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ORIGINAL ARTICLE

Thalidomide inhibits adipogenesis of orbital fibroblasts in Graves' ophthalmopathy

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Abstract The expansion of orbital adipose tissue is a main pathophysiology of Graves' ophthalmopathy (GO), which is an inflammatory autoimmune disease in the orbital region. The effects of immunosuppressive drugs on adipogenesis of orbital fibroblasts have not been determined. Thalidomide, as an immunosuppressive drug, has recently been used in the therapy of many autoimmune diseases. In this study, we analyzed the effects of thalidomide on adipogenesis and found that adipocyte differentiation from preadipocytes in the orbital region was enhanced, which was demonstrated by enhanced expression of peroxisome proliferator activated receptor γ (PPARγ), ap2, and thyroid-stimulating hormone receptor (TSHR). The expression of inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) was also increased in GO. Thalidomide dose-dependently inhibited adipogenesis of 3T3-L1 preadipocytes and orbital fibroblasts from GO patients. Along with the inhibited adipogenesis, the expression of TSHR, TNFα, and IL-6 was also down-regulated. We discovered that the mechanism for thalidomide inhibiting adipogenesis was the down-regulation of PPARγ, rather than C/EBPβ and C/EBPδ. We suggest that, besides its canonical anti-TNFα effect, thalidomide plays a role in inhibiting adipogenesis of orbital fibroblasts in GO patients.

Keywords Graves' ophthalmopathy · Adipogenesis · Thalidomide · TSHR · PPARγ

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Abbreviation

GO	Graves' ophthalmopathy
TAO	Thyroid-associated ophthalmopathy
TSHR	Thyroid-stimulating hormone receptor
TNFα	Tumor necrosis factor α
IL-6	Interleukin 6
PPARγ	Peroxisome proliferator activated receptor γ
ap2	Adipocyte protein 2
C/EBPβ	CCAAT enhancer binding protein β
C/EBPδ	CCAAT enhancer binding protein δ

Introduction

Graves' ophthalmopathy (GO), also known as thyroid-associated ophthalmopathy (TAO), is an inflammatory autoimmune disease of the periocular tissue. GO affects about 30–50% of patients with Grave's disease, and 3–5% suffer from severe eye disease [1]. Increased volume of the orbital contents characterizes GO and leads to clinical manifestations, such as exophthalmos, gritty eyes, photophobia, chemosis, diplopia, and even blindness in extreme cases [2]. Apart from the accumulation of glycosaminoglycans, edema caused by inflammation, and the swelling of the muscular apparatus, the expansion of orbital adipose tissue has also been thought of as one of the main mechanisms by which the volume of orbital contents is increased [3–7].

The expansion of the orbital adipose tissue is a process of increasing numbers of adipose cells (hyperplasia) and adipose cell size enlargement (hypertrophy), which is known as adipogenesis. Many in vitro studies also showed that adipogenesis of orbital fibroblasts in GO patients is enhanced [8], and thyroid-stimulating hormone receptor (TSHR) expression in orbital fibroblasts increases along

with enhanced adipogenesis [9]. As TSHR is a major autoantigen in the immunopathogenesis of GO, increased expression of TSHR in the orbital region is a possible factor in the process of GO progression and orbital adipose tissue expansion.

There is a lack of pharmaceutical treatment choices for this autoimmune disease. Glucocorticoids are used for treatment, but this is actually not a good choice due to side effects and high rates of recurrence following discontinuation [10]. Although some immunosuppressive agents have been used as treatments for GO, their effects were unsatisfactory. In the present study, we investigated the influence of thalidomide [2-(2,6-dioxopiperidin-3-yl) isoindoline-1, 3-dione] on adipogenesis of orbital fibroblasts in GO patients, as well as the effect on TSHR expression.

