ACTA ABP BIOCHIMICA POLONICA

Vol. 57, No 4/2010
561–566
on-line at: www.actabp.pl

Regular paper

Serum metallothionein in newly diagnosed patients with childhood solid tumours

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Tumour markers are substances produced by malignant cells or by the organism as a response to cancer development. Determination of their levels can, therefore, be used to monitor the risk, presence and prognosis of a cancer disease or to monitor the therapeutic response or early detection of residual disease. Time-consuming imaging methods, examination of cerebrospinal fluid or tumour tissue and assays for hormones and tumour markers have been used for cancer diagnosis. However, no specific marker for diagnosis of childhood solid tumours has been discovered yet. In this study, metallothionein (MT) was evaluated as a prospective marker for such diseases. Serum metallothionein levels of patients with childhood solid tumours were determined using differential pulse voltammetry — Brdicka reaction. A more than 5-fold increase in the amount of metallothionein was found in sera of patients suffering from cancer disease, compared with those in sera of healthy donors. The average metallothionein level in the sera of healthy volunteers was 0.5 ± 0.2 μmol·dm^{-3} and was significantly different (P<0.05, determined using the Schefe test) from the average MT level found in serum samples of patients suffering from childhood solid tumours (3.4 ± 0.8 μmol·dm^{-3}). Results found in this work indicate that the MT level in blood serum can be considered as a promising marker for diagnostics, prognosis and estimation of therapy efficiency of childhood tumours.

Keywords: metallothionein, cancer, serum, Brdicka reaction, marker

Received: 19 June, 2010; revised: 31 August, 2010; accepted: 03 November, 2010; available on-line: 09 November, 2010

INTRODUCTION

According to World Health Organization statistics, tumour diseases are a leading cause of death worldwide. These diseases accounted for 7.9 million deaths (around 13% of all deaths) in 2007. Lung, stomach, liver, colon and breast carcinomas cause the most cancer deaths each year, however, the most frequent types of cancer differ between men and women (Anonymous, 2008). In the Czech Republic, there live more than 300 000 persons with a tumour disease (Anonymous, 2009). More than one hundred of new reported cases of childhood solid tumours are reported per 1 000 000 children per year in the Czech Republic. Such new diagnoses represent less than 1% of the total of newly diagnosed patients with tumour diseases. Nevertheless, malignancies are the second most frequent cause of death in childhood, after accidents (Anonymous, 2009). Childhood solid tumours are biologically aggressive diseases with a high growth potential expressed as mitotic activity. Their high biological activity resulting in rapid growth leads to diagnoses being made at later stages of the disease.

Based on the great progress in the understanding of the biochemical and molecular biology pathways related to the cell cycle, signalling, apoptosis and others, on the development of various strategies for diagnosis and treatment of tumour diseases, and on screening programmes and many other efforts, the life expectancy of cancer patients has been considerably prolonged and its quality improved. However, early diagnosis is still a crucial issue, because the sooner a tumour disease is detected, the better are the chances to treat it successfully. Several different approaches to tumour disease diagnosis have been developed including those aiming at detection of tumour markers. The exact definition of this term is rather complicated, but a tumour marker can be defined as a compound, whose level in the blood, urine or tissue changes due to carcinogenetic processes. Detection of a tumour marker not only allows early diagnosis but also enables monitoring the effect of a treatment in a patient. An ideal tumour disease marker should fulfil the following requirements: easy detection, availability for whole population without sex or age limitations, high dependence on stage of disease, and presence in a body liquid or a tissue attacked by the tumour disease to specifically distinguish between potential cancer patients and those with non-malignant diseases or healthy individuals. A wide spectrum of tumour markers varying in their specificity and selectivity are known, however, new molecules serving as markers are still needed to allow earlier detection of tumour diseases (Sawyers, 2008). Unfortunately, numerous tumour

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Abbreviations: ACS, American Chemical Society labelling (chemicals meet the specifications of the American Chemical Society); BSA, bovine serum albumin; CA 15-3, cancer antigen 15-3; CNS, central nervous system; CRF-CEM, human T cell lymphoblast-like cell line; ELISA, enzyme-linked immunosorbent assay; HMDE, hanging mercury drop electrode; IGF-2, insulin-like growth factor 2; MT, metallothionein; PBS, phosphate-buffered saline; PSA, prostate-specific antigen; SDS/PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor.

