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Discrimination of hepatocyte-stimulating activity from human recombinant tumor necrosis factor α*

The involvement of tumor necrosis factor α (TNFα) in the regulation of acute-phase protein synthesis is currently under discussion. In this study the effect of human recombinant TNFα on the regulation of the 4 acute-phase proteins α_2-macroglobulin, albumin, α_1-proteinase inhibitor and α_1-acute-phase globulin was investigated in rat hepatocyte primary cultures. No changes in synthesis of any of the 4 proteins were observed. However, an acute-phase response similar to that in vivo could be generated by conditioned media from human monocytes containing natural TNFα. This response remained unchanged after neutralizing TNFα activity by the addition of a specific antibody to TNFα. It is concluded that the hepatocyte-stimulating activity synthesized by human monocytes is different from TNFα.

1 Introduction

Injuries such as bacterial or parasitic infections, physical and chemical traumata, malignant tumors and immunologic disorders lead to a highly complex reaction of the organism, the so-called acute-phase response [1–3]. The acute-phase response is characterized by fever, leukocytosis, a negative nitrogen balance and an increase in the synthesis of hepatic acute-phase proteins (APP) [4, 5]. These changes are generated by mediators secreted from mononuclear phagocytes. Many efforts have been made to identify these mediator molecules and to study their functions. Interleukin 1 (IL 1) was the first of these mediators which became available in a pure state and in large quantity by molecular cloning [6–11]. More recently, a second monokine, cachectin/tumor necrosis factor α (TNFα), has been cloned and characterized [12–16].

In previous studies we have investigated the biosynthesis and regulation of APP in vivo and in rat hepatocyte primary cultures [17–27]. Conditioned media from rat Kupffer cells [23, 28, 29] and human monocytes [23, 27, 30–34] were able to induce APP synthesis in rat hepatocytes. It was therefore of great interest to identify the factor(s) responsible for the induction of APP in hepatocytes. As a first candidate we tested murine recombinant IL 1 for its potency to stimulate the synthesis of α_2-macroglobulin (α_2M), a strong APP in the rat. Only a weak α_2M induction was observed [26]. Moreover, α_2M synthesis in rat hepatocytes was strongly stimulated by conditioned human monocyte media lacking any IL 1 activity 23, 27]. From these findings it became evident that a factor different from IL 1 is involved in the induction of APP in rat hepatocytes. This is in good agreement with the observations made by other authors [31, 32, 35, 36]. Ritchie and Fuller [30] designated the APP-inducing activity distinct from IL 1 as hepatocyte-stimulating factor (HSF).

Recently, acute-phase protein-inducing activity was attributed to cachectin/TNFα [33, 37]. Since HSF is still poorly characterized, the question arose whether or not HSF is identical with TNFα.

In the present communication we show that recombinant human TNFα as well as TNFα produced by human peripheral blood monocytes does not induce changes in the synthesis of α_2M, albumin, α_1-proteinase inhibitor (α_1PI) and α_1-acute-phase globulin (α_1APG) in rat hepatocyte primary cultures.

2 Materials and methods

2.1 Chemicals

L-[35S]Methionine (46 TBq/mmol) was purchased from the Radiochemical Centre, Amersham, GB. Protosol was obtained from New England Nuclear, Boston, MA, and protein A-Sepharose CL-4B from Pharmacia, Freiburg, FRG. RPMI 1640 medium and phenylmethylsulfonyl fluoride were from Serva, Heidelberg, FRG. Fetal calf serum was purchased from Gibco, Eggenstein, FRG. Penicillin and streptomycin were from Seromed, Berlin, FRG. Insulin, actinomycin D and crystal violet were obtained from Sigma, Deisenhofen, FRG. Kallikrein trypsin inhibitor (Trasylol®) was generously supplied by Bayer AG., Leverkusen, FRG. Lipopolysaccharide derived from Salmonella typhimurium was from Difco, Detroit, MI. Recombinant TNFα was generously supplied by BASF AG., Ludwigshafen, FRG. It contained less than 1.3 ng endotoxin per mg protein. Rabbit antisera containing polyclonal anti-TNFα antibodies were generated by immunizing rabbits (New Zealand White) with recombinant human TNFα. A serum dilution of 1:2000 neutralized completely 10 ng/ml recombinant TNFα in the TNFα cytotoxicity assay.

