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Human neuroblastoma cells produce the NF-κB-like HIV-1 transcription activator during differentiation

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Human neuroblastoma GOTO and IMR32 cells differentiate into neural cells on treatment with 5-BrdU. Here we report that in association with differentiation induced by 5-BrdU, the transcription of HIV-1 was activated in both neuroblastoma cell lines. An NF-κB-like factor was also found to be produced in association with the neural cell differentiation of these cells. These results suggest that, during differentiation of neural cells, an NF-κB-like factor was produced, and that when the neural cells are infected with HIV-1 the production of NF-κB results in activation of transcription of HIV-1. Thus this NF-κB-like factor may be involved in the mechanism causing AIDS dementia.

NF-κB; Neuroblastoma; HIV-1; AIDS; Nerve cell differentiation

1. INTRODUCTION

Human immunodeficiency virus (HIV-1) is a cytopathic retrovirus and is the etiological agent of the acquired immunodeficiency syndrome, AIDS [1,2]. Transcription of HIV-1 in stimulated T lymphocytes is activated by the binding of NF-κB to the enhancer of HIV- LTR [3]. Infection with HIV-1 results in depletion of T lymphocytes and causes AIDS, which is often associated with cerebral disturbance. Neural cells that are susceptible to HIV-1 have been detected in vitro [4], but little is known about the infected cell types or the transcription-activating factors in brain cells of HIV patients. In the present study, we demonstrated the production of a nuclear factor similar to NF-κB during differentiation of neural cells. The transcription-activating factor, NF-κB, was originally identified as an enhancer-binding protein for the immunoglobulin κ gene [5]. Its DNA binding activity has been demonstrated in cells of B-cell lineage [5], activated T cells [3], and differentiated monocytes [6], but this is the first report of the production of this factor and induction of transcription of HIV-1 in neural cells. This factor may be an important cause of AIDS dementia.

2. MATERIALS AND METHODS

2.1. *Materials*

The human neuroblastoma cell lines used in this study were obtained from the Japanese Cancer Research Resources Bank (JCRB). RPMI medium 1640 (RPMI1640). Eagle's minimum essential medium (EMEM) and fetal bovine serum (FBS) were purchased from Gibco. Plasmids pCV1 and pCD12 were provided by Dr. F. Wong-Staal. An NF-κB binding protein detection system was purchased from Gibco-BRL. Restriction endonucleases were obtained from BRL and 5-BrdU was from Sigma. All other chemicals used were of reagent grade.

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2.2. *Cell culture*

Human neuroblastoma GOTO, IMR32 and NIH3T3 cells were cultured and maintained in medium containing 45% RPMI1640, 45% EMEM and 10% FBS. The cells were cultured in the same medium with or without 5 μM 5-BrdU (5-bromo-2′-deoxyuridine) for 3 days before transfection. For examination of the effect of 5-BrdU on cell growth, cells were inoculated at a density of 5×10^4 cells per well (24-well microplates) and cultured with or without 5-BrdU in the same medium. After several treatments with 5-BrdU, cells were trypsinized and the numbers of viable cells were determined by the Trypan blue exclusion test.

2.3. *Transfection and CAT assay*

About 2×10^5 cells per 6 cm dish were cultured and the medium was changed 6 h before transfection. Cells were transfected with the plasmid pSV2-CAT [7], pCD12(HIV-LTR-CAT) [8] on pCV1(HIV-tat expression vector) [9] by calcium phosphate co-precipitation [10]. For competition assay, excess amounts of HIV-1 LTR fragments were co-transfected with pCD12. The medium was changed 18 h after transfection and culture was continued for 30 h. Then the cells were collected, washed with phosphate-buffered saline (PBS) and 2×10^5 cells were suspended in 0.25 M Tris-HCl (pH 8.0), and cellular extracts were prepared by five cycles of freezing (−80°C) and thawing.

Chloramphenicol acetyl transferase (CAT) activity was measured by incubating whole cell extracts with ^{14}C-labelled chloramphenicol and 5 mM acetyl co-enzyme A at 37°C for 18 h. Acetylated chloramphenicol was separated from non-acetylated chloramphenicol by ascending thin-layer chromatography. Chromatograms were examined with a Fuji image analyzer BA 100.

