WHOLE-GENOME EXPRESSION ANALYSIS IN THP-1 MACROPHAGE-LIKE CELLS EXPOSED TO DIVERSE NANOMATERIALS

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Abstract

From the perspective of the immune system, nanomaterials (NMs) represent invading agents. Macrophages are immune cells residing in all organs and tissues as the first line of defense. Interactions of macrophages with NMs can determine the fate of NMs as well as their potential toxic effects. In the present study, we compared toxicity of four different types of NMs [NM-100 (TiO_2, 110 nm), NM-110 (ZnO, 20 nm), NM-200 (SiO_2, 150 nm) and NM-300K (Ag, 20 nm)], towards THP-1 macrophage-like cells. Cells were incubated with non-cytotoxic concentrations (1-25 μg/ml) of NMs for 24 hours and microarray technology was used to analyze changes in whole-genome expression. Gene expression profiling revealed a substantially different molecular response following exposure to diverse NMs. While NM-100 did not exert any significant effect on gene expression profile, all other NMs triggered a pro-inflammatory response characterized by an activation of the NF-κB transcription factor and induced expression of numerous chemokines and cytokines. NM-110 and NM-300K further modulated processes such as DNA damage response, oxidative and replication stress as well as cell cycle progression and proteasome function. We suppose that genotoxicity of ZnO and Ag NMs leading to DNA damage and alternatively to apoptosis in THP-1 macrophages is probably caused by the extensive intracellular dissolution of these NPs, as confirmed by TEM imaging.

Keywords: Nanomaterials, toxicity, THP-1 macrophages, gene expression profiling

1. INTRODUCTION

Nanomaterials (NMs) represent foreign objects for the body. As such, they are recognized and processed by immune cells, especially those involved in the innate (non-specific) immune response. Macrophages, as professional phagocytes, represent a crucial component of the innate immunity. In general, macrophages are the first line of defense against invading agents, including NMs. Interactions of NMs with macrophages may determine the fate of NMs (e.g. by initiating NM clearance) as well as toxic effects of NMs (e.g. by triggering inflammation and reactive oxygen species production). As antigen presenting cells, macrophages may also affect the specific immune response. Therefore, macrophages are among the target cells for studying NM toxicity, however, the molecular mechanisms underlying macrophage-mediated toxicity of NMs have not been fully understood yet.

In the present study, we used four NM samples of the different chemical composition obtained from Joint Research Centre (JRC) Nanomaterials Repository. The JRC repository represents a reliable source of

reference NMs with detail characterization provided to partners of EU projects. In this study, we compared effects of soluble ZnO and Ag nanoparticles (NM-110 and NM-300K, respectively), and non-soluble TiO_2 and SiO_2 nanoparticles (NM-100 and NM-200, respectively).

To get insight into molecular events triggered by NMs in macrophages, we analyzed changes in whole-genome expression in THP-1-derived macrophage-like cells exposed to various types of NMs using microarrays. Observed changes can be used to elucidate NM-specific modes of action and mechanisms of potential toxicity. Changes detected on the transcriptional level may serve as predictors of cell and tissue toxicity induced by NM exposure [1].

2. METHODS

2.1. Nanomaterials

Four different types of NMs, NM100 (TiO_2), NM110 (ZnO), NM200 (SiO_2) and NM300K (Ag), were obtained from the European Commission Joint Research Centre (JRC) nanomaterials repository (http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology/nanomaterials-repository). A physico-chemical characterization of the NMs is reproduced in **Table 1**. NM dispersions were prepared in sterile-filtered water supplemented with 0.05% bovine serum albumin by sonication (16 min, 10% amplitude using a 400 W, 20 kHz Branson Sonifier S-450D (Branson Ultrasonics Corp.) equipped with a 13 mm disruptor horn. Immediately before exposure, sonicated dispersions were diluted in cell culture media (RPMI supplemented with 10% heat inactivated fetal bovine serum) to the required concentrations.

