

THE INFLUENCE OF SILVER NANOPARTICLES ON THE PROCESS OF EPITHELIAL TRANSITION IN THE CONTEXT OF CANCER METASTASES

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ABSTRACT

Background: Exposure to nanoparticles (NPs) can occur in a variety of occupational situations. Ultrafine particles of natural and anthropological origin toxicity has been described in epidemiological studies. Meanwhile, the risks associated with NPs exposure are not comprehensively assessed. A wide spectrum of NPs toxicity has been demonstrated, mainly through the induction of oxidative stress and inflammatory mediators. Among the newly described mechanisms of NPs toxicity is the induction of fibrosis via the epithelial-mesenchymal transition (EMT), which is also a key mechanism of cancer metastasis. The effect of NPs on EMT in the context of metastasis has not been sufficiently described so far, and the results of studies do not allow for the formulation of unambiguous conclusions. Therefore, the aim of the work was to determine the biological activity of silver NPs against MDA-MB-436 triple-negative breast cancer cells. **Material and Methods:** Reverse transcription real-time polymerase chain reaction was used to examine mRNA expression of EMT markers. The QCM Chemotaxis Cell Migration Assay and QCM ECMatrix Cell Invasion Assay were used to assess the level of cell migration and invasion. The tumor growth factor beta 1 (TGF-beta 1) secretion was determined by the immunoenzymatic method using the Human TGF-beta 1 ELISA Kit. **Results:** Silver nanoparticles (AgNPs) cause a statistically significant increase in relative expression of all tested mesenchymal EMT markers – cadherin 2, vimentin, matrix metalloproteinase 2 and matrix metalloproteinase 9. At the same time, reduction of epithelial cadherin 1 expression was observed. The level of MDA-MB-436 migration and TGF-beta 1 secretion was slightly increased in AgNPs-treated cells, with no influence on invasion potential. **Conclusions:** Potentially prometastatic effect of AgNPs encourages further work on the safety of nanomaterials. *Med Pr Work Health Saf.* 2023;74(6):541–8.

Key words: breast cancer, metastasis, silver nanoparticles, nanotoxicology, epithelial-mesenchymal transition, MDA-MB-436

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INTRODUCTION

Workers may come into contact with nanoparticles (NPs) primarily at the production stage, but also at various stages of use and supply chain. In 2021, based on *in vitro* studies and animal models, the United States National Institute for Occupational Safety and Health derived recommended exposure limits for silver nanoparticles (AgNPs), titanium oxide NPs and carbon nanotubes, while these values for other nanomaterials are still unknown. Unfortunately, knowledge about the harmful effects of engineered NPs,

despite many scientific reports, it still contains serious gaps and has not yet been comprehensively assessed. Meanwhile, the harmfulness of ultra-fine particles of natural and anthropogenic origin, such as erionite, asbestos, crystalline silica, particulate matter and diesel engine exhaust, has been extensively documented. *In vitro* studies, as well as *in vivo* in animal models, showed a wide spectrum of toxic effects of NPs primarily by induction of oxidative stress and inflammatory mediators, which in turn can lead to e.g., apoptosis, cell cycle disorders, genotoxicity, mutagenic and epigenetic effects [1].

Among the described mechanisms of toxicity of nanomaterials, the induction of organ fibrosis is also mentioned, the main mechanism of which is the epithelial-mesenchymal transition (EMT). The EMT is a process involving a cascade of events that enable epithelial cells to acquire a mesenchymal phenotype and consequently increase their migratory potential. This process plays a key role not only in fibrosis but also in the metastasis of malignant tumors. Therefore, it seems justified to assess the effect of NPs on the modulation of EMT in cancer models. This issue has not been sufficiently researched so far. Results that can be found in the available literature are often divergent and do not allow for the formulation of unambiguous conclusions. The research was carried out on MDA-MB-436 triple-negative breast cancer cells, which express numerous EMT factors and appropriate signaling pathways related to this process. Meanwhile, breast cancer is the most frequently diagnosed type of female cancer in the world. Unfortunately, although early detection allows for long-term survival, many patients are diagnosed when distant metastases exist. Triple-negative breast cancers are characterized by high aggressiveness and poor response to treatment, and consequently have a poor prognosis.

