ACTA ABP BIOCHIMICA POLONICA

Vol. 57, No 1/2010
99–104
on-line at: www.actabp.pl

Regular paper

Determination of metallothionein in biological fluids using enzyme-linked immunoassay with commercial antibody

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Metallothionein (MT) is a low molecular weight cysteine-rich protein with a number of roles in the pro/antioxidant balance and homeostasis of essential metals, such as zinc and copper, and in the detoxification of heavy metals, such as cadmium and mercury. Until now, detection of metallothionein in biological fluids remained difficult because of a lack of a broadly reactive commercial test. Meaningful comparison of the values of metallothionein concentrations reported by different authors using their specific isolation procedures and different conditions of enzyme-linked immunoassay is difficult due to the absence of a reference material for metallothionein. Therefore in the present study, we describe a quantitative assay for metallothionein in biological fluids such as plasma and urine performed by a direct enzyme-linked immunoassay using a commercially available monoclonal mouse anti-metallothionein clone E9 antibody and commercial standards of metallothionein from rabbit liver and a custom preparation of metallothionein from human liver. The sensitivity of the assay for the standard containing two isoforms MT-I and MT-II from human liver was 140 pg/well. The reactivity of the commercial standards and standards containing two isoforms MT-I and MT-II isolated from human liver in our laboratory with a commercial monoclonal mouse anti-metallothionein clone E9 antibody were similar. This suggests that the described ELISA test can be useful for determination of metallothionein concentration in biological fluids. The concentrations of metallothionein in human plasma, erythrocyte lysate and in urine of smoking and non-smoking healthy volunteers are reported. Tobacco smoking increases the extracellular metallothionein concentration (plasma and urine) but does not affect the intracellular concentration (erythrocyte lysate).

Keywords: metallothionein MT-I and MT-II, ELISA, human biological fluids

Received**:** 23 November, 2009; revised: 16 March, 2010; accepted**:** 20 March, 2010; available on-line**:** 22 March, 2010

INTRODUCTION

Metallothionein (MT) is a low molecular weight cysteine-rich protein affecting the redox balance and homeostasis of essential metals, such as zinc and copper, and crucial in the detoxification of heavy metals, such as cadmium and mercury (Bremner *et al.*, 1990; Lynes *et al.*, 2006). MT synthesis is induced by various stimuli such as oxidative stress, heavy metals, glucocorticoids, and cytokines (Viarengo *et al.*, 2000). There are two major isoforms of this protein — isoform I (MT-I) and isoform II (MT-II), which are expressed in most tissues and stages of development (Klaassen *et al.*, 1999). MT has been found in both extracellular spaces and in intracellular compartments (Lynes *et al.*, 2006). Immunohistochemical studies have revealed the protein also in the nucleus and, at very low concentrations, in extracellular fluids such as plasma, bile and urine (Akintola *et al.*, 1995).

Identification, quantification and structural characterization of MTs is crucial for the determination of the roles played by these metalloproteins in the organism (Dabrio *et al.*, 2002). Quantification of MT in body fluids may be useful as a biological indicator of the exposure of individuals to the mentioned earlier factors.

Methods for quantification of MT are based on a direct determination of the peptide moiety of MT or an indirect determination *via* the metal and SH content of MT (Summer & Klein, 1991). However, no commercial test for quantification of MT in biological fluids is yet available (Dabrio *et al.*, 2002).

Enzyme-linked immunoassay (ELISA) can be used to measure the concentration of metallothionein in biological fluids. There are two kinds of ELISA procedures**:** competitive ELISA (cELISA) and direct ELISA. cELISA methods for MT-I were developed using prepared a custom antibodies (Grider *et al.*, 1989; Akintola *et al.*, 1995). A direct ELISA procedure employing a commercial monoclonal anti-MT antibody for use with filtered urine has also been reported (Hirauchi *et al.*, 1999).

In the present study, we describe a quantitative direct ELISA assay for MT in biological fluids such as plasma, erythrocyte lysate and urine using a commercially available monoclonal mouse anti-metallothionein clone E9 antibody (Dako) and a commercial standard containing MT-I or MT-II from rabbit liver (Sigma-Aldrich). We also compare results obtained for the commercial metallothionein standard with those for metallothionein isolated from human liver in our laboratory.

