

Distribution of metallothioneins in the brain neoplastic cells

Bolesław Floriańczyk¹, Jacek Osuchowski², Robert Kaczmarczyk², Elżbieta Starosławska³, Tomasz Trojanowski²

¹Department of Biochemistry and Molecular Biology, Medical University of Lublin, Poland; ²Department of Neurosurgery and Children's Neurosurgery Clinic, Medical University of Lublin, Poland; ³Department of Oncology, Medical University of Lublin, Poland

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Abstract

Metallothioneins (MT) are common proteins in animal tissues. These proteins take part in the homeostasis of the ions of the metals which are necessary for the proper metabolism of the organism (zinc, copper), biosynthesis regulation and zinc-protein activity (for example the activity of the zinc-dependant transcription factors) and they also take part in the detoxication of the tissue from toxic metals. Apart from these, they also protect the tissue from reactive oxygen species, radiation, electrophilic pharmacological agents used in the cancer therapy and the mutagens. The aim of this work was to obtain cellular sub-fractions of brain tumors and to separate these proteins by SDS-polyacrylamide gel electrophoresis and Western Blotting technique and to determine the level of metallothioneins and to determine the level of metallothioneins in the cellular sub-fraction. The experimental materials were the brain neoplastic tissues resected during neurosurgical procedures. The brain tumors were divided into two groups; astrocytoma G-2 and malignant gliomas (astrocytoma GM-4, glioblastoma multiforme). The cellular fractions of tumour tissues were obtained according to Clark and Nicklas and Chauveau et al. methods. The level of the metallothioneins was determined by the cadmium-hemoglobin affinity assay using the cadmium isotope (¹⁰⁹Cd). By performing protein dissection on polyacrylamide gel (PAGE) and applying immunoidentifying technique we proved the presence of metallothioneins in all the cell sub-fractions. In GM-4 patients the total MT level increased by 12.06% whereas in the cytosol sub-fraction it increased by 17.02% in comparison with astrocytoma G-2 patients. In the mitochondrial sub-fraction the level increased by 49.09% while in the nuclear and microsomal sub-fractions the increase was by 22.38 and 8.24%, respectively.

Key words: astrocytoma, glioblastoma multiforme, metallothionein, cell sub-fractions, polyacrylamide-gel electrophoresis, immunoidentifying technique

Introduction

Metallothioneins (MT) are a widespread protein in the animal world. These proteins are characterised by a great invariability of their structure. While isolated from various animals they only slightly differ from one another in the amino acid composition. The

number of amino acids is constant in every animal group, and that is 60 (or 61) amino acids, 20 of which are the cysteins residues, which makes over 30% of the amino acids composition. Such a high amount of cysteins determines the metallothionein's functions [2,6,8,9,19,32].

Communicating author:

Floriańczyk Bolesław, Department of Biochemistry and Molecular Biology, University School of Medicine, Chodźki 1, 20-123 Lublin, Poland, tel. +48 81 740 58 45, e-mail: bolkof@poczta.onet.pl

Metallothioneins take part in the homeostasis of the ions of the metals which are necessary for the proper metabolism of the organism (zinc, copper), biosynthesis regulation and zinc-protein activity (for example the activity of the zinc-dependant transcription factors) and they also take part in the detoxication of the tissue from toxic metals. Apart from these, they also protect the tissue from reactive oxygen species, radiation, electrophilic pharmacological agents used in the cancer therapy and the mutagens [10-12,21,22,26].

The induction of metallothionein synthesis is influenced by many factors: heavy metals, inflammatory factors, reactive oxygen species, glyocortycoids and pharmacological agents [28,33].

The aim of this work was to obtain cellular sub-fractions from brain gliomas of different malignancy and to determine the level of metallothioneins. The proteins of these sub-fractions were to be separated by SDS-polyacrylamide gel electrophoresis and Western Blotting technique.

Materials and methods

Materials

The experimental materials were the brain neoplastic tissues resected during neurosurgical procedures. The brain tumors were divided into two groups; benign gliomas (*astrocytoma* G-2, n=25) and malignant gliomas (*glioblastoma multiforme*, n=30). The patients had not been exposed to any prior treatment for their tumor disease.

