Toxicity and Biomedical Imaging of Fluorescence-Conjugated Nanoparticles in Hematopoietic Progenitor Cells

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ABSTRACT

Cellular uptake of nanoparticles for stem cell labeling and tracking is a critical technique for biomedical therapeutic applications. However, current techniques suffer from low intracellular labeling efficiency and cytotoxic effects, which has led to great interest in the development of a new labeling strategy. Using silica-coated nanoparticles conjugated with rhodamine B isothiocyanate (RITC) (SR), we tested the cellular uptake efficiency, biocompatibility, proliferation or differentiation ability with murine bone marrow derived hematopoietic stem/progenitor cells. The bone marrow hematopoietic cells showed efficient uptake with SR with dose or time dependent manner and also provided a higher uptake on hematopoietic stem/progenitor cells. Biocompatibility tests revealed that the SR had no deleterious effects on cell cytotoxicity, proliferation, or multi-differentiation capacities *in vitro* and *in vivo*. SR nanoparticles are advantageous over traditional labeling techniques as they possess a high level of cellular internalization without limiting the biofunctionality of the cells. Therefore, SR provides a useful alternative for gene or drug delivery into hematopoietic stem/progenitor cells for basic research and clinical applications.

(Key words : Hematopoietic stem/progenitor cells, Silica nanoparticles, Biocompatibility, *In vivo* transplantation)

INTRODUCTION

Nanotechnology is an area of research with various applications, which in recent years, has developed useful techniques for the biomedical field. This new, small-scale technology plays an innovative role in the design and use of nanodevices for gene therapy and drug delivery, imaging, biomarker and biosensor applications (Moriguchi *et al*., 2005; Bhavsar *et al*., 2007; Suzuki *et al*., 2007; Saz *et al*., 2008). Significant efforts have been devoted to developing new nanoparticles for successful biomedical applications, particularly for cell-tracking purposes (Yang 2007). Several studies have demonstrated the use of semiconductor nanocrystals (quantum dots) and superparamagnetic iron oxide (SPIO) nanoparticles for stem cell internalization and tracking (Brucchez *et al*., 1998; Abonour *et al*., 2000; Luccardini *et al*., 2007).

Hematopoietic stem cells (HSCs), derived from bone marrow and blood, have the capacity to self-renew and differentiate into specialized blood cells (Ema *et al*., 2003; Iwama *et al*., 2004). HSCs are the critical targets of gene therapy for hematopoietic-related diseases such as cancer and immunodeficiency, as well as non-hematopoietic diseases (Barrette *et al*., 2000; Scadden *et al*., 2001; Schmitz *et al*., 2004 Zhong *et al*., 2006). In order to deliver gene or drug into hematopoietic stem cells, numerous studies were performed using viral vector (Barrette *et al*., 2000; Hanazono *et al*., 2003; Crcareva *et al*., 2005). The gene delivery, however, was limited due to a low efficiency of cellular internalization of the vectors and a cytotoxic effect. Recently, fluorescently conjugated nanoparticles have been adopted as alternative vehicles for labeling or delivery materials in stem cells, because of higher uptake efficiency of nanoparticles than viral vector into stem cells (Huang *et al*., 2005; Chung *et al*., 2007). Several report demonstrated that surface characteristics of nanoparticles may dramatically improve uptake efficiency and deleterious effects on cellular functions, especially for stem cells (Gupta *et al*., 2004; Clement *et al*., 2006; Lu *et al*., 2007). Among several nanoparticles, silica coated nanoparticles have been extensively used over the past decades and were recently synthesized with a functionalized surface for bioconjugation applications, because Silica (SiO_2) is a biocompatible material and easily functionalized for bioconjugation and targeting, so far (Lu *et al*., 2007). However, the efficiency of cell labeling with silica nanopar-

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ticles and their potential effects on biocompatibility for bone marrow hematopoietic stem cells has yet to be fully investigated.

In this paper, we test whether an organic fluorescent dye incorporated into the silica shell nanoparticle can be used to label murine bone marrow derived hematopoietic stem/progenitor cells by flow cytometry and cell proliferation. We report an enhancement of uptake efficiency of SR with bone marrow hematopoietic cells and hematopoietic stem/progenitor cells, especially without any deleterious effects.

