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Developments for the minimally invasive treatment of tumours by targeted magnetic heating

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Abstract

Purpose. Among the different minimally invasive methods for the treatment of tumours under investigation, the accumulation of magnetic material at the target region and the exposure to an alternating magnetic field comprises a highly selective approach. In the present study, we assessed if magnetic heating of tumour cells *in vitro* is feasible after binding of high-affinity magnetic nanoparticles to the tumour specific protein Her-2/neu, which is known to be expressed in 30% of breast cancers.

Material and methods. Antibodies against the Her-2/neu protein (high-affinity probe) or non-specific gamma immunoglobulins (non-affinity probe, control) were coupled to the dextran shell of magnetic nanoparticles (mean total particle diameter, 150 nm). After incubation of Her-2/neu overexpressing SK-BR-3 tumour cells with the high-affinity probe, non-affinity probe or buffer, cell labelling was verified by electron microscopy. The iron content in cells was determined by atomic absorption spectroscopy. Moreover, cells were exposed to an alternating magnetic field (amplitude, 11 kA m^{-1}; frequency, 410 kHz) for 2.8 min. Temperatures were measured using thermocouples.

Results. A distinct cell labelling was observed by electron microscopy after incubation of cells with the high-affinity probe as compared to controls. Magnetic nanoparticles were found to be localized at the cell surface as well as in granules inside the cytoplasm. The iron content of high-affinity labelled cells (e.g. 76 μg/5 $\times 10^7$ cells) was distinctly higher than in control cells (e.g. up to 25 μg/5 $\times$ 10^7 cells). During magnetic heating, temperature increases of up

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to approximately 8 °C were observed in relation to high-affinity labelled cells as compared to 1–2 °C in controls.
Conclusion. Our results show that targeted magnetic heating of tumour cells seems to be feasible. Further investigations should focus on the corresponding relationships in the *in vivo* situation.

1. Introduction

In recent years, improved detection of breast tumours has been mainly related to the use of ameliorated diagnostic systems (e.g. magnetic resonance mammography, etc). Fortunately, smaller tumours are increasingly detected, which are generally associated with a good prognosis [1]. For the treatment of small tumours, the use of minimally invasive therapies is favourable. For example, when comparing radical mastectomy (complete organ removal) with partial mastectomy, the therapeutic outcome was comparable, and the functional and cosmetic results in relation to the breast-conserving therapy are encouraging (e.g. [2]).

Among the minimally invasive therapeutic methods under investigation, selective tumour elimination can be obtained by the deposition of magnetic nanoparticles to the tumour region followed by the exposure to an alternating magnetic field. By this procedure, the magnetic material absorbs energy from the magnetic field and converts it into heat that is used to eliminate the tumour. In this regard, the structural and magnetic properties of selected fine particles and their applicability in magnetic induced heating have been documented [3, 4]. Moreover, up to now most investigators have administered magnetic nanoparticles directly to the tumour, whereby a dedicated tumour puncture is necessary [5, 6]. Whereas this procedure is very practical in relation to the treatment of uni-focal and well localized tumours, limitations will be encountered in relation to multi-focal ones. In such cases, the accumulation of the magnetic material by intravasal application seems to be feasible, because tumours with a diameter larger than 2 mm induce their own vessel system, which allows for further tumour growth due to sufficient nutrient supply [7].

Targeting could be improved when using nanoparticles with high affinity towards specific molecules on the surface of tumour cells. Interestingly, it was shown that a defined protein, the epidermal growth factor receptor Her-2/neu, is found to be overexpressed on the surface of 25–30% of breast tumours [8]. Moreover, a high-affinity antibody against the Her-2/neu protein is available. The antibody, which is currently used for therapeutic purposes [9], could also be used for a selective anchorage of sophisticated agents (magnetic nanoparticles) at the tumour after intravasal administration. The agent could be subsequently activated on site to induce heating by exposure to an external magnetic field. Therefore, in the present study we assessed the feasibility of a selected heating of Her-2/neu positive tumour cells in culture using newly designed high-affinity magnetic nanoparticles.