markers exhibit an enhanced level only in later stages of the disease. For example, in breast carcinomas, increased levels of cancer antigen 15-3 (CA 15-3) are found in 9% of breast carcinoma patients in stage I, in 19% in stage II, in 38% in stage III and in 75% in stage IV (Safi *et al.*, 1991). This makes the use of this compound as a screening diagnostic marker problematic. Levels of α-fetoprotein, β-subunit of choriogonadotropin and placental alkaline phosphatase have been found suitable for diagnosis of some tumours. Even though no specific marker has been discovered in most childhood solid tumours yet (Churackova, 2008), metallothionein (MT) might be a promising tumour marker (Jin *et al.*, 2004; Eckschlager *et al.*, 2009; Pedersen *et al.*, 2009; McGee *et al.*, 2010; Shi *et al.*, 2010). MT are low molecular mass intracellular proteins rich in cysteine, which are able to bind heavy metals. Based on their high affinity for metal ions, homeostasis of heavy metal ions is probably of the most important biological function of MT (Fig. 1). MT can also serve as "maintainers" of the redox pool of a cell. In mammals, MT has been found to be involved in apoptosis, immunomodulation, regulation of transcription, cell proliferation and activation of enzymes *via* delivery of zinc (II) atoms to proteins (Franklin *et al.*, 2007; Kimura *et al.*, 2008; Li *et al.*, 2008; Krizkova *et al.*, 2009c). Four major metallothionein isoforms have been identified in mammals: MT-1, MT-2, MT-3 and MT-4. Each isoform is encoded by multiple genes (Simpkins, 2000). MT-1 and MT-2 have ubiquitous distribution in nearly all tissues (Masters *et al.*, 1994), MT-3 is expressed mainly in the brain (Moffatt *et al.*, 1998), and the least is known about MT-4, which was discovered in epithelial cells in 1994 (Quaife *et al.*, 1994). All of the above-mentioned MT functions can substantially contribute to the typical features of cancer cell metabolism: enhanced proliferation and metabolism, and resistance to apoptosis (Krizkova *et al.*, 2009c; Pedersen *et al.*, 2009).

More than 70 years ago, Professor Rudolf Brdicka discovered catalytic evolution of hydrogen from electrolyte in the presence of proteins (Brdicka, 1933; Heyrovsky, 2005). This method was called, after its discoverer, Brdicka filtrate reaction. Brdicka himself used this method for diagnosis of patients with tumour diseases with 100% specificity (Brdicka, 1937a; 1937b; 1938; Heyrovsky, 1938; Kalous, 2004). Since then, electrochemistry has been slowly disappearing from tumour disease diagnostics due to introduction of modern techniques of analytical chemistry and molecular biology. Thus, this unique and interesting technique has not been used with several exceptions for more than fifty years. During the last decade several papers have been published on improving Brdicka reaction, its automation, and revealing the mechanism of the reaction (Raspor, 2001; Petrlova *et al.*, 2006; Krizkova *et al.*, 2009b; Adam *et al.*, 2010). Moreover, the method has been successfully employed for detection of MT in samples from patients with various tumour diseases (Kizek *et al.*, 2001; Petrlova *et al.*, 2006; Adam *et al.*, 2008a; 2010; Fabrik *et al.*, 2008a; Krizkova *et al.*, 2008; 2009a). Nevertheless, the level of MT has not been determined in childhood patients. Thus, the aim of this study was to determine the MT level in the serum of childhood patients with solid tumours by Brdicka reaction and to evaluate the usefulness of using of MT level as a new potential tumour disease marker.

MATERIAL AND METHODS

Chemicals, material and pH measurements. All chemicals of ACS purity used were purchased from Sigma (Sigma-Aldrich, USA), unless noted otherwise. Water underwent demineralization by reverse osmosis using an Aqua Osmotic 02 instrument (Aqua Osmotic, Tisnov, Czech Republic) and then was purified using Millipore RG (Millipore Corp., USA, 18 MΩ) – MiliQ water. pH was measured using a WTW inoLab pH meter (Weilheim, Germany).

Differential pulse voltammetry — Brdicka reaction. Differential pulse voltammetric Brdicka reaction measurements were performed with a 747 VA Stand instrument connected to a 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing, GPES 4.9 supplied by EcoChemie was employed. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s. For measurement the Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of –0.7 V, end potential of –1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude –250 mV, E_{ads} = 0 V, volume of injected sample: 20 μl (100 × diluted sample with 0.1 M phosphate buffer pH 7.0). All experiments were carried out at 4 °C employing a Julabo F25 thermostat (Labortechnik GmbH, Germany) (Adam *et al.*, 2008b; Fabrik *et al.*, 2008b).

