Original Article

Radiolabeling of Herceptin with ^{99m}Tc as a Her2 Tracer

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Abstract

Background: Trastuzumab is a monoclonal antibody that is used in treatment of breast cancer. We labeled this monoclonal antibody with Technetium-99m and performed in vitro and in vivo quality control tests as a first step in the production of a new radiopharmaceutical.

Materials and Methods: Trastuzumab was labeled with Technetium-99m using Succinimidyl Hydrazinonicotinamide (HYNIC) as a chelator. Radiochemical Purity and stability in buffer and serum were determined. Immunoreactivity and toxicity of the complex were tested on SKBR3, MCF7 and A431 breast cancer cell lines. Biodistribution study was performed in normal mice at 4 and 24 h post injection.

Results: The radiochemical purity of the complex was 95±1.4%. The stabilities in phosphate buffer and in human blood serum at 24 h post preparation were 85±3.5% and 74±1.2%, respectively. The immunoreactivity of the complex was 86±1.4%. The binding of labeled antibody to the surface of SKBR3, MCF7 and A431 cells were increased by increasing the human epithelial growth factor receptor 2 (Her2) concentration on the cells surface.

Conclusion: These findings showed that the new radiopharmaceutical can be a promising candidate as Her2 antigen scanning for human breast cancer.

Keywords: Herceptin, Trastuzumab, ^{99m}Tc , breast cancer, Her2 , S-HYNIC

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Introduction

HER2/neu is an Mr=185 kDa transmembrane receptor tyrosine kinase (RTK)[1] that has been extensively studied with regard to its involvement in cancer and as a therapeutic target in tumors. In breast cancer, about 25–30% of patients overexpress HER2/neu[2] mainly due to gene amplification and in these patients, HER2/neu positivity is an independent prognostic indicator of poor long-term survival[2].

HER2 is poorly expressed in normal tissue, including normal breast. HER2 plays an important role in the regulation of cell cycle, pathogenesis and biological aggressiveness of breast cancers[2]. The development of monoclonal antibodies directed to the HER2 receptor extracellular domain, such as Trastuzumab, is currently being used for the treatment of breast cancer with HER2 over-expression[3,4].

Radioimmunoimaging of breast cancer, exploiting HER2/neu expression, could allow direct assessment of the HER2/neu status of primary and metastatic lesions, and may prove to be useful for selecting patients for treatment with trastuzumab, as well as predicting response to the drug[5].

The aim of this study was labeled ^{99m}Tc with

Trastuzumab throughout HYNIC (Succinimidyl Hydrazinonicotinamide) for using as a potential radiopharmaceutical for *in vivo* minimally invasive evaluation of HER2 receptor expression in breast cancer.

Methods

Materials: Trastuzumab was purchased in 140 mg vials (Genentech, South San Francisco). HYNIC was prepared from Solulink Co. All the chemicals were purchased from Fluka chemical corp.

Cell line: SKBr3 (hormone-independent cells originally derived from a breast adenocarcinoma expressing high level of HER2), MCF7 (an estrogen-dependent mammary adenocarcinoma cells expressing medium level of HER2) and A431 (a Human epithelial carcinoma cells expressing low level of HER2) cell lines were obtained from Pasteur Institute of Iran.

The cell lines were cultured and maintained in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere (95% air and 5% CO2) at 37°C.

The HYNIC conjugate with Trastuzumab: The conjugation of the HYNIC with trastuzumab was performed using the methods described by Abrams with some modifications[6]. HYNIC (a 20 M excess to mab, 10 mg/mL in DMF) was added dropwise to the mab solution (1 mg/mL in 0.15M Carbonate buffer, pH 8.5). The solution was stirred gently for 5 h at room temperature protected from the light. The HYNIC-Trastuzumab conjugate was dialyzed against 0.15 M Na/acetate buffer (pH 5.2) overnight.

The labeling of HYNIC-Trastuzumab with ^{99m}Tc: Technetium-Tricine complex was prepared according to Larsen[7]. The ^{99m}Tc [Tricine]$_2$ was then added to the solution of HYNIC-Trastuzumab (740 MBq per each mg Ab), and the mixture was incubated for 1 h at room temperature.

Quality control: The HYNIC-conjugated Trastuzumab was measured base on determination of hydrazide groups[8]. The radiochemical purity was determined by the instant thin-layer chromatography (ITLC). Free ^{99m}Tc migrates along the solvent front while ^{99m}Tc- HYNIC-Trastuzumab complex remained at the origin. Immunoreactivity of complex was performed according to Lindmo method[9].