2. Material and methods

2.1. Magnetic nanoparticles

Magnetic nanoparticles (Innovent, Jena, Germany) were composed of both an iron oxide core (maghemite, magnetite) with a diameter of approximately 9 nm and a coating with dextrane. The corresponding total particle diameter was approximately 150 nm.

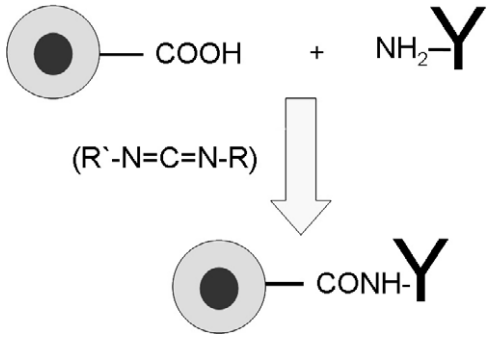

Figure 1. Scheme showing the principle of coupling high-affinity antibodies to the dextran shell of magnetic nanoparticles.

2.2. Magnetic nanoparticle probes

High-affinity probes were designed by coupling anti-Her-2/neu antibodies (Hoffmann-LaRoche AG, Grenzach-Wyhlen, Germany) (high-affinity probe) or human γ-globuline (Sigma-Aldrich, non-affinity probe, control for binding specificity) to dextrane coated magnetic nanoparticles (figure 1). Hereby, 500 μl *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimid-hydrochloride (Sigma, Aldrich, Steinheim, Germany) was incubated with 500 μl nanoparticle suspension for 30 min before adding antibodies (approximately 1 μg protein/μl). Magnetic nanoparticles were separated using a permanent magnet. The amount of coupled protein was estimated according to the method of Bradford, the iron content by atomic absorption spectrometry (AAS5 FL, Analytic Jena, Germany) as described by Preu [10]. The specific absorption rate (SAR) was determined calorimetrically according to Hilger [11].

2.3. Cell culture

The Her-2/neu expressing cell line SK-BR-3 (Cell Line Service, Heidelberg, Germany) was cultivated in Dulbecco's minimal essential medium (DMEM, Gibco, Karlsruhe, Germany) with 10% (v/v) foetal calf serum (Gibco, Karlsruhe, Germany) and 5% (v/v) HEPES (Gibco, Karlsruhe, Germany). Cells were cultured at 37 °C using standardized atmospheric conditions containing 5% CO_2.

2.4. In vitro cell labelling

Cells were labelled after trypsinization and re-suspension in buffer (phosphate buffered saline, PBS, 0.1 M, pH: 7.4, Gibco Karlsruhe, Germany) with 2 mM EDTA (Sigma-Aldrich, Steinheim, Germany) and 0.5% (v/v) BSA (Sigma-Aldrich, Steinheim, Germany). 50 μl of the HER-2/neu high-affinity probe (iron content, 0.7 mg; protein content, 3.5 μg) was added to 5×10^7 cells and incubated for 20 min at 37 °C. Controls for specific binding were performed by incubation of cells with a HER-2/neu non-affinity probe, where the antibody was replaced by γ-globulin (IgG; iron content, 0.9 mg; protein content, 4 μg; control for labelling specificity) or with buffer (PBS) only (control for effects arising from cells). After incubation, cells were washed at least three to five times in PBS in order to remove unbound magnetic nanoparticles. For macroscopic examinations, cells were embedded in 2% (w/v) agarose (Sigma, Deisenhofen, Germany) in PBS and transferred to reaction tubes.

