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Immuno-hyperthermia effected by antibody-conjugated nanoparticles selectively targets and eradicates individual cancer cells

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Abbreviations:
AMF: alternating magnetic field
Cy1: cytology-positive
DDW: double distilled water
DMEM: Dulbecco's Modified Eagle's Medium
f: frequency
FBS: fetal bovine serum

FITC: Fluorescein isothiocyanate
GFP: green fluorescent protein
H: amplitude
H&E: hematoxylin and eosin
HIPEC: hyperthermic intraperitoneal chemotherapy
Hsp: heat shock protein
MEM: Minimum Essential Medium
MHT: magnetic hyperthermia
MNPs: magnetic nanoparticles
P0: macroscopic peritoneal dissemination
PI: propidium iodide
RFP: red fluorescent protein
SPION: superparamagnetic iron oxide (Fe_3O_4) nanoparticle

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ABSTRACT

Hyperthermia has been used for cancer therapy for a long period of time, but has shown limited clinical efficacy. Induction-heating hyperthermia using the combination of magnetic nanoparticles (MNPs) and alternating magnetic field (AMF) termed hyperthermia (MHT), has previously shown efficacy in an orthotopic mouse model of disseminated gastric cancer. In the present study, superparamagnetic iron oxide nanoparticles (SPIONs), a type of MNP, were conjugated with anti-HER2 antibody, trastuzumab and termed anti-HER2-antibody-linked SPION nanoparticles (anti-HER2 SPIONs). Anti-HER2 SPIONs selectively targeted HER2-expressing cancer cells co-cultured along with normal fibroblasts and HER2-negative cancer cells and caused apoptosis only in the HER2-expressing individual cancer cells. The results of the present study show proof-of-concept of a novel hyperthermia technology, immuno-MHT for selective cancer therapy, that targets individual cancer cells.

INTRODUCTION

Hyperthermia has a long history for cancer treatment [1]. However, conventional hyperthermia treatments heat both tumor tissues as well as normal tissues and therefore have dose-limiting toxicity.

Magnetic nanoparticles (MNPs) have been used to target tumor tissues followed by applying an alternating magnetic field (AMF) from outside of the body to selectively heat tumors [2]. This treatment is called magnetic hyperthermia (MHT) [3]. MHT has been approved for glioblastoma in Europe [4]. MHT has been conducted mainly by direct injection of MNPs into tumor tissue. Our laboratory recently showed the efficacy of MHT in an orthotopic mouse model of disseminated gastric cancer using superparamagnetic iron oxide nanoparticles (SPIONs) as MNPs [5]. In the present study we have developed cancer-targeting immuno-SPIONs conjugated with an anti-HER2-antibody and demonstrated that these immuno-SPIONs caused selective targeting and eradication of individual HER2-expressing cancer cells as a novel therapy termed immuno-hyperthermia.

MATERIALS AND METHODS

Cell lines.

The human breast cancer cell line AU565 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The human gastric cancer cell line NUGC-4 was purchased from JCRB Cell Bank (National Institute of Biomedical Innovation, Osaka, Japan). The human colon cancer cell line HCT-116-RFP (AntiCancer Inc., San Diego, CA, USA), which expresses the red fluorescent protein (RFP) gene, was established previously [6,7,8]. The human fetal esophageal

fibroblasts cell line FEF3-GFP, which expresses the green fluorescent protein (GFP) gene, was established previously [9,10]. SKOV-3 was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). AU565, NUGC-4, and HCT-116-RFP cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA); and FEF3-GFP and SKOV-3 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS. All media were supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA) and 10% FBS. Cells were maintained at 37℃ in a humidified atmosphere with 5% CO_2.

SPIONs.