Table 1 NMs characteristics (as provided by the supplier)

	Chemical composition	Crystalinity (XRD)	Average diameter ± SD (TEM) [nm]	Shape (prevailing) (TEM)	Specific surface (BET) [m^2/g]
NM-100	TiO_2	anatase	190 ± 6	spherical/elipsoidal	10
NM-110	ZnO	zincite	150 (SEM)	hexagonal/cubic	12
NM-200	SiO_2	amorphous silica	50 ±51	spherical/elipsoidal	189
NM-300K	Ag	metallic silver	17±3	spherical	NA

2.2. Transmission electron microscopy

The intracellular distribution of NMs was analysed in ultrathin sections of resin-embedded cells using transmission electron microscopy (FEI Morgagni 268 equipped with Olympus Mega View III Digital Camera).

2.3. Cytotoxicity

Cytotoxicity was assessed using MTS assay (Promega) according to the recommended procedure to estimate toxic doses of all NMs. Results were expressed as the percentage of viable cells relative to unexposed controls. Non-cytotoxic doses 10, 15 and 25 µg/ml were selected for gene expression profiling. Due to the increased cytotoxicity of NM-300K, an additional non-cytotoxic dose 1 µg/ml of this NM was applied.

2.4. Gene expression analysis

RNA from samples was extracted (NucleoSpin RNA II, Macherey-Nagel), processed (In vitro transcription, Illumina), labeled with fluorescent dye and hybridized onto HumanHT-12 v4 Expression Beadchips (Illumina) to analyze gene expression (scanning of fluorescence intensities were carried out by iScan, Illumina). All steps were performed according to manufacturer's recommendations.

2.5. Statistical analysis

Raw data were summarized by GenomeStudio software (Illumina) and further processed in R statistical environment using a Limma package. Differential gene expression analysis revealed significantly upregulated (↑) and downregulated (↓) genes meeting criteria for significance (adjusted *p*-value < 0.01 and absolute fold change > 1.5). Pathway analysis was performed using gene set enrichment analysis (https://toppgene.cchmc.org/).

3. RESULTS

3.1. NM uptake

TEM imaging confirmed internalization of NM-100 and NM-200 by THP-1 cells and their localization in cell vesicles, such as phagosomes, lysosomes and endosomes (**Figure 1A** and **1C**). NM-110 particles were not detected inside cells (**Figure 1B**). Upon NM-300K uptake, structures were found inside the cells, that might represent partly dissolved NMs (**Figure 1D**).

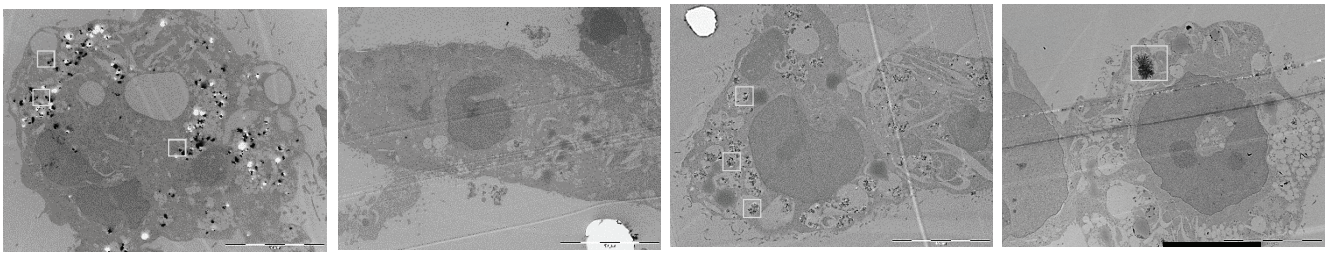

Figure 1 Cellular uptake. TEM images of THP-1 cells exposed 24h to 25 µg/ml of: A) NM-100 B) NM-110 C) NM-200 and D) NM-300K. White squares represent intracellular localization of NMs. Bar 10 µm

3.2. Cytotoxicity

No tested dose of NM-100 exhibited any effect on cell viability. On contrary, doses 50 and 100 µg/ml of all the other NMs (NM-110, NM-200 and NM-300K) significantly reduced cell viability. Decrease of cell viability was also observed for the dose 25 µg/ml of NM-300K.

3.3. Gene expression profiling

3.3.1. NM-100

No statistically significant changes in gene expression were detected under the given criteria.

