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Intratumoral Administration of Rhenium-188-Labeled Pullulan Acetate Nanoparticles (PAN) in Mice Bearing CT-26 Cancer Cells for Suppression of Tumor Growth

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Abstract The feasibility of pullulan acetate nanoparticles (PAN) with ionic strength (IS) sensitivity as a radioisotope carrier to inhibit tumor growth is demonstrated. PAN was radiolabeled with rhenium 188 (Re-188) without any chelating agents. The labeling efficiency of Re-188 into PAN (Re-188-PAN) was 49.3±4.0% as determined by TLC. The tumor volumes of mice treated with 0.45 mCi of Re-188-PAN were measured and compared with that of free Re-188 after 5 days of intratumoral injection. For the histological evaluation of apoptotic nuclei of tumor cells, hematoxylin and eosin (H&E), and terminal deoxynucleotidyl transferase biotinylated deoxyuridine triphosphate nick end labeling (TUNEL) staining were performed. The mean tumor volume of the Re-188-PAN-treated group was decreased by 36% after 5 days, whereas that the free Re-188-treated group was decreased by only 15% ($P<0.05$). The mean number of TUNEL-positive cells in Re-188-PAN-treated tumors at 144.3±79.9 cells/section was significantly greater than the control (26.7±7.9 cells/section, $P=0.03$). The numbers of leukocyte and lymphocyte were decreased in both free Re-188- and Re-188-PAN-treated mice. These results indicated that the intratumoral injection of Re-188-PAN effectively inhibits the tumor growth by prolonging Re-188 retention time in tumor site induced by the IS sensitivity.

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Key words: Pullulan acetate nanoparticles (PAN), rhenium-188, intratumoral injection, histological evaluation

Many kinds of methods have been developed for diagnosis and treatment of various cancers [11, 24, 26]. Radiotherapy by external or internal irradiation is one of the most powerful materials. The external therapy has been more widely used among them; however, its lack of selectivity leads to high systemic radiation dose, frequently resulting in irritation and damage to normal tissues and organs [25]. Therefore, many researchers have focused their attention on the internal radiation therapy, by intratumoral administration of radioisotope directly into target organ and tissue [14, 16, 28, 30].

Many beta-emitting radionuclides have been investigated for the application of internal radiation therapy [14, 16]. Specifically, rhenium-188 (Re-188) has been an excellent candidate. Its beta-emitting energy (2.1 MeV) has a maximum penetration of tissue, ranging over 10–11 mm, so that Re-188 can be used for the treatment of relatively large solid tumors. The γ-ray emission (0.155 MeV) of Re-188 could also be employed for dosimetric description and for monitoring biological distribution during the therapy [6, 15, 22]. The physical half-life of Re-188 is 16.9 h, enforcing the use of high doses and reducing the problem of radioactive waste handling and storage [5, 8–10, 32]. Furthermore, Re-188 is attractive, because it can be available

to an in-house W-188/Re-188 generator system similar to a Tc-99m generator. Because of the convenience in clinical use, Re-188 has already been tried for the treatment of tumors or benign diseases [1, 23].

In the case of internal radiation therapy, the radioactive materials injected into solid tumor have to remain at the site long enough to enhance the therapeutic effect and avoid damage to normal tissues and organs. For this reason, a carrier system for radioisotope has been investigated. Suzuki *et al.* [27] reported the biodistribution and kinetics of a Holmium 166-chitosan complex in rats and mice. They reported that the chitosan prolonged the retention time of Holmium 166 in the tumor site. Nakajo *et al.* [21] also documented a ^{131}I-labeled lipiodol for patients suffering from liver cancer. They observed that the radioactive concentration in blood after administration of ^{131}I-lipiodol was kept as low as 10×10^{-4}% ID/ml for 8 days.