The aim of the study was to evaluate the biological activity of AgNPs against triple-negative human breast cancer cells MDA-MB-436 in the context of the epithelial-mesenchymal transition. The authors examined the effect of AgNPs on the level of EMT mRNA markers – cadherin 1 (CDH1), cadherin 2 (CDH2), vimentin (VIM), matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9). The authors measured the effect of AgNPs on the migration and invasion of MDA-MB-436 cells. Finally, the authors investigated the exogenic secretion of tumor growth factor 1 beta (TGF beta 1), which is one of the most important factors inducing EMT.

MATERIAL AND METHODS

Cell culture

The MDA-MB-436 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM:F12) growth medium at a volume ratio of 1:1 supplemented with 10% fetal bovine serum, 200 mM L-glutamine and antibiotics mixture (10 000 U/ml penicillin and 10 mg/ml streptomycin). Cells were passaged when they reached about 80% confluence, 2–3 times per week, in a ratio of 1:2–1:3. Experiments were carried out between 10 and 15 passages. According to the manufacturer's instructions, cells were cultured at 37°C and in

an atmosphere containing 5% CO₂ (Cell Lines Service GmbH, Eppelheim, Germany).

AgNPs preparation

Commercially available pristine AgNPs nanopowder with an average particle size of 20 nm and 200 nm (Plasma Chem GmbH, Berlin, Germany) were used. The AgNPs solutions were prepared according to the methodology described in the previous authors' manuscript [2,3]. In brief, AgNPs (2 mg/cm³) stock solutions were prepared by dispersing 2 mg of AgNPs in 800 µl of distilled water, then sonicated on ice using a Vibra-Cell 130 probe (Sonics & Materials Inc., Newton, USA). Then, 100 µl of a 10× concentrated PBS solution and 100 µl of a 15% bovine serum albumin solution were added. Intermediate solutions were prepared by diluting the solution described above in culture medium. The AgNPs solutions were always prepared immediately before the experiment. The choice of concentrations used in further experiments – 10 µg/cm³ and 50 µg/cm³ was made on the basis of previous results of cytotoxicity studies of the AgNPs tested against MDA-MB-436 cells with the NR and MTT tests [4].

Real-time polymerase chain reaction (qRT-PCR)

After 24-h stimulation with various concentrations of AgNPs (10 µg/cm³ and 50 µg/cm³), the expression of selected epithelial and mesenchymal EMT markers was determined in MDA-MB-436 cells. The CDH2, VIM, MMP2, MMP9 and CDH1 mRNAs expression in AgNPs-treated MDA-MB-436 cells has been determined using reverse transcription real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from the cells using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. The quality and quantity of total isolated RNA was measured using a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, United States). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc., Waltham, United States) qPCR was performed using PowerUp SYBR™ Green Master Mix (Thermo Fisher Scientific, Inc., Waltham, United States). Primer pairs specific for human CDH2 (HP100018), VIM (HP100124), MMP2 (HP100168), MMP9 (HP100367), CDH1 (HP100067) and β-actin (HP100001) were used (Sino Biological Inc., Beijing, China). A melting curve was prepared for each pair of primers, to verify that non-specific amplification reaction products were not formed. Applied Biosystems® 7500 Fast Real-Time PCR System thermal cycler was used in this study (Applied

Biosystems, California, USA). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, using β -actin as endogenous control.