MATERIALS AND METHODS

Preparation of Nanoparticles

Fifty-nm size nanoparticles labeled with RITC (Sigma-Aldrich) were synthesized according to a previously described method (Yoon *et al*., 2005; Kim *et al*., 2006). In brief, trimethoxysilane modified by rhodamine B isothiocyanate (RITC) was prepared from 3-aminopropyltriethoxysilane (APS; Gelest) and RTIC under nitrogen. The organo silica cores were synthesized under nitrogen in 40 ml of ethanol and 2.1 ml of ammonia (4.2 vol%) for the SR modified nanoparticles. Reagent concentrations were chosen to prepare spheres with a radius of approximately 50 nm. The reagent tetraethoxysilane (TEOS, Glest) was added by a funnel under the solution surface while under severe mechanical stirring. After a few minutes, the solution became turbid and the synthesized nanoparticles were dispersed in ethanol and precipitated by ultra-centrifugation for 3 h. The supernatant solution was then removed and the precipitated particles were redispersed in PBS. All prepared nanoparticles were confirmed by a transmission electron microscope (TEM).

Preparation of Hematopoietic Cells

Female, 7-week-old, C57BL/6 mice were purchased from Orients Co. and used to isolate bone marrow hematopoietic cells and stem/progenitor cells and to assay *in vivo* transplantation experiment. All animals were maintained in an animal facility at the CHA Stem Cell Institute at CHA University. The mice were kept in microisolator cages and given autoclaved food and water. Mononuclear bone marrow hematopoietic cells were obtained by flushing femoral cells with a 23-gauge needle. A anti c-Kit monoclonal antibody was used to label mononuclear bone marrow cells and the positively tagged cells were purified with Magnetic cell sorting (MACS, Stem cells Technology, Vancouver, Canada), and confirmed the purity of c-Kit$^+$ cells by FACS vantage SE flow cytometry (Becton Dickinson).

Flow Cytometry Analysis

The investigation of uptake efficiency of SR was performed by flow cytometry analysis. In order to determine the uptake of SR, the cells were incubated with 200 μg/ml of a SR suspension in PBS for 1 h. After washing the cells three times with PBS, they were resuspended in staining medium containing 2% fetal bovine serum (FBS) and 0.1% sodium azide in PBS for further analysis. Dead cells were excluded using 5 μg/ ml propidium iodide (PI) before flow cytometry analysis. The red emitting rhodamine B dye incorporated in SR served as a marker to quantitatively determine the cellular uptake ability and was analyzed using FACS vantage SE (Becton Dickinson, San Diego, CA). All of the flow cytometry data collected was analyzed using a CellQuest program.

Cell Viability and Proliferation Assay

In an effort to determine the biological effects of SR on the hematopoietic cells, the cellular viability was assessed by the MTT assay. After incubation with the 200 μg/ml SR in PBS for 1 h, cells were washed three times with a PBS free solution and viability was measured via the MTT assay. The possible cytotoxic effects on the cells was measured via the addition of 10 μl of MTT solution to each well and incubated at 37℃ for 4 hr followed by the addition of 200 μl of solubilization solution. The proliferative capacity of these cells was studied as follows: after treatment with 200 μg/ml SR for 1 h, the cells were grown in a medium consisting of high glucose DMEM (GIBCO) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin for 24 hr, followed by incubation with MTT reagent. The dark blue formazan dye generated by the live cells was analyzed on a microplate reader as a measure of cell proliferation and activity. All cultures were kept in an atmosphere of 5% CO_2 at 37℃.

Cell Cycle Analysis

To assess the effects of SR on cell cycle, cells were treated with 200 μg/ml SR for 1 hr and cultured in growth medium for 24 h at 5% CO_2 and 37℃. Cells were washed twice and fixed with ice-cold 70% ethanol for more than 1 h. After washing with staining media containing 2% FBS and 0.1% sodium azide in a PBS free solution, cells were resuspended in PBS with 50 μg/ml RNase and 50 μg/ml PI and incubated for 30 min at 37℃ and then analyzed by flow cytometry. The different phases of the cell cycle were assessed by collecting and analyzing the signal on channel FL2-A. The percentage of the cell population at a particular phase was estimated by ModFit LT.

Colony-Forming-Cell Ability Assay

Colony-forming-cell capacity (CUF-C) assays were performed with hematopoietic stem/progenitor cells, which

were derived from bone marrow, to assess their multipotent differentiation capacities. Specifically, cells were plated at a density of 1×10^4 cells/ml in triplicate and cultured in a medium containing methycellulose medium 3434 (Stem Cell Technology) supplemented with rmSCF (50 ng/ml), rmIL-3(10 ng/ml), rhIL-6 (10 ng/ml), and rhEPO(3 U/ml) after reaction with or without SR. The culture dishes were incubated at 37℃ in 5% CO_2 for 14 days. After incubation, colonies were visualized microscopically and scored upon inspection.

