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Upregulation of NF-κB upon differentiation of mouse embryonic stem cells

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NF-κB is a transcriptional regulator involved in many biological processes including proliferation, survival, and differentiation. Recently, we reported that expression and activity of NF-κB is comparatively low in undifferentiated human embryonic stem (ES) cells, but increases during differentiation. Here, we found a lower expression of NF-κB p65 protein in mouse ES cells when compared with mouse embryonic fibroblast cells. Protein levels of NF-κB p65 and relB were clearly enhanced during retinoic acid-induced differentiation. Furthermore, increased DNA binding activity of NF-κB in response to TNF-α, an agonist of NF-κB signaling, was seen in differentiated but not undifferentiated mouse ES cells. Taken together with our previous data in human ES cells, it is likely that NF-κB expression and activity of the NF-κB signaling pathway is comparatively low in undifferentiated ES cells, but increases during differentiation of ES cells in general. [BMB reports 2008; 41(10): 705-709]

INTRODUCTION

Embryonic stem (ES) cells have the ability to self-renew, maintaining their stemness on mouse embryonic fibroblast (MEF) cells (1, 2), and have pluripotent differentiation ability so as to produce multiple tissue lineages under specific conditions (3-5). Studies on ES cells are producing insights into embryonic development and will provide critical information on these promising tissue sources for use in cell therapy (6, 7).

Mouse and human ES cells have differences in morphology, patterns of embryonic antigen immunostaining, expression of differentiation markers, and population doubling times (8). Mouse ES cells can be cultured and kept in an undifferentiated state and maintain their pluripotency when grown in culture medium containing leukemia inhibitory factor (LIF), but this is not the case for human ES cells (9, 10). Mouse and human ES cells also have many similar features: they express classical markers of pluripotent stem cell lines such as Oct4, Nanog, and alkaline phosphatase (AP) as well as display high levels of telomerase activity (2, 11, 12). As studies on mouse ES cells have several advantages over human ES cells, mouse ES cells are a useful model system even though the results from mouse ES cell experiments may have limited translation for application in humans (13, 14).

NF-κB, an inducible dimeric transcription factor that belongs to the Rel family of transcription factors, is a major mediator of the cellular response to a variety of extracellular stimulation and is involved in diverse biological processes including embryo development, hematopoiesis, and immune regulation, as well as neuronal functions via the induction of certain growth and transcription factors (15, 16). There are five different Rel/NF-κB proteins expressed in mammals: p65 (RelA), p50 (NF-κB1), p52 (NF-κB2), c-Rel (Rel), and RelB (17). These NF-κB proteins form homo- or heterodimers and are bound in the cytoplasm by the inhibitor of κB proteins (IκB) (18). Infection or inflammation signals activate the IκB kinase (IKK), and this activated IKK complex phosphorylates IκB at two conserved serines within the IκB N-terminus. Phosphorylated IκB becomes a target for ubiquitination and subsequent proteosomal degradation (19). Subsequently, the freed NF-κB dimers translocate to the nucleus where, as a transcription factor, they activate the expression of genes involved in cell growth, differentiation, and proliferation (20, 21). However, little is known about the role of NF-κB in ES cell functions. Recently, we reported that expression and activity of the transcription factor NF-κB was enhanced during differentiation of human ES cells (22). In this study, we investigated expression and activity of NF-kB in mouse ES cells to see if our result from humans is relevant to different species. We found that the expression and activity of NF-κB protein was up-regulated upon differentiation of mouse ES cells.

RESULTS AND DISCUSSION

Low expression of NF-κB in mouse ES cells

NF-κB complexes of p50/p65 heterodimers are rapid response transcription factors influencing the expression of multiple genes (20, 21). To evaluate the contribution of NF-κB signaling in mouse ES cells, we compaired expression of NF-κB p50 and

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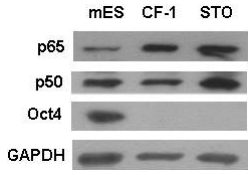

Fig. 1. NF-κB expression in mouse ES cells and mouse embryonic fibroblast cells. Protein samples from the mouse ES cell line TC-1, primary MEF cells from CF-1 mice, and the MEF cell line STO were prepared and resolved by SDS-PAGE. Protein expression levels of NF-κB p50 and p65 were determined by Western blotting. The Oct4 protein was used as a stem cell marker. The amount of GAPDH protein was used as a loading control.

