Acta Chim. Slov. **1998**, *45*(3), pp. 229-237

(Received 25. 8.1998)

MERCURY AND METHALLOTHIONEIN-LIKE PROTEINS IN THE PARTICULATE CELL FRACTION OF HUMAN CEREBELLAR NUCLEUS DENTATUS

I. Falnoga, M. Tušek-Žnidarič, R. Milačič

Jožef Stefan Institute, Jamova 39, 1111 Ljubljana, Slovenia

ABSTRACT

The particulate fraction (pellet) of human cerebellar nucleus dentatus from a retired mercury mine worker with a high mercury and selenium content (2.35 ìg/g, 1.02 ìg/g) was subjected to mild extraction of Hg binding proteins with a buffer containing 10% mercaptoethanol (ME). The amount of mercury solubilised in this procedure was very low, about 3% of the pellet Hg content. Nevertheless, in the ME extract the presence of metallothionein-like proteins (MT-LP) were found by Sephadex G-75 gel chromatography and subsequent metal analysis. They were detected as Cu, Zn, Pb, Hg MT-LP, where the binding of Cu was much the highest and Hg was present only in traces. These data indicate that in accordance with literature discussions MT in nucleus dentatus could be involved in Cu metabolism and in short term heavy metal detoxification (Pb, Hg).

INTRODUCTION

In a wider investigation of mercury (Hg) accumulation and retention in autopsy samples from Idrija mercury mine employees it was found that in certain samples of retired workers, namely thyroid, hypophysis, cerebellar tissue - nucleus dentatus and kidney cortex, almost all the retained mercury and selenium were located in the cell particulate fraction (pellet) [1,2]. Similar results have been reported for kidney tissue of a deceased dentist by Björkman et al [3].

In the cell particulate fractions mercury was accompanied by selenium in a molar ratio near to one. In the literature it is supposed that both elements can be present as insoluble Hg selenide and/or sulphide polymers [4], or as insoluble metal-protein complexes (maybe Hg-Se as a part of metallothionein or its degradation products) [5], mostly localised in lysosomes.

Metallothioneins (MTs) are a group of inducible low-molecular-weight, cystein-rich, metal binding cytosol proteins which can represent a part of the cell´s defence mechanism against metal toxicity, oxidative stress and inflammation [6,7]. Among many other functions they can serve as efficient buffers for toxic concentrations of essential and nonessential metal ions entering the system. Although metallothionein (MT) is a cytoplasmic protein, it can also accumulate in lysosomes and in some cases it has been observed in the cell nucleus [6,7,8,9]. Interesting data were found particularly with copper MT [7,8,9]. As liver copper concentrations increase, the metal often accumulates in aggregated (polymeric, granular) forms of copper MT which occur in the particulate fraction (including lysosomes) of copper- loaded liver tissue. These aggregated forms of copper can be solubilised under alkaline or reducing conditions [10].

Concerning these observations and the thesis of Suzuki et al [11] that "MT may be involved in the storage, transport, excretion, homeostasis and detoxification of heavy metals in the central nervous system", especially in nucleus dentatus and some other brain areas, we were interested in the mercury retained in the nucleus dentatus from a retired mercury worker, principally in the presence of mercury metallothionein (MT).

The aim of the present study was to extract Hg-proteins from the particulate cell fraction the way done in studies with Cu [12], and to partially characterise the metallothionein-like proteins with Hg, Cu and Zn affinity.

MATERIALS AND METHODS

Autopsy sample

The autopsy sample was obtained from the brain cerebellum (nucleus dentatus) of a retired Idrija mercury mine employee. The time between death and autopsy was not

longer than 48 hours. The subject was aged 64 years, exposed to elemental mercury vapour for 18.5 years and then retired for 16.3 years. The cause of death was heart attack. The sample was immediately frozen, and kept below - 18^{o}C until use.

Preparation of Particulate Fraction, Mercury Extraction and Sephadex G-75 Chromatography

The nucleus dentatus obtained was used for separation of pellet (particulate fraction) and supernatant. The pellet was subjected to mild Hg protein extraction with a buffer containing 10% mercaptoethanol (ME), and the MT- like proteins isolated by gel chromatography.

Tissue homogenate was prepared in nitrogen-saturated 10 mM Tris HCl buffer (pH 7.6, 4^{o}C) containing 1mM dithiothreitol (DTT), strained through a 250-nm nylon net and ultracentrifuged for 1h at 100 000g in an Centrikon T-2070 (Kontron instruments) ultracentrifuge (rotor TFT 70.38). The supernatant was removed. The pellet (nuclei, mitochondria, lysosomes, microsomes) was suspended in 10 mM Tris HCl buffer (pH 7.6) containing 10% mercaptoethanol (ME), frozen and thawed three times to aid disruption of membranes and left overnight at 4^{o}C. The mixture was then ultracentrifuged in the same way as cell homogenate to obtain an ME extract and sediment of the particulate fraction (modified procedure of Riordan and Richards) [12]. Aliquots of cell fractions were used for metal analysis.

