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Original

Effect of Short-term Tumour Necrosis Factor-alpha (TNF-α) -stimulation on the Growth and Differentiation of MC3T3-E1 Osteoblast-like Cells

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Abstract: Tumor necrosis factor-alpha (TNF-α) is an inflammatory cytokine known to cause bone resorption, swelling and edema during tissue organization. Conversely, TNF-α has also been shown to participate in tissue regeneration during the wound healing process. We have previously investigated the effects of TNF-α on human dental pulp cell differentiation. Dental pulp cells are composed of different cell types including primary odontoblasts and fibroblasts. We determined that the ratio of stem cells within the pulp cell population was increased following short-term stimulation with TNF-α. The aim of this study therefore was to investigate the effect of short-term stimulation with TNF-α on osteoblast-like MC3T3-E1 cell growth and differentiation. MC3T3-E1 cells were cultured in standard growth medium and on reaching sub-confluence were exposed to recombinant TNF-α (10 and 100 ng/ml) for 2 days prior to assessing their cell proliferation and differentiation properties in comparison to non-stimulated MC3T3-E1 cells (control). Although no significant differences in cell proliferation were observed between the TNF-α-stimulated and control groups, cell differentiation was delayed in the TNF-α-stimulated groups. In summary, short-term stimulation of cultured MC3T3-E1 cells with TNF-α had only minimal effect on their growth and differentiation.

Key words: TNF-α, Osteoblast-like cell (MC3T3-E1), Cell proliferation, Cell differentiation

Introduction

Tumor necrosis factor-α (TNF-α) elicits a variety of actions and effects such as bone and cartilage resorption, inflammation, joint destruction, swelling, edema, and enhances immune response. Conversely, an in vivo study has shown that injecting recombinant human (rh) TNF-α into the wound site significantly accelerates fracture healing and remodeling[1)].

The highly organized and efficient process of tissue injury healing is characterized by four precise but overlying phases: hemostasis, inflammation, proliferation, and remodeling[2)]. Initiation of the inflammatory cascade occurs following recruitment and activation of macrophages at the injury site and subsequent release of interleukins and TNF-α. During the peak inflammatory period, over the initial 48 hours, the process of recruitment, activation and migration of leucocytes and the surrounding connective tissue cells, including stem/progenitor cells, to the injured site contributes to tissue healing, thus representing the proliferative and remodeling phases[3)]. Conversely, suppression or termination of the inflammatory phase occurs following feedback signaling from the cells surrounding the injury site, leading to secretion of anti-inflammatory factors such as TNF-α-stimulated gene/protein 6 (TSG-6), prostaglandin E2 (PGE2), and interleukin-1 receptor antagonist (IL-1ra) from activated resident macrophages[4,5)]. It is therefore clear that the function and differentiation potential of mesenchymal stem/progenitor cells are modulated by pro-inflammatory cytokines. We have previously shown that short-term treatment of dental pulp stem/progenitor cells with TNF-α enhances their stem cell phenotype, migration ability, and differentiation potential[6)].

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TNF-α stimulation of osteoblasts hinders their bone-formative action by suppressing recruitment of osteoblasts from progenitor cells, inhibiting the expression of matrix protein genes, and stimulating expression of genes related to osteoclastogenesis[7)]. However, the effect of TNF-α is unclear in osteoblastogenesis during the inflammatory phase. The purpose of this study was to investigate the effect of short-term stimulation of TNF-α on osteoblast-like MC3T3-E1 cells.

Materials and Methods

Cell culture

Osteoblast-like cells (MC3T3-E1 derivative cell line of mouse calvaria sourced from Riken Industry, Japan) were used in this study. The cells were cultured in basal medium consisting of alpha-minimum essential medium (α-MEM) (Nacalai Tesque Inc., Kyoto, Japan), 10 % heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Massachusetts, USA), L-ascorbic acid (50 μg/ml), and β-glycerophosphate (2 mM) (Nacalai Tesque Inc.) at 37 °C in 5 % CO_2. Cells were observed by light microscopy (Nikon Eclipse TS100, Tokyo, Japan).