Superparamagnetic iron oxide (Fe_3O_4) nanoparticles (SPIONs) comprise a core of iron oxide nanoparticles surrounded by surface coatings (**Fig. 1a**). SPIONs of 10 nm core diameter with two types of surface coatings were used in the present study. Carboxydextran-coated SPIONs were kindly supplied by Meito Sangyo Co., Ltd. (Nagoya, Japan). Amphiphilic polymer-coated SPIONs were purchased from Ocean NanoTech, LLC (San Diego, CA, USA). Both surface coatings have carboxyl groups which react with EDC/NHS. The iron concentration of both types of surface coatings was adjusted to 5 mg Fe/ml. The SPION solution was sterilized through a 0.2 μm membrane filter before use.

Antibody-conjugated SPIONs.

For production of antibody-conjugated SPIONs, the EDC/NHS reaction was used to couple the carboxyl group (–COOH) of the surface coating of the SPIONs with the amine group ($–NH_2$) of the antibody [11] (**Fig. 1a**). The conjugation was performed with the Iron Oxide Nanoparticle

Conjugation Kit (ICK-10-005; Ocean NanoTech, LLC, San Diego, CA, USA) according to the manufacturer's protocol. After SPIONs (1 mg) were diluted in sodium borate, the EDC/Sulfo-NHS mixture (2 mg/ml EDC and 1 mg/ml sulfo-NHS) were added to the SPIONs. After 30 minutes reaction at room temperature, 1 mg of antibody suspended in sodium borate at 2 mg/ml concentration was added to the SPION solution, and allowed to react for 2 hours at room temperature. The solution was quenched by adding 2-(2-aminoethoxy) ethanol. Products were magnetically collected and washed twice with double distilled water (DDW) using a magnetic separator (DynaMag^{TM}-2; Life Technologies, Carlsbad, CA, USA) (**Fig. 1b**). The suspension was stored in DDW at a concentration of 1 mg/ml at 4ºC. Since the suspension aggregates easily, it was re-suspended in a bath sonicator for 1 minute before using (**Fig. 1c**). A humanized monoclonal antibody targeting HER2, trastuzumab (Chugai Pharmaceutical, Tokyo, Japan) [12], was bound to the SPIONs via a surface coating (carboxydextran and amphiphilic polymer). The resultant immuno-SPIONs were termed anti-HER2-carboxydextran-coated SPIONs and anti-HER2-amphiphilic polymer-coated SPIONs, respectively.

To evaluate the anti-HER2-SPIONs tropism toward HER2-overexpressing cancer cells, anti-HER2-carboxydextran-coated SPIONs or SPIONs without any modification were administrated to HER2-overexpressing cancer cell lines (AU565, NUGC-4 and SKOV-3). The cells were fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, #163-20145) for 15 min, permeabilized and blocked with 0.2% Triton® X-100 (Nacalai Tesque Inc., Kyoto, Japan, #35501-02) diluted by Blocking One (Nacalai Tesque Inc., Kyoto, Japan, #03953-95) for 10 min, and then were stained with Prussian blue which detects ferric iron. Prussian blue staining was performed according to the manufacturer's protocol (Iron Stain Kit, ScyTek Laboratories, Inc., Logan, UT, USA, #IRN-1-IFU).

Anti-HER2-SPION delivery to HER2-overexpressing cells.

HER2-overexpressing cells (AU565, NUGC-4, and SKOV-3) were seeded in a 35 mm glass-base dish (Iwaki, Tokyo, Japan) at a density of 1×10^5 cells per well containing 3 ml culture medium and grown for 24 hours. After 1 hour, anti-HER2-carboxydextran-coated SPIONs at 100 μg/ml concentration were added to the cells. The cells were washed twice with fresh medium to remove excess SPIONs, and the cells were incubated for another 12 hours. AU565 cells were immunostained with Alexa Fluor® 647-labeled goat anti-human IgG (H+L) antibody (Thermo Fisher Scientific, Waltham, MA, #A-21445) to visualize the Fc fragment of trastuzumab, and with fluorescein isothiocyanate (FITC) coupled anti-dextran antibody (Clone DX1; Stem Cell Technologies, Vancouver, Canada, #60026FI.1) to visualize carboxydextran of the surface coating of the anti-HER2-carboxydextran-coated SPIONs. Cellular images were acquired using an inverted microscope (IX71; Olympus Optical, Tokyo, Japan) or a confocal laser scanning microscope (FV10i; Olympus) and were analyzed using Image J software.

