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平成18年度分担研究報告書

「マイクロアレイ技術を用いたATLのゲノムワイドな解析による新規治療標的分子の探索」

「ATLモデルマウスの腫瘍細胞のmicroarrayを用いた解析に関する研究」

分担研究者：澤　洋文（北海道大学人獣共通感染症リサーチセンター　分子病態・診断部門）

研究要旨

我々はリンパ球特異的に転写を活性化する *lck* promoter の下流に、HTLV-I の転写活性化因子である Tax 遺伝子を code し、その下流にヒト growth hormone の 3'領域の塩基配列を有する construct を作製し、その construct を用いて、リンパ球特異的に HTLV-I Tax を発現するトランスジェニックマウスを作成した。本モデルは ATL と同様に、末梢血液中に分葉状の核を有する異型細胞が増殖しており、リンパ節を含む脾臓、肝臓、肺等の多臓器に浸潤していた。さらに肺には Pneumocystis carinii の感染を認め、発症個体が免疫不全状態に陥っていることが示唆された。また末梢血中に認められた異型細胞の表面に、インターロイキン-2（IL-2）受容体が発現しており、表現型が ATL と非常に類似していた。腫瘍化した細胞は CD4-CD8-のダブルネガティブな pre-T cell 由来の細胞であった。これらの結果から、本モデルマウスは現在有効な治療法が無く、また予後の極めて不良な ATL の発症機序に対する研究に有用であることが示唆された。本研究では、このマウスから腫瘍細胞を、また本マウスの background である C57BL/6 マウスからT細胞を採取して microarray による解析を行い、腫瘍細胞での遺伝子の発現様式を検討した。

A. 研究目的

ATL モデルマウスの腫瘍細胞での遺伝子発現様式を明らかにすることにより腫瘍発症の機構を明らかにすることを目的とする。

B. 研究方法

<u>材料と方法：</u>

トランスジェニックマウス作製

すべての動物実験は国立大学法人北海道大学遺伝子組換え実験等安全委員会および北海道大学医学研究科実験動物委員会の承認の元に行われた。C57BL/6 マウス（オリエンタル酵母）を用いてトランスジェニックマウスを作製した。トランスジェニックマウスは C57BL/6 を用い標準の方法に従い作製した。挿入遺伝子

である *plck*-Tax は HTLV-I Tax の配列を PCR 法により増幅し *lck* 近位プロモーターにより挿入遺伝子を発現するベクター p1017 の制限酵素 Bam HI サイトに挿入した。 *plck*-Tax プラスミドを Not I で直鎖化し 6.3 kb の遺伝子断片を挿入した。遺伝子挿入の前には Qiaex gel extraction kit (Qiagen, CA) を用いて精製した。生まれたマウスの尻尾よりゲノム DNA を採材しサザンブロット法にて遺伝子挿入を確認した。

腫瘍細胞およびT細胞の採取と RNA 抽出

ATL モデルマウスの脾臓を採取し、Sevefe Combined ImmunoDeficiency (SCID)マウスの背部皮下に移植し、経過観察を行った。移植後 1 ヶ月ぐらいで、SCID マウスには腹水貯留、食欲減少等の臨床症状が現れ、末梢血には腫瘍細胞が出現し、組織学的には脾臓を主体とした実質臓器への腫瘍細胞の浸潤が認められた。特に脾臓は通常の数倍に種大し、正常組織は腫瘍細胞でほぼ置換されていた。このようにして SCID マウスの脾臓から腫瘍細胞を採取した。次に、本 ATL モデルマウスの background である C57BL/6 マウス 2 匹の脾臓から MACS Pan T cell isolation kit (Miltenyl Biotec 社)を用いて、T 細胞を分離した。これらの腫瘍細胞および T 細胞から RNeasy kit (Qiagen 社)を用いて total RNA を抽出した。

cDNA および cRNA の合成

前述したように単離した 0.1-3.0 _g の total RNA を用いて TrueLabeling-AMP Linear RNA Amplification kit (Bioscience 社)を用いて 1st strand cDNA を合成し、その後同 kit を用いて、Biotin-16 UTP をラベルした cRNA を合成した。

Hybridization

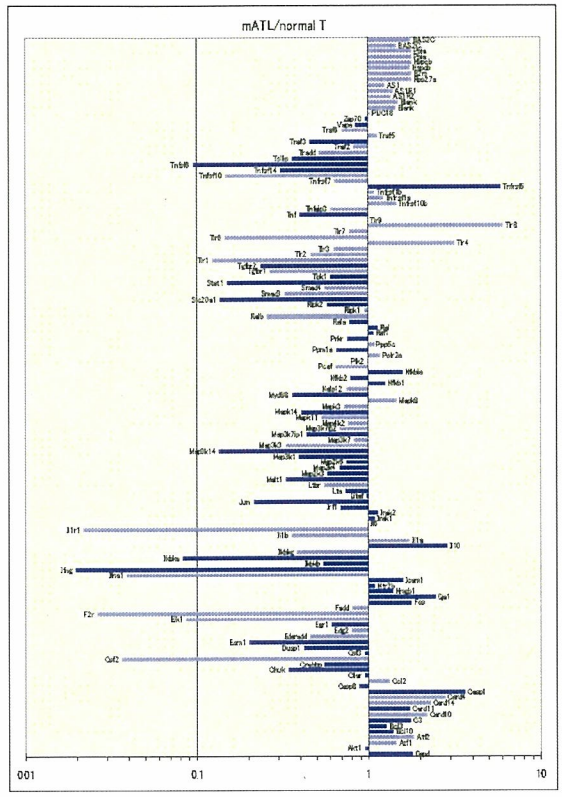
前述した方法で腫瘍細胞および C57BL/6 マウスの T 細胞から作製した cRNA を用いて、Oligo GEArray Microarrays (Bioscience 社)の hybridization を行った。Hybridization の条件は 60°C overnight で hybridization 後の洗浄は 2 x SSC, 1% SDS で 60°C, 15 分、0.1 x SSC, 0.5% SDS で 60°C, 15 分行った。その後 GEAblocking solution で blocking を行った後に、8000 倍に希釈した alkaline phosphatase-conjugate streptavidin (AP-SA) で室温で 10 分間反応を行い、その後 washing buffer を用いて washing を行った。Signal は CDP-Star と約 5 分間反応させた後に、LAS system (Fuji film 社)を用いて 10-20 分間かけて画像を撮りこんだ。

C. 結果

次ページに図として示したように今回用いた Mouse NF-_B signaling pathway Microarray を用いた検索では CD40, Caspase 1, IL-10, Cx43 が腫瘍細胞で発現の増加を認めており、逆に IFN_, IKK_, FasL, NIK, pit1, Stat1, Jun, IKK_, Myd88 , p38, IKK_, CBP 等の因子の発現の減少が確認された。現在これらの 2 倍以上の発現が認められた因子について腫瘍発症の鍵となる、もしくは治療法の開発に有用な情報をもたらす因子を選択し、詳

細な検討を加えることを予定している。

D. 考察



我々はATLモデルマウスを用いて、腫瘍発症機序を解明する為に microarray 法を行った。その結果多くの因子の発現が変化していることが示された。今後は発現の変化が認められた因子について、発祥機序の解明もしくは治療法の開発に有用である因子を選択することが重要であると考えられた。

E. 結論

ATL モデルマウスから採取した腫瘍細胞および C57BL/6 マウスから採取した T cell を用いて microarray を行い、発現の変化を認める因子の検討を行った。

F. 健康危険情報

特になし。

G. 研究発表

論文発表

1) Hasegawa H, Sawa H, Lewis MJ, Orba Y, Sherhy N, Yamamoto Y, Ichinohe T, Tsunetsugu-Yokota Y, Katano H, Takahashi H, Matsuda J, Sata T, Kurata T, Nagashima, K, Hall, WW. Development of Thymus-Derived T-cell Leukemia/Lymphoma in Mice Transgenic for the Tax gene of Human T-Lymphotropic Virus Type-I (HTLV-I). **Nat Med.** 2006 12: 466-472

2) Maeda M, Sawa H, Tobiume M, Tokunaga K, Hasegawa H, Ichinohe T, Sata T, Moriyama M, Hall WW, Kurata T, Takahashi H. Tristetraprolin inhibits HIV-1 production by binding to genomic RNA. **Microbes and Infection**. 2006 8:2647-56

3) Sunden Y, Suzuki T, Orba Y, Umemura T, Asamoto M, Nagashima K, Tanaka S, Sawa H. Characterization and application of polyclonal antibodies that specifically recognize JC virus large T antigen. **Acta Neuropathol**.2006 111: 379-387

学会発表

5. 長谷川秀樹、澤洋文、大場靖子、片野晴隆、佐多徹太郎、倉田毅、長嶋和郎　成人 T 細胞白血病(ATL)モデルマウスの解析　第 95 回日本病理学会学術集会　平成 18 年 4 月 30 日-5 月 2 日　東京

6. 川口晶、一戸猛史、澤洋文、岡田義昭、千葉丈、倉田毅、佐多徹太郎、William W.Hall、長谷川　秀樹成人 T 細胞白血病リンパ腫（ATLL）モデルマウスにおけるケモカインの発現とその機能解析　第 54 回日本ウイルス

学会学術集会　平成 18 年 11 月 19 日
-21 日　名古屋

H. 知的財産権の出願、登録状況
なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
渡邉　俊樹	HTLV-1の分子生物学：基礎と臨床をつなぐもの	渡邉　俊樹 上平　憲 山口一成	HTLV-1と疾患	文光堂	東京	2007年	280ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Horie ら	TRAF activation of C/EBPβ (NF-IL6) via p38 MAPK induces HIV-1 gene expression in monocytes/ macrophages.	Microbes and Infection,	-	-	In press
Watanabeら	IκBα independent induction of NF-κB and its inhibition by DHMEQ in Hodgkin-Reed-Sternberg cells.	Lab Invest	87	372–382	2007年
Horieら	Blocking NF-kappaB as a potential strategy to treat adult T-cell leukemia/lymphoma.	Drug News Perspect	19	201-209	2006年
Ishidaら	5' long terminal repeat (LTR)-selective methylation of latently infected HIV-1 provirus that is demethylated by reactivation signals	Retrovirology	3	69	2006年
Sugitaら	Intraocular soluble IL-2 receptor alpha in a patient with adult T-cell Leukemia with intraocular invasion	Br J Ophthalmol	90	1204-6	2006年
Miyakeら	Rapid dissemination of a psthogenic siman/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection.	J. Gen. Virol.	87	1311-20	2006年
Horieら	DHMEQ, a new NF-kB inhibitor induces apoptosis of chronic lymphocytic leukemia cells and enhances effect of fludarabine	Leukemia	20	800-6	2006年
Kamoiら	SUV39H1 interacts with HTLV-1 Tax and abrogates Tax transactivation of HTLV-1 LTR	Retrovirology	3	5	2006年
Uchiharaら	Transactivation of the ICAM-1 gene by CD30 in Hodgkin's Lymphoma.	Int. J. Cancer	118	1098-107	2006年
Hamaguchiら	Agp and Hpx are useful for pertussis vaccine safety control as vaccine toxicity-related genes.	Vaccine			In press

