Chitosan-Gadopentetic Acid Complex Nanoparticles for Gadolinium Neutron-Capture Therapy of Cancer: Preparation by Novel Emulsion-Droplet Coalescence Technique and Characterization

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Purpose. The gadopentetic acid (Gd-DTPA)-loaded chitosan nanoparticles (Gd-nanoCPs) were prepared for gadolinium neutron-capture therapy (Gd-NCT) and characterized and evaluated as a device for intratumoral (i.t.) injection.

Methods. Gd-nanoCPs were prepared by a novel emulsion-droplet coalescence technique. The effects of the deacetylation degree of chitosan and Gd-DTPA concentration in chitosan medium on the particle size and the gadolinium content in Gd-nanoCPs were examined. *In vitro* Gd-DTPA release from Gd-nanoCPs was evaluated using an isotonic phosphate-buffered saline solution (PBS, pH 7.4) and human plasma. *In vivo* Gd-DTPA retention in the tumor after i.t. injection of Gd-nanoCPs was estimated on mice bearing s.c. B16F10 melanoma.

Results. Gd-nanoCPs with the highest Gd content, which were obtained using 100% deacetylated chitosan in 15% Gd-DTPA aqueous solution, were 452 nm in diameter and 45% in Gd-DTPA content. A lower deacetylation degree of chitosan led to an increase in particle size and a decrease in Gd-DTPA content in Gd-nanoCPs. As Gd-DTPA concentration in the chitosan solution increased, Gd-DTPA content in Gd-nanoCPs increased but the particle size did not vary. Gd-DTPA loaded to Gd-nanoCPs was hardly released over 7 days in PBS (1.8%) despite the high water solubility of Gd-DTPA. In contrast, 91% of Gd-DTPA was released in plasma over 24 hours. When Gd-nanoCPs were i.t. injected, 92% of Gd-DTPA injected effectually without outflow was held in the tumor tissue for 24 hours, which was different from the case of gadopentetate solution injection (only 1.2%).

Conclusions. Gd-nanoCPs highly incorporating Gd-DTPA were successfully prepared by the emulsion-droplet coalescence technique. Their releasing properties and their ability for long-term retention of Gd-DTPA in the tumor indicated that Gd-nanoCPs might be useful as an i.t. injectable device for Gd-NCT.

KEY WORDS: nanoparticle; chitosan; gadopentetic acid; gadolinium; neutron capture therapy; emulsion-droplet coalescence.

INTRODUCTION

A drug delivery system (DDS) is most often associated with particulate carriers, such as emulsion, liposomes and nanoparticles, that are designed to localize drugs in the target site. Biodegradable nanoparticles have received considerable attention as potent vehicles for targeting a site and controlled release of a drug (1–5). Chitosan obtained by *N*-deacetylation of chitin, a polysaccharide, has favorable properties as a pharmaceutical material, in particular, for anionic drugs because of its biodegradable (bioerodible), biocompatible (nontoxic), bioadhesive (cationic) and bioactive characteristics (6); therefore, a DDS using chitosan has been widely studied (7).

Gadolinium neutron-capture therapy (Gd-NCT) is a cancer therapy that utilizes photons and electrons emitted *in vivo* as a result of the nuclear neutron capture reaction with administered gadolinium-157, a non-radioelement (8). In clinical NCT for malignant melanoma and glioma, boron-10 compounds have been generally used as short-range alpha-particle producing agents (9,10). On the other hand, NCT with gadolinium-157 has possible advantages, such as the highest thermal neutron-capture cross section among stable nuclides (255000 barns: 66 times larger than that of boron-10), and the release of the gamma-rays and Auger (and Coster-Kronig) electrons by the neutron-capture reaction (11). Consequently, Gd-NCT may reinforce therapeutic effect by increasing the chance of extensively hitting the target tumor cells with the long-range (> 100 μm) photons and/or a locally intensive destruction of DNA in neoplastic cells by short-range and high linear energy transfer electrons (8,12). In addition, since gadolinium has been used as a magnetic resonance imaging (MRI) diagnostic agent, it may be possible in the future to coordinate MRI diagnosis with Gd-NCT by using a functional gadolinium-loading particulate system or compound.

