Biosci. Biotechnol. Biochem., **75** (8), 1582–1587, 2011

An *in Vitro* Analysis System Using a Fluorescence Protein Reporter for Evaluating Anti-Inflammatory Effects in Macrophages

Tomoya SAKAMOTO,* Yuko YAMAGUCHI,* Tsuyoshi GOTO,
Nobuyuki TAKAHASHI,† and Teruo KAWADA

Laboratory for Molecular Functions of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Received April 7, 2011; Accepted April 22, 2011; Online Publication, August 7, 2011
[doi:10.1271/bbb.110278]

Monitoring of inflammation in adipose tissues, which causes insulin resistance, is valuable in evaluating insulin resistance. We developed an *in vitro* analysis system using a fluorescence protein (FP) as a reporter gene driven by pro-inflammatory cytokine promoters such as monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNFα). In the reporter-transfected RAW264 cells, the protein expression levels of green fluorescence protein (GFP) were increased by inflammatory stimulations such as lipopolysaccharide (LPS), conditioned medium prepared using hypertrophied 3T3-L1 adipocytes, and a co-culture system. The changes in fluorescence intensity were equivalent to those of the mRNA and protein expression levels for each cytokine. Moreover, the effects of 15-deoxy-12,14Δ-prostaglandine J_2, a natural anti-inflammatory compound, were detectable in this system. These data indicate that the FP system developed here is an analysis system of low cost with simple procedures for evaluating inflammation, suggesting usability in the large-scale screening of anti-inflammatory compounds.

Key words: inflammation; macrophage; adipocyte; insulin resistance; fluorescence protein

Chronic inflammation in adipose tissues contributes to the development of insulin resistance.[1,2] Macrophages infiltrate into obese adipose tissues by means of monocyte chemoattractant protein-1 (MCP-1), which is released from hypertrophied adipocytes. The infiltrated macrophages are activated by free fatty acid (FFA) released by hypertrophied adipocytes, resulting in the secretion of tumor necrosis factor-α (TNFα), which causes insulin resistance in adipocytes. In addition, TNFα induces lipolysis in adipocytes, increasing the concentration of FFA in the micro-environment of adipose tissues, which additionally activates the infiltrated macrophages, resulting in the overproduction of TNFα.[3] This interaction between hypertrophied adipocytes and infiltrated macrophages causes insulin resistance under obese conditions. Hence, inhibition of chronic inflammation is a significant goal in the treatment of insulin resistance.

Evaluation of inflammation in adipose tissues has been performed mainly by *in vitro* experiments using a co-culture system, in which macrophages are cultured together with hypertrophied adipocytes.[3,4] This is an excellent system, in which it is easy to detect the interaction between macrophages and adipocytes. We have screened anti-inflammatory food-derived compounds and investigated their effects using the co-culture system.[5–8] However, to examine the effects of anti-inflammatory compounds, it cannot be determined which type of cells the compounds mainly affect. In addition, because the indicators of inflammation are mRNA and the protein levels of cytokines, including MCP1 and TNFα, the costs of utilizing methods such as quantitative RT-PCR and ELISA are very high. Moreover, the long time, to perform them makes it difficult to evaluate inflammation in real time. Such problems result in difficulty in doing large-scale screening for anti-inflammatory compounds.

Hence, a novel analysis system following merits is required, one with the evaluation of inflammation in real time to easy identification of a target cell type for anti-inflammatory compounds in the co-culture system and suppression of the cost of an assay.

In the present study, we developed RAW264 cells transfected with a GFP reporter plasmid derived by MCP1 or TNFα promoter, which is activated by inflammatory stimulation. The screening system developed here makes it possible to evaluate inflammation in macrophages, to lower the cost, and to perform real time assay to evaluate inflammation, suggesting that it can be used as a convenient screening system for anti-inflammatory food-derived compounds.

Materials and Methods

Chemicals and cell culture. Lipopolysaccharide (LPS) and 15-deoxy-12,14Δ-prostaglandine J_2 (15d-PGJ_2) were purchased from Sigma (St. Lowis, MO). BAY11-7085 was from Alexis (CITY CA). All other chemicals were from Nacalai Tesque (Kyoto, Japan) or Sigma.

