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Metal binding of metallothioneins in human astrocytomas (U87 MG, IPDDC-2A)

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Abstract

Astroglia cells structurally and nutritionally support neurons in the central nervous system. They play an important role in guiding the construction of the nervous system and controlling the chemical and ionic environment of neurons. They also represent the major sites for accumulation and immobilisation of toxic metal ions most probably connected with metallothioneins. For this reason astroglia cells possess high cytosolic levels of metallothioneins I, II and III (MT-I,II,III). Our aim was to establish the inducibility and metal binding of MTs in two human astrocytoma cell lines, U87 MG (astrocytoma–glioblastoma, grade IV) and IPDDC-2A (astrocytoma, grade II), on exposure to cadmium chloride (1 μM). MTs were identified by molecular weight (size exclusion chromatography) and their metal content (Cd, Zn and Cu) to follow the interactions between metals. We showed that MTs are constitutively expressed in both human astrocytoma cell lines. In accordance with the higher malignancy grade of U87 MG, the amount of MTs was higher in U87 MG than in IPDDC-2A cells. After 24 hours of exposure to Cd their expression greatly increased in both cell lines and they were capable of immobilising almost all water soluble Cd. Induction of MTs in U87 MG cells was additionally followed up to 48 hours with exposure to different concentrations of $CdCl_2$ (1, 10 μM). Induction was a time dependent process throughout the period. Isoform III (identified by chromatographic separation of isoform III from I/II) was present at all exposure times, but only in traces with respect to the prevailing amounts of MT-I/II isoforms. So induction can be attributed to isoform I/II only.

Introduction

Metallothioneins (MTs) are low molecular weight non-enzymatic proteins or polypeptides with a high cysteine (Cys) content and consequently a high affinity for transition metal ions. Mammalian MTs are proteins with an absolute molecular weight of 6–7 kDa and can be found in four major isoforms: I, II, III and IV. Isoforms I and II are expressed in almost all cell types, whereas isoform III is expressed mainly in brain and isoform IV in keratinising epithelial cells (Nordberg 1998; Romero-Isart and Vašak 2002; Chian-Feng *et al.* 1996). Isoform MT-III is characterised by a slightly higher molecular mass: 68 amino acids (AA) compared to 61/62 AA in isoforms MT-I,II and IV (Kägi 1993; Vašak 2005).

Native mammalian MTs bind the essential metal ions zinc and copper and are believed to maintain their homeostasis in the cell. Under conditions of exposure to other metal ions they can also bind cadmium, lead, mercury, silver, gold,

iron and cobalt. Particularly zinc, with its lower affinity for MTs, can be replaced by other ions due to their physico-chemical similarities (Chan *et al*. 2002; Kägi & Kojima 1987; Beattie *et al*. 2005). In this way, MTs sequester toxic free metal ions and as a result lower their concentrations and protect the cell against metal toxicity. Although in the literature controversial data and opinions are found, it seems, that all isoforms, including isoform III (Satomi *et al*. 2005), can be involved in toxic metal complexation.

Isoform MT-III, originally named growth inhibitory factor (GIF), possesses an additional specific biological function which is not shared by the isoforms I and II, namely inhibition of neuronal cell growth in the brain and of some other cells and cell cultures (Gerrett *et al*. 2005; Uchida *et al*. 1991, 2002; You *et al*. 2002). The reduction of its level in Alzheimer's disease (Uchida *et al*. 1991; You *et al*. 2002; Yu *et al*. 2001; Yoshifumi & Keung 2003) indicates a possible link between this disease, MT-III and free metal ions (You *et al*. 2002; Yu *et al*. 2001; Aoki *et al*. 1998). Beside that, all three isoforms seem to have an important role in the protection of the brain following injury (Hidalgo *et al*. 2001; Chung & West 2004) and some other neurodegenerative diseases (Hozumi *et al*. 2004) what is primarily due to their free radical scavenging ability (Uchida *et al*. 2002; Køhler *et al*. 2003). Mocchegiani reported that all three isoforms, MT-I/II and MT-III, express a protective role in the hippocampus of the young rat, but with ageing the decline in the ability to release zinc from MT-I/II may lead to a decrease zinc ion bioavailability (Mocchegiani *et al*. 2004). The expression of MTs can be also involved, up or down regulated, in different human tumours depending on the differentiation status and proliferative index of tumours (Cherian *et al*. 2003; Hiura *et al*. 1998; Pattanaik *et al*. 1994). In astrocytic tumours, the aggressiveness of tumours (indicated by tumour grade) and the survival of patients can be predicted by the level of MTs in a tumour (Hiura *et al*. 1998).

Astrocytes are brain cells known to support the physiology of associated neurons but also participate in active control of neuronal activity and synaptic neurotransmission (Araque *et al*. 1999). They have been proposed as the main site of accumulation and storage of toxic metals in the brain (Tiffany-Castiglioni & Qian 2001). They possess cytoplasmic properties known as end-feet, that envelope the blood vessels at one side and neurons at the other. In this way, they are the first cells of the brain where metals crossing the blood-brain barrier encounter. As they contain high cytosolic levels of MTs they are capable of binding high amounts of metal ions (Hidalgo *et al*. 2001) including cadmium.