The potential of Gd-NCT has been reported in recent years (13–16). In our previous studies, the gadolinium delayed-releasing microcapsules were prepared using Magnevist®, a MRI contrast agent, for Gd-NCT trial (17) and they were found to be significantly effective in extending the survival time of mice inoculated with Ehrlich ascites cells (18). The previous results indicated that one of the key factors for success in Gd-NCT is the use of a device by which gadolinium can be delivered efficiently and retained in the tumor during thermal neutron irradiation.

In the present study, the biodegradable and highly gadopentetic acid (Gd-DTPA)-loaded chitosan nanoparticles (Gd-nanoCPs) were prepared as a gadolinium device for Gd-NCT and MRI diagnosis by a novel emulsion-droplet coalescence technique. The effects of experimental conditions on the particle size and gadolinium content in Gd-nanoCPs were investigated. In addition, the profile of gadolinium release from Gd-nanoCPs was analyzed *in vitro*, and gadolinium retention in tumor tissue after intratumoral (i.t.) injection of Gd-nanoCPs for simulating Gd-NCT trial on mice was examined *in vivo*.

MATERIALS AND METHODS

Chemicals

Chitosans, grade 10B (100% deacetylated; viscosity of 0.5% w/v chitosan/0.2 M acetic acid buffer (pH 4.0) solution at 20°C, 53 mPa·s), 9B (91.4% deacetylated; viscosity, 240 mPa·s) and 8B (84.9% deacetylated; viscosity, 150 mPa·s), were provided by Katokichi Bio Co., Ltd., Japan. Gd-DTPA with natural gadolinium was obtained from Aldrich Chemical Company, Inc., Australia. Liquid paraffin and sorbitan sesquioleate (Arlacel C), an emulsifier, were provided by Nacalai

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Tesque Inc., Japan. For the dissolution test, human plasma for transfusion was kindly supplied by Nihon Red Cross, Japan. For examination *in vivo*, Magnevist® (dimeglumine gadopentetate solution), a MRI diagnostic agent, was purchased from Nihon Schering Co., Ltd., Japan. The other chemicals and solvents were of reagent grade and used without further purification.

Animals and Tumors

Male C57BL/6 mice were provided by Nihon SLC, Japan. The B16F10 malignant melanoma cells were kindly supplied by the Mishima Institute for Dermatological Research, Kobe, Japan. The cells were maintained in dish for culture using Eagle's minimum essential medium supplemented with 10% fetal bovine serum under 5% CO_2 atmosphere at 37°C. For *in vivo* experiment, 0.1 ml of the cell suspension containing 3×10^6 cells, which were dispersed using an isotonic phosphate-buffered saline solution (PBS) (3×10^7 cells/ml), was carefully inoculated subcutaneously (s.c.) into the posterior flank of a six-week-old C57BL/6 mouse.

Preparation of Gd-nanoCPs

Gd-nanoCPs were prepared by a novel emulsion-droplet coalescence technique developed for the noncross-linked Gd-DTPA-loaded chitosan microparticles (Gd-μCPs) in our previous study (19). The modified preparation of Gd-nanoCPs is shown in Fig. 1. Chitosan (2.5% w/v) was dissolved in a Gd-DTPA aqueous solution (5–15% w/v) and an aliquot of 1 ml was added to 10 ml of liquid paraffin containing 5% v/v Arlacel C. The mixture was stirred to form a water-in-oil (w/o) emulsion A using a high-speed homogenizer, Polytron® with a PTA-10S generator shaft (Kinematica AG, Switzerland). Similarly, a w/o emulsion B was prepared by adding 3 N sodium hydroxide solution (1.5 ml) to liquid paraffin (10 ml) containing 5% v/v Arlacel C. As the emulsion B was added to the emulsion A, they were mixed and stirred vigorously using the Polytron®. As a result of coalescence of droplets, chitosan was deposited as nanoparticles. Gd-nanoCPs in the mixed emulsion were washed and separated by centrifugation at 3000 rpm for 60 minutes (KN-30F, Kubota, Japan) using toluene, ethanol and water successively. Finally, the Gd-nanoCPs were obtained as water suspensions or powders lyophilized after suspending in isotonic mannitol solution.