The ME extract of the particulate fraction (2.5 mL) was applied to a calibrated 1.6 x 60 cm Sephadex G-75 column, and eluted in a nitrogen atmosphere at 4^{o}C with buffer (10 mM Tris HCl, pH 7.6). The UV absorption at 280 and 254 nm and the concentrations of metals (Hg, Cu, Zn, Pb) were determined in the column eluents.

The column was standardized with marker proteins of known molecular weight (MW) (Pharmacia, Serva): blue dextran (2 000 000), bovine serum albumin (MW 67 000), ovalbumin (MW 44 000), chymotrypsinogen A (MW 25 000) myoglobin (MW 17 800), and cytochrome c (MW 12 400). Blue dextran was used to determine the void volume of the column. The standard marker proteins were eluted with 10 mM Tris HCl (pH 7.6), and the elution was monitored at 280 nm.

Metal Determination

Several methods were used for quantitative determination of total Hg, Zn, Cu, Cd and Pb in the biological samples analysed in this study: radiochemical neutron-activation analysis (RNAA), flame atomic absorption spectroscopy (flame AAS) and electrothermal atomic absorption spectroscopy (ET AAS). The analytical methods used for determination of particular elements in different samples are summarized in Table 1.

Table 1. Samples, Elements and Methods

Sample	Element	Method
Nucleus dentatus	Hg, Se Cu, Zn	RNAA [13] RNAA [14]
100 000 g supernatant	Hg, Se Cu, Zn	RNAA [13] RNAA [14]
Pellet	Hg, Se	RNA [13]
Pellet extract	Hg, Se	RNAA [13]
Fractions from the Gel- filtration column	Hg Zn Cu Pb	RNAA [13] flame AAS RNAA [14] ET AAS

RESULTS

The nucleus dentatus sample contained 2.40ìg of Hg and 1.00ìg of Se/g wet weight. The amount of tissue Hg in the 100 000g supernatant (*cytosol)* was less than 1%. The concentration of supernatant Se was under the limit of detection. Almost all Hg and Se, with an atomic ratio near 1 (0.913), were retained in pellet. In view of these results the pellet was selected for mild Hg extraction. Buffer with 10% ME was used to attempt solubilization and extraction of polymerised MT (S-S bond splitting). The extraction of Hg found was very low, about 3% of the pellet Hg content. Tissue separation and extraction data are shown in Table 2.

Using gel chromatography the presence of MT-like proteins was detected in the ME pellet extract as Cu,Zn,Pb,Hg MT-like protein, where the presence of Cu was the

most marked. Fig.1 (A,B and C) depicts the elution profiles of the ME extract of pellet on a calibrated Sephadex G-75 column where the cytochrome c molecular weight range is characteristic of MT. UV absorbances were determined at 280 and 254 nm. The lack of absorbance at 280 (the absence of aromatic amino acids) and a peak at 254 nm (the presence of metallo-mercaptide bonds) in the range of cytochrome c are additional characteristics of MT. The absence of a peak at 254 nm is the consequence of the low concentration of protein. Fig.1 (A,B,C) shows the elution profiles of mercury, lead, and the essential elements Zn and Cu. The distribution of the measured elements is mutually unlike, but in the molecular range of cytochrome c the peaks of all elements are present, although their concentrations found in this range are different in the order: Cu >Zn > Pb >Hg.