Polymeric nanocarriers with stimuli sensitivity induced by small changes in the conditions of solid tumor, such as swelling/deswelling [2, 3], particle disruption, and/or aggregation [13, 17], may provide advantages for long retention of a radioisotope in the target site and organ. In this paper, we suggest a new concept for the intratumoral delivery of radioisotopes using a self-assembled nanoparticle with ionic strength (IS)-sensitivity in the body condition. The IS-sensitive nanoparticles might exist as a stable state in distilled water (D.W., IS=0), and aggregate at ionic strength (IS) such as in human blood or other body fluids (IS= 0.15). Therefore, the carriers can be easily accumulated at the tumor site with minimal leakage after intratumoral injection. This property is expected to enhance the efficacy of local radioisotope delivery. Pullulan acetate (PA) was used to design such a nanoparticle. The PA was synthesized by the chemical modification of pullulan, a linear polysaccharide having an α-(1-6)-linked maltotriosyl repeating unit with biodegradability and biocomparability, but without any toxicity [31].

Herein, we investigated the IS-sensitivity of PAN and labeling efficiency of an isotope to demonstrate potential advantages as a tumor-targeting carrier of radioisotopes, and evaluated the therapeutic effects of intratumoral Re-188-labeled PAN (Re-188-PAN) on the inhibition of tumor growth in mice bearing CT-26 colon cancer.

Materials and Methods

Materials

Pullulan (Mw 100,000) was obtained from Hayashibara (Okayama, Japan). Acetic anhydride, formamide, dimethyl sulfoxide (DMSO), acetone, and pyridine were purchased from Sigma-Aldrich Co. (St. Louis, MI, U.S.A.). Pullulan was purified by a protocol including filtration (0.45 μm in deionized water), precipitation (70 v/v% ethyl alcohol), dialysis (MWCO 25,000), and freeze-drying, resulting in a white sponge-like product from the yellowish particle. All chemicals were purchased as reagent or spectroscopic grade and used without further purification.

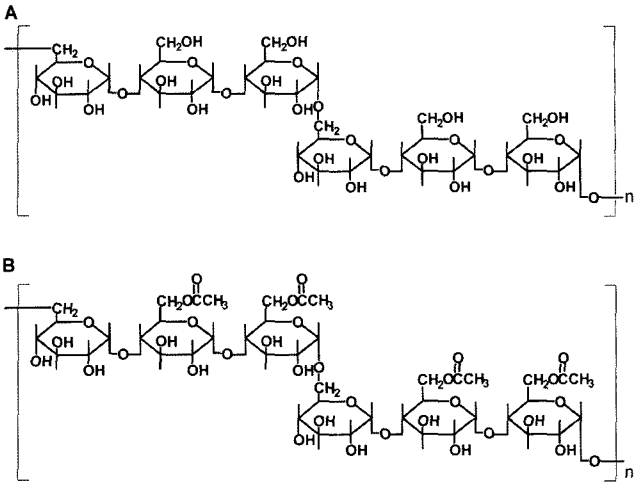

Fig. 1. Chemical structures of pullulan (**A**) and pullulan acetate (PA) (**B**).

Synthesis of Pullulan Acetate (PA)

For the acetylation of pullulan, 2 g of pullulan was suspended in 20 ml of formamide and dissolved by vigorous stirring at 50°C. Pyridine (60 ml) and acetic anhydride (150 ml) were added to the mixture and stirred at 54°C for 48 h (Fig. 1). PA was obtained after reprecipitation from 200 ml of deionized water. The synthesized PA was characterized by Fourier transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR), and gel permeation chromatography (GPC) [17, 19, 20]. The number of acetyl groups per glucose unit of pullulan was 1.16.