Masumiら	Nucleolin is involved in interferon regulatory factor-2-dependent transcriptional activation.	Oncogene	25	5113-24	2006年
Mizuochiら	Evaluation of 10 commercial diagnostic kits for in vitro expressed hepatitis B virus (HBV) surface antigens encoded by HBV of genotypes A to H	J Virological methods	136	254-256	2006年
Hamaguchiら	Loss of Tie2 receptor compromises embryonic stem cell-derived endothelial but not hematopoietic cell survival.	Blood	107	1207-13	2006年
Ohsugiら	In vitro and in vivo antitumor activity of the NF-kB inhibitor DHMEQ in the human T-cell leukemia virus type I transformed cell line, HUT-102	Leuk Res	30	90-97	2006年
Saijoら	Monkeypox Highly attenuated vaccinia vaccine, LC16m8, that lacks expression of *B5R* membrane protein protects monkeys from monkeypox	J. Virol.	80	5179-88	2006年
Maedaら	Tristetraprolin inhibits HIV-1 production by binding to genomic RNA.	Microbes and Infection	8	2647-56	2006年
Sanadaら	Unbalanced translocation der(1;7)(q10;p10) defines a unique clinicopathological subgroup of myeloid neoplasms	Leukemia			In press
Nakagawaら	AML1/Runx1 rescues Notch1-Null mutation-induced deficiency of para-aortic splanchnopleural hematopoiesis.	Blood	108	3329-34	2006年
Jacobら	Genome-wide, high-resolution detection of copy number, loss of heterozygosity, and genotypes from formalin-fixed, paraffin-embedded tumor tissue using microarrays	Cancer Res	67	2544-51	2007年
Hosoyaら	Genomewide screening of DNA copy number changes in chronic myelogenous leukemia with the use of high-resolution array-based comparative genomic hybridization.	Genes Chromosomes Cancer	45	482-94	2006年
Hasegawaら	Development of Thymus-Derived T-cell Leukemia/Lymphoma in Mice Transgenic for the Tax gene of Human T-Lymphotropic Virus Type-I (HTLV-I).	Nature Med.	12	466-472	2006年
Sundenら	Characterization and application of polyclonal antibodies that specifically recognize JC virus large T antigen.	Acta Neuropathol.	111	379-387	2006年

III. 研究成果の刊行物・別刷り

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Original article

TRAF activation of C/EBPβ (NF-IL6) via p38 MAPK induces HIV-1 gene expression in monocytes/macrophages☆

Ryouichi Horie [a,b], Takaomi Ishida [b], Masae Maruyama-Nagai [b], Kinji Ito [c], Mariko Watanabe [a], Akiko Yoneyama [d], Masaaki Higashihara [a], Satoshi Kimura [e], Toshiki Watanabe [b,*]

[a] *Department of Hematology, School of Medicine, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan*
[b] *Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*
[c] *Department of Pathology, School of Medicine, Toho University, 5-21-16 Ohmorinishi, Ohta-ku, Tokyo 143-8540, Japan*
[d] *Department of Clinical Laboratory, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8430, Japan*
[e] *AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan*

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Abstract

C/EBPβ plays a pivotal role in activation of human immunodeficiency virus type 1 (HIV-1) in monocytes/macrophages. However, mechanisms for functional regulation of C/EBPβ remain uncharacterized. Previous studies indicated that NF-κB activation by tumor necrosis factor (TNF) receptor family, which activates TNF receptor associated factor (TRAF), induces HIV-1 expression. We found that TRAF signals activate HIV-1 LTR with mutations of NF-κB sites in promonocytic cell line U937, suggesting existence of an alternative HIV-1 activating pathway. In this study, we have characterized the signal transduction pathway of TRAF other than that leading to NF-κB, using U937 cell line, and its subline, U1, which is chronically infected by HIV-1. We show that signals downstream of TRAF2 and TRAF5 activate p38 MAPK, which directly phosphorylates C/EBPβ, and that activation of p38 MAPK potently activates C/EBPβ-mediated induction of HIV-1 gene expression. We also show TRAF2 and TRAF5 are expressed in monocytes/macrophages of spleen samples from HIV-1 infected patients. Identification of TRAF-p38 MAPK-CEBPβ pathway provides a new target for controlling reactivation of latent HIV-1 in monocytes/macrophages.

Keywords: TRAF; p38 MAPK; C/EBPβ (NF-IL6); HIV-1

1. Introduction

Nowadays, highly active antiretroviral therapy (HAART) can successfully decrease and control human immunodeficiency virus type 1 (HIV-1) replication, however, complete eradication of the virus is impossible [1]. Infection by HIV-1 often results in a period of viral latency after the virus integrates into the host cell chromosome that is characterized by low levels of virus production [2]. These latently infected cells are a permanent source for virus reactivation and lead to the rebound of the viral load after interruption of HAART. Therefore, controlling virus reactivation from reservoirs after interruption of HAART is an urgent problem to be addressed.

Activation of viral gene expression can occur in response to a variety of stimuli including mitogens, cytokines and environmental stresses such as UV light, heat shock, and oxygen radicals [3]. The exact mechanism by which these stimuli activate gene expression is not completely understood. Cytokines and

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* Corresponding author. Tel.: +81 3 5449 5298; fax: +81 3 5449 5418.
E-mail address: tnabe@ims.u-tokyo.ac.jp (T. Watanabe).

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mitogens such as phorbor ester activate HIV-1 gene expression in part through the core enhancer present in the HIV-1 long terminal repeat (LTR), which contains two consensus NF-κB binding sites. Deletions or mutations in the NF-κB enhancers abolish transactivation by these stimuli [4,5]. Previous studies revealed the important roles of tumor necrosis factor (TNF) receptor family leading to activation of NF-κB in the reactivation of HIV-1 [6–8]. NF-κB activation by TNF receptor family is mediated via tumor necrosis factor receptor associated factor (TRAF) proteins [9]. Therefore, above reports underscore the importance of TRAF signals in the reactivation of HIV-1.

Monocytes/macrophages serve as a major reservoir for HIV-1 even after cytopathic treatment. Unlike T cells, monocytes/macrophages are non-lytically infected. These cells serve as viral reservoirs and vectors for virus transmissions to target cells. Therefore, the control of the reactivation from these reservoirs is an important issue to be addressed for the treatment of HIV-1 infection [10,11]. However, mechanisms of HIV-1 reactivation in monocytes/macrophage are not fully elucidated. Cytokines and cellular stress also activate p38 mitogen-activated protein kinase (p38 MAPK) leading to activation of the HIV-LTR, although the target molecule(s) of this kinase in the activation of HIV-1 has not been characterized [12]. Replication of HIV-1 in macrophages/monocytes but not in T cells requires CAAT enhancer binding proteins (C/EBP) including C/EBPβ (NF-IL6) and their binding sites present in the HIV-LTR [13,14] .

We found that TRAF signals activate HIV-1 LTR with mutations of NF-κB sites in promonocytic cell line U937, suggesting existence of an alternative HIV-1 activating pathway. In this study, we have characterized the signal transduction pathway of TRAF proteins other than that leading to NF-κB, using a promonocytic cell line, U937 and its subline, U1, which is chronically infected by HIV-1. We examined the involvement of p38 MAPK and C/EBPβ in this pathway. We also examined the expression of TRAF proteins in monocytes/macrophages of spleen samples from HIV-1 infected patients.

2. Materials and methods

2.1. Plasmids and chemicals

An expression vector for mouse C/EBPβ, pMTV-C/EBPβ, was kindly provided by Dr. S.L. McKnight (Tularik Inc. South San Francisco, CA). The luciferase reporter genes were constructed using pGL3 vector (Promega, Madison, WI). HIV-1 LTR sequence was amplified by PCR using genomic DNA of ACH-2 cell line. HIV-1 LTR sequence (8897–9621) was introduced into pGL3 vector (Promega) and resultant plasmid was named pHIV LTR-Luc. The pHIV LTR mκB-Luc construct having mutations in both NF-κB sites (GGGACTTTCC to TCTACTTTCC) was prepared with Gene Editor in vitro mutagenesis system (Promega). The pHIV LTR mC/EBPβ-Luc construct having mutations in both C/EBPβ sites (ATTTCATC in 281–288 to TGCAGGGG and GCTTGC in 339–344 to CAGCTG) was prepared according to the previous paper [15] based on the method by Kunkel et al. [16]. MAPK kinase 6 (MKK6) expression vector was constructed using pME18S vector and cDNA amplified by PCR from a control peripheral blood mononuclear cell (PBMC) cDNA. The expression vector for human CD30 and its deletion mutant, pCR-CD30 Δ, which lacks C-terminal TRAF domains consisting of 57 amino acids, was described elsewhere [17]. The p38 MAPK inhibitor SB203580 was a generous gift of Dr. J.C. Lee, King of Prussia, PA. Anisomycin was purchased from Sigma (St. Louis, MO).

2.2. Cell culture

U937 is a human promonocytic cell line, and HEK 293 cells are derived from the human embryonic kidney. Both of them were obtained through the Japanese Cancer Research Resources Bank (Tokyo, Japan). ACH-2 is a human T cell line chronically infected with HIV. U1 is a derivative of U937 line carrying latently infected HIV-1. HEK 293 cells were cultured in DMEM supplemented with 10% FCS and kanamycin. Other cell lines were cultured in RPMI 1640 supplemented with 10% FCS and kanamycin.

2.3. Transfections and luciferase assays

Activation of HIV-1 promoter was measured by reporter gene assay in U937 cells using various HIV-LTR driven Luc reporter plasmid. Renilla luciferase expression vector driven by the herpes simplex virus thymidine kinase promoter (pRL-TK) was co-transfected to standardize each experiment. Briefly, 1×10^5 cells were transfected with 25 ng of the reporter plasmids, 100 ng of pRL-TK and indicated amounts of various effector plasmids, using DMRIE-C reagent (Invitrogen, Carlsbad, CA) or Lipofectamine reagent (Invitrogen) according to the procedures provided by the manufacturer. The total amount of DNA transfected was always adjusted with an empty expression vector. About 30 h after transfection, cells were harvested and lysed, then, the activity was measured using dual luciferase assay system (Promega) according to the procedures provided by the manufacturer. Levels of activation are expressed as fold activation compared with the basal luciferase activity of the reporter constructs. Transfection was always done in triplicate and performed more than three times. The representative data were presented with the mean and standard deviation (s.d.).

