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Research paper

In vitro cellular accumulation of gadolinium incorporated into chitosan nanoparticles designed for neutron-capture therapy of cancer

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Abstract

The accumulation of gadolinium loaded as gadopentetic acid (Gd-DTPA) in chitosan nanoparticles (Gd-nanoCPs), which were designed for gadolinium neutron-capture therapy (Gd-NCT) for cancer, was evaluated in vitro in cultured cells. Using L929 fibroblast cells, the Gd accumulation for 12 h at 37°C was investigated at Gd concentrations lower than 40 ppm. The accumulation leveled above 20 ppm and reached 18.0 ± 2.7 (mean ± S.D.) μg Gd/10^6 cells at 40 ppm. Furthermore, the corresponding accumulations in B16F10 melanoma cells and SCC-VII squamous cell carcinoma, which were used in the previous Gd-NCT trials in vivo, were 27.1 ± 2.9 and 59.8 ± 9.8 μg Gd/10^6 cells, respectively, hence explaining the superior growth-suppression in the in vivo trials using SCC-VII cells. The accumulation of Gd-nanoCPs in these cells was 100–200 times higher in comparison to dimeglumine gadopentetate aqueous solution (Magnevist®), a magnetic resonance imaging contrast agent. The endocytic uptake of Gd-nanoCPs, strongly holding Gd-DTPA, was suggested from transmission electron microscopy and comparative studies at 4°C and with the solution system. These findings indicated that Gd-nanoCPs had a high affinity to the cells, probably contributing to the long retention of Gd in tumor tissue and leading to the significant suppression of tumor growth in the in vivo studies that were previously reported.

Keywords: Chitosan; Gadolinium; Neutron capture therapy; Cell accumulation of particles; Endocytosis

1. Introduction

Gadolinium neutron-capture therapy (Gd-NCT) is a cancer therapy which utlizes γ-rays and electrons emitted by ^{157}Gd (n, γ) ^{158}Gd reaction in order to kill tumor cells. The ^{157}Gd has some advantages over ^{10}B, which has been widely used, as a neutron capture element. First, neutron capture cross section of ^{157}Gd is 66 times larger than that of ^{10}B. Secondly, γ-rays have long range (> 100 μm), so that considerable tumor-killing effects can be expected even if Gd is only in the vicinity of cells. Thirdly, Auger electrons, that might exist in neoplastic cells, may lead to an efficient destruction of DNA because of its high linear energy transfer and short-range. Finally, Gd has been used as a contrast agent in the magnetic resonance imaging (MRI), making it possible to integrate Gd-NCT with MRI diagnosis.

The potential of Gd-NCT has been suggested in recent years. Akine et al. [1] revealed that upon using dimeglumine gadopentetate, growth of Gd-infused tumors was significantly suppressed in comparison to that of tumors with no Gd, when observed between 16 and 23 days after neutron irradiation. Hofmann et al. [2] recently reported that inhibition of tumor growth was observed with intratumoral (i.t.) injection of Gadobutorol (Gadovist®, Gd^{3+}-10-[2,3-dihydroxy-1-hydroxymethylpropyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetate), a neutral, macrocyclic Gd complex. However, in vivo performance in Gd-NCT has not been sufficiently established yet, because commercially available Gd agents such as dimeglumine gadopentetate aqueous solution (Magnevist®) and Gadovist® are, even if i.t. injected, eliminated rapidly from tumor tissues, probably due to their high hydrophilicity, resulting in poor accumulation and retention in tumor. Thus, one of the keys for success in Gd-NCT is to develop a device, which is able to maintain a sufficient Gd concentration in the tumor during the treatment.