Cadmium is a major industrial pollutant. Humans are exposed to cadmium through food, water, air and particularly by cigarette smoke. It has unusually long half-life (~20 years) and accumulates in the body throughout life (Kostial 1986; Gerhardsson *et al*. 2002). Part of the absorbed cadmium can also enters the central nervous system (CNS) (Falnoga *et al*. 2000) where it can act as a neurotoxic agent by changing the activity of some enzymes and/or inducing oxidative stress, and may thus represent a risk factor for the development of neurodegenerative diseases (Hidalgo *et al*. 2001). Above all the situation is critical in cases when the blood brain barrier, which usually efficiently protects brain from excessive Cd ion transport, is already damaged due to some other pathological situations. In the CNS, cadmium can accumulate in astrocytes and neurons. It is interesting that in cell culture experiments the astrocytes were found to be more resistant to acute high-dose of cadmium (300 μM for 30 minutes) than neurons, but more sensitive than neurons to long-term low-dose of cadmium (10 μM) (Im *et al*. 2006). In primary cortical mice astrocytes, cadmium-induced cell death was found to be associated with glutathion depletion (Im *et al*. 2006). In primary rat astrocytes as well as in human astrocytomas cadmium may also induces the synthesis of MTs as part of defence mechanism against cadmium toxicity (Sawada *et al*. 1994; Rising *et al*. 1995). There are many studies covering astrocytes and metallothioneins behaviour after metal exposure, but without fallowing metal binding.

The aim of present study was to establish the inducibility and metal binding of MTs in two human astrocytoma cell lines on exposure to cadmium. The test of MTs induction and cadmium binding was performed on two cell lines representing different malignancy grades of human gliomas: U87 MG (glioblastoma–astrocytoma, grade IV) and IPDDC-2A (grade II astrocytoma). In U87 MG, the higher grade cell line, we also tried

to separate MTs-I,II and MT-III isoforms with special emphasis in comparing the expression and metal binding ability of all three isoforms (MT-I,II and III) simultaneously.

Experimental

Cell cultures

Two malignant human glioma cell lines U87 MG (glioblastoma–astrocytoma, grade IV) and IPDDC-2A (astrocytoma, grade II) were grown in monolayer cultures in Minimum Essential Medium with Earle's Balanced Salt Solution (EuroClone), supplemented with 10% foetal bovine serum (EuroClone), 4 mM L-glutamine, 1% non-essential amino acids, and 1% penicillin/streptomycin at 37 °C in 5% CO_2.

Cytotoxicity assay of $CdCl_2$ in U87MG

The cytotoxicity of $CdCl_2$ was determined by MTT (methyl tetrazolium salt) assay (Mosman 1983), which is the most commonly employed procedure for detection of cytotoxicity or cell viability. MTT is a water soluble substance which is converted to insoluble formazan by mitochondrial enzymes in living cells. The concentration of formazan crystals is directly proportional to the number of living cells. U87 MG cells were seeded on 96-well microplates at a density of 15000 cells/well. After 24 hours of incubation, the medium was replaced by fresh medium containing 2, 5, 10 or 20 μM $CdCl_2$ and incubated for 21 hours. Then MTT was added (final concentration 10%) and cells were incubated for another 3 hours. The medium was removed and formazan crystals dissolved in DMSO (dimethyl sulfoxide). The absorbance (A) of each well was measured at 570 nm (reference filter 690 nm) with a GENios spectrofluorimeter (Tecan, Salzburg, Austria). Five individual wells were prepared for each treatment concentration. The survival was determined by comparing the mean value of A in the wells containing treated to those containing untreated cells.

Cell treatment

Cells were seeded in 150 cm^2 tissue culture flasks and grown to ~70–80% confluence before treatment with cadmium. Cadmium as $CdCl_2$ was dissolved in the medium to reach a final concentration of 1 or 10 μM. First both cell lines, U87 MG and IPDDC-2A, were exposed to 1 μM of cadmium for 0 and 24 hours. Secondly cell line U87 MG only was exposed to 1 or 10 μM of cadmium for 0, 3, 6, 12, 24 and 48 hours. Cells were then washed three times with Dulbecco's Phosphate Buffered Saline (EuroClone), harvested and frozen at −70 °C.

Sample preparation

U87 MG and IPDDC-2A cells (~10^6 to 10^7 cells/sample) were thawed and suspended in ice-cold nitrogen-saturated buffer (10 mM Tris–HCl, 10 mM DTT, 0.25 M sucrose, pH 7.6) to a volume of 1.5 (first experiment – MT identification) or 2 ml (second experiment – Cd exposure). Cell suspensions were frozen in liquid nitrogen and thawed three times and then centrifuged (20000 *g*, 30 minutes, 4 °C; Eppendorf AG 5804 R, Hamburg, Germany). Supernatants were used for the determination of soluble MTs. In supernatants and some pellets obtained by centrifugation the concentrations of zinc, cadmiun and copper were measured by atomic absorption spectrometry (Hitachi Z-8270 Polarized Zeeman).

For evaluation of the presence of biologically insoluble polymerised MTs, the pellet of U87 MG cells (exposed to 10 μM of cadmium for 48 hours) was resuspended in buffer containing mercaptoethanol (30 mM Tris–HCl, 1 mM DTT, pH 8.0, 1% ME) and centrifuged (20000 *g*, 30 min, 4 °C).

