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Induction of metallothionein in a human astrocytoma cell line by interleukin-1 and heavy metals

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The effects of cytokines and heavy metals on the expression and localization of metallothioneins (MTs) within U373MG astrocytoma cells were analyzed by using indirect immunofluorescence using a monoclonal anti-MT antibody (MT45). IL-1, $CdCl_2$ (50 μM) or $ZnCl_2$ (500 μM) remarkably augmented intracellular MT levels, whereas IL-6 or 10 μM of $ZnCl_2$ showed no inducing activity. From 24 to 48 h after the addition of $CdCl_2$ or IL-1, immunoreactive MTs were found in the cytoplasm and the nucleus. After 72 h, immunoreactive MTs accumulated in a granular form near the cell surfaces in the presence of $CdCl_2$ (50 μM) or IL-1 plus $ZnCl_2$ (10 μM). However, this accumulation was not observed when only IL-1 was added. Thus, Zn^{2+} facilitated the appearance of the granular form of immunoreactive MTs at a concentration where they do not induce MTs by themselves.

Metallothionein; Interleukin-1; Interleukin-6; Heavy metal; Astrocytoma cell; U373MG cell

1. INTRODUCTION

Metallothioneins (MTs) are cysteine-rich, metal-binding proteins of low molecular weight. In mammals, they are inducible by a variety of factors including heavy metals, cytokines, glucocorticoids, phorbol esters, physical stress and infection, and are recently reckoned as one of the acute-phase proteins ([1–4] for review).

The MT levels are dramatically elevated in the liver in response to lipopolysaccharide (LPS). The roles of monokines or interleukin-1 (IL-1) in the LPS-induced response has been well studied ([5] for review). IL-1 is a strong stimulator of biosynthesis of MTs and other acute-phase proteins in hepatocytes [5–7]. However, recent evidence implicates that interleukin-6 (IL-6) plays an major role in the MT-induction in the liver [8,9] as it does in the induction of other acute-phase proteins [10,11].

Several investigators have reported the expression or induction of MTs in the animal brain [12,13] and also abnormal accumulation of MTs in the brains of patients with Alzheimer's disease [14]. The localization of MT in astrocytes has been shown in the normal brain [13]. However, the available knowledge about the role and induction of brain MTs is still scarce. It is well known that, in the central nervous systems, cytokines including IL-1 and IL-6 are produced by glial cells and act as regulators of nerve cell growth and differentiation [15]. The presence of IL-1 and IL-6 within the brain implies that they might also act as MT-inducers in the brain.

As previously reported, the stimulation by IL-1 of a human astrocytoma cell line, U373MG, resulted in the induction of IL-6, IL-8 and monocyte chemotactic and activating factor (MCAF) [16,17]. Therefore, we investigated the role of cytokines and heavy metals for the MT-induction in this astrocytoma cell line.

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2. MATERIALS AND METHODS

2.1. *Cytokines and antibodies*

Recombinant human IL-1α (rhIL-1α, $1–2 \times 10^7$ U/mg protein) was provided by Dainippon Pharmaceutical Co. (Osaka). Recombinant human IL-6 (rhIL-6, 6×10^6 U/mg protein) was provided by Ajinomoto Co. (Yokohama). Preparation of the murine monoclonal antibody specific for MTs, MT45 (κ, γ 1), was reported previously [18], and the hybridoma culture supernatants were used for immunostaining. As a second antibody for indirect immunofluorescence, FITC-conjugated sheep anti-mouse Ig (Amersham, N.1031) was used.

2.2. *Cell culture*

Human astrocytoma U373MG cells were subcloned as described previously [16] and cultured in monolayers in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol.

