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Effect of All-trans Retinoic Acid-loaded Liposomes on Adipogenesis of Obese Mice

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Abstract

Background: Among the many biological functions of all-*trans* retinoic acid (RA), its inhibitory effect on adipogenesis is receiving increased attention. Taking into account the passive accumulation of nanoparticles in adipose tissues, we prepared RA-loaded liposomes (RA-liposomes) and evaluated their effect on the adipogenesis of genetic and diet-induced mice model of obesity.
Methods: RA-liposomes were prepared by thin film hydration method. Leptin-deficient *ob/ob* mice and high fat diet-fed mice were intravenously injected with RA-liposomes and their body weight was measured. One day after the last RA treatment, the weights of major fat pad were compared. The droplet size of adipocyte stained by lipophilic BODIPY was also calculated from images of confocal microscopy.
Results: RA-liposomes had the average particle size of 140 nm, and more than 90% of RA was encapsulated in liposomes. The distribution of fluorescence-labeled RA-liposomes was observed more often in the adipose tissues of *ob/ob* mice and high fat diet-fed mice than in those of lean mice. This indicated RA-liposomes could passively accumulate in obese adipose tissues. Although intravenous injection of RA did not show any effect on adiposity of *ob/ob* mice, RA-liposomes moderately inhibited body weight gain of high fat diet-fed mice. The enlargement of lipid droplet of adipocyte in high fat diet-fed mice was also inhibited by the treatment of RA-liposomes.
Conclusion: We observed RA-liposomes have moderate inhibitory effect on adipogenesis of high fat diet-fed mice but not *ob/ob* mice, although RA-liposomes reached adipose tissues of both obese mice. Further development of drug delivery carrier will enhance the inhibitory effect of RA by the appropriate delivery of RA to adipose tissue.

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Introduction

All-trans retinoic acid (RA), an active metabolite of vitamin A, induces many biological functions by regulating gene expression, mainly through activation of retinoid receptors of the nuclear receptors. RA has been used for the treatment of acute promyelocytic leukemia [1] and has also been investigated to have anticancer effects on several types of cancers [2,3]. Among the many biological functions of RA, the role in the control of lipid and energy metabolism is receiving increased attention [4]. RA affects preadipocyte survival and adipocyte differentiation (adipogenesis), lipogenesis, thermogenesis, and lipolysis [4]. RA was reported to inhibit adipogenesis of preadipocyte clonal cell line [5,6] and reduce body weight and adiposity of mice [6,7].

Nanoparticulate drug delivery system has been developed for the enhanced bioavailability of chemotherapeutic agents by reducing systemic toxicity, controlled release, and drug transport to the pathological site. There is evidence that nanoparticles passively accumulate at tumor sites owing to the high permeability of the tumor neovasculature and lack of lymphatic drainage. This phenomenon is currently known as enhanced permeability and retention (EPR) effect [8,9]. Many RA-loaded nanoformulations including liposomes [10-12], polymeric micelles [13,14], and emulsions [15] were developed for the treatment of cancer. Incorporation of RA in these nanoparticles has shown to improve poor aqueous solubility, prevent fast degradation by light or heat, maintain serum concentration after injection, and enhance the accumulation in tumor tissue [15-17].

Obesity is developed by both the hypertrophy of pre-existing adipocytes and the hyperplasia of new adipocytes from precursor cells in white adipose tissue [18]. Adipogenesis is associated with angiogenesis of new blood vessel, and adipose tissue is highly vascularized [19,20]. Recently, it has been reported that long-circulating nanoparticles passively accumulate in obese fat owing to hyperpermeability of obese vasculature [21]. This finding indicated the successful drug delivery to obese adipose tissue using nano-sized carriers by a phenomenon similar to EPR effect in tumor tissue.

Considering the liposomes with long-circulating property would be able to accumulate in obese adipose tissues, the inhibitory effect of RA on adipogenesis might be enhanced by RA-loaded liposomes (RA-liposomes). In this study, we evaluated the effect of RA-liposomes on adipogenesis of genetic and diet-induced mice model of obesity.