Isolation and characterisation of MTs; MT-I/II and MT-III separation

MTs were isolated from supernatants and mercaptoethanol extract from U87 MG cells by HPLC gel permeation chromatography (L-7100 Pump, Merck-Hitachi). Samples (0.5 ml each) were loaded on a Superdex™ 75 HR 10/30 column (Pharmacia Biotech AB, Uppsala, Sweden) and eluted with 30 mM Tris–HCl, 1 mM DTT, pH 7.6 at a flow rate of 0.75 ml/min. For the separation of isoform MT III from isoforms MT-I/II, two columns were connected in tandem and the samples (0.5 ml each) were eluted at a lower flow rate (0.5 ml/min) with 30 mM Tris–HCl, 1 mM DTT, pH 8.0 (adapted method of Yasutake *et al.* 1998).

MTs or MT-I/II retention times were determined with rabbit liver MT standards (Metallothionein and Metallothionein II, Rabbit Liver, Sigma Chemical Co., St. Louis, MO, USA) and MT-III with human recombinant MT-III standard (ZnMZ-III, ALX-201-172). On-line UV absorption at 220 or 254 nm followed (UV Detector L-7400, Merck-Hitachi) and 1 minute fractions were collected for heavy metal analysis (Fraction Collector L-7650, Merck). All elements (Cd, Zn and Cu) were measured by flame atomic absorption spectrometry (Varian AA-5), except in control samples where Cd concentrations were measured by electrothermal atomic absorption spectrometry (Hitachi Z-8270 Polarized Zeeman). The same methods were used for metal detection in supernatants and pellets, with previous acid digestion of the sample in the case of pellets.

In one sample the expression of MTs in the supernatant of U87 MG cells, exposed to 10 μM of cadmium chloride, was checked by inductively coupled plasma mass spectrometry (ICP MS).

Results

Cytotoxicity assay of U87 MG cells

Survival of U87 MG cells exposed to different concentrations of $CdCl_2$ (2, 5, 10 or 20 μM) for 21 h is shown in Figure 1. At concentrations of 2 and 5 μM of cadmium the viability of cells was not significantly different from the control. At 10 and 20 μM of $CdCl_2$ viability was reduced to about 75%. Following these results two concentrations were chosen for analysis of MT induction: 1 μM which does not affect cell viability and 10 μM which is cytotoxic.

Detection of MTs in U87 MG and IPDDC-2A cells

In the first experiment we tested the presence and inducibility of MTs in two human glioma cell lines representing various malignancy grades: grade IV of the U87 MG cell line and grade II of the IPDDC-2A cell line. Cells were exposed to 1 μM of cadmium for 24 hours. In Table 1, concentrations of Cd in control and exposed cells are shown. From Figure 2 it is obvious that constitutive MTs with bound Zn are present in both cell lines. Cu binding was present in U87 MG only, while it was under the limit of detection in IPDDC-2A cell line. Traces of Cd were also present, probably due to its presence in buffers or growth media. On a single Superdex 75 HR column they are eluted at the retention time of 20 minutes. The amount of MTs is higher in the U87 MG cell line what is in accordance with published observations that the levels of MTs expression is higher in high grade gliomas (Hiura *et al*. 1998; Bredel and Zentner 2002). After Cd exposure (1 μM) in both cell lines elevated Cd-MT appeared after 24 hours. Although the amounts of cadmium in MTs fractions of U87 MG and IPDDC-2A cell lines did not differ very much, the difference in zinc binding on MTs and other proteins through the whole chromatogram indicated that the two cell lines had a different metabolism. It seems that this points to a much lower metabolic activity in IPDDC-2A cells than in U87 MG cells.

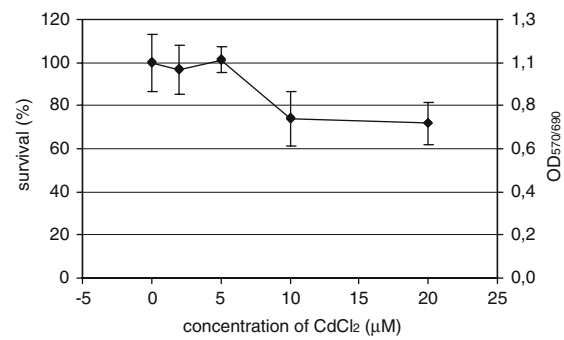

Figure 1. MTT assay of U87 MG cells treated with 2, 5, 10 and 20 μM $CdCl_2$. Survival was assessed after 21 hours and compared to control cells. The results shown are arithmetical means of five parallel measurements with standard deviation for each concentration.

Table 1. Cadmium concentrations (in ng/g) in supernatants and pellets obtained by centrifugation after exposure to 1 μM of cadmium as $CdCl_2$; in brackets are given calculated percentages regarding the total cadmium in the cells.

Sample	Exposure time (hours)	Cd conc. in supernatant; ng/g (%)	Cd conc. in pellet; ng/g (%)
U87 MG	0	*	6
	24	1220 (98.9%)	165 (1.1%)
IPDDC-2A	0	*	1
	24	956 (92.4%)	907 (7.6%)

*The concentration was under the detection limit (0.2 ng/ml).