RAW264 macrophages (RIKEN BioResource Center, Tsukuba, Japan) and 3T3-L1 preadipocytes (American Type Culture Collection, CITY VA) were cultured in DMEM with 10% FBS, 100 U/mL,

† To whom correspondence should be addressed. Fax: +81-774-38-3752; E-mail: nobu@kais.kyoto-u.ac.jp
* These authors equally contributed to this work.

Abbreviations: FP, fluorescence protein; MCP1, monocyte chemoattractant protein-1; TNFα, tumor necrosis factor-α; FFA, free fatty acid; 15d-$\Delta$$PGJ_2$, 15-deoxy-$\Delta$12,14-prostaglandin J_2; NO, nitric oxide; GFP, green fluorescence protein; LPS, lipopolysaccharide; CM, conditioned medium

penicillin, and 100 μg/mL, streptomycin (Gibco BRL, CITY NY) at 37 °C under a humidified 5% CO_2 atmosphere, as previously described.[9] Differentiation of 3T3-L1 preadipocytes was induced by treatment with adipogenic agents (0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone, and 10 μg/mL insulin) in DMEM containing 10% FBS for 2 d after the cells reached confluence (day 0), as previously described.[10] Then the medium was replaced with DMEM containing 10% FBS and 5 μg/mL, of insulin every 2 d. 20 d after the differentiation induction, the 3T3-L1 adipocytes that accumulated large lipid droplets were used as hypertrophied adipocytes. Serum-free medium of hypertrophied 3T3-L1 adipocytes cultured for 12 h was collected as a 3T3-L1 conditioned medium (CM) and stored at −20 °C until use, as previously described.[5]

Adipocytes and macrophages were co-cultured in a contact system as previously described.[5] Briefly, RAW264 macrophages (1×10^5 cells/mL) were plated onto dishes with serum-starved, hypertrophied 3T3-L1 adipocytes. The coculture was incubated in serum-free DMEM for 24 h. RAW264 and 3T3-L1 cells were separately cultured under the same conditions as for the control cultures. Each reagent was added to the co-culture at the indicated concentrations. Twenty-four h after the treatment, culture supernatants were collected and stored at −80 °C until measurements.

Construction and transfection of GFP reporter plasmid. A luciferase reporter plasmid, pGL3-MCP1 promoter, which contains approximately 3.6 kbp of rat MCP1 promoter, was a gift from Dr. Haruhiko Nishio (Shiga University of Medical Science). The reporter plasmid was digested with KpnI and BglII. After purification, the promoter region was subcloned at the sites of KpnI and BamHI in a promoter-less green fluorescence protein (GFP) expression vector, pAcGFP1-1 (Clontech, CITY CA). A plasmid containing 1.4 kbp of whole mouse TNFα promoter was a gift from Dr. Yasutomi Kamei (Tokyo Medical and Dental University). A promoter region of the plasmid was cut with XhoI and BamHI and inserted at the SalI and BamHI sites of pAcGFP1-1. The constructed GFP reporter plasmids containing rat MCP1 and mouse TNFα promoters were named pMCP1-pro-GFP and pTNFα-pro-GFP respectively.

RAW264 macrophages were transfected with pMCP1-pro-GFP or pTNFα-pro-GFP using LipofectAMINE2000 reagent (Invitrogen, CITY CA) following the manufacturer's instructions. In transfection, using 4 μg of each reporter plasmid, the transfected cells were cultured in DMEM containing 1 mg/mL, G418 for drug selection. The cells were used in experiments after the cloning procedures. The cloned RAW264 macrophages with pMCP1- and pTNFα-pro-GFP plasmids were named MCP1-pro-GFP/RAW and TNFα-pro-GFP/RAW respectively.

Activation of RAW macrophages and detection of GFP fluorescence intensity. MCP1-pro-GFP/RAW and TNFα-pro-GFP/RAW cells cultured on 24-well plates were activated with LPS (5 μg/mL) or CM for 24 h in the presence and the absence of BAY11-7085 (5 μM). To evaluate the anti-inflammatory effects of natural compounds, we used 15-deoxy-Δ12,14-prostaglandin J_2 (15d-ΔPGJ_2) in PPARγ-dependent and -independent manners,[11] which is known as an anti-inflammatory compound, at the indicated concentrations.

