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Detection of Her2 Levels in Cancerous Cells Based on Iron Oxide Nanoparticles

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ABSTRACT

In this study, we synthesized Herceptin conjugated magnetic nanoparticles (HMNs) as an alternative probe to discover the levels of HER2 (Human epidermal growth factor receptor-2) in the surface of cells. These nanoparticles can be used by magnetic resonance imaging (MRI) (non-invasive methods) for screening the patients with HER2 positive or negative tumors. Dextran coated iron oxide nanoparticles were prepared using co-precipitation method and conjugated to Herceptin antibody. The stability, cytotoxicity and HER2 specific binding of HMNs was evaluated. The HER2 expression levels of the cells were examined by measuring the signal enhancement in MRI T2 images. The core and hydrodynamic size of HMNs was 10±0.1 nm and 75±15 nm respectively. HMNs were stable up to 8 weeks in pure water and PBS buffer. The specific binding of HMNs by SKBR3, SW480, MCF7, A431 and RAJI cell lines was 19.6±2.5, 8.2±1.2, 7.3±1.3, 6.0±1.7 and 2.3±1.8 pg/cell respectively. The MRI signal enhancement of the cell lines was consistent with the specific binding results. The good characteristics of HMNs demonstrated that it can detect the HER2 levels in the cell surface and be a good candidate to use as a HER2 tracer contrast agent in MRI that needs further investigations.

Keyword: Magnetic nanoparticles; Iron oxide nanoparticle; Herceptin; HER2 antigen; MRI.

1. INTRODUCTION

Nanomedicine is the science and technology of diagnosing, treating, preventing disease and improving human health using nanoscale structured materials. The use of magnetic nanoparticles for biological and clinical applications is undoubtedly one of the most challenging research areas in the field of nanomedicine [1].

Iron oxide nanoparticles, one of the most important magnetic nanoparticles, can be used for several applications including drug delivery, nanobiosensors, cell separation, purification, magnetic fluid hyperthermia (MFH) and as contrast agents for MRI [2, 3].

HER2 is over expressed in 20-30% of breast cancer and low in certain normal tissues [4]. It contributes to tumor progression and development. Herceptin is a humanized IgG1 monoclonal antibody directed against HER2. The stable over expression of HER2 on the tu-

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mor cell surface makes it an ideal target for detection by Herceptin [4, 5]. In the present study, we synthesized a HER2 probe based on the conjugation of magnetic nanoparticles with Herceptin to detect the Her2 antigen in the cells surface.

2. EXPERIMENTAL

2.1. Materials

Ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), ammonia solution (25% wt), dextran (40 kDa) and the other chemical agents were purchased from Sigma chemical corp. The monoclonal antibody, Herceptin was purchased in 140 mg vial from Genentech Inc, South San Francisco, USA.

2.2. Synthesizing magnetic nanoparticles

Dextran coated iron oxide nanoparticles were prepared using co-precipitation method [6]. In brief; 0.5 M ferrous chloride and 1 M ferric chloride solution in water were mixed with two volumes of 25% dextran dissolved in water. Precipitation was initiated by drop wise addition of NH_4OH (3%) and continued until the pH of solution reached to 10. The solution was then heated for 70 minutes at 70°C. The nanoparticles were washed with Q water several times and the large size nanoparticles removed by centrifugation. The dextran iron oxide nanoparticles were precipitated in a non-oxidizing environment according to the following chemical reaction:

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$$

2.3. Herceptin-magnetic nanoparticles (HMNs)

Iron oxide nanoparticles (1400 mg) were dissolved in citrate buffer (2 mL, 0.02 M). Sodium Meta-periodate (30 mg) was added to the solution and kept on a magnetic stirrer at room temperature for 5 hours in dark place [6]. Then, 200 mg Herceptin was added to the activated nanoparticles solution. The solution was incubated in dark at 4°C for 16 hours. The mixture was deoxidized by adding sodium cyanoborohydride (30 mg) in dark at 4°C for 2 hours. Finally, the Herceptin nanoparticles conjugation was separated on gel column (Sephadex G50, Pharmacia, Germany) and the final antibody/nanoparticle ratio was calculated after determining the protein content with Lowry methods [7] and iron content by atomic absorption spectrophotometry (2100 UV spectrophotometer). Briefly, A sample of HMNs (100 µL) was mixed with HCl (100 µL) and H_2O_2 (10%, 100 µL) heated to allow the iron content of nanoparticles be dissolved and oxidized to Fe^{3+}. After adding potassium thiocyanate (3 mL, 3%), the Fe^{3+} formed a red complex with the thiocyanate which could be measured by a spectrophotometer at 480 nm. The standard curve for calculation of iron content was obtained measuring different concentrations (0, 250, 500, and 1000 mg/mL) of ferric chloride.