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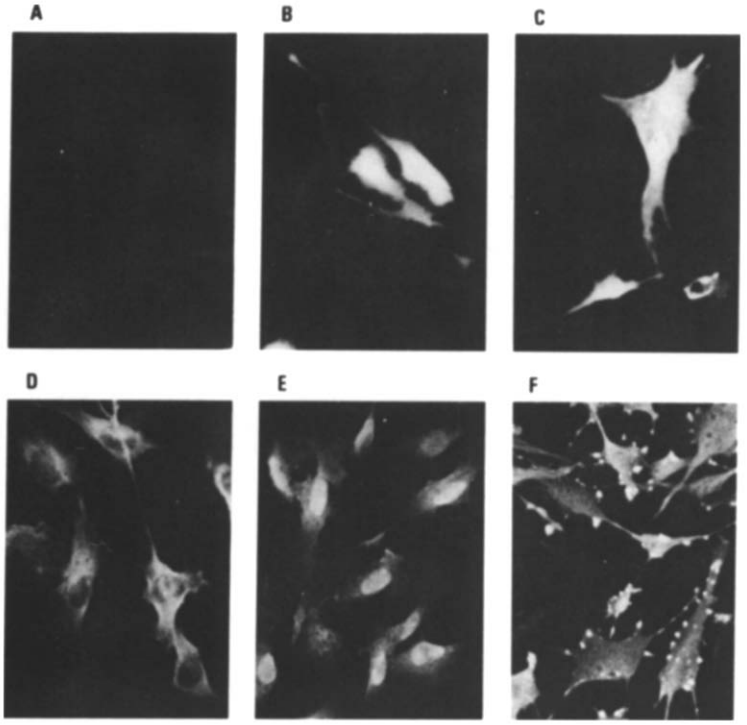

Fig. 1. Induction and localization of MTs in U373MG cells stimulated with heavy metals: A, untreated; B, cultured with 50 μM $CdCl_2$ for 48 h; C, cultured with 500 μM $ZnCl_2$ for 48 h; D, cultured with 50 μM $CdCl_2$ for 24 h; E, cultured with 50 μM $CdCl_2$ for 48 h; F, cultured with 50 μM $CdCl_2$ for 72 h. Pictures A–C and D–F were obtained from two independent experiments, respectively.

2.3. *Northern blot analysis*

A cDNA probe encoding the entire human MT 2a coding sequence was kindly provided by Dr. S. Yamazaki, Jichi Medical School, Tochigi. Other probes were prepared as described previously [17]. Northern blot analysis was carried out according to the method described previously [16].

2.4. *Indirect immunofluorescence*

Indirect immunofluorescence was carried out according to the method previously described [18]. U373MG cell monolayers (2×10^4 cells/well) grown on chamber-slides (Lab-Tek Tissue Culture Chamber/Slide, Nunc) were cultured with heavy metals and/or cytokines. The slides were centrifuged, and the cells were fixed with 3.5% formaldehyde in phosphate-buffered saline (pH 7.2) (PBS) for 20 min and then treated with 0.2% Triton X-100 in PBS for 2 min at room temperature. The fixed cells were blocked with 1.0% bovine serum albumin (BSA)-PBS for 30 min and incubated with the MT45 antibody for 45 min at room temperature. After extensive washing with PBS, the cells were treated with FITC-labeled second antibody in 1.0% BSA-PBS for 45 min at room temperature, washed with PBS, and mounted with Gel/Mount (Biomeda). The stained cells were observed and photographed under a fluorescence microscope (Olympus, Tokyo).

3. RESULTS

Northern blot analysis revealed that U373MG cells also expressed MT mRNA in response to rhIL-1α (data not shown). The kinetics of mRNA induction for metallothionein (MT) was similar to that for IL-8 or MCAF (data not shown). Under the same conditions, U373MG cells express a high level of IL-6 mRNA and release IL-6 activity into the supernatants [16]. Thus, it was considered to be necessary to determine whether or not the effect of IL-1 was indirectly mediated by the secreted IL-6. To determine the effects of IL-1 and IL-6 on MT biosynthesis, we employed the indirect immunofluorescence methods using a monoclonal anti-MT antibody (MT45) [18].

First, the induction of MTs by heavy metal ions was analyzed. A bright fluorescence was observed when U373MG cells were cultured with 50 μM $CdCl_2$ (Fig. 1B) or 500 μM $ZnCl_2$ (Fig. 1C) for 48 h, whereas the intensity of fluorescence in untreated U373MG cells was very low (Fig. 1A). The increase in fluorescence intensity was much higher than that observed in HeLa S3 and FM3A cells under similar conditions [18].

