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ORIGINAL ARTICLE

Role of c-Jun in Tumor Necrosis Factor-alpha Inhibition of Activin A-mediated Erythroid Gene Expression in Erythroleukemia K562 Cells

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Background: The activation of the tumor necrosis factor-alpha (TNF-α)/nuclear factor kappa B (NF-κB) pathway inhibits the expression of erythroid genes, whereas activin A, a member of the transforming growth factor-β superfamily, induces erythroid differentiation. The effect of TNF-α on activin A-induced erythroid gene expression has not been elucidated.
Methods: Luciferase reporter assay and reverse transcription-polymerase chain reaction (PCR) or quantitative PCR were used to investigate globin promoter activity and globin gene expression in the hematopoietic progenitor cell line K562, respectively.
Results: TNF-α inhibited the activin A-induced promoter activity of α-globin and ζ-globin in a concentration-dependent manner in K562 cells. Activin A could reverse the TNF-α-inhibited promoter activity of α-globin and ζ-globin in a concentration-dependent manner. TNF-α decreased the mRNA levels of α-globin, ζ-globin, GATA-1, and NF-E2 p45 induced by activin A. The NF-κB inhibitor, Bay117082, inhibited the TNF-α-increased c-Jun level. NF-κB p65 overexpression increased c-Jun protein and enhanced the TNF-α-increased c-Jun level. Furthermore, TNF-α inhibition of activin A-induced promoter activity and mRNA expression of α-globin and ζ-globin were abolished in cells expressing dominant-negative c-Jun. TNF-α inhibition of activin A-induced mRNA expression of GATA-1 and NF-E2 p45 was also abolished in cells expressing dominant-negative c-Jun.
Conclusion: TNF-α may inhibit activin A-induced erythroid gene expression via increases of c-Jun in K562 cells.

1. Introduction

Erythroid differentiation is regulated by multiple cytokines, such as erythropoietin (EPO), activin A, and tumor necrosis factor-alpha (TNF-α). Activin A, a member of the transforming growth factor-β superfamily, plays an important role in modulating the proliferation and differentiation of erythroid progenitor cells.[1,2] Our previous study showed that activin A induces hemoglobin synthesis in the human K562 erythroleukemia cell line.[3] We have also reported that activin A can induce the promoter activation and mRNA expression of α-globin and ζ-globin in K562 cells.[4] Activin A in cooperation with EPO increases the number of hemoglobin-synthesizing colonies derived from CFU-E progenitors and promotes the formation of BFU-E.[5,6]

The proinflammatory cytokine, TNF-α, plays a critical role in regulating the hematopoietic system and inflammatory responses. In addition, TNF-α can inhibit the erythroid differentiation of hematopoietic progenitor cells.[7] A previous study showed that TNF-α may directly inhibit erythroid differentiation via signaling through TNF-α receptors.[8] TNF-α and nuclear factor kappa B (NF-κB) p65 inhibit erythroid gene expression in K562 cells.[9] That study suggested that NF-κB acts as an erythroid differentiation inhibitor in TNF-α signaling. TNF-α suppresses erythroid differentiation of K562 cells by negatively regulating erythroid-specific transcription factors, such as GATA-1 and NF-E2 p45.[7] However, the mechanisms of TNF-α-inhibited expression of erythroid genes remain unknown. In addition, how activin A and TNF-α interact together to regulate erythroid differentiation has not been elucidated.

The proto-oncogene, c-Jun, is a major component of the activating protein (AP)-1 transcription factor, which forms homo- and heterodimers with other AP-1 family members.[10] It has been shown that NF-κB p65 transactivates and increases expression of AP-1-

Conflicts of interest: The authors have declared that there are no conflicts of interest.

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responsive genes.[11] Those results suggest that NF-κB can induce c-Jun expression. Similar to NF-κB, c-Jun was identified as participating in the inhibition of erythroid differentiation.[12] c-Jun can block hemoglobin synthesis during dimethyl sulfoxide-induced erythroid differentiation of murine erythroleukemia cells.[13] c-Jun also blocked the hemoglobin synthesis of primary human $CD34^+$ hematopoietic progenitors and K562 cells.[14] Because c-Jun proteins mediate some of the effects of TNF-α in different types of cells,[15,16] the above-mentioned studies provide an important link for c-Jun in TNF-α/NF-κB-inhibited erythroid differentiation.

Therefore, the present study was designed to test whether TNF-α inhibits activin A-induced erythroid gene expression and investigate the mechanisms of TNF-α inhibition. We used K562 cells as a model because this cell line can be triggered to undergo erythroid differentiation.[17,18] Our findings demonstrated that TNF-α can induce c-Jun expression through the NF-κB pathway. c-Jun participates in TNF-α inhibition of activin A-induced erythroid gene expression in K562 cells.