To measure GFP fluorescence intensity, we used a fluorescence microscope (IX71, Olympus, Tokyo) for general observations (data not shown), a fluorescence image analyzer (InCell Analyzer, GE Healthcare UK, Buckinghamshire, UK) to take fluorescence photographs (Fig. 1), or a fluorescence multi-well plate reader (Infinite F-200, Tecan, Männedorf, Switzerland) to quantify fluorescence signals (Figs. 2–6, Supplemental Figs. 1 and 2 see *Biosci Biotechnol Biochem.* Web site). The excitation filter was 475 nm and the emission filter was 510 nm for the detection of GFP fluorescence signals. The contrast of the fluorescence photographs taken by the fluorescence image analyzer was automatically adjusted with the attached software (Investigator IN Cell Workstation, GE Healthcare UK). For general purposes, the quantification of GFP fluorescence intensity was done using the fluorescence multi-well plate reader and analysis software attached to the apparatus. To avoid cell density-dependent dispersion of values, the GFP intensity was divided by the cell number, so that the data for fluorescence intensity were parallel to the GFP expression levels per cell.

Measurement of inflammatory mediators. The protein levels of MCP1 in the culture medium were determined by ELISA done using a Ready-Set-Go Mouse MCP1 kit (eBioscience, CA, USA), in accordance with the manufacturer's protocols. NO release was measured using Griess reagent, as previously described.[5] Total RNA preparation and quantification of mRNA expression levels were done as previously described.[12] Briefly, real-time RT-PCR was done with a LightCycler System (Roche Diagnostics, Mannheim, Germany) using SYBR Green fluorescence signals. The oligonucleotide primers of mouse *MCP1* were designed using a PCR primer selection program presented at the website of the Virtual Genomic Center from the GenBank database. All primers were previously described.[5,6] To compare mRNA expression levels among the samples, the copy numbers of all transcripts were divided by that of mouse *36B4*, which shows a constant expression level. All mRNA expression levels are presented as ratios relative to that of a control for each experiment.

Statistical analyses. Data are presented as mean ± SEM, and were statistically analyzed by unpaired *t*-test and one-way ANOVA when their variances were heterogeneous. Differences were considered significant at $p < 0.05$.

Results

Inflammatory responses of MCP1-pro-GFP/RAW cells

To determine the inflammatory responses of MCP1-pro-GFP/RAW cells, we incubated MCP1-pro-GFP/RAW cells with 5 μg/mL, LPS, a general inflammatory stimulant, for 24 h under serum-free conditions. As shown in Fig. 1, fluorescence microscopy revealed that fluorescence signals derived from GFP in MCP1-pro-GFP/RAW cells were increased by LPS treatment. To determine whether this signal is dependent on LPS-induced macrophage activation, we added BAY11-7085 (5 μM), an inhibitor of LPS-induced inflammation. The addition of BAY11-7085 suppressed the fluorescence signal induced by LPS (Fig. 1C). Quantification using a multi-well plate reader indicated that the LPS-induced increase and BAY11-7085-dependent decrease in fluorescence intensity were statistically significant (Fig. 2A). NO production, a general inflammation indicator, was also increased by LPS treatment, indicating that this experimental condition is sufficient to induce inflammation, and the increase was suppressed by BAY treatment under the same conditions. Moreover, the expression of MCP1 at both the mRNA and the protein level in MCP1-pro-GFP/RAW cells changed similarly to the fluorescence intensity (Fig. 2C and D). The TNFα-pro-GFP/RAW cells showed almost the same results as the MCP1-pro-GFP/RAW cells, as shown in Supplemental Fig. 1A–D. LPS stimulation caused on increase in fluorescence intensity, which was inhibited by BAY11-7085-treatment. Moreover, these changes were similar to those for NO production, mRNA, and the protein levels of TNFα. These results indicate that the fluorescence intensities of GFP in MCP1- and TNFα-pro-GFP/RAW cells are equivalent to mRNA expression and the protein secretion of MCP1 and TNFα under LPS-induced inflammatory conditions.

