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Superparamagnetic iron oxide nanoparticles exert different cytotoxic effects on cells grown in monolayer cell culture *versus* as multicellular spheroids

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ABSTRACT

The aim of this study was to investigate the interaction of superparamagnetic iron oxide nanoparticles (SPION) with human blood–brain barrier-forming endothelial cells (HBMEC) in two-dimensional cell monolayers as well as in three-dimensional multicellular spheroids. The precise nanoparticle localisation and the influence of the NP on the cellular viability and the intracellular Akt signalling were studied in detail. Long-term effects of different polymer-coated nanoparticles (neutral fluidMAG-D, anionic fluidMAG-CMX and cationic fluidMAG-PEI) and the corresponding free polymers on cellular viability of HBMEC were investigated by real time cell analysis studies. Nanoparticles exert distinct effects on HBMEC depending on the nanoparticles' surface charge and concentration, duration of incubation and cellular context. The most severe effects were caused by PEI-coated nanoparticles. Concentrations above 25 μg/ml led to increased amounts of dead cells in monolayer culture as well as in multicellular spheroids. On the level of intracellular signalling, context-dependent differences were observed. Monolayer cultures responded on nanoparticle incubation with an increase in Akt phosphorylation whereas spheroids on the whole show a decreased Akt activity. This might be due to the differential penetration and distribution of PEI-coated nanoparticles.

1. Introduction

Based on their unique and versatile features, nanomaterials attract widespread interest in both research and industry. In the biomedical field especially magnetic multifunctional nanoparticles (NP) composed of iron oxide cores have various applications in magnet resonance imaging, drug delivery, and hyperthermal antitumour therapy [1–3]. Whereas several iron oxide-based NP are already clinically approved as contrast agents [4], their real biological effect on distinct tissues or individual cells remains unclear. Previous studies have mostly focused on NP biocompatibility within the sense of cytotoxicity [5,6], while their effects on normal cell physiology and signal transduction of human tissue cells inevitably exposed NP upon biomedical application, remain widely unknown [7,8].

Based on their large surface to volume ratio, NP play a special role concerning reactivity and interaction with matrices. These particles may not only be capable of passive interaction with cells and cellular membranes, but may also interfere directly with various membrane receptors, thereby affecting and modulating signalling transduction pathways [9]. The membrane receptor-activated protein Akt, also referred to as protein kinase B (PKB), is a central element within the cellular signalling network [10].

This protein kinase plays a critical role in vital cellular processes, including survival, proliferation, and metabolism. Activated Akt promotes cell survival and inhibits the induction of programmed cell death, also called apoptosis. Thus, pro-apoptotic proteins like caspase-9, Bad, and forkhead transcription factors are inhibited upon Akt phosphorylation, whereas inhibitors of apoptosis such as Birc5, also known as survivin, are stimulated.

However, testing the toxicity of nanoparticles or their influence on cellular signalling in monolayers may deviate from the adequate biological effect *in vivo* due to their loss of tissue-specific properties [11]. Thus, two-dimensional cell cultures show differences in transport conditions and the absence of distinct cell–cell or cell–matrix interactions, respectively, so that contradictory

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results of toxicity studies were achieved between monolayers and *in vivo* animal models [12].

One option to bridge the gap between these two models is the application of multicellular spheroids which represent an *in vitro* 3D cell culture that consists of assembled spherical-arranged cell colonies [13]. These closely packed structures model the physiological 3D architecture of tissues, since they possess a strongly deposited extracellular matrix and biochemical gradients which influence gradient-dependent cellular responses [14]. The extracellular matrix provides cell–matrix interactions and the linking of secreted chemokines, growth factors, and a variety of signalling proteins in matrix-binding forms [14,15]. Another advantage of 3D cultures compared to monolayers is the presence of pronounced intracellular junctions mimicking physiological barriers [16]. These cellular junctions as well as the dense extracellular matrix with small pores affect the transport of drugs, nanoparticles and other compounds by reducing their penetration [12,13,17]. Therefore multicellular spheroids are also suitable as a model to test drug delivery or to investigate the toxicity and the infiltration of nanoparticles through physiological barriers [15].

As the human blood–brain barrier is a critical and sensitive interface necessarily exposed to systemically applied NP, we investigated the NP-induced effect on the cellular viability and the central Akt signalling of human blood–brain barrier-forming cells. For this purpose two distinct *in vitro* cell culture models were established: on the one hand a blood–brain barrier-representing two-dimensional cell monolayer of human brain microvascular endothelial cells (HBMEC) and on the other hand a more complex three-dimensional multicellular spheroidal system composed of HBMEC.