The effect of Cd^{2+} on the expression and localization of MTs were examined after varying the duration of incubations (Fig. 1D–F). A significant increase in fluorescence intensity was observed 12 h after the addition of the metal, and thereafter the fluorescence intensity gradually increased up to 24 h (data not shown). U373MG cells cultured for 24 h with 50 μM $CdCl_2$ showed a bright fluorescence in the cytoplasm (Fig. 1D). From 24 to 48 h after the addition of $CdCl_2$, immunoreactive MTs were found in both cytoplasm and nuclei (Fig. 1E). After 72 h, immunoreactive MTs accumulated in a granular form near the cell surfaces (Fig. 1F). Almost the same responses were observed when $ZnCl_2$ (500 μM) was added (data not shown).

Next, U373MG cells were cultured with IL-1 or IL-6 to determine their MT-inducing activity. Immunoreac-

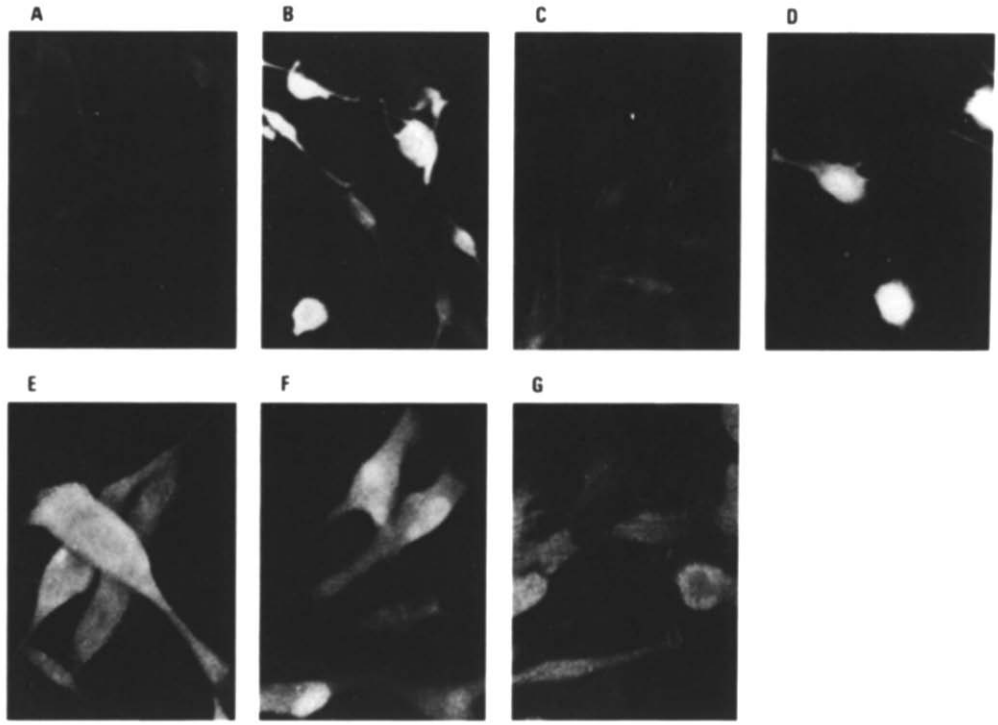

Fig. 2. Induction and localization of MTs in U373MG cells stimulated with IL-1 (100 U/ml) and IL-6 (100 U/ml); A, untreated (48 h); B, cultured with rhIL-1α for 48 h; C, cultured with rhIL-6 for 48 h; D, cultured with 50 μM $CdCl_2$ for 48 h; E, cultured with rhIL-1α for 24 h; F, cultured with rhIL-1α for 48 h; G, cultured with rhIL-1α for 72 h. Pictures A–D and E–G were obtained from two independent experiments, respectively.

tive MTs were induced in U373MG cells when incubated with rhIL-1α at more than 10 U/ml for 48 h (Fig. 2B), and the fluorescence intensity was comparable to that observed in the metal induction (Fig. 2B and D). In contrast, rhIL-6 did not augment the level of immunoreactive MTs at concentrations as high as 100 U/ml (Fig. 2C).