Responses of MCP1-promoter-GFP/RAW cells to adipocyte-induced inflammation

To determine the inflammatory responses of MCP1-promoter-GFP/RAW cells in obese adipose tissues, we prepared CM of hypertrophied adipocytes, which con-

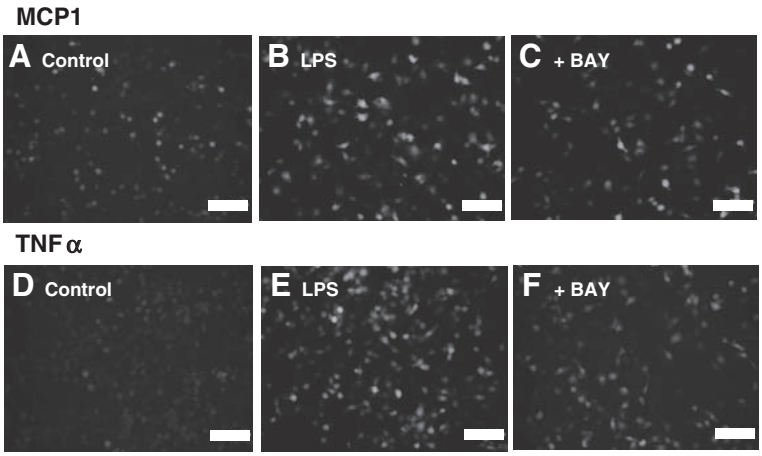

Fig. 1. Photographs of GFP Reporter-Transfected RAW Macrophages.

MCP1-pro-GFP/RAW (A–C) and TNFα-pro-GFP/RAW (D–F) cells are shown. A and D, Non-stimulated cells as control. B and E, Cells stimulated by LPS (5 μg/mL) for 24 h. C and F, LPS-stimulated cells in the presence of BAY11-7085 (5 μM). After stimulation, GFP signals were detected by fluorescence image analyzer and adjusted in contrast by attached software (see "Materials and Methods"). Representative photographs are presented. Scale bars indicate 500 μm.

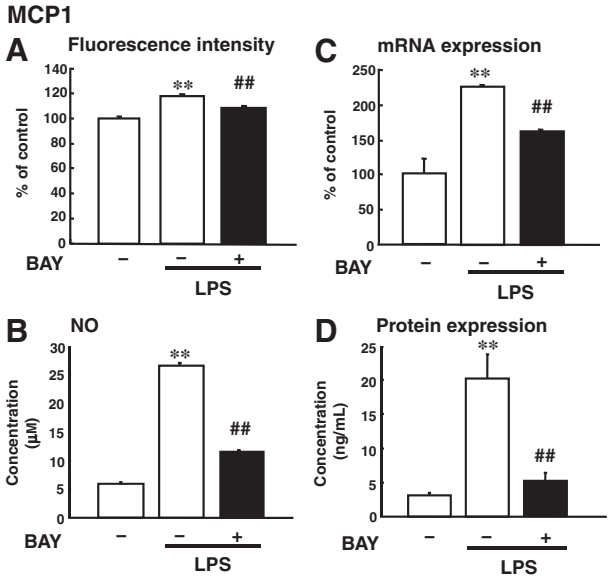

Fig. 2. Responses of MCP1-pro-GFP/RAW Cells to LPS.

A, Fluorescence intensities of MCP1-pro-GFP/RAW cells stimulated by LPS (5 μg/mL) for 24 h with and without BAY11-7085 (5 μM), quantified with a fluorescence multi-well plate reader. Values are means ± SEM for 3–4 wells. NO production (B), MCP1 mRNA (C), and protein (D) are also presented. Values are means ± SEM for 3–4 samples. $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ compared with vehicle control and LPS stimulation respectively.