Materials and Methods

Materials

Egg phosphatidylcholine (EPC, Coatsome NC-50) and methoxy-polyethylene glycol-distearoylphosphatidylethanolamine (PEG-DSPE, PEG mean molecular weight of 2000) were obtained from NOF

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Corporation (Tokyo, Japan). Cholesterol and all-*trans* retinoic acid (RA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). BODIPY® 558/568 C12 and isolectin GS-IB4 from *Griffonia simplicifolia* conjugated with Alexa Fluor® 488 were obtained from Molecular Probes (Life Technologies, Carlsbad, CA, USA). Other chemicals were of reagent grade.

Preparation and characterization of RA-liposomes

RA-loaded liposomes (RA-liposomes) were prepared using thin film hydration method followed by membrane extrusion. Briefly, EPC, cholesterol, PEG-DSPE, and RA (70/30/4/8, molar ratio) were dissolved in chloroform. The chloroform was removed using a rotary vacuum evaporator, and the thin film was hydrated with phosphate buffered saline (8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl and 2.68 mM KCl, PBS, pH 7.4). RA-liposomes were obtained after the suspensions were extruded through polycarbonate membrane (Nuclepore™, GE Healthcare UK, England) with pore sizes of 0.4 and 0.2 μm. Fluorescence-labeled RA-liposomes were prepared by the addition of 0.4mol% of CellVue® Claret (Sigma-Aldrich, St. Louis, MO, USA) to the RA-loaded liposomes.

The particle size and ς-potential of RA-liposomes were determined using a dynamic and electrophoretic light scattering instrument (ELSZ-2, Otsuka Electronics, Co. Ltd., Japan) at 25°C after dilution with an appropriate volume of Milli-Q water. To calculate the entrapment efficiency of RA, RA-liposomes was passed over the Sephadex G-50 column, and RA concentration in liposome fraction after solubilization with methanol was measured by a high-performance liquid chromatography (HPLC) method [16]. The HPLC system was composed of an LC-10AS pump (Shimadzu Co., Ltd., Kyoto, Japan), a SIL-10A auto injector (Shimadzu Co.), an UV detector (350 nm, Shimadzu Co.), and a YMC-Pack Pro C18, 150×4.6 mm I.D. column (YMC Co., Ltd., Kyoto, Japan). The mobile phase was a mixture of acetonitrile, water, and acetic acid (90:9.68:0.32, v/v/v) with a flow rate of 1.0 mL/min.

RA release from liposomes was examined by the dialysis method. RA-liposomes were placed in a dialysis tube (Spectra/Por RC membrane, molecular weight cutoff of 3500) and immersed in PBS (pH 7.4) at 37°C and shielded from light. The content of RA in the release medium was determined by HPLC method.

Animals

The animal experiments were conducted under ethical approval from the Committee on Animal Research and Ethics. Five-week-old male B6.Cg-*Lep*^{ob}/J mice (*ob/ob* mice) were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and fed standard rodent pellet diet. Four-week-old male C57BL/6J mice were obtained from Sankyo Labo Service Corp. (Tokyo, Japan) and fed a high fat diet (HFD) in which 60% of calories are derived from fat (D12492; Research Diets, Inc., New Brunswick, NJ, USA) or standard rodent pellet diet. Food and water were provided *ad libitum*.

Microscopic observation of mouse fat pad and distribution of RA-liposomes

The observation of adipose tissue was performed as reported previously [20], with some modifications. To confirm the distribution of liposome in adipose tissues, mice were intravenously administered RA-liposomes labeled with CellVue® Claret at a lipid dose of 0.1 mmol/kg. Five hours after injection, mice were killed by cervical dislocation. Epididymal fat, inguinal subcutaneous fat, and mesenteric fat were removed and minced into small pieces using a scalpel. Tissue pieces were washed with PBS and stained for 1 hour with 5 μM BODIPY 558/568 C12 to visualize fat droplet of adipocyte and for 1 hour with 20 μg/mL GS-IB4 isolectin conjugated with AlexaFluor 488 to visualize vasculature. Low-power field images were acquired by confocal laser scanning microscope (FV1200, Olympus Corp. Tokyo, Japan).