3.3.2. NM-110

The lower concentration of NM-110 (10 µg/ml) induced expression of numerous metallothioneins (*MT1M*↑, *MT1E*↑, *MT1F*↑), cysteine-rich proteins that bind metals. Upregulation of *SLC30A1*↑, a specific exporter of ions further indicates an excretion of xenobiotic metals. Incubation of THP-1 cells with a higher dose of NM-110 (25 µg/ml) resulted in a high number of mostly upregulated genes involved in regulation of cell cycle, synthesis of DNA, pro-inflammatory response, base excision/nucleotide excision/mismatch repair, replication stress response and p53 signaling. Upregulation of genes encoding mini-chromosome maintenance proteins (MCM complex) as well as other genes such as *POLA1*↑, *POLE2*↑, *PCNA*↑, *GINS2*↑, and a variety of cyclins and cyclin-dependent kinases strongly suggest stimulated replication and cell cycle phase transition (G1/S, G2/M). Activation of checkpoints (*MAD2L1*↑, *WEE1*↑, *CHEK1*↑) to control cell cycle progression and proteasome-ubiquitination system (*PSMA6*↑, *PSMB8*↑, *PSMB10*↑) was also detected. Another class of modulated genes

refers to an enhanced repair capability (*LIG1*↑, *FEN1*↑, *EXO1*↑, *POLD4*↓) and replication stress response (*RFC3*↑, *RFC4*↑, *RPA3*↑) probably elicited due to an increased rate of DNA synthesis. Activation of the p53 signaling pathway (*SIAH1*↑, *GADD45B*↑, *IGFBP3*↑) indicates a DNA damage response and cancer prevention alternatively leading to apoptosis. Elevated expression of *FOS*↑, *JUN*↑ and *EGR1*↑ is typical for immediated-early stress response regulating expression of various genes through MAPK signaling cascade. Immune response was characterized by upregulation of key component of NF-κB protein complex (*NFKB1*↑) consequently controlling transcription of various genes, increased expression of numerous chemokines and cytokines (*CCL2*↑, *CCL3*↑, *CCL5*↑, *CXCL6*↑, *CXCL12*↑, *CCL3L3*↑, *TNF*↑, *ICAM1*↑, *IL1B*↑, *IL8*↑) or suppressed interferon alpha/beta signaling (*IFITM3*↓, *MX1*↓, *STAT2*↓, *HLA-B*↓, *IRF8*↓)