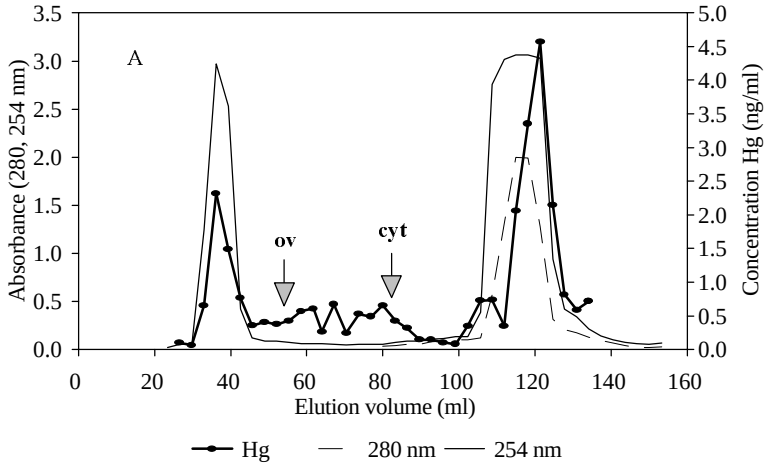
The major Hg and Zn peaks correspond to very low MW substances (< 3000), Fig.1 (A, B). The strong UV absorbance of ME (at 254 nm) can probably account for the absorbance peak detected in the same region [15]. In the study of Roesiajadi and Drum [15] related to ME buffer and mercury-binding proteins in Mytilus gills, they showed that ME can shift mercury from mercury binding proteins to low molecular weight substances. The same conclusion could also be valid in our case. It is possible that ME removed part of the bound Hg and Zn from MT-like proteins and shifted both elements to substances with very low MW (Mr < 3000, i.e. the last peaks on the chromatogram, Fig. 1 A,C). The same phenomenon is not obvious for Cu. MTs bind Cu atoms cooperatively and tenaciously [16].

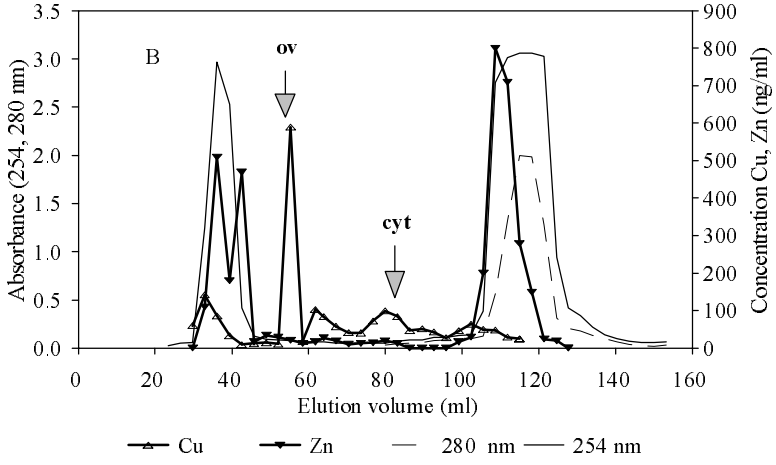
Table 2: Element concentrations in tissue and cell fractions from nucleus dentatus of retired mercury miner.[1]

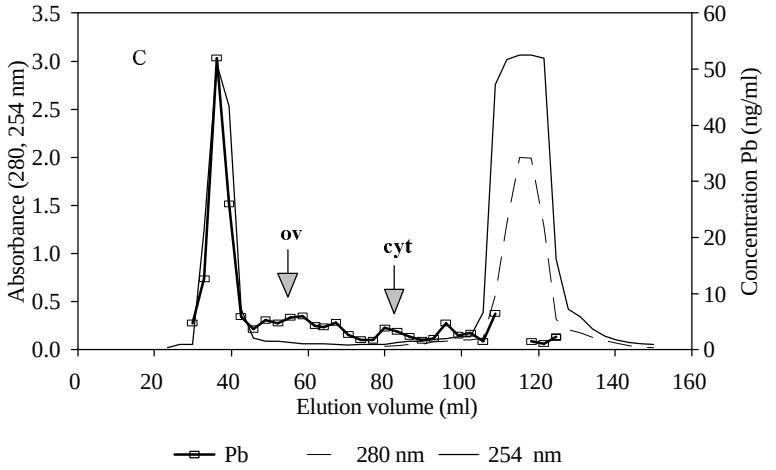
NUCLEUS DENTATUS	Hg ìg/g	Hg %	Se ìg/g	Cu ìg/	Zn ìg/
Tissue (2g)	2.36	100	1.02	6.33	4.45
100 000 g supernatant (11g)	0.003	~0.7	<0.01	0.44	0.46
Pellet (1.95g, 1.72g*)	2.35	100	1.09	-	-
100 000 g pellet ME extract (5.5g)	0.034	~3	0.034	-	-

1 - separation data are described in materials and methods, - - no data,
* - mass of the pellet used for extraction

Fig. 1 **l extract of the pellet**
The M and eluted with
10 mn for Hg (A), Cu
and Zı ı. The positions
of cyt







