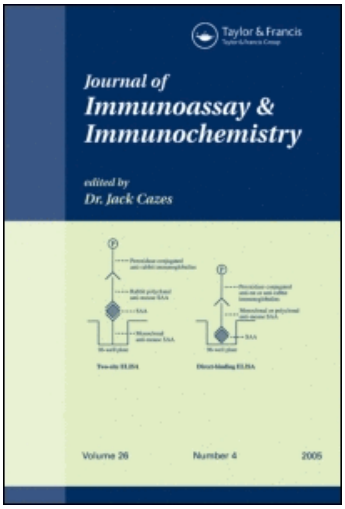

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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Online publication date: 30 December 2009

To cite this Article Mogi, Makio and Kondo, Ayami(2010) 'DOWN-REGULATION OF NF-κB LED TO UP-REGULATION OF NGF PRODUCTION IN MOUSE OSTEOBLASTS', Journal of Immunoassay and Immunochemistry, 31: 1, 92 — 101
To link to this Article: DOI: 10.1080/15321810903405142
URL: http://dx.doi.org/10.1080/15321810903405142

Journal of Immunoassay and Immunochemistry, 31:92–101, 2010

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810903405142

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DOWN-REGULATION OF NF-κB LED TO UP-REGULATION OF NGF PRODUCTION IN MOUSE OSTEOBLASTS

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□ *For investigation of the molecular cascade leading to nerve growth factor (NGF) in mouse osteoblastic MC3T3-E1 cells, pyrrolidine dithiocarbamate (PDTC), an NF-κB inhibitor, was utilized. A dose-dependent PDTC-elicited stimulation of NGF production occurred in the cells. PDTC attenuated the NF-κB nuclear translocation, whereas PDTC had no effects on the cell proliferation, apoptosis, differentiation, or cell cycle. The cell membrane-permeable SN50 peptide (a specific NF-κB inhibitor peptide) also blocked the NF-κB translocation to the nucleus, and induced NGF protein in the cells. These findings demonstrate that the suitable suppression of NF-κB nuclear translocation induced NGF production in non-neuronal osteoblastic cells.*

Keywords NF-κB, NGF, Osteoblast, PDTC, SN50

INTRODUCTION

Neurotrophins, especially NGF, are proteins known to be essential for the growth, survival, and differentiation of sympathetic and sensory neurons in vertebrates.[1,2] These physiological actions of neurotrophins are not limited to the nervous system, since several studies revealed the positive function of neurotrophins in non-neuronal cells.[3] In the case of bone morphogenesis, bone-associated neurons have been considered to regulate bone differentiation through synaptic interaction between neuronal cells and bone-forming cells, the osteoblasts.[4] The involvement of NGF in osteoblastogenesis has been only rarely studied.[5–7] Nakanishi et al. reported the presence of neurotrophins (NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin (NT)-3) and the receptor of NT-3, trk-c, in the mouse osteoblastic cell-line MC3T3-E1.[5,6] Finkelman

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et al. also reported the lack of functional receptors for NGF in bone cells from chick calvaria.[8] We also demonstrated previously that MC3T3-E1 cells could express neurotrophins (NGF and BDNF) and their receptor genes (trk-a and trk-b) and that NGF had an anti-apoptotic effect against cytokine-induced cytotoxicity in the cells.[9] However, the molecular cascade leading to NGF production in osteoblastic cells remains to be elucidated.

Mammalian NF-κB is a group of transcription factors that includes 7 members, p65 (RELA/NFKB3), c-Rel, RelB, p50/p105 (NFKB1), and p52/p100 (NFKB2).[10] Upon activation by a wide variety of stimuli (proinflammatory cytokines, growth factors, and viral proteins), NF-κB is translocated to the nucleus, where it modulates the expression of target genes involved in cell growth, survival, adhesion, and death.[11–13] Since proinflammatory cytokines can induce NGF mRNA expression and NGF protein in osteoblastic cells,[9] we hypothesized that NF-κB regulates NGF production in osteoblastic cells. Pyrrolidine dithiocarbamate (PDTC) is a thiol compound derived from dithiocarbamates, a highly characterized class of antioxidants found in both free cells and biological systems;[14] it is often used as a potent inhibitor of NF-κB.[15–18] While most studies have focused on the activation of NF-κB translocation, there is no report on the suppression of transcriptional factor-induced protein induction.