QCM Chemotaxis Cell Migration Assay

The QCM Chemotaxis Cell Migration Assay, based on the Boyden chamber principle of operation, was used to assess the effect of the AgNPs on the level of migration of MDA-MB-436 cells. According to the manufacturer, the pore size – 5 μm is suitable for testing the migration of certain types of cancer cells, including MDA-MB-436 cells. In the study 20 nm AgNPs and 200 nm AgNPs were used at 2 concentrations – 10 $\mu\text{g}/\text{cm}^3$ and 50 $\mu\text{g}/\text{cm}^3$, and the cells were incubated in the migration chamber for 24 h at 37°C and 5% CO_2 .

QCM ECMatrix Cell Invasion Assay

The QCM ECMatrix Cell Invasion Assay was used, to assess the effect of AgNPs on the relative level of MDA-MB-436 cells migration. This kit uses a reconstituted basement membrane matrix of proteins derived from the Engelbreth Holm-Swarm (EHS) mouse tumor. In the study 20 nm AgNPs and 200 nm AgNPs were used at 2 concentrations – 10 $\mu\text{g}/\text{cm}^3$ and 50 $\mu\text{g}/\text{cm}^3$, and the cells were incubated in the migration chamber for 24 h at 37°C and 5% CO_2 .

TGF-beta 1 ELISA

The TGF-beta 1 release was analyzed in the culture media of MDA-MB-436 cells exposed to 20 or 200 nm AgNPs (10 $\mu\text{g}/\text{cm}^3$ and 50 $\mu\text{g}/\text{cm}^3$) for 24 or 48 h using Human TGF-beta 1 ELISA Kit (Sigma-Aldrich, Saint Louis, USA) following the manufacturer's protocol.

Statistical analysis

All experiments were performed in at least 3 independent replicates. Statistical analysis of the obtained results was performed using GraphPadPrism 9.0 software (GraphPad Prism Software, San Diego, California, USA). The Shapiro-Wilk test was used to check the normality of data distribution. To calculate statistical significance, 1-way ANOVA was used, followed by Tukey's test as a *post hoc* test. Test results were expressed as mean values \pm standard deviation ($M \pm SD$). Differences with a $p < 0.05$ were considered statistically significant.