Chitosan (poly[β-(1-4)-2-amino-2-deoxy-D-glucopyranose]) was selected in our previous studies to prepare Gd-loaded nanoparticles (Gd-nanoCPs) as a device for NCT [3], because chitosan is bioadhesive (cationic), biocompatible (nontoxic) and biodegradable (bioerodible), and has been investigated for versatile industrial and medical applications

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[4]. A wide variety of preparation methods of chitosan particles have also been investigated, such as solvent evaporation techniques [5], multiple emulsion methods [6], spray drying methods [7], electrostatic complex-formation with anionic materials (ionotropic gelation) [8] and block copolymerization [9]. As an alternative approach, a novel emulsion-droplet coalescence technique was developed in our laboratory to prepare non-cross-linked chitosan nanoparticles [3]. The mean particle diameter and Gd content of Gd-nanoCPs prepared by this method using 100% deacetylated chitosan were 426 ± 28 nm (mean ± S.D.) and 9.3 ± 3.2%, equivalent to 32.4 ± 11.0% as gadopentetic acid (Gd-DTPA), respectively. The Gd-DTPA incorporated into Gd-nanoCPs would interact strongly with chitosan, because the highly water-soluble Gd-DTPA was hardly released from Gd-nanoCPs in an isotonic phosphate buffered saline solution over 7 days: the total Gd released was only 1.8%.

The Gd-nanoCPs injected via i.t. route exhibited much stronger tumor-killing effects in Gd-NCT trials for tumor-bearing mice by neutron irradiation, compared with the past Gd-NCT trials using Magnevist® [10]. The strong tumor-killing effects obtained with Gd-nanoCPs were attributed to the fact that Gd content in tumor tissue was about 100 times higher in Gd-nanoCPs group instantly before neutron irradiation, compared with that in Magnevist® group [10]. The question was which properties of Gd-nanoCPs made it possible to achieve such a high Gd retention in the tumor tissue.

The objective of the present study is to evaluate the bioadhesion and cellular uptake of Gd incorporated into chitosan nanoparticles by mainly using cultured L929 fibroblast cells as a model system. The results were compared to those with the cells used in our Gd-NCT trials in order to clarify mechanisms of the observed high tumor-killing effects in vivo.

2. Materials and methods

2.1. Materials

Chitosan, 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), was obtained from Katokichi Co., Ltd., Japan. Gadopentetic acid (Gd-DTPA) with natural gadolinium was purchased from Aldrich Chemical Company, Inc., Australia. Sorbitan sesquioleate (Arlacel C), an emulsifier, and liquid paraffin were obtained from Nacalai Tesque Inc., Japan. Eagle's minimum essential medium (MEM) for in vitro exposure studies using cultured cells was obtained from Nissui Pharm., Co., Ltd., Japan. Fetal bovine serum (FBS, not heat-inactivated) was purchased from JRH Biosciences, Australia. Dimeglumine gadopentetate solution (Magnevist®), an MRI contrast agent, was obtained from Nihon Schering Co., Ltd., Japan. The other chemicals and reagents were of reagent grade and used as purchased.

2.2. Cell lines

L929 mouse fibroblast cells, B16F10 melanoma cells and SCC-VII squamous cell carcinoma were employed to evaluate the accumulation properties of Gd-nanoCPs. These cells were grown and routinely maintained in Eagle's MEM medium supplemented with 10% FBS and 5% L-glutamine in a 100-mm culture dish at 37°C in an atmosphere of 95% O_2 and 5% CO_2.

2.3. Preparation of Gd-nanoCPs by emulsion-droplet coalescence technique

Gadolinium-loaded chitosan nanoparticles (Gd-nanoCPs) were prepared by the novel emulsion-droplet coalescence technique developed by Tokumitsu et al. [3]. In brief, chitosan 10B was dissolved in a 10 w/v % Gd-DTPA aqueous solution so as to obtain a polymer concentration of 2.5 w/v %. One ml of this solution was added to 10 ml of paraffin liquid containing 5 v/v % Arlacel C. This mixture was stirred using a high-speed homogenizer (Physcotron® NS-50) with a NS-10 generator shaft (Niti-on Irikaki, Japan) for 3 min to form a water-in-oil (w/o) emulsion. Similarly, another w/o emulsion consisting of 3 M sodium hydroxide solution was prepared. Then, these two emulsions were mixed and stirred using Physcotron® for 3 min. As a result of coalescence of the droplets, chitosan in the system was solidified to produce nanoparticles. The resultant Gd-nanoCPs were washed by centrifugation at 3000 rev/min for 60 min (KN-30F, Kubota, Japan) using toluene, ethanol and water, consecutively, to remove the remaining surfactant and paraffin liquid. The mean particle diameter (±S.D.) and Gd content of Gd-nanoCPs were 426 (±28) nm and 9.3 (±3.2)%, equivalent to 32.4 (±11.0)% as Gd-DTPA, respectively. Gd-nanoCPs were sterilized by autoclaving with steam at 121°C for 20 min and used in all experiments.