In Vivo Hematopoietic Reconstitution Activity

The purified c-Kit$^+$ cells were cultured with 200 μg/ml SR for 1 h at 37℃. To assay the repopulating activity of SR labeled c-Kit$^+$ cells *in vivo*, we performed by using the Ly5 congenic mouse system. SR labeled c-Kit$^+$ cells from bone marrow of B6-Ly5.1 mice were intravenously transplanted into B6-Ly5.2 recipient mice lethally irradiated at a dose of 9.5Gy together with 2×10^5 total bone marrow hematopoietic cells of B6-Ly5.2 mice as rescue cells. To confirm the mutipotential differentiation ability of SR labeled c-Kit$^+$ cells, peripheral blood cells from B6-Ly5.2 recipient mice were taken at 5 weeks after injection. The engraftment efficiency of SR uptake c-Kit$^+$ cells derived Ly5.1$^+$ mice was analyzed by flow cytometry and then determined the chimerism of myeloid (Mac-1$^+$ for monocytes and Gr-1$^+$ for granulocytes), lymphoid (B220$^+$ for B cells and CD3$^+$ for T cells) lineages.

Statistical Analysis

Results are expressed as mean±SD. All statistical analyses were preformed using students paired *t*-test. A value of $p<0.05$ is considered significant in comparison with the respective control groups.

RESULTS

Uptake Efficiency into Bone Marrow Hematopoietic Cells

SR biofunctional contained with rhodamine B isothiocyanate (RITC) were synthesized to an average core diameter of 50 nm to be used for this study (Fig. 1). Firstly, an efficient labeling time of hematopoietic cells with SR was investigated. Mononuclear hematopoietic cells from murine bone marrow were incubated with 200 μg/ml of SR in serum-free culture media over various different incubation times. After incubation for 0.5, 1, or 2 h, cells were washed and analyzed for cellular uptake efficiency by flow cytometry. It was determined that up to 87% of total mononuclear hematopoietic cells were stained with SR after 1 or 2 h incubation (Fig. 2A, B). The optimal uptake efficiency was noted after 1 h for both the SR as observed from mean value of fluorescence intensity (MFI) analysis by flow cytometry analysis (data not shown).

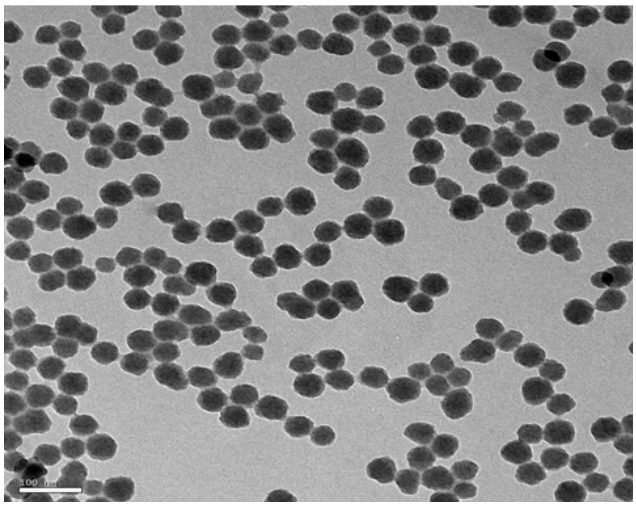


Fig. 1. TEM image of SiO_2-RITC (SR) with an average size of 50 nm.

As next trials, we investigated the most optimal dose of SR with bone marrow derived hematopoietic cells by flow cytometry. Dose of SR ranging from 0, 50, 100, 200, or 400 μg/ml were tried through 1 h incubation with bone marrow hematopoietic cells at 37℃. After incubation with the varying doses, cells were analyzed for cellular uptake frequency and fluorescence intensity of SR by flow cytometry. In the flow cytometry analysis, the uptake efficiency of SR increased with dose dependent manner (Fig. 2C,D). This study demonstrated that SR has efficient labeling capacity bone marrow hematopoietic cells. It was observed that the onset of SR cellular uptake began at approximately 0.5 h with a population of 2×10^5 bone marrow hematopoietic cells. The SR labeled hematopoietic cells was easily detectable under fluorescence microscope inspection. Upon data analysis of the incubation length and concentration dosage studies, a 1 h incubation time and a dosage of 200 μg/ml SR was selected as the conditions, which provided the most effective cellular uptake. These experimental conditions were used throughout the remainder of the study. It was determined from this data set, that the uptake efficiency of SR was both dose and time dependent.