MATERIALS AND METHODS

Preparation of metallothionein from human liver. Preparation of metallothionein was performed at 4 °C. Metallothionein was isolated from human liver according to the method described by Hidalgo *et al.* (1989).

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Abbreviations: cELISA, competitive enzyme-linked immunoassay; ELISA, enzyme-linked immunoassay; MT, metallothionein; MT-I, isoform I of metallothionein; MT-II, isoform II of metallothionein; PVDF, polyvinylidene fluoride; SDS/PAGE, polyacrylamide gel electrophoresis

Liver from a 40-years man who died in an accident was collected during autopsy at the Department of Pathomorphology, Wroclaw Medical University. The study protocol was approved by the Local Bioethics Committee (KB No 165/1999). Pieces of liver were washed several times in phosphate-buffered salne (PBS), minced and 20 g of liver was homogenized with 82 ml of buffer containing 10 mM Tris/HCl, pH 8.6, with 10 mM 2-mercaptoethanol (Sigma-Aldrich, ref. No M6259, Germany) and 25 mM sucrose (Sigma-Aldrich, ref. No 84097, Germany) using a Potter-Elvehjem homogenizer at 4°C. The homogenate (100 ml) was centrifuged immediately at 5000 × *g* for 25 min at 4°C then centrifuged for 1 h at 10000 × *g*. Supernatant (72 ml) was collected and centrifuged for 2 h at 105000 × *g* at 4°C (Sorvall Ultra 80 Combi Plus). To the obtained cytosol fraction, $CdCl_2$ (99, 999%, Sigma Aldrich, ref. No 4398000, Germany) 1 mg/ml was added to a final concentration of 25 μg $CdCl_2$/1 ml of cytosol during incubation in a water bath at 80°C for 10 min with stirring. The presence of 2-mercaptoethanol and cadmium assures that MT is not oxidized (Hidalgo *et al.*, 1989). From 69 ml of the cytosol fraction, proteins were precipitated with acetone (to 60%) and centrifuged. The pellet was discarded and the supernatant was brought to 80% acetone and the proteins were allowed to precipitate overnight at 4°C then centrifuged for 1 h at 10000 × *g*. The proteins pellet were dissolved in 7.5 ml of 10 mM Tris/HCl, pH 8.6, centrifuged and the final supernatant was stored at –80°C. A sample of 6.7 ml containing 2.15 mg of protein/ml was applied onto a Sephadex G-75 (Sigma Aldrich, ref. No G75120, Germany) column (2.6 cm × 120 cm) equilibrated with 10 mM Tris/HCl, pH 8.6. The column was run in 10 mM Tris/HCl, pH 8.6, at a flow rate of 12 ml/h at 4°C and 3-ml fractions were collected.

Absorbance at λ = 220, 250 nm and 280 nm was monitored. Fractions with the OD 250/280 ratio from 7.4 to 9 were combined and applied without prior concentration on a DEAE-cellulose (DE-52) column (2.6 cm × 120 cm) equilibrated with 10 mM Tris/HCl, pH 8.6. Elution was done with a linear gradient of 20–200 mM Tris/HCl, pH 8.6, at a flow rate of 30 ml/h. Two peaks corresponding, respectively, to MT-I and MT-II were obtained. Absorbance at 220, 250 and 280 nm was measured and the fractions with a high absorbance at 220 and 250 nm were collected and concentrated using a YM3 membrane (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane, Millipore, ref. No UFC9-003, USA).

The molecular mass of MT standards was determined by gel filtration on a Sephadex G-75 column (2.6 cm × 120 cm) equilibrated with 10 mM Tris/HCl, pH 8.6 and by SDS/PAGE (Aoki & Suzuki, 1991).

