Induction of metallothionein synthesis in cultured cells by substances released from endotoxin-activated macrophages.

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Abstract

The involvement of macrophages in the induction of metallothionein (MT) synthesis by bacterial endotoxin was studied in vitro. Rat peritoneal macrophages were incubated with endotoxin. The incubation medium from endotoxin-activated macrophages accelerated MT synthesis by human hepatic Chang cells. However, the incubation medium from non-activated macrophages did not. Endotoxin added to the culture medium of Chang cells was ineffective in inducing MT synthesis. The contents of zinc, copper and cadmium, which are primary inducers of MT, in the incubation medium of macrophages in the presence of endotoxin were not different from those in the absence of endotoxin. These results suggest that MT synthesis is induced by endotoxin-treated macrophages.

KEYWORDS: metallothionein, endotoxin, macrophages, Chang liver cells

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Induction of Metallothionein Synthesis in Cultured Cells by Substances Released from Endotoxin-Activated Macrophages

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The involvement of macrophages in the induction of metallothionein (MT) synthesis by bacterial endotoxin was studied in vitro. Rat peritoneal macrophages were incubated with endotoxin. The incubation medium from endotoxin-activated macrophages accelerated MT synthesis by human hepatic Chang cells. However, the incubation medium from non-activated macrophages did not. Endotoxin added to the culture medium of Chang cells was ineffective in inducing MT synthesis. The contents of zinc, copper and cadmium, which are primary inducers of MT, in the incubation medium of macrophages in the presence of endotoxin were not different from those in the absence of endotoxin. These results suggest that MT synthesis is induced by endotoxin-treated macrophages.

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Metallothioneins (MT) are low molecular weight heavy metal-binding proteins. The functions of the proteins have been reported to be detoxification of heavy metals, metabolism of trace metals, scavenging of free radicals, etc. (1, 2). Heavy metals and glucocorticoids are known to be the primary inducers of the proteins (3). Although bacterial endotoxin, stresses and some chemicals have also been reported to be inducers of MT in vivo (4, 5), it has not been clarified yet how they induce MT. In experiments with transgenic mice, the induction of MT by endotoxin was independent of metals and glucocorticoids (6). We have proposed a hypothetical mechanism in which macrophages may be involved in the induction of hepatic MT synthesis by endotoxin (7). In the present study, we demonstrated that the induction of MT synthesis by endotoxin was mediated by macrophages.

Materials and Methods

Male Sprague-Dawley rats weighing 200-300g were injected intraperitoneally with 10 ml of autoclaved 2% casein in phosphate-buffered saline. After 48 h, an additional 20 ml of phosphate-buffered saline was injected into the peritoneal cavity and the peritoneal exudate cells were harvested. The contaminating red cells were removed by hypotonic lysis. The resultant cells were suspended to a concentration of $3 \times 10^6$ cells/ml in Eagle's minimum essential medium containing 10% fetal calf serum (FCS-MEM). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air. After one hour of incubation, non-adhering cells were removed by aspiration. The cells which adhered to the plates were used as macrophages. Purity of these preparations was greater than 95% as assessed by latex ingestion and Giemsa stain.
morphology.

The macrophages were incubated in the FCS-MEM with or without 100 μg/ml of endotoxin \textit{(E. coli} LPS W 0127: B8, Difco Lab., Detroit, MI) for 24 h. Thereafter the macrophage incubation media were removed from the culture dishes, filtered under sterile conditions through a 0.2 μm filter (Millex-PF, Millipore Corp., Bedford, MA) and used for the stimulation of hepatocytes. Macrophage incubation medium to which endotoxin was added was designated as 'endotoxin-macrophage medium', and the macrophage incubation medium without the addition of endotoxin was designated as 'macrophage medium'.

Human hepatic Chang cells were grown in FCS-MEM for 2 days. The semi-confluent Chang cells (5×10^5 cells/5 ml of FCS-MEM/25 cm² culture dish) were replaced with 4 ml of the macrophage incubation medium, and 1 ml of fresh FCS-MEM containing 5 μCi of L-[³⁵S] cysteine (1×10⁶ Ci/mmol, New England Nuclear, Boston, MA). As the controls, Chang cells were incubated, in parallel, in 5 ml of FCS-MEM containing 5 μCi of [³⁵S] cysteine with or without 100 μM ZnCl₂.

After 18 h of the incubation, the hepatic cells were collected by centrifugation. The cell extract was prepared by freezing and thawing followed by sonication after the addition of 0.25 ml of 10 mM Tris-HCl buffer, pH 8.6. The extract was centrifuged at 15,000×g for 3 min. The resulting supernatant was chromatographed on a Sephadex G-50 column (fine, 1×90 cm) using 10 mM Tris-HCl buffer, pH 8.6 as the eluant. The radioactivity of each fraction was determined in a liquid scintillation counter, model LSC-703 (Aloka Co. Ltd., Tokyo, Japan).