2.2 Preparation of rat hepatocyte monolayers

Suspensions of rat hepatocytes were prepared as previously described by Tran-Thi et al. [38]. After washing the cells with Krebs-Henseleit buffer they were suspended in a modified Waymouth medium [39] containing 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 1 μM insulin. The high insulin concentration was chosen because insulin has been shown to enhance cell viability [40] and to be required for monolayer formation of hepatocytes [41]. Furthermore, insulin is rapidly degraded by hepatocyte cultures at 37°C [42].

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Abbreviations: APP: Acute phase protein(s) α_1APG: α_1-Acute-phase globulin α_1PI: α_1-Proteinase inhibitor HSF: Hepatocyte-stimulating factor IL 1: Interleukin 1 LPS: Lipopolysaccharide TCA: Trichloroacetic acid TNFα: Tumor necrosis factor α

Aliquots of 0.3 ml cell suspension (2.5×10^5 cells) were added to 16 mm contour-bottom Falcon plastic tissue culture dishes (Falcon, Oxnard, CA). The dishes were incubated at 37 °C in a humid atmosphere of air : CO_2 (95% : 5%) for 3 h. The plates were then washed with Krebs-Henseleit buffer and 0.3 ml culture medium was added as described [43]. Confluent monolayers were formed after an overnight incubation at 37 °C in a humid atmosphere of air : CO_2 (95% : 5%). The hepatocyte primary cultures consisted of more than 97% hepatocytes. The cultures were routinely controlled by microscopic examination prior to each experiment.

2.3 Labeling of hepatocytes

RPMI 1640 medium was used for the radioactive labeling of the hepatocytes. Three hundred kBq of [^{35}S]methionine was added to 0.3 ml of methionine-free culture medium for the labeling of proteins. After incubation at 37 °C for 3 h, the media were separated from the cells and centrifuged at $12\,000 \times g$ for 5 min and used for immunoprecipitation.

2.4 Immunoprecipitation

The preparation of antisera against rat α_2M, albumin, α_1PI and α_1-acute-phase globulin (α_1APG) has been previously described [17, 19, 24]. Immunoprecipitations were carried out in the presence of an excess of antibody. Hepatocyte medium containing 10^5 cpm of trichloroacetic acid (TCA)-precipitable radioactivity was added to 1 ml buffer A (20 mM Tris/HCl, pH 7.6, 0.14 M NaCl, 5 mM EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mg of kallikrein trypsin inhibitor. After addition of 5µl of specific antiserum and incubation at 4 °C overnight, the antigen-antibody complexes were bound to 7 mg (dry weight) of protein A-Sepharose and washed four times with buffer A and twice with 50 mM sodium phosphate buffer, pH 7.5. Elution was achieved by incubation with 0.1 M Tris/HCl, pH 6.8, 5% 2-mercaptoethanol, 5% sodium dodecyl sulfate and 10% glycerol at 95 °C for 5 min. The eluted proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [44] and subjected to fluorography according to Bonner and Laskey [45]. For the quantification of the radioactivity incorporated into the different APP, the corresponding bands identified by fluorography were cut from the gels, solubilized with Protosol/water (9 : 1, v/v) at 45 °C overnight and counted in a liquid scintillation spectrometer.

2.5 Preparation of human monocytes and conditioned media

Human mononuclear leukocytes were prepared from buffy coats of normal blood units by density gradient centrifugation [46]. Four $\times 10^6$ human mononuclear leukocytes/ml were incubated at 37 °C in a humid atmosphere containing 5% CO_2 for 2 h in plastic culture flasks (Falcon 3024F) in RPMI 1640 medium supplemented with 2% human serum. Nonadherent cells were removed by two washes with the above medium. The adherent cells were then reconstituted with the same medium, incubated for 24 h in the presence of 1 ng of lipopolysaccharide (LPS) per ml and the supernatants were harvested by centrifugation and filtration (Nalgene SCN, 45 µm, Nalge/Sybron, Rochester, NY). Alternatively, adhe-

rent cells were pre-incubated for 24 h in the absence of stimulant and then incubated with LPS for further 24 h.