Determination of HER2-targeted delivery and cellular uptake of anti-HER2-SPIONs.

One hour after AU565 cells were administrated with anti-HER2-carboxydextran-coated SPIONs, the cells were washed to remove excess SPIONs and cultured in fresh medium. The anti-HER2-SPIONs attached to the cells were observed with an inverted microscope (IX71) at different time points. To evaluate cancer antigen-specific delivery of the anti-HER2-SPIONs, the SPIONs were administrated to co-cultured AU565 (HER2-positive cancer cells), HCT-116-RFP (HER2-negative cancer cells) and HER2-negative GFP-FEF3 cells (normal cells). After 1 hour incubation, the cells are washed to remove excess SPIONs and cultured in fresh medium. The cells were observed 12 hours after administration of the anti-HER2-SPIONs.

Immuno-magnetic hyperthermia (MHT) system.

An alternating magnetic field (AMF) was produced by a 2.4 kW radiofrequency generator (EASYHEAT 2.4; Ameritherm Inc, Scottsville, KY, USA) containing a water-cooled, three-turn spiral coil with an internal diameter of 38 mm. Magnetic hyperthermia (MHT) was performed by placing a cell-containing dish over the coil that generates an AMF, with amplitude (H) = 31 kA/m and frequency (f) = 280 kHz for 30 minutes (**Fig. 3a**).

Cell viability assay.

A cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA), which is based on a sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay, was used according to the manufacturer's protocol.

Determination of therapeutic efficacy of immuno-magnetic hyperthermia.

AU565 or NUGC-4 cells were seeded in 35 mm glass-based dishes at a density of 1×10^5 cells per well containing 3 ml culture medium and grown for 24 hours. To evaluate cytotoxicity of anti-HER2-carboxydextran-coated SPIONs, the cells were cultured with 100 μg SPIONs at 100 μg/ml for 1 hour and washed twice with fresh medium to remove excess SPIONs. AMF with an H = 31 kA/m and f = 280 kHz was applied to the cells for 30 minutes.

The XTT cell-staining assay was performed at 48 hours after each treatment. Image analysis was also performed with a confocal microscope (FV10i), for determination of the cell-death index using dual staining with propidium iodide (PI) (Sigma-Aldrich, Saint Louis, MO, USA, #P4864) for nonviable cells, and Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA, #H-1399) for all cells. The percentage of cell death in each treatment group was calculated by counting the number of PI-positive nuclei and normalizing it with the number of Hoechst

33342-positive nuclei. The efficacy of MHT using SPIONs with different surface coatings as well as anti-HER2-amphiphilic polymer-coated SPIONs, was also evaluated on AU565 and NUGC-4 cells in the same way as anti-HER2-carboxydextran-coated SPIONs described above.

Determination of the cell-death mechanism and heat-shock-protein expression after immuno-magnetic hyperthermia.

AU565 cells were seeded in 35 mm glass-based dishes at a density of 1×10^4 cells per well containing 3 ml culture medium and grown for 3 days. Anti-HER2-carboxydextran-coated SPIONs (100 μg) at 100 μg/ml were applied to the cells. Five hours after administration of the Anti-HER2-SPIONs, cells were washed twice and supplied with fresh medium. Immuno-MHT was performed by applying AMF for 30 minutes. 48 hours later AU565 cells were stained using the GFP-CERTIFIED® Apoptosis/Necrosis Detection Kit (Enzo Life Science, Farmingdale, NY, USA) for 30 minutes and observed using a confocal laser scanning microscope (FV10i). The Apoptosis/Necrosis Detection kit stains early apoptotic cells with the apoptosis detection reagent (Annexin V-EnzoGold, Ex/Em: 550/570 nm, red fluorescence, Enzo Life Science, Farmingdale, NY, USA). Early necrotic cells were stained positive with the necrotic detection reagent (7-AAD, Ex/Em: 546/647 nm, Enzo Life Science, Farmingdale, NY, USA) [13]. To evaluate the expression of heat shock protein 70 (Hsp70) after immuno-MHT, the cells were stained with anti-Hsp70 antibody (Alexa Fluor® 488) (Abcam, Cambridge, MA, USA, #ab197870).

