in vivo *30*: 271-278 (2016)

Expression of Metallothionein and Vascular Endothelial Growth Factor Isoforms in Breast Cancer Cells

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Abstract. *Background: Metallothioneins (MTs) are low-molecular-weight and cysteine-rich proteins that bind heavy metal ions and oxygen-free radicals. MTs are commonly expressed in various tissues of mammals and are involved in regulation of cell proliferation and differentiation, and may be engaged in angiogenesis. Expression of MTs has been studied in many cancer types, especially breast cancer. The research results indicate that MTs may play important, although not yet fully known, roles in cancer angiogenesis. The aim of this study was to analyze the level of gene expression of selected MT isoforms induced with zinc ions in correlation with vascular endothelial growth factor (VEGF) isoforms in in vitro models of breast cancer. Materials and Methods: The studies were carried out in three breast cancer cell lines (MCF-7, SK-BR-3, MDA-MB-231). An epithelial cell line derived from normal breast tissue (Me16c) was used as a control. The levels of expression of selected MT isoforms and selected genes involved in angiogenesis were studied with real-time PCR. Results: Expression of different MT isoforms was induced by zinc ions to differing degrees in individual breast cancer cell lines. An increase in the expression of some MT isoforms was associated with a slight increase in the level of expression of VEGFA. Conclusion: The research results may indicate certain correlation between an increased expression of selected MT isoforms and a pro-angiogenic factor VEGF in specific types of breast cancer cells.*

Metallothioneins (MTs) are cysteine-rich, low-molecular-weight proteins found in human tissues and organs in four main isoforms (MTI, -II, -III and -IV) (1-3). The differences between particular isoforms refer mainly to their structure, localization and their level of expression, as well as to the mechanisms of their induction (4). In humans, the most commonly expressed is MTI isoform (which has a number of sub-isoforms MT1A, -1B, -1E, -1F, -1G, -1H and -1X), as well as MT2A isoform (2, 5). MTs are involved in the maintenance of normal cellular metal ion homeostasis, as well as in detoxification of heavy metal ions due to the presence of its thiol groups (1, 2). Moreover, MTs regulate proliferation, differentiation and apoptosis of cells. They are involved in free-radical deactivation, and therefore in protection against oxidative stress, as well as in regulation of regenerative and immunological processes (1, 6, 7). MTs also play a significant role in angiogenesis, *i.e.* formation of new network of blood vessels based on already existing ones (7, 8). It was shown that the reduction of expression of MTs in vascular endothelial cells resulted in inhibition of cell proliferation and migration, thus preventing formation of a new blood vessel network (9). On the other hand, MT overexpression or even administration of exogenous MTs into an area of injury resulted in significantly faster tissue regeneration (10, 11). It was also shown that MT expression in vascular endothelial cells was associated with the level of expression of many pro-angiogenic factors (10, 12). In line with the increase of MT level, induction was observed of vascular endothelial growth factor (VEGF), which is the most important pro-angiogenic factor determining initiation of angiogenesis (10).

The VEGF family consists of five isoforms, among which VEGFA, -B and placental growth factor (PGF) are involved in angiogenesis, and VEGFC and -D in lymphangiogenesis (13). The most important factor regulating expression of VEGFs is hypoxia-inducible factor (HIF1α). Under conditions of insufficient tissue oxygenation, HIF1α is activated and translocated from the cytoplasm to the cell nucleus, where it induces expression of VEGF (14). VEGF stimulates proliferation and inhibits apoptosis of endothelial

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Key Words: Metallothioneins, MT, angiogenesis, vascular endothelial growth factor, VEGF, breast cancer.

0258-851X/2016 $2.00+.40

cells. It is also responsible for an increase in vascular permeability during initiation of angiogenesis, which in turn facilitates migration of endothelial cells (15).