Human blood serum. Blood samples were obtained from 38 children hospitalized at the Department of Paediatric Haematology and Oncology of Faculty Hospital Motol with newly diagnosed solid tumours (medulloblastoma (n = 10), neuroblastoma (n = 12), osteosarcoma (n = 8), Ewing sarcoma (n = 4) and ependymoma (n = 4); average age 7.3 years). The blood samples were collected before chemo- and radiotherapy. Samples from healthy volunteers (n = 58, average age 27.3 years) were obtained from the Institute of Sports Medicine (Brno, Czech Republic). The samples were primarily intended for routine biochemical tests at the Department of Clinical Biochemistry and Pathobiochemistry, Faculty Hospital Motol. Serum was separated by centrifugation at 4000 × *g* for 10 min. For further investigations only sera not used for routine biochemical tests were used. The samples were stored at –80 °C until assayed.

Preparation of serum samples. The samples were kept at 99 °C in a thermomixer (Eppendorf 5430, Germany) for 15 min with shaking in order to remove ballast proteins and peptides which could influence the electrochemical response. The denatured homogenates were centrifuged at 4 °C, 15000 × *g* for 30 min (Eppendorf 5402, Germany).

SDS/PAGE. The electrophoresis was performed according to Laemmli (1970) using a Mini Protean Tetra apparatus with gel dimension of 8.3 × 7.3 cm (Bio-Rad, USA). Firstly, we poured 15% (m/v) running gel and 5% (w/v) stacking gel. The gels were prepared from 30% (w/v) acrylamide stock solution with 1% (w/v) bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 min or 30 min, respectively. Prior to analysis the samples were mixed with reducing (7.5% β-mercaptoethanol) sample buffer in 2:1 ratio. The samples were boiled for

2 min, and then the sample (4 µl) was loaded onto a gel. For determination of the molecular mass, the protein ladder "Precision plus protein standards" from Biorad was used. The electrophoresis was run at 150 V for 1 h (Power Basic, Biorad, USA) in Tris/glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 0.0035 M SDS, pH = 8.3). Silver staining of the gels was performed according to Oakley *et al.* (1980).

Western-blotting. After the electrophoretic separation the proteins were transferred onto a PVDF membrane (Bio-Rad, USA) in a Biometra Fastblot apparatus (Biometra, Germany). PVDF membranes were activated by soaking in methanol for 30 s prior to blotting. Further, the membrane was equilibrated for 5 min in blotting buffer (12.5 mM Tris-base, 75 mM glycine and 15% (v/v) methanol). The blotting was carried out for 1 h at a constant current of 0.9 mA for 1 cm^2 of the membrane. After the transfer, the membrane was blocked in 1% BSA in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4) for 30 min. The incubation with chicken primary antibody in dilution of 1:500 in PBS with 0.1% of BSA was carried out for 12 h at 4°C. After three washing with PBS containing 0.05% (v/v) Tween-20 (PBS-T) for 5 min the membrane was incubated with secondary antibody (rabbit anti-chicken labelled with horseradish peroxidase, Sigma-Aldrich, diluted 1:5000) for 1 h at room temp. Then, the membrane was washed three times with PBS-T for 5 min and incubated with chromogenic substrate (0.4 mg ml^{-1} AEC — 3-aminoethyl-9-carbazole in 0.5 M acetate buffer with 0.1% H_2O_2, pH 5.5). After adequate development the reaction was stopped by rinsing with water.

Statistics. Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Data are expressed as mean ± standard deviation (S.D.) unless otherwise noted (EXCEL®). Statistical significance of the measured data was determined using STATISTICA.CZ. Differences with $P < 0.05$ were considered significant and were determined by using one way ANOVA test (particularly Scheffe test), which was applied for means comparison.