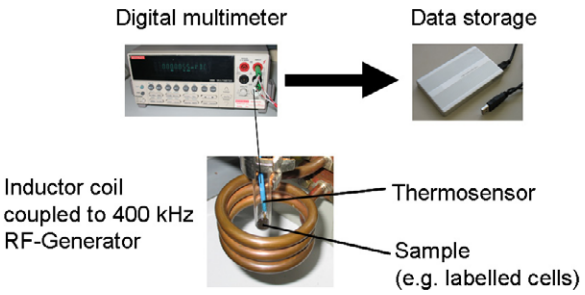

Figure 2. Experimental set-up for magnetic heating of labelled SK-BR-3 cells by exposure to an alternating magnetic field.

(This figure is in colour only in the electronic version)

Table 1. Cell number, temperature increases and amount of iron per sample after incubation of SKBR-3-cells with anti-Her-2/neu nanoparticle probes, IgG probes or PBS for 20 min at 37 °C. Temperature increases were determined during the exposure to an alternating magnetic field (amplitude, 11 kA m^{-1}; frequency, 410 kHz) for 2.8 min. MNP, magnetic nanoparticles; PBS, phosphate buffered saline; n.d., not detectable.

Cells incubated with	Cell number	Temperature increase (°C)	Amount of iron (μg)
Her-2/neu-MNP	5×10^7	7.7	76.0
	4×10^7	6.0	54.3
IgG-MNP	5×10^7	2.0	25.0
	4×10^7	1.0	18.0
PBS	5×10^7	0.7	n.d.
	4×10^7	0.4	n.d.

2.5. Verification of nanoparticle labelling

In order to verify nanoparticle labelling on cells, 0.1 ml of cell suspension was fixed in 0.9 ml of 3% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4) for 1 h. Afterwards, cells were washed in buffer, postfixed in osmium tetroxide (30 min), dehydrated and embedded in Durcupan ACM (Fluka, Switzerland). Ultrathin sections were stained according to Reynolds [12] and analysed using a Phillips CM 10 electron microscope.

2.6. Magnetic heating of labelled cells

1×10^7 cells were suspended in 100 μl PBS and exposed to an alternating magnetic field (frequency, 410 kHz; amplitude, 11 kA m^{-1}) for 2.8 min. The corresponding experimental set-up is schematically shown in figure 2. During heating, temperature was measured using a thermocouple as described previously [13].

3. Results

3.1. Magnetic nanoparticles

Quantitative analysis showed that 8 μg ml^{-1} protein was coupled to magnetic nanoparticles per 1 mg ml^{-1} iron. The corresponding specific absorption rate was found to be 100 W g^{-1} (frequency, 410 kHz; amplitude, 11 kA m^{-1}).

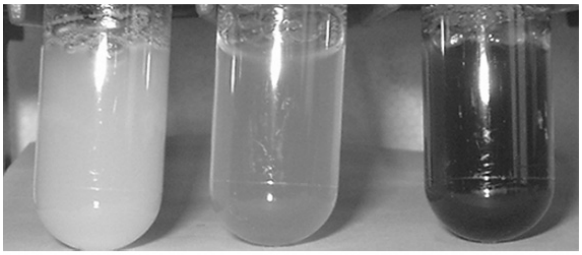

Figure 3. Microscopic view of 4×10^7 labelled SK-BR-3 cells embedded in 2% (w/v) agarose in PBS. (A) Non-labelled cells (incubation with PBS); (B), (C) cells after either labelling with the non-affinity probe (IgG magnetic nanoparticles (B)), or with the high-affinity probe (Anti-Her-2/neu magnetic nanoparticles (C)).