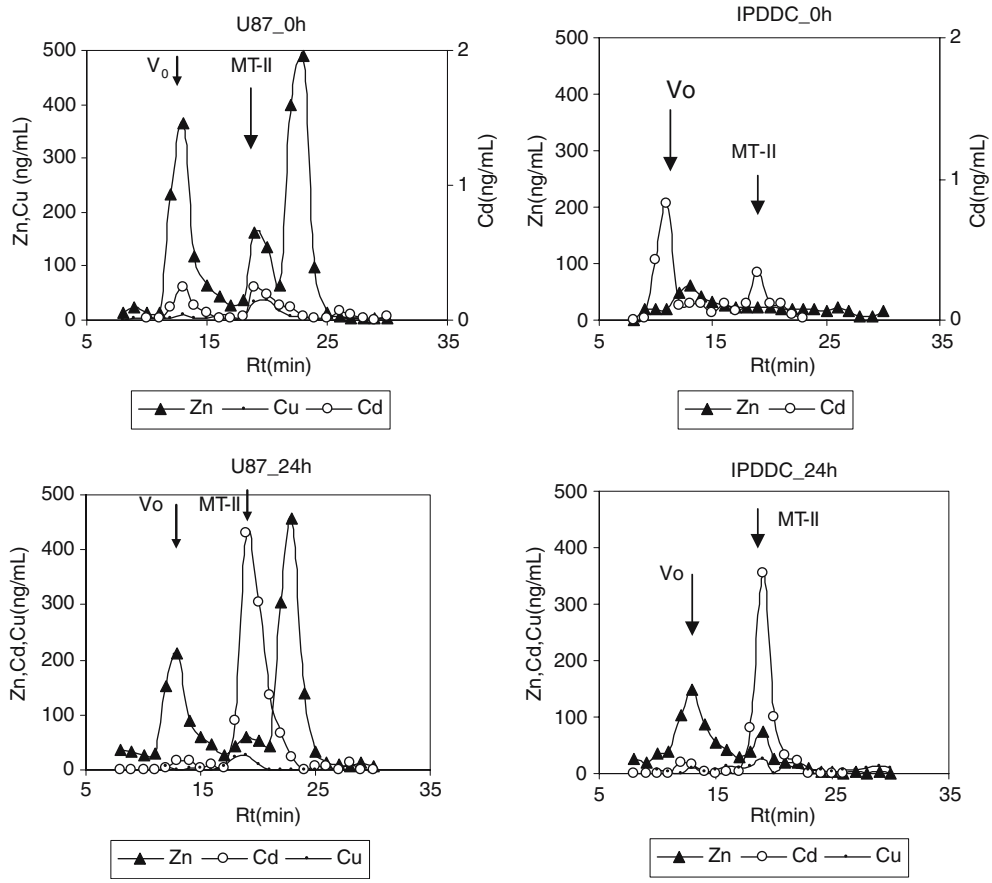

Figure 2. Superdex 75™ chromatograms of U87 MG and IPDDC supernatants (sample 0.5 ml, flow rate 0.75 ml/min, buffer 30 mM Tris–HCl, 1 mM DTT, pH 7.6, 0.75 ml fractions). Cells were exposed to 1 μM of Cd for 0 and 24 hours. Arrows indicate the elution of Blue Dextran (void volume, Vo) and rabbit liver MT-II standard, Sigma. Supernatants were obtained from 10^7 cells suspended in 1.5 ml of buffer.

There was a difference between cell lines regarding alterations in zinc after exposure. The diminution of zinc throughout the whole chromatogram – in MTs, in high molecular weight (HMW) proteins and very low molecular weight (VLW) proteins, in U87 MG proteins cannot be overlooked. The decrease of Zn after exposure (1 μM Cd, 24 hours) was due to the lower Zn concentration (0.145 mg/l) found in the medium in this case only; concentrations in the media found in all other cases were higher (0.220–0.244 mg/l).

Induction of MTs synthesis and the presence of isoform III in U87 MG cells after exposure to Cd

Figure 3 shows the induction of MTs in U87 MG cells after exposure for various times (0, 3, 6, 12, 24 and 48 hours) to 1 μM of cadmium as $CdCl_2$. Cadmium ions present in the soluble cell fraction were mosty bound to MTs. From 3 to 24 hours of exposure changes in concentrations of zinc were seen in the position of the high molecular weight proteins, only. The increase of zinc in the MTs peak at 48 hours of exposure was observed.

To separate isoform III from isoforms I/II, we applied the same sample on two columns connected in series following a slightly modified procedure of Yasutake (Yasutake *et al*. 1998). On the tandem column system MT isoforms I/II eluted at the retention time of 48 minutes (Figure 4). The expected time of elution of MT-III was estimated on the basis of the peak, which eluted before the main MT peak and contained both cadmium and zinc. The position was checked with the ZnMT-III (human, recombinant, ALX-201-172) standard. An internal control was also used – human cerebellum