2.4. Analysis of kinase activity

For in vitro kinase assays using C/EBPβ as substrate, we prepared a GST fusion protein. A fragment of C/EBPβ cDNA corresponding to nucleotide position from 575 to 887 was amplified by PCR and subcloned into pGEX5X-2 (GE Healthcare UK Ltd. Buckinghamshire, UK). The fusion protein named GST-C/EBPβ was expressed and purified by a standard procedure [18]. Using U937 cells, activation of p38 MAPK by TRAF2, TRAF5, CD30 and its deletion mutant (CD30Δ), was studied by in vitro kinase assay. Transfection

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was done by DMRIE-C (Invitrogen) using 5 μg of the each expression plasmid or a control plasmid. Forty-eight hours after transfection, cells were harvested. As controls, U937 cells treated with or without anisomycin (10 μg/ml) for 20 min were included for the assay. The cell lysates were immunoprecipitated by anti-p38 MAPK antibody (New England Biolabs, Inc. Ipswich, MA). The Immunecomplex was incubated with 2.5 μg of GST-C/EBPβ in the presence of γ-^{32}P-ATP at 37°C for 60 min and analyzed by 8% SDS-PAGE.

2.5. Measurement of HIV p24 production in U1 cells

Anti-CD30 monoclonal antibody, Ber-H2, a control monoclonal antibody against glucose oxidase (*Aspergillus niger*) (mouse-IgG1 negative control), and affinity purified anti-mouse immunoglobulin goat antibody were all purchased from DAKO (Kyoto, Japan). Cross-linking of cell surface CD30 was performed as follows: harvested U1 cells were washed in RPMI 1640 medium without fetal calf serum for 2 h, then 1×10^6 of U1 cells were incubated for 20 min at room temperature with Ber-H2 or the mouse-IgG1 negative control at the concentration of 10 μg/ml, and were washed with PBS before addition of goat anti-mouse immunoglobulin at a final concentration of 10 μg/ml. After washing with PBS and adding the cross-linking antibody, cells were cultured in RPMI 1640 medium without fetal calf serum. 1x 10^6 of U1 cells were treated with 10 ng/ml of TNFα. Samples were incubated for 48 h and harvested. ELISA assays for HIV p24 expression in U1 cells were performed using RETRO-TEK HIV-1 p24 antigen ELISA kit (Cellular Products Inc. Buffalo, NY) according to the manufacturer's instruction.

2.6. Immunohistochemistry

Sections of formalin fixed and paraffin-embedded samples were deparaffinized and treated with microwave in 10 mM sodium citrate buffer (pH7.0). Sections were treated with 5% skimmed milk in TBS and then incubated with anti-CD68 mouse antibody (Dako Cytomation, Glostrup, Denmark) at 37°C for 40 min. After wash with TBS, the sections were incubated with anti-mouse-IgG Texas Red antibody (GE Healthcare UK, Ltd.) at 37°C 40 min. For double staining with anti-TRAF2 (C-20) rabbit monoclonal antibody or anti-TRAF5 (C-19) goat polyclonal antibody (both from Santa Cruz), a modification of the tyramide signal amplification (TSA) system (NEN Life Science) was used in order to facilitate use of streptavidine–FITC instead of peroxidase-conjugated streptavidine. Signals were detected, using confocal microscopy.

Gene Bank Accession Number.

The national Cancer for Biotechnology Information human genome sequence for HIV-1 (IIIB) is K03455.2.

2.7. Statistical analysis

Differences between mean values were assessed by two-tailed *t*-test. A *P*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. TRAF signals activate HIV-LTR with mutations of NF-κB sites

Signaling from TNF receptor family leads to the induction of HIV gene expression by activating NF-κB in T cells and monocytes/macrophages [6–8]. We tried to examine whether an alternative pathway is involved in activation of HIV-LTR. For this purpose, we did reporter gene assays using a luciferase reporter construct with mutations in both NF-κB sites in HIV-LTR (pHIV LTR mκB-Luc) and a human promonocytic cell line, U937. Transient overexpression of TRAF2 or TRAF5 did activate pHIV LTR mκB-Luc, although the magnitudes were smaller than when pHIV LTR-Luc was used as the reporter (Fig. 1). The results suggested a possibility that signals emanated from TRAF proteins other than those activating NF-κB might mediate activation of HIV-LTR in monocytes/macrophages.

3.2. TRAF signals potentiate the C/EBPβ-mediated activation of HIV-LTR

We next studied whether TRAF signals can regulate C/EBPβ-mediated transactivation of HIV-1 LTR in U937 cells. In the reporter gene assays, overexpression of TRAF2 or TRAF5 along with C/EBPβ showed marked synergistic effects on HIV-LTR activation (Fig. 2a). Overexpression of TRAF2 with C/EBPβ potentiated C/EBPβ activation of HIV-LTR more than two-fold compared with C/EBPβ alone. Similarly, TRAF5 signals enhanced HIV-LTR activation by C/EBPβ up to about 50-fold compared to the basal LTR activity, which is significantly more than additive effects of C/EBPβ and TRAF5. The same effects were seen when two NF-κB sites in the construct (HIV-LTR-Luc) were mutated (HIV-LTR

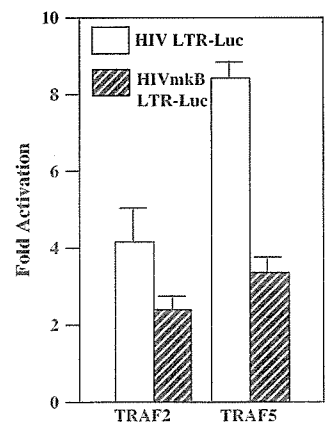

Fig. 1. Activation of HIV-LTR with mutations of NF-κB sites by TRAF signals. Transient co-transfection reporter gene assay was done in U937 cells using indicated luciferase constructs and expression plasmids of TRAF2 and TRAF5. Levels of activation are expressed as fold activation compared with the basal luciferase activity of the reporter constructs. Bars indicate standard deviation. *$P < 0.05$.

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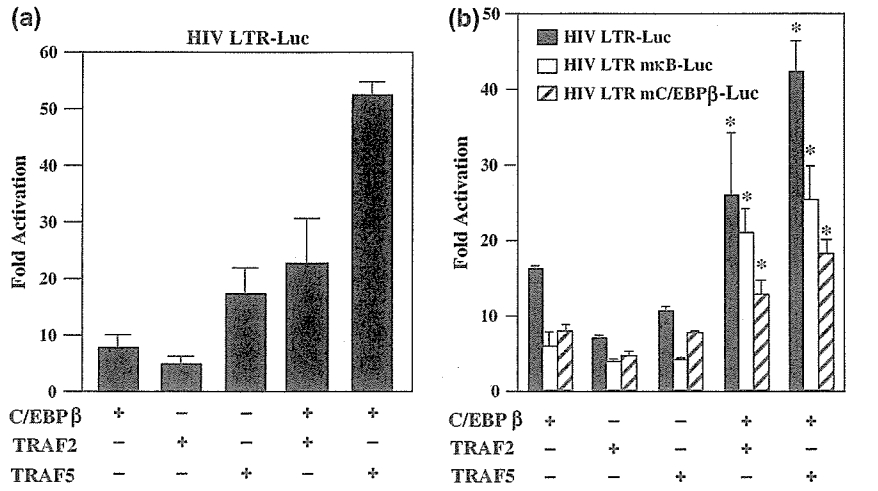

Fig. 2. Potentiation of C/EBPβ-mediated HIV-LTR activation by TRAF. (a) Effects of TRAF on C/EBPβ-mediated induction of HIV-LTR. (b) Effects of mutations in NF-κB or C/EBPβ sites of HIV-LTR on C/EBPβ-mediated HIV-LTR activation by TRAF. Transient co-transfection reporter gene assay was done in U937 cells using HIV-LTR-Luc, HIV-LTR mκB-Luc or HIV-LTR mC/EBPβ-Luc as reporters. Twenty five nanograms of reporter construct and 100 ng of pRL-TK were co-transfected into 1×10^5 cells with expression plasmids of TRAF2, TRAF5 or C/EBPβ (50 ng each). Total amount of DNA transfected was adjusted to 550 ng by an empty expression vector. Levels of activation are expressed as fold activation compared with the basal luciferase activity of the reporter constructs. Bars indicate standard deviation. $^*P < 0.05$.

mκB-Luc) (Fig. 2b). However, this effect was not completely abolished when two C/EBPβ sites in HIV-LTR-Luc were mutated (HIV-LTR mC/EBPβ-Luc). Collectively, these results indicate that signals downstream of TRAF2 and TRAF5 can potentiate C/EBPβ-mediated activation of HIV-LTR in U937 cells and that this potentiation is not completely dependent on C/EBPβ sites in HIV-LTR.

3.3. Activation of p38 MAP kinase by TRAF signaling pathway

In addition to IKK activation that leads to NF-κB activation, TRAF2 is also involved in signaling to activate other MAP kinase kinase kinases (MAPKKK), MEKK1 and ASK1, which leads to activation of stress-activated kinase (SAPK)/c-jun N-terminal kinase (JNK) and p38 MAPK [19]. On the other hand, C/EBPβ has a consensus motif for p38 MAPK phosphorylation and was shown to be a direct target of p38 MAPK in 3T3-L1 cells [20]. Therefore, we next tested the possibility that TRAF signals activate C/EBPβ through p38 MAPK pathway. For this purpose, we examined whether p38 MAPK activated by TRAF signals can phosphorylate C/EBPβ.

We prepared a GST-C/EBPβ fusion protein that contains the consensus site of p38 MAPK for a substrate of in vitro kinase assay. p38 MAPK immunoprecipitated from anisomycin-treated U937 cells directly phosphorylated GST-C/EBPβ (Fig. 3, lane 2). It was also shown that overexpression of TRAF2 or TRAF5 in U937 can activate p38 MAPK and phosphorylate GST-C/EBPβ (Fig. 3, lanes 5 and 6). Experiments using U937 cells transfected with expression vectors for CD30, a member of TNF receptor family and truncated mutants of CD30 lacking the TRAF interacting domain also showed that p38 MAPK is activated by TNF receptor family signaling pathway (Fig. 3, lanes 3 and 4). Taken together, these results demonstrate that C/EBPβ can be activated by TRAF-p38 MAPK signaling pathway.