Cell Proliferation and Cytotoxic Effects

Once it was determined that the SR were effectively taken up by the cells, the possibility of cytotoxic effects or limitations in cellular proliferation was investigated. In order to measure cytotoxic effects, hematopoietic cells were incubated with 200 μg/ml SR for 1 h at 37℃. The proliferative and cytotoxic effects were investigated by MTT assay after incubating the cells with

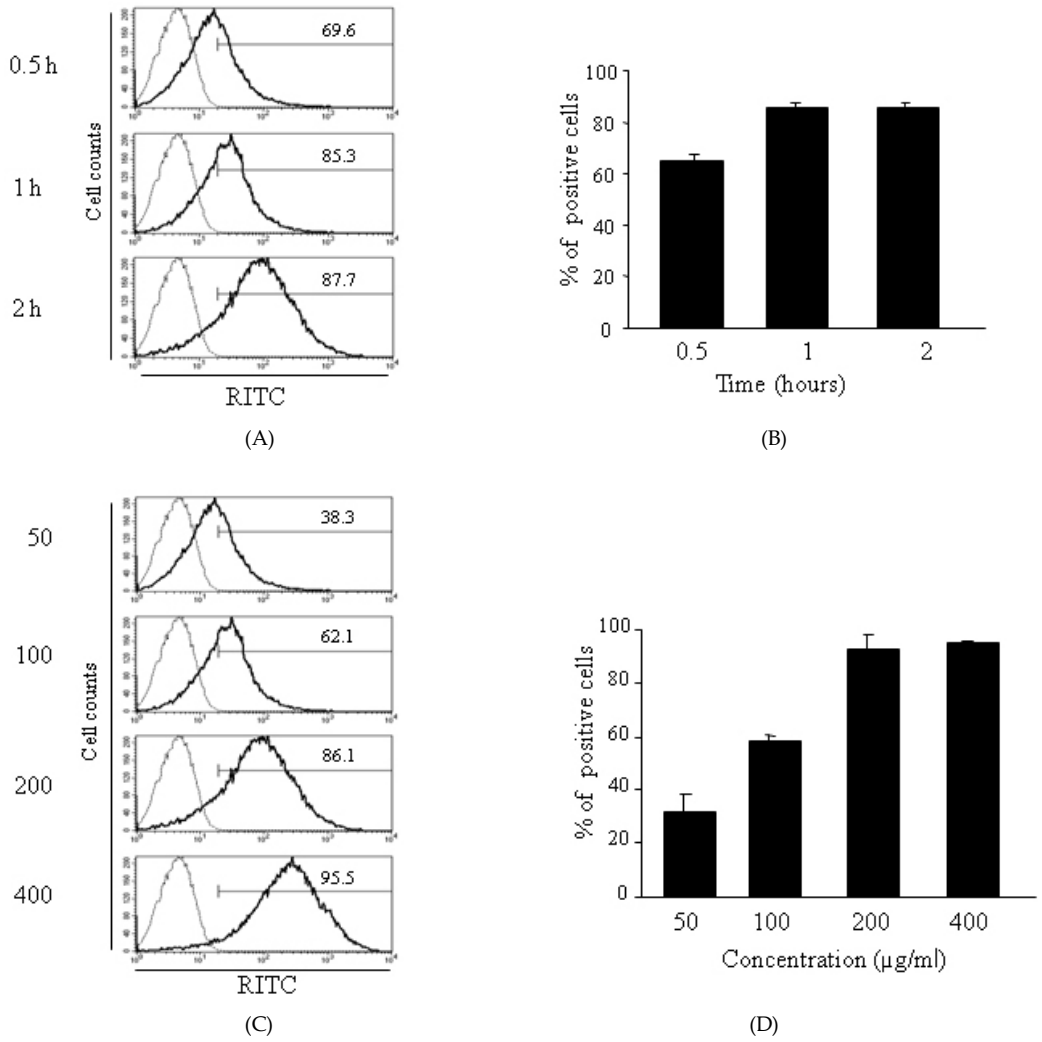


Fig. 2. Cellular uptake efficiency of nanoparticles to bone marrow hematopoietic cells. (A) Flow cytometric analysis of time dependent cellular uptake of SR nanoparticles in hematopoietic cells derived from bone marrow. Bone marrow hematopoietic cells (2×10^5 cells) were treated with SR nanoparticles for 0.5, 1, 2 h and then analyzed with flow cytometry. (B) The number of positively labeled cells was represented as the percentage of total counting cells in each panel. (C) Bone marrow hematopoietic cells(2×10^5) was reacted with 50, 100, 200, and 400 μg/ml of SR nanoparticles for 1 h and then analyzed the presence of RITC labeled cells by flow cytometry. (D) The percentage of positively labeled cells with SR nanoparticles was represented. Data are expressed as mean±SEM of three independent experiments.