2.6 TNFα assay

The cytotoxicity assay for TNFα was carried out on sensitive L929 mouse fibrosarcoma cells (2×10^4 cells/0.2 ml culture) in the presence of actinomycin D (2 µg/ml) in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. The cultures contained serial dilutions of TNFα samples. After 20 h of culture, the surviving cells were fixed and stained with crystal violet [0.5% crystal violet, 8% (v/v) formaldehyde (37%), 0.17 NaCl, 22.3% ethanol] for 15 min. Excess dye was washed off with water and the remaining dye solubilized in 33% acetic acid. Absorbance was measured at 540 nm. Concentrations of TNFα were determined by comparing the dilutions at which 50% survival was measured with a recombinant TNFα reference, kindly supplied by BASF AG.

2.7 IL 1 assay

Supernatants of stimulated human monocytes and controls were assayed for IL 1 in the costimulation assay on C3H/HeJ thymocytes as described [47].

3 Results

Rat hepatocyte primary cultures were incubated with different amounts of recombinant human TNFα ranging from 1 pg to 10 µg per ml medium for 12 h at 37 °C and proteins were labeled with [^{35}S]methionine. Three positive APP, α_2M, α_1APG and α_1PI, known to show very strong, strong or weak increases during inflammation (reviewed in [3]) and the negative APP albumin were immunoprecipitated from the media of hepatocytes incubated with TNFα. Fig. 1 shows that recombinant TNFα did not affect the synthesis of any of the 4 selected rat APP. Since we found murine recombinant IL 1 to be a weak stimulator of α_2M synthesis in rat hepatocytes [26], we studied the combined action of IL 1 and TNFα, in order to test whether TNFα enhances IL 1 activity. No synergistic effect of TNFα and IL 1 was observed (data not shown). Since recombinant TNFα may have properties different from the physiologically synthesized material, conditioned media from LPS-stimulated human monocytes containing TNFα were added to hepatocyte primary cultures. A concentration of 0.5 ng TNFα/ml was determined in the monocyte media.

As shown in Fig. 2A, last column, conditioned monocyte media stimulated α_2M synthesis 25-fold and α_1APG synthesis 2.5-fold, whereas α_1PI did not change. Albumin synthesis was decreased by 60%. These changes in the syntesis of the four APP are quite similar to those observed *in vivo* during inflammation. Thus, the conditioned medium of human monocytes contains inflammatory mediators leading to the induction of APP synthesis. Addition of a neutralizing antibody to TNFα should abolish the acute-phase response, if TNFα present in the monocyte medium is indeed the inducer of APP synthesis in hepatocytes. We therefore pre-incubated the conditioned media with diffeent amounts of anti-TNFα prior to the addition to hepatocytes. As shown in Fig. 2A, B, first 3 columns, no inhibition of the acute-phase induction was observed,
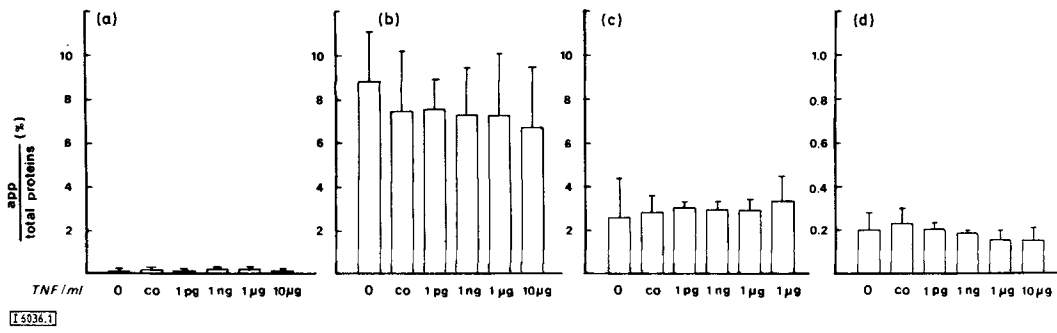
Figure 1. Effect of human recombinant TNFα on the synthesis of APP in rat hepatocyte primary cultures. Rat hepatocyte primary cultures were prepared as described in Sect. 2.2 and kept in culture for 5 days. As indicated in the figure, TNFα in different concentrations was added to the hepatocytes. The specific activity of the TNFα preparation was 2×10^7 units/mg. As a control (Co) the medium (RPMI 1640, 1% fetal calf serum) in which TNFα had been diluted was added to the hepatocytes. After incubation for 12 h, cells were labeled with [^{35}S]methionine for 3 h. α_2M (a), albumin (b), α_1PI (c) and α_1APG (d) were immunoprecipitated from the hepatocyte media containing 10^5 cpm of TCA-precipitable radioactivity and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The radioactivity of the immunoprecipitated proteins was related to the total TCA-precipitable material. Data are means ± SD of 3 different experiments.