Preparation and Physicochemical Analysis of PA Nanoparticles (PAN)

Nanoparticles were prepared by a dialysis method (molecular cutoff 2,000). PA (50 mg) was dissolved in 20 ml of dimethyl sulfoxide (DMSO), and the solution was stirred at room temperature and dialyzed against distilled water for 3 days. It was filtered with a 0.45-μm filter to remove the precipitated material. The particle size of the resulting nanoparticles was determined by dynamic light scattering (Zetasizer 3000, Malvern Instruments Ltd., U.K.) using an argon ion laser beam at a wavelength of 488 nm and a scattering angle of 90°. The sample was filtrated through a 0.45-μm filter directly into a clean cylindrical cell (10 mm diameter). The sample concentration was kept at 1.0 mg/ml. The optical transmittance of the PAN solution (1 g/l) at various IS was measured at 500 nm using a Varian CARY 1E UV/Vis spectrometer as a function of time. The relative transmittance (%) at different ionic strengths was obtained by comparing with that at 100% distilled water (D.W.).

Labeling of 188-Re into PAN

Highly pure carrier Re-188-sodium perrhenate ($NaReO_4$) was obtained in 20 ml of normal saline from an alumina-based W-188/Re-188 generator (Oak Ridge National Laboratory, TN, U.S.A.). High-performance liquid chromatographic (HPLC) analysis revealed that the Re-188 eluate was over 99% perrhenate. The W-188/Re-188 generator demonstrated consistently high Re-188 yields and low parent breakthrough for periods of at least 2 months.

Radiosynthesis of Re-188-PA was carried out in the presence of tin chloride. In short, 0.5 ml of Re-188 sodium perrhenate eluate (121–148 MBq) in 0.9% NaCl saline was mixed in a vial with 10 mg of $SnCl_2{\cdot}2H_2O$ (Sigma-Aldrich, U.S.A.) under nitrogen-purging. The vial was capped with a rubber septum and an aluminum seal under nitrogen environment, and stored in a refrigerator until use. Then, PAN (0.7 μg/μl) was added into the vial and it was incubated for 20 min at room temperature. Labeling efficiency (LE) was measured by TLC (ITLC-SG/normal saline) and radioactivity was monitored by a scanner (Imaging Scanner System 200, Bioscan, U.S.A.).

Animals Study

Six-week-old Balb/c male mice weighing 20–30 g (Semtako Bio Korea, Osan, Korea) were acclimated for a week and fed a diet of animal chow and water *ad libitum*. Using a 30-gauge needle and a 1-ml syringe, about 0.1 ml of the tumor cell [murine colon adenocarcinoma cell line (CT-26)] suspension (2×10^7 cells) was injected subcutaneously into the back of the Balb/c mice. All mice were carefully observed daily, and body weights were measured at the start and end of experiments. Tumor volumes were measured daily for 5 days with a digimatic caliper (Mitutoyo Corp. Kawasaki, Japan) and calculated using the formula: 1/2× length×width×height [26].

All animal studies were conducted according to a protocol approved by the Animal Care and Use Committee of Chonnam National University Medical School.

On separate days, a total of 27 tumor-bearing mice were randomized into three groups. In addition, samples were injected intratumorally into the central region of tumors with a sterilized syringe (32-gauge needle): Nine mice of the free Re-188 group were injected with Re-188-sodium perrhenate in 0.05 ml of normal saline solution at the dose of 0.45 mCi, 9 mice of the Re-188-labeled PA group were injected with the same dose of Re-188 as labeled into PAN, and 9 mice of the control group were injected with the same amount of PAN that was not labeled with Re-188-sodium perrhenate.

At the end of the experiment, blood cells of all mice were counted. In addition, the tumor masses were cut with razor blades and fixed in 10% buffered formaldehyde solution. The fixed tissues were embedded in paraffin and sectioned with a microtome. Hematoxylin and eosin (H&E) staining and terminal deoxynucleotidyl transferase biotinylated deoxyuridine triphosphate nick end labeling (TUNEL) were performed for the histological evaluation of apoptotic nuclei of tumor cells. TUNEL stain was performed using the TUNEL Apoptosis Detection Kit (DNA Fragmentation/Fluorescence Staining) (Upstate, N.Y., U.S.A.) according to the manufacturer's instructions. Tissue sections were deparaffinized in xylene and rehydrated in a graded concentration of ethanol and then in distilled water. The sections were then treated with proteinase K (2.5 μg/ml) for 15 min at 37°C and washed with PBS. Each section was incubated with a proteinase K-treated section with 1 μg/ml of DNase I for 60 min at 37°C and washed with PBS for 15 min. After applying equilibrium buffer, the sections were incubated with 50 μl of TdT end-labeling cocktail by respectively mixing TdT buffer, biotin-dUTP, and TdT at a ratio of 90:5:5 for 60 min at 37°C. After the sections were washed, they were incubated with 50 μl of prepared avidin-FITC solution in the dark for 30 min at 37°C. The sections were again washed with PBS for 2×15 min in the dark at room temperature. The number of TUNEL-positive cells was counted in a randomly selected field ×200 for each section by the use of a fluorescent microscopy.