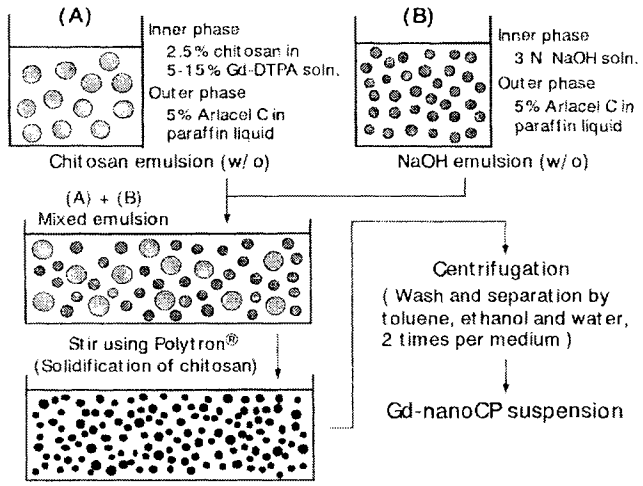

Fig. 1. Preparation process of Gd-nanoCPs by emulsion-droplet coalescence technique.

Particle Size Analysis

The mean particle diameter of Gd-nanoCPs was measured under dispersion in water using a dynamic light scattering technique (ZetaPlas with the BI-MAS option, Brookhaven Instruments Co., USA).

Gadolinium Content Assay

The Gd-nanoCP water suspension was heated and dried completely (Heating Block HF-61, Yamato Scientific Co., Ltd., Japan). Dry weight of Gd-nanoCPs was measured accurately. The weighed Gd-nanoCPs were incinerated using nitric acid under heating. The sample was dissolved in 6.6 N nitric acid and gadolinium concentration was determined by inductively coupled plasma atomic emission spectrography (ICP-AES) (P-5200, Hitachi Co., Ltd., Japan). Gadolinium content (% w/w) in Gd-nanoCPs was given on the basis of dry weight.

Gadolinium Dissolution Test *In Vitro*

Gd-DTPA release from Gd-nanoCPs that were prepared using chitosan 10B and 10% Gd-DTPA solution was examined *in vitro*. As test media, an isotonic PBS of pH 7.4 and the human plasma, as a biological medium, were used, and the method was based on a dynamic dialysis. Briefly, the Gd-nanoCPs, corresponding to 300 μg of gadolinium, were dispersed using 5 ml of the test medium inside dialytic tubing and were incubated in 50 ml of extra-dialytic tubing test medium at 37°C with shaking (Taitec Incubator Personal and Thermo Minder Mini-80, Taiyo Scientific Industries, Japan). Sampling was carried out at predetermined time intervals from extra-dialytic tubing test medium. The examination using the human plasma was performed under aseptic conditions. Released gadolinium in the sample was measured by ICP-AES after incinerating.

Intratumoral Injection Study *In Vivo*

At 10 days after tumor implantation by the above procedure, 200 μl of the Gd-nanoCP (prepared with chitosan 10B and 10% Gd-DTPA solution) suspension in isotonic mannitol solution (Gd 6000 ppm) was injected gently into a block of grown tumor having a size of about 10 mm in diameter (Gd dose, 1200 μg per mouse). In parallel, the dilute Magnevist® solution (Gd 6000 ppm) was injected in the same manner for comparison with Gd-nanoCPs. At 5 minutes or 24 hours after i.t. injection, mice were sacrificed and tumoral blocks were excised. Amount of gadolinium in the tumor tissue was analyzed by ICP-AES after incinerating.

RESULTS

Effects of Experimental Conditions on the Particle Size and Gadolinium Content

The mean particle diameter and gadolinium content (±S.D.) in Gd-nanoCPs prepared using chitosan 10B and 10%

Gd-DTPA solution as chitosan medium by the emulsion-droplet coalescence technique were 426 (±28) nm and 9.3 (±3.2)%, corresponding to 32.4 (±11.0)% as Gd-DTPA, respectively.

