Kupffer Cell Stimulation of Alpha₂-Macroglobulin Synthesis in Rat Hepatocytes and the Role of Glucocorticoid

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ABSTRACT. When stimulated with lipopolysaccharide (LPS), primary cultures of Kupffer cells from the rat secreted a hepatocyte-stimulating factor (Kupffer factor) which induced the synthesis of α_2 -macroglobulin (a₂M) by hepatocytes. The effect of this Kupffer factor was found only when dexamethasone was present in the hepatocyte culture medium. Dexamethasone alone stimulated the a₂M synthesis, to some extent, in a dose-dependent manner. When both Dex and the Kupffer factor were added to the medium, cultured hepatocytes synthesized 100-fold a₂M, quantitative evidence that explains the *in vivo* phenomenon in which the serum a₂M concentration increases more than 100-fold in the acute phase. A co-culture study with hepatocytes and Kupffer cells indicated that the latter cells have a major function in the induction of a₂M synthesis *in vivo*. A hybridization study with rat a₂M cDNA showed that the concentration of mRNA increased in cultured hepatocytes in the presence of the Kupffer factor, but only when dexamethasone was present.

From these results we concluded that Kupffer cells secrete a hepatocyte-stimulating factor when stimulated by LPS and that glucocorticoid is essential for the induction of a₂M in rat hepatocytes. The effect of the Kupffer factor and glucocorticoid appears to be regulated in the pretranslational, probably the transcriptional, phase.

Acute inflammation elicits enhanced secretion of a group of plasma proteins ("acute phase proteins") that include C-reactive protein, fibrinogen, α_1 -acid glycoprotein, α_1 antitrypsin, haptoglobin and serum amyloid A protein. The mechanisms for the synthesis of these proteins in liver has been investigated (11, 12, 20), and interleukin-1 (IL-1) appears to be an inducer (23).

We made biochemical and physiological studies of α_2 -macroglobulin (a₂M) (5, 6, 8, 9, 10, 14, 17, 18). This protease inhibitor is a typical acute phase protein found in rats, but not in humans. The serum concentration in rats increases from the normal value of approximately 30 μ g/ml (10, 17) to 4,000 μ g/ml within 24 h of a turpentine injection. The protein increases in the blood but accumulates in inflammatory or

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Abbreviations used: a₂M; α_2 -macroglobulin, Dex; dexamethasone, FCS; fetal calf serum, IL-1; interleukin-1, LPS; lipopolysaccharide, MEM; minimal essential medium.

tumor tissues (18). It acts as a protease inhibitor mainly in the circulatory system rather than in the tissues (18). It also has immunomodulative activity, suppressing the lymphocyte blastogenesis induced by lectins (14).

A rapid and marked increase of a2M in serum is caused by its increased *de novo* synthesis in the liver (6, 19) and by hepatocytes (8, 9). The synthesis of a2M is modulated by various components, including hormones and cellular factors (1, 4, 7, 8, 9). Murine IL-1 stimulates the synthesis of a2M in rat hepatocytes (2). Recently, Bauer *et al.* (1) and our group (8) have demonstrated that a culture supernatant of Kupffer cells has hepatocyte-stimulating activity that enhances the synthesis of a2M by rat hepatocytes. The role of glucocorticoid in the induction of a2M also has been investigated *in vivo* (1, 4, 7) and *in vitro* (1, 8). Little, however, is known about the regulating step of the glucocorticoid at the gene level during the induction of a2M.

A2M cDNA has been cloned by Northemann *et al.* (16) and in our laboratory (5). Using our cDNA as a probe, we demonstrated that the induction of a2M synthesis is regulated in the pretranslational stage (5). Factors such as hormones and chemical mediators would affect any experimental results gained from an *in vivo* system; therefore, the study of a2M expression with a2M cDNA in an *in vitro* system that uses a hepatocyte culture should provide an appropriate technique with which to determine the molecular mechanisms that function in the synthesis of acute phase proteins in hepatocytes.

We now have confirmed previous reports by Bauer *et al.* (1) and ourselves (8) that both glucocorticoid and a cellular factor (the Kupffer factor) are necessary for the induction of a2M. We also have shown a2M synthesis quantitatively through a radioimmunoassay that demonstrates the 100-fold induction of a2M *in vitro*. Such marked enhancement has not been reported previously (1, 8) in studies in which the immunoprecipitation of radiolabeled a2M was used to measure a2M. It provides quantitative evidence with which to explain the *in vivo* phenomenon of the increase in serum a2M. Moreover, we have demonstrated that glucocorticoid is essential for the induction of a2M synthesis by the Kupffer cell-derived factor at both the protein and mRNA levels. Glucocorticoid and the Kupffer factor affect the induction of a2M in the pretranslational, probably the transcriptional, stage.