RA Treatment for obese mice

The effect of RA-liposomes on obesity was evaluated in *ob/ob* mice that gained weight progressively on standard rodent pellet diet and C57BL/6J mice that were fed HFD. *Ob/ob* mice (7 weeks old) were divided into three groups, namely, physiological saline, RA solution, and RA-liposomes. For the treatment with RA solution, RA was dissolved in 10% Cremophor EL aqueous solution. RA solution and RA-liposomes were administered intravenously at 3 mg RA/kg/day at 2- or 3-day interval.

C57BL/6J mice were divided into four groups. One group fed standard rodent pellet diet and other three groups, namely, physiological saline, RA solution, and RA-liposomes, fed HFD for the duration of the study. After one week of feeding HFD, RA solution or RA liposomes were administered intravenously at 3 mg RA/kg/day at 2- or 3-day interval.

Body weights were measured on the day of treatment. One day following the last treatment, epididymal fat, inguinal subcutaneous fat, and mesenteric fat were removed and weighed. To measure the size of adipose droplet, fat tissues were stained for 1 hour with 5 μM BODIPY 558/568 C12 and observed by confocal laser scanning microscope. BODIPY positive areas were calculated using ImageJ software (NIH, Bethesda, MD, USA). Thirty cells in each image were measured.

Results and discussion

Preparation and evaluation of RA-liposomes

Stealth RA-liposomes were prepared by incorporating PEG-DSPE to liposome formulation because the PEG-modified stealth liposomes had been characterized for prolonged circulation and accumulation in tumor via EPR effect [22, 23]. RA-liposomes had the average particle size of 140.5 nm and the ς-potential of −1.4 mV. More than 90% of RA was encapsulated in liposomes. The release of RA from liposomes was less than 5% after 24 hours of incubation at pH7.4 (data not shown). These results corresponded well with the reports that hydrophobic RA was highly incorporated and stably retained in liposomes [11, 12]. We obtained stealth RA-liposomes with high RA loading and retention.

Distribution of RA-liposomes in mice adipose tissue

To confirm the RA delivery to fat pad by liposomes, the distribution of fluorescence-labeled RA-liposomes was evaluated in *ob/ob* mice, C57BL/6J mice pre-exposed with HFD for 3 weeks, and C57BL/6J lean mice (Figure 1). The area of droplets stained by lipophilic BODIPY was larger in *ob/ob* mice and HFD-fed mice than in lean mice. Capillaries stained by lectin were running around the adipocytes.

The fluorescence of liposomes was observed more in *ob/ob* and HFD-fed mice than in lean mice and were found to be overlapped with the signal of vessels. The stealth liposomes were reported to accumulate more preferentially in the fat pad of obese mice than normal mice [21]. Therefore, these results indicated that RA-liposomes could passively accumulate in obese adipose tissues and deliver RA to adipocytes.

Effect of RA-liposomes on obese mice

To examine whether RA-liposome inhibits adipogenesis in vivo, *ob/ob* mice were treated with RA (3 mg/kg/day) and body weight changes were observed (Figure 2A). In addition, the major fat pads were excised a day after the last treatment and their weights were compared (Figure 2B). However, there were no differences in body weight between saline-injected control mice and RA solution or RA-