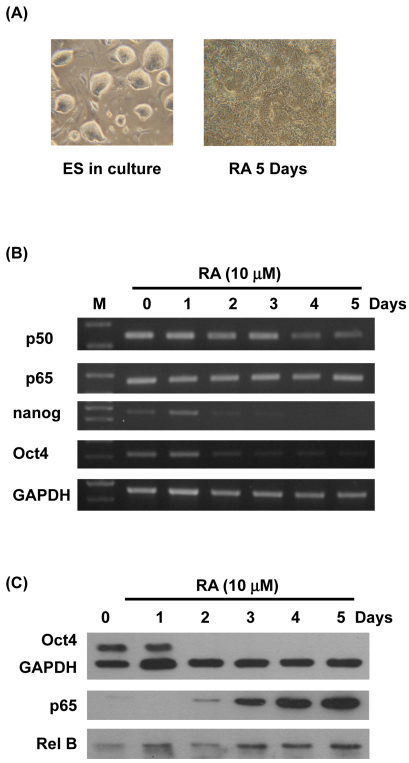

Fig. 2. Expression levels of the NF-κB protein during differentiation of mouse ES cells. Undifferentiated cells were maintained on MEFs, and differentiation of mouse ES cells was induced by 10^{-5} M of retinoic acid (ES/RA) for the indicated time periods. (A) Morphology of ES cell colonies and RA-differentiated cells. (B) RT-PCR analysis of stem cell markers and NF-κB p50 and p65 during differentiation. (C) Protein expression levels of NF-κB p65 and RelB were determined by Western blotting. Oct4 was used as a stem cell marker. The amount of GAPDH was used as a loading control.

p65 proteins in the mouse ES cell line TC-1 with primary CF-1 mouse embryonic fibroblast cells and a mouse embryonic fibroblast cell line, STO (23, 24). As shown in Fig. 1, expression levels of NF-κB p65 in the mouse ES cells was significantly lower than the other cells. Expression of NF-κB p50 was similar to that of p65. In contrast, expression of Oct4, an essential gene for ES cell self-renewal (25), was clearly detected only in the mouse ES cells. The results obtained from another mouse ES cell line, R1, were similar (data not shown).

Enhanced expression of NF-κB in differentiated mouse ES cells

As the expression levels of NF-κB p50 and p65 were lower in mouse ES cells relative to other mature cells, we decided to induce differentiation of mouse ES cells and compare expression levels of NF-κB in differentiated cells and undifferentiated ES cells. We used retinoic acid as a differentiation inducing reagent and cultured ES cells for up to 5 days. As shown in Fig. 2A, the morphology of the differentiated cells clearly differs from the undifferentiated ES cells grown on feeder cells. When we examined expression of the NF-κB p50 and p65 genes by RT-PCR, expression of NF-κB p50 and p65 mRNA in differentiated mouse ES cells was comparable to undifferentiated cells (Fig. 2B). However, expression of NF-κB p65 proteins markedly increased with time during the differentiation process (Fig. 2C). In contrast, expression of NF-κB p50 was unchanged. It is likely that post-transcriptional control is critical for the regulation of NF-κB p65 during differentiation. Additionally, we found that RelB protein levels also increased during differentiation. As expected, Oct4 protein levels were markedly decreased during differentiation in a time-dependent manner. The results from the two different mouse ES cell lines, TC-1 and R1, were similar. Therefore, we can conclude that expression of NF-κB in undifferentiated mouse ES cells is lower than other cells and gradually increases during retinoic acid-induced differentiation. Studies on the promoter regions of genes encoding the NF-κB/Rel/IκB family suggest that a high number of NF-κB binding sites can assist in their self- and inter-family regulation (26, 27). This is in accordance with the similar expression patterns observed for NF-κB p65 and relB in our study.

Expression of TNF receptor 1 and activation of the NF-κB signaling pathway in differentiated mouse ES cells

As expression of NF-κB p65 at the protein level was very low in mouse ES cells, but was increased after induced differentiation, it is likely that activation of the NF-κB signaling pathway is more prominent in differentiated cells than in undifferentiated ES cells. TNF-α is a pleiotrophic cytokine that possesses strong proinflammatory and immunostimulatory activities. TNF-α can induce various cellular responses, from proliferation to apoptosis, depending on the cell type and cellular context (28). As it is widely known that TNF-α activates NF-κB signaling as well as MAP kinase pathways in many cells, we checked the TNF-α response of ES cells before and after differentiation (28). First, we checked expression of the cellular receptors for TNF-α TNFR1 and TNFR2, by RT-PCR. We found comparable expression of TNFR1 both in undifferentiated and differentiated mouse ES cells (data not shown). Expression of TNFR2 was not detected in either of these cell types. This is in agreement with previously reported our human data (22). As TNFR1 is expressed, we consid-