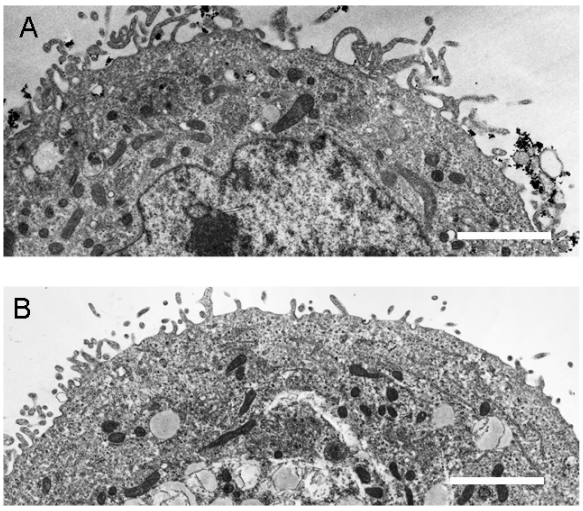

Figure 4. Transmission electron microscopy of SK-BR-3 cells labelled either with the high-affinity (Anti-Her-2/neu magnetic nanoparticles (A)) or non-affinity probe (IgG magnetic nanoparticles (B)) Bar: 2.7 μm.

3.2. Cell labelling

Macroscopic observations of labelled cells embedded in agarose showed a typical dark colour after labelling with the high-affinity probe (figure 3). In comparison to that, cell suspensions incubated with the non-affinity probe, under similar conditions, revealed a less intense colouration, whereas cells incubated with PBS were clear, as expected. Subcellular analysis of cells labelled with the high-affinity probe using electron microscopy showed the presence of magnetic nanoparticles by electron dense areas on the cell surface as well as in granules inside the cytoplasmic region (figure 4(A)). In contrast to this, almost no cell labelling was observed after incubation of cells with the non-affinity probe (figure 4(B)). Quantitative analysis of iron content per sample revealed distinctly higher values (54.3 or 76.0 μg iron/sample) after incubation of cells with the high-affinity probe as compared to the non-affinity one (18.0 or 25.0 μg iron/sample, table 1). No iron content was detectable in cells after incubation with PBS. Iron content of buffer from the last washing step was approximately 10 times lower as

compared to the iron content of the respective cell sample. 1.3 and 1.5 pg iron/cell were found after specifically labelling and comparatively lower values in the controls (lower than 0.5 pg/cell).

3.3. Magnetic heating

Cells labelled with the high-affinity probe showed a distinctly higher temperature increase (6–7 °C) during exposure to the alternating magnetic field as compared to the exposure of cells incubated with the non-affinity probe (distinctly lower temperature increases of 1 and 2 °C), together with cells that were incubated with PBS alone (0.4 and 0.7 °C, table 1).

4. Discussion

Our results indicate that a specific cell labelling is possible using a high-affinity probe against the Her-2/neu protein. This was confirmed by macroscopic and microscopic analysis as well as by quantitative iron determination. In general, without the presence of the high-affinity antibody on the surface of magnetic nanoparticles, cell labelling was distinctly lower, indicating that binding was mainly mediated by the specific antibody itself. These data are in agreement with results demonstrating the feasibility of specific labelling of Her-2/neu positive cells using fluorescence labelled high affinity antibodies [14].

When considering the amount of iron uptake per cell (up to 1.5 pg/cell), the values seem to be nearly comparable with those reported in relation to the detection of labelled cells by magnetic resonance tomography. Even though different experimental conditions have been used, iron amounts of both 1.3–2.2 pg/cell [15] as well as 0.01–2.94 pg/cell [16] after incubation with anti-Her-2/neu coupled nanoparticles were found. When using tat-peptide conjugates, 2.5 pg iron/cell [17] were obtained. Iron amounts of 17 pg/cell were determined using the periodate method [6]. These observations indicate that the designed conjugates in our study are suitable for highly efficient cell labelling procedures, being even comparable with those by which membrane translocation signals (tat-peptides) are used in order to increase levels of cell internalization.

The results of the present study are based on *in vitro* studies on cells in culture. Further investigations should show to what extent a selective labelling of tumour cells is possible in the *in vivo* situation after systemic application into the vascular system. One problem could be the sufficient extravasation of nanoparticles from the vascular system to the tumour interstitium. Indeed, when considering the total particle diameter of the affinity probe together with the estimated cut-off size of the pores of tumour vascular endothelium (between 400 and 600 nm [18]), an extravasation of the particles seems to be feasible. Nevertheless, other factors like blood flow, interstitial tumour pressure [19, 20] and high uptake of larger particles by the reticulo-endothelial system in the liver (e.g. [21]) could counteract this effect. Therefore, the further development of sophisticated magnetic nanoparticles with high tumour retention after systemic application remains a challenge in the future.