Identification of MT isoforms by SDS/PAGE and Western blotting. Samples containing isoforms of MT were dissolved in 50 mM Tris/HCl, pH 6.8, 10% glycerol, 2% SDS (Sigma-Aldrich, ref. No L3771, Germany), 2.5 mM 2-mercaptoethanol (Bio-Rad, ref. No 1610710, USA) and 0.05% bromophenol blue (Sigma-Aldrich, ref. No B0126, Germany). Protein concentration was determined by the Bradford method (Bradford, 1976). The samples were heated in a water bath at 95°C for 3 min. Sodium dodecyl sulfate/15% polyacrylamide gel electrophoresis (SDS/PAGE 15%) was performed according to the method described by Aoki and Suzuki (1991). Electrophoresis was performed for 2 h.

Gels were stained with Dodeca Silver Stain Kit (Bio-Rad, ref. No 1610481, USA). After staining the ratio of MT-I and MT-II isoforms was determinate using a Gel Analysis Software (Quantity One 1-D Analysis Software, Bio-Rad ref. No 170-9600, USA). For Western blotting, proteins from gel were transferred to a polyvinylidene fluoride membrane (PVDF, Sequi-Blot PVDF Membrane Bio-Rad, ref. No 162-0236, USA) using a Mini-PROTEAN Tetra Cell Electrophoresis Module (Bio-Rad, ref. No 165800, USA) and Mini Trans-Blot Module (Bio-Rad, ref. No 1703930, USA) according to the methods previously described (Aoki & Suzuki 1991).

The PVDF membrane was stained using Ponceau S (Sigma-Aldrich ref. No P3504, Germany). To block nonspecific binding, the membrane was preincubated with TTBS (Tris-buffered saline, pH 7.6, containing 0.05% Tween-20 and 5% nonfat milk) (Skrzycki *et al.*, 2009), then was incubated overnight at 4°C with a monoclonal mouse anti-metallothionein clone E9 antibody (DakoCytomation, ref. No M0639, Denmark) diluted 1:200 in Tris-buffered saline, pH 7.6, containing 0.05% Tween-20. The membrane was washed and incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse antibody diluted 1:1000 in Tris-buffered saline, pH 7.6, containing 0.05% Tween-20 (*GAM-AP*, ref. No 85873, Bio-Rad, USA). The color reaction was visualized using a commercial kit (AP Conjugate Substrate Kit, Bio-Rad, ref. No 1706432, USA).

Enzyme-linked immunosorbent assay. The two-step direct ELISA using a commercial MT standard (Sigma-Aldrich, ref. No M7641, Germany) according to the procedure described by Hirauchi *et al.* (1999). A dilution series of MT was performed from 1 to 100 ng/ml (100 μl) in 50 mM Tris-25 mM HCl or 100 μl of biological samples (plasma, urine or erythrocyte lysate) were incubated in microtiter plates overnight at 4°C. After incubation, the wells were washed three times using wash buffer containing 40 mM Tris-33 mM HCl with 154 mM NaCl and 0.05% (w/v) Triton X-100 (Sigma-Aldrich, ref. No X100, Germany). The microplate was then blocked using a buffer containing 3% (w/v) gelatin (Bio-Rad, ref. No 170-6537, USA) in 40 mM Tris-20 mM HCl with 154 mM NaCl and 0.05% (w/v) Triton X-100 (300 μl), and incubated for 1 h at 25°C. After washing, primary monoclonal mouse metallothionein Clone E-9 antibody (Dako, ref. No M0639, Denmark) was added in a 1:20 dilution in a buffer containing 1% (w/v) gelatin in 40 mM Tris-33 mM HCl with 154 mM NaCl and 0.05% (w/v) Triton X-100 (100 μl) and incubated overnight at 25°C. The wells were washed three times using wash buffer containing 40 mM Tris-33 mM HCl with 154 mM NaCl and 0.05% (w/v) Triton X-100. The secondary biotinylated polyclonal goat anti-mouse IgG antibody (Dako, ref. No E0433, Denmark) was then added in a 1:400 dilution in buffer containing 1% (w/v) gelatin in 40 mM Tris-33 mM HCl with 154 mM NaCl and 0.05% (w/v) Triton X-100 (100 μl) and incubated for 4 h at 25°C. The wells were then washed three times and incubated for 1 h at 25°C with HRP-Avidin (Dako, ref. No P0347, Denmark) diluted 1:800 in buffer containing 1% (w/v) gelatin in 40 mM Tris-33 mM HCl with 154 mM NaCl and 0.05% (w/v) Triton X-100 (100 μl). The reaction was visualized by ortophenylenediamine in 200 μl of 0.25 M phosphate citrate buffer (citric acid, Sigma-Aldrich, ref. No 251275, Germany; sodium phosphate dibasic, Sigma-Aldrich, ref. No S7907, Germany), pH 5.5 containing 0.006% H_2O_2. The color reaction was stopped using 50 μl of 3 M HCl. The absorbance was measured at 490 nm in an ELISA reader (STAT-FAX 2100, Analco, USA). Using the elaborated conditions, concentrations of metallothionein were determined by linear regression of a standard curve after