Changes in the level and localization of IL-1-induced immunoreactive MTs were pursued after varying the duration of incubations (Fig. 2E-G). U373MG cells cultured with rhIL-1α (100 U/ml) for 24 h showed a bright fluorescence in the cytoplasm (Fig. 2E). From 24 to 48 h after the addition of rhIL-1α, the MTs were found in both cytoplasm and nuclei (Fig. 2F). However, the fluorescence intensity was reduced nearly to the basal level after 72 h, and the granular accumulations of immunoreactivity shown in the metal induction was not detected in the IL-1 induction (Fig. 2G). Moreover, additional IL-1 at 48 h did not facilitate the appearance of the granules (data not shown).

To determine the effect of extracellular Zn^{2+} on the IL-1-induced MT expression and localization, U373MG cells were incubated in the medium containing rhIL-1α (100 U/ml) with or without 10 μM $ZnCl_2$ (Fig. 3). After 48 h of culture, immunoreactive MTs were found in both cytoplasm and nuclei (Fig. 3B and C) irrespective of the addition of supplementary Zn^{2+}. However, 72 h after the addition of rhIL-1α plus $ZnCl_2$ (10 μM), immunoreactive MTs accumulated in a granular form near the cell surfaces (Fig. 3G) while IL-1 alone did not bring about any granular accumulation (Fig. 3F). $ZnCl_2$ (10 μM) alone showed no effect (Fig. 3D and H). These data indicate that the granular accumulation of immunoreactive MTs near the cell surfaces is dependent on the extracellular Zn^{2+} concentration.

4. DISCUSSION

U373MG astrocytoma cells show strong proliferative responses to IL-1 [16,19]. Upon IL-1 stimulation, the cells also express several inflammatory cytokines [17]. U373MG cells were not stimulated by other cytokines including IL-2, IL-4, IL-5, IL-6 and interferons α and γ [16]. In the present study, Northern blot analysis and indirect immunofluorescence revealed that U373MG cells also expressed MTs in response to IL-1α. Since U373MG cells release IL-6 into the supernatants under the same conditions [16], the MT-inducing activity of IL-6 was tested. However, IL-6 did not show any augmentation of intracellular MT levels (Fig. 2C). Although IL-6 is a major cytokine mediator of MT gene expression in hepatocytes [9], the present data indicate that IL-1, but not IL-6, is an MT-inducer in U373MG cells. It cannot be ruled out that IL-1 induces MTs indirectly, e.g. via the production of IL-6 and IL-6 receptors. U373MG cells possess a small number of IL-6 receptors on their surfaces [20]. However, it seems unlikely that MT induction by IL-1 is mediated through