If the signal cascade for NGF production can be elucidated, it can open the door for a new therapy for neurodegenerative diseases. We hypothesized that inactivation of NF-κB by PDTC (a known NF-κB inhibitor) would result in the potent inhibition of NGF production in osteoblastic cells. To test this hypothesis, we first cultured MC3T3-E1 cells in the presence of PDTC to study its effects on NGF production, cell proliferation, and differentiation. Surprisingly, the present study demonstrated that the suitable suppression of nuclear NF-κB translocation by PDTC induced potent production of NGF in mouse osteoblastic cells.

EXPERIMENTAL

Materials

Purified mouse NGF was obtained from Biomed. Technol. (Stoughton, MA, USA). PDTC and SN50 (the latter being a specific NF-κB inhibitor peptide) were obtained from Calbiochem (San Diego, CA, USA).

Cell Culture

MC3T3-E1 cells, established from newborn mouse calvaria, are a clonal osteogenic cell line whose cells can differentiate into osteoblasts, and are

frequently used as a model to study the effects of proinflammatory cytokines *in vitro*.[9,19] Incubation with PDTC or SN50 was done in medium containing 0.1% FCS.

ELISA for NGF

To measure the immunoreactive NGF released into the culture medium, a two-site ELISA was used.[20] Anti-β (2.5S,7S) NGF monoclonal antibody (MoAb) and anti-β-NGF-β-galactosidase (Roche, USA) were applied, and the NGF content in the samples was determined by comparison with a mouse NGF standard curve.

ELISA for NF-κB

The NF-κB subunits p50 content in the extracts was also determined by use of a commercially available assay kit (Trans-AM, NF-κB p50 active assay, Active Motif North America, CA, USA). Nuclear protein extracts were prepared by a standard protocol. The protein concentration of the extracts was estimated by the method of Bradford, with bovine serum albumin used as a standard.[21]

Estimation of Cell Proliferation

Cell proliferation assay was performed by the detection of 5-bromo-2′-deoxy-uridine (BrdU)-labeled DNA by use of anti-BrdU MoAb labeled with peroxidase (Cell Proliferation ELISA, Roche, IN, USA).[9,22] In short, MC3T3-E1 cells grown in a 96-well microtiter plate (3×10^3cells/well) were incubated with different concentrations of PDTC (0–125 μM) for 24 hr at 37°C in a humidified atmosphere (5% CO_2), and were then labeled with BrdU for 1 hr. The amount of BrdU-labeled DNA was quantified by an ELISA.

Estimation for Cellular DNA Fragmentation

Cellular DNA fragmentation was assayed by the detection of BrdU-labeled DNA fragments in the cytoplasm of cell lysates by use of solid phase-immobilized anti-DNA MoAb and anti-BrdU MoAb labeled with peroxidase (cellular DNA fragmentation ELISA, Roche, IN, USA).[23,24] The cells were incubated in the presence of PDTC (0–125 μM) for 8 hr at 37°C in a humidified atmosphere (5% CO_2). The amount of BrdU-labeled DNA was quantified by the ELISA.

Analysis of Cell-Cycle with a Fluorescence-Activated Cell Sorter (FACS)

Cell-cycle analysis was made with the FACS and a cell-cycle analysis kit (Cycle Test plus, Becton Dickinson, San Jose, CA, USA), as described previously.[24]

Assessment of the Osteoblastic Phenotype

To assess the phenotype of the cultured cells, we examined them for alkaline phosphatase (ALP) activity and responsiveness to parathyroid hormone (PTH).[24,25] ALP activity was determined by use of Blue-Phos substrate (microwell phosphatase substrate system: Kirkegaard & Perry Lab., MD, USA). The response to PTH was determined by measuring the amount of cAMP produced by the cells, with or without exposure to 200 ng/mL human PTH (hPTH [1–34], Peptide Institute Inc., Japan) for 15 min. The concentration of cAMP in the cells was measured by an ELISA (Cayman Co., USA).

Statistical Analysis

Data were presented as the mean ± S.D. of 4–6 cultures/group. Each experiment was repeated 3 times. Differences between control and treatment groups were determined by using the paired Student's *t* test. Differences were considered significant if P was <0.05.

RESULTS

Modulation of NGF Production in MC3T3-E1 Cells by PDTC

To examine the possibility that the osteoblastic cells synthesize and release NGF in addition to PDTC, we examined the effects of PDTC on osteoblastic NGF synthesis and secretion. The potent production of NGF was induced by a relatively low concentration of PDTC (maximum at 16 μM, Fig. 1a), as determined by the use of a specific NGF ELISA. The PDTC-induced release of NGF protein into the conditioned medium was also confirmed to occur in a time-dependent manner, with the release being 50-fold higher than the control (Fig. 1b).