2.4. Quality control tests

The hydrodynamic diameter of HMNs was measured by dynamic light scattering (DLS) technique using Zeta Sizer (3000HS, Malvern, UK). The core size was determined by TEM (transmission electron microscopy, JEM 2010, JEOL, Japan). For considering the stability, without any additional agents, the HMNs were stored in purified water at 4°C for a period of 8 weeks. Once a week, an aliquot (500 µL) of the nanoparticles suspension was centrifuged and the supernatant analyzed for unbound Herceptin using Lowry methods [7]. The size of nanoparticles was also measured by Zeta Sizer every week. The measurements were repeated by storing HMNs in phosphate buffered saline (PBS) at pH = 6, pH = 7 and pH = 8.

2.5. Cell line preparation

The SKBR3, MCF7, A431, SW480 and RAJI cell lines were purchased from Pasteur Institute (Tehran, Iran). SKBR3 is a hormone-independent cell line originally derived from a breast adenocarcinoma. It has an amplified HER2 oncogene and overexpresses HER2 receptors [8] MCF7 (an estrogen-dependent mammary adenocarcinoma) [9], A431 (a human epidermoid carcinoma) [10], SW480 (a human epithelial colon adenocarcinoma) [11] and RAJI (a Burkitt lymphoma) [12] possess numerous receptors and express low levels of HER2 receptors.

The cells were grown and maintained in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient

Mixture F-12) supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 µg/mL streptomycin and incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C. Nearly confluent, cells in 75 cm^2 tissue culture flask were harvested by trypsin–EDTA solution and centrifuged at 1000 g for 10 min.

2.6. Cytotoxicity test

The cytotoxicity of HMNs was checked on SKBR3, MCF7, A431, SW480 and RAJI cells. Samples containing 6.0×10^6 cells were distributed in 96 wells plates. Different concentration (10-40 µg/mL iron) of HMNs were added to the wells and incubated for 24 hours. The cell supernatant was removed and washed three times with phosphate PBS. MTT solution (50 µL, 5 mg/mL) was added to each well and incubated for 3 hours. After that the cells were treated with dimethyl sulfoxide (50 µL). Absorption at 570 nm was measured on a plate reader. Hundred Percent viability was assumed from untreated cells.

2.7. HER2 specific binding

The HMNs cellular uptake was considered as a measure of their binding ability to HER2 oncogene on the cell surface and assessing the HER2 expression levels of the cell lines. The cells (6.0×10^6 cells/mL) were incubated with HMNs (60 µg iron, in 2 mL DMEM/F12) for 2 hours at 37ºC. The samples were centrifuged (3200 g, 10 min) and washed thrice with PBS then tested for iron content by colorimetric assay as described before.

This procedure was repeated for MRI imaging of the cells by a 1.5 Tesla clinical magnetic resonance scanner (Magnetom, Siemens Medical Systems, Erlangen and Germany) and using a knee coil. The cells (6.0×10^6 cells/mL) were resuspended in 1 mL agarose gel (PBS with 2% agarose), after 3 times washing with PBS, and transferred into plates for imaging. The plates were scanned by a fast gradient echo pulse sequence (TR = 3000, TE = 90, flip angel = 10º). Measurements of signal intensity were performed directly on the T_2 images using an operator-defined region of interest (ROI) with a constant size of pixels. The relative changes in signal intensity before (SI_{before}) and after (SI_{after}) treatments with HMNs were calculated as follows:

$$\text{Enhancement} = 100\times\frac{SI_{before}-SI_{after}}{SI_{before}}$$

2.8. Statistical analysis

Statistical analysis was performed using SPSS 10.0 considering p-value<0.05 as significant levels. The final values were presented as mean ± standard deviation (SD). The paired t-test was used to compare the results of stability, cytotoxicity, HER2 expression and signal intensity measurements.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. Particles characterization

The hydrodynamic and core size of HMNs was determined by DLS technique and TEM. The size distribution was in Gaussian form (p-value <0.05, Kolmogorov-Smirnov test) with the mean diameter 75±15 nm. The average core size of nanoparticles was 10±1.0 nm. The antibody/nanoparticle molar ratio was 3.1 to 3.5.

3.1.2. Stability of the complex

For considering the stability, the free Herceptin in the supernatant of HMNs was measured over 8 weeks. During this period no free antibody was measurable in purified water and PBS (pH = 6.0, pH = 7.0 and pH = 8.0).

