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Effective induction of death in mesothelioma cells with magnetite nanoparticles under an alternating magnetic field

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ABSTRACT

With the objective of finding an avenue for development of magnetic hyperthermia as an effective mesothelioma treatment, the influence of heating by magnetite nanoparticles (MNPs) with a diameter of ~40 nm, which were incorporated into cells and then subjected to AC magnetic field, on induction of cell death was investigated in all three histological subtypes of human mesothelioma cells (*i.e.*, epithelioid NCI-H28, sarcomatoid NCI-H2052, and biphasic MSTO-211H cells). Cellular uptake of MNPs was observed in all cell types, but the amount of MNPs incorporated per cell into MSTO-211H cells was smaller than in NCI-H28 and NCI-H2052 cells. On the other hand, cell death induced by cellular uptake of MNPs was observed specifically in MSTO-211H cells. Hence, when cells are heated by intracellular MNPs under AC magnetic field, a high degree of cell mortality in NCI-H28 and NCI-H2052 cells is induced by the temperature increase derived from the high amount of intracellular MNPs, but the combination of intracellular heating and cell-type-specific toxicity of MNPs induced high rates of cell death in MSTO-211H cells even at a lower temperature. Almost all of the heated cells were dead after 24-h incubation at 37 °C in all histological subtypes. Additionally, higher mortalities were observed in all three types of mesothelioma cells after MNPs-heating, as compared to the heating with a thermostatic bath. Herein, the significance of cellular uptake of MNPs for effectively inducing cell death in mesothelioma has been demonstrated *in vitro*.

1. Introduction

Ferrite nanoparticles with a spinel structure, especially magnetite (Fe_3O_4) nanoparticles (MNPs), are promising materials within the biomedical field and have applications in drug delivery systems, magnetic resonance imaging (MRI), and magnetic hyperthermia [1–8]. The magnetic properties of MNPs depend on their diameter [1,9–12]. The MNPs with a diameter of around 10 nm and >20 nm show superparamagnetic and ferromagnetic behavior, respectively, and possess the advantage of relatively large magnetization derived from their inverse spinel structure. Considering their application in magnetic hyperthermia, a method in which tumor cells are treated with heat generated from MNPs under an AC magnetic field (AMF), sufficient heat for inducing cancer-cell death is produced mainly by Néel and Brown relaxations of superparamagnetic MNPs or by hysteresis loss of ferromagnetic MNPs under AMF [1,12–17]. Moreover, MNPs have the advantage of high biocompatibility as demonstrated both *in vitro* and *in vivo* [18–20].

Mesothelioma is an asbestos-induced malignant pleural or peritoneal tumor [21–24]. There are three histological subtypes (epithelioid, sarcomatoid, and biphasic) of mesothelioma based on differences in cell morphology and surface proteins [24,25]. Around 60% of total mesothelioma patients are categorized as “epithelioid”, 20% are “sarcomatoid” patients, and the others are classified as “biphasic” [25]. Concerning the diagnosis and treatment of mesothelioma, diagnostic tools such as biopsy, computed tomography (CT) scan, and MRI are currently used [22,26], but no effective treatment has been established. The median overall survival periods for patients with epithelioid, sarcomatoid, and biphasic types of mesothelioma are reported to be only a few years at maximum, even when treated with primary methods of radical surgery, chemotherapy, radiation therapy, or a combination of these techniques [22,23,25–31]. Therefore, it should be emphasized here that the primary treatments for mesothelioma have a limited therapeutic effect.

We investigated the nanoparticles uptake and cell death related to the addition of MNPs in three histological types of human

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mesothelioma cells, NCI-H28 (epithelioid), NCI-H2052 (sarcomatoid), and MSTO-211H (biphasic) cells, in our previous study [32]. We demonstrated the apoptotic effect of ~10-nm MNPs specific to MSTO-211H cells, despite the fact that cellular uptake of MNPs showed a similar tendency in all cell types, and the heat-induced cell death with ~40-nm MNPs under AMF with an exposure condition of 325 kHz and ~500 Oe was observed in all cell types. Thus, we concluded that the addition of MNPs to mesothelioma can be a useful approach to mesothelioma treatment.

In the present study, in order to consider the potential of MNPs for use in magnetic hyperthermia as an effective treatment of mesothelioma, we evaluated cell death associated with cellular uptake of ~40-nm MNPs under AMF *in vitro*. We compared mesothelioma cell death induced by simple addition of MNPs by heating with MNPs internalized into cells and by thermostatic-bath heating. We also examined the change in mortality of mesothelioma cells heated by intracellular MNPs *via* 24-h incubation at 37 °C. We investigated these effects in all three major histological subtypes of mesothelioma cells to examine the differences in cellular uptake and the cell-killing effect of MNPs among three histological subtypes.

2. Experimental

2.1. Synthesis and characterization of magnetite nanoparticles

Magnetite nanoparticles (MNPs) with a diameter of ~40 nm were synthesized using the methods we outlined previously [11,32]. In short, 2.5 mmol of spermine, or *N*,*N*′-bis(3-aminopropyl)butane-1,4-diamine (Sigma-Aldrich Japan, Japan), dissolved in 50 mL of ultrapure water (pH ~ 12) was added to 1 mmol of $FeCl_2 \cdot 4H_2O$ (Kanto Chemical Co. Ltd., Japan) dissolved in 50 mL of ultrapure water, and then incubated for 4 h at room temperature with stirring. Afterwards, the black precipitates were washed with ultrapure water and ethanol, and vacuum dried to make them into a powder before use in other experiments.