3.4. p38 MAPK potentiates C/EBPβ-mediated activation of HIV-1 by TRAF signals

We next examined whether p38 MAPK mediates the synergistic effects of TRAF signals on C/EBPβ-mediated activation of HIV-LTR using a p38 MAPK specific inhibitor, SB203580. In transient co-transfection studies using U937 cells, potentiation of HIV-LTR activation by C/EBPβ observed with overexpression of TRAF2, TRAF5 or CD30 was dose-dependently inhibited by addition of SB203580 (Fig. 4a). In co-transfection of C/EBPβ with TRAF2 or CD30, addition of 40 μM of SB203580 almost abolished potentiating effects on HIV-LTR

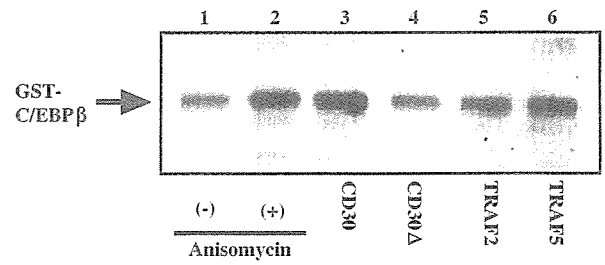

Fig. 3. p38 MAPK-mediated phosphorylation of C/EBPβ by TRAF2 and TRAF5: in vitro kinase assay. Using U937 cells, activation of p38 MAPK by TRAF2, TRAF5 and CD30 was studied by in vitro kinase assay using C/EBPβ as a substrate. U937 cells treated with or without anisomycin (10 μg/ml for 20 min) served as a positive or negative control, respectively. CD30: wild type of CD30, CD30Δ: CD30 lacking binding domain for TRAF2 and TRAF5.

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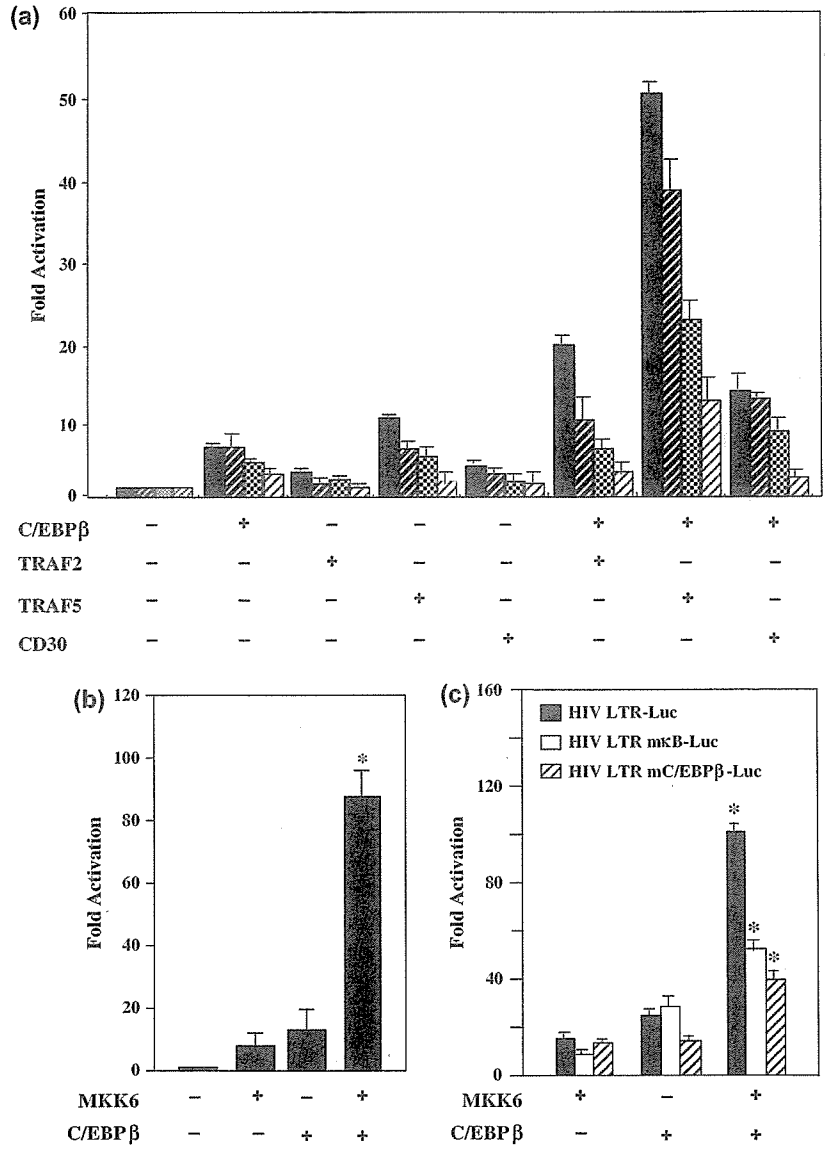

Fig. 4. p38 MAPK is involved in the potentiation of C/EBPβ by TRAF signals. (a) Effects of p38 MAPK inhibitor SB203580 on potentiation of CEBPβ mediated HIV-LTR induction by TRAF2, TRAF5 or CD30. Transient co-transfection reporter gene assay was done in 1×10^5 U937 cells using HIV-LTR-Luc constructs (25 ng) as a reporter and indicated expression plasmids for TRAF2, TRAF5 or CD30 (50 ng each) with or without indicated concentration of SB203580. One hundred nanograms of pRL-TK was also transfected to standardize the transfection efficiency. Total amount of DNA transfected was adjusted to 550 ng by an empty expression vector. Levels of activation are expressed as fold activation compared with the basal luciferase activity of the reporter constructs. Bars indicate standard deviation. (b) Effects of MKK6 on C/EBPβ-mediated induction of HIV-LTR. Transient co-transfection reporter gene assay was done in U937 cells using HIV-LTR-Luc as a reporter. Twenty five nanograms of reporter construct was co-transfected into 1×10^5 cells with an expression plasmid for MKK6 or C/EBPβ (50 ng each). One hundred nanograms of pRL-TK was also transfected to standardize the transfection efficiency. In each experiment total amount of DNA transfected was adjusted to 225 ng by an empty expression vector. Levels of activation are expressed as fold activation compared with the basal luciferase activity of the reporter constructs. Bars indicate standard deviation. $^*P < 0.05$. (c) Effects of mutations in NF-κB or C/EBPβ sites of HIV-LTR on C/EBPβ-mediated HIV-LTR activation by MKK6. Transient co-transfection reporter gene assay was done in U937 cells using HIV-LTR mκB-Luc or HIV-LTR mC/EBPβ-Luc as reporters. The experiment was performed as described in the legend for Fig. 4b. Bars indicate standard deviation. $^*P < 0.05$.

activation, whereas in co-transfection of C/EBPβ with TRAF5 suppression of the synergistic effect by SB203580 was incomplete even at the concentration of 40 μM where HIV-LTR was activated more than 10-fold (Fig. 4a). This suggests that another pathway may also be involved in C/EBPβ activation by TRAF5, which remains to be clarified. Collectively, our results strongly suggest that synergistic effects observed in HIV-LTR activation by C/EBPβ and TRAF signals are mediated mainly by p38 MAPK-mediated activation of C/EBPβ.

We further tested whether direct activation of p38 MAPK by MKK6 potentiates C/EBPβ-mediated activation of HIV-LTR. MKK6 is a MAPK kinase (MAPKK), which directly

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and specifically activates p38 MAPK [21]. In transient co-transfection assays using U937 cells, overexpression of C/EBPβ and MKK6 showed a marked activation of HIV-LTR (Fig. 4b). This synergism was still observed when we used HIV-LTR-Luc with mutations in both NF-κB sites (HIV-LTR mκB-Luc) or HIV-LTR-Luc with mutations in both C/EBPβ sites (HIV-LTR mC/EBPβ-Luc), although the magnitude of activation was a little less than that with intact NF-κB or C/EBPβ sites (Fig. 4c). The result indicates that p38 MAPK is involved in C/EBPβ-mediated activation of HIV-LTR and suggests the notion that this activation is not completely restricted by C/EBPβ sites in HIV-LTR.

To examine involvement of p38 MAPK in reactivation of the integrated HIV by TRAF signals, we next studied the effects of SB203580 on induction of HIV p24 expression in U1 cells by CD30 and TNF receptor signals. Activation of CD30 and TNF receptor resulted in two-fold induction of p24 production by U1 cells, which was blocked by inhibition of p38 MAPK (Fig. 5).

3.5. The expression of TRAF proteins in the spleen monocytes/macrophages of HIV-1 infected patients

Although TRAF proteins have been reported to be expressed in various tissues [9], their expression in monocytes/macrophages of HIV-1 infected patients has not been reported. Therefore, we examined the expression of TRAF2 and TRAF5 in monocytes/macrophages of spleen samples from HIV-1 infected patients by immunostaining. CD68 was used to identify monocytes/macrophages. Analysis by immunofluorescence confocal microscopy revealed that cells stained by CD68 also express TRAF2 or TRAF5 ($n = 5$), suggesting monocytes/macrophages of patients infected with HIV-1 expresses TRAF proteins (Fig. 6). The result supports the notion that TRAF signals can operate in the monocytes/macrophages of the patients infected with HIV-1.

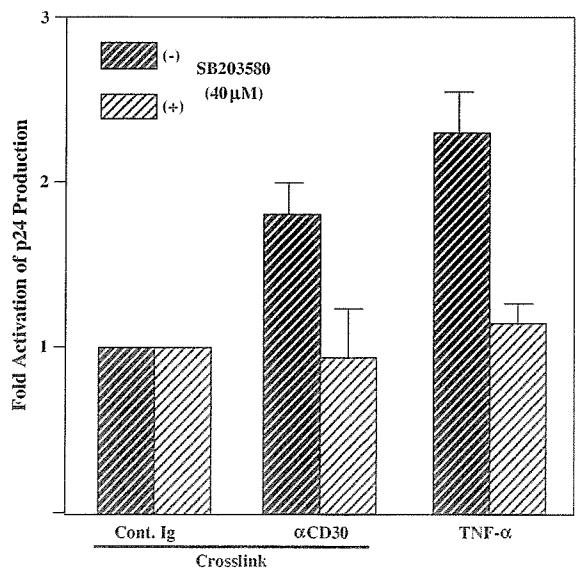

Fig. 5. Signals by TNF receptor family receptor can induce HIV-1 in U1 cells carrying latently infected HIV-1. The effects of SB203580 on induction of HIV p24 in U1 cells by CD30 and TNF receptor signals were examined by ELISA for HIV-1 p24. 1×10^6/ml of U1 cells were cross-linked by CD30 or treated with 10 ng/ml of TNFα with or without 40 μM of p38 MAPK inhibitor SB203580 for 48 h. Isotype matched IgG served as a control for cross-linking by CD30. Results of triplicated experiments are shown with standard deviation. Experiments were repeated at least three times and representative results are shown with standard deviation (s.d.). $^*P < 0.05$.