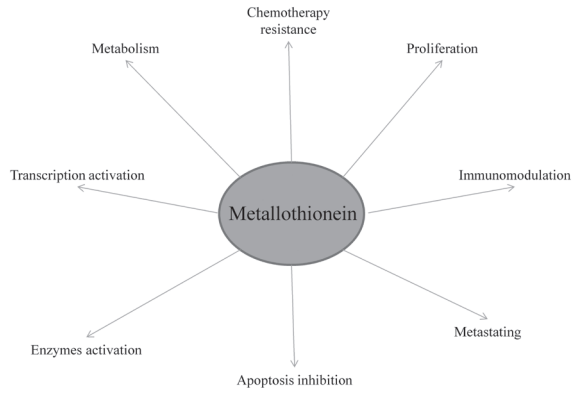

Figure 1. Metallothionein is involved in regulation of many cellular processes connected with carcinogenesis

RESULTS AND DISCUSSION

Numerous papers have been published on MT association with tumour diseases, their invasiveness and prognosis (Lara-Bohorquez *et al.*, 2008; Li *et al.*, 2008; Szelachowska *et al.*, 2008; Wei *et al.*, 2008; Pedersen *et al.*,

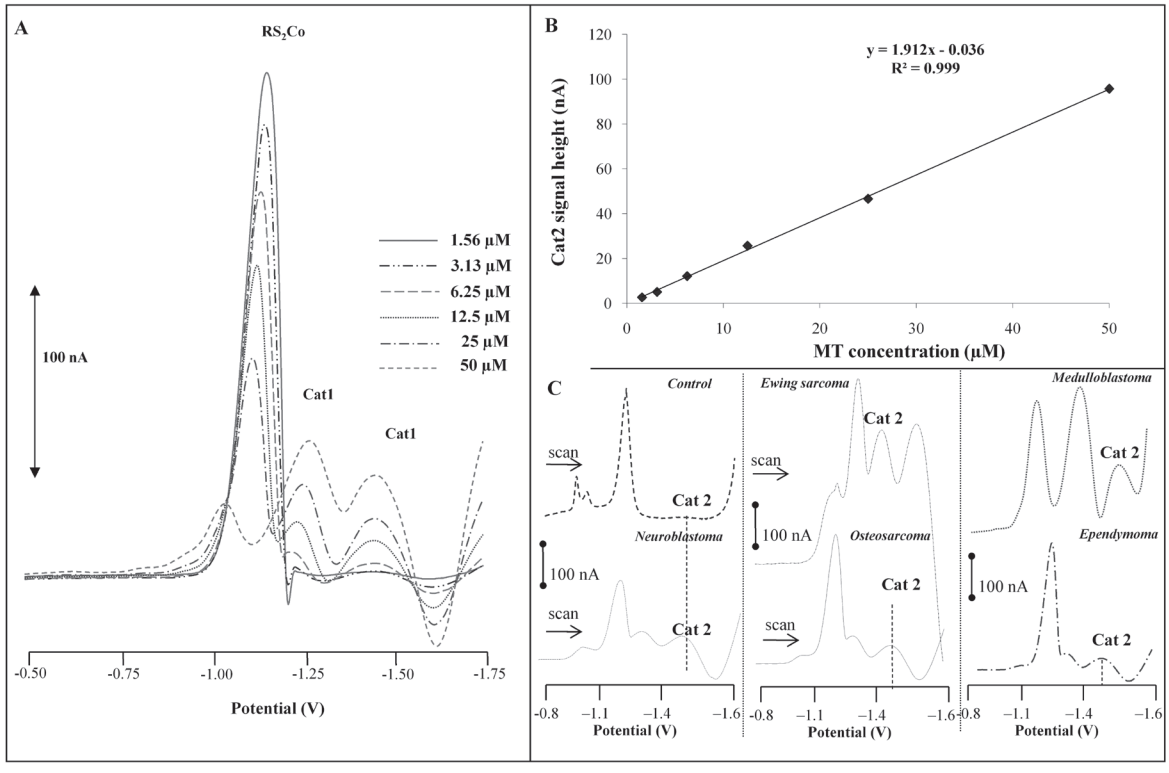

Figure 2. Electrochemical signals measured in Brdicka reaction
(A) Voltammograms of MT standards. **(B)** Calibration curve from 1.56 to 50 µM MT. **(C)** Typical shapes of voltammetric records of sera of patients with different cancer diagnoses.