3.3.3. NM-300K

Both lower concentrations of NM-300K (1 and 10 µg/ml) induced expression of metallothioneins and other genes contributing to metal binding and excretion (*MT1E*↑, *MT1F*↑, *MT1G*↑, *MT1X*↑, *HMOX1*↑, *SLC30A1*↑) while the dose 10 µg/ml itself additionally activated genes involved in Keap-Nrf2-mediated response to oxidative stress (*HMOX1*↑, *GCLM*↑) and other rapidly activated stress sensors associated with ATF-2 transcription network (*ATF3*↑, *DUSP1*↑, *IL8*↑). Besides increased expression of metallothioneins, the dose 15 µg/ml modulated transcription of numerous chemokines and chemokine receptors such as *CCR2*↓, *CCL2*↑, *CCL3L1*↑, *CCRL2*↑, *CXCL16*↑, *CCL3L3*↓, *CX3CR1*↑ or *IL8*↑. Alterations in metabolism of selenoproteins which are effective in reducing oxidative stress and antioxidant defense were further detected (*TXN*↑, *TXNRD*↑*1, CCL2*↑, *KYNU*↑, *SEPW1*↑, *LDLR, PRDX1*↑, *SOD1*↑, *SOD2*↑). Expression of various chemokine and other peptide ligands binding receptors (*CCR2*↓, *CCRL2*↑, *EDNRB*↑, *CX3CR1*↓) as well as several proteasomal components involved in protein degradation (*PSMA3*↑, *PSMB8*↓, *PSMD12*↓, *PSMD6*↑, *POMP*↑, *PSMD14*↑) were also affected. Strongly elevated expression of genes encoding proteasomal proteins was also evident upon the exposure to the highest dose (25 µg/ml) and contributed to the modulation of various pathways. Besides the activation of proteasomal complex, degradation of cell cycle proteins or regulation of mitotic cell cycle and apoptosis, these genes were also involved in G1/S DNA damage response, checkpoint trigger and p53 stabilization (together with *CDKN1A*↑) or in processing of soluble antigens destined for cross presentation by endosomes. Numerous genes related to mitosis were downregulated while others were upregulated suggesting inhibition of mitosis (*CDKN1A*↑, *CENPA*↓, *CENPF*↓, *TYMS*↓, *KIF23*↓, *KIF2C*↓, *CDCA8*↓, *CCNA2*↓, *KIF20A*↓, *LMNB1*↓, *CCNB2*↓, *CDC25B*↓, *TOP2A*↓, *PCM*↓*1, DCTN3*↓), regulation of DNA accessibility (*HIST2H2AA3*↑, *HIST1H2BK*↑) or stress response (*HSP90AA1*↑). Enhanced expression of cytokines and chemokines (*CCL2*↑, *CCL3L1*↑, *CCL20*↑, *ICAM1*↑, *CCL3L3*↑, *IL1B*↑, *IL8*↑, *IL15*↑) as well as other affected genes contributing to iron uptake and transport (*HMOX1*↑, *ATP6V1D*↑, *ATP6V1H*↑, *ATP6V0E2*↑, *ATP6V1A*↑, *ATP6V1C1*↑, *ATP6V0B*↑), Toll-like receptor signaling (*TLR8*↑, *CCL3L1*↑, *FOS*↑, *CCL4L2*↑, *CCL3L3*↑, *IFNAR2*↑, *TBK1*↑, *IL1B*↑, *IL8*↑, *IRAK1*↑, *IRF5*↑, *STAT1*↑, *JUN*↑, *NFKBIA*↑, *CD86*↑), activation of NF-κB (*NFKBIA*↑, *RELB*↑), activation of AP-1 signaling (*FOS*↑, *JUN*↑) or oxidative stress response and antioxidant defense (*HMOX1*↑, *TXNRD1*↑, *MGST1*↑, *FOS*↑, *SOD01*↑, *SOD2*↑, *NQO1*↑, *GPX1*↑, *GSR*↑) were also identified.

3.3.4. NM-200

Various cellular signaling pathways (insulin, PI3K-Akt, FoxO family, mTOR, IL-6 or FSH signaling) as well as ion channels transport and glucocorticoid receptor regulatory network were affected due to the upregulation of multifunctional serum- and glucocorticoid-induced kinase 1 (*SGK1*↑) following exposure to the lower dose of NM-200 (10 µg/ml). The hHigher dose of NM-200 (25 µg/ml) induced expression of various cytokines and chemokines (*CXCL10*↑, *CXCL16*↑, *CCL20*↑, *CCL3L3*↑, *IL8*↑, *CCL4L2*↑, *ITGAL*↓) contributing to inflammation mediated by cytokine and chemokine signaling, Toll-like receptor signaling or local acute inflammatory response. We further identified activation of NF-κB and TNFR2 signaling (*TRAF1*↑, *PLAU*↑, *TNFAIP3*↑, *CCL4L2*↑, *IL8*↑, *CXCL10*↑, *CCL20*↑) and ATF-2 transcription factor network (*PLAU*↑, *ATF3*↑, *IL8*↑).