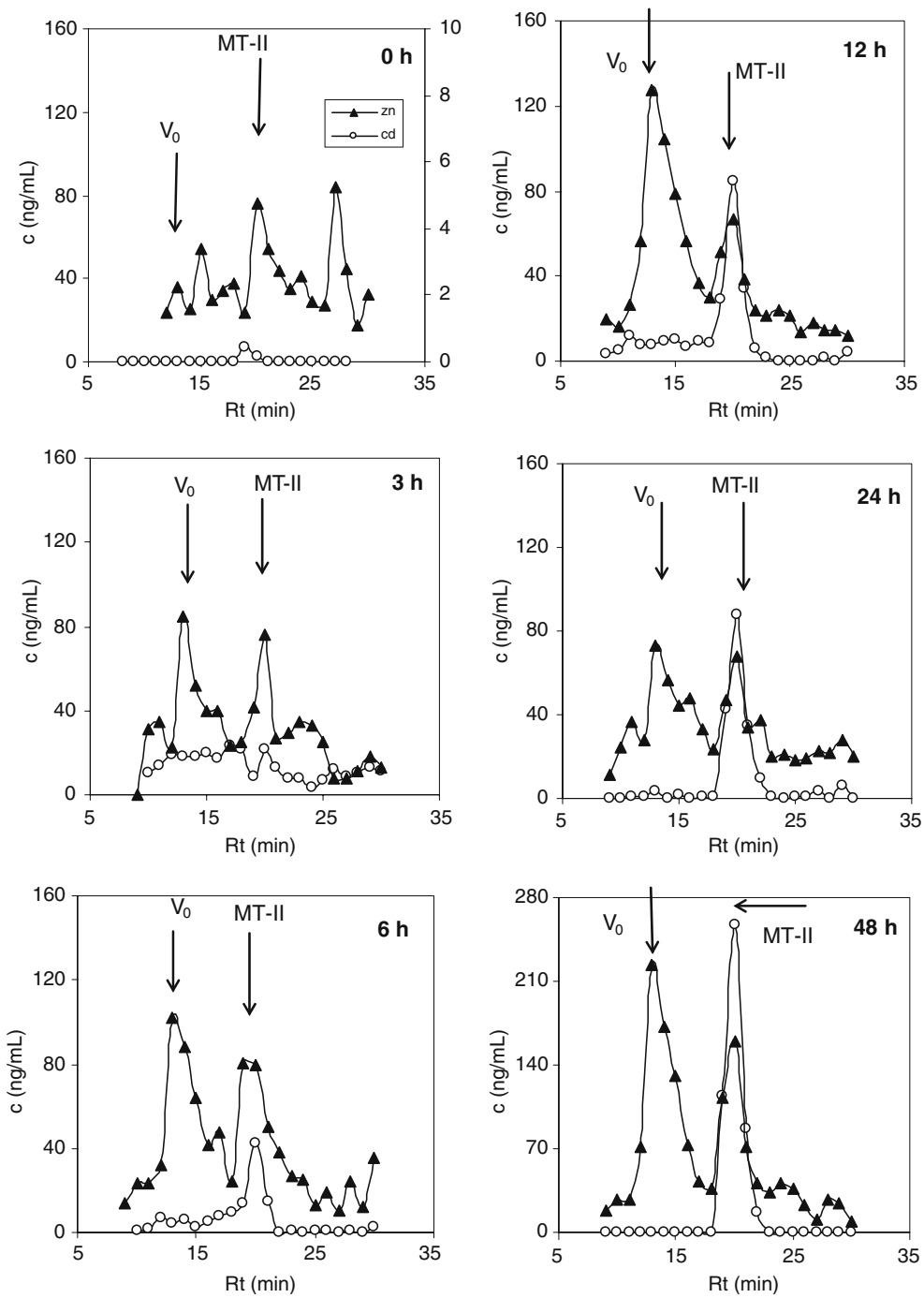

Figure 3. Superdex 75™ chromatograms of U87 MG (sample 0.5 ml, flow rate 0.75 ml/min, buffer 30 mM Tris–HCl, 1 mM DTT, pH 7.6; 0.75 ml fractions). Cells were exposed to 1 μM of $CdCl_2$ for 0, 3, 6, 12, 24 and 48 hours. Arrows indicate the positions of Blue Dextran elution (void volume, V_0) and rabbit liver MT-II standard, Sigma. Supernatants were obtained from 10^6 cells suspended in 2 ml of buffer.

homogenate containing high levels of MT-III (data not shown). The level of constitutive MT-III was very low, but slightly increased level of Zn could be seen after three hours of exposure. After six hours of exposure this level returned to control value and remained so up to 48 hours of exposure.