Table 1. TNFα activity neutralized by anti-TNFα

Dilution of anti-TNFα	TNFα activity (titer)[a]
1 : 1000	< 2
1 : 100	< 2
1 : 10	< 2
No addition	16
Control antiserum	16

a) Titers of TNFα activity are given as the reciprocal dilutions at which 50% survival was measured in the TNFα assay.

although the antibody had neutralized TNFα activity completely (Table 1).

Since the conditioned medium from LPS-stimulated freshly prepared human monocytes contains IL 1 activity, we examined whether IL 1 might be responsible for the acute phase induction. It is known that human monocytes lose their capability to secrete IL 1 after pre-incubation for 24 h prior to LPS stimulation [48–50]. When conditioned media from pre-incubated and stimulated human monocytes were added to hepatocytes, the acute-phase induction was still observed, although slightly diminished (Fig. 2B). Addition of different amounts of anti-TNFα to the pre-incubated conditioned media also did not affect the APP-inducing capacity.
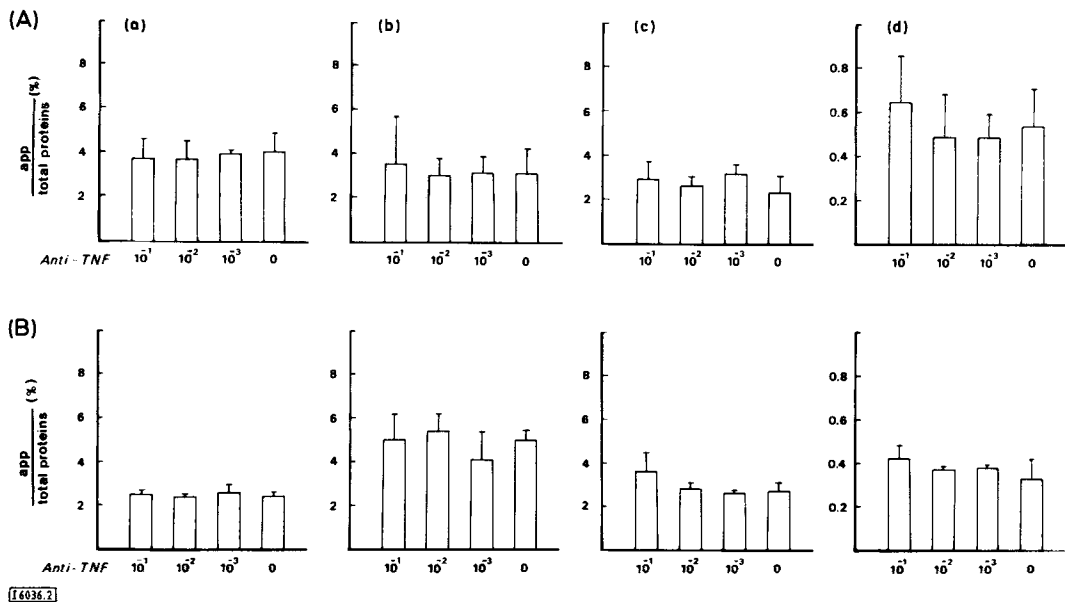

Figure 2. Effect of anti-TNFα on the synthesis of acute-phase proteins induced by conditioned media from human monocytes. Conditioned media from LPS-stimulated human monocytes 0–24 h (A) or 24–48 h (B) after preparation from human blood were incubated for 30 min at room temperature with different amounts of a monospecific antibody to human recombinant TNFα at the final concentrations indicated. The antibody-treated conditioned media were added to hepatocyte primary cultures. After incubation for 12 h, hepatocytes were labeled with [^{35}S]methionine and the synthesis of α_2M (a), albumin (b), α_1PI (c) and α_1APG (d) was measured described in Sect. 2.4. The conditioned medium from freshly prepared monocytes (A) contained IL 1 (titer 64) in the thymocyte assay, while conditioned medium from pre-incubated monocytes (B) contained no detectable IL 1 at all.

