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Gadolinium-Diethylenetriaminepenta-Acetic acid Conjugated with Monoclonal Antibody C595 as New Magnetic Resonance Imaging Contrast Agents for Breast Cancer (MCF-7) Detection

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ABSTRACT

Background: The monoclonal antibody, C595, against breast cancer cell line was conjugated with cyclic anhydride gadolinium-diethylenetriaminepenta-acetic acid (Gd-cDTPAa) to produce Gd-DTPA-C595 and used as specific breast cancer cell line (MCF-7) contrast agents in magnetic resonance imaging (MRI). **Methods:** After incubation of breast cancer cell line (MCF-7), with different contrast agents (Gd-DTPA-C595, Gd-DTPA, Gd-H and $GdCl_3$) at 37°C for 12 h and twice washing, the T_1 relaxation times and the signal enhancement of washing solutions of different contrast agents are examined by nuclear magnetic resonance and results are compared. The percent of Gd that attached into the cell membrane of MCF-7 was also measured by UV spectrophotometer. **Results:** The data indicate that the T_1 relaxation of washing solutions at 11.4 Tesla (500 MHz) in Gd-DTPA-C595 was greater than in Gd-DTPA solutions and was much greater than in control. These conjugates (Gd-DTPA-C595) show high specificity for breast cancer cell line (MCF-7). The gadolinium concentration in washing solutions measured using UV-spectrophotometer showed no gadolinium attached into the cell membrane in the $GdCl_3$ as control. **Conclusion:** Good cell membrane uptakes of Gd-DTPA-C595 indicate selective delivery of this agent into the breast cancer cell membrane and have considerable potential in diagnostic MRI. *Iran. Biomed. J. 10 (4): 209-213, 2006*

Keywords: Magnetic resonance imaging (MRI), Gadolinium, Monoclonal antibody, Breast cancer

INTRODUCTION

The development of contrast agents with tissue-specific enhancement deserves considerable attention because of their potential in early diagnosis. One approach to increasing the specificity of magnetic resonance Imaging (MRI) contrast is to use a monoclonal antibody (mAb) coupled with contrast agent Gadolinium-diethylenetriaminepenta-acetic acid (Gd-DTPA) [1-4]. For the first time, Pressman and Korngold [5] used ^{131}I-labeled polyclonal antibodies for tumors in rat. The development of mAb [6] offers the possibility of defined product for routine diagnosis. Mach *et al.* [7] published the first description of clinical studies with monoclonal anti-CEA antibodies in 1981.

Gadolinium is the element of choice for MRI enhancement due to its high number of unpaired electron [8]. The first *in vivo* studies with the gadolinium-labeled mAb were performed by Unger *et al.* [1] and Andersen-Berg *et al.* [2]. Gadolinium was attached to the mAb via the coupling of anhydride derivatives of DTPA. These studies showed that 100-1,000 gadolinium ions per antibody conjugate are needed to achieve concentrations in the tumor sufficient for signal enhancement in MRI [8]. Curtet *et al.* [3] coupled mAb to gadolinium using cyclic anhydride method and demonstrated that T_1 relaxation of water protons decreased significantly (by 15%) with mAb 19-9, which recognizes human colon adenocarcinoma. They used a 25-Gd-DTPA-antibody conjugate to reduce the effect of immunological reactivity. Implanted human colon carcinoma tumors in mice have been successfully imaged by using the mAb attached with a large number of Gd-DTPA molecule [9]. In recent years, *in vivo* studies of Gd-DTPA- mAb and Gd-

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porphyrins as MRI contrast agents for melanoma detection was investigated in nude mice by Shahbazi-Gahrouei *et al.* [10].

Nowadays, research efforts concentrated on maximizing the delivery of specific T_1 agents to tumors [11-14]. Goher-Rosental *et al.* [4] reported successful studies of Gd-DTPA mAb in subcutaneous tumor in nude mice. Matsumura *et al.* [11] also observed MRI contrast enhancement by Gd-DTPA-mAb in 9L-glioma rats. Marleen *et al.* [12] used Gadomer-17 as tissue specific dynamic T_1 MRI contrast agents in two types of glioma xenografts. However, in all mentioned studies the number of Gd attached to the DTPA-protein complex, the effect of chelation on antibody specificity, and the Gd-DTPA-antibody stability is problematic.

