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Report

Anti-Obesity Dendritic Cell Vaccine Therapy Targeting Neovascular Vessels in High-Fat-Diet-Fed Mice

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Obesity is a severe disease that causes diabetes, fatty liver, hypertension, and arteriosclerosis. It involves adipocytokines such as tumor necrosis factor- α , leptin, and resistin. The hypertrophic adipocytes induce the secretion of these adipocytokines. Regulating the growth of adipose tissue is an extremely important approach to prevent these diseases. Hypertrophy of adipose tissue is closely associated with angiogenesis, wherein neovascular vessels supply nutrients. Previous studies have shown that angiogenesis inhibitors can prevent the development of obesity in mice. In this study, we report an anti-obesity therapy targeting the neovascular vessels of adipose tissue via endothelial cell (EC) vaccination. EC vaccine was prepared using EC extract antigen, which was pulsed to dendritic cells, and was administered thrice intradermally. The anti-obesity effect was evaluated in high-fat diet-fed obesity model mice. EC vaccination significantly suppressed the increase in body weight and hyperplasia of adipocytes and improved the accumulation of hepatic lipid droplets in these mice. Adipose tissueinduced vascular-targeted vaccine therapy is a potential novel approach for obesity.

Key words obesity, vaccine, neovascular, endothelial cell, dendritic cell

INTRODUCTION

Obesity is a major cause of metabolic syndromes and predisposes an individual to stroke and heart disease. Hence, it is desirable to control obesity in the long-term. Obesity is caused by daily life activities, such as overfeeding, lack of exercise, poor sleep, and stress.

The hypertrophy of adipocytes significantly impacts the quantities of various adipokines produced by adipose tissues,¹⁻⁴⁾ and is involved in metabolic syndromes.^{5,6)} Insulin resistance is induced by several adipokines, such as tumor necrosis factor- α and resistin, which are associated with an increased risk of developing metabolic syndromes. Therefore, suppressing adipocyte hypertrophy is vital to alleviate metabolic syndromes.

Obesity is associated with the hypertrophy of adipocytes, which is further related to angiogenesis. Experiments conducted using angiogenesis inhibitors demonstrated the suppression of body weight reduction and adipose tissue loss in obese mice.^{7,8)} In adult humans, angiogenesis is only induced in limited areas such as the corpus luteum and wound healing sites. Therefore, neovascular vessel-targeted therapies may exert limited side effects and are attractive approaches for the prevention of adipocyte hypertrophy.

Vaccine therapy induces the production of antigen-specific antibodies and/or cytotoxic T lymphocytes. Several vaccines

can provide long-term antigen-specific immunity. Immune adjuvants and antigen-presenting cells are used as immunostimulators in vaccine therapy. Dendritic cells (DCs) are antigen-presenting cells often used in cancer vaccine therapy. In tumor vaccine therapy, DCs have been used to activate the immune system with antigens.^{9,10} Treating cancer-bearing mice with DC immunotherapy using endothelial cells (ECs) as antigens exerts an antitumor effect.¹¹

We conducted this study to investigate a novel approach for treating obesity using a DC vaccine with ECs as antigens in high-fat diet-fed mice.

MATERIALS AND METHODS

Obesity Model Male C57BL/6J mice (Charles River Japan, Kanagawa, Japan) aged 5 weeks were fed with High-Fat Diet 32 (HFD32) (CLEA JAPAN, Tokyo, Japan) and were used as obesity models. HFD32 was replaced thrice a week, and the mice body weight was measured for 70 days. All experimental procedures were performed in accordance with the Teikyo University guidelines for the welfare of animals.

EC Culture Human umbilical vein ECs (HUVECs) (Kurabo, Osaka, Japan) were seeded at 5×10^3 cells/cm² onto a tissue culture dish and were cultured in a 1:1 mixture of RPMI 1640 and Medium 199, supplemented with 15% fetal bovine serum (Biowest, Nuaille, France), 100 U/mL penicillin, 100 µg/mL streptomycin, and 100 μ g/mL EC growth supplement (Biomedical Technologies, Stoughton, USA) at 37°C in an atmosphere of 5% CO₂. ECs obtained from the fourth to sixth passages were used for the experiment.

DC Preparation DCs were prepared from bone marrow cells as described previously.^{12,13} Briefly, bone marrow cells were isolated from C57BL/6 mice and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 µg/mL streptomycin, 50 µM 2-mercaptethanol and 20 µg/mL mouse granulocyte-macrophage colony-stimulating factor. After 8–16 days of culture, nonadherent cells were collected and used as DCs.