For pretreatment experiments with TNF-α, cells were cultured to 60 % confluence and incubated with 10 or 100 ng/ml of recombinant mouse TNF-α (rTNF-α) (R&D Systems Inc., MN, USA) for a further 2 days. After reaching sub-confluence, cells were the passaged once with accutase (Nacalai Tesque Inc.) to completely remove the TNF-α before subsequent assaying (Fig. 1).

MTS assay

The MTS (3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl)-2H-tetrazolium) assay (CellTiter 96®Aqueous One Solution Cell Proliferation Assay, Promega, WI, USA) was performed to assess cell proliferation. The cells were incubated for 1, 3, 5, and 7 days in

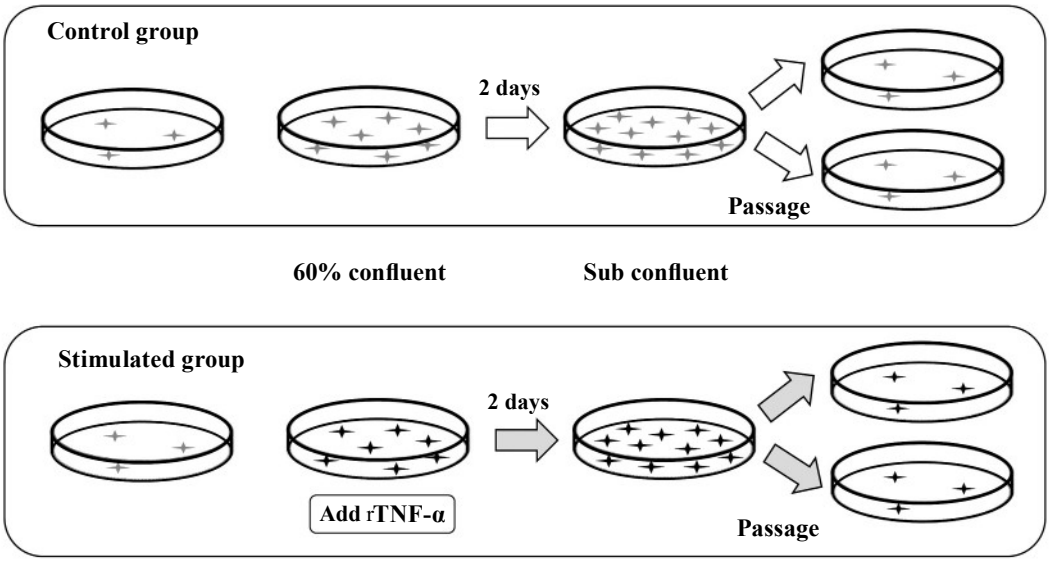

Figure 1. TNF-α-stimulation method. MC3T3-E1 cells were cultured to 60 % confluence and exposed to 10 or 100 ng/ml of recombinant mouse TNF-α for 2 days. Cells were then passaged once to remove the rTNF-α completely before applying the cells into subsequent assays.

Table 1. Quantitative Real-time PCR Primer

Runx2 Forward	aagtgcggtgcaaactttct
Runx2 Reverse	tctcggtggctggtagtga
Collagen (Type I) Forward	gagcggagtactggatcg
Collagen (Type I) Reverse	gcttcttttccttggggtt
Alp Forward	cttgactgtggttactgctgatca
Alp Reverse	gtatccaccgaatgtgaaaacgt
Gapdh Forward	gacttcaacagcaactcccac
Gapdh Reverse	tccaccaccctgttgctgta

a 5 % CO_2 atmosphere at 37 °C. The medium was discarded by aspiration, then the cells were incubated in 300 µl of α-MEM medium and 60 µl of CellTiter 96® Aqueous One Solution Reagent for 1 hour in a 5 % CO_2 atmosphere at 37 °C. Supernatants (120 µl/sample) were then transferred into a 96-well plate and read using a plate reader at a wavelength of 492 nm.