200 μg/ml SR for 24 h. As shown in Fig. 3(A) and (B), no significant effects were observed related to either cytotoxicity or a proliferative capacity with the treatment of SR. In addition, to confirm the effects on cell cycles after incubation with SR for 1 h, cells were further cultured in growth media for 24 h and then analyzed cell cycle by flow cytometry. SR uptake cells showed no alteration in cell cycle patterns as compared to control cells (Fig. 3).

In Vitro Hematopoietic Differential Effects

To evaluate the potential adverse effects following SR uptake into hematopoietic stem/progenitor cells, *in vitro* colony-forming cell capacity (CFC) assays, which can determine effective differentiate capacity of hematopoietic stem/progenitor cells, were performed. Firstly, total bone marrow hematopoietic cells were investigated multipotent hematopoietic differentiation ability after labeling with SR after incubation for 1 h. As shown Fig. 3(D), there are no significant differences of colony forming ability between SR and control cells. As next trials, c-Kit$^+$ (stem cell factor receptor/CD117) cells were purified from bone marrow, which is showing up to 87 % purity of c-Kit$^+$ cells (Fig. 4A). Purified c-Kit$^+$ cells incubated with 200 μg/ml of SR nanoparticles for 1 h. As shown in Fig. 4(B), SR was labeled significant labeling efficiency in c-Kit$^+$ cells (99.5±0.5%). From analysis of biocompatibility test by using MTT analysis, cytotoxicity and cellular proliferation ability in c-Kit$^+$ cells showed no difference compared to control cells (Fig. 4

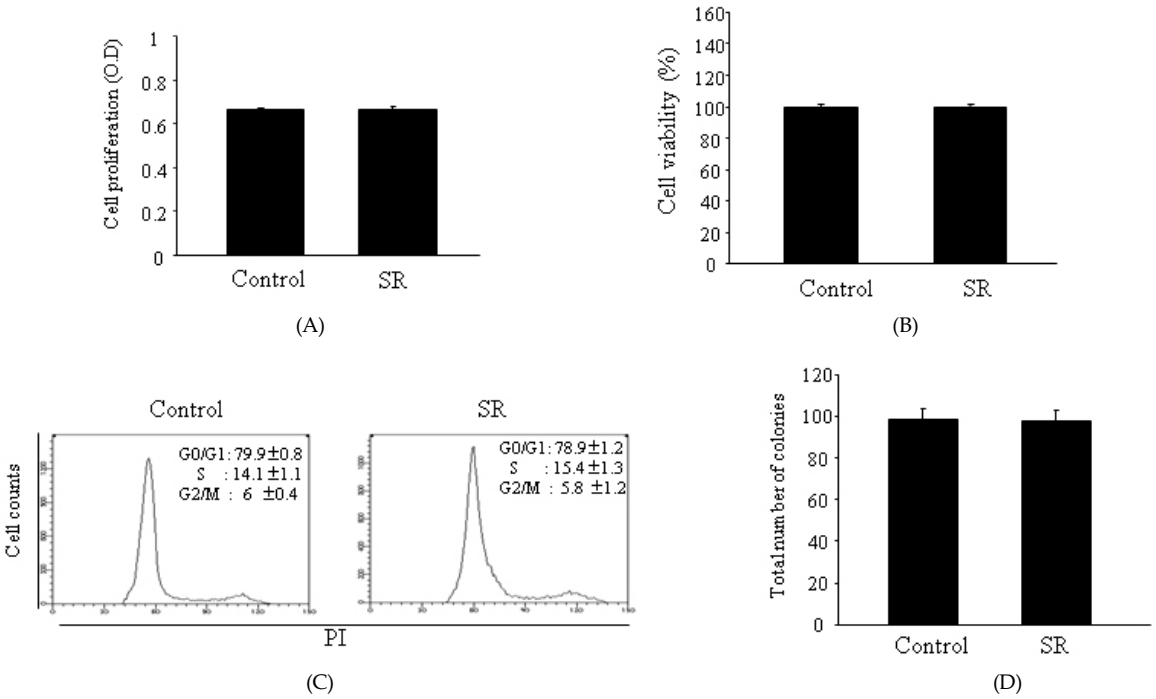


Fig. 3. Cellular effects of nanoparticles in bone marrow hematopoietic cells. The effects for proliferation (A), cytotoxicity (B), or cell cycles (C) were determined after reaction with SR nanoparticles in bone marrow hematopoietic cells for 24 hr incubation from treatment of 200 μg/ml nanoparticles for 1 h. (D)The multipotential differentiative activity of hematopoietic stem or progenitor cells was investigated after reaction with SR nanoparticles for 1 h by colony forming ability assay.