The aim of this study is to investigate the conjugation of gadolinium with mAb (Herceptin, C595) and to observe the contrast enhancement effect of Gd-DTPA- mAb as MRI contrast agent for detection of breast cancer cell line (MCF-7). All washing solutions were tested by UV spectrophotometer to determine the gadolinium concentration and the effect of the Gd-DTPA- mAb on the *in vitro* conditions.

MATERIALS AND METHODS

Samples preparation. The mAb C595, specific for breast cancer cell line (MCF-7), was covalently bound to the DTPA chelating agent using modification method of Hnatowich [15]. Cyclic anhydride DTPA (cDTPAa, 0.1 mg) was dissolved in chloroform (1 ml) and was degassed under a stream of nitrogen for 1 h. DTPA (2 mg, 1 ml) and C595 (3 mg, 1 ml) antibodies solution was added and the mixture incubated at 0°C for 45 minute. Mole ratio of DTPA to C595 was 20:1. The resulting solution was loaded on to a PD-10 column, Sephadex GM-25, (10 × 1 cm, Pharmacia, Biotech) and eluted with 0.5 M sodium acetate (pH 5.5), collecting 2 ml. The concentration of C595 in the final solution was determined by protein estimation as 2 mg/ml.

Gadolinium (III) chloride hexahydrate (1.8 mg) was dissolved in 1 ml of distilled water. To this solution, DTPA-C595 (4 mg, 2 ml) was added. The pH was adjusted to 5 by addition of 1-M sodium acetate. After stirring for 1 h at room temperature, the solution was added to a PD-10 column and eluted with sodium chloride (0.15 M, pH 5), collecting 1 ml fractions. The fourth and fifth fractions were combined to yield 2 ml of pure gadolinium conjugates. The gadolinium concentration was (protein estimate, 2 mg/ml; [Gd], 0.50 mM) in conjugate of Gd-DTPA-C595 solution.

All column fractions from the chromatography column (purification) were collected and an aliquot of each fraction was analyzed for concentration of protein and gadolinium. Concentration of protein was measured by UV-spectrophotometer absorption at 280 nm using standard protein estimation method. The fourth and fifth fractions from the column were combined as a Gd-DTPA- mAb tumor specific agent for further studies. Gadolinium-hematoporphyrin was also prepared as described previously [16].

The breast cancer cell line (MCF-7, NCBI C135, the Pasteur Institute of Iran, Tehran) was incubated with Gd-DTPA-C595 at 37°C for 12 h. The incubation time for all contrast agents was chosen as 12 h, and more than it, there was no more attachment of agents into the cell membranes. After the incubation, all the cells were washed twice with PBS/2% FCS, centrifuged, and resuspended in PBS/2% FCS solutions. In this study pure water and PBS was used as blank solution.

Nuclear magnetic resonance and UV-spectrophotometer experiments. The T_1 relaxation times and signal intensities of washing solution of discrete compounds ($GdCl_3$, Gd-DTPA, and Gd-H) and conjugates antibody (Gd-DTPA-C595) were measured using an inversion recovery pulse sequence technique using a 11.4 Tesla Bruker instrument, Germany (500 MHz, Tarbiat Modarres University, Tehran, Iran). The values of echo time and repetition time were optimized for different washing solutions. The gadolinium content of washing solution was also measured by UV-spectrophotometer (Spectronic Gene Sys2, Spectronic Instrument), using 342.249 nm for gadolinium. All sample preparations and UV-spectrophotometry experiments were done in the Department of Medical Physics, Isfahan University of Medical Sciences (Isfahan, Iran) at 2005.

T_1 relaxation times and relaxivity measurements. The relationship among (reversal of T_1) the increment of the water proton relaxation rate per unit concentration of the paramagnetic contrast agent, is called the relaxivity (R_1) and is calculated according to the following formula:

$$1/T_i = 1/T_{i\,(control)} + R_i\,C$$

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where i = {1, 2}, T_i is relaxation time of sample, $T_{i\,(control)}$ is relaxation time of blank or the system before addition of contrast agent, C is concentration of paramagnetic contrast agent or Gd, and R_i is the relaxivity (mM^{-1} s^{-1}). The measurement was performed in aqueous solution of Gd-DTPA-C595. In these experiments, the concentration of conjugated antibody was kept constant (0.01 mM), and the amount of gadolinium concentration bound to DTPA-C595 was varied.