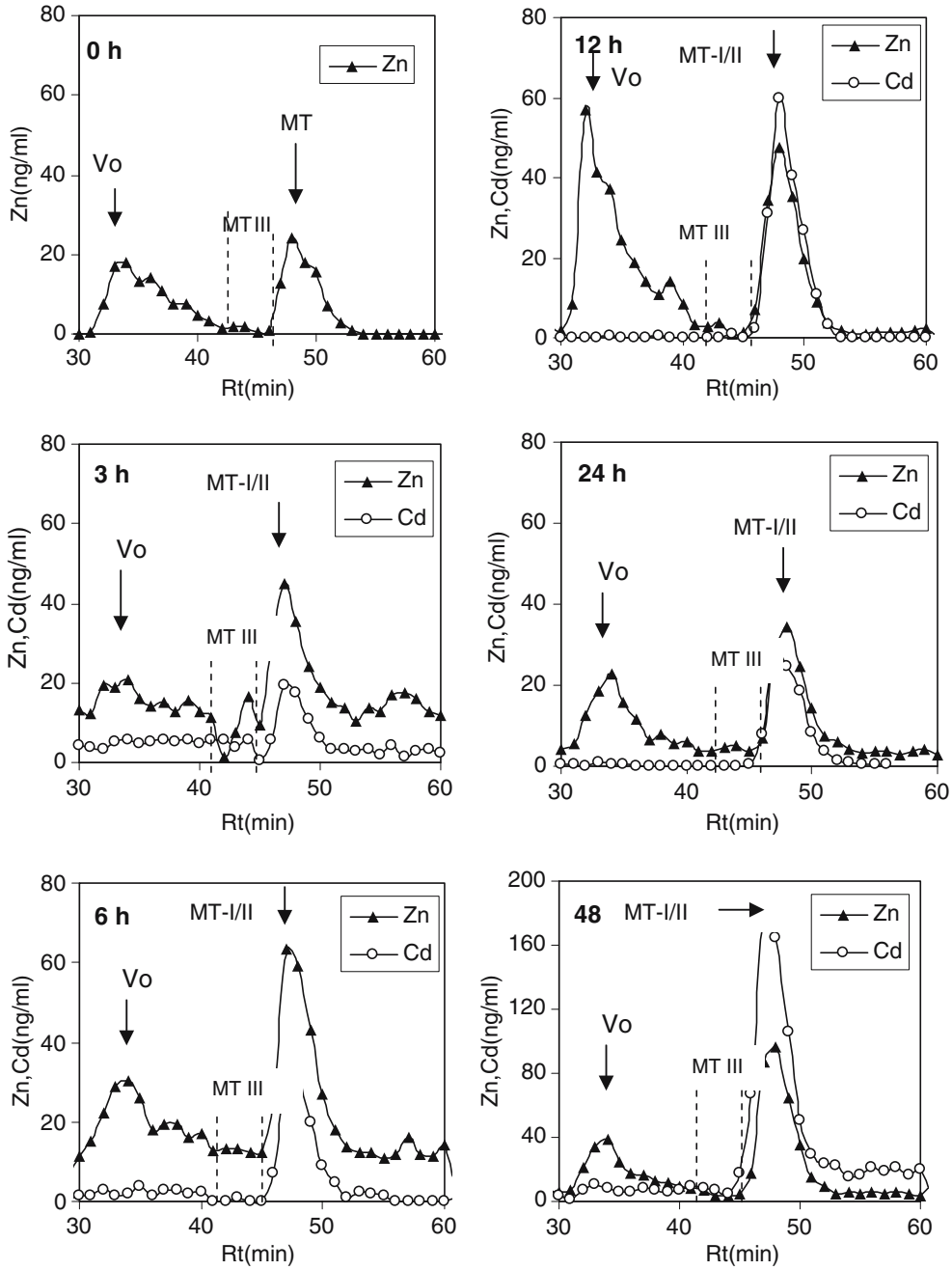

Figure 4. Chromatograms of U87 MG supernatants on a tandem column system (2× Superdex 75™, sample 0.5 ml, flow rate 0.5 ml/min, buffer 30 mM Tris–HCl, 1 mM DTT, pH 8.0; 0.5 ml fractions). Cells were exposed to 1 μM $CdCl_2$ for 0, 3, 6, 12, 24 and 48 hours. Arrows indicate the positions of Blue Dextran elution (void volume, V_0) and rabbit liver MT-I/II standard, Sigma; the dotted lines between Rt 42–45 minutes indicate the elution position of MT-III.

Under conditions of exposure to a higher cadmium concentration (10 μM) MT isoforms I/II increased drastically as CdZnMTs, but the level of isoform III stayed unchanged (Figure 5). A shift of the MT-III peak to the left after 48 hours of exposure is most probably the consequence of different amounts of MTs and HMW proteins in the sample.

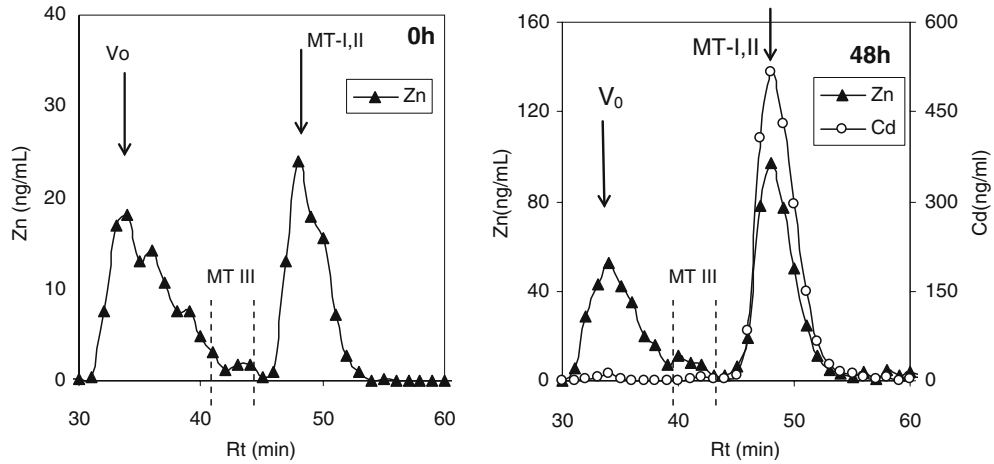

Figure 5. Chromatograms of U87 MG supernatants on a tandem column system (2× Superdex 75™). Cells were exposed to 10 μM $CdCl_2$. Arrows indicate the positions of Blue Dextran elution (void volume, V_0) and rabbit liver MT-I/II standard, Sigma; the dotted lines between Rt 42–45 minutes indicate the elution position of MT-III.