The stability of the labeled antibody in phosphate buffer saline (pH 7.4) at room temperature was assessed in time intervals up to 24h and was assessed same as the procedure described above by ITLC. For estimate the stability of the ^{99m}Tc-HYNIC-Trastuzumab complex in 37°C, fresh human serum after 24h. The labeled antibody was added at the concentration of 10µg/ml in serum and analyzed by ITLC.

MTT assay: The cytotoxicity of the complex was analyzed on the SKBR3, MCF7 and A431 cell lines by colorimetric assay based on MTT assay[10].

Cell-binding assay: The SKBR3, MCF7 and A431 cell samples (1.0×10^6 cells/well) were seeded for 24h. ^{99m}Tc- HYNIC-Trastuzumab at concentrations of 0-100ng was added to the samples and incubated for 1 h at 37ºC. The medium was then aspirated and the cells were counted for ^{99m}Tc radioactivity using a γ-counter.

Biodistribution: The *in vivo* behavior of ^{99m}Tc-HYNIC-Trastuzumab was evaluated in BALB/c mice weighing 20–25 g. The tissue biodistribution experiments were performed in two groups of six female BALB/c mice. The labeled antibody was injected via the tail vein (100µL, 30µg mab

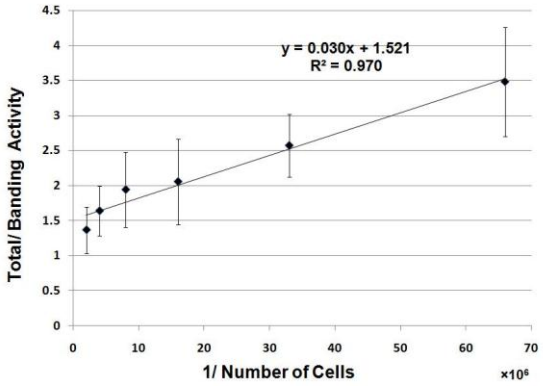

Figure 1. The immunoreactivity fraction shows the ability of the complex to bind to SKBR3 cells.

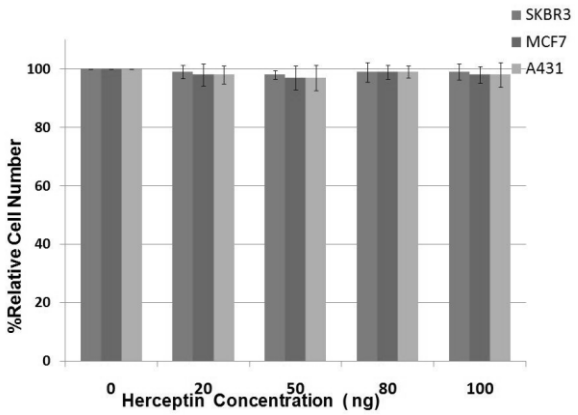

Figure 3. The results of MTT colorimetric assay after 24h of treatment with different concentrations of the complex.

equivalents to 3.7-5.5 MBq). At 4 and 24 h post injection, groups of mice were sacrificed by ether anesthesia. The vital organs were dissected and weighed. Activities of all samples were measured in a γ-counter, and organ activity was expressed as a mean percentage of injected doses per gram of tissue (%ID/g).

Animal experiments were performed in compliance with the regulation of our institution and with generally accepted guidelines governing such work.

Mice bearing Her2 tumor: The tumors were originally established from a murine mammary carcinoma in an inbred female BALB/c mouse. The breast tumor was produced by implantation of the tumor fragments (≈1 mm^3) in the right flank of healthy inbred female BALB/c mice (20–25g, 8–10 weeks old). The biodistribution study was performed by the tumors with volumes reached 7-8mm^3. To finding what is the reaction between trastuzumab and the mice tumor cells, immunohistochemistry was applied[11].

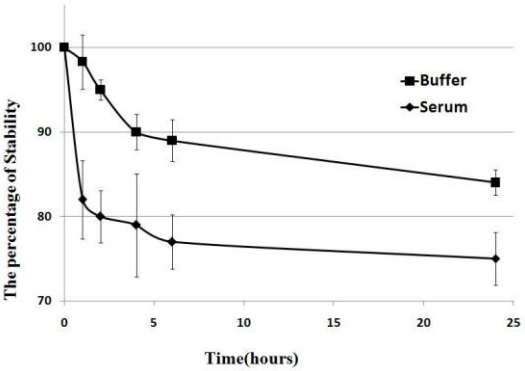

Figure 2. The stability of the complex in phosphate buffer and human blood serum at different times after labeling.