Statistical Analysis

Statistical analyses were performed using the SPSS software package (Version 11.0, SPSS Inc., Chicago, U.S.A.). Descriptive data are presented as mean±standard deviation. Continuous variables, tumor volumes, apoptotic cells, and hematological parameters of three groups (PAN-, free Re-188-, and Re-188-PAN-treated groups) were compared using the ANOVA test. The difference was considered to be significant if the p value was less than 0.05.

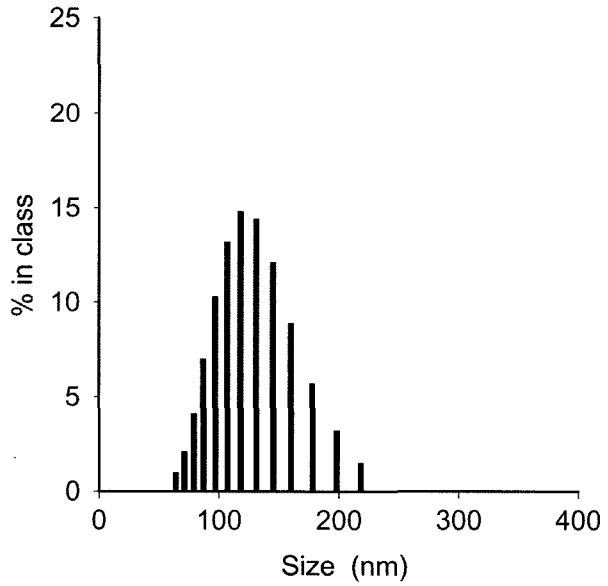

Fig. 2. The particle size distribution of pullulan acetate nanoparticle (PAN) prepared in distilled water.

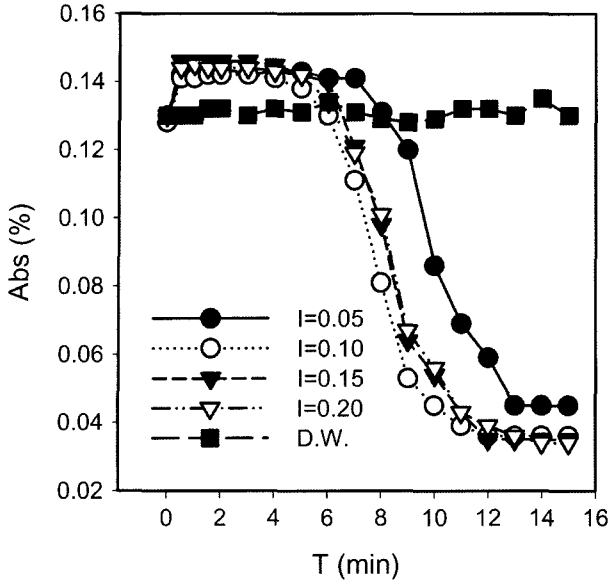

Fig. 3. Changes in turbidity of PAN solutions depending on ionic strength (IS) as a function of time.