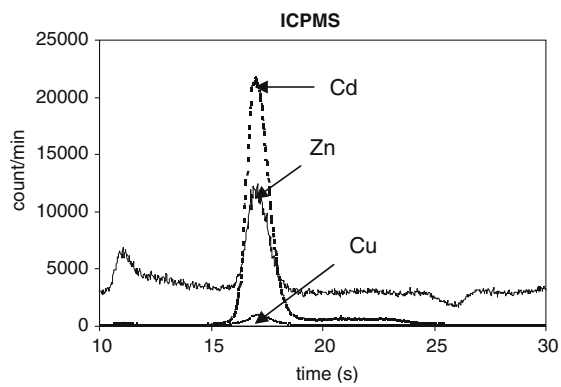

Figure 6. HPLC/ICP-MS chromatogram of U87 MG supernatant. Cells were exposed to 10 μM $CdCl_2$ for 48 hours. Arrows indicate the positions of ^{111}Cu, ^{66}Zn and ^{65}Cd MT peaks.

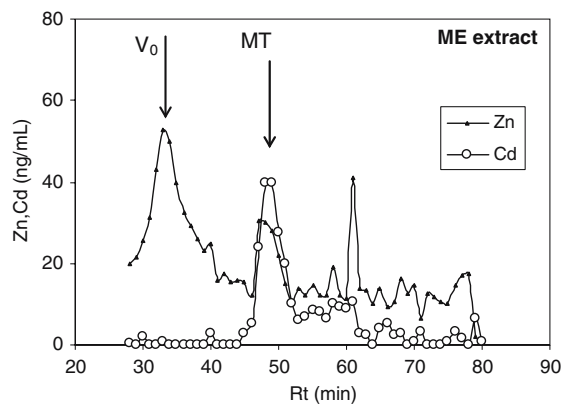

Figure 7.Chromatogram of U87 MG mercapthoethanol extract on a tandem column system (2× Superdex 75™, sample 0.5 ml, flow rate 0.5 ml/min, buffer 30 mM Tris–HCl, 1 mM DTT, pH 8.0, 0.5 ml fractions). Cells were exposed to 10 μM $CdCl_2$ for 48 hours. Arrows indicate the positions of elution of Blue Dextran (void volume, V_0) and rabbit liver MT-I/II standard, Sigma.

Figure 6 shows metal elution profiles of the supernatant of U87 MG cells exposed 10 μM $CdCl_2$ for 48 hours separated by size-exclusion HPLC and connected with ICP MS. Elution of ^{66}Zn and ^{65}Cd were detected at the same position, but here we also detected the presence of ^{111}Cu, although in much lower level. So it seems that Cd induced MTs are mainly occupied by Cd and Zn.

The presence of insoluble MTs was evaluated in the pellet of U87 MG cells treated with the toxic cadmium concentration (10 μM) for 48 hours. The mercaptoethanol extract of this pellet was applied to the tandem column system. Figure 7 clearly demonstrates the presence of MT isoforms in pellets.

Discussion

Regulation of divalent metal ions in astroglia cells is rather complex and depends on many factors. Among others, the availability of zinc ions and the concentrations of other divalent metals like cadmium are of great importance. Astrocytes, which support neurons must maintain the cellular zinc concentration within a narrow range, because low basal zinc levels inhibit cell growth and division, while high zinc levels are toxic (Tapiero & Tew 2003). MT-I/II

are known to regulate Zn homeostasis and participate in protective mechanisms against non-essential transition metal ions (Cd, Hg) toxicity. So beside involvement in astrocytes Zn metabolism, MT-I/II can act as a metal trap for non-essential metals and at the same time as a protective barrier against metal toxicity to neurons.

In this study we used the two glioma cell lines U87 MG and IPDDC-2A as models to follow the capability of two different astrocytomas to synthesis MTs and bind cadmium after cadmium exposure. We clearly showed that cadmium (1 μM, 24 hours) can induce the synthesis of MTs in both examined cell lines (Figure 2; the number of cells was between 10^6 and 10^7). The observed induction and Cd binding confirms that astroglia is able to regulate and also chelate cadmium. This ability of both cell lines is highly significant in relation to resistance to anticancer agents, distinctly to cisplatin (Bredel & Zentner 2002).

MTs are able to interfere with the growth and proliferation of most carcinomas and can contribute to the resistance of cells to variety anticancer drugs (Bredel 2001; Miles *et al.* 2000). Sometimes their involvement is highly dependent on tumour grade (Cherian *et al.* 2003; Hiura *et al.* 1998; Bredel & Zentner 2002; Sens *et al.* 2000). Tumour grades represent the degree of abnormality of cancer cells, and are a measure of differentiation; the highest grade (IV) is usually connected with the lowest differentiation and generally also the most aggressive behaviour. The cell lines we examined were of grade II and IV: IPDDC-2A and U87 MG, respectively. In U87 MG the basal level of MTs were relatively high and definitely higher than that found in the IPDDC-2A cell line (Figure 2). This is in accordance with the finding of Hiura (Hiura *et al.* 1998) that the low grade astrocytomas (I, II) possess a lower level of MTs in comparison with the high grade (III, IV) astrocytomas. In fact the results of a number of investigators suggest that MTs play an important role in cell proliferation (Miles *et al.* 2000). In our study the concentrations of zinc and cadmium in fractions obtained after SEC confirmed that after exposure to cadmium (1 μM) the synthesis of MTs was highly induced in both cell types, and their binding of water soluble cadmium was almost complete. In IPDDC-2A cells the basal metabolism was obviously very low, as is evident from the low zinc concentrations in the supernatant and pellet (data not shown) and the low zinc peaks throughout the whole chromatogram of the control cells. After Cd exposure the uptake of cadmium was associated with uptake of zinc, which appeared in increased peaks of MTs and HMW proteins.