log transformation. The sensitivity of the ELISA for a sample containing isoforms MT-I or MT-II was found to be 140 pg/well.

We compared the affinity of the commercial monoclonal mouse anti-metallothionein clone E9 antibody to the commercial standards of MT I (Sigma-Aldrich, ref. No M5267, Germany) or MT II (Sigma-Aldrich, ref. No M5392, Germany) isolated from rabbit liver and a standard containing two isoforms MT-I and MT-II or only MT-I isolated from human liver in our laboratory.

Analytical procedure. The concentration of MT was measured in plasma, erythrocyte lysate and urine. Blood and urine from 45 men were assayed**:** 17 smoking (aged 40.8 ± 10.4 years) and 28 non-smoking (aged 41.3 ± 6.7 years). The study protocol was approved by the Local Bioethics Committee of Wroclaw Medical University (KB No 94/2007).

Whole blood was drawn into trace element-free tubes containing heparin and centrifuged at 2500 × *g* for 15 min to separate the plasma from cell pellet. The pellet was washed in equal volume of ice-cold 0.9% NaCl. This process was repeated twice. The washed cells were lysed by addition of ice-cold, double distilled water (Hydrolab, HLP Smart 1000, Poland) (1**:**1.4), and the resulting lysate was used for the ELISA (Thomas *et al.*, 1992). Plasma and the erythrocyte lysates were frozen at –80 °C until used. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

Spot urine samples were collected in acid-washed containers and stored at –80 °C until required for analysis. Urinary creatinine levels were determined by the Jaffe reaction (Butler, 1975).

Statistical analysis. The data are expressed as mean ± S.D. values. The normality of the variable was tested by the Shapiro-Wilk *W* test. Differences between the smoking and non-smoking groups were tested using Student's *t*-test. In all instances, $P < 0.05$ was considered as statistically significant. Statistical calculations were done using STATISTICA version 6.0 (Polish version; StatSoft, Kraków, Poland).

RESULTS

MT was purified from human liver by Sephadex G-75 chromatography and the absorbance was measured at λ = 220 nm, λ = 250 nm and λ = 280 nm. As expected, the low molecular-weight protein peak showed a high absorbance at 250 nm, a feature indicating the binding of cadmium to MT. The low absorbance at 280 nm confirmed a lack of aromatic amino acids, as it had already been shown for MT from various species (Hidalgo *et al.*, 1989). The 250/280 absorbance ratio for the MT peak of the gel filtration chromatography was from 7.4 to 9 in collected fractions. We assayed for sulfhydryl groups with the Ellmann method in collected fractions (Ellman, 1959). The result confirmed the presence of numerous sulfhydryl groups in our samples. The molecular mass determined by gels filtration was about 8 kDa. Determination of the molecular mass using ProteomeLab™ SDS-MW Analysis Kit (Beckamn Coulter, ref. No P/N 390963AB, USA) on capillary electrophoresis (ProteomeLab™ PA 800, Beckman-Coultur) confirmed the presence of a pure protein with molecular mass of about 9 kDa (unpublished), in agreement with earlier results of Kriskova *et al.* (2009).