RESULTS

T_1 relaxation time and signal intensity measurements. For $GdCl_3$, no gadolinium was detected in the cancer cells and all gadolinium was found in washing solutions. For Gd-DTPA and Gd-H, some gadolinium was attached to the cells. However, a larger fraction of gadolinium complex was observed in washing solutions.

Table 1 shows the T_1 relaxation time of washing solution of breast cell line with different contrast agent at room temperature. As this Table shows the amount of gadolinium ions which attached into the breast cancer cell membranes resulted in an increase in T_1 relaxation time.

Table 1. T_1 relaxation times of different contrast agents in washing solution (mean ± SEM of values obtained from an average of five samples).

Contrast agent	T_1 ± SD (ms)
Gd-DTPA-C595	1123.0 ± 26.9
Gd-DTPA	933.0 ± 31.0
$GdCl_3$	560.0 ± 42.1
Gd-H	1026.0 ± 63.7
Water [a]	2048.3 ± 00.0
PBS[a]	1013.8 ± 00.0

[a]pure

The T_1 relaxation times of the intact cells reflected the amount of gadolinium absorbed into or fixed to the cell membranes. As can be seen from this Table, the lowest amount of gadolinium and the highest T_1 relaxation time are observed for the washing solution of specific conjugated mAb, Gd-DTPA-C595. Figure 1 shows the graph of signal intensity plotted against the gadolinium quantity in different washing solution of different studied agents. $GdCl_3$ as control had high MRI signal intensity of washing solutions, but the lowest MRI signal intensity was observed for the washing solutions of Gd-H and Gd-DTPA-C595. Since higher signal intensity is due to larger gadolinium concentrations, this indicated that lower concentration of gadolinium was found in washing solutions of Gd-H and Gd-DTPA-C595, resulted approximately all gadolinium remained into the cell membranes. This result is in good agreement with previous studies [10, 15].

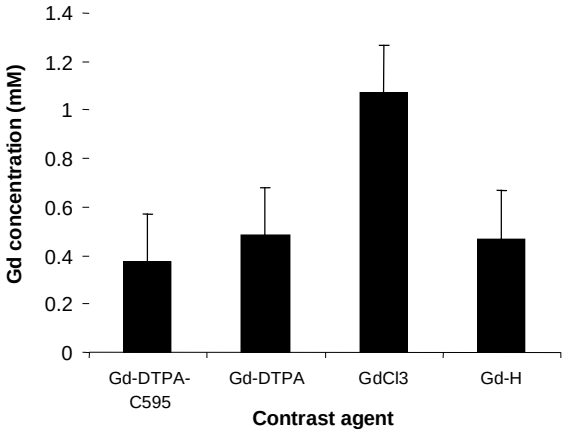

Fig. 1. Signal intensity of different contrast agents in washing solution of different contrast agents.

Determination of gadolinium concentrations. The gadolinium concentration in washing solutions of mentioned contrast agents ($GdCl_3$, Gd-DTPA, Gd-H, and Gd-DTPA-C595) was measured by UV-spectrophotometer and results are shown in Table 2.

For $GdCl_3$, most of this material was found in the washing solution. This indicated that during incubation $GdCl_3$ remained in solution and did not become fixed to the breast cancer cell membranes. Gd-DTPA showed some uptake into the MCF-7 cell membranes with incubation, however, its amount was significantly lower than that of the mAb conjugate. Gd-H showed a greater uptake into the cells compared to Gd-DTPA, but still not as high as the uptake of the antibody conjugate.

Table 2. Gadolinium concentration of different contrast agents in washing solution (mean ± SEM of values obtained from an average of five samples).

Contrast agent	Gd concentration ± SD (mM)
Gd-DTPA-C595	0.372 ± 0.06
Gd-DTPA	0.482 ± 0.21
$GdCl_3$	1.070 ± 0.34
d-H	0.468 ± 0.02

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Table 3. $1/T_1$ relaxation rates of water for different number of gadolinium bound to Gd-DTPA-C595 in aqueous solution. Gadolinium ions inserted by reaction of DTPA-C595 with $GdCl_3$.