There are numerous ongoing studies on the expression of MT in various cancer types. It has been shown that the level of expression of individual MT isoforms differs significantly and depends on cancer type, as well as on the stage of tumor development and the grade of differentiation (16-18). It is considered that during the initial stages of cancer development, MTs may play a suppressor role, thereby protecting the cell against cytotoxic and genotoxic factors, while during later stages, MTs promote tumor growth by their proliferation-stimulating and apoptosis-inhibiting properties (6, 16, 17). Breast cancer was one of the first tumor types studied for the level of MT expression. In most cases, an increase of MTI/II was observed, that was associated with faster tumor progression, more frequent metastasis to lymph nodes, and poorer prognosis (16-20). The level of MT expression was closely associated with the grade of histological malignancy, and increased expression of MT was shown in less differentiated tumors. Moreover, correlation between increased MT expression and reduced efficacy of chemotherapy was found (19).

Previous studies have showed that MTs may play an important role in physiological angiogenesis (6). In many types of cancer, including breast cancer, high MT expression indicated that MTs may also take part in cancer cell-induced angiogenesis (6). It was shown that the formation of blood vessel networks within a tumor determines the supply of sufficient oxygen and nutrients essential for further growth of the cancer and enables metastasis (21). Taking into account that adequate tumor vascularization determines its progression and spread, determination of the potential role of individual MT isoforms in cancer angiogenesis seems of importance.

The aim of this work was to analyze the level of gene expression for selected MT isoforms in correlation with the most important pro-angiogenic factors of the VEGF family (A, B, and C) in selected breast cancer cell lines. Many previous experiments showed that MT expression might be induced by metal ions, particularly by zinc ions (Zn^{2+}). In this study, we attempted to evaluate whether an increase of expression of individual MT isoforms following Zn^{2+} stimulation is associated with changes in expression of pro-angiogenic genes at the mRNA level.

Materials and Methods

Cell line. Three breast cancer cell lines were used in the experiments, representing types of tumors of increasing aggressiveness (MCF-7, SK-BR-3, MDA-MB-31), and a normal cell line – immortalized breast epithelial cell line (Me16c). All cell lines were provided by the American Type Culture Collection (LGC Standards, Lomianki, Poland). The cells were cultured in an incubator at 37˚C under 5% CO_2. Culture media (Lonza, Walkersville, MD, USA) contained 10% fetal bovine solution (FBS) and 1% mixture of streptomycin and L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The cells were passaged with the use of TrypLE™ (Gibco).

Zinc accumulation. In order to determine if Zn^{2+} is transported into the cancer cells, Zinquin reagent (MelliTech, Grenoble, France) was used. Zinquin is quinoline-derived fluorescent dye which can form a complex with Zn^{2+} because of two nitrogen atoms in the molecule. Zinquin has the ability to cross the cell membrane and selectively bind Zn^{2+} ions, which facilitate their detection within the cell. In the experiment, model breast cancer cells were used (MCF-7 cell line). The cells were seeded in 8-well slides and pre-incubated in medium with Zn^{2+} at 75 μM for 24 h at 37˚C, followed by 30 min with Zinquin reagent (30-60 μM). The controls were cells not treated with Zn^{2+} and incubated with Zinquin reagent, as well as cells treated with Zn^{2+} that were not incubated with Zinquin. Once the incubation was finished, the slides were washed with phosphate-buffered saline (PBS; Lonza) and coverslips were applied. The preparations were analyzed using a BX51 fluorescence microscope and CellF software to assess specimens qualitatively (Olympus, Tokyo, Japan).

Extraction of total RNA and reverse transcriptase reaction. Cells incubated in medium enriched with Zn^{2+} (75 μM) were harvested successively after 6 and 24 h of incubation. Cells incubated in medium without additional Zn^{2+} were used as the control. Hydrated zinc sulphate ($ZnSO_4 \cdot 7H_2O$; Sigma-Aldrich, St Louis, MO, USA) dissolved directly in culture medium was used in the experiments. Suitable cytotoxicity tests (sulforhodamine B protein stain assay, data not shown) were used to determine the optimal, subtoxic concentration of Zn^{2+} to be added to the medium. Total RNA was isolated from cell pellets with the use of RNase Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The concentration and purity of obtained RNA was determined based on spectrophotometric measurements made with NanoDrop (Thermo Scientific, Thermo Fisher Scientific). Reverse transcriptase reaction was carried out on RNA material isolated from cell pellets, with the use of High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Thermo Fisher Scientific).