Effect of Gd-DTPA Concentration in Aqueous Chitosan Solution

The effects of Gd-DTPA concentration in the solution on particle size and gadolinium content in Gd-nanoCPs were studied using chitosan 10B and the results are shown in Fig. 2. In Gd-nanoCPs prepared using 5% and 15% Gd-DTPA solution, the mean particle diameters were 461 (±15) nm and 452 (±25) nm, respectively, and the gadolinium contents were 7.7 (±1.7)% and 13.0 (±1.8)% (corresponding to 26.9% and 45.3% as Gd-DTPA), respectively. Thus, as the Gd-DTPA concentration in the solution of chitosan 10B increased, the gadolinium (Gd-DTPA) content increased, but the particle size was not significantly influenced.

Effect of Deacetylation Degree of Chitosan

The Gd-nanoCPs prepared at 10% Gd-DTPA concentration using different chitosan grades, 10B, 9B and 8B, were examined. Figure 3 shows the mean particle diameter (top) and gadolinium content (bottom) in Gd-nanoCPs for each chitosan grade. When chitosan 9B and 8B were used, the mean particle diameters were 594 (±96) nm and 750 (±77) nm, respectively, and the gadolinium contents were 4.1 (±1.0)% and 3.3 (±0.8)% (corresponding to 14.2% and 11.6% as Gd-DTPA), respectively. Namely, as the deacetylation degree of chitosan decreased, the particle size increased gradually and, in contrast, Gd content decreased markedly.

Gd-DTPA Releasing Property of Gd-nanoCPs

Figure 4 shows the release of gadolinium from Gd-nanoCPs, prepared with chitosan 10B and 10% Gd-DTPA solution, in PBS and human plasma at 37°C *in vitro*. In PBS, Gd-nanoCPs released only 1.5% of gadolinium (Gd-DTPA) up to

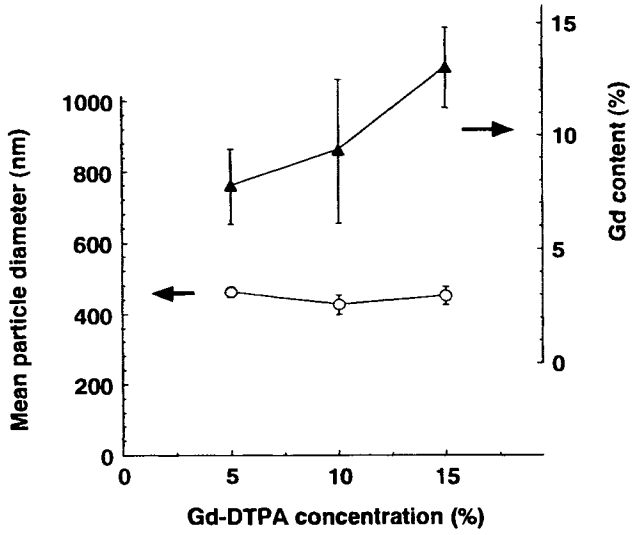

Fig. 2. Effects of Gd-DTPA concentration in chitosan medium on the particle size and gadolinium content of Gd-nanoCPs with chitosan 10B. ○, mean particle diameter, ▲, gadolinium content. The results are shown as average ± S.D. of six experiments.

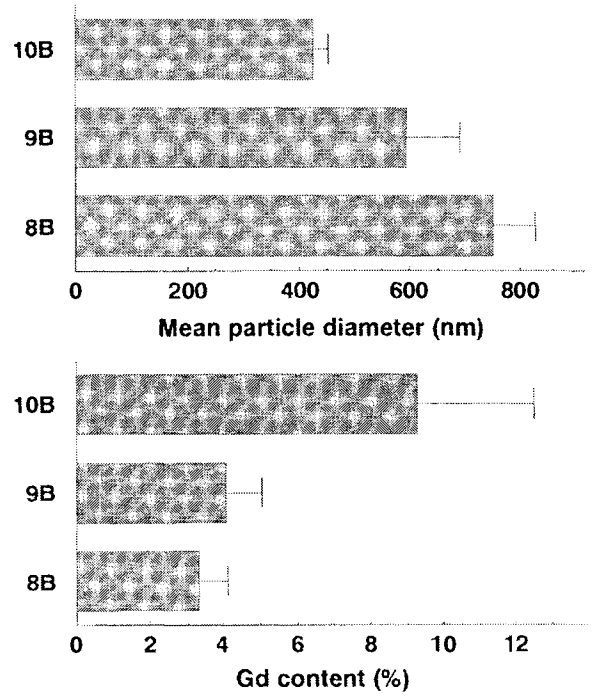

Fig. 3. Effects of chitosan grade on the particle size (top) and gadolinium content (bottom) of Gd-nanoCPs. The results are shown as average ± S.D. of six experiments.