4. Discussion

In the present study, we have focused on the signal transduction pathway of TRAF other than NF-κB activation pathway in monocytes/macrophages and the results clearly showed that C/EBPβ is a target molecule of p38 MAPK activated by TRAF signals, providing evidence for another pathway of TRAF signals other than NF-κB activation to induce HIV gene expression in monocytes/macrophages.

Our initial data that TRAF activation of HIV-LTR with mutations in both NF-κB sites led us to study signaling pathways other than that for NF-κB. Involvement of p38 MAPK in activation of HIV-LTR has been documented and C/EBPβ transcription factor was shown to be involved in the activation of HIV-LTR in U937 cells [12,15,22]. Furthermore, activities of C/EBP family transcription factors are regulated by phosphorylation by MAPK. For example, CHOP was shown to be activated by p38 MAPK [23]. Another example is C/EBPβ that is activated by ras-signaling pathway, which was assumed to be mediated by ERK [24]. C/EBPβ and C/EBPδ have been reported to be regulated by phosphorylation dependent manner [25,26]. These backgrounds prompted us to focus on p38 MAPK and C/EBPβ, and test whether these molecules were involved in TRAF mediated activation of HIV-LTR.

Our results show the importance of TRAF-p38 MAPK-C/EBPβ pathway in activation of HIV-LTR in monocytes/macrophages. C/EBP factors can associate with members of the NF-κB/Rel family, generating C/EBP-NF-κB complexes, which efficiently activate transcription of cellular genes [27]. Accordingly, both the C/EBP and NF-κB *cis* sequences have been identified in the regulatory regions of many genes involved in inflammation and immune regulation [28]. In HIV-LTR, two NF-κB sites and two CEBPβ sites are also located in a neighboring region. Therefore, NF-κB and CEBPβ can be assumed to cross-talk and co-operate each other in HIV-LTR activation, as was indicated by previous reports [15,29]. Our data presented in Figs. 2b and 4c indicate induction of HIV-LTR mediated by C/EBPβ is dependent not only on C/EBPβ sites but also on NF-κB sites, supporting the notion that C/EBPβ can also activate HIV-LTR in association with NF-κB. However, this redundancy does not minimize the role of TRAF-C/EBPβ pathway in induction of HIV-LTR. The inhibition of p38 MAPK pathway significantly reduced the levels of TRAF-C/EBPβ-mediated activation of HIV-LTR (Fig. 4a), and inhibition of p38 MAPK almost completely abrogated CD30- and TNFR-signal mediated induction of HIV-1 in U1 cells (Fig. 5). Furthermore, previous reports showed that replication of HIV-1 requires C/EBPβ in

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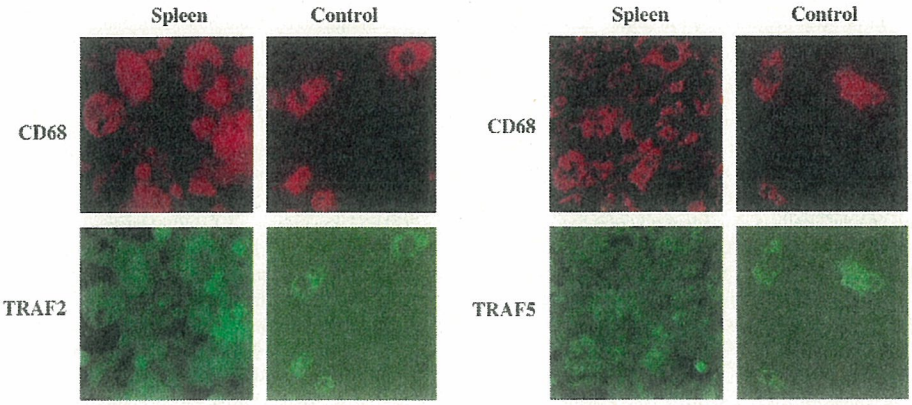

Fig. 6. The spleen macrophages of HIV-1 infected patients express TRAF2 and TRAF5. Spleen samples from HIV-1 infected patients were immunostained by TRAF2 or TRAF5 (green) and monocytes/macrophages marker CD68 (red), and observed by confocal immunofluorescence microscopy. The staining of normal tonsil served as a control.

monocytes/macrophages [13,14]. These observations strongly support the pivotal roles of TRAF-p38 MAPK-C/EBPβ pathway in HIV-LTR activation in monocytes/macrophages.

The data presented in this study also suggested that C/EBPβ is regulated by p38 MAPK dependent phosphorylation. It is well established that regulation of phosphorylation state is an important regulatory mechanism for function of transcription factors, including C/EBPβ. Multiple phosphorylation sites have been characterized on C/EBPβ and are reported to be phosphorylated by Ras signals (Thr 235), calcium/calmodulin-dependent protein kinase (Ser 276), protein kinase C (Ser 105, Ser 240, and Ser 299) and protein kinase A (Ser 105, Ser 173, Ser 233, and Ser 299) [25,26,30]. These reports indicate that multiple phosphorylation sites on C/EBPβ are available for its regulation. Our study added another example of the signal, which phosphorylates and regulates C/EBPβ function, although detailed analyses of phosphorylation sites remain to be done.

We showed that p38 MAPK is involved in the induction of HIV p24 in U1 cells by TNF receptor family signals. U1 cell line, which is chronically infected with HIV-1, is a derivative of monocytic cell line, U937 and carries latently infected HIV-1. Chronically HIV-1 infected cell lines have been used as a model for studying HIV-1 latency and reactivation. The results indicate that TRAF-p38 MAPK-C/EBPβ pathway is involved in the reactivation of the latently infected HIV-1 in monocytes/macrophages. We also showed that TRAF2 and 5 are expressed in monocytes/macrophages of spleen samples from HIV-1 infected patients. Collectively, these results indicate that the signal transduction pathway leading to HIV-1 LTR reactivation that consists of TRAF-p38 MAPK-C/EBPβ operates in monocytes/macrophages of HIV-1 infected patients.

This study underscores the significance of p38 MAPK-C/EBPβ pathway in reactivation of HIV-1 and suggests that p38 MAPK-C/EBPβ pathway is the potential target of prevention of HIV-1 reactivation in monocytes/macrophages. Recent understandings for the pathogenesis of HIV-1, and the virological and immunological responses to HAART, along with the numerous drawbacks of HAART, have clearly demonstrated that the eradication of the virus is not a feasible therapeutic goal, and that there is an urgent need to develop other approaches to control HIV-1 infection and obtain clinical benefit. Several attempts for this purpose include the use of immunosuppressive drugs as an adjuvant to HIV-1 treatment [31]. To this end, molecularly targeted treatments based on understanding of molecules involved in the reactivation of the virus from the reservoir pool will be required for efficient regulations of latent virus after interruption of HAART. The results of this study propose the TRAF-p38 MAPK-C/EBPβ pathway as a potential target of intervention in HIV-1 reactivation by compounds with low molecular weight.

In conclusion, the results in this study indicate an alternative new signal transduction pathway of TRAF proteins leading to HIV-1 LTR activation that consists of TNF receptor family-TRAF-p38 MAPK-C/EBPβ. Identification of a new pathway of TRAF signals that activate C/EBPβ, a key cellular transcription factor regulating HIV-LTR, provides a new target for controlling reactivation of latent HIV-1 in monocytes/macrophages.

References

[1] F.J. Palella Jr., K.M. Delaney, A.C. Moorman, M.O. Loveless, J. Fuhrer, G.A. Satten, D.J. Aschman, S.D. Holmberg, Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N. Engl. J. Med. 338 (1998) 853–860.

[2] T.W. Chun, A.S. Fauci, Latent reservoirs of HIV: obstacles to the eradication of virus. Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 10958–10961.

[3] O. Kutsch, E.N. Benveniste, G.M. Shaw, D.N. Levy, Direct and quantitative single-cell analysis of human immunodeficiency virus type 1 reactivation from latency. J. Virol. 76 (2002) 8776–8786.

[4] M. Mallardo, E. Dragonetti, F. Baldassarre, C. Ambrosino, G. Scala, I. Quinto, An NF-kappaB site in the 5′-untranslated leader region of the human immunodeficiency virus type 1 enhances the viral expression in response to NF-kappaB-activating stimuli. J. Biol. Chem. 271 (1996) 20820–20827.

[5] R. Lapointe, R. Lemieux, A. Darveau, HIV-1 LTR activity in human CD40-activated B lymphocytes is dependent on NF-kappaB. Biochem. Biophys. Res. Commun. 229 (1996) 959–964.