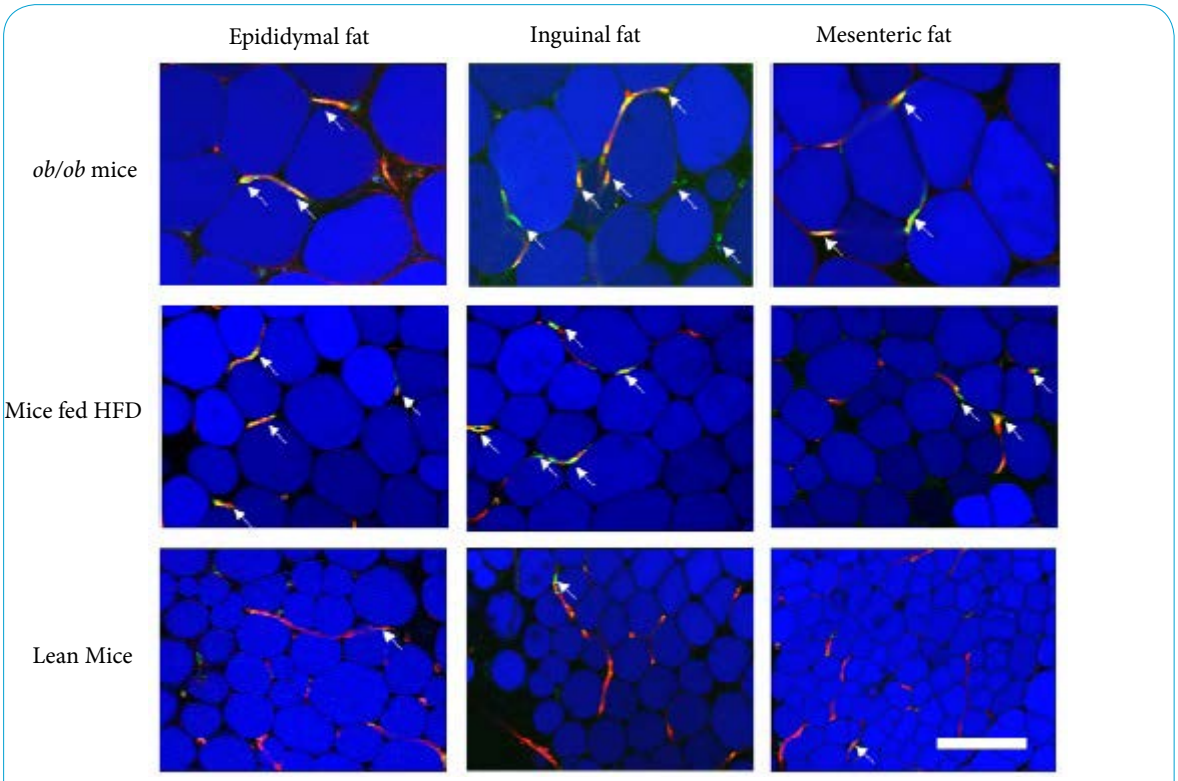

Figure 1: Distribution of fluorescence-labeled RA-liposomes in mice fat pad. Five hours after intravenous injection of fluorescence-labeled RA-liposomes, excised fat pads were stained with BODIPY (blue) to visualize fat droplet and lectin (red) to visualize endothelial cells. Arrows indicate distribution of fluorescence-labeled liposomes (green) or those colocalized with endothelial cells (yellow). bar = 100 μm

