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In vivo imaging of obesity-induced inflammation in adipose tissue

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ABSTRACT

Obesity is associated with low-grade inflammation in adipose tissue, which contributes to the development of obesity-related diseases such as insulin resistance, hypertension and arteriosclerosis. Here we developed an animal model that non-invasively monitors inflammation in adipose tissue using *in vivo* bioluminescent imaging (BLI) technique. *In vitro*, stimulation with TNFα or co-culture with RAW264 macrophages increased bioluminescence in 3T3-L1 adipocytes expressing NF-κB-mediated luciferase gene (3T3-L1/NF-κB-re-luc2P). *In vivo*, lipopolysaccharide increased bioluminescence in mice transplanted with 3T3-L1/NF-κB-re-luc2P cells. Moreover, light emission derived from implanted cells was significantly higher in diet-induced obese mice transplanted with 3T3-L1/NF-κB-re-luc2P than in lean mice. Our results showed that BLI technique and 3T3-L1/NF-κB-re-luc2P cells provide a useful approach to non-invasively monitor obesity-induced inflammation in adipose tissue in *in vivo*.

Introduction

Obesity has recently become a matter of great concern, because it is considered as one of the highest risk factors for insulin resistance, type 2 diabetes mellitus, hypertension and arteriosclerosis [1,2]. In obese patients and animal models, secretion of adipocyte-derived hormones is dysregulated. Moreover, evidence has accumulated that obesity is associated with a state of chronic, low-grade inflammation in adipose tissue characterized with infiltrated macrophages [3]. Adipocytes as well as accumulated macrophages release inflammatory cytokines such as tumor necrosis factor-α (TNFα), chemokine (C–C motif) ligand 2 (CCL2) known as monocyte chemoattractant protein-1 (MCP-1), and interleukin-6 (IL-6) [4]. Since inflammation is a hallmark of arteriosclerosis and insulin resistance, it has been now recognized that inflammatory changes within adipose tissue may critically contribute to the development of many aspects of obesity-related diseases [5–10].

It is well established that nuclear factor-kappa B (NF-κB) is a key regulator in several inflammatory responses. Various stimuli from extracellular environment activate NF-κB, including pro-inflammatory cytokines such as CCL-2 and IL-6, bacteria and virus infections, endotoxin and several stresses [11]. It has been also demonstrated that in obese adipoise tissues, inflammation stimuli derived from adipocytes themselves and infiltrated macrophages activate NF-κB [12]. As the result of NF-κB activation, production of pro-inflammatory cytokines from adipocytes and proliferation of adipocytes is further enhanced [13]. Moreover, inhibition of NF-κB signaling by salicyrates improved obesity-induced inflammation and insulin resistance in mouse models, implicating that NF-κB pathways are activated [14].Therefore, the monitoring of the NF-κB activity in obese adipose tissues may be useful for the evaluation of obesity-associated diseases.

Imaging technology has been playing a large role in *in vivo* real-time and non-invasive monitoring of disease diagnoses and/or evaluation of the efficacy of therapeutic approaches [15]. Optical imaging modalities are recently developed and widely spread in several research areas [16–21]. Among them, bioluminescence imaging (BLI) has been applied to quantitative monitoring with advantages in high-sensitivity and ease to use [22].

In this study, we developed an animal model to evaluate and monitor obesity-induced chronic, low-grade inflammation in adipose tissue through NF-κB activation by combining implantation of 3T3-L1 pre-adipocytes and *in vivo* BLI technique.

Materials and methods

Materials. Tumor necrosis factor-α (TNF-α) from mice, lipopolysaccharides (LPS) from Escherichia coli 0128:B12, insulin, and dexamethazone, 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma–Aldrich (St. Louis, MO). pGL4.32 [luc2P/NF-κB-RE/Hygro] vector and Steady-Glo® Luciferase Assay System were obtained from Promega (Madison, WI).