DTPA-C595 (mM)	$GdCl_3$ (mM)	T_1 (ms)	$1/T_1$ (1/s)
0.01	00.0	2760 ± 32.0	0.36 ± 0.02
0.01	00.1	1000 ± 12.0	1.00 ± 0.07
0.01	00.5	521 ± 2.10	1.90 ± 0.10
0.01	10.0	159 ± 3.60	6.20 ± 0.60
0.01	30.0	74 ± 6.10	13.0 ± 0.30
0.01	60.0	43 ± 0.40	23.0 ± 0.80
0.01	70.5	35 ± 0.70	28.0 ± 0.20
0.01	10.0	30 ± 0.30	33.0 ± 0.40

Effect of Gd-DTPA-C595 on relaxation time T_1. Table 3 shows the effect of Gd-DTPA-C595 on water relaxation time T_1 at room temperature. The number of bound gadolinium ions increases result in a decrease in T_1 relaxation time. At high concentrations of gadolinium, the effect on T_1 relaxation time was reduced.

DISCUSSION

Several approaches might be considered to increase gadolinium concentration in the tumor. It is possible to increase the amount of DTPA coupling per molecule of mAb, although with a corresponding loss of immunoreactivity [1, 3, 10]. In order to improve the quality of imaging and increase efficacy of therapy using radiometal-labeled mAb, cyclic DTPA anhydride has been developed and well characterized. Hnatowich *et al.* [16] reported a successful conjugation of DTPA to human albumin (18.8 mg/ml, 2.8×10^{-4} M) using cDTPAa as an acylating agent. In this work, this agent was used to conjugate DTPA to a practical concentration (300 μg/ml) of mAb (C595). The choice of anhydride is sensible. Cyclic DTPA anhydride forms one of the strongest chelates known for a large number of metals [16].

Before assessing the potential clinical value of gadolinium coupled mAb for MRI of tumors, it was necessary to ensure that the antibody did not modify the paramagnetic properties of gadolinium in the complex. The studies performed on aqueous solutions of Gd-DTPA confirmed that T_1 is inversely proportional to the concentration of the paramagnetic agent. When gadolinium was conjugated with the antibody by means of the chelator cDTPAa, a linear relationship was observed between the reversal of T_1 relaxation time and the gadolinium concentration. The measurement of different gadolinium concentration versus reversal of T_1 was performed and results are showed in Table 3. The T_1 relaxation time of pure water was also measured to compare the accuracy of the results of this study and their agreements with other studies [10, 15].

The results of incubation of breast cancer cells with discrete compounds and conjugates mAb contrast agents showed that no gadolinium was uptake using $GdCl_3$. This also showed that any gadolinium released from their complexes either in the discrete compounds or the antibody conjugate will end up in the washing solutions. Therefore, any gadolinium found inside the cell lines must have been delivered to the cell membrane as the intact gadolinium complex. The assumption is the volume of the breast cancer cells has not changed during the incubation with the contrast agents. Hence, the amount of gadolinium in the washing solutions reflects the concentration of gadolinium that did not attaché to the cells. The lack of any uptake by MCF-7 for $GdCl_3$ was consistent with this finding that antibody remaining selective to breast cancer cells and the results are in good agreement with *in vivo* findings in nude mice [10].

Recently, it has been shown that other derivative complex of gadolinium is also applied as tumor-specific MRI contrast agents [12-14]. Because of differences between these gadolinium complexes and gadolinium complex here, it is not possible to compare results. As results showed, the highest T_1 relaxation time, reflecting the lowest gadolinium accumulation was observed for specific conjugated mAb (Gd-DTPA-C595). This is because the mAb binds the gadolinium to the cell membrane and/or internalizes the gadolinium into the cell membranes.

This result indicates that the mAb conjugates (Gd-DTPA-C595) is potential MRI contrast agents for detection of cancer at early stages. Gd-H also has a similar potential application as a breast-specific MRI

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contrast agent [17].

Overall, with the satisfactory low levels of gadolinium in the washing solutions, gadolinium antibody conjugates have considerable potential for further diagnostic applications of MRI.

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