Time-lapse imaging after immuno-magnetic hyperthermia.

HER2-overexpressing AU565 cancer cells (3×10^4) and normal GFP-FEF3 fibroblasts (2×10^4) were seeded together in 35 mm glass-based dishes containing 3 ml culture medium. The co-cultured cells were administrated anti-HER2-conjugated amphiphilic polymer-coated SPIONs

（200 μg）and AMF was applied for 30 minutes. Starting at 30 minutes after application of AMF, time-lapse images were acquired using a confocal microscope (FV10i, Olympus).

Statistical analysis.

Statistical analysis was performed using the Student's t-test in Excel (Microsoft, Redmond, USA). All values are presented as mean ± standard deviation (SD) of at least three independent experiments. *p*-values of 0.05 or less between groups were considered statistically significant.

RESULTS

Targeted delivery of anti-HER2-conjugated SPIONs to HER2-expressing cancer cells.

When administered with anti-HER2-conjugated-carboxydextran-coated SPIONs, Prussian-blue stained particles were observed in the peri-nuclear area of AU565, NUGC-4 and SKOV-3 cells as early as 12 hours (**Fig. 1d**), indicating the SPIONs have entered the cancer cells. No blue particles were observed in the cells administrated SPIONs without antibody conjugation, indicating the selective targeting of anti-HER2-conjugated-carboxydextran-coated SPIONs.

Blue stained particles were confirmed as anti-HER2-conjugated-carboxydextran-coated SPIONs by immunostaining with Alexa Fluor® 647-labeled goat anti-human trastuzumab IgG on the anti-HER2-conjugated SPIONs, and with FITC-labeled anti-dextran antibody for detecting surface coating of the SPIONs (**Fig. 1e**).

The localization of anti-HER2-conjugated-carboxydextran-coated SPIONs after administration to HER2-overexpressing AU565 cells was determined at different time points by Prussian-blue staining before and after cell permeabilization. Prussian-blue staining, after cell permeabilization,

detected intracellular ferric iron by the brown SPIONs turning blue, indicating that the SPIONs were in the cytoplasm (**Fig. 2b**).

Cancer-associated-antigen-dependent delivery of anti-HER2-conjugated SPIONs.

To demonstrate the specific targeting of the anti-HER2-SPIONs, they were administrated to a co-culture of AU565 (HER2-positive cancer cells), HCT-116-RFP (HER2-negative cancer cells) and FEF3-GFP cells (HER2-negative normal cells). Prussian-blue staining at 12 hours after administration showed that the anti-HER2-conjugated SPIONs were specifically delivered only to AU565 cells, demonstrating they were specific for HER2 (**Fig. 2c**).

Cytotoxicity of immuno-MHT.

To evaluate the cytotoxicity of immuno-MHT, AU565 cells were first administrated anti-HER2 antibody or anti-HER2-conjugated-carboxydextran-coated SPIONs without applying AMF, or AMF alone. There was not a significant decrease of cell viability with anti-HER2 antibody alone, anti-HER2-conjugated SPIONs-administration alone, or AMF alone, in either cell line (**Fig. 3b**). AMF with anti-HER2-conjugated SPIONs caused a significant increase of death of AU565 cells (**Fig. 3c, 3d**). AMF with anti-HER2-conjugated-amphiphilic polymer-coated SPIONs also caused AU565 cell death (**Fig. 4a, 4b**).

Kinetic response to immuno-magnetic hyperthermia.

