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Original Paper

Enhanced Invasiveness of Tumour Cells After Host Exposure to Heavy Metals

A. Haga, H. Nagase, H. Kito and T. Sato

Department of Public Health, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502, Japan

The invasiveness of tumour cells to heavy metal-exposed host cells or tissues was investigated. Human fibrosarcoma cell invasion of heavy metal-treated fibroblast or endothelial cells was enhanced in a treatment-time-dependent manner although tumour cell attachment to host cells was not affected. This enhancement was correlated with an increase in metallothioneins in the cytosol of fibroblasts or endothelial cells. Mouse melanoma cell invasion of organ samples obtained from syngeneic mice who had been administered heavy metals was also enhanced. The results suggest that heavy metal-induced metallothioneins serve as a host-derived factor in malignant disease and closely relate to metastasis. Copyright © 1996 Elsevier Science Ltd

Key words: heavy metals, tumour cells, invasiveness

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INTRODUCTION

TUMOUR CELL invasiveness is a critical factor in metastasis [1, 2]. Several processes are involved in malignant invasion, including motility by chemotactic mechanisms [3], adhesion to various substratum glycoproteins or glycosaminoglycans [4], and degradation of these molecules by different classes of enzymes [5]. Primary tumour cell invasion of surrounding tissue is the first stage of a metastasis cascade, and many factors such as matrix metalloproteinases (MMPs) are associated with this stage [6].

Both tumour and host factors influence metastasis, and a greater understanding of these factors could further anti-metastasis research. Our investigation focused on the relationship between tumour cell invasiveness and host exposure to heavy metals. Various studies have indicated such a relationship. High tissue zinc (Zn) concentration has been observed in malignant disease in humans [7, 8] and in experimental tumour-bearing mice [9]. Futhermore, cadmium (Cd) loading promotes experimental carcinogenesis in rats [10], and the metal containing anticancer drug, cisplatin (CDDP), is administered to patients who have malignant tumours [11].

Heavy metals induce the synthesis of metallothioneins (MTs) or heat shock proteins [12], which lead to the alteration of normal and tumour tissues. In cancer chemotherapy, the association of drug resistance with drug-induced MT synthesis is a critical problem [13]. However, there is little information on the effect of heavy metals on tumour metastasis so in this paper we used *in vitro* assays to investigate the effect of heavy metals, zinc, cadmium and CDDP on metastasis.

Correspondence to A. Haga.
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MATERIALS AND METHODS

Cell cultures

Human fibrosarcoma HT-1080 cells [14], human fibroblast WI-38 cells [15], and bovine carotid artery endothelial HH cells [16] were provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). Highly metastatic mouse melanoma B16-BL6 cells [17] were kindly provided by Dr I.J. Fidler, M.D. Anderson Cancer Center, Houston, Texas, U.S.A. through Dr I. Saiki. HT-1080 cells and B16-BL6 cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). WI-38 and HH cells were maintained as monolayer cultures in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% FBS.

Tumour cell invasion of host cell monolayers

Tumor cell invasiveness was assayed according to Akedo and associates [18]. WI-38 cells were pretreated with 10 μM Cd, CDDP or 100 μM Zn, and HH cells were pretreated with 1 μM Cd, CDDP or 10 μM Zn in serum-free DMEM

for 1, 3, 6, 12, or 24 h in a confluent culture, grown in 3.5 cm dishes. The medium was removed and the pretreated host cell monolayer was washed with PBS three times. HT-1080 cells (1×10^5) in 2 ml serum-free EMEM were applied to pretreated WI-38 or HH cell monolayers. After 12 h incubation at 37°C, the uninvaded HT-1080 cells were removed with PBS and the invaded cells were fixed with 10% formalin and the number of penetrated HT-1080 cells in 50 different fields was counted by phase contrast microscopy at a magnification of 200×.