Materials and methods

Human orbital fibroblast isolation and culture

Orbital adipose tissue explants were obtained from euthyroid GO patients who had orbital decompression surgery for severe exophthalmos ($n = 3$) and from normal controls that had orbital surgery for some medical condition without orbital tissue involvement ($n = 3$). One of three patients received glucocorticoid therapy for GO before decompression surgery, while the other two patients had stopped glucocorticoid therapy for more than 6 months before the surgery was performed. Both received anti-thyroid drugs at the time of surgery (5 mg methymazol for two patients and 100 mg thiouracil for one patient). Informed consent was obtained from all participants in the study, and the ethics board at Nanjing Medical University approved the study. The tissue was minced into pieces and placed in 6-cm dishes, allowing the fibroblasts to proliferate, as indicated by Bahn [11]. In brief, with 199 medium supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and 20% fetal bovine serum (Gibco), the explants were cultured at 37°C in a 5% CO^2 humidified incubator. When confluent, cultures were removed and transferred to DMEM medium with 10% fetal bovine serum, and cells were passaged with the same medium until the differentiation experiment was performed.

Cell culture and adipocyte differentiation

3T3-L1 cells were obtained from the American Type Culture Collection and cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen). 2 days after confluence, the medium was replaced with a differentiation induction medium containing 0.5 mM 3-isobutyl-methylxanthine (Sigma), 1 mM Dexamethasone (Sigma), 10 μg/ml insulin (Eli Lilly), and 10% FBS (15%FBS for MEFs), and the time point was marked as day 0. At day 2, cells were changed to a medium containing 10 μg/ml insulin. At day 4 and on following days, cells were changed to a medium that was used before day 0, and the medium was renewed every other day. At day 8, oil red O staining was conducted to evaluate the lipid laden. Thalidomide was added to the medium at day 0 and every time afterward when the medium was renewed. Samples of protein and RNA were harvested at different time points as indicated.

Real-time PCR

Total mRNA was isolated from tissues or cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of RNA was carried out using the Reverse Transcription System (Promega). Real-time PCR was performed on an Applied Biosystems 7300 Real-Time PCR system according to the manufacturer's instructions, using a SYBR PrimeScript Real time PCR Kit (Takara). The primer that was used is listed in Table 1. Values are shown from three separate experiments, each of which being done in triplicate.

Western blotting

Equal amounts of protein were loaded onto 10% SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked with 10% nonfat milk and then incubated with different primary antibodies, anti-TSHR (Abcom), and anti-GAPDH (Millipore), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The specific immune complexes were visualized with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Little Chalfont Buckinghamshire, UK) according to the manufacturer's protocol.

Results

Enhanced adipogenesis and increased pro-inflammation molecule expression in orbital tissue from GO patients

To verify that GO patients have enhanced adipogenesis, we compared the mRNA levels of mature adipocyte markers in orbital tissues from GO patients to those of normal controls. The expression of PPARγ (Fig. 1a) and ap2 (Fig. 1b) was increased significantly in GO patients. TSHR expression was also increased in the orbital tissue from GO patients as detected by real-time PCR (Fig. 1c) and by protein blots (Fig. 1f). As TNFα and IL-6 were considered to be among the orbital inflammation causative cytokines [7], we checked their expression by real-time PCR, and the results showed the mRNA levels of TNFα (Fig. 1d) and