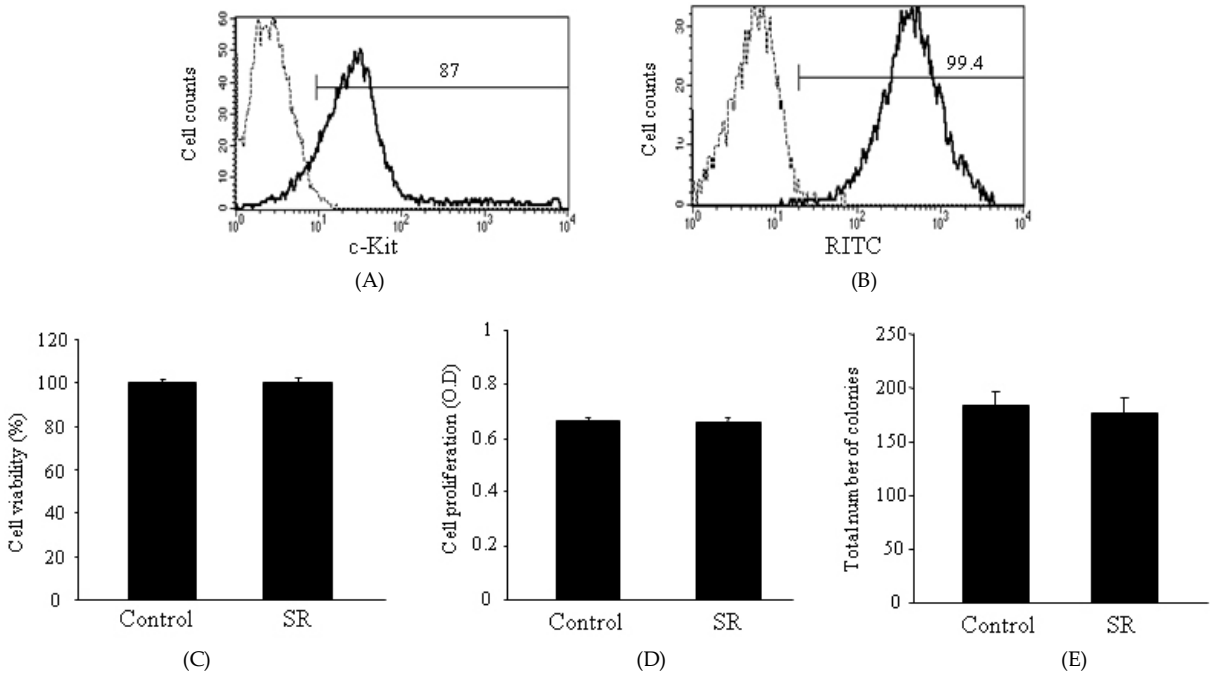


Fig. 4. Cellular uptake and biological effects of nanoparticles with c-Kit$^+$ bone marrow hematopoietic stem/progenitor cells. (A) The c-Kit$^+$ cells was purified with bone marrow and analyzed the purity using flow cytometry. (B) The cellular uptake efficiency of SR nanoparticles was determined by flow cytometry analysis after incubation with 200 μg/ml nanoparticles and 1×10^5 c-Kit$^+$ cells. The proliferation (C) and cytotoxic (D) effects was investigated by MTT assay after 24 h incubation from 1 h reaction with 200 μg/ml SR nanoparticles to purified 1×10^5 of c-Kit$^+$ cells. (E) The assay of colony forming ability was measured by reaction of 200 μg/ml nanoparticles with 1×10^5 c-Kit$^+$ cells for 1 h.