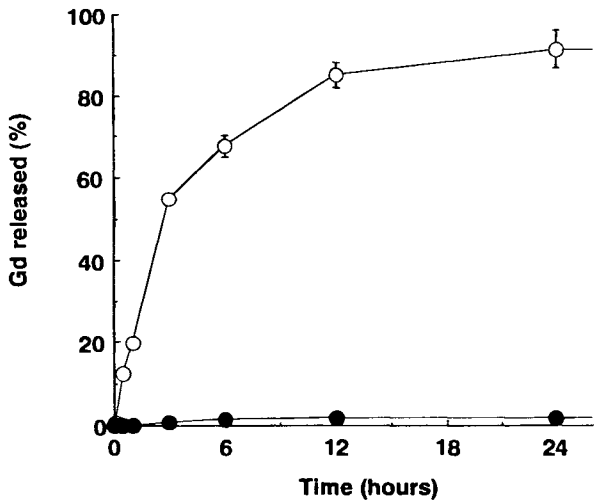

Fig. 4. Release of Gd from Gd-nanoCPs in phosphate-buffered saline solution (pH 7.4) and human plasma at 37°C *in vitro*. ●, phosphate-buffered saline solution; ○, human plasma. The results are shown as average ± S.D. of three experiments.

6 hours and, thereafter, 1.8% up to 7 days. On the other hand, 67.9 and 91.5% of gadolinium (Gd-DTPA) were eluted from Gd-nanoCPs in human plasma for 6 and 24 h, respectively. The gadolinium (Gd-DTPA) releasing behavior was significantly different between Gd-nanoCPs in PBS and those in the human plasma.

Gd-DTPA Retention in Tumor Tissue After Intratumoral Injection

The quantity of gadolinium in the melanoma tissue on mice after i.t. injection of each Gd-DTPA dosage form containing 1200 μg as gadolinium is shown in Fig. 5. When a

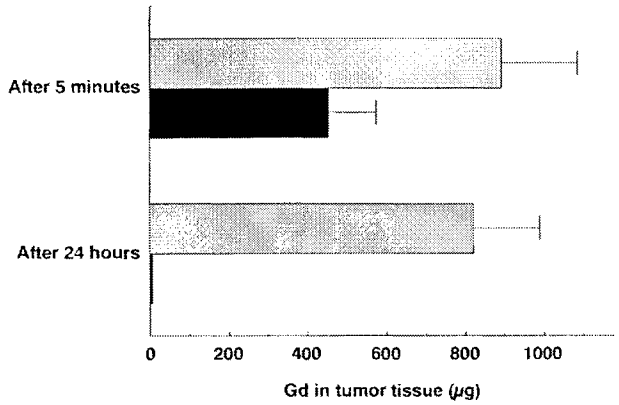

Fig. 5. Amount of gadolinium in B16F10 melanoma tissue on C57BL/6 mice after intratumoral injection with the Gd-nanoCP suspension and dilute Magnevist® solution. ▒, Gd-nanoCP suspension; ■, dilute Magnevist® solution. The results are shown as average ± S.D. of five experiments.

dilute solution of Magnevist®, which had been used in conventional Gd-NCT trials, was administered, the quantities of gadolinium in a tumor block were 451.7 μg (37.6% of dose) and 5.3 μg (0.4%) 5 minutes and 24 hours after injection, respectively. In contrast, 891.7 μg (74.3% of dose) and 820.6 μg (68.4%) of gadolinium remained in the tumor block 5 minutes and 24 hours after administration of Gd-nanoCP suspension, respectively. Based on the larger amount of gadolinium remaining 5 minutes after injection, it was suggested that Gd-nanoCP suspension was smaller in the loss of gadolinium dose from the tumoral block by outflow during i.t. injection than dilute Magnevist® solution. The remaining proportion of the gadolinium effectually injected into the tumor block without outflow was 1.2% in Magnevist® solution but 92.0% in Gd-nanoCPs during 24 hours (from 5 minutes to 24 hours) after i.t. injection. When Gd-DTPA was administered as a solution, it was rapidly eliminated from the tumor tissue. In contrast, the Gd-nanoCPs retained the Gd-DTPA for a longer time in the tumor tissue.