Based on previous literature apart from particles' material composition, size, and shape especially the NP surface charge plays a pivotal role in determining the particular interaction with biological systems as well as their cellular uptake [18–21]. That is why in this study expediently, analysed spherical NPs of similar size were chosen according to their surface charge resulting from different polymer coatings: neutral starch-coated (fluidMAG-D), cationic polyethylenimine-coated (fluidMAG-PEI), and anionic carboxymethyldextran-coated (fluidMAG-CMX) iron oxide cored NP. Results show a concentration-dependent cytotoxic effect of cationic fluidMAG-PEI in both 2D and 3D cellular systems. Regarding Akt signalling pathway analysis a strong global Akt activation is found in cell monolayers especially for the cationic NP, whereas investigations in 3D spheroids reveal deviating outcomes, which might be substantiated in time-dependent spatial distributions and indicating a context-dependant effect of NPs on cellular processes.

2. Experimental methods

All chemicals were purchased by Sigma-Aldrich (Taufkirchen, Germany) or Life technologies (Darmstadt, Germany) if not otherwise stated.

2.1. Cell culture

Human brain microvascular endothelial cells (HBMEC) represent a reasonable and widely used *in vitro* cell culture model of the human blood–brain barrier. The cell line immortalised by the introduction of the SV40 large T antigen [22], was cultured at 37 °C and 5% CO_2 in RPMI 1640 medium supplemented with GlutaMAX™ and 10% foetal calf serum (Biochrom-Seromed, Berlin, Germany) in a humidified atmosphere. Cell cultures with 90% confluency were split in ratios between 1:8 and 1:12 and supplied with fresh medium. Cell cultures were used up to passage 30.

Multicellular spheroids were created with the hanging-drop method [23]. Medium drops with a volume of 20 µl containing 20,000–30,000 cells were disseminated on a petri dish lid (28.3 cm^2mm) and the lid was turned upside down. Due to gravity multicellular spheroids formed at the bottom of each drop within 4–5 days in an incubator at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. Nanoparticles and free polymers

All nanoparticles were provided by Chemicell GmbH, Berlin. They possessed a core made of iron oxide and a shell of differently charged polymers. According to the manufacturer's specifications the hydrodynamic diameters of neutral starch-coated fluidMAG-D, cationic polyethyleneimine (PEI, MW = 750 kDa)-coated fluidMAG-PEI, and anionic carboxymethydextran (CMX)-coated fluidMAG-CMX nanoparticles were 150 nm, 100 nm, and 150 nm, respectively. The hydrodynamic diameters of the fluorochrome-labelled nano-screen MAG/G-D, nano-screen MAG/G-PEI and nano-screen MAG/G-CMX were 150 nm. The cores are additionally covered with a lipophilic dye (ex = 476 nm, em = 490 nm).

2.3. Real time cell analysis

The viability of incubated HBMEC was monitored via real time cell analysis (RTCA) using the xCELLigence system by Roche Applied Science (Mannheim, Germany). This non-invasive approach is based on electronic impedance measurements accomplished by gold microelectrode sensor arrays integrated into each bottom of 16 well E plates. As cells altering the local ion environment and adhering to the electrode-covered surface, act as insulators, relative changes in the measured impedance can be associated with variations in cell number, morphology, degree of adhesion, and cellular viability represented by the dimensionless parameter cell index (CI). Thus, rising numbers of vital cells are accompanied by increasing impedance values, whereas decreasing impedances are associated with cytotoxic events [24].

64,000 cells/cm^2 were seeded in a well of a 16 well E plate, each. After a 30-min sedimentation period cells were monitored in real time at 37 °C in a humidified atmosphere with 5% CO_2. Following 24 h of cultivation the compounds were added to the cells and mixed carefully before RTCA was continued for up to 72 h. Medium-treated cells and cell-free compound-treated wells served as controls.

2.4. Cell lysis and immunoblotting

HBMEC monolayers cultured in 6-well plates (64,000 cells/cm^2, Greiner Bio-One, Frickenhausen, Germany) or a pool of 90 spheres incubated with respective compounds for indicated durations were washed twice with D-PBS, harvested, and lysed in 20 mM HEPES, 150 mM NaCl, 10 mM EDTA (pH 8.0), 2 mM EGTA (pH 8.0), 1% (v/v) Triton X 100, 10 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM Na_3VO_4 supplemented with aprotinin (10 µg/ml), pepstatin (1 µM), leupeptin (10 µM), and PefaBloc (500 µg/ml). After a centrifugation step (20 min at 16,200*g*, 4 °C) total protein was quantified using the Bradford method [25]. Proteins were separated by SDS–PAGE (4–12% Criterion™ XT precast gel, BioRad) and transferred to Immobilon membranes (BioRad Laboratories, Munich, Germany). Immobilised proteins were multistrip-probed with 1000-fold diluted specific antibodies against phospho-Akt (Ser473) and pan-Akt (Cell Signaling Technologies, Heidelberg, Germany). Staining with actin (α,β,γ)-directed antibodies (1:5000 dilution, Santa Cruz Biotechnology, Heidelberg, Germany) served as loading control.