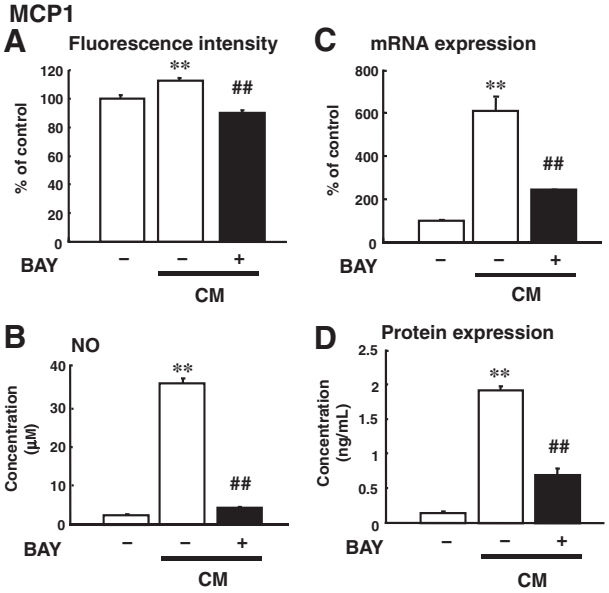

Fig. 3. Responses of MCP1-pro-GFP/RAW Cells to Conditioned Medium (CM).

A, Fluorescence intensities of MCP1-pro-GFP/RAW cells stimulated by CM prepared using hypertrophied 3T3-L1 adipocytes at 20 d for 24 h with and without BAY11-7085 (5 μM), quantified by fluorescence multi-well plate reader. The values are means ± SEM of 3–4 wells. NO production (B), MCP1 mRNA (C), and protein (D) are also presented. Values are means ± SEM of 3–4 samples. $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ compared with vehicle control and LPS stimulation respectively.

tains high concentrations of FFA, produced by lipolysis in hypertrophied adipocytes, and incubated MCP1-pro-GFP/RAW cells in the CM for 24 h. As shown in Fig. 3A, CM significantly increased the fluorescence intensity of GFP in MCP1-pro-GFP/RAW cells. The significant increase in GFP fluorescence intensity was inhibited by treatment with BAY11-7085. These changes were equivalent to those for NO production, MCP1 mRNA expression levels, and protein levels (Fig. 3B, C, and D respectively). As well as the MCP1-pro-GFP/RAW cells, the TNFα-pro-GFP/RAW cells demonstrated the same responses for GFP intensity, NO production, TNFα mRNA expression levels, and protein levels, as shown in Supplemental Fig. 2. This indicates that GFP-derived fluorescence intensities in both cell lines are useful in the evaluation of responses to adipocyte-derived stimuli.

To confirm the responses of MCP1-pro-GFP/RAW cells to inflammatory stimulation, dose-dependency was examined. As shown in Fig. 4A, the CM-induced GFP fluorescence intensity in the MCP1-pro-GFP/RAW cells increased in a dose-dependent manner (CM was diluted by fresh serum-free DMEM at the indicated magnification). These changes were equivalent to those in MCP1 protein secretion from the cells (Fig. 4B). The data indicate that changes in the fluorescence intensities in reporter-transfected RAW cells are parallel to those for cytokine expression.

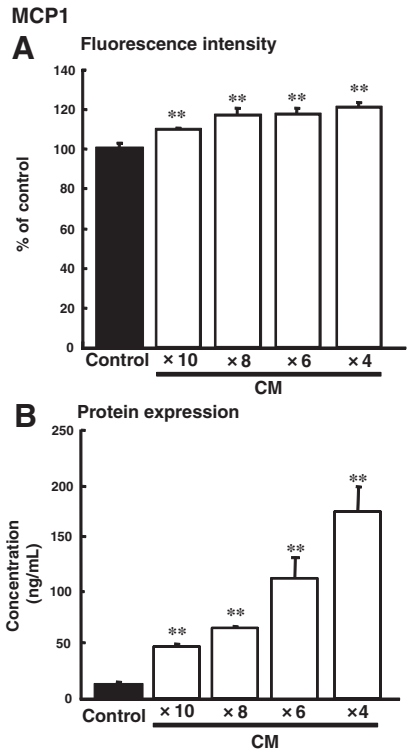

Fig. 4. Dose-Dependency in the Responses of MCP1-pro-GFP/RAW Cells to Conditioned Medium (CM).