To evaluate the heating capacity of MNPs, a sample of 1.0 mg of MNPs dispersed in 500 μL of water was prepared. The sample was subjected to AC magnetic field (AMF) induced using an EASYHEAT (Alonics, Ltd., Tokyo, Japan) device including a 3-turn coil with an outside diameter of 40 mm, at a frequency of 325 kHz with a current of 569.1 A for 20 min. The strength of the magnetic field was calculated to be 536 Oe. During the exposure to AMF, the temperature of the sample was recorded every a second with an Anritsu FL-2000 fiber thermometer (Tokyo, Japan), and cooling water was circulated in the coil using a DC inverter chiller RKE1500B1-V (Nagano, Japan) to prevent the influence of the heat from the coil on the temperature of a sample. Specific absorption rate (SAR) were determined from the slope between 0 and 100 s in the recorded temperature curves using parameters of specific heat capacity (C_{pi}) and mass for each substance (m_i) or MNPs (m_{MNPs}), using the following equation.

$$\mathrm{SAR} = \frac{\sum_i C_{pi} m_i}{m_{MNPs}} \cdot \frac{\Delta T}{\Delta t} \quad (1)$$

A magnetization curve of the sample as a powder at the magnetic field of −10,000 to 10,000 Oe at 300 K was measured using a BHV-35 vibrating sample magnetometer (VSM; Riken Denshi Co. Ltd., Tokyo, Japan). Additionally, the zeta potential of MNPs in ultrapure water at pH ~ 7 was measured using an ELS-8000 light-scattering photometer (Otsuka Electronics, Osaka, Japan).

2.2. Cell culture

Human mesothelioma NCI-H28, NCI-H2052, and MSTO-211H cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Biowest, Nuaillé, France) and 1% penicillin-streptomycin (Sigma-Aldrich) at 37 °C under a 5% CO_2 atmosphere.

2.3. Evaluation of cellular uptake

The amount of MNPs incorporated into a cell and percentage of cells containing MNPs were evaluated as a cellular uptake study using the same procedures described in our previous reports [17,19,32]. In brief, 5×10^5 (for NCI-H28 and MSTO-211H) or 1×10^5 (for NCI-H2052) cells, allowed to adhere for 24 h in advance, were further incubated for 24 h with MNPs at concentrations of 0, 133, and 267 μg/mL. The numbers of cells cultured with MNPs were determined considering cell conditions such as viability and density of cells after 48-h cultivation. A range of MNPs concentration was determined considering the experimental condition in our previous report [32] and the mortality of normal cells (human umbilical vein endothelial cells; HUVEC) with MNPs: the mortalities of HUVEC was below 10% following the addition of MNPs in the concentration range from 0 to 267 μg/mL (as shown in Fig. A1). The excess MNPs not incorporated into cells were removed by washing with Dulbecco's phosphate buffered saline (DPBS; Gibco, Life Technologies) before both evaluations. In the evaluation of uptake-quantity, the cells containing MNPs were collected using a magnet followed by complexation of Fe^{3+} with thiocyanate. The absorbance at 480 nm measured by a V-550 spectrophotometer (JASCO International Co. Ltd., Tokyo, Japan) was used with a calibration curve to determine the amount of intracellular MNPs. On the other hand, the percentage of MNP-containing cells was evaluated *via* a FACSCanto II flow cytometer (BD Biosciences, Becton, Dickinson and Co., Franklin Lakes, NJ) at concentrations of 0, 67, 133, 200, and 267 μg/mL. Cells containing MNPs were discriminated from others by the intensity of side scatter (SSC) because the SSC indicates intracellular complexity. Cells containing MNPs are SSC positive, whereas MNPs alone or cells not containing MNPs are SSC negative. Cellular uptake at a MNPs concentration of 67 μg/mL was investigated by microscopic observation using an Iron Stain Kit (Sigma-Aldrich) with a Nikon TE2000-U microscope (Tokyo, Japan).

2.4. Exposure of cells containing MNPs to AC magnetic field

NCI-H28, NCI-H2052, and MSTO-211H cells with MNPs at the concentrations of 0, 133, and 267 μg/mL were prepared as described in Section 2.3. Then, 5×10^5 cells containing MNPs were collected by magnetic separation, dispersed in 500 μL of medium, and then subjected to 20 min of AMF (325 kHz, ~500 Oe), measuring the sample temperature every a second. Cell mortality immediately after exposure to AMF was evaluated by cell counting with trypan blue (TB; Gibco) staining. Prior to the exposure, cell mortality of samples was evaluated. Live cells were TB-negative, whereas dead cells were TB-positive; hence, cell mortality was calculated by the following formula:

$$\text{Cell mortality}(\%) = \frac{\text{Counts of TB positive cells}}{\text{Counts of all cells}} \times 100 \quad (2)$$

The percentage of non-viable cells, which was evaluated by cell counting after the 20 min of exposure, was compared with that evaluated after 20-min heating using a THERMAL ROBO TR-1A thermostatic water bath (AS ONE Corporation, Osaka, Japan). Temperatures in the thermostatic bath were set to the average temperatures between 800 and 1200 s obtained from the temperature curves of AMF-applied samples.

The time course of cell mortality after the exposure of cells containing MNPs to AMF was also investigated. After exposure, the cells were further incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. The same experiment was performed on the cells containing MNPs without AMF application. Cell morphology after the 24-h incubation was observed using a Nikon TE2000-U microscope.