Attachment of tumour cells to the host cell monolayer

WI-38 cells were pretreated with 10 μM Cd, and HH cells were pretreated with 1 μM Cd in serum-free DMEM for 1, 3, 6, 12 or 24 h in a confluent culture in 24-multiwell plates. The medium was removed, and the pretreated host cell monolayer was washed with PBS three times. HT-1080 cells (1×10^5) labelled with 2′,7′-bis (carboxyethyl)-5(6)-carboxyfluorescein penta(acetoxymethyl) ester (BCECFAM) (Wako Pure Chemical Co., Tokyo, Japan) were seeded on to WI-38 or HH cell monolayers. After 60 min incubation at 37°C, the cultures were washed and then HT-1080 cells that had attached to the monolayer were removed by treatment with 1% Triton X-100, and fluorometric intensities were measured using a microplate fluorophotometer (MILLIPORE CytoFluor 2300) [19].

Measurement of cellular metallothioneins

A cadmium saturation assay [20] was performed to determine the total amount of MTs in cells. Confluent WI-38 or HH cells in 90 mm dishes were treated with serum-free DMEM containing 10 μM Cd for various lengths of time. After treatment, Cd was removed by exchanging the medium, and the cells were cultured with serum-free DMEM for 12 h. Cells were washed with PBS (−) three times, scraped with a silicon cell scraper, and lysed. The cell lysate in RIPA buffer (25 mM Tris–HCl, pH 7.4, containing 25 mM NaCl, 0.5 mM ethylene glycol tetraacetic acid, 10 mM sodium pyrophosphate and 10 mM sodium fluoride), was incubated with 0.2 μg Cd at room temperature for 10 min. Bovine haemoglobin was then added to the mixture to bind the excess cadmium. The mixture was placed in a 100°C water bath for 1 min and centrifuged at 10 000*g* for 3 min. The cadmium content of the supernatant was measured by atomic adsorption spectrophotometry (Shimadzu AA-6500).

Tumour cell invasion of organ samples

Tumour cell–organ interaction was investigated according to the method of Easty and associates [21, 22]. Eight week-old female C57BL/6 mice were treated daily s.c. with 50 μg Cd, CDDP or 500 μg Zn for 3 days to induce metallothionein synthesis [23]. The animals were killed by dislocation of cervical vertebrate and 1 mm^3 pieces of heart, lung, liver, ovary were placed in vials and cultured with HEPES-buffered DMEM supplemented with 10% FBS for 20 h. The control group consisted of C57BL/6 mice given 50 μl saline daily for 3 days. 5×10^5 B16-BL6 cells (0.5 ml) were added to the vials along with 2.5 ml HEPES-buffered DMEM. After 24 or 48 h incubation at 37°C, the tissue pieces were fixed in Bouin's solution and embedded in paraffin. Sections (5 μm) were prepared and stained with haematoxylin and eosin. The number of B16-BL6 cells that invaded the tissues were counted for 50 different fields using a light microscope at a magnification of 100×.

Statistical analysis

The statistical significance of differences between control and treatment in each assay were determined by the Student's *t*-test ($n > 3$).

RESULTS

HT-1080 cell invasion of host cell monolayers

With 24 h pretreatment in serum-free DMEM, WI-38 cells pretreated with 100 μM Cd, CDDP or 1 mM Zn, and HH cells pretreated with 10 μM Cd, CDDP or 100 μM Zn, were resistant to invasion by HT-1080 cells (data not shown). When WI-38 cells were pretreated with 10 μM Cd, CDDP or 100 μM Zn, and HH cells were pretreated with 1 μM Cd, CDDP or 10 μM Zn, in serum-free DMEM for 1, 3, 6, 12 or 24 h, HT-1080 cell invasiveness was enhanced in a pretreatment-time-dependent manner. The difference between untreated and pretreated host cell at each treatment time was statistically significant at 6 h ($P < 0.01$), and at 12 and 24 h ($P < 0.001$) for WI-38, and at 6 and 12 h ($P < 0.01$), and at 24 h ($P < 0.001$) for HH. Pretreatment