MATERIALS AND METHODS

Animals. Pathogen-free, male Wistar King A rats (200–300 g) housed in the Animal Center of Kyushu University were used in all our experiments.

Primary culture of hepatocytes. Hepatocytes were isolated from rat liver, according to Seglen (21) with minor modifications (8). These hepatocytes were suspended at a concentration of 8×10^5 cells/ml (1 ml per 35-mm Falcon dish) in Eagle's modified essential medium (MEM) containing 10% fetal calf serum (FCS), 1 μ M insulin and 1 μ M dexamethasone (Dex). The medium was changed 4 h after plating and every 24 h thereafter with the fresh medium containing 1 μ M of Dex. When studying the effect of Dex on a2M synthesis, that compound was omitted from the medium for 24 h before the experiment.

To obtain a pure hepatocyte preparation, the cell suspension was layered on 50% isotonic Percoll solution then centrifuged at $2,000 \times g$ for 30 min at 4°C. Hepatocytes were collected from the fraction with a specific gravity between 1,080 to 1,090, just above the lowest layer formed by the red blood cells.

Primary culture of Kupffer cells. Non-parenchymal cells were isolated from rat liver

by perfusion of 0.2% pronase *in situ* (8) then fractionated on a Percoll self-generated gradient. The liver cell suspension was mixed with 35 ml of 50% isotonic Percoll solution diluted with phosphate-buffered saline and the culture medium. The centrifuge tube containing this suspension was then spun at $20,000 \times g$ for 30 min at 4°C . The fraction containing nonparenchymal cells was collected and washed twice with Hanks' solution, after which the concentration was adjusted to 5×10^5 cells/ml in RPMI 1640 containing 10% heat-inactivated FCS. Samples of 1 ml were placed in a 35-mm plastic dish and, after 24 h of incubation, the dish was rinsed with Ca^{2+} , Mg^{2+} -free phosphate-buffered saline, which removed all nonadhering cells. Fresh medium was then added, and incubation continued in 5% CO_2 /air at 37°C . The adhering cells had Fc and C_3 receptors, and therefore were considered Kupffer cells (15).

Co-culture of hepatocytes and Kupffer cells. Parenchymal cells were seeded at a concentration of 5.0×10^5 cells per ml in MEM containing 10% FCS, $1 \mu\text{M}$ Dex and $1 \mu\text{M}$ insulin. After incubation for 48 h, the culture medium was removed and freshly isolated Kupffer cells were added at a concentration of 5×10^5 per ml in RPMI 1640 containing 10% FCS, after which incubation was continued for 24 h. The following experiments were performed thereafter:

Preparation of the Kupffer cell-derived hepatocyte-stimulating factor (Kupffer factor). Kupffer cells obtained as described above were cultured for 24 h with $50 \mu\text{g}$ of lipopolysaccharide (LPS). Supernatants were collected and pooled for use as the Kupffer factor.

Radioimmunoassay of $\alpha_2\text{M}$. The concentration of $\alpha_2\text{M}$ in the culture medium was measured by a radioimmunoassay as described elsewhere (10).

Isolation of RNA. The total cellular RNA in the cultured hepatocytes was extracted by the guanidium thiocyanate/cesium chloride procedure of Chirgwin *et al.* (3).

Dot blot hybridization. The total RNA was denatured with glyoxal (13) then applied to a nylon membrane as described elsewhere (5). These membranes were baked at 80°C for 2 h then hybridized with nick-translated DNA at 42°C in $5 \times \text{SSC}$, 50 mM sodium phosphate (pH 6.5), 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone and $250 \mu\text{g/ml}$ of sonicated salmon sperm DNA (24). After hybridization, the nylon membranes were washed 4 times with $2 \times \text{SSC}$ and 0.1% SDS for 5 min at room temperature then twice with $0.1 \times \text{SSC}$ and 0.1% SDS at 50°C for 15 min, after which they were exposed to X-ray film at -70°C .

RESULTS

Because the $\alpha_2\text{M}$ secreted in the culture medium represents the $\alpha_2\text{M}$ synthesized *de novo* in cultured hepatocytes (8) and because the fresh culture medium contained no $\alpha_2\text{M}$ (as determined by the radioimmunoassay), the $\alpha_2\text{M}$ in the medium was considered to be $\alpha_2\text{M}$ synthesized by the hepatocytes.

We first studied the daily changes in the amount of $\alpha_2\text{M}$ synthesized by primary cultures of rat hepatocytes. With $1 \mu\text{M}$ Dex in the culture medium, the synthesis of $\alpha_2\text{M}$ was $127.0 \pm 8.0 \mu\text{g}/10^6$ hepatocytes/24 h on the first day, but this value decreased rapidly (Table 1); on the 5th day $\alpha_2\text{M}$ synthesis was only $0.5 \mu\text{g}/10^6$ hepatocytes/24 h.