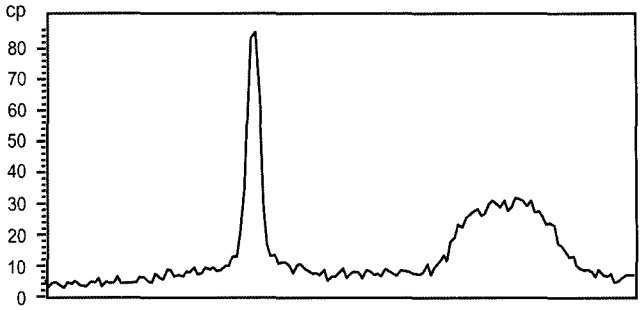

Fig. 4. Labeling efficiency of 188-Re into PAN.
Labeling efficiency was measured by using thin-layer chromatography (TLC) coupled with a radioisotope scanner.

RESULTS

Physicochemical Characterization of the PAN

The size of PAN ranged from 60 to 230 nm and had a unimodal distribution (Fig. 2). To observe the IS-sensitivity of PAN prepared in D.W. (IS=0.0), aggregation behavior was studied by examining the turbidity according to time course (Fig. 3). When PAN solution was added into the solutions with various ISs, the turbidity of all samples dropped dramatically in just 6 min, except in the case of D.W., indicating that the state of particles was stable in D.W. but aggregated with increasing IS from 0.05 to 0.10. The tendency of the IS sensitivity might originate from the breaking of various interactions between PA molecules induced by the attacking of ions.

Labeling Efficiency (LE) of Re-188 into PAN

To closely estimate the potential of the PAN as a radioisotope carrier, labeling of 188-Re into PAN was carried out without any chelating agents. Fig. 4 illustrates the LE of PAN with Re-188 perrhenate, and the LE of Re-188-PAN determined by TLC was 49.3±4.0%.

Therapeutic Effects of 188-Re-PAN

The distribution of Re-188-PAN was examined in the Balb/c mice using whole-body imaging (Fig. 5). In the animal experiments, no mortality was observed at the dose of 0.45 mCi, and the body weights of mice did not show any statistical differences between each group at 5 days. Daily growth observation revealed no side effects. After one hour post-injection, the Re-188-PAN was found to be retained in the tumor mass, and no migration into other organs was observed (Fig. 5A). Even after 2 h, the radioactivity was still detected at the tumor site, although small activity was found in the kidney (Fig. 5B). The radioactivity at the tumor site was clearly observed up to 12 h (Fig. 5C). By treating the mice only once with Re-188-PAN, the growth of tumor was effectively inhibited

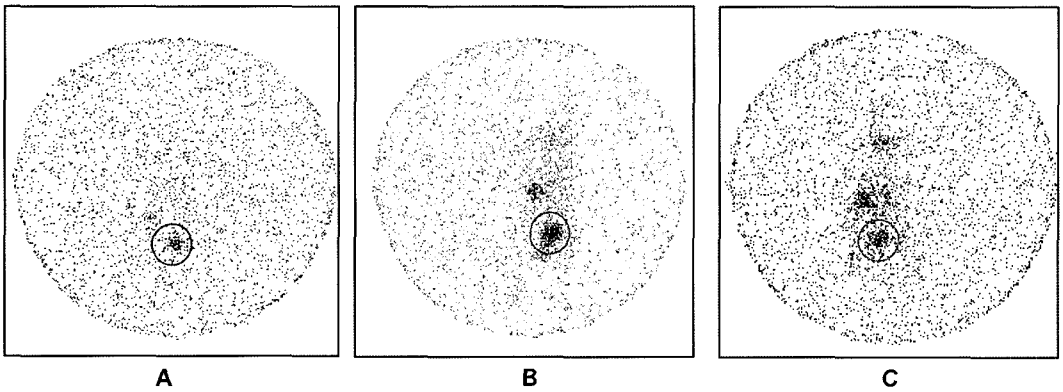

Fig. 5. Biodistribution images at various time points after intratumoral injection of Re-188-labeled PAN into the CT-26 tumoral masses inoculated on the right thigh.
Open circle indicates the tumor site. **A.** 1 h; **B.** 2 h; **C.** 12 h.