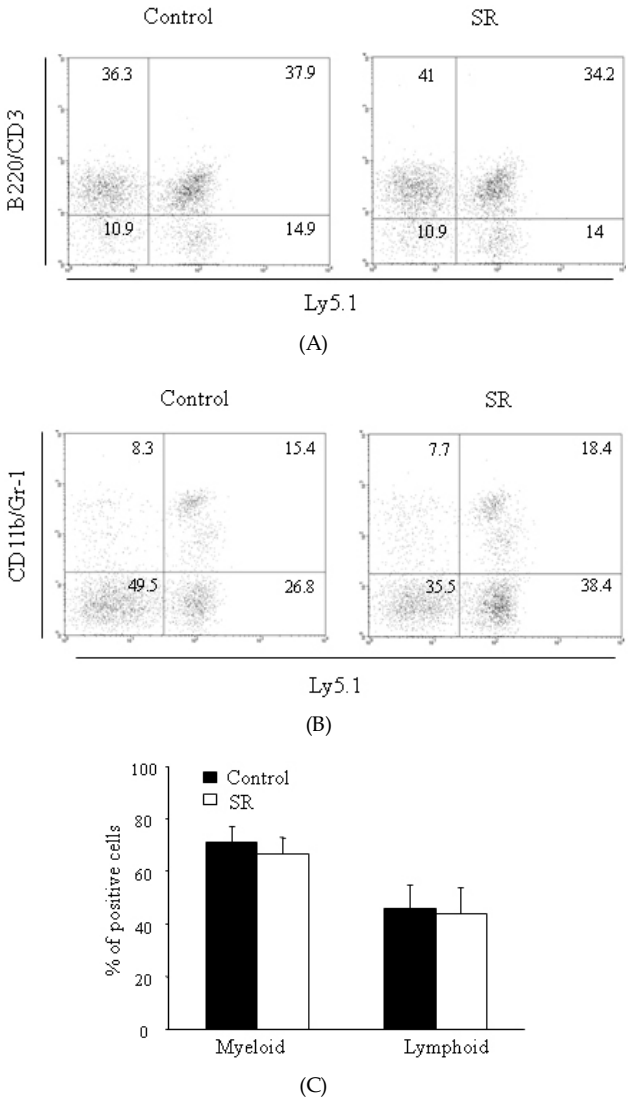


Fig. 5. *In vivo* hematopoietic reconstitution effects of nanoparticles uptaked c-Kit$^+$ cells. Purified 2×10^4 c-Kit$^+$ cells isolated from Ly5.1 mice bone marrow was treated with 200 μg/ml SR nanoparticles for 1 h, and then transplanted into lethal irradiated Ly5.2 recipient mice with 2×10^5 total bone marrow cells as rescue cells. After 5 weeks from injection, the engraftment efficiency was analyzed in peripheral blood by flow cytometry. The reconstitution ability of lymphoid (A) and myeloid (B) cells was determined and showed the representative flow cytometry data. (C) The percentage of engraftment efficiency of SR nanoparticles labeled LY5.1 positive donor cells was calculated.

C, D). The hematopoietic multipotent differentiation capacities of c-Kit$^+$ cells were showed no significant difference between SR and control cells (Fig. 4E).

In Vivo Hematopoietic Reconstitution Effects

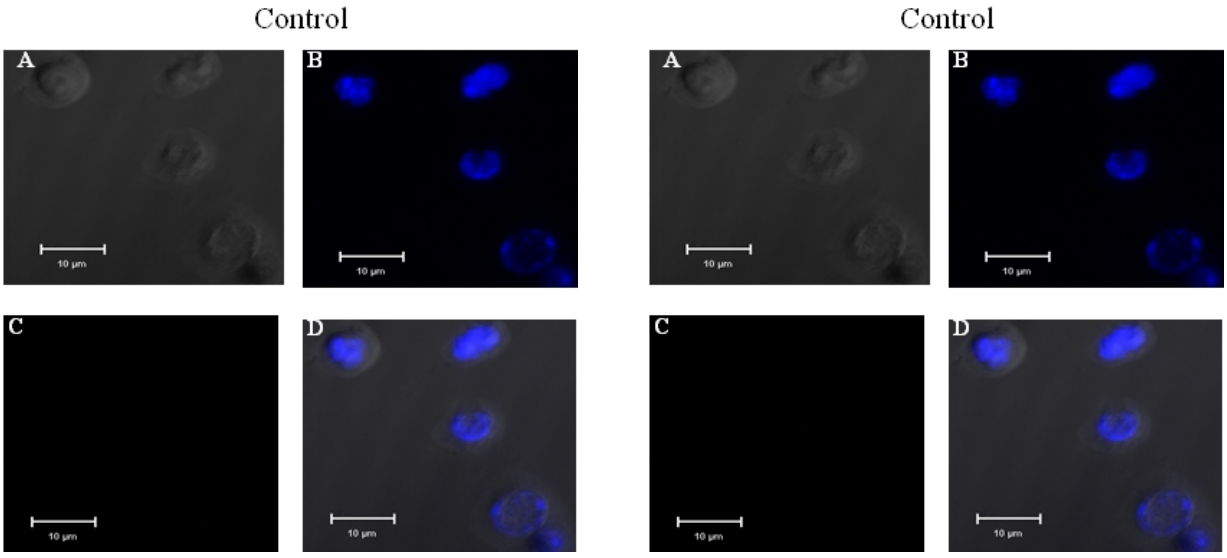
To confirm *in vivo* multipotential hematopoietic differentiation capacity with c-Kit$^+$ cells, purified c-Kit$^+$ cells (2×10^4 cells/mouse) with or without SR uptake for 1 h were injected into lethal irradiated Ly5.1 congenic mice with 2×10^5 total bone marrow cells from Ly5.2 mice as rescue cells. After 5 weeks from injection with c-Kit$^+$ cells, peripheral blood from mice was investigated to determine their multi-lineage differentiation capacity by flow cytometry. All of the mice studied showed similar engraftment efficiency between SR nanoparticles labeled cells injected mice and control mice. The engraftment of donor derived CD45$^+$ cells and the ratio of mature myeloid and lymphoid cells revealed no difference in SR uptake c-Kit$^+$ cells injected mice compared to control mice (Fig. 5). These results directly demonstrate that SR nanoparticles had no effect on the multipotential hematopoietic reconstitution ability of c-Kit$^+$ cells *in vivo*.