2.4. *Assay of binding of nuclear proteins to the NF-κB binding motif*

Proteins binding to the NF-κB DNA motif were detected by the

Gibco-BRL protocol. Briefly, 42mer DNA containing two NF-κB DNA motif (GGGGACTTTCC), was end-labeled with [γ-^{32}P]ATP for binding with nuclear extracts. Nuclear extracts were prepared by the method of Dignam et al. [11] after treatment with 5-BrdU for 3 days. Samples of 5 ng of end-labeled DNA fragments were bound with 3 μg of nuclear proteins in a solution of 20 mM HEPES buffer (pH 7.9), 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 125 mM spermidine and 3 μg poly(dI–dC) for 20 min. The preparations were then separated by electrophoresis in a 4% polyacrylamide gel in Tris-borate-EDTA buffer and autoradiographed. For competition assays, excess amounts of cold 42mer fragments and synthesized mutant of NF-κB motif (TCGACAGAATTCACTTTCCGAGAGGCTCGA) [12] were used for binding assays.

2.5. *Binding assay of nuclear proteins with HIV-LTR DNA*

HIV-LTR DNA was digested with *Sac*I and *Pvu*II to a 120 bp DNA fragment (fr120). This 120 bp fragment, which contained the NF-κB binding motif, was end-labelled with [γ-^{32}P]ATP for binding with nuclear extracts. Band shift assays for binding with nuclear proteins from neuroblastoma cells treated with 5-BrdU were performed as described above.

After band shift assay, shifted bands were removed and eluted from the gel by electrophoresis. The obtained protein–DNA complexes were immunoprecipitated by anti-NF-κB p65 rabbit serum (Santa Cruz Biotech. Inc.), and the recovery of the radioactivities of ^{32}P-labelled DNA was determined.

3. RESULTS AND DISCUSSION

3.1. *Cell growth on 5-BrdU treatment*

Human neuroblastoma GOTO [13] and IMR32 [14] cells are known to be prevented from growth and differentiation into glial or nerve cells by treatment of 5-BrdU [15,16]. We examined the effects of 5-BrdU on the growth of GOTO, IMR32 and NIH3T3 cell lines. As shown in Fig. 1, 5-BrdU at 5 μg/ml inhibited the growth of NIH3T3 cells slightly and the growth of GOTO and IMR32 cells moderately. GOTO and IMR32 cells grew slowly from day 2 of treatment, reaching only about 15% of the control growth level on day 5. The viabilities of the 3 cell lines treated with 5-BrdU were about 90% or more. Before treatment, GOTO cells were small and spindle shaped, but during 3 days of treatment with 5-BrdU they became larger and flattened out (cf. [16]). This flattening was thought to represent differentiation of the cells into glial cells. These cells were observed by phase-contrast microscopy after 3 days treatment with 5-BrdU. In subsequent experiments we used GOTO and IMR32 cells after the appearance of large, flattened cells.

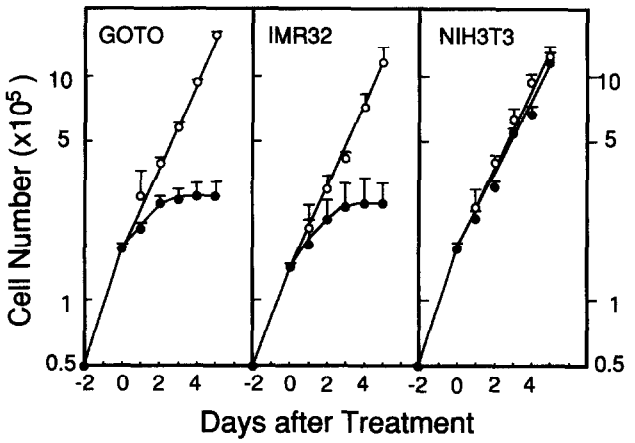

Fig. 1. Effect of 5-BrdU on the growth of GOTO, IMR32 and NIH3T3 cells in culture. Cells were subcultured at 5×10^4 cells/flask for 2 days and then incubated with (●) or without (○) 5 μg/ml 5-BrdU for 5 days. Numbers of viable cells were determined by the Trypan blue exclusion test. Values are means ± S.E.M. (bars) for 4 experiments.