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[6] O. Kutsch, D.N. Levy, B.R. Kosloff, G.M. Shaw, E.N. Benveniste, CD154-CD40-induced reactivation of latent HIV-1 infection, Virology 314 (2003) 261–270.
[7] P. Biswas, C.A. Smith, D. Goletti, E.C. Hardy, R.W. Jackson, A.S. Fauci, Cross-linking of CD30 induces HIV expression in chronically infected T cells, Immunity 2 (1995) 587–596.
[8] M.A. Munoz-Fernandez, J. Navarro, A. Garcia, C. Punzon, E. Fernandez-Cruz, M. Fresno, Replication of human immunodeficiency virus-1 in primary human T cells is dependent on the autocrine secretion of tumor necrosis factor through the control of nuclear factor-kappa B activation, J. Allergy Clin. Immunol. 100 (1997) 838–845.
[9] R.H. Arch, R.W. Gedrich, C.B. Thompson, Tumor necrosis factor receptor-associated factors (TRAFs)—a family of adapter proteins that regulates life and death, Genes Dev. 12 (1998) 2821–2830.
[10] G. Herbein, Cytokines, viruses and macrophages: an interactive network. An immune dysregulation involving the members of the tumor necrosis factor (TNF) receptor superfamily could be critical in AIDS pathogenesis, Pathol. Biol. (Paris) 45 (1997) 115–125.
[11] J.M. Orenstein, C. Fox, S.M. Wahl, Macrophages as a source of HIV during opportunistic infections, Science 276 (1997) 1857–1861.
[12] P.S. Cohen, H. Schmidtmayerova, J. Dennis, L. Dubrovsky, B. Sherry, H. Wang, M. Bukrinsky, K.J. Tracey, The critical role of p38 MAP kinase in T cell HIV-1 replication, Mol. Med. 3 (1997) 339–346.
[13] M.R. Nonnemacher, T.H. Hogan, S. Quiterio, B. Wigdahl, A. Henderson, F.C. Krebs, Identification of binding sites for members of the CCAAT/enhancer binding protein transcription factor family in the simian immunodeficiency virus long terminal repeat, Biomed. Pharmacother. 57 (2003) 34–40.
[14] E.S. Lee, D. Sarma, H. Zhou, A.J. Henderson, CCAAT/enhancer binding proteins are not required for HIV-1 entry but regulate proviral transcription by recruiting coactivators to the long-terminal repeat in monocytic cells, Virology 299 (2002) 20–31.
[15] A.J. Henderson, X. Zou, K.L. Calame, C/EBP proteins activate transcription from the human immunodeficiency virus type 1 long terminal repeat in macrophages/monocytes, J. Virol. 69 (1995) 5337–5344.
[16] T.A. Kunkel, J.D. Roberts, R.A. Zakour, Rapid and efficient site-specific mutagenesis without phenotypic selection, Methods Enzymol. 154 (1987) 367–382.
[17] R. Horie, S. Aizawa, M. Nagai, K. Ito, M. Higashihara, T. Ishida, J. Inoue, T. Watanabe, A novel domain in the CD30 cytoplasmic tail mediates NFkappaB activation, Int. Immunol. 10 (1998) 203–210.
[18] R. Horie, M. Watanabe, T. Ishida, T. Koiwa, S. Aizawa, K. Itoh, M. Higashihara, M.E. Kadin, T. Watanabe, The NPM-ALK oncoprotein abrogates CD30 signaling and constitutive NF-kappaB activation in anaplastic large cell lymphoma, Cancer Cell 5 (2004) 353–364.
[19] T. Yuasa, S. Ohno, J.H. Kehrl, J.M. Kyriakis, Tumor necrosis factor signaling to stress-activated protein kinase (SAPK)/Jun NH2-terminal kinase (JNK) and p38. Germinal center kinase couples TRAF2 to mitogen-activated protein kinase/ERK kinase kinase 1 and SAPK while receptor interacting protein associates with a mitogen-activated protein kinase kinase kinase upstream of MKK6 and p38, J. Biol. Chem. 273 (1998) 22681–22692.
[20] J.A. Engelman, M.P. Lisanti, P.E. Scherer, Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis, J. Biol. Chem. 273 (1998) 32111–32120.
[21] J. Raingeaud, A.J. Whitmarsh, T. Barrett, B. Derijard, R.J. Davis, MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway, Mol. Cell. Biol. 16 (1996) 1247–1255.
[22] L. Shapiro, K.A. Heidenreich, M.K. Meintzer, C.A. Dinarello, Role of p38 mitogen-activated protein kinase in HIV type 1 production in vitro, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 7422–7426.
[23] X.Z. Wang, D. Ron, Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase, Science 272 (1996) 1347–1349.
[24] T. Nakajima, S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, S. Akira, Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 2207–2211.
[25] H.J. Tae, S. Zhang, K.H. Kim, cAMP activation of CAAT enhancer-binding protein-beta gene expression and promoter I of acetyl-CoA carboxylase, J. Biol. Chem. 270 (1995) 21487–21494.
[26] C. Trautwein, P. van der Geer, M. Karin, T. Hunter, M. Chojkier, Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements, J. Clin. Invest. 93 (1994) 2554–2561.
[27] S.M. Dunn, L.S. Coles, R.K. Lang, S. Gerondakis, M.A. Vadas, M.F. Shannon, Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter, Blood 83 (1994) 2469–2479.
[28] S. Akira, T. Kishimoto, NF-IL6 and NF-kappa B in cytokine gene regulation. [Review] [215 refs], Adv. Immunol. 65 (1997) 1–46.
[29] G. Nabel, D. Baltimore, An inducible transcription factor activates expression of human immunodeficiency virus in T cells, Nature 326 (1987) 711–713.
[30] C. Trautwein, C. Caelles, P. van der Geer, T. Hunter, M. Karin, M. Chojkier, Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain, Nature 364 (1993) 544–547.
[31] G.P. Rizzardi, A. Lazzarin, G. Pantaleo, Potential role of immune modulation in the effective long-term control of HIV-1 infection, J. Biol. Regul. Homeost. Agents 16 (2002) 83–90.

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IκBα independent induction of NF-κB and its inhibition by DHMEQ in Hodgkin/Reed-Sternberg cells

Mariko Watanabe[1,*], Md Zahidunnabi Dewan[2,3,*], Miyako Taira[1], Momoko Shoda[4], Mitsuo Honda[3], Testutaro Sata[5], Masaaki Higashihara[1], Marshall E Kadin[6], Toshiki Watanabe[4], Naoki Yamamoto[2,3], Kazuo Umezawa[7] and Ryouichi Horie[1]

Constitutive nuclear factor κB (NF-κB) activation characterizes Hodgkin/Reed-Sternberg (H-RS) cells. Blocking constitutive NF-κB has been shown to be a potential strategy to treat Hodgkin lymphoma (HL). Here, for the first time we show that although constitutive NF-κB level of H-RS cell lines is very high, topoisomerase inhibitors further enhance NF-κB activation through IκB kinase activation in not only H-RS cell lines with wild-type IκBα, but also in those with IκBα mutations and lacking wild-type IκBα. Thus, both constitutive and inducible NF-κB are potential targets to treat HL. We also present the data that indicate the involvement of IκBβ in NF-κB induction by topoisomerase inhibitors. A new NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ) inhibited constitutive NF-κB activity and induced apoptosis of H-RS cell lines. DHMEQ also inhibited the growth of H-RS cells without significant systemic toxicity in a NOD/SCID/γc^{null} (NOG) mice model. DHMEQ and topoisomerase inhibitors revealed enhancement of apoptosis of H-RS cells by blocking inducible NF-κB. Results of this study suggest that both constitutive and inducible NF-κB are molecular targets of DHMEQ in the treatment of HL. The results also indicate that IκBβ is involved in NF-κB activation in H-RS cells and IκBβ substitutes for IκBα in H-RS cells lacking wild-type IκBα.

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Advances in chemotherapy and radiotherapy regimens for treating Hodgkin lymphoma (HL) represent a significant breakthrough in clinical oncology and have increased the long-term survival rate from 5% in the 1960s to greater than 80%. Today, problems of late side effects by chemotherapy such as secondary malignancies, myelodysplasia and cardiotoxicities and chemotherapy-resistant cases with poor prognosis have become important issues to still be resolved.[1] Recently, a strategy that targets the molecules critical for maintenance and growth of the tumor cells has been thought to be a key to develop more effective treatment with less undesirable effects.[2] This strategy intensifies the specificity of treatments to tumor cells and minimizes undesirable effects to normal cells. To establish a molecular target strategy for HL, it appears critical to identify the biological basis involved in anti-apoptosis of HL and develop specific agents that target this pathway.

It was found that constitutively activated nuclear factor-κB (NF-κB) is a molecular hallmark and survival mechanism for Hodgkin/Reed–Sternberg (H-RS) cells. Defective IκBα has been reported to be a cause of constitutive NF-κB activation in H-RS cells bearing IκBα gene mutations.[3–7] We also reported that ligand-independent signals from overexpressed CD30 is responsible for constitutive NF-κB activation.[8]

[1]Department of Hematology, School of Medicine, Kitasato University, Kitasato, Sagamihara, Kanagawa, Japan; [2]Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan; [3]AIDS Research Center, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo, Japan; [4]Laboratory of Tumor Cell Biology, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan; [5]Department of Pathology, National Institute of Infectious Diseases, Toyama, Shinjuku-ku, Tokyo, Japan; [6]Department of Pathology, Harvard Medical School and Beth Israel Deaconess Medical Center, Boston, MA, USA and [7]Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Hiyoshi, Kohoku-ku, Yokohama, Kanagawa, Japan

Correspondence: Dr R Horie, MD, PhD, Department of Hematology, School of Medicine, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan.
E-mail: rhorie@med.kitasato-u.ac.jp or

Dr N Yamamoto, MD, PhD, Department of Molecular Virology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan.
E-mail: yamamoto.mmb@tmd.ac.jp

*These authors contributed equally to this work.

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Constitutively activated NF-κB is considered to be responsible for aberrant growth and cytokine gene expression of H-RS cells.[9,10] We reported that adenovirus-mediated gene transfer of a dominant IκBα resistant to phosphorylation-mediated degradation or a decoy CD30 lacking a tumor necrosis factor receptor associated factor (TRAF) binding domain can block the constitutive NF-κB activity and induce apoptosis in H-RS cell lines.[8] These observations suggest that NF-κB, which is strongly and constitutively activated in H-RS cells, provides a molecular target for the treatment of HL.

Recently, NF-κB activation has also been connected with chemo-resistance of tumor cells.[11] Topoisomerases are essential for cell survival and crucial for multiple aspects of DNA metabolism. Topoisomerases control the degree of supercoiled DNA, and whereas type I is crucial for transcription, type II is necessary for DNA replication. In both situations, the enzyme creates transient single-strand breaks in DNA, and the inhibitors prevent the replication of the strand breaks. SN-38, a powerful active metabolite of CPT-11, targets topoisomerase I activity, whereas daunorubicin and etoposide are topoisomerase II inhibitors. Treatments with topoisomerase inhibitors induce transient NF-κB activation via IκB kinase (IKK), which makes tumor cells resistant to induction of apoptosis.[12]

Previous reports suggested that blockade of the SN-38-induced activation of NF-κB by adenovirus-mediated gene transfer of IκBα super-repressor enhanced anti-tumor activity.[13] Therefore, if topoisomerase inhibitors induce NF-κB activation in H-RS cells, inducible NF-κB also becomes a potential molecular target to treat HL. As tumor cells used in previous experiments did not show constitutive NF-κB activity, it remains to be examined whether the same strategy can be applied to tumor cells of HL with constitutive activation of NF-κB, especially for those with IκBα mutations.

IκBα mutations result in production of truncated, nonfunctional IκBα protein. Existence of IκBα mutations and lack of wild-type IκBα has been thought to make NF-κB activity uncontrolled by upstream IKK signals, leading to constitutive activation of NF-κB.[3–5,7] NF-κB induction by topoisomerase inhibitors has been reported to be mediated by IKK.[12,14] Therefore, it can be hypothesized that treatment with topoisomerase inhibitors will fail to induce NF-κB activity in H-RS cell lines with IκBα mutations.