We conclude from these results that TNFα is not the main mediator of the acute-phase response in rat hepatocyte primary cultures. Furthermore, it is evident that a mediator different from IL1 is present in conditioned media from freshly prepared as well as from pre-incubated human monocytes after LPS induction.

4 Discussion

At first sight, our finding that human recombinant TNFα is not involved in the induction of seveal APP in rat hepatocytes is in contrast to the observations of Perlmutter et al. [37] and Darlington et al. [33]. These authors described stimulation of complement factor C3, factor B and α_1-antichymotrypsin in HepG2 and Hep3B2 cells by human recombinant TNFα. However, several other APP such as complement factors C2 and C4, α_1PI, fibrinogen, α_1-acid glycoprotein, C-reactive protein and haptoglobin did not respond to TNFα. For the negative APP albumin Perlmutter et al. [37] observed a decrease in synthesis, whereas Darlington et al. [33] did not detect a change after addition of human recombinant TNFα to human hepatoma cells. Taken together, TNFα was found to induce only a very limited number of APP.

Both groups used tumor cell lines for their experiments. Therefore, their data should be interpreted with reservation. Tumor cells, as dedifferentiated cells, may not be the ideal model system to study the regulation of APP synthesis. In our study we avoided the use of a tumor cell line. Since human hepatocyte primary cultures are hardly available, we have used rat hepatocyte primary cultures. In response to conditioned medium from human monocytes rat hepatocytes synthesize APP similarly to the rat liver in vivo during inflammation. Thus, it is unlikely that species specificity is responsible for the lacking effect of human recombinant TNFα on rat hepatocytes. This view is supported by experiments of Tracey et al. [51] who found that human recombinant TNFα exerts a strong action in the rat. Recently, Smith et al. [52] described human and mouse TNFα to be active in heterologous cell systems, although the concentrations of TNFα needed were higher than in the homologous system.

From the fact that human recombinant TNFα did not induce α_2M, α_1APG or α_1PI synthesis in rat hepatocytes, while conditioned medium from human monocytes was a potent stimulator, it must be concluded that TNFα is distinct from HSF.

IL1 was reported to induce several APP in rat hepatocyte primary cultures. Previously we described a 2-fold stimulation of α_2M synthesis by murine recombinant IL1 [26]. Ramadori et al. [53] described the induction of serum amyloid A and factor B by murine recombinant IL1 in vivo and in hepatocyte primary cultures from mice. Increases in the synthesis of α_1-antichymotrypsin, factor B and C3, but not C2, C4 and α_1PI were observed in Hep3B cells upon addition of human recombinant IL1 [37]. Using HepG2 cells, Karin et al. [54] induced metallothioneine mRNA by recombinant murine IL1. As in the case of TNFα, Darlington et al. [33] reported that among several APP tested in Hep3B2 cells mouse recombinant IL1 induced only the synthesis of C3. In rat hepatocytes Fuller et al. [31] did not observe any stimulation of fibrinogen synthesis by murine recombinant IL1.

From the fact that some APP are inducible by IL1, whereas others are not, it is evident that IL1 is not an universal mediator in APP induction.

In this study we have presented data which shows that TNFα did not induce rat α_2M synthesis, whereas conditioned media of human monocytes devoid of TNFα and IL1 activities were still capable of stimulating α_2M synthesis 10-fold. Thus, a factor distinct from TNFα and of much higher potency than IL1 is involved in the induction of APP.

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Note added in proof: After submission of this manuscript Koj, A. et al. (*Biochem. Int.* 1987. *14:* 553) also excluded TNFα as a major acute phase mediator in rat hepatocytes.