Imaging studies: The biological behavior of ^{99m}Tc- HYNIC-Trastuzumab was also ascertained by carrying out simultaneous scintigraphic imaging studies in mice bearing Her2 tumor (n=5). ^{99m}Tc-HYNIC-Trastuzumab (3.7-5.5 MBq /0.1mL) was injected intravenously via the tail vein. Serial scintigraphic images were recorded at 4h after injection using a low-energy, high-resolution collimator (LEHR). Prior to the acquisition of images, the animals were anesthetized using a combination of xylazine hydrochloride and ketamine hydrochloride. All the images were recorded by acquiring 300 K counts per pixel in 512×512 matrix size.

Statistical analysis: All statistical analyses were performed using SPSS 13.0. Each in vitro experiment was performed three times, each times the values from eight wells were determined and the final values were presented as mean±standard deviation (SD). The paired t-test was used to compare the results and $p<0.05$ were considered to indicate statistical significance.

Results

Quality control tests: The radiochemical purity of the complex was 95±1.4% at 1h after labeling. The results of the immunoreactivity tests are presented in figure 1. The immunoreactivity curve is the double inverse plot of the total applied radioactivity divided by the cell bound radioactivity against the inverse of the cell concentrations[12] . The equation of the linear curve fitted to data is $Y=0.030X+1.521$ ($R^2=0.97$, $p<0.05$). The inverse of the intercept (1/1.521=86%) is the immunoreactivity[9]. The graph shows that the binding of ^{99m}Tc to trastuzumab significantly affects the immunoreactivity of the antibody. The percentage of immunoreactivity was 86±1.4%.

The stability of the complex in human blood serum and in phosphate buffer at 1, 2, 4, 6 and 24h is presented in figure 2. The complex showed good stability up to 24 h in both of the media. On the average 84±1.2% and 75±3.5% of the complex was stable in the phosphate buffer and in human blood serum at 24h, respectively. The stability of the complex in buffer was higher than that in human blood serum due to the lack of blood enzymes in the buffer.

Cytotoxicity (MTT colorimetric assay): The cytotoxicity of the complex on the SKBR3, MCF7 and A431 cell lines are shown in figure 3. No cytotoxicity at equal concentrations of 100ng of

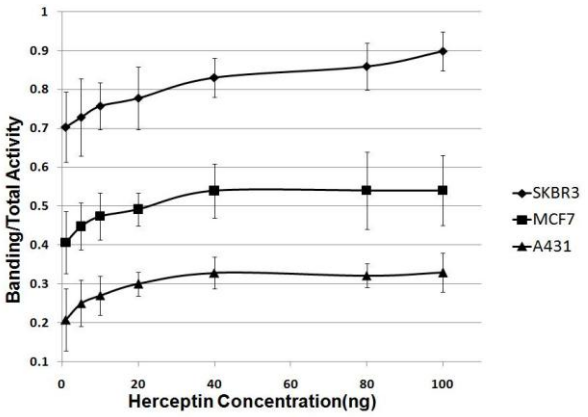

Figure 4. The cell-binding assay with SKBR3, MCF 7 and A431 cells with different concentration of ^{99m}Tc-S-HYNIC-Trastuzumab.

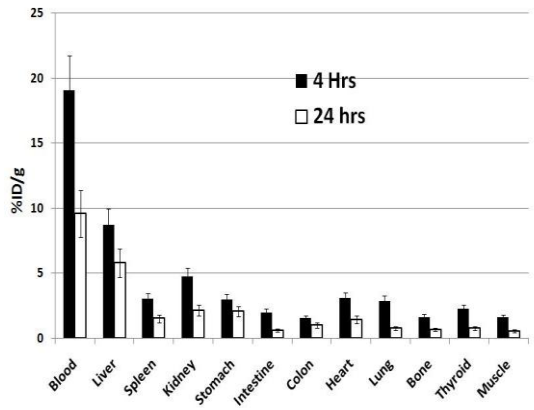

Figure 5. Biodistribution study of ^{99m}Tc- HYNIC-trastuzumab in female BALB/c mice performed at 4 and 24 h post injection.

