Volume 298, number 2,3, 165–168 FEBS 10732 February 1992

Synthesis of α_1-microglobulin in cultured rat hepatocytes is stimulated by interleukin-6, leukemia inhibitory factor, dexamethasone and retinoic acid

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Received 4 December 1991

The secretion of α_1-microglobulin by primary cultures of rat hepatocytes was found to increase upon the addition of interleukin-6 or leukemia inhibitory factor, two mediators of acute phase response. This stimulatory effect was further enhanced by dexamethasone. α_1-Microglobulin is synthesized as a precursor also containing bikunin, and the precursor protein is cleaved shortly before secretion. Our results therefore suggest that both α_1-microglobulin and bikunin are acute phase reactants in rat hepatocytes. Furthermore, we found that retinoic acid, previously shown to be involved in the regulation of cell differentiation and development, also stimulated α_1-microglobulin synthesis. Only free, uncomplexed α_1-microglobulin (28,000 Da) was detected in the hepatocyte media, suggesting that the complex between α_1-microglobulin and α_1-inhibitor 3, found in rat serum, is formed outside the hepatocyte.

Interleukin-6; Retinoic acid; Rat hepatocyte; Acute phase; α_1-Microglobulin; Bikunin

1. INTRODUCTION

α_1-microglobulin (α_1-m) is a low molecular weight plasma protein, belonging to the lipocalin family [1–3]. It is synthesized by hepatocytes as a precursor containing also a proteinase inhibitor, recently named bikunin, which is identical to the light chain (HI-30) of inter α-trypsin inhibitor (ITI) or pre-α-trypsin inhibitor [4–7]. Cleavage of the precursor occurs intracellularly shortly before secretion [8–10]. In plasma, α_1-m exists in free form but also in a complex with the heavy chain of IgA in man [11], and with α_1-inhibitor 3 in rat [12].

The plasma levels of α_1-m in man (approximately 0.03 mg/ml) has been reported to be unaffected by neoplastic diseases and inflammatory conditions [13], although some contradictory reports exist [14]. For bikunin, an inflammation-associated increase in plasma and urine has been demonstrated [3,5,15,16], but it has not been clear whether this is caused by increased synthesis or increased release from higher molecular mass complexes. To resolve the question whether α_1-m/bikunin are positive acute phase reactants, we have studied the synthesis of α_1-m in primary cultures of rat hepatocytes incubated with factors known to induce typical acute phase response [17–19].

It was shown previously [20] that the levels of fetal and adult rat liver α_1-m-encoding mRNA vary with age. The plasma levels of α_1-m have also been reported to vary with age and development [13,21]. This suggests a developmental regulation of the expression of the α_1-m/bikunin gene. Retinoic acid has been shown to be involved in cell growth and differentiation of embryonic and adult cells [22–24], and experiments in this work demonstrate an influence of retinoic acid on the hepatocyte expression of α_1-m.

Abbreviations: α_1-m, α_1-microglobulin; IL-6, interleukin-6; LIF, leukemia inhibitory factor; CPI, cysteine proteinase inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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2. MATERIALS AND METHODS

2.1. *Materials*

Williams E medium and foetal calf serum were purchased from Gibco (Paisley, Scotland); collagenase (type IV), dexamethasone, bovine serum albumin and vitamin A acid all-*trans* (retinoic acid) were from Sigma Chemical Co. (St. Louis, MO, USA); human recombinant interleukin-6 (IL-6) was a gift of Dr. W. Fiers (Gent, Belgium) and human recombinant leukemia inhibitory factor (LIF) from Dr. H. Baumann (Buffalo, NY, USA). Rat α_1-m was prepared from rat serum as described [12]. Rabbit anti-rat α_1-m and goat anti-rabbit immunoglobulins were prepared using conditions as described [12,25]. Antibodies against the rat proteins α_1-inhibitor-3, α_2-macroglobulin, α_1-cysteine proteinase inhibitor (CPI), and albumin were prepared in rabbits as described [26].

2.2. *Hepatocytes*

Hepatocytes were isolated under sterile conditions from liver of

adult Wistar rats after perfusion with collagenase and cultured in Williams E medium containing 9 mM $NaHCO_3$, 2 mM glutamine, 1 μM insulin and initially 5% foetal calf serum [27]. Hepatocyte monolayers were obtained by pipetting 1×10^6 cells in 1.25 ml medium into 35 mm dia culture dishes (Flow Laboratories, McLean, VA, USA) coated with collagen.