Table 1 The primer used in this article for real time PCR

Gene name	Accession number	Species	Primer sequence
TSHR	NM_172671	Mouse	5′-GAGTGTGCGTCTCCACCCTGTGA-3′
			5′-TTCCAGCCGCTGCAGAGTTGCAT-3′
PPARγ2	NM_011146	Mouse	5′-CCATTCTGGCCCACCAAC-3′
			5′-AATGCGAGTGGTCTTCCATCA-3′
ap2	NM_024406	Mouse	5′-AAGGTGAAGAGCATCATAACCCT-3′
			5′-TCACGCCTTTCATAACACATTCC-3′
C/EBPδ	NM_007679	Mouse	5′-CGACTTCAGCGCCTACATTGA-3′
			5′-CTAGCGACAGACCCCACAC-3′
C/EBPβ	NM_009883	Mouse	5′-TGGACAAGCTGAGCGACGAG-3′
			5′-TGTGCTGCGTCTCCAGGTTG-3′
36B4	NM_007475	Mouse	5′-AGATTCGGGATATGCTGTTGGC-3′
			5′-TCGGGTCCTAGACCAGTGTTC-3′
GAPDH	NM_008084	Mouse	5′-AGGTCGGTGTGAACGGATTTG-3′
			5′-TGTAGACCATGTAGTTGAGGTCA-3′
TSHR	NM_000369	Human	5′-GGAATGGGGTGTTCGTCTCC-3′
			5′-GCGTTGAATATCCTTGCAGGT-3′
PPARγ2	NM_015869	Human	5′-CCTATTGACCCAGAAAGCGATT-3′
			5′-CATTACGGAGAGATCCACGGA-3′
ap2	NM_003220	Human	5′-CCAGGGACTTTGGGTACGTG-3′
			5′-GGTTGAGAAATTCAGCTACTGCT-3′
TNFα	NM_000594	Human	5′-ATGAGCACTGAAAGCATGATCC-3′
			5′-GAGGGCTGATTAGAGAGAGGTC-3′
IL-6	NM_000600	Human	5′-AAATTCGGTACATCCTCGACGG-3’
			5′-GGAAGGTTCAGGTTGTTTTCTGC-3′
GAPDH	NM_002046	Human	5′-ATGGGGAAGGTGAAGGTCG-3′
			5′-GGGGTCATTGATGGCAACAATA-3′