Preparation of Antigen Antigens were extracted from HUVECs using the butanol extraction method.¹⁴⁾ HUVECs were washed twice with phosphate-buffered saline (PBS) and were then incubated with PBS containing 2.5% (v/v) 1-butanol. The solution was collected and centrifuged twice at 1600 × g and 4°C for 20 min. The resulting supernatant was dialyzed with water using a Spectra/Por Dialysis Membrane (MWCO: 10,000; Spectrum Laboratories, CA, USA). The dialysate was further centrifuged at 1600 × g and 4°C, and the resulting supernatant was freeze–dried.

Vaccination DC vaccine was prepared as described previously.¹⁵⁾ Briefly, to prepare a lipoplex, 720 µg of freeze–dried antigen was rehydrated with 1 mL of Opti-MEM and was incubated with 100 µL lipofectin (Thermo Fisher Scientific KK, Tokyo, Japan) at room temperature for 20 min. DCs (1×10^6 cells) were pulsed with the lipoplex in a CO₂ incubator for 5 h. The cells were washed with PBS, and the DCs (1×10^6 cells/100 µL) were injected intradermally into the back of each C57BL/6 mouse thrice at weekly from day 1 of HFD32 feeding.

Measurement of Blood Glucose, Aspartate Aminotransferase (AST), and Alanine Aminotransferase (ALT) Levels Mice were fasted for 20 h on the 70th day of HFD32 feeding, and whole blood was collected under anesthesia. The collected blood was left undisturbed at room temperature for 30 min to allow the formation a blood clot. The blood clot was removed via centrifugation at $1500 \times g$ for 10 min, and the serum was collected. Blood glucose levels were measured using a glucose assay kit via the glucose oxidase method (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). AST and ALT were measured using a transaminase assay kit by the pyruvate oxidase method (FUJIFILM Wako Pure Chemical Corp.).

Histopathological Observation The mice epididymal adipose tissue and liver were removed 10 weeks after initiating HFD32 feeding and were fixed with 10% formalin neutral buffer solution. The fixed tissues were embedded in paraffin, cut into 4-µm thick sections, and deparaffinized. The sections were stained with hematoxylin and eosin (HE; Kureha Special Laboratory, Fukushima, Japan).

Calculation of Adipocyte Average Area HE sections of epididymal adipose tissues were observed under a microscope (magnification: 400 ×). The microscopic images were printed, and the number of adipocytes in a 500- μ m square area was counted. The adipocytes present on or touching the top and left lines were counted, but those on or touching the right or bottom lines were not considered. The average area of adipocytes was calculated by dividing 250,000 μ m² by the number of adipocytes counted.

Statistical Analysis Data were analyzed using Tukey's test. The level of statistical significance was set at p < 0.05.

RESULTS

Anti-Obesity Effect of EC-Targeting DC Vaccine The HFD32-fed mice vaccinated with the EC vaccine showed a slower increase in body weight than the nontreated HFD32fed mice. This demonstrated a significant suppression of the increase in mice body weight 10 weeks after the initiation of HFD32 feeding. In contrast, the DC vaccine-treated group without antigen pulse showed almost no weight suppression (Fig. 1A). Additionally, the appearance of the EC-vaccinated mice was almost similar to that of the normal diet-fed mice (Fig. 1B). Moreover, the EC vaccine suppressed epididymal fat tissue growth (Fig. 2B). The EC-targeting vaccine significantly suppressed adipose tissue growth; furthermore, to verify whether it could affect the size of adipose cells, HE sections of adipose tissues were prepared for the HUVEC/DC group and observed under a microscope. The hyperplasia of adipocytes was suppressed by the EC-targeting vaccine, and the average area of adipocytes in the HUVEC/DC group was the same as that in the normal diet (ND) group (Fig. 2A and 2C). The HUVEC/DC group showed no statistically significant difference in blood glucose level compared with the ND group but showed a tendency to improve to the same blood glucose



Fig. 1. Suppression of Body Weight via Endothelial Cell (EC) Vaccination (A)

Body weight was measured thrice a week for 70 days, and the average weekly body weight was plotted in a graph. Mice were vaccinated thrice weekly. (B) The body size at 70 days after the initiation of HFD32 feeding. Non/dendritic cell (DC) (\diamond); DC vaccination without antigen group; human umbilical vein endothelial cells (HUVEC)/DC (\blacksquare); DC vaccination with HUVEC antigen group; high-fat diet (HFD) (*); unvaccinated group; normal diet (ND) group (\times). Data are expressed as mean \pm SD. **p < 0.01 compared with HFD group (n = 8).