Determination of alkaline phosphatase activity

Measurement of alkaline phosphatase (ALP) activity was performed using the p-Nitrophenyl Phosphate Substrate method (LabAssay™ ALP, Wako, Osaka, Japan) in accordance with the manufacturer's instructions. Cell cultures were examined at 3, 5 and 7 days in confluent monolayers.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the cultured cells after incubation on day 3 and day 7 using TRIzol reagent (Life Technologies, CA, USA). The final total RNA was dissolved in DEPC-treated water and absorption at 260 nm and 280 nm was measured by a spectrophotometer (NanoDrop® ND-1000, Nanodrop Technologies, Inc., DE, USA) to calculate quantity and purity. Total RNA (1 µg) was then treated with RNase-free DNase I (Invitrogen, Thermos Fisher Scientific, CA, USA) to remove DNA contaminants. Complementary DNA (cDNA) was then synthesized from the RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Life Technology, CA, USA). Real-time PCR was carried out using Step One Plus (Applied Biosystems, Life Technology). The cDNA amplification reactions were performed for *Runx2*, *Collagen (Type I)*, and *Alp* genes, and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Real-time PCR primers (Fasmac, Kanagawa, Japan) are shown in Table 1. The real-time PCR reaction was performed in a total volume of 20 µl PCR mixture containing Fast SYBR® Green Master Mix (Applied Biosystems), 0.5 µM of each PCR primer and 2 µl of cDNA sample. Targets were amplified, after initial denaturation at 94 °C for 3 min, with 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. At the end of the PCR cycles, melting curve analysis was performed to identify specificity of the PCR produced by heating the samples from 65 °C to 95 °C at rate of 0.5 °C/s. Reaction products were quantified using serial dilutions of each sequence of known concentration to generate a standard curve. The mRNA content of each target was normalized to the amount of *Gapdh* mRNA in each sample.

Alizarin red staining

Calcification content was verified using Alizarin red staining. The cells were washed with PBS and stained with 1 % Alizarin red solution (Muto Pure Chemicals Co., Japan) for 10 min. The culture plates were then washed 3 times with distilled water and calcification was observed.

Scanning Electron Microscopy

Cell morphology was analyzed using scanning electron microscopy (SEM) at after incubation for 1 day post-TNF-α exposure. Samples were fixed with 2 % gluteraldehyde in 0.1 M of Na-Cacodylate-HCL buffer and 0.1 M sucrose. Fixative solution (250 µl/well) was added into the medium and the cells were incubated for 5 minutes at 37 °C. The medium and fixative solution was then removed from each well and replaced with 500 µl of fixative solution prior to incubation at room temperature for 1 hour and then at 4 °C for 24 hours. The samples were then dehydrated with a series of ethanol gradients (70 %, 80 %, 90 %, and 100 %) and immersed in 1–2 ml of t-butyl alcohol

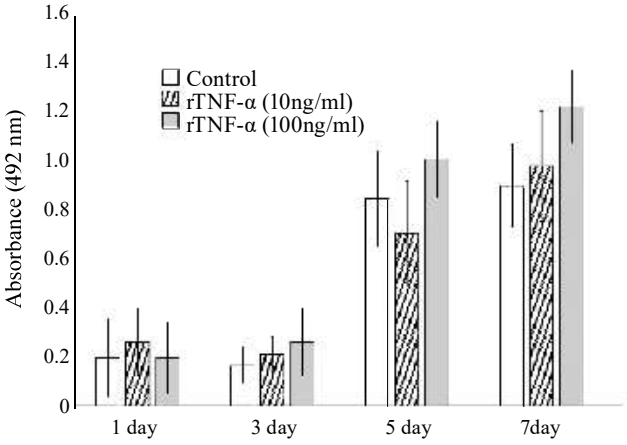

Figure 2. MTS assay. There was no significant difference in cell proliferation between the rTNF-α and control groups. (ANOVA and post hoc test)