RESULTS

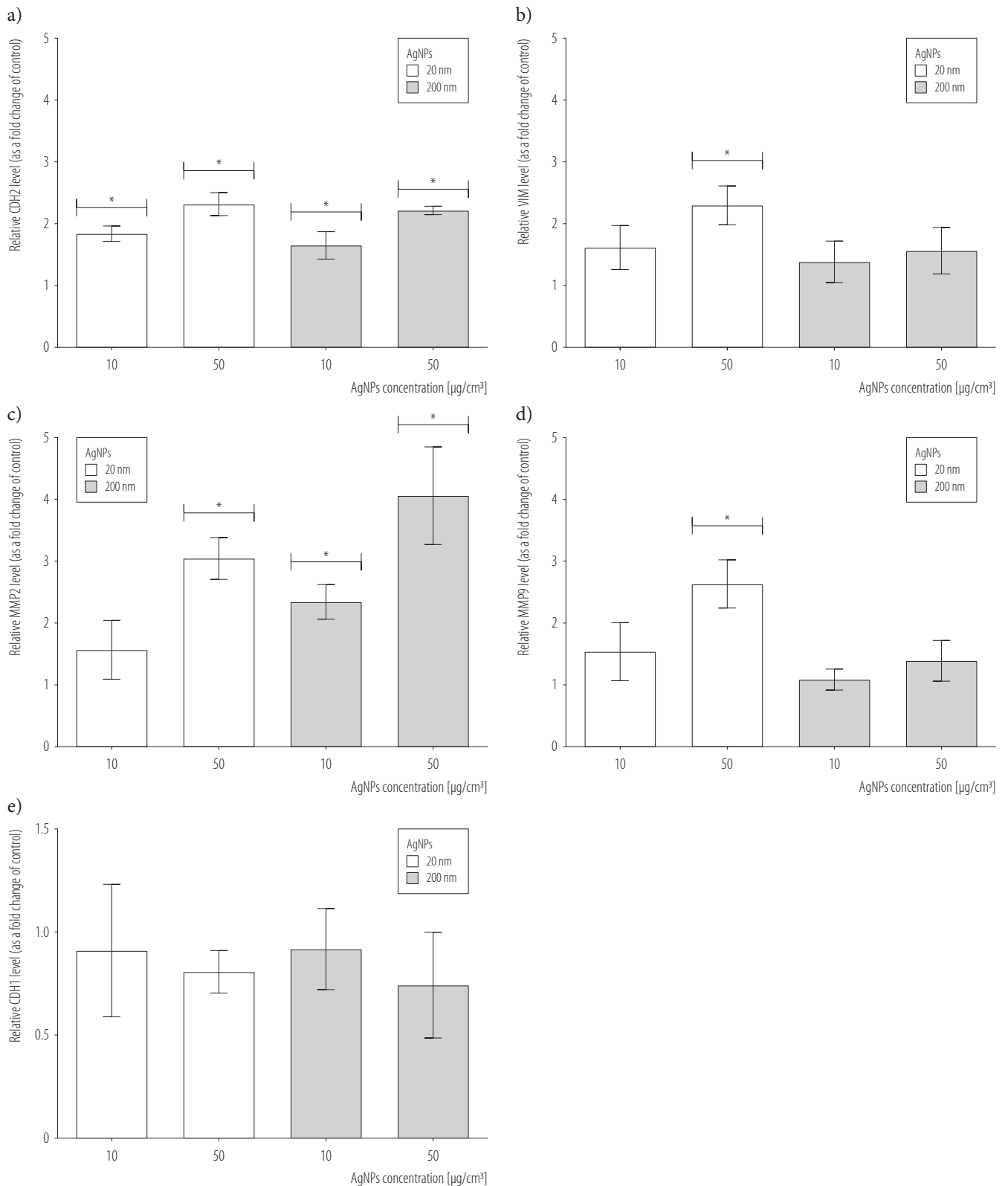
As part of the experimental work, the effect of AgNPs on the level of mRNA expression of EMT process markers

was determined, then the level of migration and invasion of MDA-MB-436 cells, and the results were supplemented with the assessment of paracrine secretion of TGF-beta 1.

The influence of AgNPs on the level of mesenchymal EMT markers – CDH2, VIM, MMP2 and MMP9, as well as epithelial CDH1 was examined. Incubation of cells led to a statistically significant, concentration-dependent increase in CDH2 expression. Levels were similar for 20 nm and 200 nm AgNPs (Figure 1a). Moreover, 20 nm AgNPs significantly increased relative VIM expression levels in a concentration-dependent manner (Figure 1b). The strongest effect was noted when examining the effect of AgNPs on the level of MMP2 expression. Both tested sizes of AgNPs strongly increased the relative level of MMP2 expression in a concentration-dependent manner. The study showed that 50 $\mu\text{g}/\text{ml}$ 20 nm AgNPs resulted in a 3-fold increase in expression, and 200 nm AgNPs an almost 4-fold increase in the relative expression level of MMP2 mRNA (Figure 1c). The relative expression of the last mesenchymal marker tested, MMP9, was also increased in MDA-MB-436 cells incubated with 20 nm AgNPs (Figure 1d). The study also showed that 20 nm AgNPs as well as those with a size of 200 nm reduced the expression of CDH1 – an epithelial EMT marker. However, due to high standard deviations, probably associated with high CT values in the qRT-PCR study, these relationships were not statistically significant (Figure 1e).