2.4. In vitro accumulation in cultured cells

The cells were seeded at a density of 5×10^5 cells in a 100-mm culture dish and incubated for 48 h under 5% CO_2 atmosphere at 37°C. After the culture medium was aspirated, the cells were incubated with 10 ml of autoclaved Gd-nanoCP suspension or with Magnevist® in the fresh culture medium at a Gd concentration ranging from 0 to 40 ppm for a predetermined time at 37 or 4°C. The cells were twice washed with 5 ml of Dulbecco's PBS (NaCl 137.0 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM) of pH 7.4 to remove free Gd and Gd-nanoCPs. After counting the number of the cells, the samples were incinerated two or three times using 60 v/v % nitric acid under heating (Heating Block HF-61, Yamato Scientific Co., Ltd., Japan). The samples thus incinerated were dissolved in 20 v/v % nitric acid. The amount of Gd in the sample, providing the cellular accumulation, was determined by inductively coupled plasma atomic emission spectrography (ICP-AES) (P-5200, Hitachi Co., Ltd., Japan). Percentage of the cell

number of Gd-group to that of non-Gd-group was defined as cell viability. All experiments were conducted in triplicate.

2.5. Transmission electron microscopy

L929 cells were observed using transmission electron microscope (TEM, JEF-2000EXII, JEOL. Ltd., Japan). Following exposure to the autoclaved Gd-nanoCP suspension (40 μg Gd/ml) in culture medium at 37°C for 12 h, L929 cells were washed with Dulbecco's PBS to remove the free Gd-nanoCPs being not adhered on and not endocytosed into the cells. Then, the cells were fixed with formaldehyde solution and epoxy resin, thinly sliced with a microtome, and observed by TEM.

2.6. In vitro Gd-DTPA release from Gd-nanoCPs

The release study was carried out in a cell-free culture medium to ensure that Gd detected in the cell-accumulation experiments was not due to the uptake and adhesion of free-Gd released from Gd-nanoCPs. Five millilitres of Gd-nanoCP suspension (60 μg Gd/ml) was directly placed into 5 ml of MEM containing 20% FBS and incubated for 0–180 min under 5% CO_2 atmosphere at 37°C. Then, this mixture was centrifuged at 3000 rev/min for 5 min. The supernatant was collected and the Gd concentration in the supernatant was determined by ICP-AES after incinerating. The experiments were performed in triplicate.

3. Results

3.1. Effect of Gd concentration on cellular accumulation

Fig. 1 shows Gd accumulation in the L929 cells incubated with Gd-nanoCPs at various Gd concentrations for 12 h at 37°C. The Gd amount existing in and on the cells was increased with the feed concentration of Gd in the range of 0–20 ppm. The accumulated amount of Gd reached a plateau at 20 ppm of the feed Gd concentration and this value was unchanged up to 40 ppm. The cell viability was kept around 80% at 10–40 ppm (Fig. 2). Therefore, the following studies were performed at a Gd feed concentration of 40 ppm.

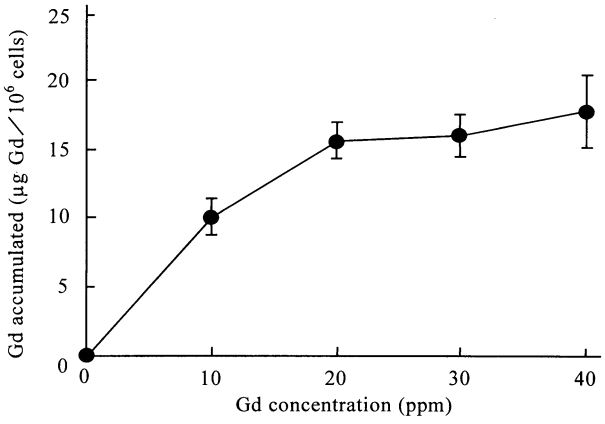

Fig. 1. Effect of Gd concentration on Gd accumulation in L929 fibroblast cells incubated with Gd-nanoCPs for 12 h under 5% CO_2 atmosphere at 37°C. Each value represents mean ± S.D. ($n = 3$).