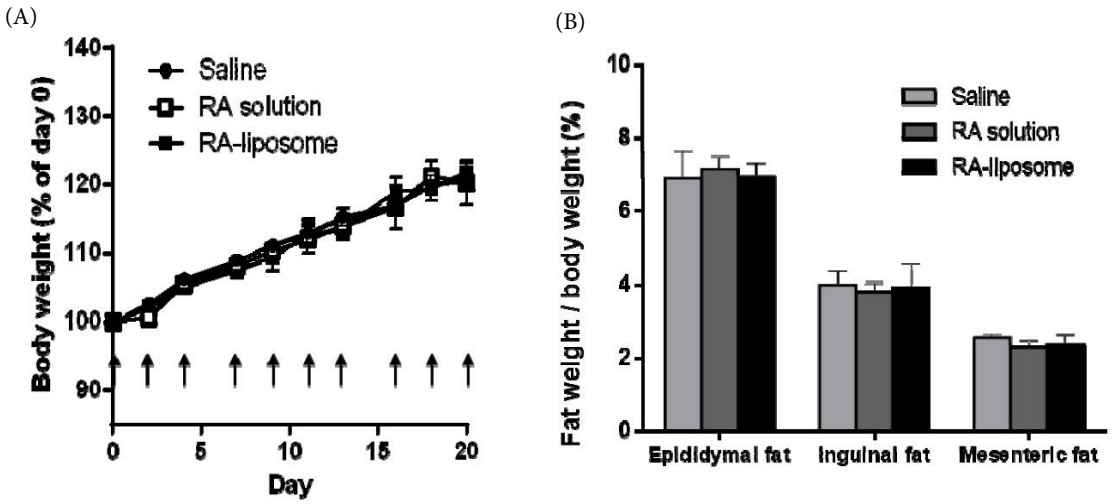

Figure 2 Effect of RA-liposomes on *ob/ob* mice
(A) Body weight curves of male *ob/ob* mice injected with saline (closed circle), RA solution (open square), or RA-liposome (closed square). Arrows indicate the day of drug injection. Each value represents the mean ± S.D. (n=3).
(B) Percentage of fat weight against body weight of *ob/ob* mice one day after the last injection of saline, RA solution and RA-liposome. Each value represents the mean ± S.D. (n=3).

liposome injected mice. These results indicate the intravenously injected RA solution and RA-liposomes did not show any inhibitory effect on adipogenesis of *ob/ob* mice. *Ob/ob* mice are known to show progressive weight gain on standard rodent pellet diet. At the time of starting RA treatment (7 weeks old), *ob/ob* mice developed excess body weight of around 40g, which was approximately 1.8 times higher than that of age- and sex-matched C57BL/6J mice. Therefore we concluded that the effect of RA on large adipose tissue was not fully induced by intravenous injection of RA in both solution and liposomal form.

From the studies in preadipocyte 3T3-L1 cells, RA is known as a potent inhibitor of differentiation of preadipocyte to mature adipocyte when applied at relatively high doses (0.1-10 μM) and at early stages of adipogenesis [5]. However, it is reported that RA promotes adipogenesis when applied at low doses (1pM to 10 nM range) [24]. It is suggested that the amount of RA in adipose tissues was not enough to inhibit adipogenesis, even after the preferential accumulation of RA-liposomes in adipose tissue of *ob/ob* mice (Figure 1).

Further experiments were performed on the HFD-fed mice to evaluate the effect of RA-liposomes on adipogenesis (Figure 3). After being pre-exposed to HFD for one week, mice were treated with RA (3 mg/kg/day) at 2- or 3-day interval. Continuous weight gain by HFD-fed mice was relatively suppressed by RA-liposome treatment compared to RA solution treatment (Figure 3A). However, the weight of major fat pad was not significantly different (Figure 3B). Suppressed body weight gain by RA-liposome treatment was not induced by the inhibition of weight gain in the fat pads. To determine whether RA-liposomes have an effect on adipocyte, the size of fat droplets was calculated from the confocal microscopic images of fat pad stained with lipophilic BODIPY (Figure 4A and Figure 4B). Surprisingly, the fat droplet size in RA-liposome treated mice was relatively smaller than that in untreated and RA solution treated mice. These results indicate that RA-liposomes may have inhibitory effect on hypertrophy of pre-existing adipocytes in HFD-fed mice.

Although it is not clear why the size of adipocyte did not reflect in the weight of the fat pad, the liposomal formulation of RA did prevent

