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Preparation and Bioevaluation of ^{177}Lu-labelled Anti-CD44 for Radioimmunotherapy of Colon Cancer

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Abstract - CD44 is a particular adhesion molecule and facilitates both cell-cell and cell-matrix interactions. In particular, splice variants of CD44 are particularly overexpressed in a large number of malignancies and carcinomas. In this study, the ^{177}Lu-labelled CD44 targeting antibody was prepared and bioevaluated *in vitro* and *in vivo*. Anti-CD44 was immunoconjugated with the equivalent molar ratio of cysteine-based DTPA-NCS and radioimmunoconjugated with ^{177}Lu at room temperature within 15 minutes. The stability was tested in human serum. An *in vitro* study was carried out in HT-29 human colon cancer cell lines. For the biodistribution study ^{177}Lu-labelled anti-CD44 was injected in xenograft mice. Anti-CD44 was immunoconjugated with cysteine-based DTPA-NCS and purified by a centricon filter system having a molecular cut-off of 50 kDa. Radioimmunoconjugation with ^{177}Lu was reacted for 15 min at room temperature. The radiolabeling yield was >99%, and it was stable in human serum without any fragmentation or degradation. The radioimmunoconjugate showed a high binding affinity on HT-29 colon cancer cell surfaces. In a biodistribution study, the tumor-to-blood ratio of the radioimmunoconjugate was 43 : 1 at 1 day post injection (p.i) in human colon cancer bearing mice. The anti-CD44 monoclonal antibody for the targeting of colon cancer was effectively radioimmunoconjugated with ^{177}Lu. The *in vitro* high immunoactivity of this radioimmunoconjugate was determined by a cell binding assay. In addition, the antibody's tumor targeting ability was demonstrated with very high uptake in tumors. This radioimmunoconjugate is applicable to therapy in human colon cancer with highly expressed CD44.

Key words : Lutetium-177 (^{177}Lu), Anti-CD44, Radioimmunotherapy, Tumor targeting

INTRODUCTION

Chemotherapy can be efficient, but a lack of specificity often causes an indiscriminate toxicity. One way to reduce the toxicity is to selectively accumulate cytotoxic substances in malignant tumors by targeting molecular structures, which are overexpressed by the cancer cells. Using monoclonal antibodies as a targeted therapy can be done only for cancers in which the antigens have been identified. Radioimmunotherapy (RIT) uses an antibody labeled with a radioisotope to deliver cytotoxic radiation to a target cell. The use of a radionuclide can be advantageous owing to a lack of multidrug resistance and the so-called cross-fire effect (i.e., irradiation of cancer cells by nuclides delivered to their malignant neighbors) (Volkert *et al*. 1999; Smith-Jones, 2004; Goldenberg *et al*. 2006; Jain *et al*. 2007). The radionuclide-labeled anti-lymphoma antibodies, Zevalin (^{90}Y) and Bexxar (^{131}I), showed clear improvement in response rates in comparison with their non-radiolabeled counterparts (Hagenbeek *et al*. 2005). In contrast to the overwhelming success of RIT in treating NHL, only modest success has been achieved in the RIT of solid tumors. This can be partially explained in that lymphomas are generally

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less radio-resistant. The RIT of solid tumors still remains a challenge. The important step is using an appropriate combination of radionuclide and antibody (Jain *et al*. 2007; Salaun *et al*. 2010; Huang *et al*. 2012).

Colorectal cancer compromises a transformation of the normal colonic epithelium to an adenoma and then to a carcinoma. This process is characterized by diverse molecular alterations including epithelial cell differentiation, proliferation, migration, and apoptosis. The conservation of colonic epithelium is facilitated by adhesion molecules. CD44 is a particular adhesion molecule that facilitates both cell-cell and cell-matrix interactions. In particular, splice variants of CD44 are particularly overexpressed in a large number of malignancies and carcinomas (Lakshman *et al*. 2004; Richter *et al*. 2012). In a colon cancer animal model, it was observed that CD44 regulates cell migration via Lyn kinase and AKT phosphorylation (Shao *et al*. 2011). Intratumoral RNAi of CD44 has also been shown to suppress tumor growth and increase apoptosis in animal models (Subramaniam *et al*. 2007). It is certain that the targeting of human CD44 is a potential therapeutic approach for colon cancer.