Methods

Obtaining Cell Sub-fractions

A fragment of tissue was homogenised in a buffer for initial homogenising (10 mM Tris-HCl pH 7.4 0.25 M saccharose, 4 mM magnesium chloride, 0.5 mM PMSF) in a glass homogeniser with teflon pistle and then centrifuged for 10 mins at 4 000 rev/min. Supernatant (a) was centrifuged again (at 12 000 rev/min) to get mitochondrial sediment. The sediment, which included mitochondria, was suspended in a 3% solution of ficol and layered on a 6% solution of ficol and centrifuged for 30 minutes at a speed of 12 000 rev/min. The act of suspending mitochondrial solution in the 3% solution and layering it on the 6% solution of ficol was repeated again and the mitochondrial solution was centrifuged keeping the same conditions [5].

The supernatant (a) was then centrifuged at a high speed of 40 000 rev/min ($\approx 100\ 000 \times g$) for an hour to obtain microsomal and cytosol sub-fraction.

The sediment of cell nuclei obtained after the initial homogenising at first centrifuging was subjected to further procedure [3]. The sediment of nuclei in a cleansing buffer was ultracentrifuged at 30-40 000 rev/min).

Microsomal and nucleic sediments were suspended in a buffer for initial homogenising and left for further analysis.

Polyacrylamide-Gel Electrophoresis (PAGE)

The electrophoresis was done according to Laemmli [25]. First dissecting then coagulating gels were applied to electrophoretic plates. The process of polymerisation of gel lasted 24 hours and was undergoing at a temperature of 4°C. The protein solutions (that included standard proteins, tissue homogenates or sub-cellular fraction solutions) were diluted in a lysing buffer (20 μ l examined protein sample, 20 μ l lysing buffer and 2 μ l dichlorofenoloindofenole stain) before they were applied on the gel. The process took place in a boiling bath at 100°C.

Thus prepared samples were incubated at a temperature of 100°C for 1 min. The electrophoretic dissection was conducted at a room temperature with 80 V during the protein passage through the coagulating gel and with 12 V during their passage through the dissecting gel. The gel was being stained for 10 mins then the solution was poured out for staining and the gel was rinsed with water. The process of gel staining took place with four-time replacement of the staining solution. After the staining the gel was submerged in a pre-prepared immersion liquid (with 1- hour immersion time) and then dried in a Dryout kit for 24 hours at 30°C.

Western Blotting (transfer of proteins from polyacrylamide gels onto the membranes)

The transfer of proteins from polyacrylamide gels onto the nitrocellulose was done after Towbin [40].

Using a Western-blotting apparatus we put 6 pieces of Whatman blotting paper soaked with Solution I onto the anode (Solution I included 0.3 M Tris-HCl pH 7.4 20% methyl alcohol). Then we put there 3 layers soaked with Solution II (0.025 M Tris-HCl pH 10.4 plus 20% methyl alcohol). Next a sheet

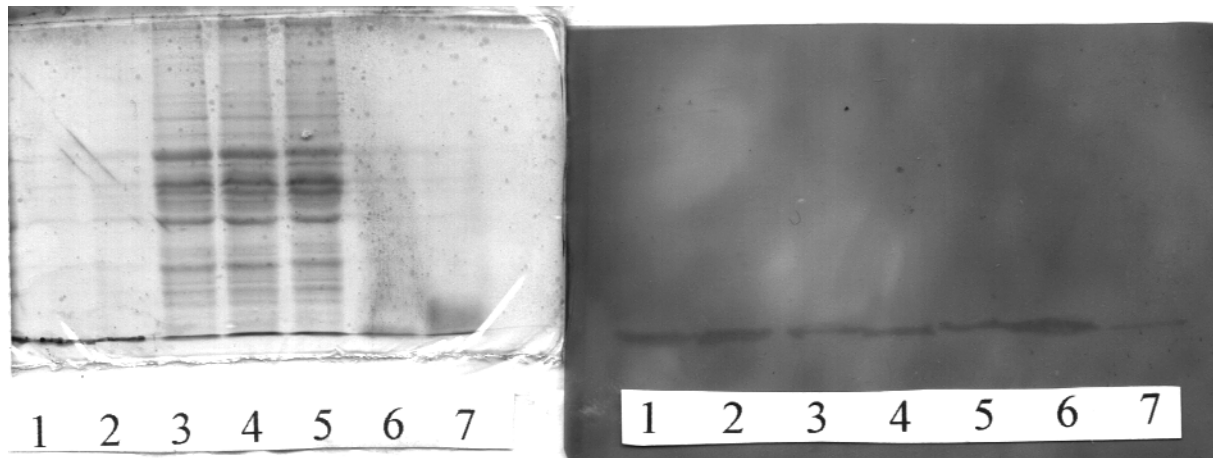


Fig. 1. Polyacrylamide gel electrophoresis and immunoidentifying technique. Proteins obtained from astrocytoma G-2; 1 – metallothionein type I, 2 – metallothionein type II, 3 – crude homogenate of tissue, 4 – cytosol sub-fraction, 5 – microsomal sub-fraction, 6 – nuclear sub-fraction, 7 – mitochondrial sub-fraction