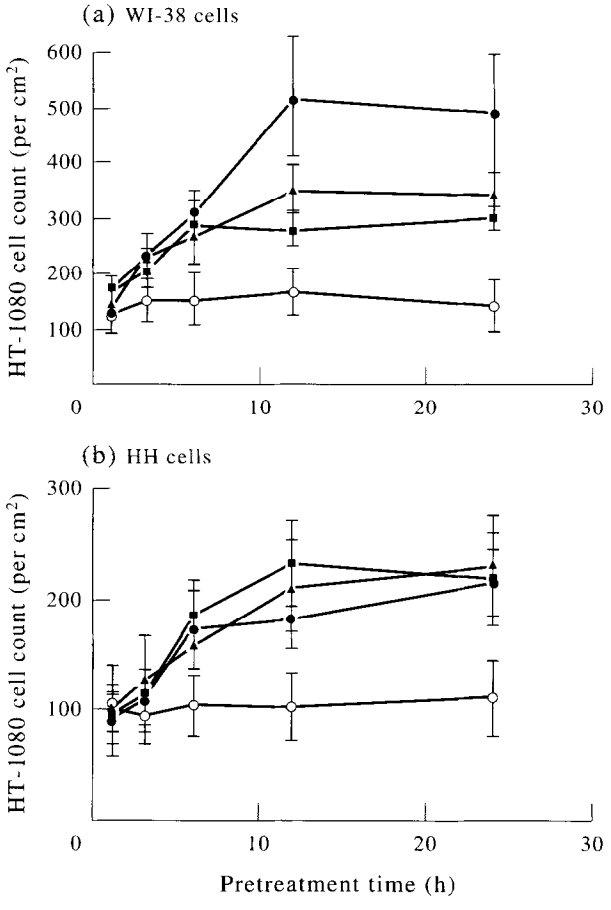

Figure 1. Invasiveness of HT-1080 cells in to heavy metal-treated WI-38 or HH cells. (a) WI-38 cells were pretreated with serum-free DMEM (○) or serum-free DMEM containing 10 μM Cd (●), CDDP (■) or 100 μM Zn (▲) for various lengths of time. (b) HH cells were pretreated with serum-free DMEM (○) and serum-free DMEM containing 1 μM Cd (●), CDDP (■) or 10 μM Zn (▲) for various lengths of time.

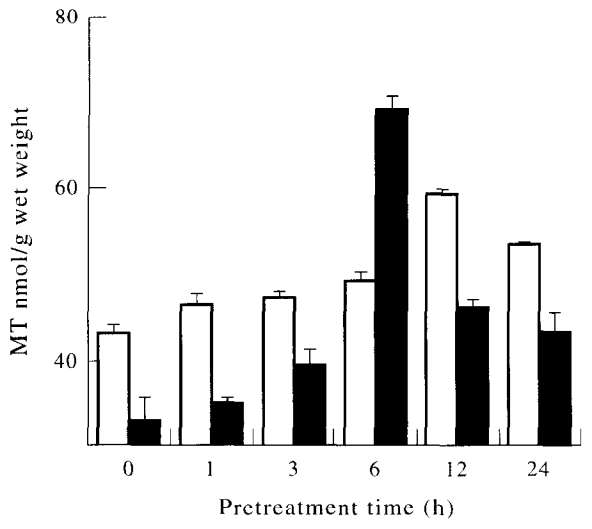

Figure 2. Metallothionein contents of Cd-treated WI-38 or HH cells. WI-38 (□) or HH (■) cells were treated with serum-free DMEM containing 10 μM Cd for various lengths of time.

