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INVOLVEMENT OF TRANSFORMING GROWTH FACTOR-α SECRETED BY MACROPHAGES IN METALLOTHIONEIN INDUCTION BY ENDOTOXIN

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SUMMARY: The mechanism of metallothionein (MT) induction of the liver by endotoxin, which is mediated by a factor secreted by endotoxin-stimulated macrophages, was studied in vitro. MT induction of the liver cells by the endotoxin-stimulated macrophage conditioned medium was inhibited by a monoclonal anti-epidermal growth factor (EGF) / transforming growth factor-α (TGF-α) receptor antibody, which acts as an antagonist of EGF and TGF-α. MT was induced by the substance, which was adsorbed by polyclonal antibody to TGF-α, but not by a monoclonal antibody to EGF, in the conditioned medium of endotoxin-stimulated macrophages. These results suggest that TGF-α secreted by macrophages is involved in MT induction by endotoxin.

Endotoxicemia or inflammation induces a decrease in serum zinc concentration, an increase of zinc content in the liver and synthesis of metallothionein (MT), which is a heavy metal binding protein (1-3). The mechanism of MT induction by endotoxin and the relationship between MT and zinc metabolism in endotoxicemia must be studied for clarifying the physiological function of MT. We have reported that MT induction by endotoxin is mediated by a factor secreted by macrophages (4, 5) and that zinc accumulation into the liver cells by endotoxin occurs subsequent to MT synthesis by the factor (6).

MT is inducible by heavy metals, glucocorticoid hormones, interferon and interleukin-1 in vitro. We have already reported that the macrophage-derived MT inducing factor is distinct from these inducers (4). Here we report that transforming growth

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factor-α (TGF-α) secreted by macrophages is involved in MT induction by endotoxin.

MATERIALS AND METHODS

Macrophage-conditioned medium: Rat peritoneal exudate macrophages isolated as in (4) were cultured in serum-free defined medium with 100 μg/ml of endotoxin (Escherichia coli lipopolysaccharide W 0127: B8; Difco Lab., Detroit, MI) for 24 hr. The serum-free medium used was a 1:1 mixture of RPMI 1640 medium and Dulbecco's modified Eagle's medium containing 15 mM Hepes, 2.2 mg/ml of sodium bicarbonate, 5 μg/ml of insulin, 5 μg/ml of transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol, 1 nM sodium selenite and 0.5 mg/ml of bovine serum albumin. The endotoxin-stimulated macrophage conditioned medium was sterilized by 0.2 μm filter and stored at -30°C until use.

Preparation of monoclonal antibodies: Hybridomas producing the anti-EGF receptor antibody were cultured in the above serum-free medium. The secreted antibodies were purified by Protein A-agarose chromatography. The purity of the monoclonal antibodies was confirmed by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

MT induction in the liver cells: The liver cells used were Chang liver cells (7) and RL34 cells (8) derived from a human and a rat, respectively. The semi-confluent liver cells in 6-well plates were cultured with 1 ml of a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DME/F12) containing 15 mM Hepes, 10% fetal bovine serum and 5 μCi of [^{35}S]cysteine and 1 ml of the macrophage conditioned medium for 18 hr. The [^{35}S]cysteine-labeled cell extracts were carboxymethylated and analyzed by SDS-PAGE according to the method of Koizumi et al. (9).

Immunoaffinity chromatography: A monoclonal antibody to rat EGF and rabbit antiserum to rat TGF-α were purchased from Ohtsuka Assay Lab. (Tokushima, Japan) and Peninsula Lab, Inc. (Belmont, CA), respectively. The antibody to TGF-α was purified by anti-rabbit immunoglobulin column. These antibodies were coupled to CNBr-activated Sepharose 4B (Pharmacia, Sweden) according to the manufacturer's instructions. The conditioned medium of endotoxin-stimulated macrophages was applied to the above columns and the columns were washed with 10 times volume of phosphate-buffered saline. The eluants by sodium-citrate buffer (pH 3.0) were dialyzed against DME/F12. MT inductions by the eluants and the conditioned medium treated with immunoaffinity column were studied by the above method.

RESULTS AND DISCUSSION

MT of human Chang liver cells and rat liver RL34 cells was induced by the endotoxin-stimulated macrophage conditioned medium (Fig. 1a, lane 3 and Fig. 1b lane 3). The bottom bands were confirmed to be MT by an immunoadsorption method using anti-rat