of nitrocellulose moistened with distilled water and acrylamide gel with dissected protein was applied. Then again we added 3 layers of Whatman blotting paper soaked with Solution II (0.035 M Tris-HCl pH 10.4 20% methyl alcohol, 0.04 M ϵ -amino-n-caproic acid). Transfer conditions: the transfer area was about 24 cubic centimetres; $I=120-65$ mA; the tension was 26 V and transfer time – 1.5 h. After the transfer the nitrocellulose was stained with Ponceau S stain. The redundant stain was rinsed off with distilled water and the nitrocellulose was incubated in a blocking solution of 1% BSA in PBS buffer of pH 7.2) for 0.5-1 h, then again rinsed with water. Next metallothioneins were identified using antibodies.

To do that we incubated the membrane in a solution including the first antibody (monoclonal mouse anti-metallothionein at 1:25-1:200 concentration) diluted in PBS buffer with 0.1% BSA/Tween20 added for 1 h using a stirrer. After the incubation the membrane was rinsed 3-5 times (10 mins each) in PBS solution of pH 7.2 containing 3% Tween20, and incubated in a solution containing the second antibody (anti-mouse/HRP) diluted in PBS buffer with 0.1% BSA and 0.1% Tween added (where the concentration of antibodies was 1: 20 000). This was held for 1 h including the shaking on a stirrer.

After the incubation with the second antibody the nitrocellulose membrane was rinsed 3-5 times (10 mins each) in PBS solution of pH 7.2 containing 3% Tween-20, then immersed in ECL I and ECL II Dako solutions mixed together in the proportion of 1:1.

Metallothioneins level

The level of the metallothioneins was determined by the cadmium-hemoglobin affinity assay [7], using the cadmium isotope from Du Pont.

Quantitative Protein Assay

The proteins were determined with a method described by Shakir et al. [35].

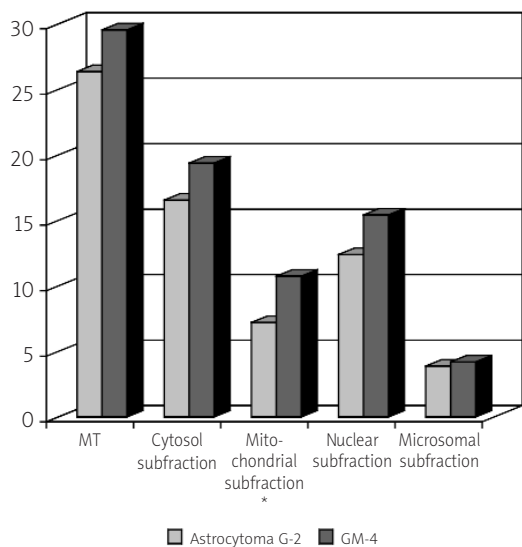
Statistical Analysis

The statistical analysis of the results was conducted using SPSS 8.0 pack. To evaluate the significance of variable discrepancies of standard distribution we used t-Student test for independent trials. The interdependencies of particular parameters in the groups were examined by means of Pearson correlation co-efficient and linear regression. The assumed hypotheses were verified on the significance level of $p < 0.05$.

Results

Electrophoretic dissection of proteins contained in cellular sub-fractions and metallothionein immunoidentification.

By performing protein dissection on polyacrylamide gel (PAGE), we proved that in all the sub-fractions there were proteins showing mobility similar to that of



* difference significant both ways on the level of 0.05

Fig. 2. The total content of MT (in ug/g of tissue) and their amount in cellular sub-fraction (in ug/g of protein) obtained from brain neoplastic tissues

standard proteins (type 1 and type 2 metallothioneins and aprotynine with the molecular weight of 6.3 kDa). Applying immunoidentifying technique in sub-fractions obtained from brain neoplastic tissues we proved the presence of metallothioneins (Fig. 1).

Quantitative assay of metallothioneins

Quantitative assay of metallothioneins by means of cadmium-haemoglobin method provided information concerning their quantitative distribution in cellular sub-fractions. The results of metallothionein assay in the sub-fractions can be seen in Fig. 2.