After exposure to an alternating magnetic field for 2.8 min, cells labelled with high-affinity magnetic nanoparticles showed a distinctly higher temperature increase as compared to controls. In general, the results from heating experiments are in agreement with the observations related to iron contents of the different samples. The low-temperature increase in non-labelled cells (incubation with buffer) is related to the occurrence of eddy currents. In contrast to this, a temperature increase of only 2 °C was observed when binding magnetic nanoparticles to cells by virtue of the periodate method [6]. Compared to the data of the present study, the discrepancy in the temperature increases could possibly be based on the different amounts of iron bound on cells and the different study design as well as the specific absorption

rate of the magnetic nanoparticles used. Since the labelled cells were washed several times with buffer and the iron amount of buffer from the last washing step was approximately ten times lower compared to the iron content of the respective cell sample, the influence of free magnetic nanoparticles on the observed temperature increases should be rather low (almost 10%). With regard to the *in vivo* situation, magnetic nanoparticles with appropriate pharmacokinetic and pharmacodynamic properties should be used in order to achieve a highly selective heating which is restricted to the tumour region.

The data revealed a temperature increase between approximately 6 and 8 °C. Considering the body temperature of 37 °C, one would expect a mean temperature increase up to 43 and 45 °C in the *in vivo* situation. In fact, one has to take into consideration that the conditions associated with thermal convection and conduction are quite different from the *in vitro* experimental set-up used in the present study. Particularly, the thermal conduction due to blood flow is known to restrict the local temperature increases *in vivo*, especially in organs with a rich vascularization such as the liver. In contrast to this, the treatment of tumours in the breast seems to be favourable, since this part of the body is known to be mainly composed of insulating fat tissue and to present a comparatively low degree of vascularization.

According to the aforementioned relationships, after specific labelling of Her-2/neu tumour cells *in vivo* hyperthermic temperatures (up to approximately 42–43 °C) rather than thermoablative (over 50 °C) ones are expected to be obtained. The cytotoxic effects of hyperthermic temperatures are mainly associated with impact on the cell membrane, cytoskeleton, cellular proteins and nucleic acids [22]. Since the effects seem to be reversible (e.g. 50% or tumours regress, [23]), combinations with classical oncological methods (chemotherapy, radiotherapy) will probably be necessary.

Due to the main core diameter of 9 nm of nanoparticles and the parameters of the magnetic field used in the present study, magnetic induced heating should mainly be generated by relaxational processes [4]. The temperature increases observed in the present study were comparatively lower than those which were reported using the direct deposition of the magnetic material within the tumour [5, 11]. Hereby, comparatively high iron oxide amounts could be accumulated in the tumour region leading to thermoablative temperatures over 55 °C with the corresponding impact on the target cells such as DNA degradation [24]. Moreover, the intratumoural deposition of the magnetic material seems to be better manageable as compared to the specific labelling of cells with magnetic nanoparticles, where the magnetic material has to be administered systemically.

The reported temperature increases are based on targeting of the Her-2/neu protein on tumour cells. Many other tumour proteins are known with potential suitability for specific labelling with magnetic nanoparticles. Hereby, since the amount of target molecules is limited, the use of magnetic materials with high heating potential seems to be important. The increase of the heating potential of magnetic nanoparticles by optimization of the magnetic field parameters was already described by Hergt *et al* [3, 4].

In summary, the present investigation shows that a specific magnetically induced heating is possible after labelling of Her-2/neu positive cells with high-affinity magnetic nanoparticles. Further investigations are needed to assess the feasibility for a highly selective heating of tumours in the *in vivo* situation.

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