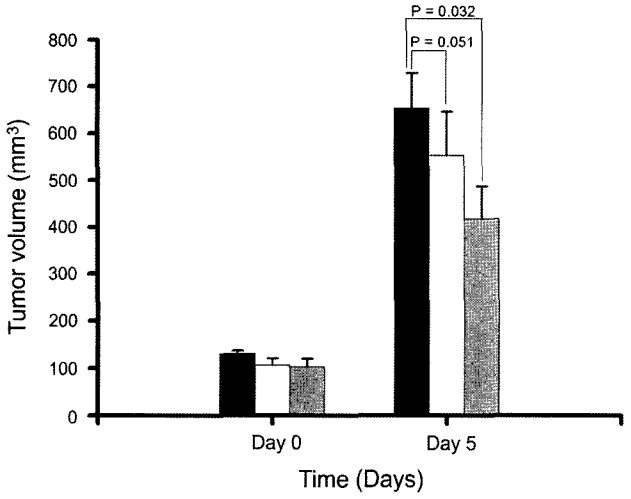

Fig. 6. Inhibition of tumor growth.
Solid bar: control PAN; patterned bar: free Re; and open bar: Re-188-labeled PAN (n=9, respectively). At 5 days post-injection, tumor growth of the mice treated with Re-labeled PAN was significantly inhibited, compared with that of the control mice ($p<0.05$).

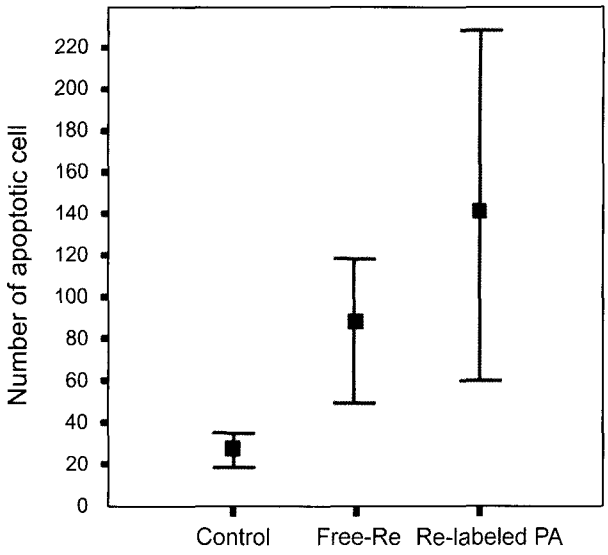

Fig. 8. Number of apoptotic cells in three groups (control PAN, free Re, and Re-labeled PAN, n=9, respectively).
At 5 days post-injection, the mean number of apoptotic cells in tumor mass of the mice treated with Re-labeled PAN was significantly higher than that of the control mice ($p=0.03$).

during the experimental period: The mean tumor volume of mice in the Re-188-PAN group was decreased to 36% ($p=0.03$ vs. control) at 5 days post-injection, whereas the mean tumor volume of mice in the free Re-188-treated group was decreased only to 15% ($p>0.05$ vs. control, Fig. 6).

Histology Studies

Both necrotic and apoptotic cells were observed more frequently at tumor tissues of both free Re-188-treated and Re-188-PAN-treated groups than that of control group, evidenced by H&E staining (Figs. 7A–7C). Similar findings were also detected clearly in TUNEL-stained samples (Figs. 7D–7F). The number of TUNEL-positive cells of PAN (control), free Re-188, and Re-188-PAN-treated groups were 26.7±7.9, 84.0±33.1, and 144.3±79.9 cells/section, respectively ($p=0.03$ by ANOVA test) (Fig. 8).