Discussion

Although factors inducing the synthesis of MT are known, the mechanisms responsible for MT distribution have not yet been well documented. The synthesis of the MT is induced by the ions of metals, hormones, inflammatory factors, free radicals, physical stress and some pharmacological agents [1,4,14,29].

The intensive expression of intracellular MTs has been shown in many kinds of human and animal neoplasms [13,15,17,20,24,37-39]. The studies have shown that the following factors induce the synthesis

of MT in the neoplasm cell: tumor necrosis factor (TNF), interferon α , interleukin-1, interleukin-2) [34].

The synthesis and intracellular distribution of MT is a very important aspect in oncology, because these proteins not only indicate a protective role in environmental factors, but also are responsible for the cell's resistance to pharmacological medications [30].

It follows from the studies conducted that metallothioneins are present in all the examined cell sub-fractions. In astrocytoma G-2 it is the cytosol sub-fraction that is characterised by a highest MT content. Slightly lower MT level is present in the nuclear and mitochondrial sub-fractions and the lowest level is observed in the microsomal sub-fraction. Also an uneven increase of MT content in the particular sub-fractions was noted in GM-4 patients. In this group the total MT level increased by 12% whereas in the cytosol sub-fraction it increased by 17%, in the mitochondrial sub-fraction the level increased by 49% while in the nuclear and microsomal sub-fractions the increase was by 22 and 8%, respectively.

Some other researchers have also found changes in the organisation of the cell distribution MTs during the neoplastic proliferation process [27,36,41-44]. The studies conducted so far, of the intracellular distribution of MT show that some deciding factors of the cell metallothioneins compartmentation exist [27,41].

Nartey et al. [27], Wong and Klaassen [45] and Tsuikawa et al. [41] found, while studying rats, that during the embryonic development and the infancy period of rats metallothioneins are localised mainly in the nuclear sub-fraction. MT content in the nuclear sub-fraction of their livers was the highest shortly after birth to become lower and lower with time up to the moment where it became typical of adults.

The significance of these changes in MTs distribution remained unclear. Such a localisation was at first thought to protect the proteins against degradation [27]. Then it turned out that large quantities of MT in the nucleus were connected with increased demand for zinc during the period of intensive synthesis of nucleic acids. Subsequent studies confirmed the latter hypothesis [18,31,46]. It was also proved that MT concentration in the nucleus of hepatocytes was higher during liver tissue repair, too.

Literature does not provide us with reports concerning the conditions in which MT translocate

to mitochondrial sub-fraction. In our research we stated considerably noticeable translocation of MT to this sub-fraction both in breast cancer [16].

The presence of MT in mitochondria also is justified because of their antyoxidant function. In the respiratory chain apart from four-electron reaction a slight one electron reaction of oxygen reduction takes place (consumption 1-4% general oxygen) in the process of which reactive oxygen species are created, whose precursor is the superoxide anion radical.

Simpkins et al. [36] examined *in vitro* the influence of MT on mitochondrial function. The authors proved that MT-1 (appearing in stress) modifies oxygen consumption in mitochondria isolated from rat liver. The above mentioned authors stated that the primary result of MT action is de-polarisation of internal mitochondrial membrane which is followed by increase in permeability, the result of which is a swelling of mitochondria.

The influence of metallothioneins on the transport of electrons in the respiratory chain is possible thanks to thiolic clusters – SH in MT, which may be electron donors for cytochrome C or for other electron acceptors. The authors have also shown that the effects of MT influence on mitochondria were hindered by cyclosporin – the compound responsible for the sealing of internal mitochondrial membrane.

The essential role in the process of metallothionein removal is played by lysosomal sub-fraction. It turned out while applying different inhibitors for cathepsins that the greatest role in the process is held by cathepsin B. Also examined was the decomposition speed of MT depending on whether it was a free protein (apometallothionein) or a protein containing metals. The former was faster affected by decomposition when compared to zinc- or cadmium-bound metallothionein (where it was stated that Cd-MT decomposed slower than Zn-MT). The research indicate a possibility of protective influence of metals on metallothionein decomposition processes. For instance, metal content of at least 5 eq Zn/mol of protein drastically inhibits metallothionein decomposition [23].

Conclusions

1. The highest concentration of metallothionein was found in cytosol fraction.

2. The significant changes in the distribution of metallothionein was found in the mitochondrial fraction.
3. The changes in the distribution of MT may affect the metabolism of a neoplastic cell.

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