Medium-energy beta emitters, such as ^{177}Lu are better suited for the irradiation of small-sized, disseminated metastases, while the high energy of ^{90}Y makes it suitable for the irradiation of large lesions. ^{177}Lu is an ideal radionuclide for RIT owing to its excellent characteristics such as a 6.7-day half-life and $E_{\beta\text{-max}}$ of 0.5 MeV, corresponding to a mean path length of 0.7 mm. On the other hand, ^{90}Y needs a surrogate for diagnosis or dosimetry such as with ^{111}In. Its decay is also associated with the emission of γ photons of 210 keV, which is useful for monitoring the *in vivo* localization of the injected radioactivity (Mausner *et al*. 1993; Choi *et al*. 2006).

In this study, we prepared a ^{177}Lu-labeled CD44 targeting antibody using a bifunctional chelating agent, and carried out *in vitro* and *in vivo* evaluations for potential application in the radioimmunotherapy of human colon cancer.

MATERIALS AND METHODS

1. Materials

^{177}Lu was produced at the HANARO research reactor (30 MW) installed at the Korea Atomic Energy Research Institute (KAERI) using the neutron irradiation of natural ^{176}Lu [^{176}Lu (n, γ) ^{177}Lu]. After the irradiation of a double capsulated $^{176}Lu_2O_3$ target for 5 days at a neutron flux of 1.0×10^{14} n cm^{-2}, it was cooled for 48 hrs and dissolved in 3 ml of 0.05 N HCl solution. Anti-CD44 monoclonal Ab was purchased from Santacruz, the mAb was purified using protein A/G spin column (Pierce) before use. After immunoconjugation the Centricon filter system (Millipore) was used for purification. Cysteine based DTPA-NCS was synthesized according to published procedures by producing 4-Ehhylaniline-DTPA-L-Cysteine (Choi *et al*. 2006). The other chemicals used in this study were all reagent grade. The radioactivity was measured using an ionizing chamber (Capintec 115R, BIODEX Atomlab 200) by setting the calibration value for ^{177}Lu. The radiolabeling yield and radiochemical purity (RCP) were determined using ITLC (Instant Thin-Layer Chromatography). ITLC silica gel paper (Gelman Science Inc.) was analyzed with a Cyclone Storage Phosphor System (PerkinElmer).

2. Immunoconjugation of anti-CD44 with cystein based DTPA-NCS

The anti-CD44 mAb was purified by protein A/G spin column before immunoconjugation. The purified anti-CD44 mAb was conjugated with cysteine based DTPA-NCS dissolved in a 50 mM Na-acetate buffer (pH 5.5). The molar ratio was 1 : 1 at room temperature for 15 min. Unbound cystein based DTPA-NCS was removed using a centricon filter system (Millipore) after immunoconjugation. The concentration of the immunoconjugate was calculated by Bradford assay and human IgG solution (0.01∼0.1 mg ml^{-1}) was used for the standard curve.

3. Radiolabeling with ^{177}Lu

Purified immunoconjugate was reacted with 3.7MBq ml^{-1} (100 μCi ml^{-1}) of ^{177}Lu solutions during 15 minutes at room temperature. The radiolabeling yield was determined using ITLC. iTLC paper was developed with saline as the mobile phase and analyzed using a Cyclone Storage Phosphor System. For radioimmunoconjugation the purified immunoconjugates including ten micrograms of anti-CD44 were added to 3.7MBq ml^{-1} of the ^{177}Lu solutions. The reaction mixtures were stirred gently and allowed to stand at room temperature for 10 min.