To examine the effect of Kupffer cells on hepatocytes *in vivo*, we co-cultured pure hepatocyte preparations with Kupffer cells, then examined the effect of LPS on the synthesis of $\alpha_2\text{M}$. In the pure hepatocyte culture, LPS did not enhance the synthesis of $\alpha_2\text{M}$, in the presence or absence of Dex, but Dex alone slightly enhanced this

TABLE 1. TIME COURSE OF a2M SYNTHESIS BY PRIMARY CULTURES OF RAT HEPATOCYTES

Culture Day	a2M synthesis ($\mu\text{g}/10^6$ hepatocytes/24 h)
1	127.0 ± 8.0
2	87.4 ± 15.4
3	42.3 ± 5.6
4	16.7 ± 2.1
5	0.5 ± 0.1

Following the first medium change 4 h after plating, the hepatocytes were incubated in MEM containing $1 \mu\text{M}$ Dex. The medium was exchanged every 24 h thereafter with fresh medium as described in MATERIALS AND METHODS. The a2M secreted into the medium was measured by a radioimmunoassay. Each value is the mean $\pm$ S.D. of four determinations.

synthesis. In contrast, when hepatocytes and Kupffer cells were co-cultured, $50 \mu\text{g}/\text{ml}$ of LPS markedly stimulated the synthesis of a2M when Dex was added to the medium (Fig. 1). When no Dex was present, there was little increase in a2M synthesis.

We next analyzed the effects of Dex and the supernatant of LPS-activated Kupffer cells (the Kupffer factor) on a2M synthesis in cultured hepatocytes. Dex alone slightly enhanced the synthesis of a2M, which effect was dose-dependent above 10^{-9} M (Fig. 2 and 3). The Kupffer factor alone had little effect on the enhancement of the synthesis of a2M (Fig. 3), but it did have a potent stimulatory effect on a2M synthesis by hepatocytes when Dex was present. This effect appeared to depend on the concentration of the Kupffer factor present (Table 2). When cells were treated with $50 \mu\text{g}/\text{ml}$ of LPS, a2M was synthesized at $117.8 \mu\text{g}/10^6$ hepatocytes/24 h, almost 100-fold the amount synthesized in the absence of both the Kupffer factor and Dex.

We then determined whether these factors act in the pretranslational stage. We

Fig. 1. Effects of LPS on the synthesis of a2M in a pure hepatocyte culture (left) and in a co-culture of hepatocytes with Kupffer cells (right). In the pure hepatocyte culture, 4-day-old cultures of hepatocytes were incubated with, or without, $1 \mu\text{M}$ of Dex, $50 \mu\text{g}$ of LPS or both. In the co-culture system, 4-day-old cultures of hepatocytes were incubated with Kupffer cells, in the presence of $50 \mu\text{g}$ LPS. Dex was left out of the medium in both culture systems 24 h before the experiments were done. The a2M secreted in the medium was measured by a radioimmunoassay. Each column represents the mean $\pm$ S.D. of 4 determinations.

Fig. 2. Dose-response curve showing the effect of Dex on a2M synthesis. Hepatocytes from 5-day-old cultures were used. Dex was omitted from the medium 24 h before the experiments were done. Each point and bar represents the mean $\pm$ S.D. of 4 determinations.

isolated the total RNA from hepatocytes (stimulated by Dex, the Kupffer factor, or both) and measured the amount of a2M mRNA present by dot blot analysis. The concentration of a2M mRNA was increased approximately 125-fold by an addition of the Kupffer factor in the presence of Dex (Fig. 4), but an addition of the Kupffer factor alone did not increase the mRNA. An addition of 1 μ M of Dex alone produced 8- to 16-fold enhancement. We concluded that the Dex and the Kupffer factor affect a2M induction in the pretranslational stage, possibly in the transcriptional phase.