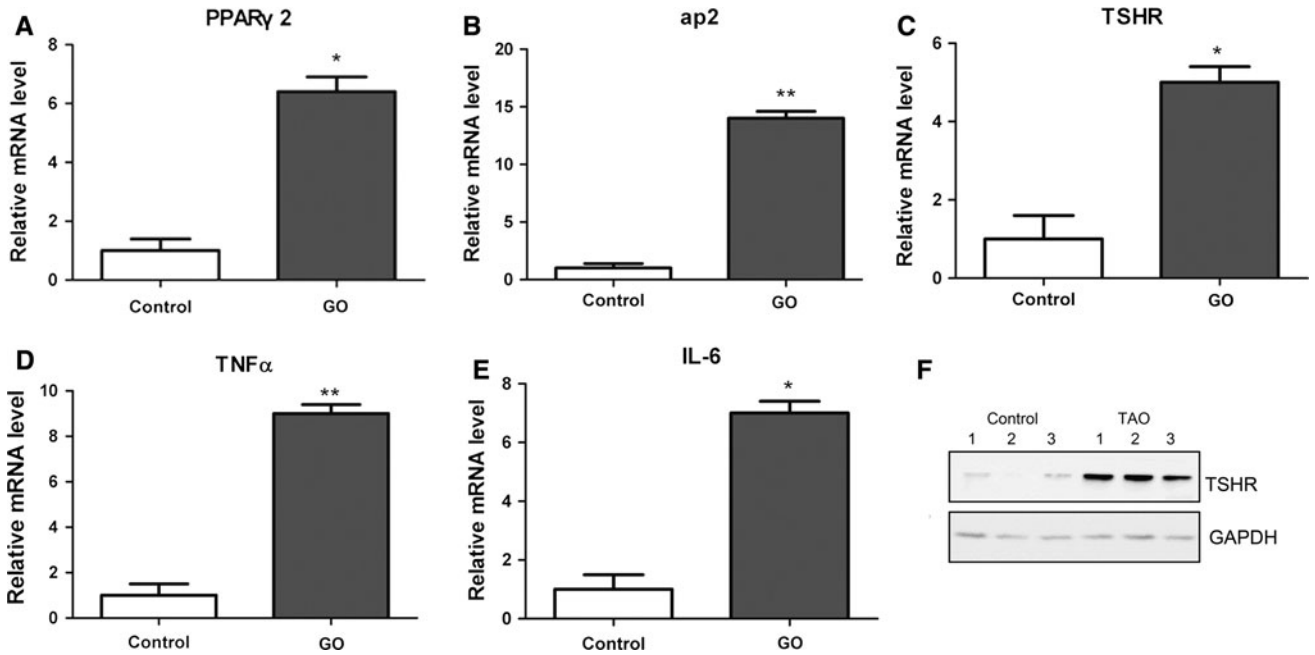

Fig. 1 Real time PCR results for the expressions of PPARγ (**a**), ap2 (**b**), TSHR (**c**), TNFα (**d**), and IL6 (**e**) in orbital tissue from controls ($n = 3$) and GO patients ($n = 3$). 36B4 was used as an internal control. Values are from three separate experiments, each done in triplicate, $*P < 0.05$, $**P < 0.001$. Western blot results show the expression of THSR, with GAPDH as a loading control (**f**)