A, Fluorescence intensities of MCP1-pro-GFP/RAW cells stimulated by CM prepared using hypertrophied 3T3-L1 adipocytes at 20 d for 24 h, quantified by fluorescence multi-well plate reader. Values are means ± SEM for 3–4 wells. B, Protein expression levels of MCP1 derived from MCP1-pro-GFP/RAW cells stimulated by CM prepared using hypertrophied 3T3-L1 adipocytes at 20 d for 24 h. Values are means ± SEM of 3–4 samples. $^{**}p < 0.01$ compared with vehicle control.

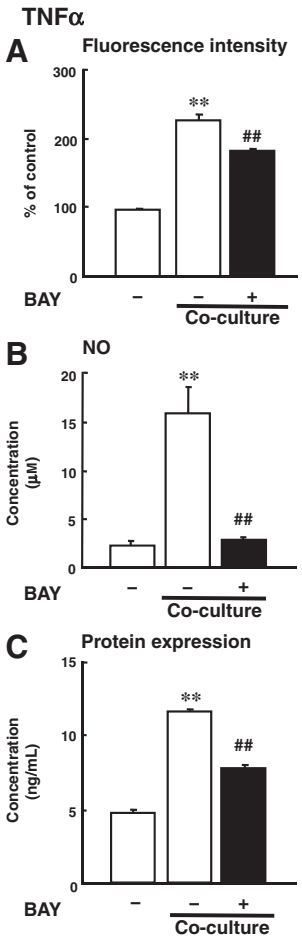

Fig. 5. Responses of TNFα-pro-GFP/RAW Cells in Co-Culture with Hypertrophied 3T3-L1 Adipocytes.

A, Fluorescence intensity of MCP1-pro-GFP/RAW cells stimulated by co-culture with hypertrophied 3T3-L1 adipocytes at 20 d for 24 h, quantified by fluorescence multi-well plate reader. Values are means ± SEM for 3–4 wells. B, NO production of TNFα-pro-GFP/RAW cells. C, Protein expression levels of TNFα derived from TNFα-pro-GFP/RAW cells. Values are means ± SEM for 3–4 samples. $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ compared with vehicle control and co-culture stimulation respectively.

In addition to dose-dependency, we studied the responses of reporter-transfected cells in a co-culture system of macrophages together with hypertrophied adipocytes. As shown in Fig. 5A, the fluorescence intensities of the TNFα-pro-GFP/RAW cells were increased by co-culture with hypertrophied 3T3-L1 adipocytes, which were cultured for 20 d as described in "Materials and Methods." This increase was inhibited by BAY11-7085 treatment. These changes in fluorescence intensity were equivalent to those for NO production and TNFα protein expression levels (Fig. 5B and C). This suggests that reporter-transfected cells respond properly to inflammatory stimuli in adipose tissues.

Detection of moderate anti-inflammatory effects of a natural compound

To determine whether this system can detect moderate effects of natural compounds in comparison to synthetic compounds, we examined the effects of 15d-PGJ_2, which is considered an anti-inflammatory natural compound.[13] As shown in Fig. 6A and B, 10 μM 15d-PGJ_2 inhibited fluorescence intensity increase due to either LPS or CM in the TNFα-pro-GFP/RAW cells. This inhibition was statistically significant in the cases of increase due to LPS or CM. Moreover, 15d-PGJ_2 significantly inhibited fluorescence intensity in a co-culture of TNFα-pro-GFP/RAW cells with hypertrophied 3T3-L1 adipocytes (Fig. 6C). The changes were equivalent to those for TNFα mRNA and protein levels (data not shown). These findings indicate that this system detects moderate anti-inflammatory effects of natural compounds such as 15d-PGJ_2, suggesting the system is useful for evaluating the anti-inflammatory effects of natural compounds.

Discussion

In the present study, we developed a fluorescence protein (FP) reporter system for evaluating inflammation in macrophages. In adipose tissues, macrophages are activated by FA derived from hypertrophied adipocytes, it then secretes various pro-inflammatory cytokines, such as TNFα and MCP1. TNFα induces additional lipolysis in hypertrophied adipocytes, in which lipolysis activity is originally high, and additional expression of MCP1. These additional effects of FA and MCP1 cause further activation and infiltration respectively of macrophages in adipose tissues. This mechanism contributes to the development of insulin resistance under obese conditions.[14] Hence, monitoring of the production of