In the untreated control group, AU565 cancer cells showed active cell growth. Treatment of AU565 cells with immuno-MHT resulted in cell death as shown by round, highly light-refractile cells under phase-contrast microscopy with the co-cultured FEF3-GFP cells over-growing the culture.

DISCUSSION

In the present study, MNPs with a specific tumor-targeting antibody directed to overexpressed HER2, showed high targeting specificity. SPIONs as MNPs, were used for their high heating capacity and safety [14]. SPION-alone treatment did not decrease viability of any cell type tested, indicating their non-toxicity. Trastuzumab is an antibody with high binding affinity to HER2 [15]. We bound trastuzumab to the outer surface (–COOH) of the SPIONs using EDC/NHS coupling. The resultant anti-HER2 SPIONs selectively targeted HER2-positive cancer cells when administrated in vitro, and were internalized inside the cells as early as 12 hours after administration as shown in **Fig. 1d**. Non-conjugated SPIONs did not targeted HER2-positive cancer cells and were not internalized. Anti-HER2 SPIONs alone were not cytopathic.

In the present study, we proved the novel concept of immuno-hyperthermia. Future studies will use other cancer-specific antibodies bound to SPIONs to demonstrate the generality of the new concept of immuno-hyperthermia. Our previous study showed SPION-based hyperthermia was effective against disseminated gastric cancer in a mouse model [5]. Future studies will test immuno-SPIONs in vivo as a bridge to the clinic.

Authors' contributions

H.K. designed research; T.K. performed research; T.K., Y.M., H.A., H.T., K.S., S.Y., T.O., T.F., R.M.H. and H.K. analyzed data; and T.K., R.M.H. and H.K. wrote the paper.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1

Production and delivery of HER2-targeted antibody-conjugated superparamagnetic iron oxide nanoparticles (SPIONs).

(**a**) Conjugation of SPIONs with antibody was achieved with amide bonds between the surface coating of SPIONs and the antibody, using the EDC/NHS reaction.

(**b**) Magnetic separation of antibody-conjugated SPIONs.

(**c**) Antibody-conjugated SPIONs were re-suspended before use by sonication.

(**d**) Intracellular delivery of anti-HER2 antibody-conjugated SPIONs (coated with carboxydextran): anti-HER2-conjugated SPIONs (blue particles) in HER2-overexpressing cancer cells (AU565, NUGC-4, SKOV-3). Prussian-blue staining. Scale bar: 20 μm.

(**e**) Prussian blue staining and immunofluorescence staining of AU565 cells after administration of anti-HER2-conjugated SPIONs. Treatment schedule (**e1**). Brown particles under bright filed (**e2**) turned blue after Prussian-blue staining (**e3**), indicating that they were SPIONs. Carboxidextran was identified with anti-dextran antibody (**e4**, green). Humanized anti-HER2 antibody was identified with goat anti-human IgG secondary antibody (**e5**, red). Co-localization of green and red immunofluorescence signals at the area of SPIONs (**e6**), indicated that SPIONs were successfully conjugated with the anti-HER2 antibody. Scale bar: 10 μm.

Figure 2

Cancer-associated-antigen-dependent delivery and cellular internalization of anti-HER2-conjugated SPIONs.

(**a**) Anti-HER2-conjugated SPIONs were seen on the surface of HER2-overexpressing AU565

cancer cells at 1 hour after administration of anti-HER2-SPIONs (**a1**). SPIONs were internalized by 12 hours post-incubation (**a2**).

(**b**) The cells of **Fig. a2** were stained with Prussian blue. Brown SPION particles were not stained by Prussian blue without cell-membrane permeabilization (**b1**). The particles turned blue after permeabilization (**b2**). Nuclear fast-red staining of cells of **Fig. b2** (**b3**).

