Activity modulation of the nuclear factor kB by post-translational modifications in association with granulocytic differentiation of HL-60 cells

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The nuclear factor κB (NFκB) is one of the important transcriptional regulatory proteins interacting with many genes and has been implicated in cellular programs such as growth and differentiation. The present study examines the influence of post-translational modifications (phosphorylation, acetylation, glycosylation) on activation of the NFκB during HL-60 cell differentiation induced by retinoic acid (RA). We have shown that the binding properties of NFκB to DNA elements in promoter/enhancer regions of myeloid genes gradually increased during maturation of leukemic cells to granulocytes. Treatment of HL-60 cells with specific inhibitors of protein kinases and protein phosphatases (lavendustin, Go6976, Go6983, sodium vanadate), or histone deacetylases (sodium butyrate), or inhibitors of O- and N-glycosylation (IPTG, tunicamycin) resulted in a marked influence on cell growth, differentiation and binding activity of NFκB, which was modulated predominantly in the commitment stage of HL-60 cell differentiation. These results suggest that post-translational modifications may regulate myeloid genes by involvement of NFκB.

Key words: transcription factors, leukemia, differentiation

INTRODUCTION

Granulocytic differentiation includes the production of myeloblasts, promyelocytes, myelocytes, and then neutrophils. Leukemia occurs when the homeostasis of normal hematopoiesis is disrupted. The factors that regulate these events have not been completely elucidated but include growth factors that permit cell proliferation and nuclear regulators (transcription factors) that activate lineage-specific genes. Diverse transcription factors perform important regulatory functions in myelopoesis. NFκB proteins are one of the regulators of cellular programs [1]. In mammals this protein family includes p50, p52, p65 (Rel), c-Rel, and Rel B [2]. In the inactive state, NFκB proteins occur as homodimeric or heterodimeric complexes in the cytoplasm bound to inhibitory IκB proteins. After appropriate stimulation, IκB is phosphorylated, ubiquinated, and degraded, which allows translocation of NFκB to the nucleus and transcription of NFκB-target genes [2,3]. It has been documented that activation of NFκB plays a pivotal role in many cellular processes, including inflammation, cell proliferation and apoptosis [4]. Stimulus-dependent post-translational modifications of NFκB complex has been reported to modulate transcriptional activation of NFκB-dependent genes and to provide a further point of regulation in this pathway [5]. Here we examined the role of post-translational modifications on NFκB activity in association with granulocytic differentiation of promyelocytic leukemia HL-60 cells and demonstrated the involvement of NFκB in the process of myelopoiesis.

MATERIALS AND METHODS

Cell culture. Human promyelocytic HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C and used for assays during the exponential phase of growth. The degree of differentiation was assayed by the ability of cells to reduce nitro blue tetrazolium (NBT) after stimulation with PMA [6].

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Electrophoretic mobility shift assays (EMSA). Nuclear extracts from exponentially growing HL-60 cells induced to differentiate by 1 μM retinoic acid (RA) were prepared according to previously described methods [7]. EMSAs were performed by incubating 10 μg nuclear extracts with the double-stranded oligonucleotide containing consensus binding sites for NFκB labeled with [γ-^{32}P-ATP] by using T4 polynucleotide kinase as previously described [8]. For competion analysis, a 50-fold molar excess of unlabeled oligonucleotide was added to the nuclear extracts before addition of a labeled probe. Binding reactions were resolved on a 5% nondenaturating polyacrylamide gel and electrophoresed in 0.5x Tris-borate buffer. The dried gels then were exposed to X-ray films overnight at –70 °C.

RESULTS AND DISCUSSION

To determine whether NFκB induction was associated with granulocytic differentiation, we exposed HL-60 cells to 1 μM RA for 5 days and performed EMSA daily. In uninduced HL-60 cells, there was a low level of nuclear binding to the NFκB consensus sequence, as determined by EMSA (Fig. 1A). In contrast, treatment of these cells with RA was associated with gradual increase in nuclear protein binding to the NFκB oligonucleotide. The intensity of the retarded fragment related to the p50 component of the complex increased during 5 days of induction by RA. Furthermore, addition of the unlabeled NFκB oligonucleotide at a 50-fold excess, compared with the labeled fragment, resulted in complete disappearance of the retarded band.