3.2. Transmission electron microscopy

Transmission electron microscopy was carried out to confirm bioadhesion and endocytosis of Gd-nanoCPs. The micrographs of L929 cells incubated with Gd-nanoCP at Gd concentration of 40 ppm for 12 h at 37°C are shown in Fig. 3. These clearly indicated that Gd-nanoCPs adhered to L929 cells (Fig. 3a) and some were incorporated within the cells (Fig. 3b).

3.3. Gd-DTPA release from Gd-nanoCPs in culture medium

In human plasma, Gd-nanoCPs released 67.9 and 97.8% of Gd-DTPA for 6 and 24 h, respectively [3]. From this previous finding, it was anticipated that Gd-DTPA could also be released in the culture medium. To confirm this, the release property of Gd-nanoCPs in the culture medium used in this study was examined under in vitro conditions. Fig. 4 shows time course of Gd-DTPA release from Gd-nanoCPs. The amount released was about 5% for the first 30 min and remained nearly the same over 180 min. Thus, it was confirmed that most Gd-DTPA incorporated in Gd-nanoCPs was not released in the culture medium.

3.4. Effects of incubation time and temperature on cellular accumulation

The Gd accumulation in L929 cells, B16F10 melanoma cells and SCC-VII squamous cell carcinoma incubated with Gd-nanoCPs at 37 and 4°C is shown in Fig. 5 as a function of incubation time. The accumulation at 37°C seemed to have leveled off after 12 h. When incubated with Gd-nanoCPs for 12 h at 37°C, the total Gd amounts were 18.0 ± 2.7 (mean ± S.D.), 27.1 ± 2.9 and 59.8 ± 9.8 μg Gd/10^6 cells with L929 fibroblast cells, B16F10 cells and

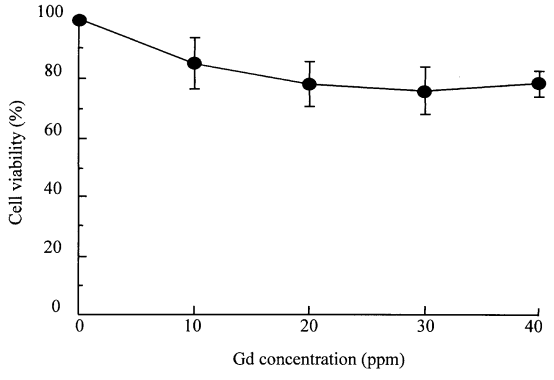

Fig. 2. Effect of Gd concentration on the growth rate of L929 fibroblast cells incubated for 12 h under 5% CO_2 atmosphere at 37°C. Each value represents mean ± S.D. ($n = 3$). The results are shown as cell viability, percentage of the growth rate of Gd-group to that of non-Gd-group.