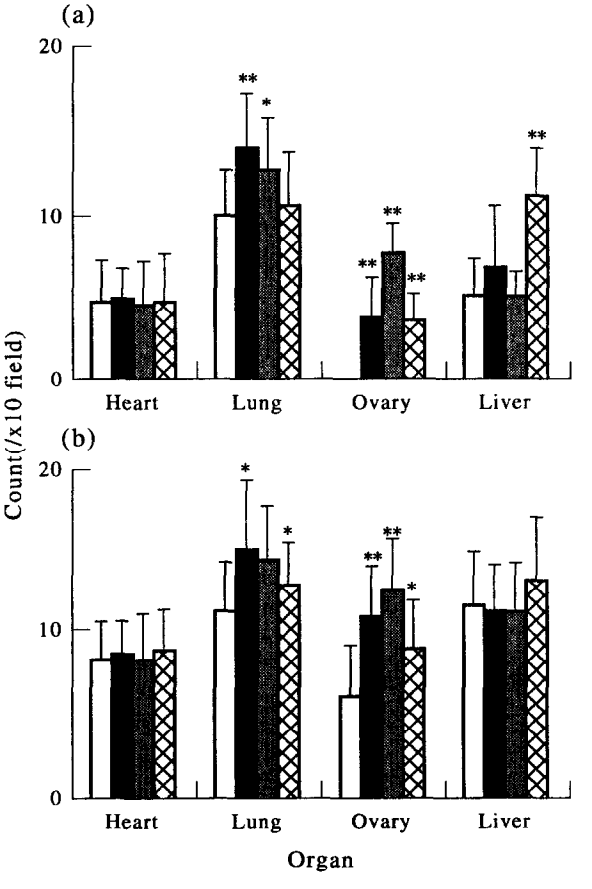

Figure 3. Invasiveness of B16-BL6 cells to organ tissue samples from heavy metal-exposed C57BL/6 mice. Organ pieces from C57BL/6 mice administered saline (□), 50 μg Cd (■), CDDP (grey area) or 500 μg Zn (▩) daily for 3 days were cultured with 5×10^5 B16-BL6 cells for 24 h (a) or 48 h (b). Statistical significance was calculated by Student's *t*-test. *$P < 0.05$, **$P < 0.01$.

with serum-free DMEM alone did not affect invasiveness (Figure 1). This enhancement was related to the total MT content of the Cd pretreated WI-38 cells. A peak in total MT induction occurred after 6 h in Cd pretreated HH cells (Figure 2). Cd treatment of WI-38 or HH cell monolayers did not affect the ability of HT-1080 cells to adhere to them (data not shown).

B16-BL6 cell invasion of organ tissue pieces

B16-BL6 cell invasion into the organ samples obtained from the heavy metal-exposed mice increased compared with the control group. The increase was statistically significant, except for the heart tissues, particularly in the 24-h incubation samples (Figure 3). In the ovary tissue control, no invasiveness was observed in the 24-h incubated samples, although attachment of B16-BL6 cells to the tissue was observed; this finding has also seen reported by Nicolson and associated [22]. Enhanced invasiveness was observed in ovary samples from the heavy metal-treated mice (Figure 4).

DISCUSSION

HT-1080 cell invasiveness to fibroblast or endothelial cell monolayers exposed to cadmium, zinc or platinum salts was enhanced with an increase in metallothionein induction in the host cells, although tumour cell attachment to those cells was not changed by exposure. A similar phenomenon was observed in B16-BL6 melanoma cell invasion of organ tissues from isogeneic mice exposed to heavy metals. These results suggest that some host-derived factor, such as metallothioneins induced by heavy metal treatment, acted on the tumour and host proteinase (matrix metalloproteinases, MMPs) or promoted tumour cell motility.