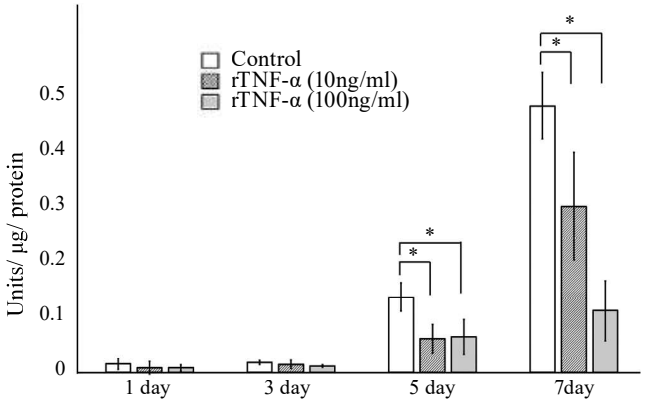

Figure 3. ALP assay. In both groups ALP activity increased after 1, 3, 5, and 7 days. ALP activity was significantly lower in the rTNF-α-treated group compared with the control group on days 5 and 7; *P < 0.05 (by post hoc test).

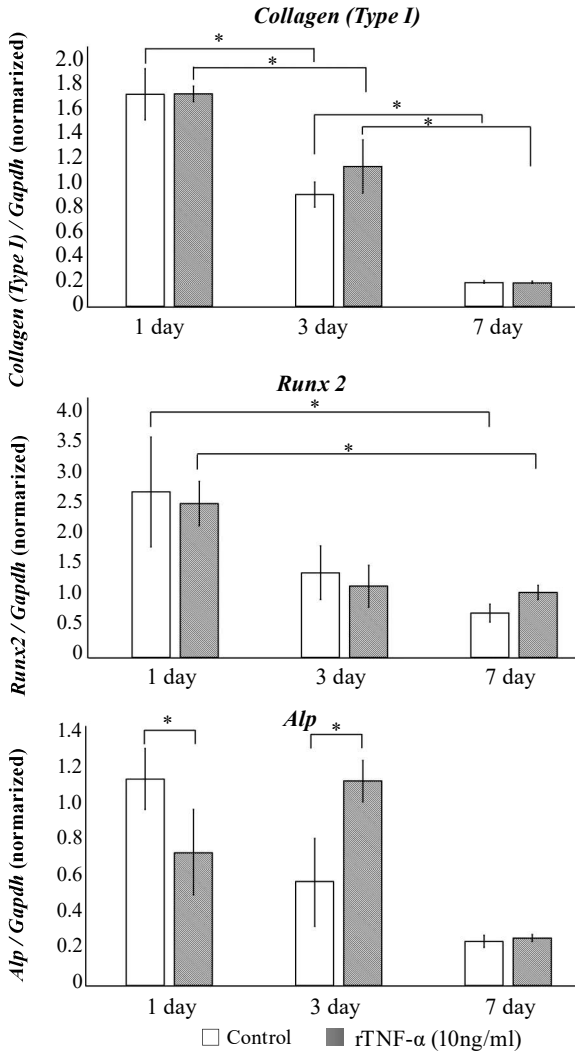

Figure 4. Quantitative real-time PCR of bone differentiation genes. Both *Collagen (type-I)* and *Runx2* were decreased over time, with no differences in their expression observed between the rTNF-α and control groups. Interestingly, *Alp* expression was suppressed in the rTNF-α group on day 1, but elevated on day 3. At day 7 however, a comparable decrease in *Alp* gene expression level was observed in both groups; *P < 0.05 (by post hoc test).

for 15 minutes. Samples were then transferred into a vacuum freeze drying evaporator (12 CR-QD; The Virtis Company Inc. Gardiner, NY, USA). The t-butyl alcohol was completely sublimated during a 3–4 hour evacuation. After vacuum drying, the samples were mounted and fixed on an aluminum stub using a carbon tape and sputter-coated with Au. A 6 mA charge was applied for 60 seconds using an iron coating apparatus (IB-3, Eiko Engineering Co. Ltd., Tokyo, Japan). Finally, samples were observed and imaged under SEM (JCM5700, Jeol Co. Ltd., Tokyo, Japan).