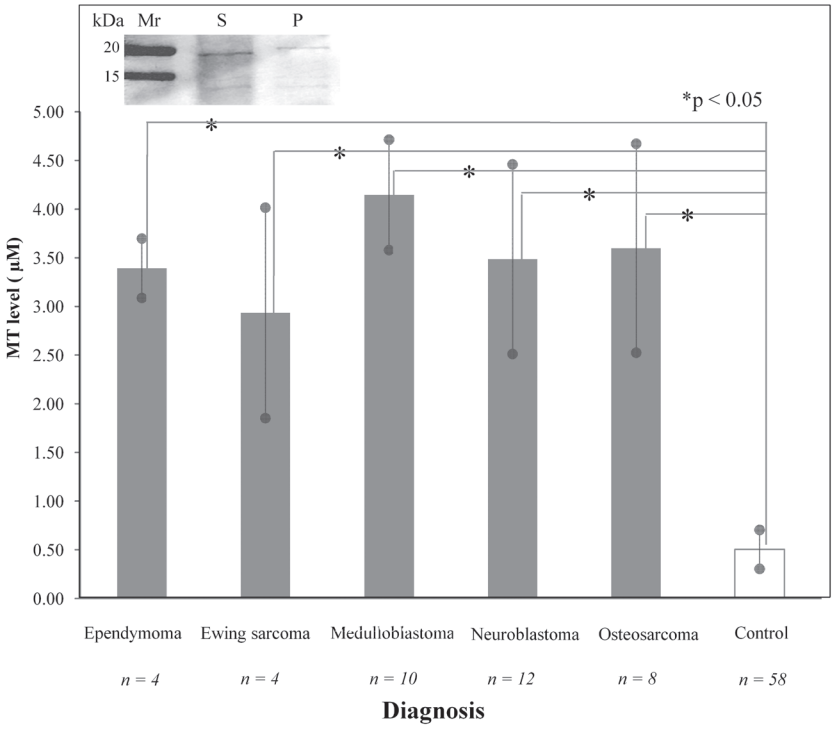

Figure 3. MT levels in sera of patients with anaplastic ependymoma, Ewing sarcoma, medulloblastoma, neuroblastoma, osteosarcoma and those of control human donors
Results were statistically treated with ANOVA, Scheffe test. Differences with $P < 0.05$ were considered significant. Inset: detection of metallothionein by Western-blotting. S, standard; P, patient.

2009). It has also been shown that the relation of MT level and resistance to cytostatics ought to be considered (Bacolod *et al.*, 2009; Knipp, 2009), because it may be used for changing the therapy (Penkowa *et al.*, 2009; Szelachowska *et al.*, 2009; Telang *et al.*, 2009; Yap *et al.*, 2009). Immunochemical and indirect heavy metal-based assays are the most commonly used ones for MT determination in clinical practice (Milnerowicz *et al.*, 2010). Electrochemical methods are used rarely, although they allow simple routine determination of MT in clinical sample. Krizkova *et al.* (2009b) found excellent agreement (more than 90%) between the results obtained by ELISA employing chicken antibodies and Brdicka reaction.

Metallothionein determination

To analyze clinical samples, a robust and automated analytical system must be used. Therefore, we utilized an automated electrochemical analyser comprised of an autosampler with a cooled sample holder, a VA Stand instrument using a standard cell with three electrodes, and a 746 VA Trace Analyzer for detection of MT (Fabrik *et al.*, 2008b). Primarily we measured various concentrations of MT. The measurements gave three signals RS_2Co, Cat_1 and Cat_2, which differed with increasing MT concentration (Fig. 2A). We found that the height of the Cat_2 signal was proportional to MT concentration. The resulting dependence was strictly linear ($y = 1.912\ x - 0.037$; $R^2 = 0.999$) and is shown in Fig. 2B. This method was subsequently used for analysis of blood serum samples from child patients with tumour diseases. The samples of newly diagnosed patients were collected during 2008–2010 at the largest and the most specialized institution for children medicine in the Czech Republic, the Faculty Hospital Motol in Prague. After processing the samples according to the protocol shown in Material and Methods, electrochemical analyses were carried out. The obtained voltammograms are shown in Fig. 2C. The average metallothionein level in healthy volunteers was 0.5 ± 0.2 $\mu mol \cdot dm^{-3}$. Sigh *et al.* (2006) found that the serum level of metallothioneins in children of similar age (7.4 ± 3.4 years) was 2.1 μg/ml, i.e., 0.31 μM, which is comparable to our results. However, in patients with solid tumours the average MT level was 3.4 ± 0.8 $\mu mol \cdot dm^{-3}$ (Fig. 3). The presence of MT was confirmed by Western-blotting (Fig. 3). Despite the relatively high standard deviations and high variations among the MT levels in cancer samples, the MT level in all newly diagnosed patients was significantly higher than in the control samples (tested by Schefe test, $P < 0.05$). However, metallothionein can be induced by zinc and other dietary factors, inflammation, and by many forms of environmental stress, e.g., exposition to heavy metals or after liver injury (Afridi *et al.*, 2009). Therefore, we analysed also samples from patients suffering from inflammation (n = 20, average age = 12). The level of MT was enhanced, but, after statistic evaluation, no significant differences between the level of MT in the control and the inflammation patients were found (not shown). In addition to quantification of MT, we found that the shape of the voltammograms of cancer samples differed from the voltammograms of controls (patent PV 2007-568) (Vyzkumny ustav pletarsky *et al.*, 2009). Particularly, in patients with Ewing sarcoma, medulloblastoma and ependymoma the voltammetric curves were deformed.