HT-1080 cells secrete two types of MMPs, MMP-2 and MMP-9, identified as 72 kDa gelatinase A and 92 kDa gelatinase B, respectively [6]. Mouse melanoma B16-BL6 cells secrete a 103 kDa gelatinase considered to be MMP-9 [24]. Since these MMPs are secreted as inactive zymogens, activation is important for the expression of enzymic potency. This latency is maintained by chelation between a zinc atom in the active centre and a thiol group of its N-terminal peptide [25], so MMPs can be experimentally activated by thiol-reaction reagents or protein-degeneration reagents such as aminophenylmercury acetate [26]. Furthermore, serine proteases such as trypsin, chymotrypsin and cathepsin G, and matrix metalloproteinase such as stromelysin 1 (MMP-3) are endogenous activators of MMP-9 [26]. We have also reported that the activity of MMPs secreted from human fibrosarcoma HT-1080 is enhanced by addition of metallothioneins, and the invasiveness of HT-1080 and B16-BL6 cells to reconstituted basement membrane Matrigel is promoted [27]. Sato and associates have reported the membrane-type matrix metalloproteinase (MT-MMP) as a critical activator of MMP-2, and the possibility of a new cascade in tumour invasion, wherein tumour MT-MMP activates the pro-gelatinase A secreted from host cells, and the gelatinase A degrades the host tissue itself [28]. That high expression of MMP-2 mRNA was recognised in the interstitial fibroblast rather than in the tumour cell itself [29, 30] supports this possibility.

Human normal fibroblasts also secrete the MMP-2 [31–33]. We have previously found that Cd and Zn 24 h exposure of the WI-38 fibroblast cell monolayer transform the