Statistical analysis

Statistical differences among groups with respect to cellular proliferation, ALP activity and real-time PCR gene expression levels were determined using one-way analysis of variance (ANOVA) and post-hoc test (Bonferroni) with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). EZR is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria), and more precisely, is a modified version of R commander designed to add statistical functions frequently used in biostatistics. A value of $p < 0.05$ was considered to indicate statistical significance.

Each experimental sample (10 and 100 ng/ml rTNF-α exposure) was compared with the control group using Bonferroni. A p-value of 0.05 was considered statistically significant.

Results

MC3T3-E1 cell proliferation

Cell proliferation was detected using the MTS assay at 1, 3, 5, and 7 days and no significant differences were observed between the rTNF-α (10 and 100 ng/ml) and control groups (Fig. 2).

MC3T3-E1 cell differentiation

Cell differentiation was determined by ALP activity, quantitative real-time PCR and Alizarin red staining. Fig. 3 shows Alp activity of MC3T3-E1 cells at 1, 3, 5, and 7 days. On days 1 and 3, there were no significant differences in ALP activity between all concentrations of rTNF-α and the control group. However, on days 5 and 7, ALP activity was significantly reduced in the rTNF-α-treated groups compared with the control group.

Quantitative real-time PCR showed that *Collagen* (*type-I*) and

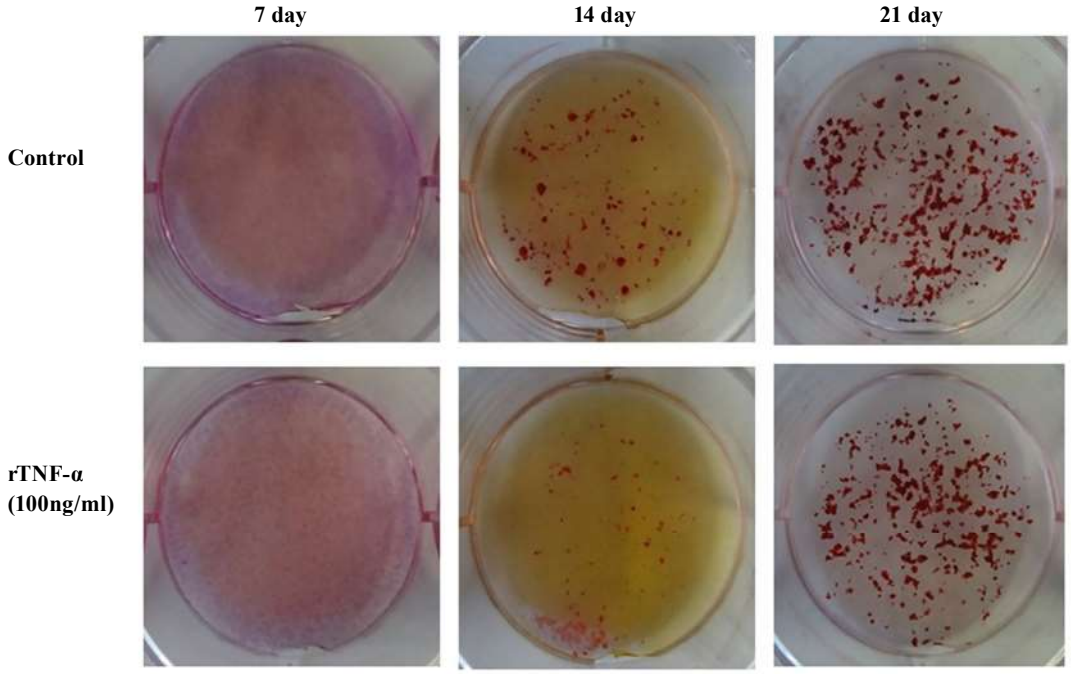

Figure 5. Alizarin red staining. Alizarin red staining was enhanced in the control group compared with the rTNF-α group at days 7 and 14. At day 21, Alizarin red staining was comparable between both the control and TNF-α groups.

