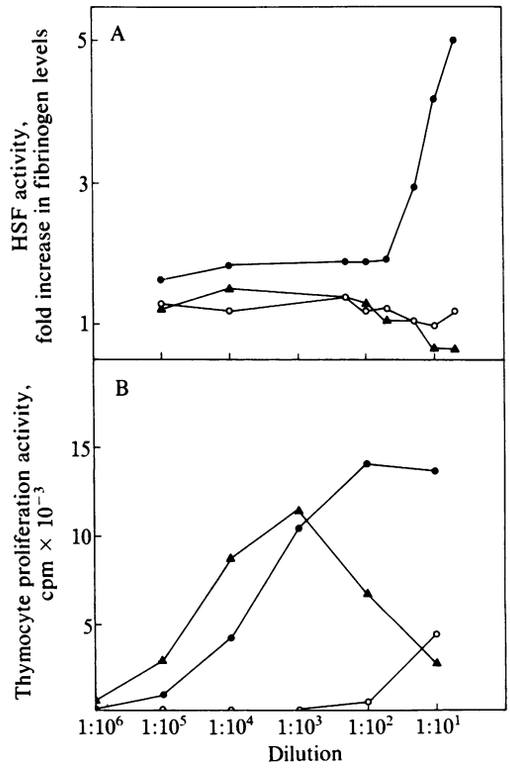

FIG. 4. HSF and thymocyte proliferation assay of superinduced and nonsuperinduced P388D$_1$-C medium. P388D$_1$ cells were cultured at 2×10^6 cells per ml in the presence (●) or absence (○) of 30 ng of PMA per ml for 24 hr or in superinduction medium (▲). HSF and IL-1 activities were determined by bioassays. Results shown are the means of three to five analyses; standard errors were <10% of the means.

ate into macrophages (27, 28). These macrophages can be distinguished from monocytes and other progenitor cells of the monoblast series by properties that include increased adherence to substratum, increased cytotoxicity due to increased phagocytosis and a respiratory burst that produce active oxygen species, elevated levels of cell surface enzymes such as ecto-5'-nucleotidase, increased levels of lysosomal hydrolases, and elevated secretion of a number of proteins (25, 29–31). Two of the products of these cells that have been identified are the monokines IL-1 (25) and HSF (10, 13).

Differentiation of progenitor cells into macrophage-like cells has been extensively studied in recent years by using leukemia cells blocked at various stages of development (17). The cell lines U-937, P388D_1, PU5-18, WEI-3, and THP-1 acquire features of macrophages, including IL-1 production, when treated with stimulating agents such as PMA (19, 20, 27, 28, 32). The present study demonstrates that the U-937, P388D_1, and HL-60 cells produce HSF in addition to IL-1 upon PMA treatment. The different levels of production of monokines by these cells may be related to the stage of maturation they have achieved in culture. HL-60 cells are a promyelocyte-like cell line and can be induced to differentiate into granulocyte-like or macrophage-like cells by different treatments (17). On the other hand, U-937 cells are a monocytoid cell line already committed to the monocyte/macrophage lineage (17). Finally, P388D_1 cells are a histiocytic cell line that has acquired many macrophage features such as phagocytosis and adherence to glass and plastic surfaces (18). Surprisingly, the U-937 and HL-60 cells, which have features of cells at earlier stages of monoblast maturation, are better producers of HSF than the P388D_1 cells; P388D_1 cells are high producers of IL-1 (20). Also, superinduction culture conditions that lead to a 1400-fold increase in IL-1 production by P388D_1 cells also lead to a complete loss of HSF production. These observations suggest that the monokines IL-1 and HSF may not be produced simultaneously but rather at different stages of maturation. However, other possibilities exist for these results, such as different stabilities of mRNA for the different monokines.

Much of the HSF from human monocytes chromatographed with an apparent molecular mass of 25–30 kDa when examined by gel filtration (10) and gel permeation chromatography (this work). The leukemia cells studied here all produced HSF with an apparent molecular mass of 25–30 kDa, although activity was also seen at an apparent molecular mass of 50 kDa. Baumann *et al.* (12) have recently confirmed our previous finding of the presence of a HSF from MC medium with an apparent molecular mass of 25–30 kDa. They also reported that human keratinocytes and lipopolysaccharide-stimulated mouse peritoneal macrophages have a hepatocyte-stimulating activity in the 50- to 60-kDa range in addition to the 25- to 30-kDa activity. Our data demonstrate the presence of HSF activity in higher molecular mass ranges also. These observations suggest that there are several species of HSF produced by cells. IL-1 was present in C medium with apparent molecular masses of 9–15 and 7–8 kDa as well as higher molecular weights of 20, 50, and 70 kDa. IL-1 has been reported to have a molecular mass of 11–15 kDa when isolated from several sources (8, 14, 19, 20, 25, 31); higher molecular mass species have also been reported (8, 14, 25, 31). The smaller molecular mass species identified here are interesting in light of a recent observation by Kimball *et al.* (33) of IL-1-like species with molecular masses of 2 and 4 kDa present in human urine. These authors suggested that these small species may result from proteolytic degradation of larger species (33). It may be that the 7- to 8-kDa species with thymocyte proliferation activity represents an intermediate in this processing.

Our results demonstrate that stimulated leukemia cell lines produce HSF; this factor is clearly different from the monokine IL-1. Also, superinducing culture conditions for IL-1 resulted in a loss of HSF production, although cells had been exposed to stimulating levels of PMA. It has been shown that a specific receptor for PMA in leukemia cells is protein kinase C and that phorbol esters activate this enzyme by dramatically increasing the affinity for calcium (34). Presumably PMA stimulates monokine production by mimicking the synergistic action of phospholipids and calcium on this intermediate of the cell-surface signal transduction; further work is necessary to understand the role of protein kinase in IL-1 and HSF induction. This work also demonstrates that the *in vivo* effects of partially purified IL-1 on fibrinogen production (6, 16) are not due to direct effects of the monokine on hepatocytes; superinduced P388D_1 C medium is a highly active source of IL-1 but does not elevate hepatocyte fibrinogen biosynthesis and secretion. Increased hepatic production of the plasma proteins α_1-acid glycoprotein, albumin, and sialyltransferase is characteristic of the acute-phase response in the rat (35, 36); these proteins are also influenced by leukocytic factors (5, 12, 37). It will be of interest to determine whether HSF, IL-1, or other monokines regulate the response of these parameters by direct action on hepatocytes.

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