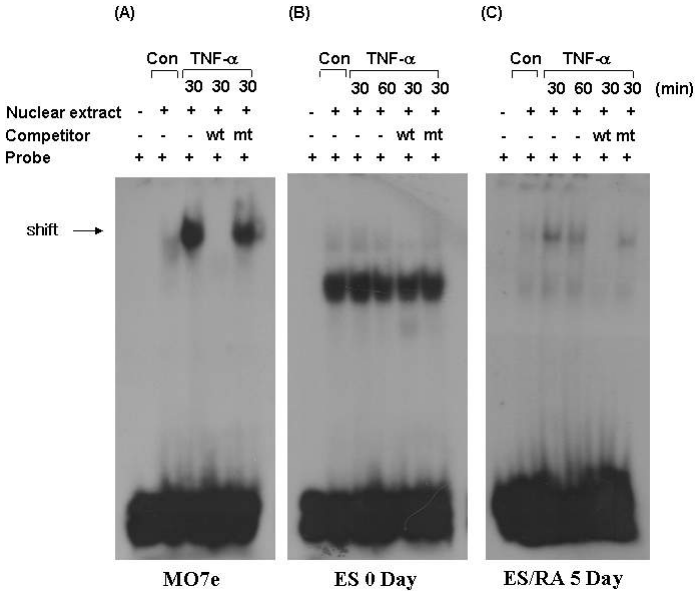

Fig. 3. DNA binding activity of NF-κB in differentiated TC-1 cells after stimulation with TNF-α. MO7e cells before and after 30 min of stimulation with TNF-α (10 ng/ml) were used as positive controls (A). TC-1 cells maintained on MEFs (B) and the cells differentiated from TC-1 cells in the presence of retinoic acid for 5 days (C) were stimulated with control medium or TNF-α for the indicated time periods. Nuclear extracts were prepared and gel shift assays were performed using consensus oligonucleotides for the NF-κB binding site. Competition assays were performed with cold probe or mutant oligonucleotides.

ered that TNF-α might initiate upstream signaling both in undifferentiated and differentiated mouse ES cells.

In order to investigate NF-kB activation in response to TNF-α, we investigated the DNA binding activity of NF-κB after stimulation with TNF-α (29, 30). A human growth factor dependent cell line, MO7e, was used as a positive control (Fig. 3A). DNA binding activity of NF-κB was clearly detected after TNF-α stimulation in MO7e cells (30). In undifferentiated mouse ES cells, DNA binding activity of NF-κB was not detected before or after stimulation with TNF-α (Fig. 3B). Interestingly, strong non-specific bands were consistently observed in repeated experiments. In contrast, an increase in NF-κB binding activity following TNF-α stimulation was clearly seen in ES-derived differentiated cells (Fig. 3C) These results suggest that lower expression of NF-κB may be associated with lower activity of NF-κB signaling in undifferentiated ES cells relative to differentiated cells. As we can see in Fig. 3A and 3C, induced NF-κB binding activity of ES-derived differentiated cells was much weaker than that of MO7e cells. This may be in part a result of lower expression of NF-κB proteins or lower expression of the TNF receptor as previously reported (31).

In this study, we investigated the expression levels of NF-κB in undifferentiated mouse ES cells and found comparatively low expression of NF-κB in mouse ES cells and upregulation of NF-κB during differentiation induced by retinoic acid. This is exactly the same conclusion that we previously drew from experiments in human ES cells (22). Therefore, it is possible that this phenomenon is common to ES cells from different species. Even though NF-κB expression and its activity are low, we still cannot exclude the possibility that NF-κB plays some role in undifferentiated ES cells. A potential contribution of NF-κB signaling to self renewal of human ES cells was suggested based on the higher expression level of phospho-p65(Ser536), as determined by immunostaining and confocal microscopy, along with the results obtained from inhibitor studies (32). NF-κB signaling was also suggested to be involved in the increase of glucose uptake upon hydrogen peroxide treatment in mouse ES cells, which was determined by immunostaining and inhibitor studies (33). Taken together with its low expression in ES cells, an attempt to increase NF-κB expression or activity in ES cells without impairing their stemness may be one of strategy for achieving better maintenance of ES cells.

MATERIALS AND METHODS

Maintenance of mouse ES cells

Mouse ES cell lines TC-1 and R1 were maintained on mouse embryonic fibroblasts (MEF) in ES medium, which contains Dulbecco's modified Eagle's medium (DMEM), 15% fetal bovine serum (FBS, Hyclone Inc., Logan, UT, USA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cultures were passaged as cells became confluent (i.e., about two or three times a week). ES cells were dissociated with 0.025% trypsin-EDTA and then seeded on MEF prepared as follows. MEF cells were harvested and irradiated with 30 Gy and seeded at a density of $\sim 5.5 \times 10^4$ cells/ml in MEF medium (DMEM, 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids) a day before ES cell seeding.