(**c**) HER2-overexpressing cells (AU565, blue arrowheads) were co-cultured with HER2-negative cells (FEF3-GFP: yellow arrowheads, and HCT-116-RFP: red arrowheads). After the cells were administrated with anti-HER2-conjugated SPIONs, nuclei were stained with Hoechst 33342. Only AU565 (fluorescence negative) cells contained SPIONs in their cytoplasm (**c1**, blue particles), indicating that anti-HER2 antibody-conjugated SPIONs were specifically delivered to HER2-overexpressing cells. Fluorescence image of the cells of **Fig. c1** (**c2**). Scale bar: 20 μm.

Figure 3

Immuno-magnetic hyperthermia (MHT) system with anti-HER2-conjugated SPIONs heated by an alternating magnetic field (AMF).

(**a**) AMF generator (arrow). Water-cooled, three-turn spiral coil (circle) delivers AMF to a 35-mm dish placed over the coil (arrowhead).

(**b**) Cytotoxicity of anti-HER2 antibody-conjugated SPIONs (anti-HER2-SPIONs) or AMF with amplitude (H) = 31 kA/m and frequency (f) = 280 kHz for 30 minutes on AU565 and NUGC-4 cells, determined with the XTT cell-viability assay, 48 hours after each treatment. The cell viability of mock-treated cells was considered 100%, and the percent viability of treated cells was thereby calculated. Data are presented as mean ± SD. No significant cytotoxicity was observed, suggesting that anti-HER2-conjugated SPIONs alone and AMF alone are non-toxic.

(**c**) Cell viability was assessed using propidium iodide (red, dead cells) and Hoechst 33342 dye

(blue, whole cells) after AU565 cells were treated with anti-HER2 antibody alone, anti-HER2-conjugated SPIONs alone, and anti-HER2-conjugated SPIONs and AMF. Scale bar: 200 μm.

(**d**) The percentage of cell death in each treatment group was calculated by counting the number of PI-positive nuclei and normalizing it to the number of Hoechst 33342-positive nuclei. Data are presented as mean ± SD. Statistical significance was $P < 0.01$ (single asterisk).

Figure 4

Intra-cellular magnetic hyperthermia using antibody-conjugated SPIONs with an amphiphilic polymer coating.

(**a**) Anti-HER2-amphiphilic polymer-coated SPIONs were delivered to HER2-overexpressing AU565 cancer cells for immuno-magnetic hyperthermia (MHT). Bright field (**a1**). Prussian-blue staining (**a2**). Immunostaining with goat anti-human IgG secondary antibody (red) and Hoechst 33342 dye (blue) (**a3**). Scale bar: 20 μm.

(**b**) XTT assay at 48 hours after immuno-MHT. Viability of mock-treated cells was considered 100%, and the percent cell viability of treated cells was thereby calculated. Data are presented as mean ± SD. Statistical significance was $P < 0.01$ (single asterisk).

Figure 5

Immuno-MHT induces apoptosis of HER2-expressing AU565 cells.

AU565 cells were treated with immuno-MHT at an amplitude = 31 kA/m and frequency = 280 kHz for 30 minutes. The cells were observed using the Apoptosis/Necrosis Detection Kit with an anti-Hsp70 antibody 48 hours after MHT.

Immuno-MHT treated cells showed predominant red fluorescence, indicating early apoptosis,

rather than green fluorescence which indicates early necrosis. Scale bar: 50 μm.

Figure 6

Kinetic response to cancer-specific immuno-MHT.

(**a**) Outline of time-course experiment.

(**b**) HER2-overexpressing cancer cells (AU565, blue arrowhead) and normal fibroblasts (FEF3-GFP, white arrow) were co-cultured. Anti-HER2 antibody-conjugated SPIONs were used for immuno-MHT. The anti-HER2-conjugated SPIONs were specifically delivered to AU565 cells (red arrowhead). An alternating magnetic field (AMF) at amplitude = 31 kA/m and frequency = 280 kHz for 30 minutes was applied to the cells. In the control group, AU565 cancer cells were proliferating. Immuno-MHT treated AU565 cells were eradicated, and GFP-FEF3 fibroblasts cells overgrew the culture.