The next stage of the research was to determine the impact of the AgNPs on the level of migration and invasion of MDA-MB-436 cells. The relative level of migration of 20 nm AgNPs was only slightly increased in cells incubated with 20 nm AgNPs at a concentration of 50 $\mu\text{g}/\text{ml}$. The relative migration observed in cells incubated with 200 nm AgNPs remained within the control values and did not reach statistical significance (Figure 2a). Moreover, AgNPs did not significantly affect the level of invasion of MDA-MB-436 TNBC cells at any of the tested concentrations and sizes (Figure 2b).

The last stage of the research was to determine the effect of AgNPs on the exogenous secretion of TGF-beta 1. The research showed only a slight effect of 20 nm AgNPs after 48 h incubation of MDA-MB-436 cells. The relative level of TGF-beta 1 secretion by cells treated with 200 nm AgNPs at all tested concentrations and incubation times was close to control values and not statistically significant (Figure 3).



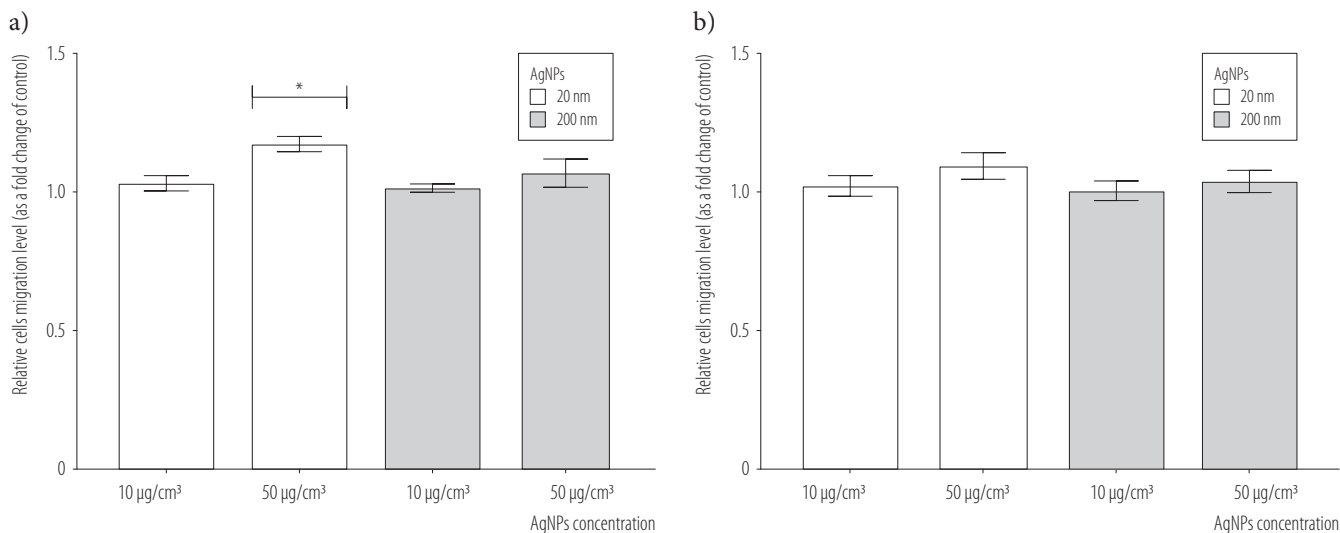
Measured by real-time quantitative reverse transcription PCR.

The graph presents the fold change of marker level calculated for samples incubated with AgNPs relative to the untreated control; β -actin was used as a reference gene.

Data are expressed as $M \pm SD$ ($N = 3$).

* Statistical significance $p < 0.05$.

Figure 1. Relative mRNA expression level of a) cadherin 2 (CDH2), b) vimentin (VIM), c) matrix metalloproteinase 2 (MMP2), d) matrix metalloproteinase 9 (MMP9) and e) cadherin 1 (CDH1) in MDA-MB-436 cells treated with silver nanoparticles (AgNPs)



Measured by QCM Chemotaxis Cell Migration Assay and QCM EMatrix Cell Invasion Assay. The graph presents the fold change of migration/invasion level calculated for samples incubated with AgNPs relative to the untreated control. Data are expressed as M±SD (N = 3). * Statistical significance p < 0.05.