After overnight incubation at 37°C in air/CO_2 (19:1), the unattached cells were aspirated and 1 ml of serum-free medium containing 0.2% bovine serum albumin added. This medium was changed after 4 h, and 0.25 ml of suitable dilutions of IL-6, LIF or retinoic acid was added. Control cultures received 0.25 ml of the medium alone. The media were changed daily with appropriate supplementation of the tested factors. Media collected after 24 or 48 h of culture were dialyzed against 15 mM NH_4HCO_3 and concentrated 10 times.

2.3. *Electroimmunoassay and radioimmunoassay*

The concentrations of the five proteins α_1-m, α_1-inhibitor 3, α_2-macroglobulin, CPI and albumin in the concentrated media were estimated by electroimmunoassay using monospecific antisera to rat proteins [27]. The concentrations of α_1-m in gel chromatography fractions were determined by radioimmunoassay [28]. Proteins were labelled with ^{125}I (Nordion Int. Co., Canada) using chloramine T [29] to a specific activity around 0.2 MBq/μg.

2.4. *Gel chromatography*

Concentrated hepatocyte culture medium was applied to a column (1.2 × 90 cm) packed with Sephadex G-200 (Kabi-Pharmacia, Sweden). The column was eluted with 20 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3, at a flow-rate of 1.5 ml/h. Fractions of 0.75 ml were collected and analyzed by determination of the absorbance at 280 nm, and by radioimmunoassay.

2.5. *Electrophoresis and immunoblotting*

Concentrated hepatocyte culture media were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions [30]. The separated proteins were then either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes (Bio-Rad, Palo Alto, CA) [31]. The membranes were blocked, incubated with anti-rat α_1-m overnight and ^{125}I-labelled goat-anti-rabbit immunoglobulins for one hour, washed, dried and autoradiographed as described [32].

3. RESULTS

In agreement with earlier findings [19], we observed that rat hepatocytes cultured in the presence of LIF (500 units/ml) or IL-6 (50 ng/ml) synthesize more of the positive acute phase proteins CPI and α_2-macroglobulin, especially in the presence of 1 μM dexamethasone (Table I). Under the same conditions, the synthesis of two negative acute phase proteins, α_1-inhibitor 3 and albumin, was suppressed. The synthesis of α_1-m showed the same pattern of stimulation as CPI and α_2-macroglobulin.

The effect of the addition of 1.0 μM retinoic acid, in the presence or absence of dexamethasone, was also studied. The synthesis of α_1-m, CPI and α_1-inhibitor 3 was found to be stimulated by this factor (Table I). Contrary to IL-6, the stimulation was reduced by the presence of dexamethasone. The synthesis of α_2-macroglobulin and albumin was not significantly affected by retinoic acid. A positive dose-response was achieved with 0.1, 1.0 and 10 μM retinoic acid on the synthesis of α_1-m and α_1-inhibitor 3 (Fig. 1).

Table I

Effects of LIF (500 units/ml), IL-6 (50 ng/ml) and retinoic acid (1 μM) on the synthesis of CPI, α_2-macroglobulin (α_2-M), α_1-inhibitor-3 (α_1-I_3), albumin and α_1-m by cultured rat hepatocytes

Culture	CPI Dex		α_2-M Dex		α_1-I_3 Dex		Albumin Dex		α_1-m Dex	
	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)	(–)	(+)
Control	100	100	100	100	100	100	100	100	100	100
LIF	132	142	143	240	87	91	92	94	135	159
IL-6	145	206	228	383	75	82	93	89	154	205
Retinoic acid	142	121	105	120	176	114	99	94	155	140

Cells were cultured for 48 h in the medium without (–) or with (+) 1 μM dexamethasone. The results are expressed as percent of control culture and are the means of at least 2 experiments.

The size and molecular distribution of α_1-m secreted into the medium by the cultured hepatocytes was analyzed by gel chromatography of the IL-6-containing medium after dialysis and concentration (Fig. 2). Proteins of various sizes were present as evident from the total protein profile of the eluate. The major peak, which was eluted at the same position as bovine serum albumin, probably corresponds to rat serum albumin. Analysis of α_1-m in the fractions by radioimmunoassay showed that this protein was mainly eluted in a position expected from monomeric α_1-m. This major peak was immediately preceded by a shoulder in the α_1-m-contents, however, suggesting the presence of other forms of the protein.