4. Serum stability assay

The serum stability was determined based on the incu-

bation in two-hundred microliters of 25% human serum in PBS at 37°C for 24 hours. Thirty microliters of radioimmunoconjugate was mixed with a sample buffer (5× containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 1 μg ml^{-1} of bromophenol blue) and analysed using SDS-PAGE. The gel was analyzed with a Cyclone Storage Phosphor System to determine the radioactive band and then it was visualized through Coomassie brilliant blue R-250 staining.

5. Cell binding assay

HT-29 human colon cancer cells were purchased from the Korea Cell line Bank and cultured in RPMI1640 (LONZA) with 10% fetal bovine serum, 300 mg l^{-1} L-glutamine, 25 mM HEPES, 25 mM $NaHCO_3$, 100 units ml^{-1} penicillin, and 100 g ml^{-1} streptomycin (SigmaAldrich) in an atmosphere of 5% CO_2 in air at 37°C. 5×10^5 HT-29 cells were incubated in 12-well plates for 12~16 hrs. After washing once with a cold minimum essential medium, the cells were incubated at room temperature for 2 hr with 500,000 cpm of ^{177}Lu-cysteine-DTPA-NCS or ^{177}Lu-cysteine-DTPA-NCS-anti CD44. The cells were washed with PBS (phosphate buffered saline) pH 7.4 twice and lysed in 0.2 ml of 1 N NaOH for 5 min. The percentage of radioactivity associated with the cell pellet was calculated based on results of the gamma counts (Perkin Elmer Life science, Massachusetts, U.S.A).

6. Biodistribution assay

The biodistribution proterity of the radioimmunoconjugate was studied in female Balb/c nude mice (Orient Co., Republic of KOR.) bearing HT-29 xenografts at least 7 weeks of age. HT-29 tumor xenografts were established in the hind flank by subcutaneous injection of 1×10^6 cells in 100 μl of 50 : 50 Matrigel (BD Bioscience) and a serum-free media. The tumor volume was measured by a bilateral Vernier caliper measurement, using the formula; Volume = 0.5 $(width)^2\times length$, where the length was the longest diameter across the tumor, and the width was the corresponding perpendicular value. The mice were randomized into groups of 5 prior to treatment at a point when the tumors reached a volume of 0.55~0.70 cm^2. For the biodistribution studies, 0.185 MBq (5 μCi) of ^{177}Lu-cysteine based DTPA-NCS-anti CD44 was injected intravenously into the tumor-bearing mice. The mice (n = 5) were sacrificed 24 hours after injection, and the radioactivities in the tumor, kidney, liver, spleen, heart, small and large intestine, lung, stomach, and blood were determined using a gamma coumter and expressed as a percentage of the injected dose per gram of tissue (% ID g^{-1}).

RESULTS

The immunoconjugation steps using cysteine-based DTPA-NCS were already optimized by varying the incubation times, molar DTPA-NCS to Ab ratio, and the method of purification (Lee *et al*. 2009). The purified anti-CD44 monoclonal Ab was conjugated with this BFCA by mixing for 10 min at room temperature at a pH 7.4 with a molar ratio of 1:1. We easily obtained a high radiolabeling yield (>98%) of ^{177}Lu-cysteine-based DTPA-NCS-anti-CD44.

The ^{177}Lu-labeled anti-CD44 was stable in 25% serum at 37°C for 1 day. No free ^{177}Lu or byproduct formations were observed with ITLC (Fig. 1). The Rf values of the ^{177}Lu- and ^{177}Lu-cysteine based DTPA-NCS, and ^{177}Lu-cysteine based DTPA-NCS-anti-CD44 were 1, 0.8~0.9, and origin, respectively. It also was analyzed in a non-reducing SDS-PAGE. The gel was visualized by Coomassie blue R-250 and showed numerous bands in serum. The gel was also determined by autoradiography and detected only one radioactive band with a high molecular weight (Fig. 2). From this result, we reconfirmed that the radioimmunoconjugation with ^{177}Lu was very stable without degradation.