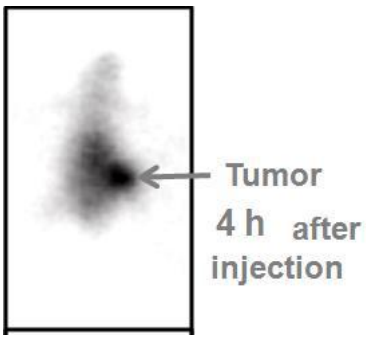

Figure 6. The gamma camera images were acquired at 24 and 168 h after injection of ^{177}Lu-trastuzumab.

complex was seen on the cells.

Cell-binding assay: The affinity of the ^{99m}Tc-HYNIC-Trastuzumab for the target HER2 was determined with cell-binding assay. The binding of labeled antibody to the surface of SKBR3, MCF7 and A431 cells were increased by increasing concentration of the Her2 on the cells surface (Figure 4). The ability of this complex to compete with complex to bind to the cell receptors showed specific binding of the ^{99m}Tc-HYNIC-Trastuzumab onto the human breast cancer cell lines.

Biodistribution: The result of the ^{99m}Tc-HYNIC-Trastuzumab biodistribution in the mice bearing breast tumors at 4 and 24h after IV administration, expressed as percentage of injected dose per gram of tissue (%ID/g) is shown in figure 5.

Gamma camera imaging: The gamma camera images at 4h after injection of ^{99m}Tc- HYNIC-trastuzumab are shown in Figure 6. The tumor accumulated large amounts of ^{99m}Tc. Small amounts of radioactivity could also be found in the liver area. A region of interest analysis of the tumor and corresponding lateral region gave a tumor to background ratio of 20±3.5 for 4h.

Discussion

It has been found that 20-30% of all breast cancers overexpress Her2[13]. These patients with HER2 overexpression are associated with a poor prognosis, more disease relapse, and distant metastasis. These patients may be candidates for trastuzumab treatment in the postsurgical or metastatic setting[14].

Trastuzumab is a humanized antibody binds to the extracellular domain of the HER2 receptor. This antibody arrests the treated cells during the G1 phase of the cell cycle and exhibit reduced proliferation[1]. Unfortunately, Trastuzumab treatment is expensive and has been associated with cardiac toxicity[15]. For these reasons, it is very important to evaluate HER2 expression. Currently, it is evaluated by Fluorescence in situ hybridization (FISH) or immunohistochemistry from a biopsy sample. A minimally invasive *in vivo* characterization of the HER2 status of these patients would represent a qualitative advantage. This approach would be possible with the development of radiolabeled Trastuzumab.

The strategy could provide information about HER2 expression, the receptor distribution and a whole body scan for HER2 tracing in positive metastasis. There are currently different strategies to label monoclonal antibodies with ^{99m}Tc. A bifunctional agent widely used currently is the SHYNIC[16-18]. The main advantages of HYNIC as a bifunctional agent are high labeling efficiency, rapid and high yield radiolabeling[7].

In this study, the labeling procedure of ^{99m}Tc-S-HYNIC-Trastuzumab is achieving radiochemical purity higher than 95%. ^{99m}Tc-HYNIC-Trastuzumab showed *in vitro* stability in buffer (84±1.2%) and blood serum (75±3.5%) for 24h post-labeling, allowing enough time for managing controls and clinical application.

The prepared complex showed good immunoreactivity (86±1.4%) no cytotoxicity and specific binding onto the SKBR3, MCF7 and A431 cells.

In a study, trastuzumab was labeled with ^{99m}Tc by Hynic (the radiochemical purity: 90%, stability was checked only in buffer: 95%, immunoreactivity: 66±0.8%, with no cell interaction checking) and the biodistribution was checked in mice bearing spontaneous breast tumor mice[19]. Their biodistribution findings in normal mice are similar to this study with slight differences. Their result of blood clearance at 24 h is about 5±2.5% and the liver uptake is increased by time (~11±2.5%). While the liver uptake of our

complex (7.7±1.6%) is slight lower and can produce the blood clearance 9.6 ±%2.2 after 24 h. This indicates no statistical significance ($p<0.05$).

Conclusion

From these results we can conclude that ^{99m}Tc-HYNIC-Trastuzumab may be considered for further evaluation in animals and possibly in humans as a new radiopharmaceutical for HER2 detection in breast cancer. This has the potential to substitute breast biopsy methods to establish HER2 status and also to be used to detect HER2 distant metastasis and evaluate Trastuzumab therapy response.

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