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Animals. Male C.B.17/scid mice (5 weeks old) were purchased from CLEA Japan, (Tokyo, Japan). All mice were maintained under conditions of controlled temperature (23 ± 2 °C) and illumination (07:00–19:00). Water and food (CE-2, CLEA Japan) were available *ad libitum* unless otherwise noted. 13-week-old mice were divided into two groups and given a high fat diet (D12492; Research Diets Inc, New Brunswick, NJ) to one group for 2 months to obtain diet-induced obesity (DIO) mice. All animal procedures complied with National Institutes of Health guidelines and were approved by Banyu Animal Care and Usage Committee.

Cells. 3T3-L1 pre-adipocytes were maintained and cultured in culture medium (high-glucose DMEM supplemented with 10% bovine serum and penicillin/streptomycin) at 37 °C in 5% CO_2 atmosphere. Three days after seeding 3T3-L1 cells into 96-well plates at 10,000 cells, differentiation was induced by changing the culture media to medium containing insulin (10 μg/mL)/IBMX (0.2 mM)/dexamethazone (1 μM) for two days, and then switching medium containing insulin (10 μg/mL) alone. Three days after changing media, cells were cultured with the culture medium for five days. To establish constitutively luciferase expressing 3T3-L1cells, cultured cells were infected with LV-luc at a moi of 500 for overnight in a volume of 500 μL as described before [23].To establish NF-κB-mediated luciferase gene expressing 3T3-L1 cells, pGL4.32 vector was transfected into 3T3-L1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were selected with Hygromycin B (500 μg/mL) and subcloned. RAW264 cells were cultured in RPMI-1640 medium supplemented with 10% bovine fetal serum and penicillin/streptomycin at 37 °C in 5% CO_2 atmosphere. For co-culture system, 3T3-L1/NF-κB-re-luc2P cells were harvested with 0.05% of trypsin containing with 0.1 mM EDTA (Invitrogen), washed with PBS, and resuspended in the culture medium. Cell suspension of RAW264 cells was added into differentiated 3T3-L1 cells in 96 well plates and cultured at 37 °C in 5% CO_2 atmosphere.

Luciferase assay. To evaluate luciferase activity *in vitro*, differentiated 3T3-L1 cells were treated with TNF-α or macrophage and the luciferase activity was measured with Wallac 1420 ARVO-sx (PerkinElmer Life Sciences, Boston, MA) using Steady-Glo® Luciferase Assay System according to the manufacturer's protocol.

Cell implantation in epididymal white adipose tissue (eWAT). 3T3-L1/NF-κB-re-luc2P cells were harvested with 0.05% of trypsin containing with 0.1 mM EDTA (Invitrogen), washed with PBS, and resuspended (2×10^7 cells/mL) in PBS. C.B.17/scid mice were anesthetized with isoflurane (2–3%) and were made a small incision at center abdomen. Exposing right epididymal white adipose tissue (eWAT), 50 μL of cell suspension were directly injected into the eWAT with a 27 gauge needle attached with 1 ml of syringe (Terumo, Japan). The incision was closed with a surgical skin clip or a 4–0 silk and 3 mg cefamezine® (cephalosporin) was given subcutaneously (Astellas, Japan).

Bioluminescent imaging. Bioluminescent imaging was performed as previously reported [23]. Briefly, prior to acquiring images, 15 mg/kg of D-luciferin (Xenogen Corporation, Hopkinton, MA) was administered by intra peritoneal injection to all animals. Ten minutes after administration of substrate, bioluminescent imaging was measured using the IVIS-200 System (Xenogen Corporation, Hopkinton, MA). To induce NF-κB activity *in vivo*, LPS (20 mg/kg) was administrated by intra peritoneal injection. For each mouse, all values were determined as photons per sec (photon/s). Relative NF-κB activity in DIO mice was determined by measuring light emission in DIO mice divided by that in lean mice.