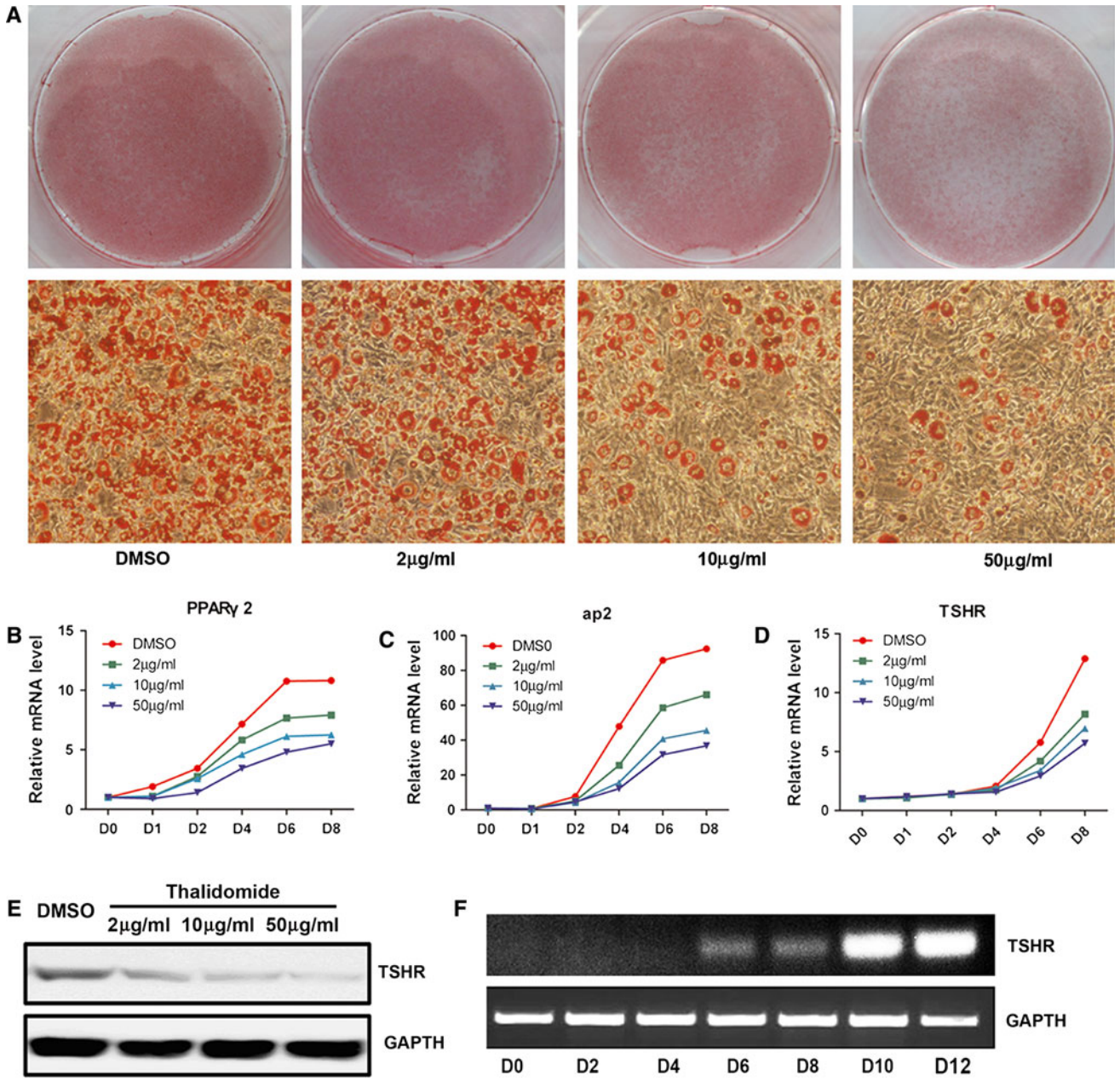

Fig. 2 Oil Red O staining showed that the adipocyte differentiation from 3T3 L1 preadipocytes was inhibited by thalidomide dose-dependently (**a**). Results are shown as a whole (*up*) and at a magnification of ×40 (*below*). The expressions of PPARγ (**b**), ap2 (**c**), and TSHR (**d**) also were inhibited by thalidomide dose-dependently at indicated time points, as shown by real time PCR, with 36B4 as an internal control. **e** Western blotting showed the expression of THSR at day 8, with GAPDH as a loading control. **f** TSHR expressions at different times of adipogenisis. RT-PCR results for 3T3-L1 cells, with GAPDH as an internal control

IL-6 (Fig. 1e) were comparatively higher in the orbital tissues from GO patients.

Thalidomide inhibited adipogenesis of 3T3-L1 preadipocytes

3T3-L1 preadipocytes are used as a model for adipogenesis study. We evaluated the function of thalidomide on adipogenesis of this cell model using different concentrations. As indicated in Fig. 2a, thalidomide inhibited drug-induced adipogenesis of 3T3-L1 preadipocytes dose-dependently, and the ultimate inhibiting effect in the study was seen at a dose of 50 μg/ml. The expression of PPARγ (Fig. 2b) and ap2 (Fig. 2c) at different time points of adipogenesis was also inhibited by thalidomide dose-dependently.