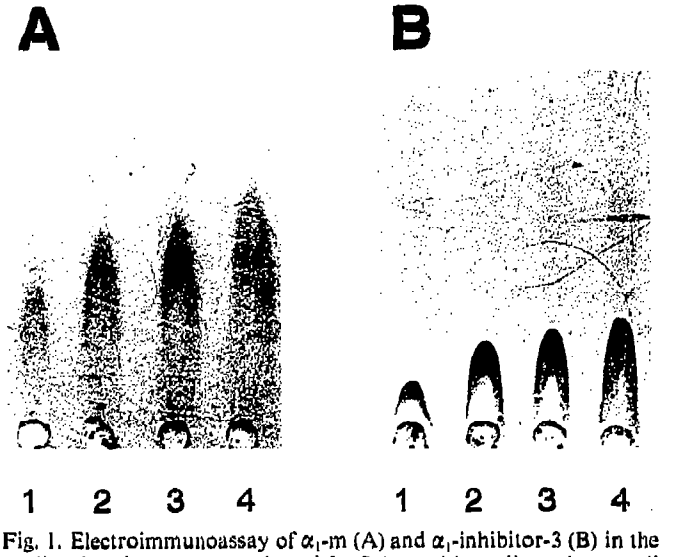

Fig. 1. Electroimmunoassay of α_1-m (A) and α_1-inhibitor-3 (B) in the media of rat hepatocytes cultured for 2 days with medium alone (well 1), with 0.1 μM retinoic acid (2), 1 μM retinoic acid (3), or 10 μM retinoic acid (4). Cells were cultured in Williams E media containing 0.2% BSA and 1 μM insulin, but no dexamethasone. For other details, see section 2.

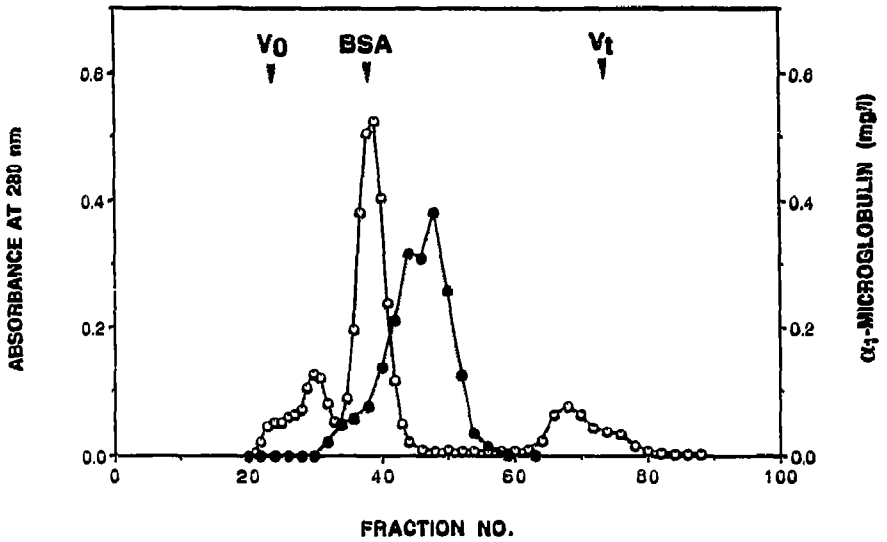

Fig. 2. Gel chromatography of hepatocyte culture medium, containing 50 ng/ml IL-6, collected after 48 h of culture. The medium was concentrated 10 times and 0.2 ml was applied to a column (1.2 × 90 cm) packed with Sephadex G-200, 0.75 ml-fractions of the eluate were collected, and analyzed for total protein by absorbance at 280 nm (open symbols), and α_1-m concentrations by radioimmunoassay (closed symbols). The elution volumes of Blue dextrane, bovine serum albumin, and dinitrophenyl-alanine are marked with V_0, BSA, and V_t, respectively.

The α_1-m released from the hepatocytes was finally analyzed by SDS-PAGE and immunoblotting of the media. Figure 3 shows the result of control hepatocyte cultures, or cultures with either IL-6 alone, dexamethasone alone, or both IL-6 and dexamethasone. Only one protein band, with a molecular mass of 28 kDa, was bound by anti-α_1-m antibodies in the immunoblotting, demonstrating that mainly the un-complexed, free form of α_1-m is secreted from the hepatocytes. The results suggest that the small amounts of high molecular weight forms of α_1-m which were displayed by the gel chromatography (Fig. 2) represent dimers or higher orders of α_1-m aggregates. Moreover, the results confirmed those presented in Table I, with an enhanced hepatocyte production of α_1-m in the presence of IL-6.