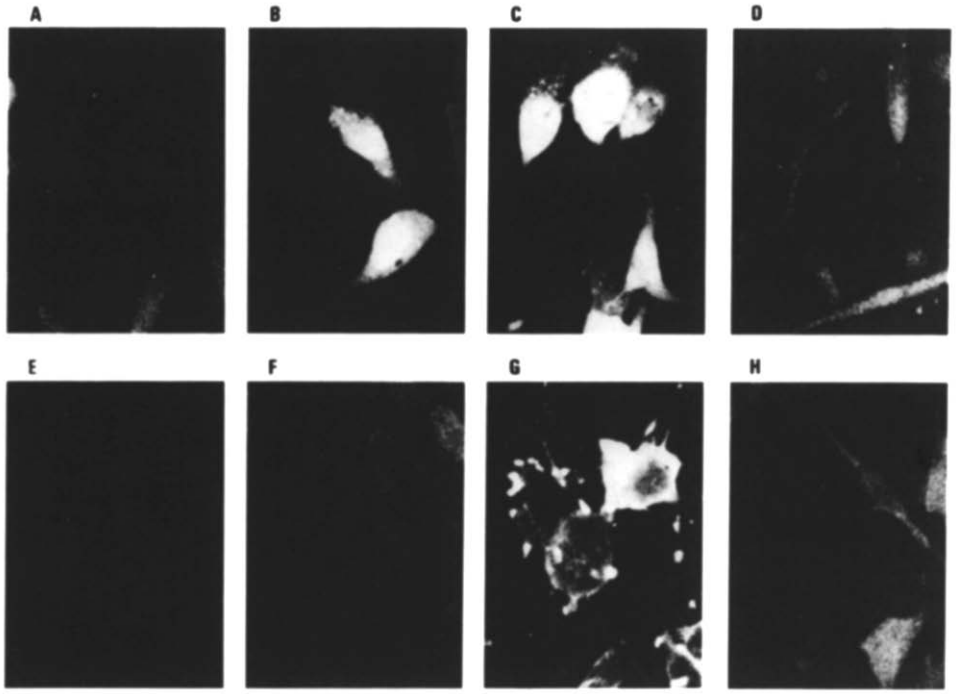

Fig. 3. Effect of $ZnCl_2$ (10 μM) on the expression and localization of MTs induced by IL-1 (100 U/ml) in U373MG cells: A, untreated (48 h); B, cultured with rhIL-1α for 48 h; C, cultured with rhIL-1α plus $ZnCl_2$ for 48 h; D, cultured with $ZnCl_2$ for 48 h; E, untreated (72 h); F, cultured with rhIL-1α for 72 h; G, cultured with rhIL-1α plus $ZnCl_2$ for 72 h; H, cultured with $ZnCl_2$ for 72 h.

IL-6 receptors, since IL-6 itself does not activate this cell line in terms of cytokine production and proliferation [16] and anti-IL-6 antibody did not suppress the IL-1-induced cytokine production (our unpublished data).

At present, it is not certain that astroglial cells in vivo produce MTs upon IL-1 stimulation. However, the fact that both IL-1 receptors [21,22] and MTs (unpublished data) are localized in the brain hippocampal regions at higher levels implies the role of IL-1 for MT induction in these regions. The MT-induction by IL-1 might be part of a protective response to minimize damage by free radicals generated during the IL-1-induced responses [3].

Intraperitoneal administration of zinc or cadmium does not increase the brain MT levels [23,24] probably due to the blood–brain barrier [25]. However, MTs are induced by intracerebral zinc administration [26]. Therefore the primary function of MTs in the brain was suggested to be zinc homeostasis rather than metal detoxification ([27] for review). In fact, the zinc content of the brain is greater than those of other tissues or organs. On this point, it is noteworthy that the Zn^{2+} concentration required for MT induction is much higher than the Cd^{2+} concentration.

Several investigators have reported the intranuclear localization of MTs, especially, in developing tissues and genital organs [28–30]. We also observed a transient but marked localization of MTs in the nuclei of U373MG cells. The physiological role of the nuclear translocation of MTs and its mechanism are entirely obscure. One likely possibility is that MTs carry Zn^{2+} ions into the nucleus and supply the ions to nuclear zinc-requiring enzymes or factors under certain specific conditions.

It is interesting that immunoreactive MTs accumulate after a prolonged incubation with heavy metal ions. This accumulation was not observed when only IL-1 was added. The addition of a low concentration of Zn^{2+} with IL-1 resulted in the appearance of the granular accumulations. Several investigators have reported on particulate forms of Zn- and Cu-MTs [31–33]. At present, however, the physiological relevance and mechanism of the formation of particulate MT is not clear. The granular accumulation might be a step before the MTs are excreted. It is suggested that MT polypeptides are degraded mainly in lysosomes and excreted outside the cell. The apo-MTs have been shown to be even more sensitive to proteolysis than metal-including MTs ([34] for review). Therefore, without supplemental $ZnCl_2$, the newly synthesized apo-MTs following stimulation by IL-1 might be rapidly degraded.

In conclusion, the induction of MTs by IL-1 and heavy metals in the astrocytoma cell line, U373MG, is remarkable, and the basal MT expression is low. Thus, this in vitro induction system provides a useful model for elucidation of the mechanisms of the induction and intracellular metabolism of MTs.

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