INTRODUCTION

188Re (Rhenium) is a short-lived beta emitting radionuclide (physical half-life=17 hr, E\text{max}=2.11 \text{MeV}). It has an average beta particle penetration of 3.3 mm (maximum=10.8), providing a tightly circumscribed region of high-energy deposition with little damage to the adjacent cells and organs. In addition, 188Re is easily obtained from an in-house 188W/188Re generator that is similar to the current 99Mo/99mTc generator, making it very convenient for clinical use. This characteristic makes this radionuclide a promising candidate as a therapeutic agent. Polyethylenimine (PEI) is a cationic polymer and has been used as a gene delivery vector. Positively charged materials interact with cellular blood components, vascular endothelium, and plasma proteins. In this study, the authors investigated whether intratumoral injection of 188Re labeled transferrin (Tf)-PEI conjugates exert the effect of radionuclide therapy against the tumor cells. When the diameters of the Ramos lymphoma (human Burkitt’s lymphoma) xenografted tumors reached approximately 1 cm, 3 kinds of 188Re bound compounds (HYNIC-PEI-Tf, HYNIC-PEI, 188Re perrhenate) were injected directly into the tumors. There were increases in the retention of 188Re inside the tumor when PEI was incorporated with 188Re compared to the use of free 188Re. The 188Re HYNIC-Tf-PEI showed the most retention inside the tumor (retention rate=approximately 97%). H&E stain of isolated tumor tissues showed that 188Re labeled HYNIC-PEI-Tf caused extensive tumor necrosis. These results support 188Re HYNIC-PEI-Tf as being a useful radiopharmaceutical agent to treat tumors when delivered by intratumoral injection.

Key Words: Rhenium; Polyethylenimine; Transferrin; Lymphoma

MATERIALS AND METHODS

Synthesis of HYNIC-PEI-Tf conjugates

Tf-PEI (branched PEI, 25 kDa) conjugates were synthesized as described by Kircheis and coworkers (16). Branched PEI was purchased from Aldrich (WI, U.S.A.). Briefly, human apo-transferrin in 30 mM sodium acetate buffer (pH 5.0) was subjected to gelfiltration on a Sephadex G-25 superfine column (Pharmacia, Uppsala, Sweden). The resulting solution was cooled to 0°C and sodium periodate (in 30 mM pH 5 sodium acetate buffer) were added. The mixture was kept in an ice bath in the dark for 90 min and promptly added to
PEI solution and mixed at room temperature. After 30 min, four portions of sodium cyanoborohydride were added at 1 hr intervals. After 18 hr, 3 M sodium chloride was added. The reaction mixture was loaded on a cation-exchange column, Macro-Prep high S HR 10/10 (Bio-Rad, Hercules, CA, U.S.A.) and was fractionated with a salt gradient from 0.5 M to 3.0 M sodium chloride in 20 mM HEPES (pH 7.3). A 3 mol excess of dissolved succinimidyl 6-hydrazinonicotinate hydrochloride (HYNIC) (5 mol% of PEI amino group) in 30 mM dimethyl-formamide (DMF) was added dropwise to a stirred solution of Tf-PEI conjugates. The solution was stirred gently for 24 hr at 4°C protected from light. This was followed by dialysis against HBS (pH 7.3, 150 mM sodium chloride, 20 mM HEPES) at 4°C (six buffer changes for 72 hr). The iron incorporation was performed by addition of 1.25 μL 10 mM iron (III) citrate buffer per mg transferrin content. The conjugates were divided into convenient small aliquots and kept at -20°C.

**Labeling with 188Re**

188Re-perrhenate was obtained in 20 mL of normal saline from 188W/188Re generator (Shanghai Ke Xing Pharmaceuticals, Shanghai, China). The labeling process was carried out in the presence of stannous chloride dehydrate. 188Re sodium perrhenate eluate (370 MBq) in 0.9% normal saline was mixed in a vial with 10 mg of ascorbic acid, 40 mg of SrCl2 (Sigma, U.S.A.), and 500 μL of HYNIC-PEI-Tf (1 μg/μL) or HYNIC-PEI. Then, the vial was reacted at 37°C for 1 hr. The labeling yield was determined by ITLC-SG (Gelman Science, Ann Arbor, MI, U.S.A.) using acetone as the mobile phase.