Aliquots of the extract were subjected to 0.1% sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE) after carboxymethylation according to the method of Koizumi \textit{et al.} (8). The gels were run for 12 h at 3 V/cm after an initial one hour at 1.25 V/cm. Gels containing [³⁵S]-labeled compounds were fixed for one hour in 30% methanol-10% trichloroacetic acid-10% acetic acid. After fixation, the gels were washed with water and soaked for one hour in Enhance solution (New England Nuclear, Boston, MA). Gels were dried in a press under vacuum, and fluorograms were prepared by exposure to Kodak X-Omat AR medical X-ray film at −70°C for a week.

The concentrations of zinc, copper and cadmium were measured with an atomic absorption spectrophotometer, model AA-640-12 (Shimadzu Corp., Kyoto, Japan).

\textbf{Results and Discussion}

Fig. 1 shows typical elution profiles of the Chang cell extracts on Sephadex G-50. The second peak at Ve/Vo = 1.6 was MT. A high MT peak was seen on the chromatogram of the extract of Chang cells cultured

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Elution profiles of [³⁵S] cysteine-labeled Chang cell extracts on Sephadex G-50 (1×90 cm). Semi-confluent Chang cells (5×10^5 cells / 5 ml of FCS-MEM/25 cm² culture dish) were incubated at 37°C for 18 h in (a) endotoxin-macrophage medium, (b) macrophage medium, and (c) a medium containing 100 μM ZnCl₂. For details, see Materials and Methods.}
\end{figure}
in endotoxin-macrophage medium (Fig. 1a). Only a very low MT peak was seen in the extract of cells cultured in macrophage medium (Fig. 1b). The typical elution pattern of the extract of cells incubated in the medium containing ZnCl₂ is shown in Fig. 1c. The peak of MT in Fig. 1c was higher than that of Fig. 1a. In order to analyze further the MT formed, the extract was carboxymethylated and applied to SDS-PAGE as shown in Fig. 2. Carboxymethylated-MT migrated faster than expected in running gels as shown by an arrow (Fig. 2) because of the presence of many negatively charged groups of carboxymethyl-cysteine as discussed previously (9). The putative MT band, with a relative mobility of 0.62, has an electrophoretic mobility identical to purified rat liver MT which was confirmed by amino acid analysis (our unpublished data). The MT level in the hepatic cells cultured in endotoxin-macrophage medium (lane 3) was higher than that of control cells (lane 1) or that of cells cultured in macrophage medium (lane 4). Thus the MT synthesis in Chang cells was evidently accelerated by endotoxin-macrophage medium. The mechanism of this acceleration was further studied.

![Fig. 2](image_url)

**Fig. 2** Electrophoretic analysis of [³⁵S] cysteine-labeled products after carboxymethylation. Chang cells were incubated at 37°C for 18 h in (1) control medium (FCS-MEM), (2) medium containing IL-1 (4 u/mL), (3) endotoxin-macrophage medium, (4) macrophage medium, (5) the same medium as (3) heated at 80°C for 15 min, and (6) medium containing 100 µM ZnCl₂. The Chang cell extracts were carboxymethylated and subjected to electrophoresis on 15% polyacrylamide-0.1% SDS slab gels. Fluorography was carried out as described in Materials and Methods. The arrow indicates carboxymethylated metallothionein.
Table 1 shows the concentrations of MT-inducing metals in endotoxin-macrophage medium, macrophage medium and FCS-MEM. No significant difference in the concentrations of copper and cadmium was seen between these media. The concentration of zinc increased by about 15% in the presence of macrophages. The zinc concentrations both in endotoxin-macrophage medium and in macrophage medium were far less than the minimum level (40 μM) to induce MT (3, and our unpublished results).

Fetal calf serum in the medium may contain a small amount of glucocorticoids, but this was not enough to induce MT (Fig. 2, lane 1). Endotoxin alone did not induce MT (7), indicating that it was not contaminated with hormones or metals.

The present results suggest that some mediator(s) released from endotoxin-treated macrophages induces the synthesis of MT by Chang cells.

Interleukin-1 (IL-1) is known to be a mediator protein released from macrophages, and to be associated with the induction of acute-phase proteins (10). These proteins play an important role in host defense. Recently, Karin et al. (11) reported that IL-1 regulated MT gene expression in three cell lines. In our preliminary experiments, no MT was synthesized when two lots of commercially available human IL-1 (4 units/ ml, Genzyme, Boston, MA) were added to the culture medium of Chang cells (Fig. 2, lane 2). Since IL-1 was reported to require cofactor(s) to induce acute-phase proteins (10) and to be lot-dependent (12), we could not conclude that IL-1 was associated with the synthesis of MT in this cell line. Because MT was also synthesized in endotoxin-macrophage medium heated at 80°C for 15 min (Fig. 2, lane 5), the mediator might be a heat-stable factor, which differs from IL-1.

The macrophage preparation used in the present experiments contained a small amount (less than 5%) of other types of cells. The possibility of the involvement of these cells in MT synthesis should be kept in mind. The slight increase in MT of Chang cells cultured in macrophage medium might be attributed to the stimulation of casein during macrophage preparation.

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