DISCUSSION

Recent investigations have shown that the synthesis of acute phase proteins is elicited in liver by IL-1, a macrophage-derived cytokine (22, 23). Because Kupffer

TABLE 2. DOSE DEPENDENCE OF THE EFFECT OF THE KUPFFER FACTOR ON a2M SYNTHESIS

Kupffer factor (μ l/ml)	Dex (μ M)	a2M synthesis (μ g/10 ⁶ hepatocytes/24 h)
0	0	1.2 $\pm$ 0.2
50	0	1.4 $\pm$ 0.2
0	1	18.6 $\pm$ 5.3 ^a
5	1	36.5 $\pm$ 2.7 ^a
10	1	48.0 $\pm$ 3.0 ^a
20	1	61.0 $\pm$ 4.0 ^a
30	1	99.8 $\pm$ 24.2 ^a
50	1	117.8 $\pm$ 16.6 ^a

Various concentrations of the Kupffer factor were added to 4-day-old hepatocyte cultures with, or without, 1 μ M of Dex. The a2M secreted in the medium was measured by a radioimmunoassay. The Kupffer factor was prepared as described in MATERIALS AND METHODS. Dex was omitted from the hepatocyte culture medium for 24 h before each experiment. Values are the means $\pm$ S.D. of 4 determinations. ^aSignificantly different ($p < 0.001$) when compared with the top row (Student's test).

Fig. 3.

Fig. 3. Effects of Dex and the Kupffer factor on a2M synthesis. A: no additions (control), B: plus 50 μ l/ml of the Kupffer factor, C: plus 1 μ M of Dex, D: plus 1 μ M of Dex and 50 μ l/ml of the Kupffer factor. Hepatocytes from 5-day-old cultures were incubated with, or without, 50 μ l of the Kupffer factor, 1 μ M of Dex, or both. The a2M secreted in the medium was measured by a radio-immunoassay. Each column represents the mean $\pm$ S.D. of 4 determinations.

Fig. 4.

Fig. 4. Dot blot analysis of the a2M mRNA. The total RNA isolated from primary cultures of hepatocytes was placed on a nylon membrane. The uppermost row shows the standard RNA extracted from the liver of a turpentine-injected rat in a 5-fold serial dilution (the first dot contains 1 μ g of total RNA). RNA preparations A to D were taken from the hepatocytes described in Fig. 3. The first dot contains 1 μ g of total RNA, the others sequential 2-fold serial dilutions.

cells are the major macrophage-phagocyte system in the liver and are in close proximity to hepatocytes, interest has been focused on their role in acute phase responses. Recently, Bauer *et al.* (1) as well as our group (8) found that supernatants from LPS-stimulated Kupffer cells from rats show hepatocyte-stimulating activity by inducing a2M synthesis. Recombinant IL-1 also has been demonstrated to stimulate a2M synthesis by directly affecting hepatocytes (2).

Co-culture experiments demonstrated that an addition of LPS to the hepatocyte culture medium stimulated the synthesis of a2M, which suggests a role for LPS. LPS, itself, did not, however, directly cause hepatocytes to begin a2M synthesis, rather it worked via the Kupffer cells by stimulating them to secrete a hepatocyte-stimulating factor into the culture medium.

Our study has provided quantitative evidence for a2M synthesis by hepatocytes upon stimulation with Dex, the Kupffer factor or both. When both Dex and the supernatant of Kupffer cells were added to the medium, cultured hepatocytes synthesized a2M that was 100-fold the value found for the control hepatocytes incubated without a combination of the Kupffer factor and Dex. This marked difference was not found when a2M synthesis was measured by the immunoprecipitation of radiolabeled a2M (1, 8). Thus, we here have shown an important function of glucocorticoid and a cellular factor (the Kupffer factor) in the induction of a2M *in vivo* which shows that serum a2M increases more than 100-fold in the acute phase.

The stimulatory effect of the Kupffer factor on a2M synthesis was found only when there was Dex in the culture medium. When Dex was not present, no increase in a2M synthesis took place with the Kupffer factor alone. Similar results have been reported for other assay methods (1, 8) and *in vivo* (1, 7, 17). In adrenalectomized or hypophysectomized rats, which have few endogenous glucocorticoids, no increase in serum a2M took place after a turpentine injection, but with a supplement of Dex, the response became normal (1, 7).

We confirmed this by measuring the a2M mRNA present in the hepatocytes with a2M cDNA. This mRNA increased to 125-fold the value for the control in the presence of the Kupffer factor and Dex, but there was no increase with the Kupffer factor alone. Quantitative differences in the increases in the a2M protein and the mRNA produced by this stimulation are explained by differences in the turnover rate, the rate for mRNA being higher than that for the protein.

The mechanism by which glucocorticoid induces the synthesis of acute phase proteins is postulated to be 1) production of an increase in the rate of transcription of specific genes, or 2) the stabilization of specific mRNA. Recent studies with α_1 -acid glycoprotein have demonstrated that this glucocorticoid stimulates the accumulation of α_1 -acid glycoprotein mRNA by inducing an RNA-processing factor that facilitates the production of stable transcripts (25). Our study demonstrates that the expression of the effect of Dex on a2M synthesis takes place in the pre-translational stage, possibly in the transcriptional phase. Further investigation of cloned a2M genomic DNA coupled with the *in vitro* system is being made.

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