4. DISCUSSION

The main finding in this study is that IL-6 and LIF increase the release of free α_1-m into the medium, and that the stimulation is enhanced in the presence of the dexamethasone. The results indicate that the expression of the α_1-m/bikunin gene in hepatocytes is regulated by cytokines and glucocorticoid hormones, and strongly suggests that an increased synthesis of α_1-m and bikunin is part of the accute phase response of the liver. The findings are in agreement with the demonstration of several sequence elements in the α_1-m/bikunin gene, including potential glucocorticoid receptor binding sites, proposed to be important in the activation of known acute phase proteins [6,7].

To our knowledge, the effect of retinoic acid on the acute phase response has not previously been evaluated. We used retinoic acid in the range of concentrations from 0.1 to 10 μM as recommended by other authors who studied the induction of hepatic enzymes [24], expression of proto-oncogenes in a human embryonal cancer cell line [22] or synthesis of heparin-binding proteins with growth and neurotrophic activities [23]. Retinoic acid enhanced the synthesis of CPI, α_1-inhibitor 3 and α_1-m, but had no effect on the synthesis of α_2-macroglobulin and albumin, suggesting that the re-

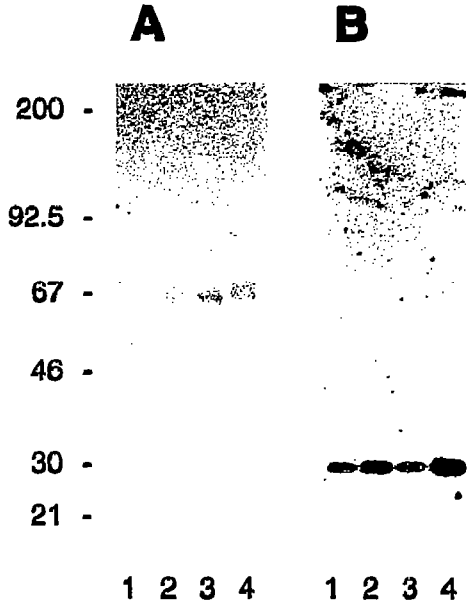

Fig. 3. SDS-PAGE and immunoblotting of hepatocyte culture media. 0.1 μl of concentrated control medium (lane 1), medium with 50 ng/ml IL-6 (lane 2), medium with 1 μM dexamethasone (lane 3), or medium with 50 ng/ml IL-6 and 1 μM dexamethasone (lane 4), were applied to SDS-PAGE (T = 8.5%), stained with Coomassie brilliant blue (panel A), or transferred to nitrocellulose membranes and incubated overnight first with rabbit anti-rat α_1-m, diluted 500 times, and then with ^{125}I-labelled goat anti-rabbit immunoglobulins, 10^6 cpm/ml, (panel B). The molecular masses of standard proteins are given in kDa.

gulatory role of this molecule is different from the acute phase response. Retinoic acid is thought to be involved in the development and differentiation of various cell-types, acting through specific receptors that belong to the steroid/thyroid receptor gene superfamily [33,34]. Consequently, retinoic acid is a possible candidate for the regulation of the liver expression of α_1-m, which, together with the plasma concentrations, was reported to fluctuate during development and ageing of humans and rats [13,20,21], although the physiological function of this fluctuation is not known. The effect of retinoic acid on α_1-m synthesis is also interesting due to the fact that proteins belonging to the lipocalin superfamily have a hydrophobic pocket able to bind several prosthetic groups, including derivatives of vitamin A [35]. However, further studies are required to elucidate the reason for the regulation of the synthesis of α_1-m.

The results indicate that the hepatocytes secrete only free α_1-m. No trace was found in the hepatocyte medium of the complex between α_1-m and α_1-inhibitor 3, reported to be present in the blood of rats [12], indicating that hepatocytes contribute very little to the formation of the complex. Instead, this complex seems to be formed outside the hepatocyte. Further studies of the mechanisms of the formation of this complex are needed to explain the apparent discrepancies that the synthesis rate of α_1-m is increased, whereas the synthesis rate of α_1-inhibitor 3 is decreased under the influence of IL-6 and LIF (Table I). Furthermore, the almost constant plasma levels of α_1-m during inflammation, in spite of an increased release of the protein from hepatocytes, as suggested by the results of this work, needs to be explained.

Acknowledgements: This work was supported by Grant DNS-P/01/078 from the Ministry of National Education, Grant 7144 from the Swedish Medical Research Council, a grant from the Swedish Natural Science Research Council, and by grants from the Foundations of Johansson, Kock and Österlund, and King Gustav V's 80-year Foundation. The authors are grateful to Dr. W. Fiers for a sample of human recombinant IL-6, to Dr. H. Baumann for human recombinant LIF, and to Ms. Maria Allhorn for excellent technical assistance.

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