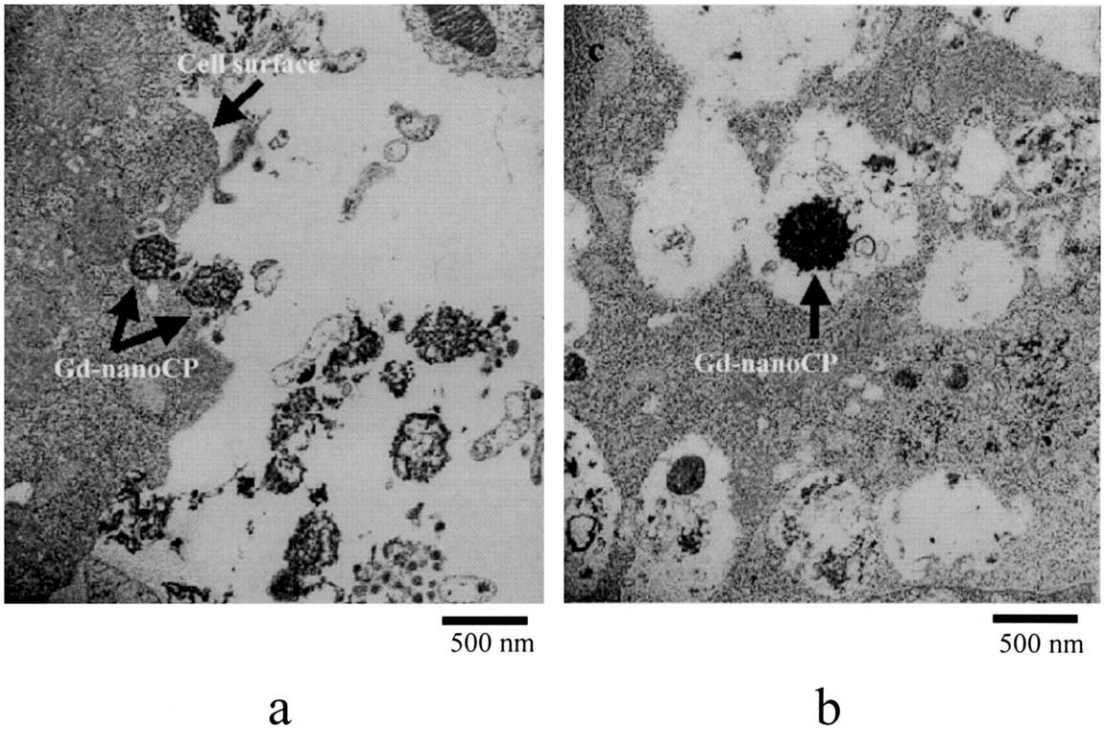

Fig. 3. Transmission electron micrographs of L929 fibroblast cells incubated with Gd-nanoCPs for 12 h at 37°C.

SCC-VII cells, respectively. In contrast, the Gd accumulation at 4°C achieved lower plateau levels more rapidly, 5 or less μg Gd/10^6 cells. Thus, this significantly higher cellular-accumulation at 37°C, which was attributed to endocytosis that was active at 37°C, but suppressed at 4°C, indicated that the endocytic uptake of Gd-nanoCPs made a major contribution to the total accumulation in the cells incubated for long time periods, e.g. up to as 12 h.

3.5. Comparative study with Magnevist®

Fig. 6 summarizes the effect of cell type on the Gd accumulation in the cells incubated with Gd-nanoCPs for 12 h at 37 and 4°C in comparison to those incubated with Magne-

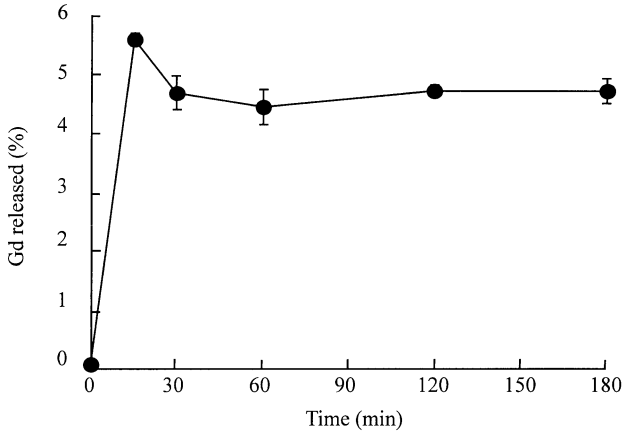

Fig. 4. In vitro release of Gd from Gd-nanoCPs in Eagle's MEM medium containing 10% fetal bovine serum at 37°C. Each value represents mean ± S.D. ($n = 3$).