Real-time polymerase chain reaction (PCR) analysis. Real-time PCR reactions were carried out with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), that included polymerase and reaction buffer, as well as TaqMan Gene Expression Assay including a mixture of commercially available primers suitable for *MT1E, -1F, –1G, -1X* and *-2A*, and *VEGFA ,-C* and *-D* genes, and fluorescence TaqMan minor groove binder probes (all reagents were provided by Applied Biosystems). Reactions were conducted in 384-well plates with 7900HT Fast Real-Time PCR System apparatus from Applied Biosystems. Actin (*ACTB*) was used as an endogenous control. Cells that had not been stimulated with Zn^{2+} were used as the calibrator. Results obtained by real-time PCR were processed according to the $\Delta\Delta C_T$ (21) method, using $2^{-\Delta\Delta Ct}$ to determine the final level gene expression normalized against the endogenous control (actin), and refer to the value of calibrator (cells that were not stimulated with Zn^{2+} ions).

Statistical analysis. Statistical analysis of results obtained with real-time PCR was performed with Prism 5.0 (GraphPad, La Jolla, CA,

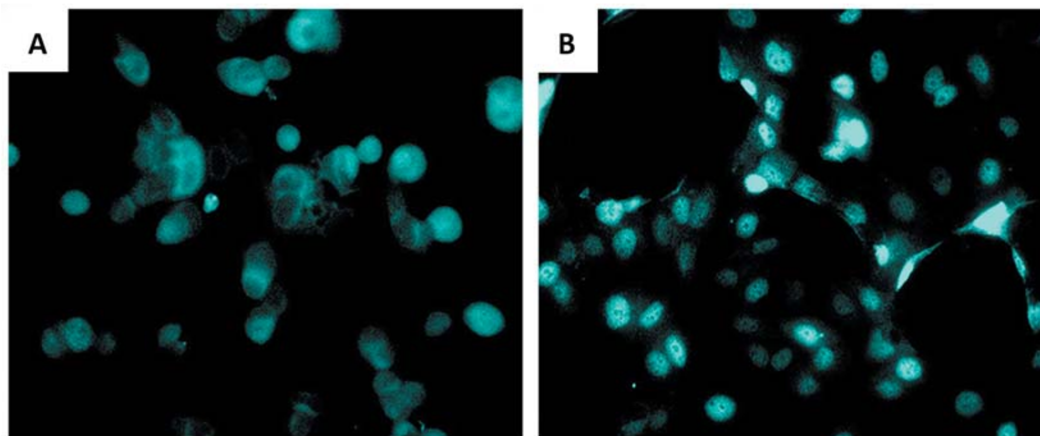

Figure 1. *MCF-7 cell line incubated in zinc-enriched medium (75 μM) and with Zinqiun reagent at 30 μM (A) and 60 μM (B). Fluorescent signal was localized inside the cells. The higher the concentration of Zinqiun reagent used, the more intense was the fluorescence.*

USA). In order to compare the differences between mean relative quantities of mRNA values for studied genes in individual groups, Kruskall–Wallis test and *post hoc* Bonferroni test were used. Spearman's rank correlation test was used for correlation analysis. All results were statistically significant when $p<0.05$.

Results

Analysis of Zn^{2+} ion accumulation. Zinquin reagent enabled the detection and determination of Zn^{2+} localization. Zn^{2+} ions accumulated inside the cancer cells and their concentration in culture medium was found to be significantly lower. Culture media contained trace amounts of Zn^{2+}, resulting in a low level of fluorescence. In the case of cells cultured in medium enriched in Zn^{2+}, the fluorescent signal was much more intense and indicated Zn^{2+} accumulation in cells. The higher the concentration of Zinquin reagent, the more intense was the fluorescence observed in cells (Figure 1) at the same concentration of Zn^{2+} (75 μM) in medium. The lack of fluorescence in the case of cells incubated with Zn^{2+} and not treated with Zinquin excludes the possibility of non-specific fluorescence being caused by culture medium enrichment in Zn^{2+}.