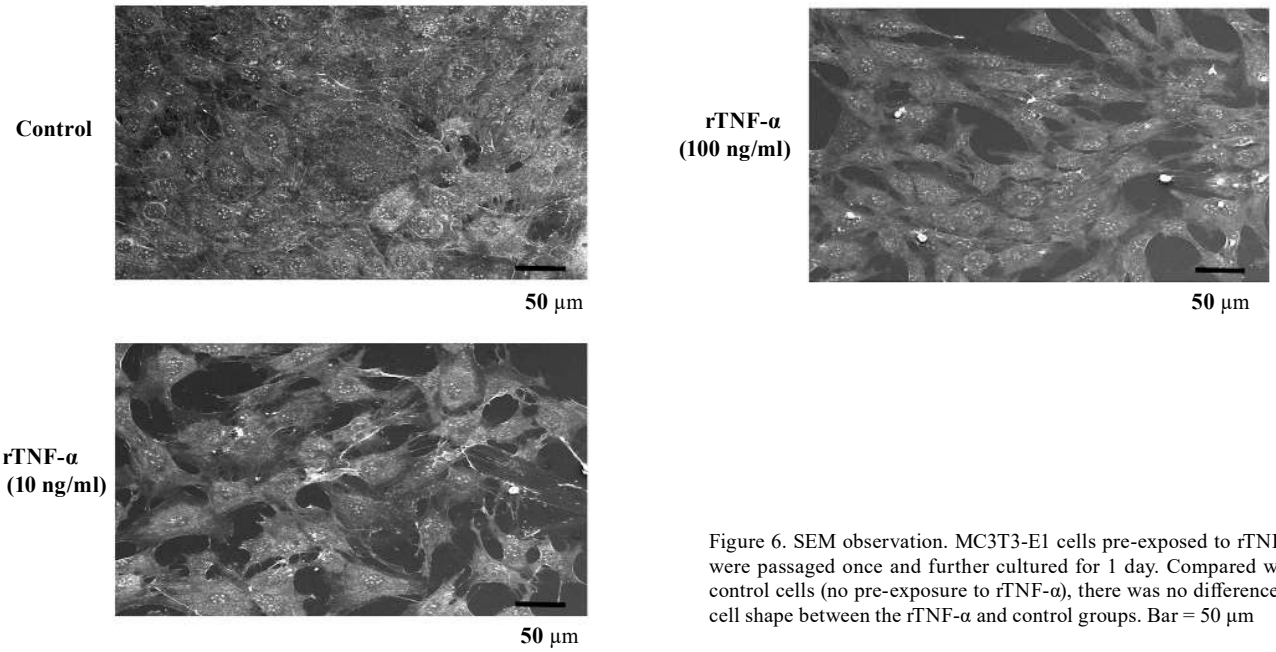

Figure 6. SEM observation. MC3T3-E1 cells pre-exposed to rTNF-α were passaged once and further cultured for 1 day. Compared with control cells (no pre-exposure to rTNF-α), there was no difference in cell shape between the rTNF-α and control groups. Bar = 50 μm

Runx2 decreased with time, but no significant differences in the levels of these markers were observed between the rTNF-α and control groups. Although *Alp* expression was suppressed at day 1, this level was elevated in the rTNF-α group at day 3. At day 7, a similar decrease in *Alp* expression level was observed in the control and rTNF-α groups (Fig. 4).