For evaluation of the potential and direct relationship between NGF production and NF-κB signaling pathways in MC3T3-E1 cells, an ELISA for NF-κB subunits p50 was performed. The addition of PDTC (16 μM) to MC3T3-E1 cells resulted in the suppression of NF-κB/p50 translocation to the nucleus ($^*P < 0.05$, Fig. 1c), thereby suggesting a role for the NF-κB

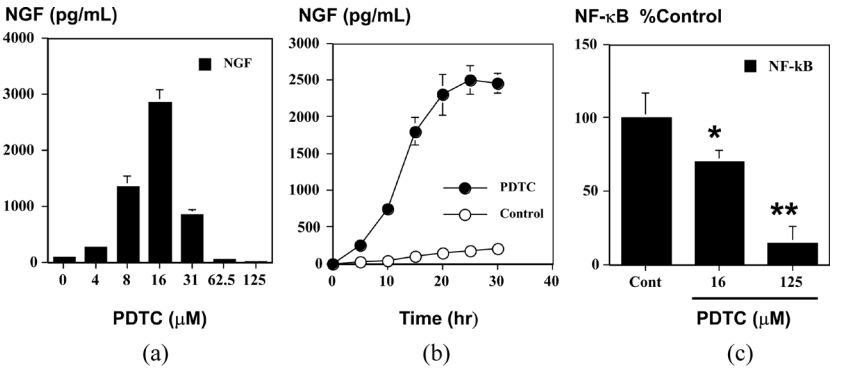

FIGURE 1 Induction of NGF in MC3T3-E1 cells by PDTC. (a) Concentration-dependent production of NGF. MC3T3-E1 cells were cultured for 24 hr in the presence of PDTC (0–125 μM). Conditioned medium was harvested from cultured cells. The content of NGF in the conditioned medium was determined by an ELISA. Values are expressed as the mean ± S.D. of 6 wells (ng/mL). Each experiment was repeated 3 times, and the results shown are representative of these 3 independent experiments. (b) Time-dependent production of NGF. Cells were incubated for the indicated times in the presence or absence of PDTC (16 μM). The content of NGF in the conditioned medium was determined by an ELISA. Values are expressed as means ± S.D. of 6 wells. (c) The involvement of NF-κB on the NGF production induced by PDTC Cells were incubated for 24 hr in the presence or absence of PDTC (16 μM or 125 μM). The NF-κB/p50 content in the extracts was determined by use of a commercially available assay kit. Nuclear protein extracts were prepared by a standard protocol. Values are expressed as means ± S.D. of 6 wells. Differences between the control and PDTC (16 μM or 125 μM) were evaluated by using the paired Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

cascade in regulating the induction of NGF in osteoblastic cells. In contrast, PDTC (125 μM) completely inhibited both NF-κB/p50 translocation and NGF production in the cells with statistical significance.

Modulation of Proliferation and Apoptosis in MC3T3-E1 Cells by PDTC

Upon activation by a wide variety of stimuli, NF-κB is translocated to the nucleus, where it modulates the expression of target genes involved in cell growth, survival, adhesion, and death.[10–13] Furthermore, it is widely known that PDTC itself has several toxic effects, especially as an apoptosis inducer in different cell types.[15–17] Recent studies have demonstrated that the induction of PDTC-mediated apoptosis occurs through a pro-oxidative pathway.[18] In accordance with previous reports, a high concentration of PDTC (125 μM) resulted in statistical significant inhibition of cell proliferation and induction of DNA fragmentation, as an index of apoptosis, in the cells (Fig. 2a and 2b). However, a relatively low concentration of PDTC (4–31 μM), which was suitable for NGF induction, had no effect on proliferation or apoptosis in the cells.