**In vivo animal model**

To generate tumors, three 5- to 6-week-old female BALB/c nude mice (Orient Co. Ltd., Seoul, Korea) were injected subcutaneously in the left thigh with Ramos cells (ATCC CRL 1596, human Burkitt’s lymphoma, 5 × 10⁶ cells/100 μL). Tumor size was measured using a vernier caliper across its gama, U.S.A.), and 500 L of HYNIC-PEI-Tf (1 g/L) or three times into the tumors, respectively.

Histologic evaluation with H&E staining was performed 2 days after injection. The frozen sections were washed with tap water for 5 min, immersed in hematoxylin for 2 min and checked for complete staining in tap water. Eosin staining was carried out for 3 min. Sections were dehydrated through a graded series of alcohol (70 to 100% ethanol, 3 min each), cleared in xylene, cover-slipped and observed with a light microscope.

**Image analysis and dosimetry**

Region of interest (ROI) were drawn manually on 10 min image around the tumor and whole body. ROIs of the same size and shape were applied to 2 hr and 12 hr images. Retention rates of 3 radiotracers were calculated. A half-life for 188Re-HYNIC-PEI-Tf was also calculated in the form of effective half-life and the residence time was defined as half-life/ln 2. In 2. Tumor self-radiation S-value of 188Re-HYNIC-PEI-Tf was obtained using the Nodule Module in MIRDOS3.1 software (Oak Ridge Associated University) (17). From the calculated S-values and residence time in the mouse model, the dose of 188Re-HYNIC-PEI-Tf to obtain 100 Gy of tumor irradiation was calculated.

**Reverse transcription-polymerase chain reaction**

Total RNA isolated from Ramos cells with Trizol reagent (Invitrogen, U.S.A.) was used for RT-PCR. First-strand cDNA synthesis was performed at 42°C for 50 min, followed by 70°C for 15 min using Superscript II reverse transcriptase and oligo (dT)₁₀ as the primer. The cDNA was amplified using the following primers: Tf-R F 5′-CACCCACACTGTGCCCATCTACA-3′; Tf-R R 5′-CTCATGACGATCATTGAG 3′. A pair of primers specific to β-actin (F 5′-TGACGGGGTGCCACACCACACTGTCGTTGCA-3′; R 5′-CTGAGCAGGAGCATTTGGAG-GG-3′) was used as a control. PCR was performed in a DNA thermal cycler using 94°C melting, 45°C annealing, and 72°C extension temperatures for 33 cycles. The PCR products were loaded on agarose gel (1%) containing ethidium bromide and electrophoresis was performed at 100 V for 20 min.

**RESULTS**

The labeled 188Re-HYNIC-PEI-Tf or HYNIC-PEI remained localized at the origin of injection and radiochemical purities of these labeled compounds at 15 min and 1 hr were 97% and 80%, respectively.

Fig. 1A, B showed that when PEI was incorporated with 188Re, the retention of 188Re inside the tumor was increased compared with free 188Re. The 188Re-HYNIC-PEI-Tf mostly

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**Hematoxylin & eosin staining**

Histologic evaluation with H&E staining was performed 2 days after injection. The frozen sections were washed with tap water for 5 min, immersed in hematoxylin for 2 min and checked for complete staining in tap water. Eosin staining was carried out for 3 min. Sections were dehydrated through a graded series of alcohol (70 to 100% ethanol, 3 min each), cleared in xylene, cover-slipped and observed with a light microscope.
Intratumoral Injection of $^{188}$Re Conjugates

remained in the tumor and showed a higher retention rate than $^{188}$Re HYNIC-PEI (approximately 97% vs. 85%). $^{188}$Re HYNIC-PEI released from the tumor accumulated in the liver and lungs. Fig. 1C showed that free $^{188}$Re escaped from the tumor over time and that there was no remaining $^{188}$Re 12 hr after injection. The $^{188}$Re released accumulated in the stomach, thyroid, and bladder in much the same way that $^{99m}$Tc pertechnetate did.

Effective half-life of $^{188}$Re HYNIC-PEI-Tf was assumed to be same to the physical half-life. The calculated residence time of this conjugate was 24.5 hr. The nodular self-dose S-value was estimated as 0.343 mGy/MBq·h. using MIRDOS3.1. The radioactivity required for a target irradiation dose of 100 Gy for 0.97 to 1.23 cm tumor was calculated 6.4 to 11.9 MBq for $^{188}$Re HYNIC-PEI-Tf from this S-value.