Expression of the MT isoforms. It was observed that there are many ways in which zinc may affect the expression of particular MT isoforms in selected cell lines. In normal breast epithelial cell line Me16c, the strongest expression was observed for *MT1G* (Figure 2A). Its expression was increased significantly after 6, as well as 24 h of incubation with Zn^{2+}. Zinc ions also induced expression of other isoforms, however, their levels were significantly lower in comparison with *MT1G* (Figure 3A). It seemed that among all isoforms, *MT2A* was of the least importance and its expression stayed at the lowest level, and was induced by Zn^{2+} ions only to a small degree.

In the case of MCF-7 breast cancer cell line, the predominant MT isoform was *MT1G* (Figure 2B), similarly to Me16c cells. Zn^{2+} stimulated expression of other MT isoforms at a very low level (Figure 2B). In most cases, the mRNA level of the different isoforms was increased after 6 hours of incubation with Zn^{2+}, while after 24 h, it had relatively decreased. The expression of all investigated MT isoforms was maintained at a very low level in comparison to that in the normal Me16c cell line.

Expression of the MT isoforms in both SK-BR-3 and MDA-MB-231 cancer cell lines was within a similar range. In SK-BR-3 cell line, the highest level of expression following Zn^{2+} stimulation was observed for *MT1F*, as well as *MT1E* and *MT1X* (Figure 3C). *MT2A* was stimulated by Zn^{2+} to a smaller extent, whereas *MT1G* remained at a very low level and was not induced by Zn^{2+} (Figure 2C).

In MDA-MB-231 cancer cell line, Zn^{2+} stimulated *MT1G* to the highest degree in comparison to all other MT isoforms (Figure 2D). Expression of *MT1E* and *MT1F* was stronger than that of isoforms *MT1X* and *MT2A* (Figure 3D). The level of *MT1E, -F* and *MT2A* increased after 6, as well as after 24 h of incubation, while the level of *MT1X* expression after 24 h was significantly reduced relative to that after 6 h.

To summarize, *MT1G* seems to be the dominant MT isoform expressed after induction by Zn^{2+} and its level of expression after zinc stimulation was the highest in comparison with all other isoforms in the case of Me16c, *MCF-7* and MDA-MB-231 cell lines. One exception was the SK-BR-3 cancer cell line, where the level of MT1G expression was very low, and that of *MT1E, MT1F* and *MT1X* was dominant.

Expression of VEGF isoforms. The expression of investigated pro-angiogenic factors was altered to different extents in the individual cell lines following Zn^{2+}