In view of its observed metabolic vigour the U87 MG cell line was used for further studies. The induction of MT was followed during 2 days of exposure (Cd, 1 μM; 3, 6, 12, 24 and 48 hours) and special attention was paid to the presence of the brain-specific isoform III.

As expected, MTs synthesis was time dependent (Figure 3), but in comparison with the human hepatoma cell line HepG2 checked previously (Fatur *et al.* 2002), the induction process in astrocytoma–glioblastoma U87 MG proceeds slower. While in hepatoma cells the highest Cd and Zn binding on MTs was reached after 12 hours (Fatur *et al.* 2002), in astrocytoma–glioblastoma cells the highest concentrations were not observed before 48 hours. But we cannot conclude that this was a plateau (the maximal concentration). Unfortunately we did not follow the process for long enough to see the fate of the MT peak in the long term. Nevertheless, between 12 and 48 hours the concentration of zinc in MTs and also in the region of the void volume increased significantly. The longer was the exposure, the more Cd was bound on MTs and after 24 hours Cd was bound exclusively on MTs. It can be assumed that at this time the balance between MT synthesis and the influx of Cd was reached. With repetition of exposure to a ten times higher concentration (10 μM Cd, 48 h) it turned out that the capacity for MTs expression was high enough to chelate almost all the Cd in the supernatant even in that case (Figure 5). At the same time, Cd was also deposited in the insoluble part of the cell (pellet, Table 1) and here at least partly in the form of Cd,Zn,Cu-MTs (Figure 7). Regarding the procedure used (addition of mercaptoethanol), it is possible that originally insoluble polymerised MTs were present in the pellet (George 1983).

MT-III isoform was proposed to exert an inhibitory effect on the neuronal cell and to have a very poor inducibility by non-essential metals (Yasutake *et al.* 1998). But it takes an active part in zinc ion homeostasis in astrocytes. Although its metal binding affinity is lower than MT-I/II,

undoubtedly its metal binding capacity is higher (Palumaa *et al*. 2005). Figure 4 shows a very low basal level of MT-III in U87 MG cells (detected as ZnMT-III) which is in accordance with the observations of Amoureux *et al*. (1995). They reported very low MT-III mRNA levels in transformed astrocytes, in normal astrocytes the levels are higher. A slight increase of Zn and traces of Cd in isoform III was detected after three hours of exposure to Cd (1 μM) (Table 1). After 6 hours of exposure these levels of metals returned to their starting values and remained at the same level for up to 48 hours of exposure. Similarly they stayed unchanged after exposure to ten times higher concentrations of Cd (10 μM, 48 hours; Figure 5). The slight increase of Zn (and Cd) concentrations in the MT-III peak after 3 hours of exposure occurred together with the induction of MT-I/II isoforms. Regarding the high metal binding capacity of isoform III (Palumaa *et al*. 2005), which is higher than that of other isoforms, it could be assumed that MT-III picked up the free zinc ions appearing in cytoplasm after Zn substitution by Cd on MT-I/II. MT-III also bound traces of cadmium. Probably some Cd remained unbuffered due to the low amount of isoforms I and II synthesised up to that time. But this happened only transiently, and later the MT-III peak returned to its basic level. This phenomenon supports the idea of Palumaa (Palumaa *et al*. 2005), that MT-III could serve as a buffer for fluctuating concentrations of zinc and for other metals, if the capacity of the other two isoforms is too low. The *in vivo* capability of MT-III for binding exogenous metals (Hg) was recently shown by Satomi (Satomi *et al*. 2005). In MT-I/II null mice exposed to mercury vapour (known to penetrate the blood brain barrier), MT-III incorporated a larger amount of mercury, while in wild type mice the majority of mercury was bound to MT-I/II. So the absence or deficiency of MT-I/II is supposed to give rise to the binding of metal ions to MT-III.

Taken together the results of our study showed that in U87 MG (human astrocytoma–glioblastoma) cells MT isoforms I/II and III are constitutively expressed, although isoform III was found in traces only and should be quantitatively checked in future by recently developed method of Meloni *et al*. (2005). MT-I/II were identified as Zn,CuMT. During cadmium exposure (1 and 10 μM, up to 48 hours) the synthesis of MTs isoforms I/II were induced in a time and concentration dependent manner. Under the conditions examined, glioma cell metallothioneins I/II were capable of immobilising all water soluble cadmium together with increased binding of Zn and without significant changes in copper. MT-III isoform was present at all exposure times, but only in much lower amounts with respect to the prevailing amounts of MT-I/II isoforms. The transient increase of metals bound on isoform III at the beginning of exposure may suggest the possibility of its involvement in Zn (and Cd) metabolism in astrocytomas. So the study of all three isoforms simultaneously is important, because it seems that they actually function in a synchronised way.

The experiment should be repeated with higher Cd concentration, maybe for short time up to 300 μM (27) to find the limit of binding of cadmium. Regarding isoform III the research should be done on primary culture of astrocytes and astrocytomas to compare its metal binding ability in normal and malignant cells. Isoform III should also be checked and quantified by recently developed method (Meloni *et al*. 2005).

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