DISCUSSION

In the past, Gd-NCT trials using an MRI contrast agent such as Magnevist®, a major problem was that a sufficient quantity of gadolinium could not be retained in the tumor tissue during neutron irradiation (13). The commercially available gadolinium agent does not exhibit such a selective accumulation in the tumor after i.v. injection as boronophenylalanine in B-NCT (10) and is eliminated rapidly from the tumor tissue after i.t. injection. Therefore, gadolinium compounds that can be efficiently accumulated in the tumor have been sought (20). In the present study, following the previous research on Gd-μCPs (19), novel Gd-nanoCPs were produced in order to retain gadolinium in the tumor tissue during a Gd-NCT trial.

The emulsion-droplet coalescence technique (Fig. 1) was developed to prepare the biodegradable chitosan particles without a cross-linking agent such as glutaraldehyde which blocks the free polyamino groups of chitosan molecules and would diminish the incorporation of Gd-DTPA, a two-valent anionic compound (19). In this technique, the following property of emulsion was utilized; when two emulsions of the same type with the same continuous phase were mixed and stirred vigorously, droplets of each emulsion would collide at random, coalesce and split, and all of the droplets would finally be made uniform in content. Thus, the Gd-nanoCP generation was triggered by neutralization of acidic chitosan-dissolving droplets of emulsion A with sodium hydroxide in droplets of emulsion B. The nanoparticle generation consequently occurred within the emulsion-droplets. Then, the size of nanoparticles did not reflect the droplet size, whereas the size of previously reported Gd-μCPs, which were about 4 μm in mass median diameter, corresponded to the droplet size of emulsion A. It was confirmed by scanning electron microscopy that Gd-nanoCPs prepared using chitosan 10B were almost the same in particle size as the primary particles of agglomerated Gd-μCPs. This indicated that the present process, in which the continuous phase was changed from chloroform to paraffin liquid, made it possible to prevent nanoparticles from agglomerating.

The phenomena that occurred during the generation of nanoparticles were not simple. The Gd-DTPA concentration in aqueous chitosan solution and the chitosan grade with different deacetylation degree, which might influence neutralization and electrostatic interaction, were changed to investigate the effects of preparation conditions on the size and the gadolinium content in Gd-nanoCPs. With 100% deacetylated chitosan (10B), the gadolinium content in Gd-nanoCPs increased with Gd-DTPA concentration, with the particle size being constant (Fig. 2). In this case, the calculated contents of gadolinium and Gd-DTPA were 18% and 63%, respectively, when the 1:1 ion-pair formation of the amino groups and the carboxylic groups occurred completely. Figure 2 indicates that the amino groups of chitosan were not saturated in the experimental range. Therefore, an increase in Gd-DTPA concentration could still have a potential to not only increase the mass of the complex, but also decrease its volume by deswelling due to the cross-linking effect. It is possible that these two competitive effects would lead to unchanged particle size (430–460 nm). The effects of the deacetylation degree of chitosan on the particle size and the gadolinium content (Fig. 3) could be similarly explained: as the deacetylation degree decreases, the capacity of ion-pair formation also decreases, resulting in reduced incorporation of gadolinium (lower gadolinium content) and diminished deswelling (larger particle size). In addition, the particle size would also be highly related to different precipitation properties of each chitosan grade.

The results from the present process were different from the previous ones. The gadolinium content in Gd-μCPs was not affected by Gd-DTPA concentration and was only slightly increased with a decrease in the deacetylation degree of chitosan, as reported previously (19). Gd-μCPs were agglomerates and their gadolinium content prepared using 100% deacetylated chitosan was about 1/4 of that in the corresponding Gd-nanoCPs at maximum. The possibly complicated mechanisms of Gd-nanoCP and Gd-μCP generation were not clear in detail at present.