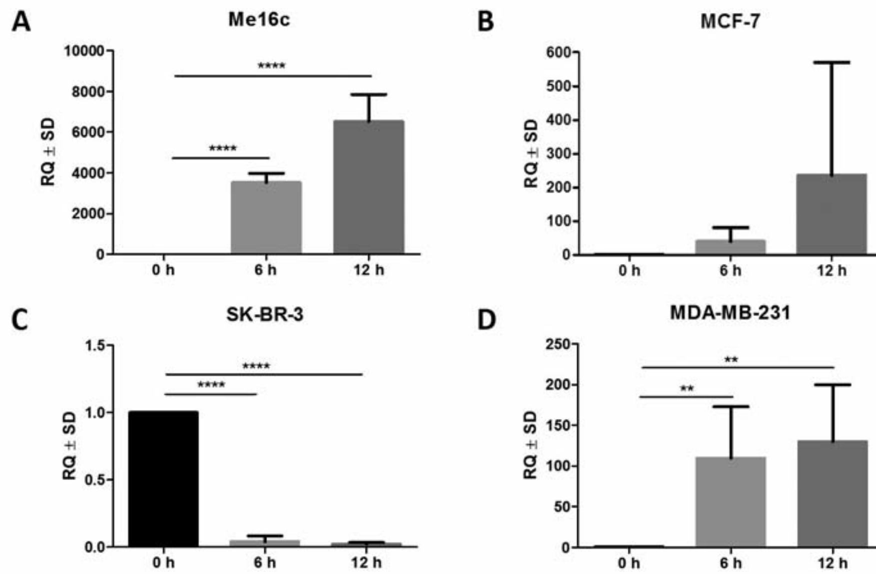

Figure 2. *The level of mRNA expression for metallothionein 1G (MT1G) in normal Me16c cells (A), and MCF-7 (B), SK-BR-3 (C) and MDA-MB-231 (D) breast cancer cell lines. **p<0.01, ****p<0.0001. RQ: Relative quantity; SD: standard deviation.*

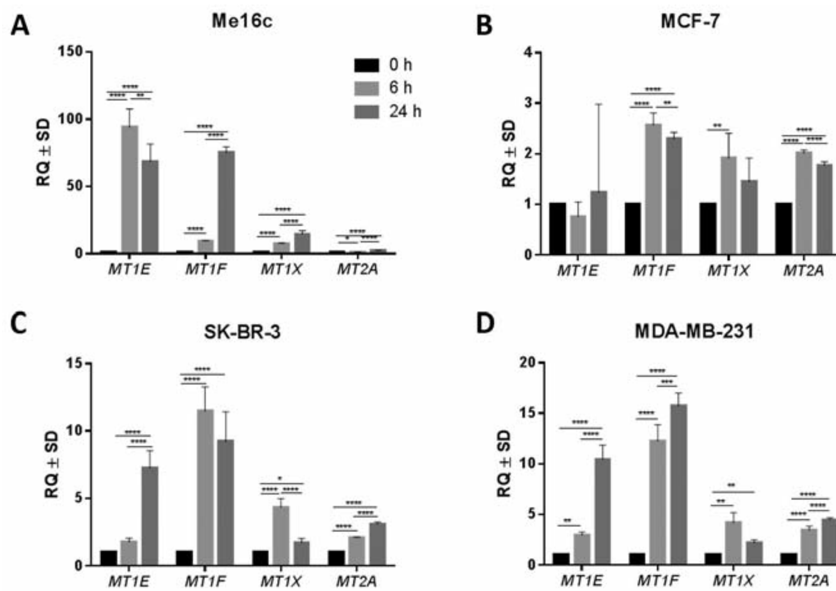

Figure 3. *The level of mRNA expression for selected metallothionein (MT) isoforms in normal Me16c cells (A), and MCF-7 (B), SK-BR-3 (C) and MDA-MB-231 (D) breast cancer cell lines. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. RQ: Relative quantity; SD: standard deviation.*

stimulation. In the case of the normal Me16c cell line (Figure 4A), the level of *VEGFA* and *VEGFC* did not change significantly following stimulation by Zn^{2+}, whereas expression of *VEGFD* increased mainly after 6 h of incubation. In the MCF-7 cell line, zinc had only a small effect on the expression of VEGFs (Figure 4B). Among all VEGFs, only *VEGFA* was stimulated by Zn^{2+}. In the case of the SK-BR-3 cell line, both *VEGFA* and *VEGFC* were slightly stimulated by zinc ions (Figure 4C). In the MDA-MB-231 cell line, the level of *VEGFA* expression also did not increase significantly and slight stimulation by Zn^{2+} was only observed for *VEGFA* (Figure 4D).