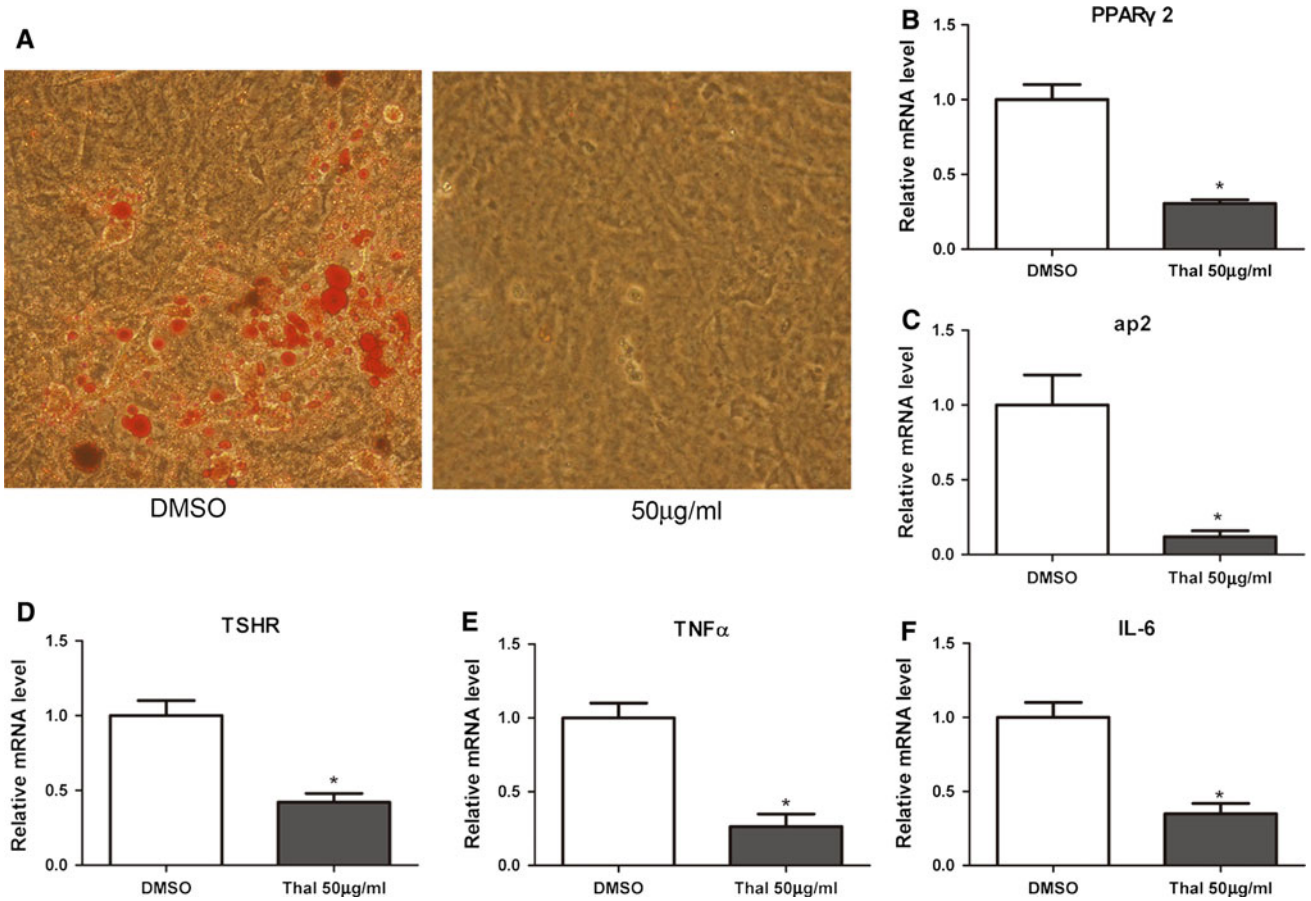

Fig. 3 Oil Red O staining showed that adipocyte differentiation from orbital fibroblasts of GO patients was inhibited by 50 μg/ml thalidomide (**a**). Results are shown for the whole (*upper*) and at a magnification of ×40 (*below*). Real time PCR results showed that the expressions of PPARγ (**b**), ap2 (**c**), TSHR (**d**), TNFα (**e**), and IL6 (**f**) were inhibited by thalidomide, with 36B4 as an internal control. Values are shown from three separate experiments, with each done in triplicate, $*P < 0.05$

The TSHR expression in 3T3-L1 preadipocytes could be detected by real time PCR at day 4 of adipogenesis and peaked at day 10 of adipogenesis (Fig. 2f). With inhibition of adipogenesis by thalidomide, TSHR expression was also down-regulated, as shown by real time PCR (Fig. 2d) and Western blotting (Fig. 2e).

Thalidomide inhibited adipogenesis of orbital fibroblasts from GO patients

We next examined whether thalidomide could inhibit adipogenesis of orbital fibroblasts from GO patients, as indicated in Fig. 3a. 50 μg/ml of thalidomide dramatically inhibited the fat content of orbital fibroblasts from GO patients. PPARγ (Fig. 3b) and ap2 (Fig. 3c) expressions decreased significantly at day 10 of drug-induced adipogenesis. Along with inhibited adipogenesis, the expression of TSHR, TNFα, and IL-6 from adipogenic orbital fibroblasts at day 10 also decreased comparatively (Fig. 3d–f).

Thalidomide did not influence cell numbers and earlier molecule expression in the process of adipogenesis

In the first 2 days of adipogenesis, there is a stage called clone proliferation that is critical for thorough adipogenesis. We deduced that thalidomide inhibited adipogenesis through proliferation suppression. The results of our study did not show any difference in the 3T3 L1 cell numbers between the groups with different doses of thalidomide at differentiation day 0 (Fig. 4a) or day 2 (Fig. 4b). The orbital fibroblast cell numbers at each adipogenic time point were not influenced by thalidomide either (Fig. 4c). We also checked the expression of earlier adipogenesis modulators, C/EBPβ, and C/EBPδ, and found that there was no difference in the expressions between the cultures with and without thalidomide (Fig. 4d, e).