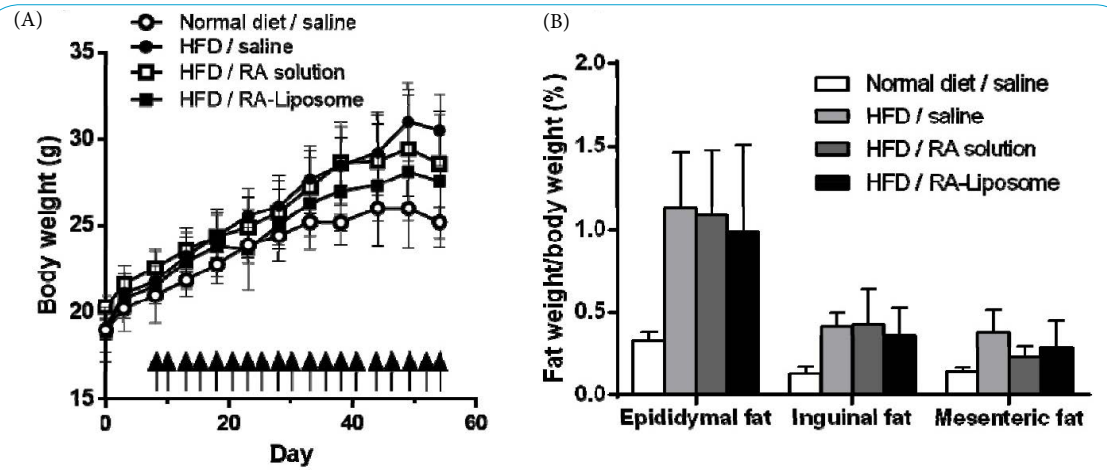

Figure 3 Effect of RA-liposomes on the high fat diet (HFD)-fed mice.
(A) Body weight curves of mice fed with normal diet (open circle) or HFD injected with saline (closed circle), RA solution (open square), or RA-liposome (closed square). C57BL/6J mice were fed HFD from Day 0 and throughout the study period. Arrows indicate the day of drug injection. Each value represents the mean ± S.D. (n=3-6).
(B) Percentage of fat weight against body weight of HFD-fed mice one day after the last injection of saline, RA solution and RA-liposome. Each value represents the mean ± S.D. (n=3-6).

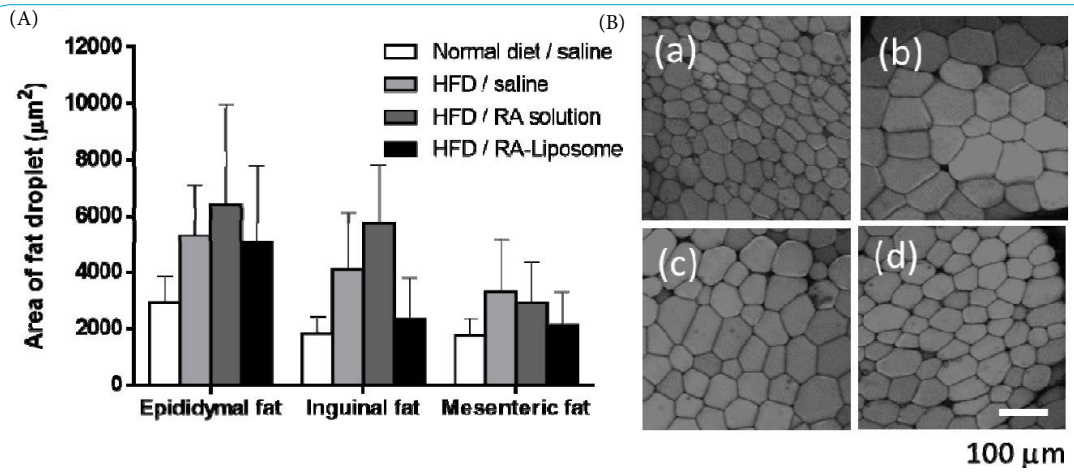

Figure 4 Fat droplet size of fat pads after the RA treatment to HFD-fed mice.
(A) Average area of fat droplet calculated from three images of each animal. Each value represents the mean ± S.D. (n=180 cells).
(B) Representative image of fat droplet stained with BODIPY of mesenteric fat from mice fed with normal diet (a) or HFD injected with saline (b), RA solution (c), and RA-liposome (d).

diet-induced weight gain more than RA solution. RA has been shown to reduce the adiposity by depression of adipogenic/lipogenic potential of adipose tissues via depression of peroxisome proliferator activated receptor γ levels and increment of thermogenic potential in brown adipose tissue and muscle with increased expression of uncoupling proteins [25]. The suppression of body weight gain and hypertrophy of adipocyte by RA-liposomes might be, in part, a result of enhanced energy expenditure in adipocyte and muscle of HFD-fed mice.

Conclusions

We evaluated the effect of RA-liposomes on adipogenesis of obese mice. Although intravenous injection of RA did not show any effect on adiposity of *ob/ob* mice, RA-liposomes moderately inhibited body weight gain of HFD-fed mice. Further development of drug delivery carrier will enhance the inhibitory effect of RA by the appropriate delivery of RA to the adipose tissue.

Competing Interests

The authors declare that they have no competing interests in this work.

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