Quantitative RT-PCR. Left eWAT was isolated and stored at −80 °C until RNA isolation. Total RNA from eWAT tissue was extracted using a miRNeasy Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. cDNA synthesis from total RNA and Taqman Gene Expression Assays were performed as previously described with a little modification [24]. In brief, reverse transcription of total RNA was performed using random hexamers and a High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed using ABI Prism 7700 cycler (Applied Biosystems). All primers and probe sets for the detection of the expression of Ccl2 (Mm00441242_m1), Il10 (Mm00439616_m1), Il1b (Mm00434228_m1), Il6 (Mm00446190_m1), Leptin (Mm00434759_m1), and Tnf (Mm00443258_m1) were purchased from Applied Biosystems.

Statistical analysis. Data are presented as means ± SEM. For *in vitro* study, unpaired Student's *t*-test or one-way ANOVA followed by Dunnett's test was used to compare two or more than two groups to control. For *in vivo* study, Repeated Measures ANOVA followed by Dunnett's test was used to compare to control. Values of $p < 0.05$ were considered statistically significant.

Results and discussion

Establishment of NF-κB-mediated luciferase gene expressing 3T3-L1 cells

Reporter gene assay has been used for measuring the activity of transcriptional factors with high-sensitivity. NF-κB, one of the transcription factors, regulates the production of many kinds of cytokines, therefore evaluating NF-κB activity is useful to determine inflammatory state. In order to detect inflammation signals, we established NF-κB-mediated luciferase gene expressing 3T3-L1 cells (3T3-L1/NF-κB-re-luc2P). We investigated the response of TNF-α in differentiated 3T3-L1/NF-κB-re-luc2P adipocytes in i*n vitro*. Results showed that TNF- α treatment (10 ng/mL) highly

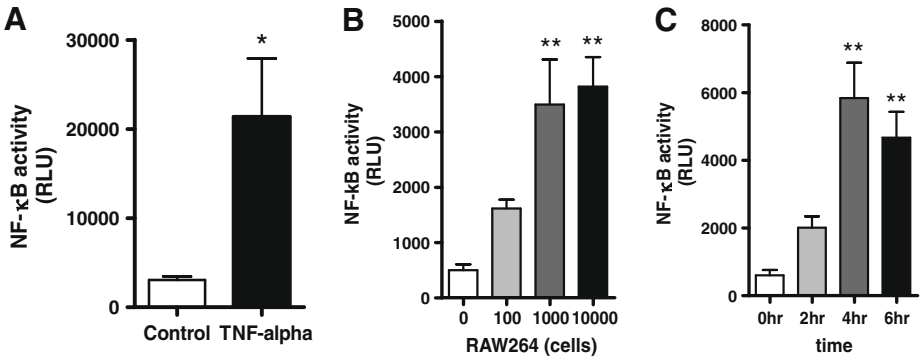

Fig. 1. Establishment of NF-κB activity-mediated luciferase expressing 3T3-L1 cells. Differentiated 3T3-L1/NF-κB-re-luc2P cells were stimulated with 10 ng/mL of murine TNF-α (A). 3T3-L1/NF-κB-re-luc2P adipocytes were co-cultured with mouse macrophage-derived RAW264 cells. The luciferase activity on (B) cell dependency of macrophages, and (C) time course of co-culture were shown. Data represent means + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, comparing with control by Student *t*-test or one-way ANOVA followed by Dunnett's test.

induced the luciferase activity in 3T3-L1/NF-κB-re-luc2P cells (Fig. 1A). We next investigated the effects of the presence of macrophage on luciferase activity in 3T3-L1/NF-κB-re-luc2P cells, since adipose tissues under the condition of obesity are characterized with increased infiltration of macrophages [4]. Co-culture of RAW264 macrophages with differentiated 3T3-L1/NF-κB-re-luc2P cells induced the luciferase activity in 3T3-L1/NF-κB-re-luc2P cells in dependent of the number of RAW264 cells and incubation time (Fig. 1B and C). These results clearly indicated that 3T3-L1/NF-κB-re-luc2P cells respond to inflammatory signals in *in vitro*.