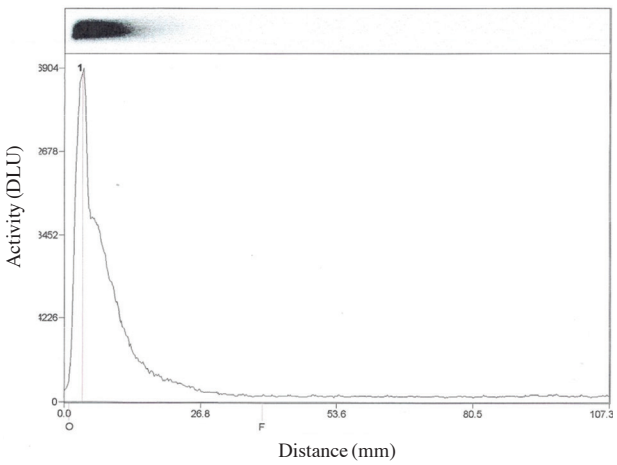

Fig. 1. ITLC profiles of ^{177}Lu-cysteine derivative DTPA-NCS-anti CD44 after storing in 25% serum at 37°C for 24 hours. The x-axis shows the distance (mm) from the origin (left); the y-axis shows the proportional activity count.

The cell binding property of ^{177}Lu-cysteine-based DTPA-NCS-anti-CD44 to the HT-29 human colon cancer cells is shown in Fig. 3. The figure shows the percentage of ^{177}Lu-cysteine-based DTPA-NCS-anti-CD44 binding to the cell surface and compares it with that of ^{177}Lu-cysteine-based DTPA-NCS only. The high binding affinity was 5.1% on the surface of the HT-29 cell, in comparison to radiolabeled BFCA with 2.4% cell binding.

The results of a biodistribution study of ^{177}Lu-cysteine-based DTPA-NCS-anti-CD44 in Balb/c nude mice bearing HT-29 human colon cancer cells showed a significant uptake in kidney and liver, tumor (Fig. 4). There was a markedly higher tumor uptake than blood uptake (3.05 ± 0.18%ID g^{-1} and 0.06 ± 0.02%ID g^{-1}, respectively). The tumor-to-blood ratio is 43 : 1 at 24 h p.i. The ^{177}Lu-labeled anti-CD44 was highly retained in the kidney and liver at 7.08 ± 0.3%ID g^{-1} and 7.15 ± 0.7%ID g^{-1}, respectively.

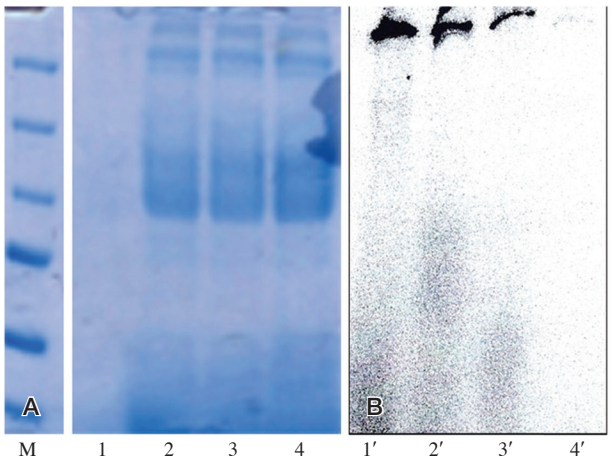

Fig. 2. Electrophoresis of ^{177}Lu-cysteine derivative DTPA-NCS-anti CD44 in 25% serum. Gel colored by Coomassie blue (A) and radiography (B). The protein size markers (m) include protein sizes of 250, 150, 100, 75 and 50 kDa. Lane 1, 1′: Before adding 25% serum; lane 2, 2′: 0 hrs; lane 3, 3′: 24 hrs at 37°C, lane 4, 4′: 25% serum.

DISCUSSION

Recently, the development of targeted agents such as bevacizumab and cetuximab has changed the treatment strategies for patients with colorectal cancer although 5-fluoreouracil (5-FU) is still important in chemotherapy. The targeted agents also play an important part in the treatment of metastatic colorectal cancer. In this study, we found a biomarker with potential as a radioimmunotherapeutic agent.