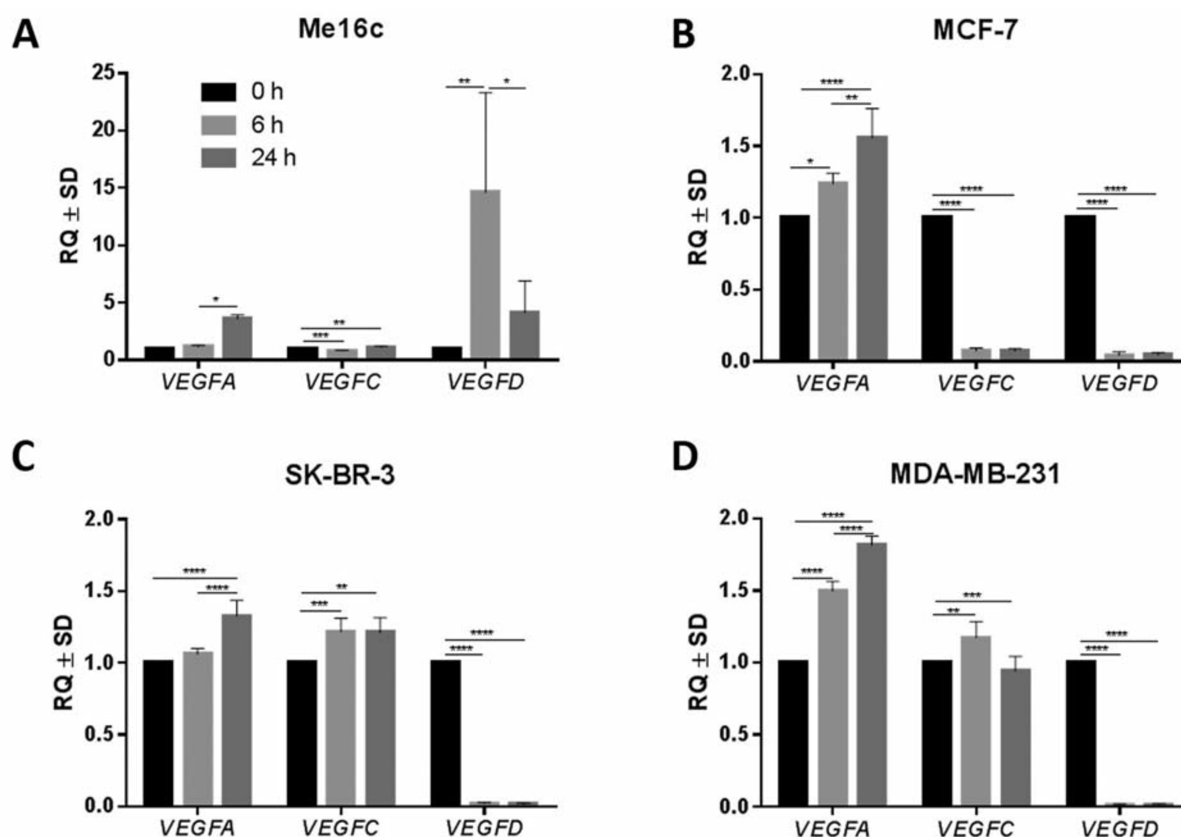

Figure 4. *The level of mRNA expression for vascular endothelial growth factor (VEGF) isoforms in normal Me16c cells (A), and MCF-7 (B), SK-BR-3 (C) and MDA-MB-231 (D) breast cancer cell lines.* *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. *RQ: Relative quantity; SD: standard deviation.*

Correlation analysis of gene expression. Spearman's rank correlation analysis was also performed for the expression of *VEGFA* and *VEGFC* with MT isoforms (Table I). Correlation analysis for the Me16c cell line showed that *MT1G, MT1X* and *MT2A* positively correlated with *VEGFA*, whereas *MT1F, MT1G* and *MT2A* positive correlated with *VEGFC*. In the MCF-7 cancer cell line, only *MT1X* positively correlated with VEGFC. On the other hand, in the SK-BR-3 cell line, *MT1E* and *MT2A* were positively correlated with *VEGFA*. In the case of the most malignant cell line, MDA-MB-231, expression of *MT1E, MT1F* and *MT2A* isoforms was positively correlated with *VEFGA*.