Establishment of transplantation of 3T3-L1 cells using in vivo BLI monitoring

To confirm the successful engraftment of 3T3-L1 cells in immunodeficient C.B.17/scid mice, we examined transplantation using 3T3-L1 cells expressing luciferase gene driven by the SV40 early enhancer/promoter region. And then, we determined *in vivo* BLI in order to check the viability of implanted 3T3-L1 cells in mice. Regarding to chronological monitoring of BLI, bioluminescence at abdomen of mouse was detected over one month (Fig 2A). To check the engraftment of cells at adipose tissue, we determined *in vivo* and *ex vivo* BLI analysis 38 days after implantation. The photon signals in mouse abdominal cavity was observed exclusively at eWAT on *ex vivo* BLI (Fig 2B, right panel), where the bioluminescence on *in vivo* BLI was also found at the same position (Fig 2B, left panel). Setting regions of interest (ROI) at mice abdomen, we quantified the light emission from implanted cells. As shown in Fig 2C, the photon counts were detected until day 38 with no statistical difference compared to day 0. These results suggested that the cells implanted into visceral adipose tissue was successfully engrafted over one month and that the cells implanted into eWAT had not been spread to other organs.

In vivo monitoring of LPS-induced acute inflammation in adipose tissue

LPS induced-inflammation model was employed to monitor the activation of NF-κB in *in vivo*. 3T3-L1/NF-κB-re-luc2P cells were inoculated into right eWAT in C.B.17/scid mice. Ten days after the transplantation, LPS (20 mg/kg) was administrated and BLI

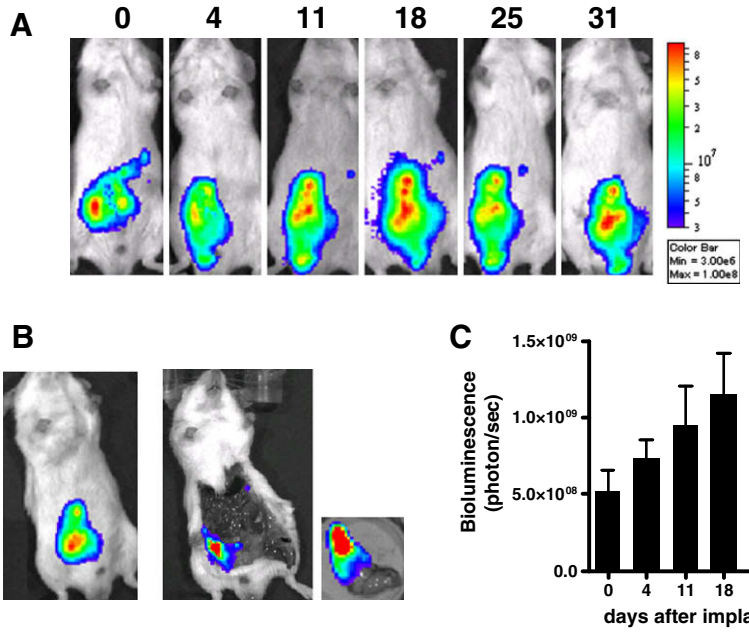

Fig. 2. *In vivo* monitoring of transplanted 3T3-L1 cells. C.B.17/scid mice were inoculated into right eWAT with 3T3-L1/luc2 cells (1×10^6 cells in 50 μl volume) on day 0. BLI were taken on indicated days after 3T3-L1 cells inoculation. Representative images of *in vivo* BLI at day 0 to day 31 in a series of one mouse (A), *in vivo* and ex vivo BLI images of whole body and eWAT at day 38 (B), and quantitative data of *in vivo* BLI (C) are shown. Data represent means + SEM.