The cross-linking (21,22) and electrostatic interaction by anionic materials such as alginate (23) have been generally utilized in order to prepare the solid chitosan particles, since it is difficult to form microparticles using only chitosan and a drug. In addition, the precipitation technique (24,25) and block copolymerization (25) have been studied to produce chitosan nanoparticulate carriers in recent years. One of the major problems with the clinical application of nanoparticles is that the drug load is too low to deliver an effective dose; the performance of the above chitosan particles in the past studies was also

unexceptional. In the present emulsion-droplet coalescence technique, the Gd-DTPA appears to strongly interact electrostatically with amino groups of chitosan in the deposition of Gd-nanoCPs. This would contribute to the extraordinarily high Gd-DTPA content (45.3%) and their small particle size, which was reduced to i.v. injectable size (452 nm), in Gd-nanoCPs prepared using chitosan 10B and 15% Gd-DTPA solution.

The gadolinium (Gd-DTPA) release profile of Gd-nanoCPs prepared using chitosan 10B in PBS was very different from that in human plasma (Fig. 4). This again suggests a strong complex formation of Gd-DTPA with chitosan in a simple aqueous medium, because highly water-soluble Gd-DTPA was hardly eluted for a long time. This releasing property might be advantageous to Gd-NCT trial by i.t. injection into a solid tumor. The mechanism of fast, not bursting, release of gadolinium (Gd-DTPA) from Gd-nanoCPs in human plasma was not clearly demonstrated *in vivo*. However, Gd-DTPA release might be greatly related to the stability of Gd-nanoCPs as an electrostatic complex in human plasma; since it was clear from the previous study (6) and our observation in the present study that 100% deacetylated chitosan was not easily degraded, the rapid release of Gd-DTPA from Gd-nanoCPs probably occurred without the degradation of chitosan. This rapid release in plasma might be unfavorable for delivering gadolinium to tumor via i.v. injection (or infusion) in Gd-NCT and MRI diagnosis, since Gd-DTPA would not be distributed preferentially to target tissues and cells via i.v. injection, as is well known. For that purpose, the dissolution properties of Gd-nanoCPs must be improved, for example, by heat treatment (26) and surface modification.

The retention of gadolinium (Gd-DTPA) in tumor tissue was examined by i.t. injection of Gd-nanoCPs into an experimental solid tumor *in vivo* (Fig. 5). The results indicated that Gd-nanoCPs had an excellent retention property when applied by i.t. injection. Since the gadolinium, effectually injected into the tumor block without outflow using Gd-nanoCPs, leaked hardly to the surrounding normal tissues, in particular, to subcutaneous space on tumor tissue, over 24 hours, the damage beyond the tumor part by gadolinium neutron-capture reaction would be kept to a minimum. The Gd-DTPA nonrelease property of Gd-nanoCPs in the aqueous medium might contribute to prolonged retention in the tumor tissue. In addition, it was confirmed by our study *in vitro*, using cultured L929 fibroblast cells, that Gd-nanoCPs exhibited a bioadhesion property and a possibility to be intracellularly taken up by endocytosis (data not shown). This retention extended by Gd-nanoCPs may lead to enhancement of the antitumor effect as compared to the past Gd-NCT trials in which Magnevist® was used, whose half-life of wash-out from tumor tissue after i.t. injection was about 20 minutes (13). This will also allow for the flexible adaptation of duration and frequency of neutron irradiation in the Gd-NCT trial.

In summary, the present study demonstrated that Gd-nanoCPs, which were high in gadolinium content and about 450 nm in mean particle diameter, could be successfully prepared as a device for Gd-NCT by a novel emulsion-droplet coalescence technique. The ion-complex formation between chitosan and Gd-DTPA probably contributed to the generation of Gd-nanoCPs. Gd-nanoCPs hardly released gadolinium in PBS and retained gadolinium in the tumor for a longer period *in vivo* after i.t. injection. These results suggest that Gd-nanoCPs might be a useful device for i.t. injection into a solid tumor in Gd-NCT trial, which might enhance the therapeutic effect.

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