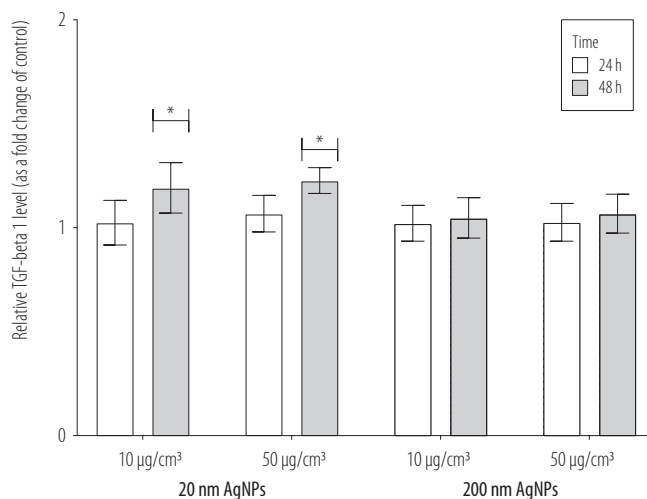
Figure 2. Relative level of silver nanoparticles (AgNPs) treated MDA-MB-436 cells a) migration and b) invasion

DISCUSSION

In the literature, we can find a description of a wide spectrum of toxic effects of nanoparticles, including the induction of oxidative stress and inflammation mediators. Among the newly described mechanisms of toxicity, the induction of organ fibrosis and the related participation in the modulation of the epithelial-mesenchymal transition process, which is also crucial in the metastasis of malignant tumors, are mentioned. The studies carried out in this manuscript aimed to determine the effect of AgNPs on EMT modulation in MDA-MB-436 cells. It was shown that in MDA-MB-436 cells incubated with AgNPs, the expression of mesenchymal markers – CDH2, VIM, MMP2 and MMP9 increased, while the expression of epithelial CDH1 decreased (Figure 1a–e), which indicates the EMT-stimulating effect of AgNPs in the studied model. Nevertheless, the influence of the tested AgNPs on the level of migration and invasion of MDA-MB-436 cells was only slightly increased (Figure 2a and 2b), as well as the endogenous secretion of TGF-beta 1 (Figure 3).

The obtained results are, according to authors' knowledge, the first ones assessing the effect of AgNPs on the level of EMT markers in MDA-MB-436 cells. Roszak et al. incubated TNBC cells of the MDA-MB-231 line with AgNPs, showing increased invasion, without a significant impact on migration, but the effect of AgNPs on EMT markers was not determined [5]. In addition, the MDA-MB-231 model incubated with AgNPs

showed an increased percentage of binuclear cells, characteristic of EMT [6]. In estrogen-dependent breast cancer cells of the MCF-7 line, AgNPs increased migration and caused a significant increase in the level of EMT markers – β-catenin and Snail transcription factor [7]. In studies prepared by Martin et al., mammary gland-derived MCF10A cells treated with AgNPs showed decreased CDH1 while increasing CDH2 expression and



Measured by Human ELISA TGF-beta 1 Kit. The graph presents the fold change of TGF-beta 1 level calculated for samples incubated with AgNPs, relative to untreated control. Data were expressed as M±SD (N = 3). * Statistical significance p < 0.05.

Figure 3. The TGF-beta 1 level in growth media of MDA-MB-436 cells treated with silver nanoparticles (AgNPs)

TGF-beta 1 secretion [8,9]. As mentioned before, EMT is also a key mechanism in the process of organ fibrosis. Assar et al. described the effect of AgNPs on the induction of liver fibrosis [10]. In addition, AgNPs stimulated EMT also in cells derived from the respiratory system – BEAS-2B [8,9,11,12] and A549 [13], as well as derived from the digestive system – HepG2 [14], Caco2 [15] and CCD18-Co [8,9].