Discussion

Induction of MT expression by metal ions, including Zn^{2+}, has been well documented in various cell lines (23-27), at both mRNA and protein levels. However, MT overexpression induced by Zn^{2+} in breast cancer cell lines has not been analyzed to our knowledge. Experiments conducted here with the use of the Zn^{2+}-selective fluorescent marker Zinqiun

Table I. *Spearman's correlation analysis between expression of vascular endothelial growth factor A and C (VEGFA and VEGFC) and metallothionein (MT) isoforms in selected cell lines.*

	Spearman's correlation	
Cell line, isoform	r	*p*-Value
Me16c		
VEGFA vs. MT1G	0.815	0.0022
VEGFA vs. MT1X	0.876	0.0002
VEGFA vs. MT2A	0.935	<0.0001
VEGFC vs. MT1F	0.800	0.0031
VEGFC vs. MT1G	0.954	<0.0001
VEGFC vs. MT2A	0.762	0.0039
MCF7		
VEGFC vs. MT1X	0.755	0.0045
SK-BR-3		
VEGFA vs. MT1E	0.944	<0.0001
VEGFA vs. MT2A	0.643	0.0240
MDA-MB-231		
VEGFA vs. MT1E	0.790	0.0022
VEGFA vs. MT1F	0.863	0.0006
VEGFA vs. MT2A	0.937	<0.0001

showed that breast cancer cells accumulate Zn^{2+} from culture medium, that may result in overexpression of specific genes in those cells.

Previous studies have shown that among main MT isoforms, only expression of MTI/II is induced by metal ions, while MTIII and MTIV are relatively insensitive to such stimulation (1, 2, 5). Differences in this type of regulation of individual sub-isoforms within MTI/II group by metal ions are still insufficiently understood. From the limited research performed so far, one may conclude that individual MTI and -II isoforms are stimulated by metal ions differently, and this regulation varies depending on the cell type (28). Our study showed that there are five functional MTI/II isoforms expressed in the three breast cancer cell lines analyzed after Zn^{2+} stimulation: *MT1E,-F,-G,-X* and *MT2A*. No expression of *MT1B* and *MT4* was found. This is consistent with the results obtained by Thirumoorthy *et al.*, showing the lack of expression of these isoforms in breast cancer cells (29). Therefore it seems that *MT1B* and *MT4* are not induced by Zn^{2+} (2, 5). Moreover, no expression of MT3 was observed in our study after Zn^{2+} stimulation.

Expressions of the investigated MT isoforms were time-dependent and differed from one another, which was also shown by other authors (27, 30). In the case of some isoforms, the level of expression of mRNA began to rise significantly no earlier than after 24 hours of incubation with Zn^{2+}, which is exemplified by *MT1E* in all investigated cancer cell lines. However, for most of the analyzed isoforms, significant changes in the level of expression were observed as early as after 6 hours of incubation with zinc. The highest level of expression among Zn^{2+}-induced isoforms was found for *MT1G*, both in the normal epithelial breast cell line Me16c, and in two cancer cell lines: MCF-7 and MDA-MB-231. These results suggest that *MT1G* is the dominant isoform, with the highest increase of expression upon Zn^{2+} stimulation. Similar results were obtained while carrying out research on thyroid papillary carcinoma cell line stimulated by cadmium ions – among all induced functional isoforms, *MT1G* was the one exhibiting the highest stimulation by ions of this metal (29). Similar results were also shown in studies on cadmium-stimulated lymphocytes, where once again, *MT1G* was indicated as the isoform stimulated to the highest degree (27). This may suggest that *MT1G* is the MT isoform most sensitive to stimulation by zinc and cadmium ions and that it is responsible for the observed increase in the level of MTI/II proteins in various cell types. From research on the other MT isoforms, it is suggested that the differences in expression of individual isoforms in response to stimulating factors are due to point changes in TATA sequences within the gene promoter regions (27). Regulation of expression of *MT1F* has been investigated and it was shown that it is induced by cadmium, and zinc, as well as copper (31). In the case of the promoter of *MT1A* gene, it was shown that it is stimulation by cadmium ions only, while the promoter of the gene for *MT2A* is sensitive to stimulation by cadmium, zinc and glucocorticoids (27, 32). The results of the present study suggest that the promoter of *MT1G* is more sensitive to stimulation by Zn^{2+} than are the other isoforms, which is consistent with observations of other authors (33).