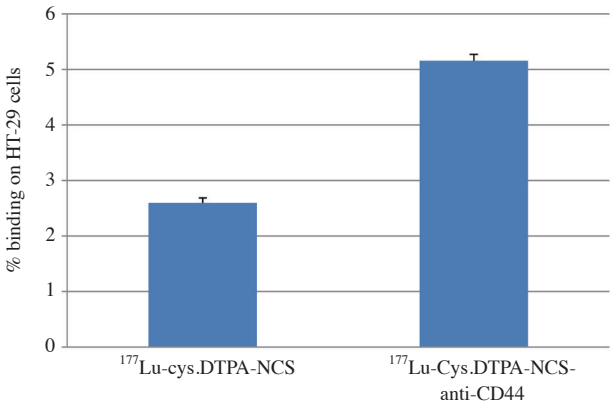

Fig. 3. Binding percentages of the ^{177}Lu-cysteine derivative DTPA-NCS and ^{177}Lu-cysteine derivative DTPA-NCS-anti-CD44 mAb to HT-29 cells.

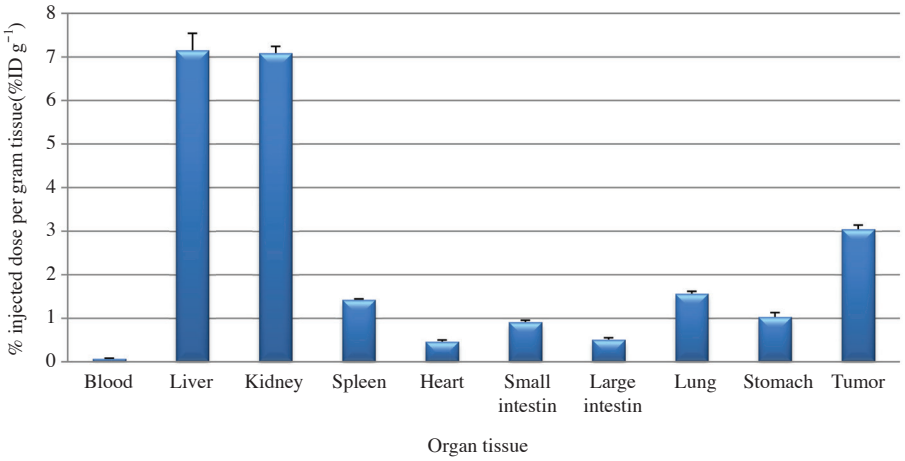

Fig. 4. Biodistribution of ^{177}Lu-cysteine-based DTPA-NCS-anti CD44 mAb in HT 29 bearing mice 24 hrs post injection. Values are percentages of the injected dose per gram tissue (% ID g^{-1}).

To maximize the therapeutic efficacy of radioimmunotherapy, the choice of an appropriate biomarker overexpressed on the cell surface is one of the most important aspects.

Interestingly, the overexpression of CD44 isoforms is found in 80% of colonic adenomas. The CD44 proteins are a ubiquitously expressed family of cell surface adhesion molecules involved in cell-cell and cell-matrix interactions and are well-characterized members of the hyaluronate receptor family. The expression of CD44 isoforms and a hyaluronate binding profile can influence tumor growth and development (Wong *et al*. 2003; Shao *et al*. 2011; Richter *et al*. 2012). Tiffany Shao et al suggested that CD44 may play a role in the directional motility of human colon cancer cells (Shao *et al*. 2011), CD44 may be involved in alteration of cell migration through the regulation of cofilin (Subramaniam *et al*. 2005), and CD44 overexpression provides resistance to apoptosis in both mouse and human colon cancers (Weilenga *et al*. 1993). Ulrich Richter et al observed the expression of hyaluronan binding CD44 on a tumor cell surface using flow cytometry. The melanoma cell line, MeWo, adhered best to hyaluronan followed by HT 29 (human colon cancer cell) (Richter *et al*. 2012). The authors demonstrated that a significant decrease in p53 expression is associated with the inhibition of CD44 expression in tumors derived from human colon cancer cells. These results show that CD44 plays an anti-apoptotic role as an oncogenic protein in cancer metastasis (Lakshman *et al*. 2004). Some positive therapeutic effects have been achieved by siRNA or anti-CD44 treatments in tumor xenograft models. Subramaniam et al reported that the intratumoral RNAi of CD44 has been shown to suppress tumor growth and increase apoptosis in nude mice (Subramaniam *et al*. 2007). The specific anti-CD44 monoclonal antibodies (H90 and A3D8) can inhibit the proliferation of acute myeloid leukemia (AML) cells and induce their apoptotic death (Gadhoum *et al*. 2004).