Several studies have reported results that contradict authors' own. None of these were performed on MDA-MB-436 cells. Mouse TNBC cells of the 4T1 line treated with AgNPs showed reduced migration and invasion at high concentrations of AgNPs, but the expression of EMT markers did not change [16]. The AgNPs also reduced the migration of breast cancer cells of the MCF7 [17] and A549 [18] lines, however, the level of EMT markers was not determined in these studies. Authors believe that the different results may be due to a number of factors. First of all, it is difficult to compare research conducted on different cell lines, even those derived from breast cancer, because the composition of their surface receptors and mutation status is of key importance. Moreover, it should be remembered that the biological activity of NMs depends on numerous factors and should be assessed individually for each experimental model. It may vary depending on parameters such as the culture medium used, exposure time, concentration, distribution and aggregation state of particles, and above all on physicochemical parameters, which include among others size, shape or type of functionalization [19]. Finally, it is worth noting that 2 sizes of AgNPs were used in these studies – those with a diameter of 20 nm and 200 nm. In the case of 20 nm AgNPs, their statistically significant effect on almost all tested parameters was proven, while 200 nm AgNPs had no effect, for example in the case of cell migration or TGF beta secretion. The observed differences in the effect of AgNPs of different sizes are consistent with observations from the literature. It is described that the biological activity of AgNPs increases as their diameter decreases. Taking into account the same mass, smaller NMs have a larger specific surface area on which interactions with cellular components can occur. The smaller size also facilitates the entry of NMs into the cell [20].

Summary, studies have shown that AgNPs increase the expression of mesenchymal EMT markers in the investigated cell model. Simultaneously, only a slight increase in the relative level of cell migration and TGF-beta 1 secretion was observed, without affecting their invasion potential. The obtained results are preliminary

studies. However, they encourage further, more detailed analyzes to assess the mechanisms causing changes in the expression of EMT markers by the tested AgNPs. It is worth confirming the mRNA expression of the tested EMT markers at the level of their corresponding proteins. Interestingly, the TGF-beta 1 factor, which is a key regulator of EMT and also a strong profibrogenic factor [21], did not show a significant increase in secretion as a result of the action of AgNPs in authors' studies. This indicates an alternative mechanism responsible for the increase in the expression of mesenchymal markers in the tested system. Several signaling pathways are involved in EMT in breast cancer, not only the one related to the TGF-beta 1 factor, but also Notch, Wnt, tumor necrosis factor alpha (TNF- α), Hedgehog and receptor tyrosine kinases (RTKs) [22]. Further studies are recommended to assess the involvement of those potential signaling pathways, e.g. it is worth assessing the change in β -catenin phosphorylation and its possible intracellular translocation. In further stages, it is also worth analyzing the expression of EMT transcription factors, including Snail, Slug, Twist and ZEB1/2 proteins.

CONCLUSIONS

As a result of experimental work carried out in this manuscript, the biological activity of AgNPs against TNBC cells of the MDA-MB-436 line was demonstrated for the first time in the literature, indicating that they increase the expression of mesenchymal markers, which may suggest the stimulation of the EMT process. The results presented in this manuscript enrich the current state of knowledge on the safety of nanomaterials, focusing on their potential prometastatic effect, which has not yet been comprehensively described in the literature. This seems important in the light of significant exposure of workers to NMs and the fact that distant metastases are the most common cause of cancer-related deaths.

Author contributions

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Research methodology: Magdalena Matysiak-Kucharek

Collecting material: Magdalena Matysiak-Kucharek

Statistical analysis: Magdalena Matysiak-Kucharek, Krzysztof Sawicki

Interpretation of results: Magdalena Matysiak-Kucharek, Jacek Kurzepa, Lucyna Kapka-Skrzypczak

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