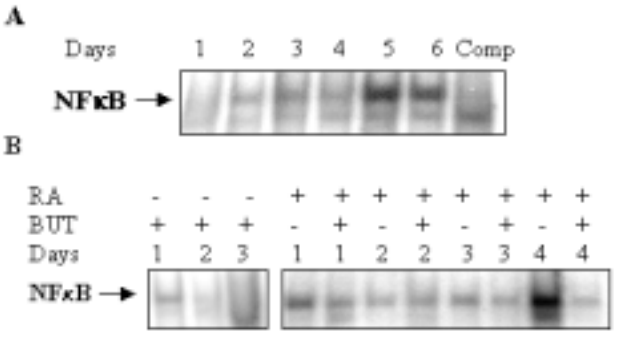

Fig. 1. NFκB-binding activity in HL-60 cells exposed to RA or sodium butyrate during granulocytic differentiation. Nuclear extracts were prepared from HL-60 cells induced by RA for 6 days (A) or from proliferating (first panel) and differentiating (second panel) cells exposed to histone deacetylases inhibitor But (B) for 3 and 4 days, respectively. EMSAs were performed using 10 μg of nuclear proteins and 1 pM ^{32}P-labeled oligonucleotide containing the NFκB consensus sequence. Arrows indicate the NFκB specific complexes abolished by addition of 50-fold molar excess of cold competitor (comp)

Since transcription factors consist of various members of proteins, which are translated on cytoplasmic ribosomes, post-translational modifications of these proteins in the nucleus may be responsible for altered activity of these factors. Previous work [5] has indicated that protein phosphorylation is at least one of such modifications. In order to verify this assumption, similar studies were performed with nuclear proteins from RA-induced cells exposed to

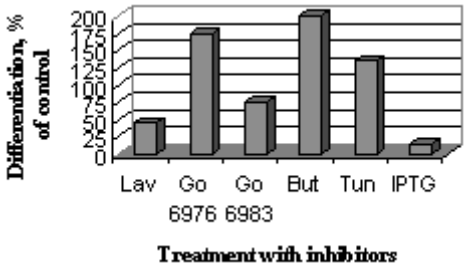

Fig. 2. Modulation of the level of HL-60 cell differentiation by post-translational modifications. HL-60 cells induced by RA were treated for 5 days with specific inhibitors of phosphorylation (Lav, Van, Go6976, G6983), glycosylation (Tun, IPTG) or histone deacetylases (But). Cell differentiation was determined by NBT reducing ability on day 5

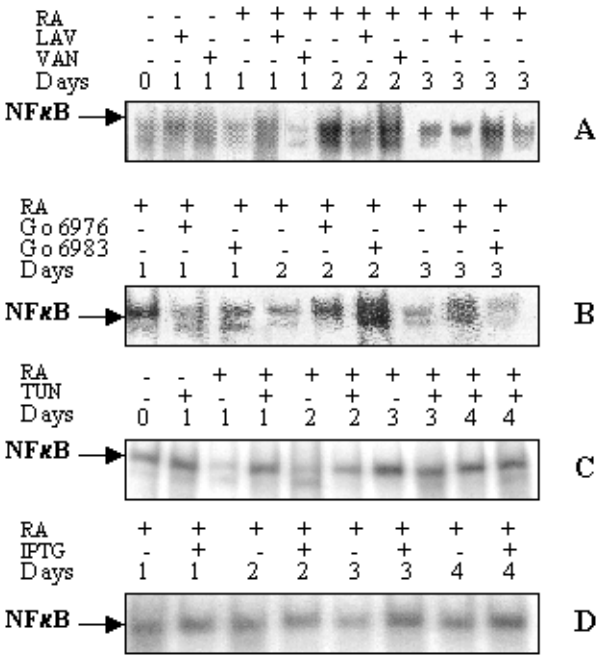

Fig. 3. Modulation of NFB-binding activity by inhibitors of phosphorylation and glycosylation. HL-60 cells induced by RA were treated for indicated time with specific inhibitors of phosphorylation: Lav or Van (A), Go6976 or Go6983 (B); or glycosylation: Tun (C) or IPTG (D). EMSAs were performed using 5–10 μg of nuclear proteins and 1 pM ^{32}P-labeled oligonucleotide containing the NFκB consensus sequence. Arrows indicate the NFκB specific complexes

the inhibitor of tyrosine protein kinase, 25 μM lavendustin (Lav), or inhibitor of tyrosine phosphatase, 100 μM sodium vanadate (Van). Lav about 2-fold inhibited RA-induced HL-60 cell differentiation (Fig. 2), while Van caused a drastical decrease of cell viability at day 3 (data not shown). Treatment with Lav of uninduced or RA-induced HL-60 cells was associated with an increase in nuclear protein binding to NFκB oligonucleotide during 2 days of exposure and a decrease at day 4 (Fig. 3A). Exposure to Van for 48 h did not noticeably alter the binding pattern of NFκB in any treatment of cells. This shows that inhibition of tyrosine phosphorylation, which is important for growth of leukemic cells, upregulated the activity of NFκB binding to myeloid promoters during commitment stage of differentiation, and this is negatively associated with granulocytic differentiation. In the cells with dephosphorylated status NFB complexes appear to participate in the abrogation of a signal for differentiation.