4. DISCUSSION

In the present study, we compared transcriptional changes following exposure to four NMs with different physicochemical properties in human macrophage-like THP-1 cells. After 24h exposure we observed internalized NM-100, NM-200 and NM-300K but not NM-110 nanoparticles. We suppose that these nanoparticles might be rapidly dissolved following cellular uptake, however also NM-300K nanoparticles were partly dissolved and probably released intracellularly toxic ions as supported by gene expression data. The lower concentration of both NM-110 and NM-300K induced expression of metallothioneins and solute carrier proteins indicating an effective detoxification and excretion of dissolved Zn^{2+}/Ag^{+} ions [2]. Higher doses of NM-110 and NM-300K resulted in modulation of various other processes. We found enhanced expression of genes encoding proteasomal proteins. The proteasome is abundant in cells and regulates the majority of cellular processes. Metal NMs has been identified as modulators of proteasome activity thus posing a toxicity concern [3]. The dose 25 µg/ml NM-300K further elicited transcription of genes involved in oxidative stress response and antioxidant defense and also in G1/S checkpoint activation, p53 stabilization and inhibition of mitosis strongly suggesting DNA damage response signaling. Excessive ROS production and consequent DNA damage have been proposed as an important mechanism of toxicity of numerous NMs [4, 5]. On the other hand, THP-1 cells upon NM-110 exposure exhibited transcriptional changes related to increased replication rate, activation of checkpoints, induction of mini-chromosome maintenance protein-coding genes, replication stress response and enhanced repair capability. This possibly indicates DNA damage involving impairment of DNA replication and loss of chromosomal stability [6]. Numerous studies have evidenced a toxic effect of NMs related to the immune system. NMs toxicity was intimately linked to oxidative stress and induction of inflammation through release of inflammatory cytokines. Metallic NMs are widely known to exert their toxicity through the induction of ROS [7]. The NMs-induced increase in ROS levels could lead to activation of NF-κB family transcription factors, critical regulators of multiple biological functions. NF-κB transcription factors are responsible for induction of pro-inflammatory gene expression leading to the synthesis of inflammatory cytokines such as IL-6, IL-1β and TNF-α, chemokines (IL-8), adhesion molecules, growth factors and enzymes [8]. Although we observed activation of NF-κB and enhanced expression of various chemokines and cytokines by all toxic NMs (NM-110, NM-200 and NM-300K), a diversity in immune responses was also detected. NM-200 induced expression of genes involved in TNFR2 signaling indicating regulation of a number of critical cell functions including cell proliferation, survival, differentiation, and apoptosis through modulation of NF-κB [9]. NM-300K activated genes associated with Toll-like receptors, key pattern recognition molecules that initiate downstream signaling events [10], or genes related to interferon-alpha/beta signaling which has been linked to NF-κB activation and promotion of cell survival [11]. In contrary, NM-110 suppressed expression of genes participating in IFN alpha/beta signaling possibly suggesting an opposing effect on NF-κB function and consequent cell fate.

5. CONCLUSIONS

We found diverse gene expression profiles induced by various NMs indicating the distinct mechanism of toxicity in THP-1 macrophage-like cells. A dose-response toxic effects were exerted by all NMs excepting NM-100 whose threshold toxic dose was probably much higher than all the tested doses. While the lower doses of toxic NMs activated mostly excretion- and detoxification-related genes, the higher doses activated stress response and cellular damage indicating exceeded detoxification capacity. Genotoxicity was characteristic for NM-300K and NM-110, both metal containing NMs which undergo an intracellular dissolution thus causing DNA damage, interference with DNA synthesis and activation of stress response related to disruption of normal cell cycle progress. Importantly, a “Trojan horse” mechanism of metallic NMs including endocytosis-driven cellular uptake followed by lysosome degradation and ion release which is harmful to the intracellular environment has been proposed [12]. NM-300K further triggered strong antioxidant response suggesting the increased formation of ROS while NM-110 rather induced replication stress response indicating different mechanism of DNA damage. Pro-inflammatory response characterized by a widely recognized toxic

mechanism of NMs, was identically modulated by NM-110, NM-200 and NM-300K. Overall, our toxicogenomic approach combining gene expression profiling and physicochemical characterization of NMs may help to elucidate their toxic mechanisms and contribute to risk assessment.

ACKNOWLEDGEMENTS

The authors acknowledge the assistance provided by the Research Infrastructure NanoEnviCz, supported by the Ministry of Education, Youth and Sports of the Czech Republic under Project No. LM2015073. The project was supported by the Operational Program Prague Competitiveness under Project No.CZ.2.16/3.1.00/24507. The Microscopy Centre - Electron Microscopy CF, IMG AS CR is supported by the Czech-BioImaging large RI project (LM2015062 funded by MEYS CR) and by OP RDE (CZ.02.1.01/0.0/0.0/16_013/0001775 "Modernization and support of research activities of the national infrastructure for biological and medical imaging Czech-BioImaging").

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