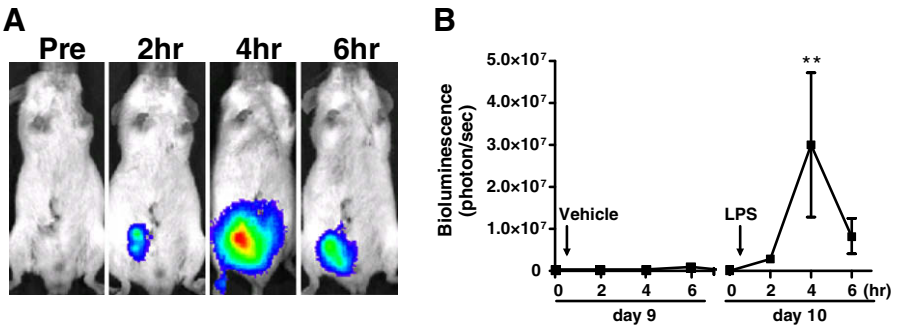

Fig. 3. Activation of NF-κB activity in 3T3-L1 cells *in vivo*. C.B.17/scid mice were inoculated into right eWAT with 3T3-L1/NF-κB-re-luc2P cells (1×10^6 cells in 50 μL volume) on day 0, and monitored bioluminescence on day 9 and day 10 after pre-adipocytes inoculation. On day 9 and 10, mice were administrated with vehicle and LPS (20 mg/kg), respectively. Representative images of BLI on day 10 (A) and quantitative data (B) are shown. Data represent means + SEM. $^{*}p < 0.05$, comparing with the value before administration of vehicle or LPS on the day, by repeated measures ANOVA followed by Dunnett's test.

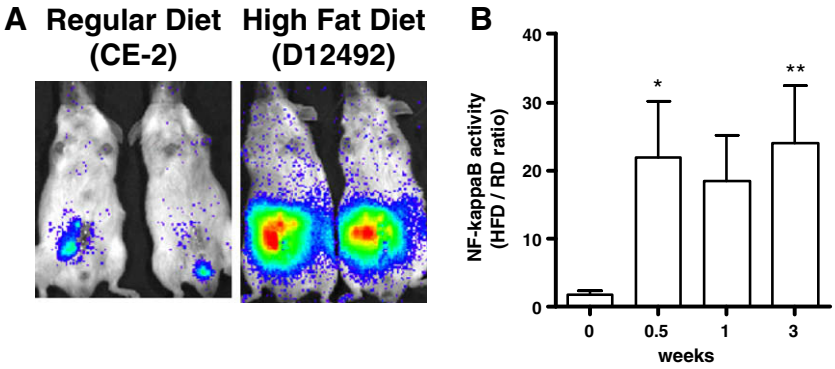

Fig. 4. Real time monitoring of NF-κB activity in lean and DIO mice. Mice were received regular diet or high fat diet for 8 weeks. Then these mice were inoculated 3T3-L1/NF-κB-re-luc2P cells into right eWAT (1×10^6 cells in 50 μL volume) on day 0, and then monitored bioluminescence on indicated days after pre-adipocytes inoculation. Representative BLI images on day 21 (A), and quantitative data (B) are shown. Data represent means + SEM. $^*p < 0.05$, comparing with the value at day 0, by repeated measures ANOVA followed by Dunnett's test.