DISCUSSION

Our investigation revealed that buffer (10 mM Tris HCl) containing 10% mercaptoethanol (ME) dissolved only a small proportion of retained Hg and Se from nucleus dentatus pellet, about 3% and 7%. We found a similar result for a thyroid pellet sample from a retired mercury mine worker where the buffer contained 1% SDS (sodium dodecyl sulphate) (our unpublished data). These low extraction results are in concordance with another extraction study of the kidney of a deceased dentist where a buffer (10 mm Tris HCl) containing a mixture of two reagents, 5 ìmol/ml ME and 1% SDS was used [3]. But considering the Hg extraction efficiencies of different buffers used for dolphin liver samples [17] it would be sensible to repeat the procedure with 20 mm ammonium acetate containing 4% SDS. In any case it is evident that in tissues from subjects with former exposure to Hg the deposits of Hg and Se are almost insoluble by mild extraction procedures. Regarding these data the suggestion that both elements can be present as insoluble Hg selenide polymers [4] seems more convincing than storage in aggregated metallothioneins [5].

In the ME pellet extract MT-like proteins were identified as Cu,Zn,Pb,Hg MT-LP. The binding of copper was outstanding and Hg was present only in traces, as seen from Fig. 1 (A,B,C). In view of literature data about liver granular Cu MT [8,9,10]and a study of granular forms of MTs accumulated in astrocytoma cells in the presence of interleukin-1 and heavy metals [18], we suppose that the nucleus dentatus cell pellet contained MT aggregates, which were dissolved by mercaptoethanol (S-S bond cleavage). As ME can act both as a reductant and a chelating agent, it is possible that the height of the Hg and Zn MT peaks might be diminished (discussed in Results).

Despite some procedural uncertainties connected with the effects of mercaptoethanol on Hg and Zn, it is significant that this is the first observation of nucleus dentatus MT-like proteins by gel-filtration chromatography and subsequent metal analysis of the MT fractions. Until now they were noticed and localized only by immunohistochemical techniques in nucleus dentatus of *macaca fascicularis* [18], and in nucleus dentatus of human brain [20, 11, 21] in the nuclei, cytoplasm and vascular feet

of astrocytes. In brain three MT isoforms (MT-I, MT-II, MT-III) exist, but in astrocytes MT-I and MT-II appear to be abundant and MT-III conspicuously absent [22]. In recent discussions about MT-I and MT-II isoforms within astrocytes, both forms have been associated with compartmentalization of zinc, and other metals (Cu) between neurons and astrocytes, regulation of metal intracellular concentrations and with tolerance to heavy metal and free radical neurotoxicity [22, 23, 24]. The data from our investigation indicate that MT in nucleus dentatus could be involved in Cu, Zn metabolism and in short term heavy metal detoxification (Pb, Hg) (Fig.1). Their involvement in the long term storage of Hg seems very questionable.

Undoubtedly it should be pointed out that it is becoming more difficult to operate with only a partial characterisation of Cu MTs, as recently (1997) a new family of Cu proteins has been discovered. Within the cell, Cu appears to be distributed among Cu-requiring proteins (enzymes) through the use of so-called Cu chaperons [16]. Until now in humans chaperons have been identified as cytosolic soluble copper binding low molecular weight proteins with cysteine residues (HAH 1 with 68 amino acids, COX 17 with 63 amino acids) [16, 25]. It could be said that MTs are important for Cu sequestration in a nonexchangeable form and chaperons are involved in intracellular Cu distribution [16]. As the connection of chaperons with Cu storage or aggregation in cellular organelles is not known, we suppose that their discovery does not affect the interpretation of results made in our investigation.

ACKNOWLEDGMENTS

We thank to Dr. Josip Sabolić from the Department of Pathology of the Jože Goričar Hospital (Nova Gorica) and Mrs. Vesna Lupšina-Miklavčič M.Sc., from the Idrija Mercury Mine for their generous assistance in providing the autopsy sample and the personal data of the case examined. The work was supported by the Slovenian Ministry of Science and Technology.

REFERENCES

[1] I. Falnoga, I. Kregar, P. Stegnar, and M. Tušek-Znidarič, in: P.W.F. Fischer, M.R. L´Abbé, K.A. Cockell, and R.S. Gibbson, *Trace Elements in Man and Animals - 9. Proccedings of The Ninth International Symposium on Zrace Elements in Man and Animals;* NRC Ressearch Press, Ottawa,