Separation of human MT by DEAE-cellulose (DE-52) ion-exchange chromatography gave two major peaks of high Cd content representing metallothionein isoforms MT-I and MT-II. The separation was based on their single charge difference. The presence of the metallothionein isoforms and the molecular mass were confirmed by polyacrylamide gel electrophoresis and Western blotting. The detection limit of SDS/PAGE silver staining was about 100 ng/lane. The results highligheted two important points**:** the isoforms could separated by SDS/PAGE and metallothionein stains strongly using silver. Metallothionein is substantially more enhanced by silver staining than are other low-molecular-mass, heat stable proteins (McCormick & Lin, 1991).

We analyzed the ratio of MT isoforms I and II using commercial Quantity One 1-D Analysis Software. The results showed that MT-I constituted approx. 75% of the total amount of metallothionein.

Figure 1 shows a gel after silver staining and PVDF membrane after Ponceau S staining. The presence of metallothionein in samples was confirmed by Western blotting analysis using a monoclonal antibody against MT, Clone E-9 (DakoCytomation, ref. No M0639, Denmark). The results are shown in Fig. 2. The obtained image also confirms the presence of two isoforms, MT-I and MT-II, in our samples.

We compared the affinity of different isoforms (MT-I and MT-II) of metallothionein to the commercial antibody by direct ELISA analyses performed in the same experimental conditions. We analyzed the commercial standard and samples isolated in our laboratory containing two isoforms, MT-I and MT-II and those containing only one isoform, MT-I or MT-II. There were slight differences in the affinity of the commercial monoclonal mouse anti-metallothionein clone E9 antibody to samples containing different isoforms of MT isolated from human liver or from rabbit liver (Fig. 3).

We assayed the concentration of MT in erythrocyte lysate, plasma and urine of smoking and non-smoking healthy men using the elaborated ELISA procedure. We observed a significantly higher concentration of MT in the plasma and urine of smokers. In contrast, the concentration of metallothionein in erythrocyte lysates was similar in the two groups. The results are shown in Table 1.

DISCUSSION

The quantification of MT is challenging and has led to the development of various techniques for that purpose.

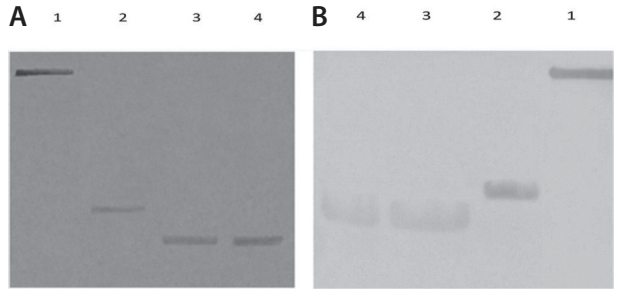

Figure 1. Localization of metallothionein
(A) SDS/PAGE electrophoresis, silver staining (amount of proteins about 100 ng/lane). **(B)** PVDF membrane staining with Ponceau S (amount of proteins about 20 µg/lane). (1) Albumin human electrophoretic marker + vitamin B12, molecular weight 67 kDa, (Serva, ref. No. 11885, Germany), (2) myoglobin from human heart, molecular mass 17 kDa (Sigma-Aldrich, ref. No M0636, USA); (3–4) samples containing two isoforms of MT from human liver, molecular mass approx. 6.5 kDa.

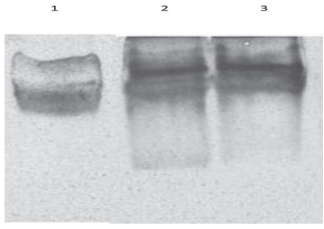

Figure 2. Western blot analysis of purified human hepatic MT-I and MT-2.
After separation by SDS/PAGE proteins were transferred on PVDF membrane. The presence of MT was visualized using commercial monoclonal mouse anti-metallothionein clone E9 antibody. Commercial standard of MT-II from rabbit (2–3) samples containing two isoforms of MT isolated in our laboratory from human liver.