Previous studies have implicated serin/threonin protein kinase C (PKC) in the activation of NFκB by phosphorylation of IκB [9]. The cells express different isozymes of PKC with respect to activation that may mediate distinct cellular events [9]. To assess the influence of serine/threonine phosphorylation on NFκB binding activity, HL-60 cells induced to differentiate by RA were exposed to PKC inhibitors: 25 nM Go6976, which specifically inhibits conventional, Ca^{2+}-dependent isoforms (PKC α, β and μ) or 80 nM Go6983, which inhibits PKC α, β, γ, μ and Ca^{2+}-independent isoforms PKC ι, δ. As is shown in Fig. 2, Go6976 enhanced RA-induced HL-60 cell differentiation (170% of control), while Go6983 decreased it by 25%. As is shown, NFκB binding activity decreased following treatment with Go6976 (Fig. 3B) during the first 24 h and increased after the commitment stage. In contrast, treatment with Go6983 for 24 h increased NFκB binding compared to that of Go6976-treated cells, but had a similar response in respect to control. At the beginning of the cell maturation stage on day 3 Go6976-mediated NFκB binding activity was changed *versus* Go6983. These results allow to suggest that conventional isoforms of PKC are responsible for cell maturation but not for the commitment of granulocytic differentiation.

Modification of histones, DNA-binding chromatin proteins, by addition of acetyl groups is associated with transcriptionally active DNA [10]. In understanding how acetylation may alter interactions between the nuclear transcription factor κB and DNA, we incubated HL-60 cells with 250 nM sodium butyrate (But) in the presence or absence of RA. The But alone induced cell differentiation to 40%, and in combination with RA the number of mature cells increased two-fold (Fig. 2). As is shown in EMSA, hyperacetylation caused a direct decrease in NFκB-DNA binding at the beginning of about 24 h of exposure to But of proliferating cells and disappearance of the complex at day 3 (Fig. 1B). The formation of NFκB-DNA complexes was downregulated at day 1 of simultaneous exposure to But and RA, and a slight decrease in binding intensity was seen in the course of differentiation. These data therefore indicate a positive role of acetylation in granulocytic differentiation, but through downregulation of the transcriptional function of NFκB.

O- or N-glycosylation are the modifications that can influence transcription and differentiation processes [11]. For this purpose, HL-60 cells were incubated with the inhibitor of N-glycosylation, 1 μM tunicamycin (Tun) or inhibitor of O-glycosylation, 2 mM isopropylthiolgalactoside (IPTG). IPTG drastically inhibited RA-induced HL-60 cell differentiation (to 15% of control), while Tun increased it to 135% (Fig. 2). DNA binding of NFκB was increased during 48 h of treatment of proliferating and RA-induced cells by Tun. The signal intensity did not decrease during the next 2 days of exposure (Fig. 3C). Thus, binding intensity by Tun was associated with induction of HL-60 cell differentiation. In contrast, there were no significant changes in nuclear protein binding to specific sites of NFκB regulated-myeloid promoters in the cells during 2 days of treatment with IPTG, but such a treatment did produce a greater band intensity in respect to control cells at the stage of cell maturation (Fig. 3D). This implies the possibility that the function of O-linked glycosylation is to remodel the architecture of promoter-bound transcription complexes and to influence the transcription and differentiation processes by recruitment of NFκB.

In summary, the results suggest that post-translational modifications may regulate the activity of myeloid genes by involvement of NFκB.

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POSTTRANSLIACINIŲ MODIFIKACIJŲ POVEIKIS TRANSKRIPCIJOS FAKTORIAUS NFκB AKTYVUMO MODULIAVIMUI IR RYŠYS SU HL-60 LĄSTELIŲ GRANULIOCITINE DIFERENCIACIJA

S a n t r a u k a

Branduolio faktorius κB (NFκB) yra svarbus transkripcijos reguliatorinis baltymas, sąveikaujantis su daugeliu genų, dalyvaujantis ląstelės augime ir diferenciacijoje. Šiame darbe buvo tiriamas posttransliacinių modifikacijų (fosforilinimo, acetilinimo, glikozilinimo) poveikis NFκB aktyvavimui retinoine rūgštimi (RA) indukuotų HL-60 ląstelių diferenciacijos metu. Paaiškėjo, kad NFκB ir DNR sekų mieloidinių genų promotorių srityse ryšio efektyvumas palaipsniui didėja leukeminėms ląstelėms bręstant link granuliocitų. Veikiant HL-60 ląsteles specifiniais proteino kinazių, fosfatazių (lavendustinas C, Go 6976, Go 6983, natrio vanadatas), histonų deacetilazių (natrio butiratas) bei O- ar N-glikozilinimo (tunikamicinas, IPTG) inhibitoriais pastebėtas ryškus poveikis ląstelių augimui, diferenciacijai ir NFκB ryšio efektyvumui, ypač HL-60 ląstelių diferenciaciją nulemiančioje stadijoje. Rezultatai leidžia manyti, kad posttransliacinės modifikacijos gali reguliuoti mieloidinių genų veiklą įtraukiant NFκB.