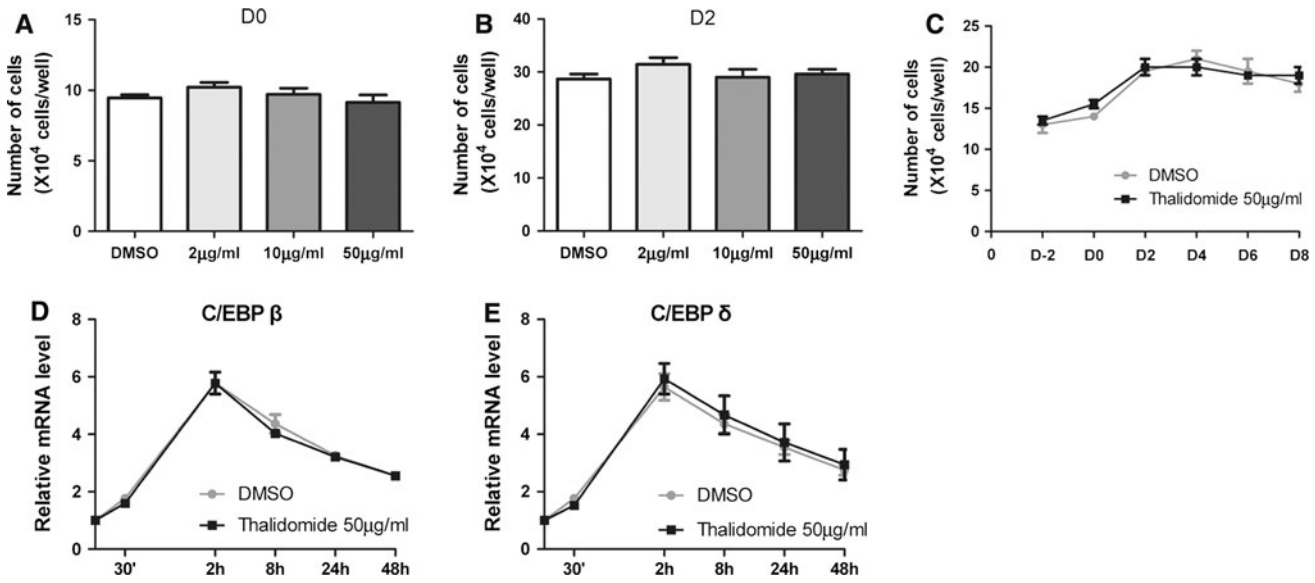

Fig. 4 At day 0 (**a**) and day 2 (**b**) of MDI-induced 3T3 L1 differentiation, the cell number counted by CCK8 (DOJINDO Japan) did not change with different doses of thalidomide, at the different time points indicated. 50 μg/ml of thalidomide did not change cell numbers (**c**). Realtime PCR results showed that C/EBPβ (**d**) and C/EBPδ (**e**) expression did not change with the use of thalidomide at the various time points indicated, with 36B4 as an internal control. Values are from three separate experiments, each done in triplicate

Discussion

In the present study, we showed enhanced adipogenesis of orbital fibroblasts in GO, accompanied by increased expression of TSHR, IL6, and TNFα. We report here that thalidomide inhibited adipogenesis of not only 3T3-L1 cells, but also orbital fibroblasts from GO patients. Along with impaired adipogenesis, the expressions of PPARγ and TSHR were downregulated significantly as was the expression of pro-inflammation factors IL6 and TNFα. The expression of earlier adipogenic factors, C/EBPβ and C/EBPδ, did not change, and the cell numbers in adipogenesis did not change with use of thalidomide as well.

Thalidomide was first introduced in 1953 as an oral sedative hypnotic and was then removed from the market in 1961 when it was found to cause limb malformation in newborns after usage by the mother during pregnancy. Owing to its anti-inflammatory and antiangiogenic properties, there is new interest in thalidomide that is currently being used as a treatment for several autoimmune diseases, including rheumatoid arthritis [12], sarcoidosis [13], Sjögren's syndrome [14], and chronic cutaneous lupus [15]. The immunoinhibitory and anti-inflammation effects of thalidomide are complex and incompletely understood. Many mechanisms that have been reported include angiogenesis inhibition and the influence of cytokine production [16, 17].