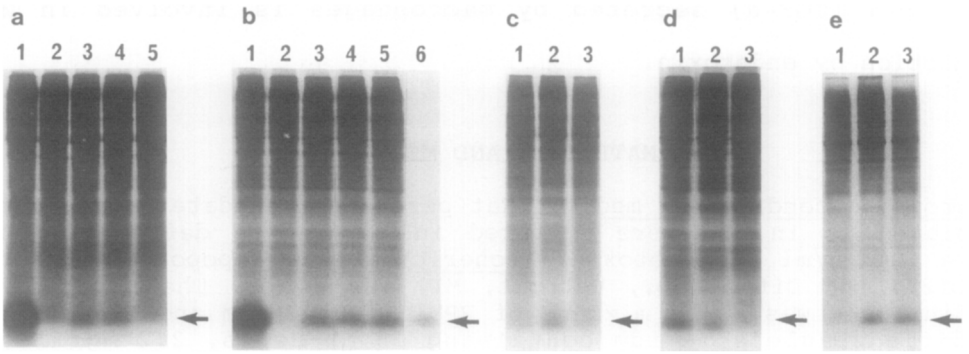

Figure 1: Fluorograms of [^{35}S]cysteine labeled cell extracts and metallothionein (MT).

a, Human Chang liver cells were treated with medium containing 100 μM zinc (lane 1), control medium (lane 2), 50% of the endotoxin-stimulated macrophage conditioned medium (lane 3), 20 μg/ml of the 455 monoclonal antibody and 50% of the endotoxin-stimulated macrophage conditioned medium (lane 4), or 20 μg/ml of the 528 monoclonal antibody, which acts as an antagonist of EGF and TGF-α, and 50% of the endotoxin-stimulated macrophage conditioned medium (lane 5).

b, Rat liver RL34 cells were treated under the same conditions as in Fig. 1a (lane 1-5). The cell extract of lane 3 was immunoadsorbed by an anti-rat MT polyclonal antibody. The adsorbed protein was carboxymethylated and electrophoresed (lane 6).

c, Chang cells were cultured with control medium (lane 1), the substance adsorbed by anti-TGF-α antibody (lane 2), or the substance adsorbed by anti-EGF antibody (lane 3).

d, Chang cells were cultured with the endotoxin-stimulated macrophage conditioned medium (lane 1), the endotoxin-stimulated macrophage conditioned medium adsorbed by anti-TGF-α antibody column (lane 2), or control medium (lane 3).

e, Chang cells were cultured with control medium, medium containing 1 ng/ml of EGF, or medium containing 1 ng/ml of TGF-α.

After an 18 hr incubation, the cells were harvested and extracted by sonication. The cell extracts were carboxymethylated and applied to SDS-15% polyacrylamide gel electrophoresis. The arrows indicate carboxymethylated MT.

MT antibody (Fig. 1b, lane 6). MT induction by the endotoxin-stimulated macrophage conditioned medium was completely inhibited by the 528 monoclonal antibody to the EGF receptor (Fig. 1a, lane 5), which acts as an antagonist of EGF, but not by the 455 monoclonal antibody (Fig. 1a, lane 4), which is not an antagonist of EGF (10). Inhibition of MT induction of rat liver RL34 cells by the 528 monoclonal antibody was not seen (Fig. 1b, lane 5), because the 528 monoclonal antibody does not bind to the rodent EGF receptor (J. D. Sato, private communication). This result

shows that the inhibition of MT induction by the 528 monoclonal antibody is independent of neutralizing of the MT-inducing factor(s) of the macrophage conditioned medium. Since TGF-α is a potent agonist for the EGF receptor, which is a transmembrane glycoprotein with intrinsic protein kinase activity (11), the above results suggest that the MT-inducing factor(s) of the conditioned medium are EGF and/or TGF-α.

We attempted to adsorb the MT-inducing factor of the macrophage conditioned medium by immunoaffinity chromatographies of anti-EGF antibody or anti-TGF-α antibody. MT was induced by the substance which was adsorbed by polyclonal antibody to rat TGF-α (Fig. 1c, lane 2), but not by a monoclonal antibody to rat EGF (Fig. 1c, lane 3). These results suggest that TGF-α is involved in MT induction by endotoxin. Treatment with the anti-TGF-α antibody column reduced MT inducibility of the macrophage conditioned medium by 22% densitometrically (Fig. 1d, lane 2). We could not conclude that TGF-α was the only MT-inducing factor of the endotoxin-stimulated macrophage conditioned medium, because the rate of MT induction by the conditioned medium was relatively low (22%) inhibited by immunoadsorption using anti-TGF-α antibody and MT was synergestically inducible by two different inducers (Fukushima and Iijima, unpublished data).

Imbra and Karin (12) have reported that growth factors including hepatocyte growth factor and EGF are inducers of MT. TGF-α can be a primary inducer of MT, because TGF-α as well as EGF has a mitogenic effect through the EGF receptor. We confirmed that EGF and TGF-α are primary inducers of MT (Fig. 1e).

Recently, Rappolee et al. (13) reported that TGF-α was expressed by endotoxin-stimulated macrophages, but that EGF expression was not found. Our failure to adsorb EGF by the anti-EGF antibody from the macrophage conditioned medium is consistent

with the report made by Rappolee et al. Furthermore, we have reported that the MT-inducing factor of the conditioned medium is a heat stable, low pH stable protein and has no species specificity (4). These characters of the factor are identical with those of TGF-α.

Although TGF-α is well known as an autocrine factor (14), our observation suggests that TGF-α is one of the inflammatory cytokines and that MT may play a host defense role against inflammation.

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