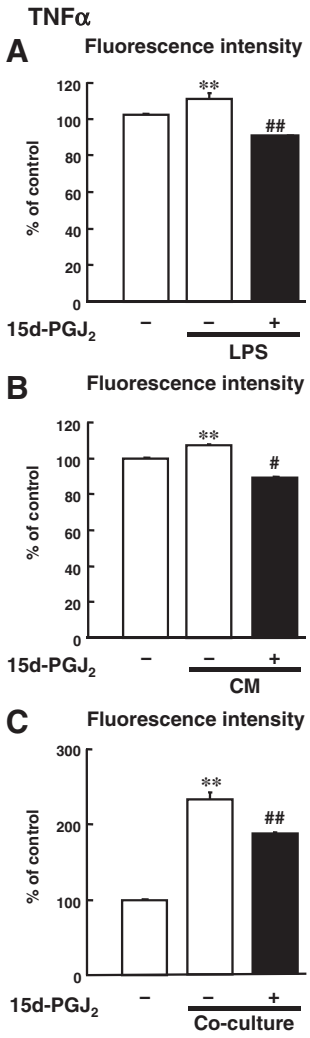

Fig. 6. Evaluation of the Anti-Inflammatory Effects of 15d-PGJ_2 in Various Stimulations Using TNFα-pro-GFP/RAW Cells.

TNFα-pro-GFP/RAW cells were stimulated by LPS (5 μg/mL) (A), CM (B), or co-culture with hypertrophied 3T3-L1 adipocytes for 24 h. 15d-PGJ_2 (10 μM) was added during stimulation of the macrophages. Fluorescence intensity was quantified by fluorescence multi-well plate reader. Values are means ± SEM for 3–4 wells. $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ compared with vehicle control and stimulation respectively.

these cytokines is very important in evaluating inflammation in adipose tissues. To date, monitoring has been performed at the mRNA and protein levels using quantitative RT-PCR and ELISA respectively. However, these assays require costly complicated procedures. Hence, a simple, economical assay system is needed. Promoter-driven reporter systems are useful for examining gene expression *in vitro* and *in vivo*. Promoter activities can be evaluated by the expression levels of a reporter gene. A luciferase gene is widely used as a sensitive reporter. In previous studies, we have characterized various food-derived compounds, activating nuclear receptors as ligands by a luciferase reporter system.[6,9,12,15–17] However, the luciferase reporter system is also costly, and complicated, although its sensitivity is very high. On the other hand, the FP-reporter system developed here is simple and economical. Therefore, this system should make it easy to screen huge chemical libraries to identify anti-inflammatory compounds.

In the present study, changes in GFP fluorescence intensities were equivalent to those of mRNA and protein expression levels as quantified by real-time RT-PCR and ELISA respectively, but the changes in the FP-reporter system relatively smaller than those in real-time RT-PCR or ELISA. This might have been due to the high levels of the base lines, meaning that a GFP protein is present under non-stimulated conditions. In the co-culture system, the basal fluorescence signals appeared to be lower than those in other systems, using LPS or CM (Fig. 5). This was due to higher induction of reporter GFP caused by positive feedback stimulation in macrophage-adipocyte interactions. That is partly because the half-life of a GFP protein is relatively long (approximately 48 h), resulting in the accumulation of GFP proteins even if macrophages are not activated. This problem can be solved using a short half-life FP as a reporter gene, such as DD-AcGFP (Clontech, CITY CA), whose half-life is less than 12 h. In our preliminary experiments, base lines became lower when such a short half-life FP was used as reporter gene (data not shown), although further experiments are needed to examine details. Nevertheless, the present study indicates that the FP-reporter system is useful for evaluating inflammation along with real-time RT-PCR and ELISA. In addition, the system is sufficient to detect the inhibitory effects of anti-inflammatory compounds not only such as BAY11-7085 but also such as 15d-PGJ_2. Whereas BAY11-7085 is a reagent for research, 15d-PGJ_2 is a natural compound with anti-inflammatory effects.[13] Such natural compounds generally show fewer effects than chemically synthesized compounds. Using this system, we performed large-scale screening to identify natural compounds including food-derived ones with anti-inflammatory effects. However, it might be better for characterizing the details of the effects to use a more sensitive system, such as a luciferase reporter, after first screening by the FP-reporter system.