2. Methods

2.1. Cell line and reagents

The human K562 erythroleukemia cell line was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). K562 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a 5% CO_2 incubator at 37°C. Recombinant human activin A and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). Bay117082 was purchased from Sigma (St. Louis, MO, USA). Antibodies specific for NF-κB p65, c-Jun, and B23 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-tubulin monoclonal antibodies were obtained from Abcam (Cambridge, MA, USA).

2.2. Transfection of K562 cells

For K562 cell transfection, 2×10^6 cells were transfected with pCMV4-NF-κB p65 plasmids (2 μg) or an empty vector using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested to analyze c-Jun expression using Western blotting. Dr Warner C. Greene provided pCMV4-NF-κB and p65.[19] For the luciferase reporter assay, transfection of K562 cells was also accomplished using lipofectamine 2000. Two micrograms of plasmids and 0.05 μg of the pRL-TK internal control vector were used in each transfection experiment.

2.3. Luciferase reporter assay

Promoter activity was detected with a reporter assay using previously described procedures.[4] After transfection of cells for 5 hours, the indicated cytokines were added to the culture medium, incubated for 24 hours, and then harvested. Luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Relative luciferase activity was adjusted by normalizing the ratio of firefly luciferase activity to Renilla luciferase activity generated by pRL-TK (Promega). Human c-Jun mutant cDNA was generated by deleting the transactivation domain (TAM-67).[22] Dr Michael J. Birrer provided pCMV-c-Jun and pCMV-TAM-67.[20,21]

2.4. Reverse transcription and quantitative real-time polymerase chain reaction

Total RNA was isolated from K562 cells using the TRIzol reagent (Gibco, Life Technology, Grand Island, NY, USA) according to the manufacturer's instructions. After purification, 1 μg of RNA was reverse-transcribed at 42°C for 60 minutes with an oligo dT_{18} primer, followed by enzyme inactivation at 70°C for 15 minutes. The resulting cDNA was amplified and quantified by performing a quantitative polymerase chain reaction (qPCR). The primers used here were as follows: α-globin sense strand 5′-GACAAGACCAACGTCAAGGCCGCC-3′ and antisense strand 5′-CAGGAACTTGTCCAGGGAGGC-3′; ζ-globin sense strand 5′-GCCACCCGCAGACCAAGACC-3′ and antisense strand 5′-TAGGCGTGCAGCTCGCTCAG-3′; Bmi1 sense strand 5′-CAGCT CATCCTTCTGCTGATGC-3′ and antisense strand 5′-CATTGCTGCTGGG CATCGTAAG-3′; MDR1 sense strand 5′-GCCCTGTTTGCGGTGATCTC-3′ and antisense strand 5′-CGCCTCCTTCTCAGTCTCTG-3′; and CDCP1 sense strand 5′-CTTCAGCCTGGACGAGGATG-3′ and antisense strand 5′-CGAGGGCAGACAGCAGTAAG-3′. qPCR was performed using a QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) on a Roter-Gene Q real-time PCR machine (Qiagen). The normalized gene expression was calculated relative to GAPDH. GAPDH primers were purchased from Qiagen (cat. no.: QT01192646).

2.5. Subcellular fractionation

Cells pellets were resuspended in hypotonic buffer [20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.4, 1mM $MgCl_2$, 10mM KCl, 0.5% NP-40, 0.5mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride (PMSF), and 2 μg/mL leupeptin] at 4°C for 30 minutes. After centrifugation at 4000 × *g* for 10 minutes at 4°C, supernatants contained the cytoplasmic proteins. Pellets containing the nuclei were resuspended in hypertonic buffer (20mM HEPES at pH 7.4, 20% glycerol, 0.4M NaCl, 1mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreito, 1mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 100mM sodium fluoride) and then incubated at 4°C for 15 minutes. After centrifugation at 8000 × *g* for 15 minutes at 4°C, the supernatants contained the nuclear proteins.

2.6. Western blot analysis

Cells were lysed in lysis buffer (1% Triton X-100, 20mM Tris at pH 7.5, 150mM NaCl, 1mM ethylene glycol tetraacetic acid, 1mM EDTA, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM PMSF, 1 μg/mL of leupeptin, and 1mM Na_3VO_4) at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to resolve protein lysates, the protein bands were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and the membranes were probed with primary antibodies. After binding with the appropriate secondary antibodies (GE Healthcare, Waukesha, WI, USA), the blots were visualized using an enhanced chemiluminescence detection system (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA).

2.7. Statistical analysis

Quantitative data are presented as the mean ± standard error. Statistically significant differences between groups were identified using the Student *t* test. A p value <0.05 was considered statistically significant.

3. Results

3.1. TNF-α inhibited activin A-mediated promoter activation and gene expression of globins

Our previous study showed that activin A induced erythroid differentiation of K562 cells.[3] Erythroid differentiating cells express globins, an erythroid marker. We first analyzed whether TNF-α inhibited activin A-induced α-globin and ζ-globin promoter activity