Dehydroxymethylepoxyquinomicin (DHMEQ) is a new NF-κB inhibitor that is 5-dehydroxymethyl derivative of a novel compound epoxyquinomicin C having a 4-hydroxy-5,6-epoxycyclohexenone structure like panepoxydone.[15] Panepoxydone had been found to inhibit TNF-α-induced activation of NF-κB. We have shown that DHMEQ inhibits NF-κB at the level of nuclear translocation.[15]

In the present study, we demonstrate that topoisomerase inhibitors induce NF-κB in not only H-RS cell lines with wild-type IκBα but also in H-RS cell lines lacking wild-type IκBα by activating IKK and constitutive as well as inducible NF-κB are the molecular target of HL treatment by DHMEQ. The results also indicate that NF-κB activity in H-RS cells with IκBα mutations is regulated by IκBβ, which is a substitute for IκBα.

MATERIALS AND METHODS

Cell Cultures

H-RS cell lines (KMH2, L428, HDLM2 and L540) were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell lines were cultured in RPMI 1640 with supplementation of recommended concentrations of fetal bovine serum (FBS) and antibiotics. Peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers were cultured in RPMI 1640 supplemented with 20% FBS and antibiotics.

Chemicals

DHMEQ is a NF-κB inhibitor that acts at the level of nuclear translocation of NF-κB.[15,16] Daunorubicin and etoposide were purchased from SIGMA (Toyko, Japan). SN-38, an active metabolite of camptothecin-11, was provided by Yakult (Tokyo, Japan). Compounds were dissolved with DMSO and used for experiments at the indicated concentrations. Bisbenzimide H 33342 fluorochrome (Hoechst 33342) was purchased from CALBIOCHEM (Bad Soden, Germany).

Electrophoretic Mobility Shift Analysis

Electrophoretic mobility shift analysis (EMSA) was carried out according to the methods described previously.[17] Double-stranded oligonucleotide probes containing the mouse immunoglobulin kappa (Igκ) light-chain NF-κB consensus site and Oct-1 were purchased from Promega (Madison, WI, USA). Antibodies used for super-shift assays were as follows: NF-κB p50 (C-19) goat polyclonal antibody, RelB (C-19) rabbit polyclonal antibody and mouse antibodies for NF-κB p65 (C-20), NF-κB p52 (C-5) and c-Rel (B-6) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

In Vitro Kinase Assay

Cell extracts prepared from equivalent numbers of cells were subjected to immunoprecipitation with anti-IKKα monoclonal antibody in TNT buffer (20 mM TrisHCl (pH 7.5), 200 mM NaCl, 1% Triton X100, 0.5 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 100 μM Na_3VO_4 and 20 mM β-glycerophosphate). Immunoprecipitates were collected on Protein G-Sepharose beads (Amersham Biosciences Corp., Piscataway, NJ, USA), which were then washed three times with TNT buffer and three times with kinase reaction buffer (20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 100 μM Na_3VO_4, 20 mM β-glycerophosphate, 2 mM DTT, and 20 mM ATP). Kinase reactions were performed for 30 min at 30°C using 5 μCi of [γ-^{32}P]ATP and glutathione S-transferase (GST)-IκBα protein (amino acids 1317) (Santa Cruz Biotechnology, Inc.) as substrates. The reaction products were separated on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and revealed by autoradiography.

Antibodies for IKKα (B78-1) (BD Biosciences Pharmingen, San Diego, CA, USA) and IKKα/β (H-470) (Santa Cruz Biotechnology, Inc.) were used for immunoprecipitation and immunoblot of IKKα respectively.

Coimmunoprecipitation Analysis

Cell extracts were subjected to immunoprecipitation with anti-IκB-β (S-20) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) in TNE buffer (10 mM Tris–HCl, pH 7.8, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA). Immunoprecipitates were collected on Protein G-Sepharose beads (Amersham Biosciences Corp.), which were then washed three times with TNE buffer.

Immunoblot Analysis

Immunoblot analysis was performed as described.[17] Antibodies used were as follows: NF-κB p65 (F-6) mouse monoclonal antibody, NF-κB p50 (C-19) gout polyclonal antibody, IκB-β(S-20) rabbit polyclonal antibody, α tubulin (TU-02) mouse monoclonal IgM antibody (all from Santa Cruz Biotechnology, Inc.) and phosphoserine mouse monoclonal IgM antibody (Biomol, Meeting, PA, USA). Alkaline phosphatase-conjugated secondary antibodies are as follows: donkey anti-goat IgG antibody, donkey anti-mouse IgG antibody, donkey anti-rabbit IgG antibody (all from Chemicon International, Temecula, CA, USA) and goat anti-mouse IgM antibody (Santa Cruz Biotechnology, Inc.).

Cell Viability Assay

Effects of DHMEQ on cell viability were assayed by the MTT method as described previously.[8] After incubation with DHMEQ or DMSO alone at the indicated concentrations and time points, 5×10^4 cells treated with MTT solution were measured by a microplate reader (Bio-Rad, Richmond, CA, USA) at a reference wavelength of 570 nm and test wavelength of 450 nm. The cell viability was expressed as a percentage of the DMSO-treated control samples.

Immunohistochemistry

Immunohistochemical analyses were carried out as described.[17] Primary antibodies used were as follows: mouse monoclonal antibody for activated NF-κB p65 (Chemicon International), rabbit polyclonal antibodies for cleaved caspase-3 (Asp-175) (Cell signalling, Beverly, MA, USA), NF-κB p65 (C-20), Bcl-xL (H-62), FLIPs/l (H-202), GAPDH (FL-335) and mouse monoclonal antibody for α tubulin (TU-02) (all from Santa Cruz Biotechnology, Inc.). Fluorochrome-labeled secondary antibodies used in these studies are as follows: FITC-labeled anti-goat immunoglobulin donkey antibody, FITC-labeled anti-rabbit immunoglobulin goat antibody and FITC-labeled anti-mouse immunoglobulin goat antibody (all from Santa Cruz Biotechnology, Inc.).

Northern Blotting

Northern blot analysis was carried out essentially as described.[17] Briefly, 2 μg of poly(A)-selected RNA was size-fractionated on 1% formalin agarose gel electrophoresis and subsequently blotted onto Hybond-C extra-nitrocellulose membranes (Amersham Bioscience Corp.). Filters were hybridized in 4 × SSC, 1 × Denhardts, 0.5% SDS, 0.1 M $NaPO_4$ (pH 7.0), 10% Dextran-Na at 65°C with 1.0×10^6 cpm/ml of random prime-labeled probes. After washings to a final stringency of 0.2 × SSC and 0.1% SDS at 65°C, filters were exposed to XAR-5 films (Eastman Kodak, Rochester, NY, USA) at −80°C. RT-PCR amplified cDNA fragments of human Bcl-xL, human FLIPs/l and human GAPDH were used as probes.

Apoptosis and Analysis of Caspase Activity

Cells were labeled with FITC-conjugated Annexin V (BD Biosciences, Pharmingen) and followed by flow cytometric analysis. For analysis of morphological changes of nuclei, cells were stained by 10 μM Hoechst 33342, and photographed through a UV filter. Activation of caspases was examined by detecting decrease of uncleaved fragments or appearance of cleaved fragments with immunoblot analysis. Antibodies used in these experiments were as follows: mouse monoclonal antibodies for caspase-3/CPP32 (BD Biosciences Pharmingen), cleaved caspase-8 (Asp384) 11G10 and rabbit polyclonal antibodies for caspase-9 (both from Cell Signaling). Alkaline phosphatase-conjugated secondary antibodies are as follows: anti-mouse IgG (H&L) antibody and anti-rabbit IgG (Fc) antibody (both from Promega). caspase activity was blocked using cell-permeable irreversible caspase-3 inhibitor II (z-DEVD-FMK), caspase 8 inhibitor II (z-IETD-FMK) or caspase-9 inhibitor I (z-LEHD-FMK) (all from CALBIOCHEM).

In Vivo Therapeutic Effect of DHMEQ

NOG mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). The Ethical Review Committee of the National Institute of Infectious Diseases approved the experimental protocol. H-RS cells were inoculated subcutaneously into the post-auricular area of NOG mice. DHMEQ was administered intraperitoneally three times a week for 1 month to mice at doses of 12 mg/kg, beginning on either day 0 or day 5 when tumors were palpable. The control mice were inoculated RPMI 1640 (200 μl). Mice were sacrificed at 1-month follow-up period after inoculation. Tumor tissues were fixed with Streck Tissue Fixative (STF) and processed to paraffin wax-embedded sections for staining with hematoxylin and eosin (HE), as described.[18]

Statistical Analysis

Differences between mean values were assessed by two-tailed *t*-test. A *P*-value <0.05 was considered to be statistically significant.

RESULTS

Topoisomerase Inhibitors Induce Transient NF-κB Activation in H-RS Cell Lines Independent of IκBα Mutations

We first examined effects of topoisomerase inhibitors on the levels of NF-κB activity in H-RS cell lines without IκBα mutation (L540 and HDLM2) as well as in H-RS cells with IκBα mutations and lacking wild-type IκBα (KMH2 and L428). Treatment by SN-38, daunorubicin and etoposide further induced transient NF-κB activation, which peaked at 1 or 2 h after treatment in L540 without IκBα mutations (Figure 1a top). HDLM2, which also lacks IκBα mutation, showed a similar result (data not shown). Surprisingly, NF-κB induction, which peaked later at 4 or 5 h, was observed in H-RS cell line KMH2 bearing IκBα mutations (Figure 1a bottom). L428 bearing IκBα mutations showed transient NF-κB activation, which peaked at 1 h after treatment (data not shown). Supershift analyses using nuclear extracts prepared from H-RS cell lines at the time point of maximal NF-κB induction revealed that NF-κB includes p50 and p65. The representative results of KMH2 cells are shown in Figure 1b. Next we examined whether NF-κB induction by topoisomerase inhibitors in H-RS cell lines is mediated by IKK activation. *In vitro* kinase assay clearly showed transient activation of IKK after SN-38 treatment in both L540 and KMH2 cells (Figure 1c). These results indicate that transient NF-κB induction, which is induced independent of IκBα mutations by topoisomerase inhibitors is mediated by activation of the IKK pathway and not only constitutive, but also inducible NF-κB is a potential molecular target for treatment of HL.

Involvement of IκBβ in the Topoisomerase Inhibitor-Mediated Induction of NF-κB in H-RS Cells with IκBα Mutations

Previous reports indicated the functional redundancy and similar kinetics of IκBα and IκBβ.[19] Therefore, we examined the involvement of IκBβ in the topoisomerase inhibitor-mediated induction of NF-κB in H-RS cells with IκBα mutations and lacking IκBα. To address this point, we examined the binding of p65 and p50 with IκBβ, and the degradation and phosphorylation of IκBβ after SN-38 treatment. Previous reports indicated that like IκBα, IκBβ is also regulated by the phosphorylation of serine residues near the N terminus.[20] We used anti-phosphoserine antibody to detect phosphorylation of IκBβ. We also examined by confocal microscopy the distribution of activated NF-κB p65 and IκBβ after SN-38 treatment.