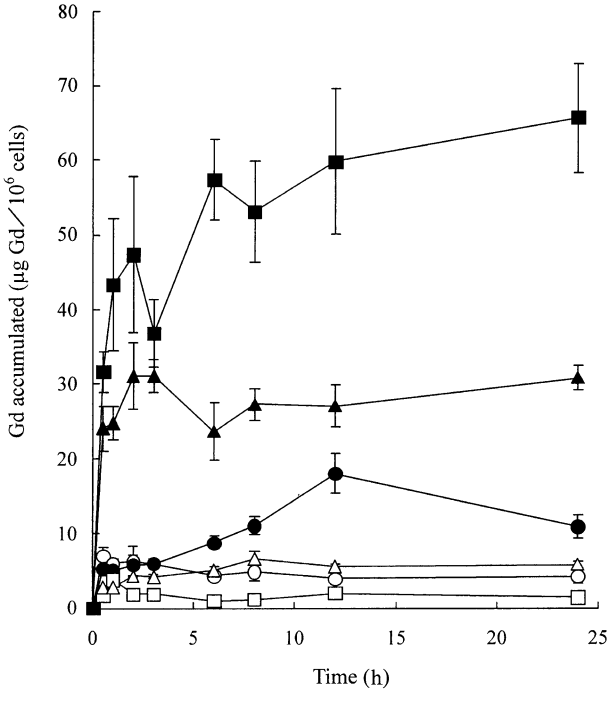

Fig. 5. Effect of incubation time and temperature on Gd accumulation in L929 fibroblast cells (○ ●), B16F10 melanoma cells (△ ▲) and SCC-VII squamous cell carcinoma (□ ■) incubated with Gd-nanoCPs at Gd concentration of 40 ppm under 5% CO_2 atmosphere at 37°C (closed symbols) or 4°C (open symbols). Each value represents mean ± S.D. ($n = 3$).

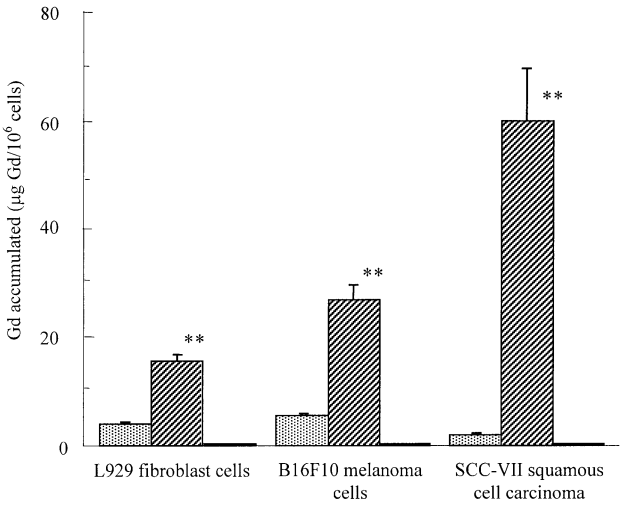

Fig. 6. Effect of cell type on Gd accumulation in cultured cells incubated for 12 h at Gd concentration of 40 ppm under 5% CO_2 atmosphere, shaded bar, Gd-nanoCP at 4°C; hatched bar, Gd-nanoCP at 37°C; solid bar, Magnevist® at 37°C. **, significantly different from the value at 4°C, $P < 0.001$. Each value represents mean ± S.D. ($n = 3$).

vist® for 12 h at 37°C. These results indicated that the use of Gd-DTPA solution led to a significantly lower Gd accumulation in the cells. The accumulated Gd in Magnevist® group was 0.15 ± 0.06, 0.21 ± 0.04, 0.28 ± 0.02 (mean ± S.D.) μg Gd/10^6 cells, respectively.

4. Discussion

The extracellular matrix is composed of sulfated glycosaminoglycans and polysaccharide acids, which form hydrophilic, negatively-charged gels over the cell membrane. Membrane glycoproteins, most often bearing sialic acid residues, also contribute to the negative charge of the cell surface. On the other hand, it is well known that chitosan is a cationic polyelectrolyte. Thus it has a strong electrostatic interaction with negatively charged cell surfaces. Therefore, the use of cationic polymers, such as chitosan, in preparing particulate carriers would give rise to higher drug retention on cells and in tissues because of the electrostatic interaction between particle surfaces and cell surfaces. Indeed, many researchers have reported that bioadhesive properties of chitosan would originate in the electrostatic interaction between positively charged amino groups in chitosan and negatively charged sialic acid residues on cell surfaces [11,12]. Chatelet et al. demonstrated the effect of deacetylation degree of chitosan on the bioadhesive property using chitosan film [13]. They showed that the adhesion of chitosan to fibroblasts isolated from foreskins of children was increased as the deacetylation degree of chitosan was increased. Therefore, the present Gd-nanoCPs comprising 100% deacetylated chitosan, which was unsaturated with Gd-DTPA [3], probably utilizing such functions fully, were also expected to show a bioadhesive property.