SK-BR-3 cancer cell line representing the human epidermal growth factor receptor 2 (HER2)-positive molecular type of breast cancer (34) was the only exception in our experiment. In the case of SK-BR-3 cells, *MT1G* was not stimulated by zinc ions and dominant isoforms were *MT1F, MT1E* and *MT1X*. Such different regulation of expression of MT1G between the investigated breast cancer cell lines may be associated with specific modifications within regulatory sequences for *MT1G*, which are characteristic for cells of HER2-positive breast cancer only. In some cancer cells, it was shown that MT expression could be silenced by hypermethylation within the promoter (17). In one of the latest findings, it was shown that the level of expression of *MT1G* gene in liver cancer is lowered, most probably due to methylation within the gene promoter (35). Similar mechanism may also be present in the case of the SK-BR-3 breast cancer cell line.

According to the literature data, *MT2A* is the dominant isoform in cells of liver cancer (36). Our research suggests that this isoform did not show such strong response upon Zn^{2+} stimulation as the other studied isoforms. The level of expression of *MT2A* isoform, both in normal cells stimulated by Zn^{2+} ions and in cancer cells, was lower than the level for most of the other isoforms, and was comparable in the case of MCF-7 cell line.

There is evidence showing differences in the profile of expression of *MT1E* in different breast cancer types according to the presence of estrogen receptor (ER) (16, 17, 36). Significantly higher *MT1E* expression was observed in ER-negative *vs.* ER-positive cancer cells (37). This observation was confirmed by studies carried out on cell lines, as well as on tissue material from patients. The level of ER expression in breast cancer is a very important prognostic factor associated with poorer prognosis, as ER-negative tumors are not susceptible to hormone therapy (36, 38). Transformation of breast cancer cells from type ER-positive to ER-negative seems to be a very important stage in cancer progression. It is suggested that in ER-negative cells, it may be *MT1E* which plays the role of proliferation-stimulating factor previously played by estrogen (17, 37). We observed that in the MCF-7 ER-positive cell line, the level of *MT1E* in cells stimulated by Zn^{2+} was much lower than in both the ER-negative cell lines (SK-BR-3 and MDA-MB-231). The results obtained are therefore consistent with observations described by others (36, 39).

Angiogenesis in breast cancer remains a subject of many studies. Many pro-angiogenic factors have been described that are overexpressed in cancer cells, and which contribute

to the initiation of cancer angiogenesis. Among the most important proteins analyzed are transcription factor HIF1α and VEGF, specific for epithelial cells (40). The level of HIF1α is associated with the grade of cancer differentiation and tumor proliferative potential. In more invasive breast cancer, the level of HIF1α increases, which is also related with changed expression of VEGF. A number of authors showed that an increased level of VEGF correlates with poorer prognosis and is associated with higher resistance to chemotherapy (39, 40).

In the case of VEGF, we showed that expression of mRNA for *VEGFA* was slightly increased in all investigated cell lines subjected to Zn^{2+} stimulation. This suggests that an increase of MT expression upon Zn^{2+} stimulation is associated with the induction of *VEGFA* expression at the mRNA level, that is confirmed by other authors (41, 42). Moreover, expression correlation analysis for individual MT isoforms and *VEGFA* showed that depending on the cell line tested, expression of different MT isoforms positively correlated with *VEGFA* expression.

Based on our research, one can conclude that relations between increased expression of individual MT and VEGF isoforms are very complex and are probably strictly dependent on cell type. It can be assumed that in different types of breast cancer cells, some MT isoforms may stimulate an increase in the expression of *VEGFA* in a minor way. Further research is needed to explain the exact mechanisms of regulation of expression of genes associated with angiogenesis by MT in various types of breast cancer and analyze the potential importance of those relationships in cancer progression.

Acknowledgements

This work was funded by scientific grant no. ST 540 of Wroclaw Medical University.

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Received January 20, 2016
Revised March 11, 2016
Accepted March 16, 2016