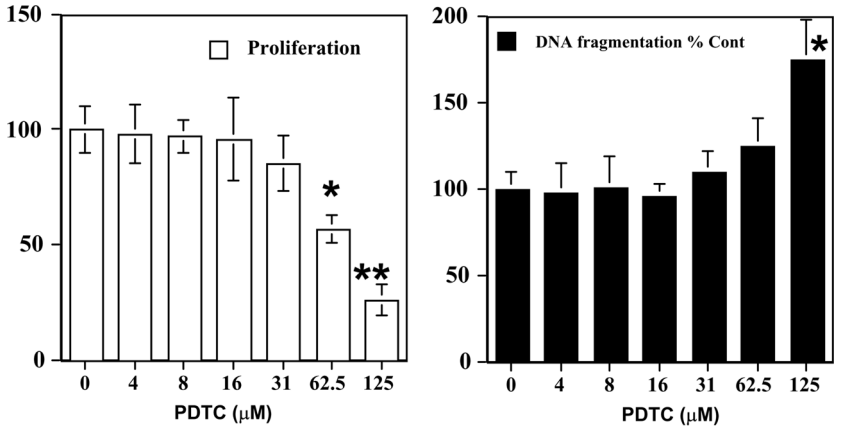

FIGURE 2 (a) Proliferation of MC3T3-E1 cells induced by PDTC. MC3T3-E1 cells were incubated for 24 hr in the presence of PDTC (0–125 μM). The cells were then incubated with BrdU for 1 h, and the incorporated BrdU was detected with anti-BrdU antibody labeled with peroxidase (Cell proliferation ELISA: Roche, USA). Values represent the mean ± S.D. of 4 independent determinations performed. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (PDTC: 0 μM). (b) PDTC induced-DNA fragmentation in MC3T3-E1 cells. MC3T3-E1 cells were incubated for 8 hr in the presence of PDTC (0–125 μM). Levels of apoptosis were quantitatively determined by cellular DNA fragmentation ELISA. Values represent the mean ± S.D. of 4 independent determinations performed. $^{*}P < 0.05$ vs. control (PDTC: 0 μM).

Modulation of Differentiation in MC3T3-E1 Cells by PDTC

To examine the possibility that PDTC induces the differentiation of osteoblastic cells, we performed further experiments. When a cell moves into a quiescent and/or terminally differentiated state from G1, it clearly enters a different metabolic state. In general, G0/G1 arrest in the cell cycle is induced by apoptotic stimulators and/or occurs in cells in the terminally differentiated state. FACS cell-cycle analysis demonstrated that treatment with PDTC (16 μM:maximum and suitable concentration for NGF induction) did not result in potent G0/G1 arrest in MC3T3-E1 cells (population rate: 64.5 ± 5.5% in control vs. 65.8 ± 6.8% in 16 μM PDTC). In contrast, a relatively high concentration of PDTC (125 μM) induced substantial G0/G1 arrest in the cells (78.9 ± 6.5%; $^{*}P < 0.05$ compared with the control). Taken together with Figs. 1 and 2, PDTC (125 μM) resulted in apoptotic cell death in the cells and led to potent suppression of NGF synthesis.

Further, PDTC (16 or 125 μM) had no effects on ALP expression or responsiveness for PTH-elicited cAMP production as phenotypes of osteoblastic cells (data not shown), suggesting that PDTC had no effects on the differentiation in MC3T3-E1 cells.

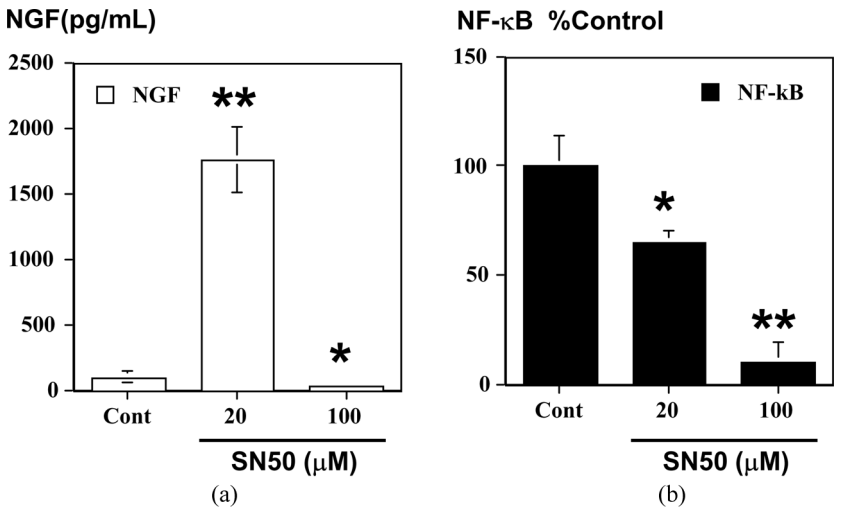

FIGURE 3 Involvement of NF-κB in the NGF production induced by cell membrane-permeable SN50 peptide. (a) Cells were incubated for 24 hr in the presence or absence of SN50 (20 μM or 100 μM). The content of NGF in the conditioned medium was determined by an ELISA. Values are expressed as means ± S.D. of 6 wells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. (b) The NF-κB content in the extracts was determined by use of a commercially available assay kit. Nuclear protein extracts were prepared by a standard protocol. Values are expressed as means ± S.D. of 6 wells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