Intracellular Uptake into Hematopoietic Stem/Progenitor Cells

In usual, bone marrow derived hematopoietic stem/progenitor cells have little gene delivery efficiency and endocytic activity. To confirm the possibility of SR uptake in c-Kit$^+$ hematopoietic stem/progenitor cells, we conducted confocal laser scanning microscopy (CLSM) analysis. The c-Kit$^+$ cells also revealed the presence of SR nanoparticles in intracellular cytoplasmic regions of the cells (Fig. 6).

DISCUSSION

Recently, several nanoparticles have been developed for labeling of specific target cells, such as mesenchymal stem cells (MSCs), neural progenitor cells, embryonic stem cells, tumor cells and lymphocytes (Gupta *et al*., 2004; Lu *et al*., 2007). Compared with other organ stem cells, bone marrow derived hematopoietic stem cells (HSCs) showed a very low gene or drug incorporation efficiency, due to cellular quiescent states or the high multi-drug efflux out of the cell (Barrette *et al*., 2000). To overcome these problems, in this study, we tested the incorporation ability of SR using murine bone marrow hematopoietic cells and hematopoietic stem/progenitor cells, c-Kit$^+$, for successfully uptake. Without any modification, we confirmed that SR was effectively internalized into bone marrow hematopoietic cells with a dose- and time-dependant manner (Fig. 2). The SR internalization occurred over very short times, reaching 70% labeling within the first 30 min of treatment and achieving maximum labeling efficiency after 1 h of incubation. Confocal microscopic analysis showed that the labeling of nanoparticles in cells was derived from uptake into the cytoplasm by internalization, instead of labeling on the cell surface (Fig. 6). Moreover, we investigated the cellular uptake efficiency with various organs including hematopoietic or non-hematopoietic or-



(A) (B)

Fig. 6. Analysis of intracellular localization of nanoparticles in c- Kit+ cells. Purified c-Kit$^+$ cells was reacted with 200 μg/ml SR nanoparticles for 1 h and then analyzed intracellular uptake with confocal laser scanning microscope.

gans, and revealed that the bone morrow hematopoietic cells showed the most effective binding efficiency compared other organ, such as thymus, spleen, lymph node, liver, heart, kidney, and lung (data not shown). Collectively, this data suggests that SR could be a powerful candidate for biological applications in the labeling of bone marrow hematopoietic stem cells.

In addition, it was confirmed that internalization of SR did not affect cell cytotoxicity, proliferation, or multi-differentiation capacities of bone marrow hematopoietic cells (Fig. 3). Furthermore, in purified c-Kit$^+$ hematopoietic stem/progenitor cells, SR revealed over 97% labeling efficiency without adverse effects on cell viability, proliferation, or multilineage differentiation capacities (Fig. 4). This data implies that SR might be useful for applications involving drug or gene conjugation by labeling with hematopoietic stem/progenitor cells. In addition, the analysis of the mutipotential hematopoietic differentiation ability using *in vitro* CFC assay and the *in vivo* transplantation showed that SR uptake c-Kit$^+$ hematopoietic stem/progenitor cells maintained the ability to differentiation into multiple lineages mature hematopoietic cells (Fig. 5). In conclusion, our result suggests that SR may have great future potential for gene or drug delivery into bone marrow hematopoietic stem/progenitor cells.

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