In conclusion, we developed a system using a FP-reporter gene that was simpler than the system used currently. Using this system, we evaluated levels of inflammation and effects on anti-inflammation in macrophages. This system is useful for large-scale screening of natural compounds such as food-derived compounds with anti-inflammatory effects, although additional improvements are needed in reporter selection and experimental protocols to obtain higher sensitivity.

Acknowledgments

We thank Dr. Y. Okada and M. Tominaga (National Institute for Physiological Sciences) for support in cell analysis using IN Cell Analyzer 1000, S. Shinotoh and Y. Tada for secretarial and technical supports respectively. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sport, Science, and Technology of Japan (22228001 and 22380075).

References

1) Fernández-Real JM and Ricart W, *Endocr. Rev.*, **24**, 278–301 (2003).
2) Lyon CJ, Law RE, and Hsueh WA, *Endocrinol.*, **144**, 2195–2200 (2003).

3) Suganami T, Nishida J, and Ogawa Y, *Arterioscler. Thromb. Vasc. Biol.*, **25**, 2062–2068 (2005).
4) Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, Aoe S, Kamei Y, and Ogawa Y, *Arterioscler. Thromb. Vasc. Biol.*, **27**, 84–91 (2007).
5) Hirai S, Kim YI, Goto T, Kang MS, Yoshimura M, Obata A, Yu R, and Kawada T, *Life Sci.*, **81**, 1272–1279 (2007).
6) Kang MS, Hirai S, Goto T, Kuroyanagi K, Ezaki Y, Takahashi N, and Kawada T, *Biochem. Biophys. Res. Commun.*, **369**, 333–338 (2008).
7) Ando C, Takahashi N, Hirai S, Nishimura K, Lin S, Uemura T, Goto T, Yu R, Nakagami J, Murakami S, and Kawada T, *FEBS Lett.*, **583**, 3649–3654 (2009).
8) Uemura T, Goto T, Kang MS, Mizoguchi N, Hirai S, Lee JY, Nakano Y, Shono J, Hoshino S, Tsuge N, Narukami T, Makishima M, Takahashi N, and Kawada T, *J. Nutr.*, **141**, 17–23 (2011).
9) Takahashi N, Kawada T, Goto T, Kim CS, Taimatsu A, Egawa K, Yamamoto T, Jisaka M, Nishimura K, Yokota K, Yu R, and Fushiki T, *FEBS Lett.*, **550**, 190–194 (2003).
10) Takahashi N, Kawada T, Yamamoto T, Goto T, Taimatsu A, Aoki N, Kawasaki H, Taira K, Yokoyama KK, Kamei Y, and Fushiki T, *J. Biol. Chem.*, **277**, 16906–16912 (2002).
11) Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, and Evans RM, *Nat. Med.*, **7**, 48–52 (2001).
12) Takahashi N, Kawada T, Goto T, Yamamoto T, Taimatsu A, Kimura K, Saitoh M, Hosokawa T, Miyashita K, and Fushiki T, *FEBS Lett.*, **514**, 315–322 (2002).
13) Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, and Glass CK, *Proc. Natl. Acad. Sci. USA*, **97**, 4844–4849 (2000).
14) Hirai S, Takahashi N, Goto T, Lin S, Uemura T, Yu R, and Kawada T, *Mediators Inflamm.*, in press.
15) Goto T, Takahashi N, Kato S, Egawa K, Ebisu S, Moriyama T, Fushiki T, and Kawada T, *Biochem. Biophys. Res. Commun.*, **337**, 440–445 (2005).
16) Kuroyanagi K, Kang MS, Goto T, Kusudo T, Hirai S, Yu R, Yano M, Sasaki T, Takahashi N, and Kawada T, *Biochem. Biophys. Res. Commun.*, **366**, 219–225 (2008).
17) Takahashi N, Goto T, Taimatsu A, Egawa K, Katoh S, Kusudo T, Sakamoto T, Ohyane C, Lee JY, Kim YI, Uemura T, Hirai S, and Kawada T, *Biochem. Biophys. Res. Commun.*, **390**, 1372–1376 (2009).