was measured. As shown in Fig 3A, the luciferase activity was exclusively detected in the right side of abdomen, indicating that the engrafted 3T3-L1/NF-κB-re-luc2P cells locate in eWAT and respond to LPS in *in vivo*. Quantitative analysis showed that the photon emission in adipose tissue immediately increased and reached maximum 4 h after administration of LPS while no change was observed after vehicle administration in the same mouse (Fig 3B). These results indicate that BLI measuring of mice implanted 3T3-L1/NF-κB-re-luc2P cells is applicable for a real-time monitoring of the adipose inflammation in living animals in a non-invasive manner. It has been reported that LPS-induced inflammation is monitored with BLI technique using transgenic mice systemically expressing NF-κB-mediated luciferase gene [25]. Although inflammatory responses in liver, ling and kidney were nicely detected in that study, it seemed to be hard to monitor inflammation in adipose tissue because of feeble emission of bioluminescence and scant induction of luciferase activity in adipose tissue. In contrast, our method employing transplantation of adipocytes expressing NF-κB-mediated luciferase gene was able to clearly detect the NF-κB-dependent BLI signals and show significant induction of luciferase activity by LPS specifically in adipose tissue. These benefits attempted us to monitor low-grade inflammation, observed in obese adipose tissues.

In vivo monitoring of obesity-induced chronic inflammation in adipose tissue in DIO mice

We investigated whether NF-κB activity in adipose tissue increased in diet-induced obesity (DIO) mice using BLI with 3T3-L1/NF-κB-re-luc2P cells. The mice given high fat diet became significantly heavier than the mice on regular diet in 8 weeks (body weight were 37.9 g ± 1.30 g in DIO mice and 29.8 g ± 0.22 g in Lean mice, $p < 0.001$). Lean and DIO mice were implanted with an equal number of 3T3-L1/NF-κB-re-luc2P cells in right eWAT. By analyzing photon counts of BLI, there were no differences in the bioluminescence between the groups soon after implantation, confirming that there was no difference in the number of implanted cells (Fig 4B, time 0). As shown in Fig 4A, however, bioluminescence from implanted 3T3-L1 cells in eWAT in DIO mice was much higher than that in lean mice 1 week after implantation. Quantitative analysis of BLI data showed that the photon emission in DIO mice was increased within several hours after the implantation and remained more than 20-fold compared with that in lean mice for three weeks after the implantation (Fig 4B). To know whether the higher BLI signals in DIO mice is actually associated with increased pro-inflammatory signals in adipose tissue, we performed quantitative real-time RT-PCR analysis on left eWAT in which 3T3-L1/NF-κB-re-luc2P cells were not implanted. The results showed that mRNA expressions of interleukin 6 (Il6) and chemokine (C–C motif) ligand 2 (Ccl2) in DIO mice tended to be higher than those in lean mice group (Table 1). These results suggest that obesity-associated inflammation leading to NF-κB activation in adipose tissue is successfully detected.

Table 1
mRNA expression profiles in eWAT on representative pro-inflammatory genes and Leptin. Gene symbol and relative gene expression in DIO mice compared with control group are shown. Values are means ± SEM.

Gene symbol	Means ± SEM
Ccl2	2.646 ± 0.90
Il10	0.977 ± 0.59
Il1b	1.081 ± 0.22
Il6	4.203 ± 2.66
Tnf	0.832 ± 0.15
Lep	2.309 ± 0.44

We observed that *in vitro* differentiation of 3T3-L1 cells by differentiation inducers (Ins/Dex/IBMX cocktails) gave only a weak induction of NF-κB activity (data not shown), the result which is consistent to a previous report showing that differentiated 3T3-L1 cells did not induce any inflammatory cytokines [3]. Given that obese adipose tissues are characterized with inflammatory cytokines leading to NF-κB activation, the increased NF-κB activation in 3T3-L1 of DIO mice is highly likely to result from the chronic inflammation in adipose tissue. The experiments that inhibition of IKKb by salicylates or anti-inflammatory response by PPARγ agonists are to be needed to clarify the relevance between NF-κB dependent BLI at adipose tissue and inflammatory phenotypes in obesity.

In conclusion, we developed a non-invasive and real-time monitoring method of inflammation in living animals. Furthermore, obesity-induced inflammation in visceral adipose tissue was able to be monitored with this animal model. This animal model would provide an opportunity for elucidating the relevance of adipose inflammation and obesity-associated diseases and be useful as preclinical *in vivo* model to develop drug candidates.

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