Representative hematoxylin and eosin-stained sections of the isolated tumors injected with $^{188}$Re HYNIC-PEI-Tf and $^{188}$Re perrhenate were shown in Fig. 2. In the tumor injected with $^{188}$Re HYNIC-PEI-Tf, histological changes were found in contrast with $^{188}$Re perrhenate. $^{188}$Re HYNIC-PEI-Tf made extensive central necrosis inside the tumor and remained the small portion of viable tumor tissue around the tumor mass.

The result of RT-PCR represented the Ramos Burkitt's lymphoma cells had Tf-R mRNA and the possibility of expression of Tf-R protein on the cell membrane (Fig. 3).

DISCUSSION

Our preliminary results show that intratumoral injection with $^{188}$Re HYNIC-PEI-Tf caused extensive necrosis in the xenografted tumor without a significant leakage of radioactive compound. Although PEI could chelate with $^{99m}$Tc or $^{188}$Re because of the secondary or tertiary amine groups in this polymer, in this study HYNIC was introduced as a bi-conjugate for higher and safer labeling.

$^{188}$Re has not only strong potential for therapeutic use but also excellent imaging characteristics because of its $\beta$ energy...
(2.1 MeV), its short physical half-life (17 hr) and its 155 keV γ-ray emission; often used for dosimetric and imaging purposes. Therefore many researchers have used $^{188}$Re in a variety of fields such as intravascular radiation, radioimmunotherapy using monoclonal antibodies, and metastatic bone lesions (2–4). Radiotherapy using $^{188}$Re needs novel therapeutic strategies, which can facilitate an increase in the intratumoral uptake or a retention and reduction in its systemic levels. Radiotherapy with intratumorally-injected radiopharmaceuticals is a promising approach to some kinds of tumor such as head and neck cancers because it offers the potential to localize the radiation inside the tumor. Because potential leakage of therapeutic radionuclide causes serious problems in the site of accumulation, there is a need to minimize leakage of the injected compound. Intratumoral injection of $^{188}$Re HYNIC-PEI-Tf resulted in the highest retention in the tumor mass indicating this approach had strong potential for the treatment of solid tumors.

PEI is a cationic polymer and has been used as a gene delivery vector. Positively charged materials interact with cellular blood components, vascular endothelium, and plasma proteins when they are injected systemically (18, 19). In this study, $^{188}$Re HYNIC-PEI leaked out from injected site showed the liver and lung activity, this might be explained that positive surface charge of PEI led to interactions with lung and hepatic endothelium. Until now, there have been few reports about intratumoral injection using $^{188}$Re labeled cationic polymer. Therefore, despite of incomplete and preliminary data, our result is thought to be introduced the new, useful compound to the field of radionuclide therapy.

High retention of $^{188}$Re HYNIC-PEI in the tumor demonstrates that the intratumoral injections of positively charged radioactive conjugates bind to tumor cells adjacent to the injection sites. Based on the fact that Tf-PEI derivatives/DNA complexes have been known to deliver the gene efficiently through the Tf-Tf receptor system, in the intratumoral approach of $^{188}$Re HYNIC-PEI-Tf, Tf-Tf receptor mediated tumoral uptake is thought to play an additional role for higher retention of radiocompound in the tumor than that of $^{188}$Re HYNIC-PEI. We certified the existence of Tf-receptor mRNA through RT-PCR method in the Ramos lymphoma cell line. Another role of Tf conjugate as a ligand may be that Tf enlarged the size of $^{188}$Re labeled polymer because the molecular weight of Tf is relatively heavy, about 80 kDa.

As shown in Fig. 1, 2, high retention of $^{188}$Re labeled cationic polymers in the tumor were not only inspected through nuclear imaging of good quality because of appropriate gamma energy for imaging, but also predicted extensive necrosis around the sites of injection. We verified that injected and retained dose, 11.1 MBq, of $^{188}$Re HYNIC-PEI-Tf was enough to cause the necrosis to approximately 1cm-sized tumor through calculated results for dosimetry using the Nodule Module in MIRDOS3.1 software.

In conclusion, the results of this preliminary study show that $^{188}$Re labeled HYNIC-PEI-Tf can be a useful radiopharmaceutical agent to treat solid tumors when delivered by intratumoral injection.

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