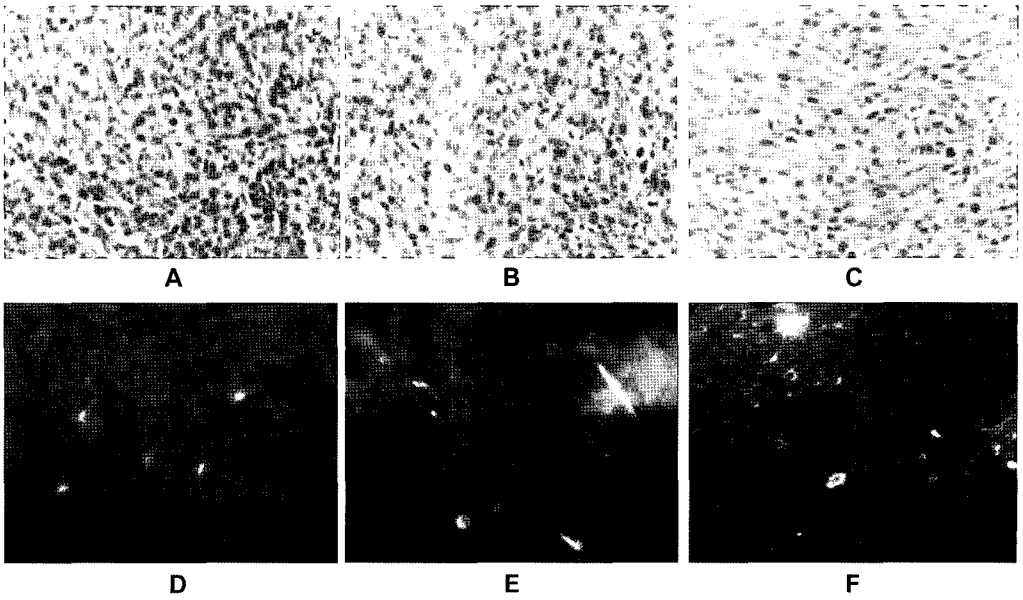

Fig. 7. H&E and TUNNEL staining in 5 days after intratumoral injection of PAN as control, free Re, and Re-labeled PAN.
Positive nuclei (apoptotic cells) were markedly increased in the Re-labeled PAN group (Magnification ×400). H&E staining of PAN (**A**), free 188-Re (**B**), 188-Re-PAN (**C**). TUNNEL staining of PAN (**C**), free 188-Re (**D**), 188-Re-PAN (**E**).

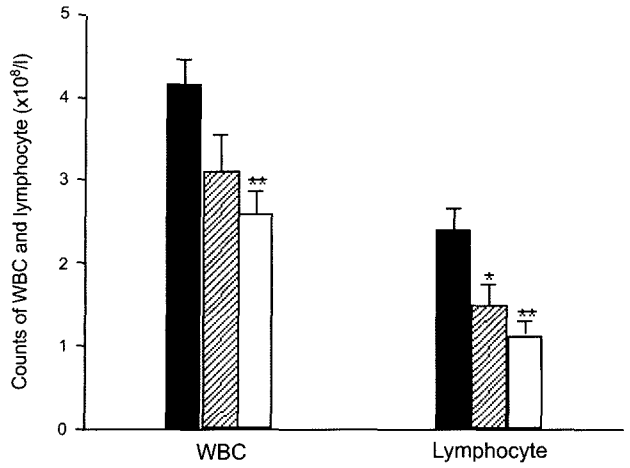

Fig. 9. Blood cell counts measured at the end of the experiment. Solid bar: control PAN; patterned bar: free Re-188; and open bar: Re-188-labeled PAN (n=9, respectively). At 5 days post-injection, WBC counts were decreased in the free Re-treated mice (p=0.061) and Re-188-labeled treated mice (**p<0.01), compared with that of control mice. Lymphocyte counts were also decreased significantly in both Re-treated groups (*p<0.05, **p<0.01). No significant difference in the WBC and lymphocyte counts was found between the Re-188-treated groups.

Hematologic analysis at 5 days post-injection showed that the number of WBC was decreased in both free Re-188- and Re-188-PAN-treated groups. Lymphocyte counts were also decreased significantly in both groups (Fig. 9). However, no significant differences in terms of the WBC and lymphocyte counts were found between the Re-treated groups.