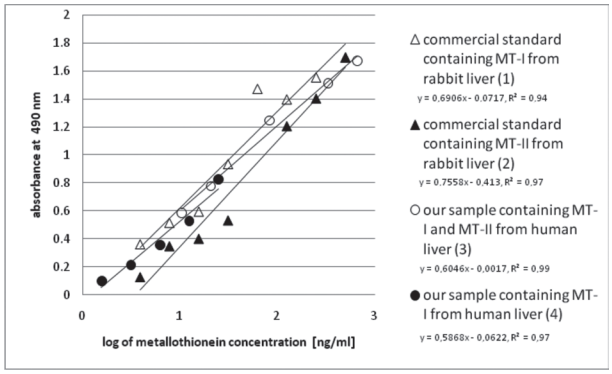

Figure 3. Comparison of standard curves obtained for different isoforms of MT.
Direct ELISA two step assay was run with commercial anti-MT antibody Clone E-9.

Changes in MT concentration have been observed in various diseases, therefore MT quantification may be a useful indicator of pathologic states, which calls for a convenient procedure for quantitative analysis of MT (Andrews, 2000). The sensitivity of immunoassay primarily depends on the specificity and affinity of the antibody, therefore the type of antibody is crucial for ELISA assay. The reproducibility of ELISA assays using antibodies purified in different laboratories is not always satisfactory because the availability of the antibodies is limited (Dabrio *et al.*, 2002). In many studies MT isolated from different mammalian tissues, containing variable ratios of MT isoforms, was used as a standard. Different authors have elaborated specific procedures in each laboratory. Therefore a robust comparison of the results of MT quantification with the use of different standards, antibodies and different conditions of ELISA is virtually imposible.

The large number of isoforms and subisoforms within the MT family makes application of immunological techniques to biological fluids difficult. In order to increase the selectivity and sensitivity of these methods, the specificity of the antibodies is important. However, the entire MT family lacks common antibodies for the different isoforms and subisoforms, which could result in underestimation when total MT is measured, for example by ELISA (Dabrio *et al.*, 2002).

Hirauchi *et al.* (1999) have elaborated a direct ELISA procedure using commercial reagents but the biological fluids were filtered using a microcentrifuge tube with a filter.

The purpose of this report was to elaborated an ELISA procedure using commercial standard and antibody without any specific preparation of samples.

In our earlier studies we assayed the concentrations of MT in human milk and fluid collected intrasurgically from pancreatic cysts of patients with chronic pancreatitis using a ^{109}cadmium/haemoglobin assay. The level of MT in breast milk of smoking women was 5.1 ± 1.9 μg/ml (Milnerowicz & Chmarek, 2005) and 13.8 ± 3.3 μg/ml in fluids collected intrasurgically from pancreatic cysts of patients with acute or chronic pancreatitis (Milnerowicz *et al.*, 2004). Those values of MT concentrations were many times higher than the concentrations of MT in the plasma or urine (Akintola *et al.*, 1995), therefore quantification of MT in plasma and urine using those methods is impossible. In the present study we used a two-step direct ELISA assay with commercial standard and antibodies. We compared the affinity of commercial monoclonal antibodies to different isoforms of MT isolated from human or rabbit liver using this ELISA assay. We used MT-I and MT-II isoforms because there were recent reports that the MT-III and MT-IV isoforms were tissue-specific, MT-III was specifically expressed in the brain and MT-IV was expressed only in differentiating cells of stratified squamous epithelia, and presence of MT-III and MT-IV in the plasma or serum has not yet been demonstrated (Palimter *et al.*, 1992; Quaife *et al.*, 1994). Our investigation showed only a slight difference in the affinity of the commercial MT antibody to samples containing different isoforms of MT isolated from human liver and rabbit liver. The reactivity of the commercial standard of metallothionein and metallothionein isolated in our laboratory suggests that the described ELISA procedure can be useful for researchers who need a simple tool to determine of metallothionein concentration in biological fluids.