Fig. 2. Suppression of Adipocyte Size via Endothelial Cell (EC) Vaccination

(A) Epididymal fat pads were observed using hematoxylin and cosin (HE) staining (magnification: $100 \times$). Scale bar = $200 \ \mu m$. (B) The weights of the epididymal fat pads on both sides were measured (n = 8, each group). (C) The number of adipocytes was counted on HE-stained sections in an area of $250,000 \ \mu m^2$. The average adipocyte area was calculated (n = 4, each group). (D) Mouse serum blood glucose levels were measured (n = 7–8, each group). Data are expressed as mean \pm SD. **p < 0.01.



Fig. 3. Suppression of Lipid Droplet Accumulation in the Liver via Endothelial Cell (EC) Vaccination

(A) Accumulation of lipid droplets in the liver was observed via hematoxylin and eosin (HE) staining (magnification: $100 \times$). Scale bar = 100μ m. (B) Mouse serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured. Data are expressed as mean \pm SD. (n = 7–8, each group).

level as the ND group (Fig. 2D).

Dietary Fatty Liver Suppression Effect of EC-Targeting DC Vaccine Feeding mice with HFD32 did not significantly affect liver weight (Fig. 3B). The HE-stained liver sections of in the HFD group showed an accumulation of lipid droplets, whereas those of HUVEC/DC group showed almost no accumulation of lipid droplets (Fig. 3A). The level of liver damage markers AST and ALT showed no statistically significant differences between the groups. However, although these levels tended to increase in the HFD group, those in the HUVEC/DC group were suppressed to almost the same level as in the ND

group (Fig. 3C).

DISCUSSION

HUVECs were used as a model for neovascular ECs. The DC vaccine pulsed with EC antigen showed a significant antiobesity effect in HFD32-fed mice. However, to induce immunity in mouse vascular ECs, it is generally necessary to use mouse vascular ECs as antigens. Wei *et al.* reported the induction of immunity in the neovascular vessels of tumors in tumor-bearing mice model using HUVECs as an antigen; further, they reported that the HUVEC antigen and the mouse EC antigen crossed over.¹¹) It is also known that proteins showing high homology are expressed in human and mouse ECs.¹⁶) Therefore, in the present study as well, a crossover was observed between the HUVEC antigen and the neovascular EC antigen of adipose tissue. Immunity was induced in the neovascular vessels of the adipose tissue.

In this study, we did not measure food intake. HFD32, a short-lived feed, was changed thrice a week, and the amount of food consumed was almost constant regardless of vaccination. It remains unclear why vaccination reduced body weight and adipose tissue weight, despite the constant intake of high-fat diet. The destination of the surplus energy ingested is uncertain. One hypothesis is that the surplus energy is used continuously to induce immunity to these neovascular vessels. To verify this hypothesis, future studies must evaluate the amount of food intake, the amount of feces, the amount of exercise, and the amount of basal metabolism using mass analysis measurement of oxygen–carbon dioxide in exhaled breath.

We observed that the vaccine therapy did not decrease body weight but suppressed the increase in body weight. We speculated that the immunity induced by the EC vaccine affects only the neovascular vessels induced during the growth of the adipose tissue, resulting in the suppression of in increase in body weight. Adipocytes are essential cells in the body and are thus not suitable targets. Nevertheless, if the EC vaccine targets only new blood vessels, it may only suppress hypertrophy in adipocytes during energy overdose and rebound. Therefore, EC vaccines may represent a suitable approach for the long-term control of obesity. However, Nomura et al.17) reported that the use of ECs isolated from solid tumors of mice as an antigen in DC vaccine therapy, which targets neovascular vessels in cancer, show a high antitumor effect. Furthermore, it has been reported that the DC vaccine targeting neovascular vessels does not affect the time required for wound healing. However, the DC vaccine developed using cultured mouse ECs as an antigen delayed wound healing. Therefore, the HUVEC/DC vaccine used in this study may inhibit physiological angiogenesis. In future research, we will investigate the development of a vaccine that enables long-term control of obesity without affecting physiological angiogenesis using ECs isolated from visceral fat of obese mice as an antigen.

Clarifying the specificity of a vaccine targeting adipose tissue neovascularization toward target cells, its effect on physiological angiogenesis, and its anti-obesity mechanism can help indeveloping treatment for obesity.

Conflict of interest The authors declare no conflict of interest.

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