DISCUSSION

The purpose of this study was to develop a stimuli-sensitive nanoparticle for an effective radiotherapy of solid tumor. The carrier with a radioisotope was expected to stay in the tumor mass when injected directly into the tumor, because the carrier might be aggregated inside the tumor (IS=0.15). We tried to intratumorally inject Re-188-PAN in order to examine the feasibility of the above hypothesis. Several articles have already reported the potential radiotherapeutic value of the tumoral injection of radioisotope using polymers [21, 27]. However, the polymer supporters (chitosan or lipiodol) examined may be easily broken down by radioactivity from the radioisotope; any interaction between polymers is broken, except the original bond of the polymer. On the contrary, the breaking time in the case of self-assembled nanoparticles is expected to be longer than that of reported polymers, because the self-assembly property of a nanoparticle is an entanglement induced by hydrophobic interaction. Therefore, the radiotherapy using polymeric nanoparticle as a carrier could provide a new modality for cancer treatment.

Wang *et al.* [30] intratumorally injected Re-188 microspheres in an animal model with hepatoma, and found that 60 percent of animals studied showed significant decrease in tumor size, and survival rate was significantly prolonged over 60 days. According to our results, the mean tumor volume of mice in the Re-188-PAN group decreased to 36% in 5 days, whereas that of mice in the free Re-188 group decreased to only 15%. In this respect, our results are quite similar to the results of Lee *et al.* [12],who reported that 67–74% of animals with malignant melanoma were alive when treated with intratumoral injection of Ho-166, whereas all the control animals died. The above two studies show that intratumoral injection of unsealed beta-emitting radioisotopes such as Re-188 or Ho-166 has advantages for local treatment of tumors, because of their physical characteristics.

H&E and TUNEL stains of Re-188- and Re-188-PAN-treated tumor tissues showed some necrotic cells and many apoptotic cells, suggesting that the major therapeutic effect induced by radiation of Re-188 is apoptosis. Lee *et al.* [12] reported that the main cell death mechanism by intratumoral injection of Ho-166 seems to be either radiation-induced central necrosis and peripheral growth arrest, or secondary necrosis of tumor cells, rather than apoptosis. However, Friesen *et al.* [7] reported that beta-irradiation induces apoptosis and activates the apoptosis pathway in leukemia cells, depending on dose, time points, and dose rates. Therefore, inhibition of tumor cell growth or cell death in radiotherapy appears to be dependent on the physical characteristics of the beta-emitter, absorbed doses, and injection number.

Hematological toxicity was observed in this study. WBC and lymphocyte counts were reduced in both groups treated with either free Re-188 or Re-188-PAN in comparison with that of the control group. These findings indicated that the Re-188 suppressed the bone marrow by diffusion through the bone marrow, as seen on the biodistribution images. This hematological toxicity has already been noted in several clinical studies using Re-188 [15, 22], and is dependent on a dose of Re-188. These studies reported that blood count values reached baseline levels or at least normal levels at week 8 [22] or within 12 weeks [15].

The present study, however, has several questions to be solved. First of all, the LE of Re-188-PAN was only about 50%, which was significantly lower than that of Tc-99m-PAN (data not shown), even though the same labeling method was employed for both radioisotopes. It could be due to a subtle difference in physical characteristics between Re-188 and Tc-99m. Further study to improve radiolabeling of Re-188 into PAN is needed, possibly using several chelating agents such as DTPA or MAG_3 [4]. Secondly, we did not perform dosimetry in this study. However, our results showed significant inhibition of tumor growth with Re-188-PAN, indicating adequate doses absorbed.

In conclusion, we observed that the IS-dependent PAN was successfully retained with carrying radioisotope in the tumor sites. Moreover, intratumoral injection of PAN labeled with beta-emitting Re-188 effectively inhibited the tumor growth. Therefore, therapeutic application of PAN, which is labeled with beta-emitting radionuclide Re-188, can be expected in a variety of tumors.

Acknowledgment

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