GO is an autoimmune disease and high-dose systemic corticosteroids alone or in combination with orbital irradiation remains the main option for GO treatment. However, such treatment is imperfect due to the side effects of high-dose systemic corticosteroids and high recurrence rates following discontinuation of the therapy [18]. With the use of thalidomide, the inflammatory cytokines expressed in orbital fibroblasts cultured in vitro decreased significantly, which demonstrated the anti-inflammatory effects of the drug. TNFα, *o*ne of the most potent inflammation factors that plays an important role in GO, was also decreased significantly in thalidomide-treated 3T3-L1 and orbital fibroblasts. In orbital tissue, TNFα increases the production of glycosaminoglycans, as well as the expression of other immunomodulatory proteins that are important in the pathogenesis of the disease.

It had been reported that, through suppression of PPARγ expression, TNFα by itself has only a weak effect in inhibiting adipogenesis [19, 20]. Therefore, lowering TNFα may enhance adipogenesis. In the present study, TNFα expression in cultures of orbital fibroblasts was inhibited by thalidomide, but this TNFα lowering did not lead to enhanced adipogenesis. Instead, we found that with the decrease in TNFα, the adipogenesis of orbital fibroblasts was inhibited. From the results of the study, we conclude that the effect of anti-adipogenesis by thalidomide is potent enough to overcome the effect of TNFα on adipogenesis. The potent anti-adipogenesis effects of thalidomide have also been demonstrated in experiments with 3T3-L1 cells. Thalidomide dose-dependently inhibited adipogenesis and

downregulated PPARγ and aP2, which are two molecular markers of mature adipocytes.

PPARγ is a key molecule in adipogenesis. Through PPARγ, many molecules play roles in adipogenesis regulation [21]. C/EBPβ and C/EBPδ are expressed at every early stage of adipogenesis, and regulate adipogenesis by modulating PPARγ expression as well. In the present study, thalidomide did not influence the expression of C/EBPβ and C/EBPδ at different adipogenic time points, while the expression of PPARγ was inhibited dramatically. These results suggest that thalidomide plays a role in the regulation of adipogenesis by PPARγ in late stages, rather than by C/EBPβ and C/EBPδ in early stages. Since we did not find any influence of thalidomide on cell numbers in adipogenesis, we do not propose that thalidomide inhibits adipogenesis with an anti-proliferation effect, as indicated in its anti-tumor therapy activity [22, 23]. Other mechanisms for the downregulation of PPARγ by thalidomide have not been disclosed in recent studies, and this is worthy of further study. Along with inhibited adipogenesis, TSHR expression decreased dramatically. This is of vital significance since TSHR plays an important role in triggering autoimmune reactions and enhancing adipogenesis in the orbital region [4, 24, 25].

There are some limitations of this study. First, only three GO patients had been recruited in the study, and one patient was taking glucocorticoids when orbital surgery was performed, which may influence the homogenicity of the sample, further study is needed to confirm that if the adipogenesis inhibiting effects of thalidomide applies to all GO patients. Second, we did not test the doses of thalidomide beyond 50 μg/ml, which could be one of the reasons that the cytotoxicity effect of thalidomide exhibited in anti-tumor experiment has not been found in the present study. Third, the experiment that tests the effect of thalidomide on other lineage differentiation was not done in this study, and it has significance in delineating the whole picture of thalidomide on cell differentiation and could give clues to explain the mechanisms of thalidomide's inhibiting effect on adipogenesis.

In conclusion, we discovered that thalidomide inhibits adipogenesis of orbital fibroblasts from GO patients. Although the mechanism of the anti-adipogensis effect of thalidomide should be studied further, the coexistence of the effects of anti-adipogenesis and anti-TNFα suggests that thalidomide might be better than other immunosuppressive agents in GO therapy.

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Conflict of interest None.

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