The effect of Gd concentration in the culture medium on the Gd accumulation indicated that it was saturated at 18 μg Gd/10^6 cells in the range of 20–40 ppm (Fig. 1). At first for the saturation of Gd accumulation in the cells was thought to be due to lowering of cell viability after exposure of the cells to Gd-nanoCPs, because cationic macromolecules such as polylysine have, in general, been known to affect the cell viability [14]. However, incubation with Gd-nanoCPs in this concentration range exhibited no serious cytotoxicity with respect to cellular growth (Fig. 2) and did not reveal morphologic change during experiments. These results indicated that the cell viability, although a little reduced, was not directly related to the saturation phenomenon.

It is interesting to note that intra- and extra-cellular accumulation of Gd takes place in the form of particles. The TEM observations showing Gd-nanoCPs bound to the cell surfaces and located, to some extent, in the intracellular spaces are a strong indication of this event (Fig. 3). This is further supported by the fact that only 5% of Gd-DTPA loaded into Gd-nanoCPs was released in the culture medium (Fig. 4).

It is generally accepted that endocytic activity is significantly suppressed at 4°C [15]. From this point of view, it is conceivable that the Gd accumulation at 4°C shown in Fig. 5 would reflect the amount of Gd-nanoCPs adhering to the cell surfaces. The fact that it occurred instantly, within the initial 30 min, and was saturated soon (Fig. 5) would also support this speculation. Thus, these results indicated that endocytosis contributed largely to the increased Gd accumulation in incubation of the cells at 37°C.

The size and bioadhesive property of Gd-nanoCPs would be related to the endocytic uptake by the cells. Indeed, Green et al. [16] examined the particle size dependence of endocytosis of polyethylene particles in C3H murine peritoneal macrophages. They found that polyethylene particles in the range from 0.3 to 10 μm in diameter were endocytosable. The size of the present Gd-nanoCPs, 430 nm, was included in this range. In addition, Lee et al. [17] demonstrated that a high level uptake of liposomes in J774 cells, a murine macrophage-like cell line, could be obtained by altering the lipid formulation to provide an adhesive property on their surfaces, as might be achieved by the present Gd-nanoCPs (Fig. 3). These results suggested that the adhesion of optimally sized Gd-nanoCPs on the cell surface might be an important step in the subsequent uptake by endocytosis.

The accumulation behavior of Gd-nanoCPs, in and on cells, varied with cell types (Figs. 5 and 6). In fact, the accumulation of Gd-nanoCPs in B16F10 cells and SCC-VII cells was significantly higher than that in L929 cells. In particular, a two-fold increase in accumulation of Gd-nanoCPs in SCC-VII cells was observed, when compared to B16F10 cells: the accumulation in SCC-VII cells after exposure to Gd-nanoCPs for 12 h at 37°C was 60 μg Gd/10^6

cells, whereas that in B16F10 cells was 27 μg Gd/10^6 cells (Fig. 6). On the contrary, no meaningful difference was observed among three cell lines in regard to the adhesion of Gd-nanoCPs on their cell surfaces at 4°C where endocytic activity would not exist. Such a varied accumulation behavior of Gd in these three cell lines was attributed to their difference of endocytic activity.

The cellular accumulation behavior observed in vitro may also be related to the tumor-killing effects in vivo. B16F10 cell- or SCC-VII cell-bearing mice were employed in our Gd-NCT trials in vivo as reported earlier [10,18]. The tumor-killing effects observed in SCC-VII cell-bearing mice [18] were nearly identical to those achieved with B16F10 cell-bearing mice [10] at the same i.t. Gd dose of 2400 μg/tumor in spite of the fact that the thermal neutron fluence in the Gd-NCT trials using SCC-VII cell-bearing mice (3.31×10^{12} neutrons/cm^2) was only a half of that in the cases of B16F10 cell-bearing mice (6.32×10^{12} neutrons/cm^2). From the present results, at least one reason for the relatively higher tumor-killing effects obtained with SCC-VII cell-bearing mice in vivo was their higher endocytic activity.