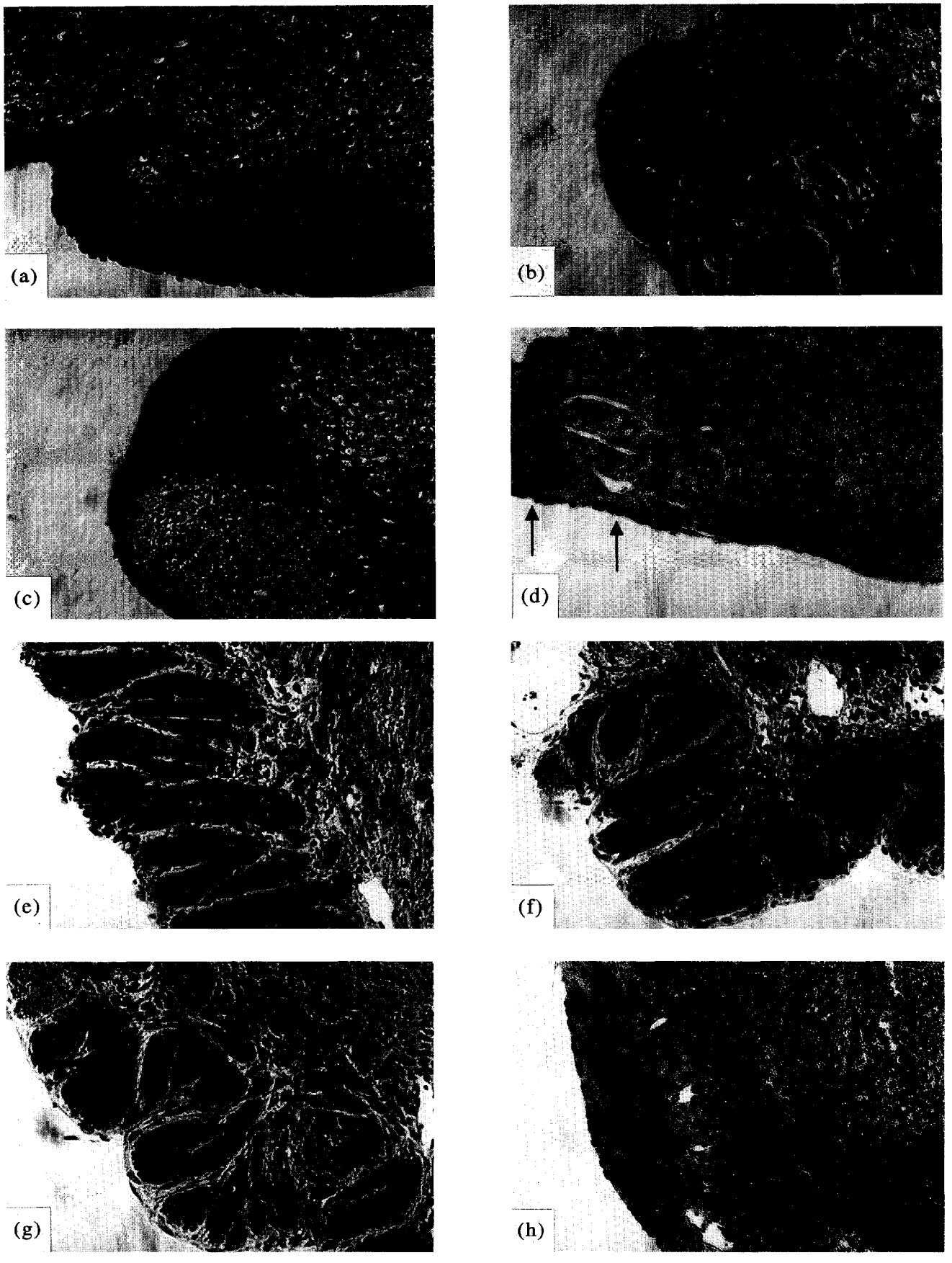

Figure 4. Invasiveness of B16-BL6 cells into ovary tissue pieces. Ovary tissue pieces were incubated with B16-BL6 cells for 24 h. (a) CDDP-treated section (400 ×), (b) Cd-treated section (400 ×), (c) Zn-treated section (400 ×), (d) control section (200 ×). Arrows indicate the attached tumour cells. Ovary tissue pieces incubated with B16-BL6 cells for 48 h, (e) CDDP-treated section (400 ×), (f) Cd-treated section (400 ×), (g) Zn-treated section (400 ×), (h) control section (200 ×).

inactive 62 kDa intermediate species of MMP-2 secreted from WI-38 cells to the 59 kDa active enzyme, and enhance its collagenolytic activity without any changes in the secretion volume of MMP-2. This transformation and enhancement have also been observed in the reactions between the conditioned medium of WI-38 cells and purified MTs [34]. Such enhancement of the enzymic activity in host cells may promote tumour cell invasiveness in this investigation. The result that heavy metal exposure enhances tumour invasiveness to lung or ovary tissue may support this possibility because they abound in fibroblasts. A peak of MT induction on various cultured cells has been known to occur after 6–12 h exposure to heavy metals [35, 36]. Transformation and activity enhancement of MMP-2 secreted from WI-38 have been recognised after affecting MTs for 6 h. [34]. These phenomena are considered to differ in cell lines, therefore, we concluded that there was no correlation between MT induction and HT-1080 invasiveness in the HH cell experiment. Onosaka and Cherian found that cadmium exposure induced approximately 800 times murine hepatic MTs and 13 times heart MTs, respectively, but not in the lung [37]. In this investigation, a significant increase of tumour invasiveness was not observed at 48 h in liver and 24 and 48 h in heart tissue. This means host-derived MTs may affect the host MMPs rather than the tumour itself.

MTs are inducible by a wide variety of agents including heavy metals or stress, surgery [12] and the tumour [9]. MTs are also known to be a cause of drug resistance in cancer chemotherapy. Satoh and associates reported the possibility of overcoming cisplatin resistance and cancer spread by controlling hepatic MT synthesis using propargylglycine (PPG), known as a cystathionase inhibitor [38]. Most MT binding of heavy metals is intracellular, but some MTs are released extracellularly from injured or dead cells [12]. Tumour cells cause injury and death in surrounding tissues, thereby increasing the amount of MTs in the extracellular space. If host MT levels can be controlled by using PPG and other suppressors of MT induction, tumour metastasis may be influenced.

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