PH.D. THESIS

INVESTIGATION OF THE ANTI-CANCER ACTIVITY OF GOLD AND SILVER NANOPARTICLES IN P53 DEFICIENT CANCER CELLS AND IN TUMOR METASTASIS MODEL

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1. Introduction

Although new-generation cancer medicines provide good alternatives to conventional cytotoxic compound-based chemotherapy, the worldwide mortality data on cancerous diseases indicate that there is still a constant demand for new therapeutic strategies and for novel pharmaceutical agents to overcome cancer. Due to revolutionary improvements in numerous nanotechnological methods, today it seems possible to develop nano-sized materials for clinical applications as well. Metal nanoparticles represent one of the major classes of nanomaterials, and owing to their unique phyisco-chemical nature they provide an ideal platform for the development of therapeutically useful anti-cancer agents. While silver-based nanoparticles trigger apoptosis efficiently and inhibit tumor cell growth both in *in vitro* and *in vivo* model systems, gold-based nanoparticles are considered as biologically inert moieties and their radio- and photosensitizing activities render them attractive therapeutic tools for cancer treatment modalities. Although several research groups are recently focusing on metal nanoparticle treatment-triggered cellular responses, it is still not exactly understood how the basic physical and chemical properties – such as particle size and chemical composition – influence the interaction of such nanoparticles with living organisms.

To develop new treatment strategies and to identify novel therapeutic targets, it is indispensable to understand cancer at a molecular, cellular and also at tissue organization levels. During cancerous transformation, tumor cells accumulate numerous genetic alterations which allow them to proliferate in an uncontrolled fashion. As a result of this high genetic plasticity, cancer stem cells appear early in the tumor mass and constantly produce cells with novel genomic variations. One of the most important hallmarks of cancer cell development is the ability to avoid programmed cell death. Cancer cells generally achieve this feature by the inactivation of tumorsuppressors, such as p53. Normally, p53 serves as a multifunctional platform to detect cellular impairments, like DNA damage or stress. As a transcriptional activator, p53 induces the expression of various repair genes, the maturation of anti-proliferative miRNAs, and in case of severe cellular damages, p53 has the potential to initiate apoptotic cell death. However, cancer cells without functional p53 can respond to apoptotic stimuli with survival, rendering the chemotherapeutic elimination of malignant cells rather challenging.

Once the constantly proliferating cancer cell population is grounded, cancer cells establish a sophisticated communication network with other cells residing in the neighboring tissues. As a result of an intensive recruiting activity on the part of cancer cells, solid tumors can grow in a highly supportive microenvironment containing various cell types such as fibroblasts, macrophages and even

adipocytes. The most abundant components of the reactive tumor stroma are the re-programmed fibroblast cells, which support cancer cell proliferation via secretion of various soluble growth factors, exosomes and cytokines. Furthermore, owing to the secreted factors and the tissue remodeling activity of cancer-associated fibroblast, invasion of the surrounding tissues and intravasation into blood vessels is facilitated, which might lead to the metastatic spread of the primary tumor.

2. Aims

Given the moderate success rate of conventional chemotherapy in p53-deficient cancer cells, we investigated whether silver nanoparticles have the capability to induce programmed cell death in both p53-expressing and p53-deficient osteosarcoma cells. We also wished to examine whether 5 nm sized and 35 nm sized silver nanoparticles induce cancer cell apoptosis via identical or different intracellular mechanisms. To answer these questions, we treated wild type p53-expressing U2Os and p53-deficient Saos-2 osteosarcoma cells with either 5 nm or 35 nm sized nanoparticles and we compared the apoptotic responses triggered by the applied nanoparticle treatments.

It is well established that the supportive tumor stroma has a determining role in both the invasive feature and the metastatic activity

of the malignant cancer cells. Therefore, targeting the stroma cell-tumor cell crosstalk seems to be a promising strategy to treat cancer and suppress metastasis. In line with this, we decided to investigate whether the presence of metallic nanoparticles in the tumor microenvironment influences the crosstalk between cancerous cells and tumor-associated fibroblasts. Furthermore, we wished to find out whether the chemical composition of the applied metal nanoparticles influences their effect on the communication network within the tumor microenvironment. For this, we employed *in vitro* tumor cell-fibroblast co-cultures and *in vivo* 4T1 metastasis animal models and investigated the effects of gold and silver nanoparticles in these systems. In order to reduce the toxicity, but in the same time exploit the unique apoptotic features of silver nanoparticles, moreover, to take advantage of the biocompatible nature of gold nanoparticles, we synthetized gold-core, silver-shell hybrid nanoparticles and tested their effects on the above described *in vitro* and *in vivo* models.