Differentiation of mouse ES cells with retinoic acid and stimulation of the differentiated cells with TNF-α

Plates were coated with gelatin (Sigma Aldrich, St. Louis, MO,

USA) diluted at a ratio of 1:20 (final 0.1%) in PBS at room temperature for 1hr. MEF-conditioned medium (CM) was prepared by harvesting the overnight culture medium from the irradiated MEF cell cultures. $5 \times 10^5 \sim 1 \times 10^6$ mouse ES cells were placed into gelatin-coated 6-well plates and grown overnight with MEF-CM in feeder free condition (24). Differentiation of mouse ES cells was stimulated by treating the cells in MEF medium with 10^{-5} M retinoic acid (*all-trans*, Sigma-Aldrich) for 5 days.

In order to examine intracellular signaling in mouse ES cells in response to TNF-α, cells were washed with MEF medium and treated with TNF-α at a concentration of 10 ng/ml (CytoLab, Rehovot, Israel).

RT-PCR analysis

Total RNA was isolated using TRI REAGENT® according to the instructions provided by the manufacturer (MRC, Cincinnati, OH, USA). 5 μg of total RNA was reverse-transcribed in first-strand buffer containing 6 μg/ml oligo (dT) primer, 50 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 2 mM dNTP, and 40 U RNase block ribonuclease inhibitor. The reaction was conducted at 42 °C for 1 h. One microliter of the cDNA synthesis was subjected to the standard PCR reaction for 30 cycles of denaturation for 60 sec at 95 °C, annealing for 60 sec at 58 °C, and elongation for 60 sec at 72 °C. The primer sequences used were as follows. GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (anti-sense). Oct4, 5′-GAGAACAATGAGAACCTTCAGGAGA-3′ (sense) and 5′-TTCTGGCGCCGGTTACAGAACCA-3′ (antisense). Nanog, 5′-AGGGTCTGCTACTGAGATGCTCTG-3′ (sense) and 5′-CAACCACTGGTTTTTCTGCCAC-3′. NF-κB p65, 5′-CTTGGCAACAGCACAGACC-3′ (sense) and 5′-GAGAAGTCCATGTCCGCAAT-3′ (antisense). NF-κB p50, 5′-AGGAAGAAAATGGCGGAGTT-3′ (sense) and 5′-GCATAAGCTTCTGGCGTTTC-3′ (antisense). TNFR1, 5′-GTGTCCCCAAGGAAAATATATCCAC-3′ (sense) and 5′-AAAGGCAAAGACCAAAGAAAATGAC-3′ (antisense). TNFR2, 5′-GGATAAAGGAGAAGGCATGAAATTG-3′ (sense) 5′-AACTTTCATTGTCTTGGGATCAACA -3′ (antisense).

Western blotting

Cells on culture plates were washed with PBS once and directly lysed in 50 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/mL aprotonin, 10 mM EDTA, 10 μg/mL leupeptin, 100 mM NaF, 2 mM Na_3VO_4, and 1% NP-40). Samples were resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp, Bedford, MA, USA). Membranes were blocked with 5% dry milk and probed with the appropriate primary antibody. Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody and an ECL reagent (iNtRon, Seongnam, Korea). Membranes were stripped and then probed with another primary antibody when necessary. Antibodies to NF-κB p65 (C20) were purchased from Delta Biolabs (Vandell Way Campbell, CA, USA). Antibodies to GAPDH (6C5) and NF-κB p50 (E-10) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The antibody against RelB was acquired from Cell Signaling Technology (Beverly, MA).

Electrophoretic mobility shift assay

Nuclear extracts from untreated and TNF-α-treated cells were prepared as previously described (34). The electrophoretic mobility shift assay (EMSA) was performed using a Nushift NF-kB assay kit (Active Motif, Tokyo, Japan) according to the manufacturer's protocol. 20,000 cpm of ^{32}P-labeled consensus NF-kB binding sequence (5′-AGCTTGGGGTATTTCCAGCCG-3′) was incubated with 20 μg of nuclear-extract proteins at 4°C for 30 min and then resolved on 4% polyacrylamide gels containing 0.25X TBE (1X TBE: 89 mM Tris borate and 1 mM EDTA, pH 8.0) and 2.5% glycerol. The oligonucleotide competition assay was performed by preincubating nuclear extracts with 100-fold excess cold NF-kB consensus or mutant (5′-AGCTTGGcaTAggTCCAGCCG-3′, mutated sequences are shown in lower case) oligonucleotides for 30 min at 4 °C before the addition of labeled probe.

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