Canada, 1997, pp 489-490.
[2] I. Falnoga, *Absorption, distribution and elimination of mercury in experimental animals exposed to mercury vapour*, *Ph.D. Thesis*, University of Ljubljana, 1995 (in Slovene).
[3] L. Björkman, B. Palm, M. Nylander, and M. Nordberg, *Biol. Trace Element Res.*, **1994**, 40, 255-265.
[4] G. Dancher, and B. Mřller-Madsen, *J. Histochem. Cytochem.*, **1985**, 33, 219-228.
[5] T. Aoi, T. Higuchi, R. Kidokoro, R. Fukumura, A.Yagi, S. Ohguchi, A. Sasi, H. Hayashi, N. Sakamoto, and T. Hanaichi, *Human Toxicol.*, **1985**, 4, 637-642.
[6] J. H. R Kägi, and A. Schäffer, *Biochemistry*, **1988**, 27, 8509-8515.
[7] M. G. Cherian, and H. M. Chan, in K.T. Suzuki, N. Imura, and M. Kimura, *Metallothionein III*; Birkhäuser Verag, Basel-Boston-Berlin, 1993, pp 87-109.
[8] I. Bremner, in J.F. Riordan, and B. L. Vallee, *Methods in Enzimology*; Academic Press, Inc., pp. 1991, vol. 205, pp 584-599.
[9] I. Bremner, in K.T. Suzuki, N. Imura, and M. Kimura, *Metallothionein III*; Birkhäuser Verag, Basel-Boston-Berlin, 1993, pp 112-114.
[10] I. Bremner, in M. Anke, D. Meissner, and C. F. Mills, *Trace Elements in Man and Animals - TEMA 8*, 1993, pp 507-515.
[11] K. Suzuki, K. Nakajima, N. Otaki, M. Kimura, U. Kawaharada, K. Uehara, F. Hara, Y. Nakazato, and M. Takatama, *Path. Int.*, **1994**, 44, 20-26.
[12] J.R. Riordan, and V. Richards, *J. Biol. Chem.*, **1980**, 255, 5380-5383.
[13] A.R. Byrne, and L. Kosta, *Talanta*, **1974**, 21, 1083-1092.
[14] M. Dermelj, A. Vakselj, V. Ravnik, and B. Smodiš, *Radiochem. Radioanal. Lett.*, **1979**, 41, 149-160.
[15] G. Roesijadi, and A.S. Drum, *Comp. Biochem. Physiol.*, **1982**, 71B, 455- 459.
[16] K.A. Koch, Ma O. Pena, and D.J. Thiele, *Chemistry & Biology*, **1997**, 4, 549-560.
[17] Y. Shibata, M. Morita, and K. Fuwa, *Adv. Biophys.*, **1992**, 28, 31-80.
[18] Y. Kikuchi, M. Irie, T. Kasahara, J. Sawada, and T. Terao, *FEBS Lett.*, **1993**, 317, 22-26.
[19] K. Suzuki, K. Nakajima, U. Kawaharada, F. Hara, K. Uehara, N. Otaki, M. Kimura, and Y.Tamura, *Acta Histochem. Cytochem.*, **1992**, 25, 609-616.
[20] H.G.T. Blaauwgeers, P.A.E. Sillevis Smitt, J.M.B.V. De Jong, and D. Troost, *Glia*, **1993**, *8*, 62-70.
[21] K. Nakajima, and K. Suzuki, *Neurochem. Int.*, **1995**, 27, 73-87.
[22] M. Aschner, *FASEB J.*, 1996, 10, 1129-1136.
[23] M. Aschner, *NeuroToxicology*, **1996**, 17, 93-106.
[24] J.W. Neal, S.K Singhrao, B. Jasani, and G.R. Newman, *Neuropathol. Appl. Neurobiol.*, **1996,** 22, 243-247.
[25] I.H. Hung, R.L.B. Casareno, G. Labesse, F.S. Mathews, and J.D. Gitlin, *J. Biol. Chem.*, **1998**, 273, 1749-1754.

POVZETEK

Iz avtoptičnega vzorca nukleusa dentatusa malih možgan upokojenega rudarja smo pripravili partikularno celično frakcijo. Vsebovala je visoko vsebnost živega srebra in selena (2.35 ìg/g, 1.02 ìg/g). Z milo ekstrakcijo (pufer z 10% merkaptoetanolom/ME) smo poskusili raztopiti beljakovine z vezanim živim srebrom. Količina tako raztopljenega Hg je bila zelo nizka, okoli 3% vsebnosti Hg v partikularni frakciji. V ekstraktu smo s pomočjo gelske filtracije na Sephadex G-75 koloni in določitve kovin v eluatu identificirali metalotioninom podobne beljakovine (MT-PB). Zaznali smo jih kot Cu,Zn,Pb,Hg MT-LP, kjer je bila vezava bakra najbolj izstopajoča, živo srebro pa je bilo prisotno le v sledovih. Glede na to in na literaturne podatke previdevamo, da so MT v nukleusu dentatusu lahko vpleteni v metabolizem Cu ter kratkoročno detoksifikacijo težkih kovin (Hg, Pb).