Immunological studies using rabbit polyclonal antibody and murine monoclonal antibodies raised against rat MT showed that they were cross-reactive with various kinds of mammalian MTs (Nakajima *et al.*, 1991). In a sensitive competitive radioimmunoassay for MT using both polyclonal and monoclonal antibodies, full cross-reactivity was observed for MT isolated from the livers of rat, rabbit and human (Nakajima *et al.*, 1991). Our method was also used to determine the concentrations of MT in the plasma, erythrocyte lysate and urine in

Table 1. Metallothionein concentrations in blood and in urine of smoking and non-smoking healthy volunteers

Concentration of metallothionein in biological fluids			
Healthy volunteers n = 35	plasma (ng/ml)	erythrocyte lysate (μg MT/g of protein)	urine (μg MT/g of creatinine)
smoking n = 17	4.40 ± 2.76*	14.70 ± 4.05	1.47 ± 0.99**
non-smoking n = 28	3.42 ± 2.30*	14.42 ± 2.39	1.08 ± 0.46**

*Statistically significant differences for metallothionein in plasma between smoking and non-smoking groups, $P < 0.02$; **Statistically significant differences for metallothionein in urine between smoking and non-smoking groups, $P < 0.04$. Values are expressed as X ± S.D..

smoking and non-smoking healthy volunteers. Whole blood was drawn on heparin, because earlier study was shown, that addition of EDTA displaced heavy metals from MTs and renders them susceptible to tryptic digestion (Wang *et al.*, 2007). In all cases the concentrations of MT in the blood and urine were higher in smokers than in non-smokers. The highest concentrations of MT were found in erythrocyte lysate of smoking (14.70 ± 4.05 μg/g of protein) and non-smoking (14.42 ± 2.39 μg/g of protein) healthy volunteers.

The concentration of MT in the plasma of smokers was statistically significantly increased (4.40 ± 2.76 ng/ml, $P < 0.02$) in comparison to non-smokers (3.42 ± 2.30 ng/ml). The increase of MT concentration in plasma suggests induction of the synthesis of MT in the liver and confirms the circulation of the MT-cadmium complex from liver to kidney (Park *et al.*, 2001, Nordberg, 2009). In the urine of smoking, healthy volunteers the concentration of MT was also increased (1.47 ± 0.9 μg/g of creatinine, $P < 0.04$) compared with non-smokers (1.08 ± 0.46 μg/g of creatinine). The increase of MT concentration in urine of healthy smokers persons confirms the accumulation of cadmium in the kidney and nephrotoxicity of cadmium deriving from tobacco smoking (Ronco *et al.*, 2005). The obtained results show that tobacco smoking in healthy persons influences extracellular MT concentration (plasma and urine) but does not change the intracellular MT concentration (erythrocyte lysate).

Akintola *et al.* (1995) developed an ELISA assay for measuring MT-I in plasma and urine. The mean concentrations of MT-I in plasma and urine in man were 32 ± 16 ng/ml and 10 ± 6 ng MT-I/μmol of creatinine, respectively. Investigations performed by Nordberg *et al.* (1982) using radioimmunoassay showed that MT concentrations in plasma and urine ranged between 2–11.3 ng/g of protein and 2–155.2 ng/g of protein, respectively. MT levels were measured by a two-step competitive ELISA procedure in urine of two groups of children living in areas of mild and high environmental pollution due mainly to heavy metals (Swierzcek *et al.*, 2004). The MT concentrations in that study ranged between 3–30 μg/g of creatinine. Grider *et al.* measured MT concentrations in erythrocyte lysate using human MT-I as a standard in a competitive ELISA assay. The MT concentrations was 34 ± 6 μg/g of protein (Grider *et al.*, 1989). However, a reliable comparison of the results of MT quantification obtained by different authors using their specific isolation procedures and various types of ELISA assay is not easy due to the absence of a reference material for MT.

CONCLUSIONS

Our investigation showed similar affinity of commercial monoclonal mouse anti-metallothionein clone E9 antibody to MT-I and MT-II isoforms isolated from human and rabbit liver.

The similar affinity of isofoms MT-I and MT-II to the antibody ensures accurate determination of total MT concentration regardless of the proportion of the MT isoforms in biological samples.

The detection limit (140 pg/100 μl) of this ELISA procedure using commercial reagents allows determination of the concentration of metallothionein in biological fluids such as plasma, urine and erythrocyte lysate without any specific pretreatment.

Tobacco smoking in healthy persons increases extracellular metallothionein concentration (plasma and urine) but does not change intracellular metallothionein concentration (erythrocyte lysate).

Acknowledgements

This research was supported by the State Committee of Scientific Research, Poland (grant No 0552/B/P01/2009/36).

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