Modulation of NF-κB and NGF Production in SN50-Treated MC3T3-E1 Cells

Since little insight is available concerning the signal cascade triggered by PDTC in osteoblastic cells, further experiments were performed. Cell membrane-permeable SN50 (a specific NF-κB inhibitor peptide: 20 μM) also resulted in a suppression of NF-κB/p50 subunits translocation to the nucleus ($^{*}P < 0.05$ compared with the control; Fig. 3b). In accordance with data obtained by using PDTC (16 μM), as shown in Fig. 1, SN50 (20 μM) induced NGF production in MC3T3-E1 cells ($^{**}P < 0.01$ compared with the control; Fig. 3a). Further, SN50 (100 μM) potently inhibited both NF-κB/p50 translocation and NGF production with statistical significance (Fig. 3b).

DISCUSSION

The results of the current study show that the suitable suppression of nuclear NF-κB translocation induced the profound production of NGF in mouse osteoblastic cell line MC3T3-E1. Although MC3T3-E1 cells produce only a small amount of NGF under the basal condition, a relatively low concentration of PDTC (16 μM) induced NGF protein up to 50-fold higher

than the control. Although several inducers of NGF are widely known, such as tumor necrosis factor-α, interleukin-1β, and basic-fibroblast growth factor,[26–28] we demonstrated for the first time that PDTC has the potential to induce NGF in osteoblastic cells. In general, NF-κB nuclear translocation is accompanied by apoptosis, and/or inflammation; whereas, the present experimental design (low concentration of PDTC: 4–31 μM) had no effects on apoptosis, differentiation, the cell cycle, or proliferation in mouse MC3T3-E1 cells (Fig. 2). Since treatment with cell membrane-permeable SN50 (a specific NF-κB inhibitor peptide) also resulted in a profound suppression of NF-κB translocation to the nucleus and induced NGF production (Fig. 3), we confirmed that the down-regulation of NF-κB up-regulated NGF production in this mouse osteoblastic cell line. In addition, since PDTC also induced NGF protein in mouse stromal cell line ST2, this effect is not limited to mouse osteoblastic MC3T3-E1 cells (data not shown).

As a novel feature of mouse osteoblastic cells, the cells continuously express the neurotrophins of the NGF family (NGF and BDNF), as well as the neurotrophin receptors trk-a, and trk-b.[9] Because NGF was also shown to rescue other cultured cells from apoptosis,[29,30] the released NGF might play an important role as an endogenous anti-apoptotic effector. NGF might, thus, have a special and unknown physiological significance in mouse osteoblastic cells.

What is the means of suppression of NF-κB nuclear translocation in the cells? The prototypical NF-κB complex is a heterodimer composed of p50 and p65.[10] Activated NF-κB (p50 and p65) translocates to the nucleus and promotes transactivation of target genes and release cytokines. Depending on the cell type, NF-κB has been reported to mediate or prevent apoptosis.[11–13] Previous findings from our laboratory showed that application of exogenous proinflammatory cytokines could specifically initiate apoptotic cell death in MC3T3-E1 cells and induce NGF.[9,19,22,23] We also confirmed that proinflammatory cytokine induced NF-κB/p50 translocation to nucleus in the cells (data not shown). On the contrary, we found the phenomenon that the suitable suppression of NF-κB/p50 translocation triggered potent NGF production in the cells. Although we have definitely no answer about it, the pathway (the suppression of NF-κB/p50 leading to NGF induction) is very unique. Further, previous evidence indicates that mice deficient in both NF-κB subunits p50 and p52 have retarded growth and shortened long bones,[13] suggesting that NF-κB exerts a regulatory role in bone growth and development. In accordance with those findings,[13] we confirmed that PDTC (125 μM, a relatively high concentration) induced potent down-regulation of NF-κB that resulted in the suppression of proliferation and the induction of apoptosis in the cells (Figs. 1 and 2). PDTC (125 μM) completely inhibited the

production of NGF and NF-κB translocation to the nucleus (Fig. 1), suggesting that the sensitive and suitable regulation of NF-κB translocation contributed to NGF production in osteoblastic cells. Since PDTC is reported to inhibit ubiquitin-proteasome-mediated proteolysis, and antioxidant, the signal cascade for NGF production in detail remained to be elucidated.

In conclusion, the present results underline the unique cascade leading to NGF production, suggesting that mouse osteoblastic MC3T3-E1 cells secrete NGF through the suppression of NF-κB nuclear translocation. Thus, NF-κB emerges as a central and endogenous mediator of NGF production in osteoblastic cells.

ACKNOWLEDGMENTS

This study was partly supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (no.18592052 to M.M. and no.19791377 to A.K.).

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