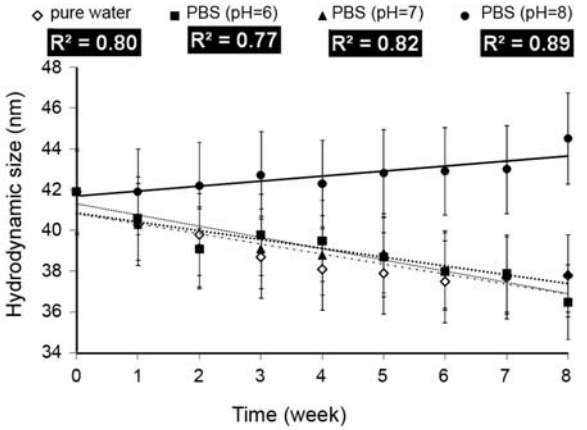

Figure 1: *Herceptin-nanoparticles were dispersed in purified water and PBS buffer with different pH (6, 7 and 8) show a very good stability up to 8 weeks.*

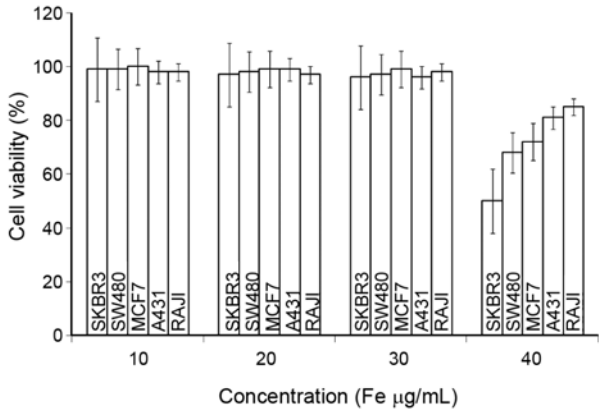

Figure 2: *The graph shows the relative number of the cells treated with different concentration of Herceptin nanoparticles as measured by MTT assay.*

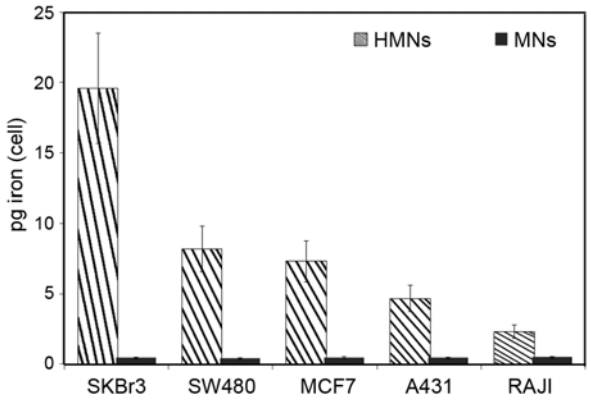

Figure 3: *The iron content of the cells after 2 hours treatment with MNs and HMNs at concentration of 30 µg(Fe)/mL in medium.*

The average hydrodynamic size of HMNs in purified water and PBS over this period was also determined. The result of measurements is presented in Figure 1. After 8 weeks of storage in purified water there was only 12% reduction in HMNs size. The best stability for HMNs was occurred in PBS at pH = 7 with 7% size increasing.

3.1.3. Cytotoxicity of the complex

The relative number of cells at 24 hours post treatment with HMNs that measured by MTT assay are presented in Figure 2. The HMNs up to concentration of 30 µg(Fe)/mL did not significantly (p-value < 0.05) inhibit any variations in the cell growth. They were toxic for all the cell lines at the concentration of 40 µg(Fe)/mL of the complex.

3.1.4. HER2 specific binding

The HMNs and MNs uptakes by the cells are presented in Figure 3. The graph shows the iron content of the cells (pg/cell) 2 hours post incubation of HMNs and MNs at equal concentration (30 µg/mL iron in the medium). The maximum and minimum uptake were observed with SKBR3 (19.6±2.5 pg/cell) and RAJI (2.3±1.8 pg/cell) cells respectively. The uptakes of SW480 (8.2±1.2 pg/cell), MCF7 (7.3±1.3 pg/cell) and A431 (4.6±1.7 pg/cell) cells were also low. This observation was consistence with the HER2 expression levels of the cell lines reported previously [15-19]. The T2-weighted images of the cells before and after treating with HMNs are shown in Figure 4-A. In all the cell lines, the signal intensity significantly (p-value<0.05) decrease after treatment with HMNs by showing a negative enhancement. The maximum signal enhancement was observed with SKBR3 (75±2.1%) cells and the minimum with RAJI cells (5.4±1.3%). The signal enhancement with SW480, MCF-7 and A431 cells were 31±2.1%, 26±1.5% and 11±1.7% respectively. It can be concluded that signal enhancement was proportional to the cells HER2 expression levels. The iron content in cells after treatment with HMNs versus the signal enhancement is presented in Figure 4-B. The results showed a linear relative between iron quantity and signal enhancement ($R^2 = 0.99$).