In previously published studies the increased level of MT in serum from patients with lymphoid leukaemia, lung carcinoma, thyroid carcinoma (Adam *et al.*, 2008b), spinocellular carcinoma (Vajtr *et al.*, 2008), head and neck cancer (Fabrik *et al.*, 2008a) and in pigs with hereditary melanoma (Krizkova *et al.*, 2008) has also been found. Nevertheless, other papers do not support the clinical importance of MT level (Eckschlager *et al.*, 2009), which indicates that more research in this area is required. Identification of individual MT isoforms seems to be a promising way. Other authors published that MT-1 and MT-2 and their receptor megalin were significantly altered in central nervous system (CNS) lymphoma compared with controls. MT-1 and MT-2 were secreted in the CNS and were found mainly in lymphomatous cells, while the concentration of megalin was increased in cerebral cells. Those authors suggest that this feature likely reflects the CNS lymphoma microenvironment and molecular interactions between lymphomatous and neuronal cells (Pedersen *et al.*, 2010). It is known that MT chelates zinc under physi-

ological conditions; Takeda *et al.* (2001) used ^{65}Zn for imaging of rat brain tumours.

Besides clinical samples, studying cell lines can also help in elucidating the usefulness of MT as a new promising tumour disease marker. An increased MT level was found in melanoma cell lines compared with non-malignant control cell lines (Krizkova *et al.*, 2008). A different expression of MT in cisplatin-resistant and sensitive neuroblastoma cells was observed (Fabrik *et al.*, 2008a). Those authors confirmed the results of Yasuno *et al.* (1999), who found that a considerably higher MT level with π-isomer of glutathione-*S* transferase contributed to cisplatin resistance in cisplatin resistant neuroblastoma cell lines. Besides cisplatin, Bacolod *et al.* (2009) found that sequestration of the antitumour drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) by MT may contribute to resistance in a medulloblastoma cell line and in rhabdomyosarcoma (Bacolod *et al.*, 2002). Gallium nitrate used as an antineoplastic agent induced expression of metallothionein-2A in human T-lymphoblastic leukaemia/lymphoma CCRF-CEM cells. A role of metallothionein in modulating the antineoplastic activity of gallium was confirmed by the fact that the induction of metallothionein expression by zinc provided partial protection against the cytotoxicity of gallium and by showing that the level of endogenous metallothionein in lymphoma cell lines correlated with their sensitivity to gallium nitrate (Yang *et al.*, 2007). Genes associated with growth, survival, and aggressive behaviour of tumour cells (metallothioneins, vascular endothelial growth factor (VEGF), neuropilin 1, adrenomedullin, and IGF-2) were induced in human neuroblastoma cell lines cultivated under hypoxic conditions (Jogi *et al.*, 2004).

The results found in this work, showing differences in the level of MT in the serum of healthy humans and patient with cancer diseases, are consistent with existing knowledge on a connection of MT with several types of tumours.

CONCLUSIONS

The results of this study demonstrate that differential pulse voltammetry — Brdicka reaction is a promising tool for determination of MT level in serum from patients with tumour disease. They also indicate that MT levels in the serum can be a suitable indicator for diagnosis, prognosis and selection of efficient therapy.

Acknowledgements

Supported by grants GACR (P301/10/0356 and 301/09/P436), GA AV IAA401990701, IGAMZ (10200-3) and Ministry of Education of the Czech Republic (MSM0021620808).

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