Both p65 and p50 were immunoprecipitated along with IκBβ in L428 cells (Figure 2a). In L428 cells, decreased expression of IκBβ was observed from 0.5 to 1 h after SN-38 treatment (Figure 2b). The decreased expression of IκBβ was preceded by phosphorylation of IκBβ (Figure 2c). Stimulation of L428 with SN-38 induced NF-κB activity, which peaked at 1 h (Figure 2d). Analysis by confocal microscopy

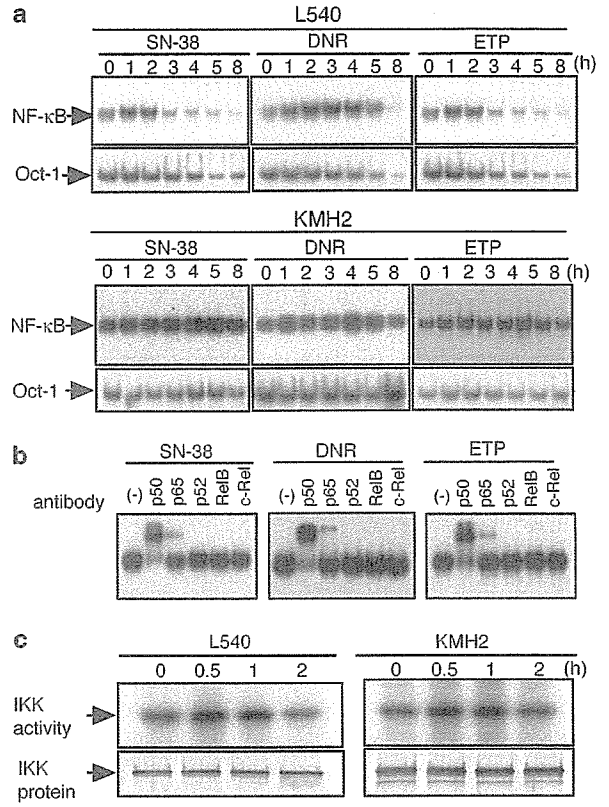

Figure 1 Treatment by topoisomerase inhibitors further induces NF-κB activity via IKK in H-RS cell lines with or without IκBα mutations. (**a**) The effect of SN-38, daunorubicin and etoposide on NF-κB activity of H-RS cells with or without IκBα mutations. H-RS cell lines; L540 cells without IκBα mutations or KMH2 cells with IκBα mutations were treated with 100 ng/ml of SN-38, 2 μM daunorubicin or 50 μM etoposide for indicated hours. Nuclear extracts (1 μg) were examined for NF-κB-binding activity by EMSA with radiolabeled NF-κB specific probe. Topoisomerase inhibitors used are indicated above. DNR, daunorubicin; ETP, etoposide. Lower panels show results of EMSA with a control probe, Oct-1. (**b**) Analysis of NF-κB subcomponent after treatment with topoisomerase inhibitors by supershift assay. KMH2 cells were treated with topoisomerase inhibitors for 5 h and harvested.Two micrograms of nuclear extracts were subjected to analysis. Antibodies used are indicated above. (**c**) *In vitro* kinase assay of H-RS cell lines with or without IκBα mutations. L540 cell or KMH2 cells treated with 100 ng/ml of SN-38 for indicated hours were immunoprecipitated by anti-IKK antibody and subjected to *in vitro* kinase assay using IκBα as substrate. Phosphorylation of IκBα by IKK, which represents IKK activity, is shown in the upper panels. Immunoblot of immunoprecipitates by anti-IKK antibody in lower panels shows an equal amount of IKK was used in each reaction.

revealed that active p65 NF-κB, which showed diffuse distribution before treatment, was concentrated in the nucleus 1 h after SN-38 treatment and was redistributed to the cytoplasm 2 h after SN-38 treatment (Figure 2e, top panels). IκBβ decreased the expression 1 h after SN-38 treatment (Figure 2e, bottom panels). These results indicate that IκBβ is involved in the topoisomerase inhibitor-mediated induction of NF-κB in H-RS cells lacking wild-type IκBα.

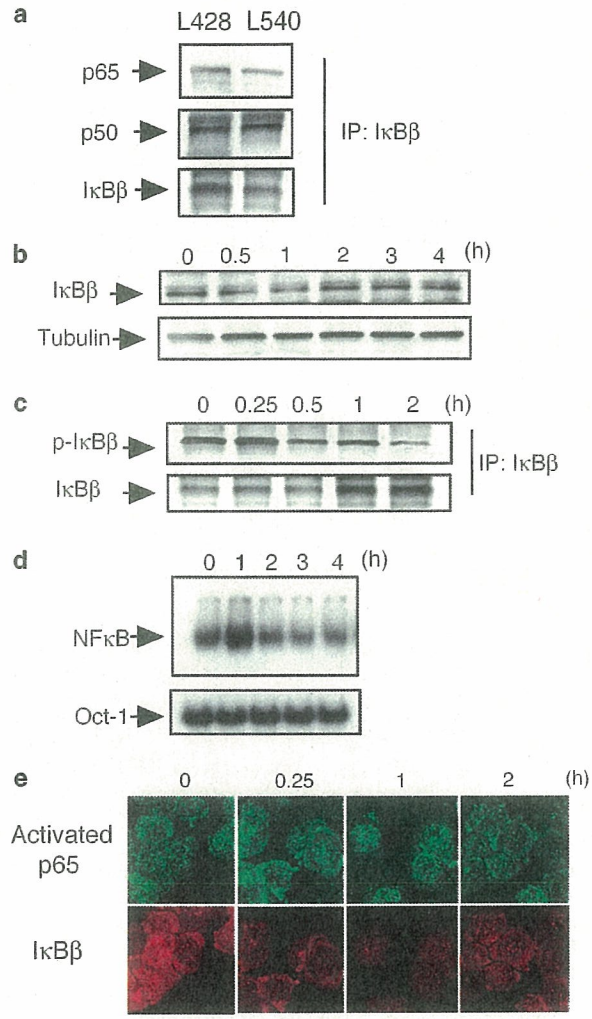

Figure 2 Topoisomerase inhibitor-mediated induction of NF-κB is mediated by the phosphorylation and degradation of IκBβ in H-RS cells with IκBα mutations. (**a**) Coimmunuprecipitation analysis of NF-κB p50 and p65 with IκBβ. Immunoprecipitates of anti-IκBβ antibody were blotted with anti-NF-κB p50 or p65 antibodies (upper panels). Immunoprecipitates were blotted with anti-IκBβ antibody (bottom panel). IP, immunoprecipitation. (**b**) Expression level of IκBβ protein in L428 cells after treatment with topoisomerase inhibitor. Whole cell lysates of L428 treated with SN-38 for the indicated number of hours were blotted with anti-IκBβ antibody (upper panel) or anti-α tubulin antibody (bottom panel). (**c**) Phosphorylation of IκBβ protein in L428 cells after treatment with topoisomerase inhibitor. L428 cells were treated with 100 ng/ml of SN-38 for the indicated number of hours. Immunoprecipitates of anti-IκBβ antibody were blotted with anti-phosphoserine antibody (upper panel) or anti-IκBβ antibody (bottom panel). (**d**) The effect of SN-38 on NF-κB activity in L428 cells. L428 cells were treated with 100 ng/ml of SN-38 for the indicated number of hours. Nuclear extracts (1 μg) were examined for NF-κB-binding activity by EMSA with radiolabeled NF-κB-specific probe. (**e**) Localization of activated NF-κB p65 and IκBβ after treatment with topoisomerase inhibitor. L428 cells were treated with 100 ng/ml of SN-38 for the indicated number of hours. Confocal immunofluorescence microscopic analysis was performed on cytospin samples stained with antibodies against activated NF-κB p65 and IκBβ.

DHMEQ Reduces Cell Growth and Induces Apoptosis of H-RS Cells Through Inhibition of Constitutive NF-κB Activity

We next examined the effects of DHMEQ on constitutive NF-κB activity in H-RS cell lines. Treatment with DHMEQ at a concentration of 10 μg/ml abrogated constitutive NF-κB-binding activity in these cell lines (Figure 3a). Inhibition of constitutive NF-κB activity by DHMEQ was observed in all H-RS cell lines examined irrespective of presence (L428 and KMH2) or absence (L540 and HDLM2) of IκBα mutations. Analysis by confocal microscopy revealed accumulation of active form of NF-κB p65 in the cytoplasm after DHMEQ treatment in KMH2 and L540, indicating action of DHMEQ at the level of translocation of NF-κB into the nucleus (Figure 3b). Time-course studies showed that DHMEQ almost completely abrogated NF-κB binding activity at 1 hour after DHMEQ treatment and thereafter for 16 h (Figure 3c). The supershift assays revealed that the affected NF-κB components include p50 and p65 as reported previously[9] (Figure 3d).

We next examined the effect of DHMEQ on viability of H-RS cell lines (Figure 4). Results of MTT assays showed that DHMEQ treatment reduced cell viability of all four H-RS-derived cell lines in a dose-dependent manner, whereas it did not show a significant effect on the viability of PBMC even at higher concentrations (Figure 4a). Furthermore, we examined whether DHMEQ induces apoptosis of H-RS cell lines by analyzing Annexin V reactivity and nuclear fragmentation. Based on the results obtained from MTT assays, L428 and KMH2 cells were treated with 20 μg/ml, and L540 and HDLM2 cells were treated with 10 μg/ml of DHMEQ. Flow cytometric analysis showed a significant increase in the number of Annexin V-positive cells after DHMEQ treatment in H-RS cell lines, but not in PBMC (Figure 4b). Hoechst 33342 staining showed fragmentation and condensation of the nucleus of H-RS cell lines, suggesting induction of apoptosis in these cells, but not in normal PBMC (Figure 4c).

Taken together, these results indicate that DHMEQ selectively targets constitutive NF-κB activity in H-RS cells and induces apoptosis of these cells independent of IκBα mutations.

DHMEQ-Induced Apoptosis Involves Activation of Caspases 3, 8 and 9

To examine whether induction of apoptosis upon inhibition of constitutive NF-κB activity in H-RS cells by DHMEQ is caused by the activation of the caspase pathway, we first studied activation of caspase 3 by immunoblot analysis. Results clearly showed cleavage of caspase 3, suggesting DHMEQ-induced apoptosis is associated with activation of the caspase pathway (Figure 5a, top panel). To differentiate the membranous and mitochondrial pathways, we next examined activation of caspase 8 and 9 that are upstream of caspase 3 by immunoblot analysis. DHMEQ-treated H-RS cells showed activation of both caspases 8 and 9 (Figure 5a