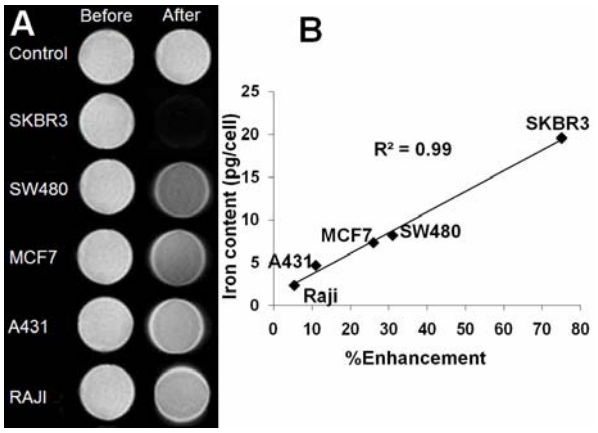

Figure 4: *T2-weighted MRI images of the cells before and after 2 h treatment with the complex (A). The SKBR3 cells showed maximum difference in signal intensity compared to the other cell lines tested. The relation between iron content of the cells and signal enhancement in T2 images was linear (B).*

3.2. Discussion

Cancer tissues differ from normal in their anatomy and protein expression patterns [13]. They often over express receptors for peptides, hormones and antibodies. These receptors provide an opportunity for the active and specific targeting [13].

The HER2 protein is a receptor on the surface of the cells that controls the cells growth and division. In HER2-positive cancer, the amount of HER2 protein in the cells increases and tumor growth can be very fast and more aggressive [13].

Herceptin is a monoclonal antibody against HER2 used in treatment of HER2 positive cancers [5]. It is an expensive drug and can have serious side effects. To increase the effectiveness of treatment and reducing the side effects and cost, it is important to have tests that accurately determine the HER2 tumor status [14]. For selecting the Herceptin therapy for treatment, the biopsy exam must be performed for the patient tumors (it's an invasive method) and the samples referred to pathology lab. There are two main methods for testing the in vitro HER2 expression. Immunohistochemistry (IHC) measures the levels of HER2 protein present in the tumor sample grading it from 1+ to 3+ [14]. A test result showing the score 1+ means the cells are HER2 negative, 3+ means they are HER2 positive and score 2+ means the result is not clear. Another test,the fluorescence in-situ hybridization (FISH) test measures the amount of the HER2/neu gene in each cell and the result is either FISH positive, or FISH negative [14].

In this study, we synthesized Herceptin conjugated magnetic nanoparticles as an alternative probe to discover the levels of HER2 in the surface of the cells. These nanoparticles can be used by MRI imaging (non-invasive methods) for screening the patients with Her2 positive or negative tumors.

Dextran coated iron oxide nanoparticles were synthesized with hydrodynamic size of 50-100 nm and core size of 9-11 nm. The nanoparticles were conjugated to Herceptin, checked its toxicity and uptake in SKBR3, SW480, MCF7, A431 and RAJI cell lines.

HMNs successfully demonstrated that SKBR3 cells considerably overexpressed HER2. It also showed that the HER2 expression levels was lower in SW480, MCF7, A431 and RAJI cell lines as expected [8-12]. The result of iron content measurement was quite consistence with MRI T2 images. The signal intensity of SKBR3 (HER2 positive) cells in the presence of HMNs was significantly (p-value<0.05) lower than the other cells at the same conditions. Moreover, HMNs had the ability to target and differentiate human cancer cells with different HER2 expression levels as proven by in vitro MRI studies. Its good stability in various pHs and low cytotoxicity (up to 30 μg(Fe)/mL) suggests the possibility of external imaging of HER2 positive tumors with magnetic resonance imaging (MRI).

4. CONCLUSIONS

In this study we synthesized Herceptin conjugated iron oxide nanoparticles as a probe for HER2 detecting. We considered its HER2 binding ability in cancer cells based on the iron content detection and MR imaging of the target cells. The good characteristics of HMNs demonstrated that it can detect the HER2 levels in the cell surface and be a good candidate to use as a HER2 tracer contrast agent in MRI that needs further investigations.

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