Alizarin red staining was more intense in the control group compared with the rTNF-α groups at days 7 and 14. However, Alizarin red staining was comparable in both groups at day 21. Mineralized nodule formation was delayed in the rTNF-α-treated groups. This result indicates that short-term stimulation of rTNF-α delayed osteoblast differentiation (Fig. 5).

Observation of MC3T3-E1 cells

MC3T3-E1 cells were observed using light microscopy and SEM. In all groups, cells were observed to be flattened and spreading. rTNF-α did not affect cell shape and surface morphology after 1 day (Fig. 6).

Discussion

Inflammatory cytokines play a primary role during the process of wound healing and normal tissue regeneration. However, the

mechanisms behind the cascade of biochemical events related to tissue inflammation/regeneration remain unclear. The effect of short-term TNF-α-stimulation in osteoblast cells has never been analyzed. This study aimed to investigate the effect of short-term TNF-α stimulation on the growth and differentiation of MC3T3-E1 osteoblast-like cells. We found that after short-term TNF-α stimulation of MC3T3-E1 cells: (1) cell proliferation was not significantly affected over 7 days of subsequent culture; (2) cell differentiation was delayed; (3) cell morphology was not affected.

Similar to our previous study using human dental pulp cells, we found that short-term TNF-α stimulation did not affect MC3T3-E1 cell proliferation[6]. Bin *et al*. showed that high concentrations of TNF-α promoted apoptosis in a dose-dependent manner (10, 20, 40 ng/ml) after 24 h of treatment[8]. During TNF-α stimulation in the present study, apoptosis was observed by light microscopy. However, the TNF-α was completely removed after 48 hours of stimulation. Analysis of the expression of caspase-3 using real-time PCR found no increase in apoptosis in the rTNF-α-stimulated group compared with the control group (data not shown).

Quantitative real-time PCR expression of *Alp* showed that there was a delay of cell differentiation in the TNF-α-stimulated groups compared with the control group.

Nanes *et al*.[7] reported that TNF-α inhibits the production of type-I collagen from cultured calvaria and long bone explants, isolated fetal calvaria osteoblasts, and osteoblastic clonal cell lines[8-10]. TNF decreases the expression of *OCN* mRNA in primary cultures of osteoblasts and in clonal osteoblastic cell lines, and its action due to transcriptional repression by *NF-κB*[11-13]. ALP is inhibited by TNF via a complex mechanism[9-11, 14-18]. TNF regulation of ALP occurs at several levels. Although TNF will decrease ALP mRNA and protein expression, TNF can also stimulate ALP release. TNF-induced apoptosis of osteoblasts is associated with an immediate release of soluble ALP[19]. The steady-state number of osteoblasts is a function of their rate of differentiation and their rate of loss[7]. TNF inhibition of osteoblast differentiation suggests that these critical differentiating factors are direct or indirect targets of TNF regulation[20]. Using three experimental models (fetal calvaria pre-osteoblasts, bone marrow stromal cells, and clonal MC3T3-E1 pre-osteoblasts) Gilbert *et al*. have shown that TNF blocks osteoblast differentiation[20]. Analysis revealed a critical period of sensitivity to TNF during the stage of phenotype selection, in which the precursor cells commit to differentiate along an osteoblastic trajectory rather than one of other cell types.

MC3T3-E1 cell morphology was maintained following short-term stimulation with rTNF-α. Cell surface is known to be affected by high concentrations of magnesium and fluoride in odontoblast-like cells[21]. The morphological features of the MC3T3-E1 cells in media containing 10 mM magnesium and 0.1 mM fluoride showed an abundance of filopodia. In our study there was no change in filopodia at 1 day post-TNF-α exposure (Fig. 6).

This study concludes that short-term stimulation of TNF-α on MC3T3E1 cells had no effect on cell proliferation and cell morphology, but cell differentiation was delayed in these osteoblast-like cells.

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Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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