In the comparative study using the solution system, the most important point to be emphasized again was that Gd accumulation in all cell lines utilized in the present study was mostly achieved in the form of particles by cell-surface adhesion and endocytosis of Gd-nanoCPs, though the activity varied. In fact, Magnevist® solution scarcely showed the intra- and extra-cellular Gd accumulation in all cell lines (Fig. 6). The Gd amount detected in Magnevist® group after 12 h at 37°C was less than 1% of that in Gd-nanoCP group (Fig. 6), probably relating to the well-known fact that Magnevist® was eliminated from the tumor tissue immediately after i.t. injection [10]. On the other hand, bioadhesion, endocytosis and strong Gd-DTPA-binding of optimally sized Gd-nanoCPs would significantly extend the elimination half-life of i.t. administered Gd [10]. The present results evidenced that Gd-nanoCPs had a great potential to accumulate Gd into tumor tissue and/or cells, consequently leading to improvement of the therapeutic efficiency in our past Gd-NCT trials [10].

A major concern in the past Gd-NCT trials using cultured cells had been the necessity of a large amount of Gd for cell-growth suppression. Akine et al. [19] reported that 10% survival level of the cultured Chinese hamster cells was obtained with a fluence of 1.55×10^{12} neutrons/cm^2 in the presence of Gd of 5000 ppm. Tokuuye et al. [20] evaluated the effect of radiation released during neutron capture reaction by ^{157}Gd of 800 ppm, ^{10}B of 51 ppm or their combination, using Magnevist® and sodium borocaptate sodium, on cell survival. Then, 1.13, 1.69 and 0.95×10^{12} neutrons/cm^2 were required for 10% survival levels, respectively. Further, Hofmann et al. [2] demonstrated using the macrocyclic Gadobutrol (Gadovist®) that at a fluence of 3.6×10^{12} neutrons/cm^2, Gd concentration of 10 mmol Gd/l, equivalent to 1570 ppm, was required to obtain about 50% survival level of Sk-Mel-28 cells, a melanoma cell line of human origin. A possible problem of these studies was that they had to apply high Gd concentrations, because only Gd-agents that show no affinity to the tumor cells, possibly including Gadovist®, were available. As a result, cells were unable to suffer sufficient neutron-irradiation because of obstruction by the presence of excess neutron-capture elements in medium. Unlike such a solution-based formulation, Gd would be concentrated on and in cells by using the present Gd-nanoCPs. This situation is clearly favorable for obtaining effective cell-growth suppression.

It has been believed that a contact between the cells and Gd is not always necessary for the tumor cell inactivation in Gd-NCT, because of the long-range γ-rays emitted as a result of Gd neutron-capture reaction. Akine and his-coworkers, however, described that the electrons might also play an important role in the tumor-killing effect in Gd-NCT [19]. Therefore, the presence of Gd in the intracellular space may be rather desirable for Gd-NCT, since the electrons, especially Auger electrons, have a short-range and a high linear energy transfer. As shown in the present study, Gd-nanoCPs could bind to the cell surfaces, as a consequence of their cationic nature, and subsequently be endocytosed. These intra- and extra-cellular accumulation behaviors would provide a compatible way to accomplish the high suppression of tumor growth in Gd-NCT.

5. Conclusions

In spite of the low Gd concentrations, e.g., 20–40 ppm in the present study, the use of Gd-nanoCPs resulted in high Gd-accumulation due to their bioadhesion to the cell surface and subsequent endocytosis. The accumulation was 100–200 times larger in comparison to the case of Gd-DTPA solution, Magnevist®, in spite of cell-type variation in endocytic activity. This lets us reach the conclusion that Gd-nanoCPs are a very promising Gd delivery device for Gd-NCT.

This study provides two key pieces of information: first, the bioadhesive and endocytic properties of Gd-nanoCPs are crucial factors to obtain the high tumor-killing effects by i.t. injection in Gd-NCT, and second, Gd-nanoCPs i.t. injected may be effective for a wider type of cancers, different from B-NCT where certain ^{10}B compounds that can only accumulate in specific cancers have been used.

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