In this study, to develop a new radiopharmaceutical for colon cancer, anti-CD44 and ^{177}Lu were chosen for targeting and therapy, respectively. ^{177}Lu is an ideal radionuclide for radioimmunotherapy (RIT) owing to its favorable decay characteristics. ^{177}Lu decays with a half-life of 6.73 d by the emission of beta particles with maximum energies of 497 keV (78.6%) and gamma photons of 208 keV (11%) with relatively low abundances that allow simultaneous scintigraphic studies (Mausner *et al*. 1993; Choi *et al*. 2006). Rhona Stein et al compared the efficacy of ^{177}Lu-labeled RS7 mAb with that of RS7 mAb labeled with ^{90}Y and a residualizing form of ^{131}I in preclinical targeting and therapy studies. From their results, ^{177}Lu-labeled mAb is an effective radioimmunoconjugate for therapy of even relatively large tumor xenografts (Stein *et al*. 2001).

Radioimmunoconjugation using cysteine-based DTPA-NCS was achieved by the simple mixing and reaction for 10 minutes at room temperature. The stable chelating power was also determined by retaining the radioimmunoconjugate in spite of being stored in serum and undergoing electrophoresis.

Surprisingly, radioimmunoconjugated anti-CD44 with ^{177}Lu had a high targeting property against tumors in a human colon cancer xenograft. The tumor-to-blood ratio was 43 : 1. This result shows a higher uptake to tumors than other developing radioimmunoconjugates. For example, the tumor-to-blood ratio of ^{111}In-labeled anti-CD20 is only 0.5 : 1 in a Ramos lymphoma xenograft (Oliver *et al*. 2001). And the ratio of ^{177}Lu-labeled anti-VEGFR 1 was 3.25 : 1 in a Calu6 non-small lung cancer xenograft (Lee *et al*. 2009). The ^{177}Lu-labeled anti-CD44 having a high targeting ability has a high potential for the diagnosis and treatment of tumors overexpressing CD44.

However, almost all radioimmunoconjugates accumulate in the liver and kidneys to catabolize protein and eliminate the nonspecific radioactivity, as in ^{177}Lu-labeled anti-CD44. This undesirable behavior can be minimized through various strategies including pretargeting and fragmentation of Ab (Boswell *et al*. 2007).

In conclusion, the anti-CD44 monoclonal antibody for the targeting of colon cancer was stably radioimmunoconjugated with ^{177}Lu, and its high specificity in accumulating in tumor tissues is a strong evidence of its clinical potential. The therapeutic efficacy of this radioimmunoconjugate will be determined in further studies.

CONCLUSION

The anti-CD44 monoclonal antibody for the targeting of colon cancer was stably radioimmunoconjugated with ^{177}Lu, and its high specificity in accumulating in tumor tissues is a strong evidence of its clinical potential. The therapeutic efficacy of this radioimmunoconjugate will be determined in further studies.

Abbreviations used: RIT: Radioimmunotherapy, mAb: monoclonal Antibody, Ab: Antibody, BFCA: Bifunctional chelating agent, DTPA: diethlyenetriaminepentaacetic acid, p.i.: post injection, iTLC: instant Thin Layer Chromatography, SDS-PAGE: Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

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