3. Materials and methods

During our investigation, we applied several *in vitro* based mono- and co-cultures of human and mouse derived cancer and fibroblast cells. Cells were exposed to nanoparticles or treated with various cytotoxic drugs. To determine cellular viability and plasma membrane leakage, MTT and LDH enzyme activity assays were performed. Cell proliferation and migration was investigated using BrdU incorporation and wound healing assays. Flow cytometry experiments were carried out to analyze the apoptotic response of the nanoparticle exposed cells. Caspase 3 cleavage was detected with immunostaining techniques and cytoplasmic Cytochrome c levels were quantified with western blot experiments. Cell surface attached and internalized nanoparticles were visualized by scanning and transmission electron microscopy, while epifluorescence microscopy-based methods were used to measure changes in mitochondrial membrane potential. Gene expression analysis has been accomplished with RT-qPCR measurements and transcriptome analyses was performed using Illumina MiSeq platform based RNA sequencing. To investigate the *in vivo* effects of the metal nanoparticles on tumor growth and metastases, a 4T1 cell-based tumor model was applied in Balb/c mice.

4. Major findings

1. 5 nm and 35 nm sized silver nanoparticles are taken up by osteosarcoma cells, and induce cell death both in p53-expressing U2Os and in p53-defficient Saos-2 cells with comparable degree.

2. Silver nanoparticles can trigger apoptotic cell death in the absence of functional p53.

3. In U2Os cells, 5 nm and 35 nm sized silver nanoparticles stimulate p53 signaling.

4. 5 nm and 35 nm sized silver nanoparticles induce mitochondrial dysfunction in osteosarcoma cells.

5. In the examined osteosarcoma cells, 5 nm and 35 nm sized silver nanoparticles induce apoptotic cell death via the activation of identical molecular pathways.

6. Silver and silver-gold hybrid nanoparticles possess higher cytotoxicity to cancer cells than to non-cancerous fibroblast cells.

7. Silver and gold-silver hybrid nanoparticles decrease the proliferation, migration and invasion of metastatic 4T1 cells, while gold nanoparticles do not affect these functions.

8. Silver and gold-silver hybrid nanoparticles suppress the cancer cell proliferation-promoting activity of tumor-associated fibroblast cells.

9. Silver and gold-silver hybrid nanoparticles induce similar transcriptomic changes in tumor-associated fibroblasts and decrease the expression of several extracellular growth factors.

10. Locally administrated gold-silver hybrid nanoparticles suppress the metastatic activity of 4T1 tumors *in vivo*, and increase the efficacy of intravenous doxorubicin therapy.

5. Discussion

We investigated the biological effects of different metal nanoparticles in p53-deficient tumor cells as well as in *in vitro* tumor stroma and *in vivo* metastasis models. Similarly to the results of other research groups, we verified that smaller, 5 nm sized silver nanoparticles possess higher cytotoxicity than larger counterparts. Additionally, we found that silver nanoparticles have the capability to induce apoptosis-dependent programmed cell death in the absence of the tumor suppressor p53. As conventional cancer therapy often fails to induce cell death in p53-deficient cancer cells, these results indicate the unique chemotherapeutic potential of such nanomaterials. We concluded that 5 nm and 35 nm sized nanoparticles primarily induce cell death through targeting of mitochondrial structure and function. Although we found that smaller nanoparticles are more cytotoxic, the mechanism of the apoptotic action of both 5 nm and 35 nm sized silver nanoparticles is proved to be identical.

We also found, that the cytotoxic features of silver and gold-silver hybrid nanoparticles are cell-type dependent, as we observed higher cytotoxicity in cancer cells than in non-cancerous fibroblasts. Our results indicate, that the stimulation of tumor-associated fibroblast cells with metal nanoparticles could be an ideal therapeutic strategy, since we proved that silver and gold-silver hybrid nanoparticle treatments suppress the cancer-cell promoting activity of such tumor-associated fibroblasts. Our results are also supported by *in vivo* data, as we found that locally administrated gold-silver hybrid nanoparticles inhibit the metastatic spreading of 4T1 tumors in Balb/c mice and could also enhance the therapeutic efficacy of intravenously administrated doxorubicin therapy. By the application of the gold-silver hybrid nanoparticles, the unique anti-cancer potential of silver nanoparticles was exploited while we took advantage of the biocompatible nature of gold nanoparticles. We found that the active component of the tested gold-silver hybrid nanoparticles is the silver shell, as the transcriptomical profiles of silver nanoparticle- and gold-silver hybrid nanoparticle-treated fibroblast cells were largely similar.

With our work, we provide further data on the interaction of metal-based nanomaterials with living cells. Our results give support to the potential application of metal nanoparticles in the clinical practice of cancer treatment and strengthen the fact that these nanoparticles represent ideal platforms for the development of therapeutically useful anti-cancer agents.

List of publications

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