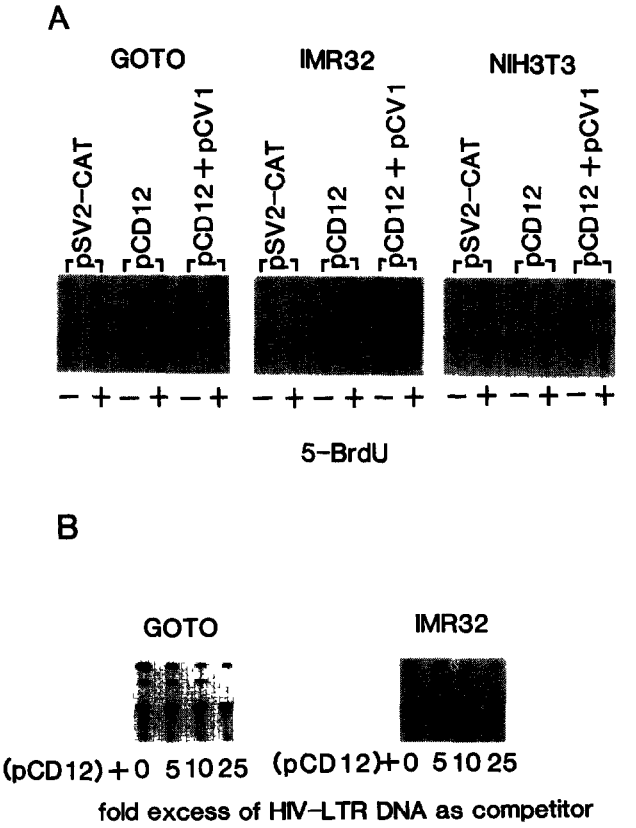

Fig. 2. Transcriptional activation of HIV-LTR by 5-BrdU treatment in GOTO, IMR32 and NIH3T3 cells measured as CAT activity. (A) Thin-layer chromatography after analysis of CAT activity in cells transfected with pSV2-CAT, pCD12(HIV-LTR-CAT) or pCD12 plus pCV1(HIV-tat expression vector). (B) Decrease of CAT activity by competition with excess amounts of HIV-LTR fragments. CAT activities were measured in 5-BrdU-treated GOTO and IMR32 cells transfected with pCD12 with excess amounts of HIV-LTR DNA (HIV-3'LTR *Eco*RI–*Hin*dIII fragment, cf. Fig. 4A).

3.2. *Transcriptional activation of HIV-1 in 5-BrdU-treated neuroblastoma cells*

Cells were cultured in the presence or absence of 5-BrdU for 3 days and then transfected with DNAs of pCD12(HIV-LTR-CAT), pCD12 with pCV1(HIV-1 tat expression vector), or pSV2-CAT. The cells were harvested 48 h after transfection and 2×10^5 cells were collected, and their CAT activities were measured (Fig.

2A). The CAT activity, the percentage conversion of the acetylated form of chloramphenicol, of GOTO cells transfected with pCD12 cultured in the presence of 5-BrdU was about 63.5%, and the same cells in the absence of 5-BrdU was 4.8%. Transfection with the *tat* gene together with pCD12 increased the percentage conversion of the acetylated form by 51.3% (Fig. 2A). Treatment with 5-BrdU also elevated the CAT activity (89.2%). On transfection with the same genes, IMR32 cells before and after differentiation induced by 5-BrdU showed the same response, i.e. elevation of CAT activity, as those in GOTO cells. In NIH3T3, CAT activity did not increase on transfection of pCD12 after treatment with 5-BrdU. This result supports the findings that the elevation of CAT activity that appeared in GOTO and IMR32 cells was not induced by 5-BrdU but by cell differentiation triggered by 5-BrdU. Once differentiation had been induced by 5-BrdU, cells showed similar high CAT activities in media with and without 5-BrdU (data not shown). To exclude the possibility that these results were due to 'read through' by the pCD12 DNA, we co-transfected the GOTO cells and IMR32 with pCD12 and HIV-LTR DNA as a competitor. An increase in the amount of competitor DNA (HIV-LTR) resulted in a marked decrease in CAT activity by competition with HIV-LTR (Fig. 2B). Thus the increase of CAT activity observed in differentiating GOTO and IMR32 cells was concluded not to be due to 'read through', but to transcriptional activation. These results suggested that during differentiation, GOTO and IMR32 cells may produce a transcription-activating factor(s) that associates with HIV-LTR.

3.3. *NF-κB DNA motif binding protein induced in 5-BrdU-treated neuroblastoma cells*

Binding proteins with a NF-κB DNA motif were detected with a Gibco-BRL kit. A 42mer DNA containing two GGGGACTTTCC motifs was end-labeled with

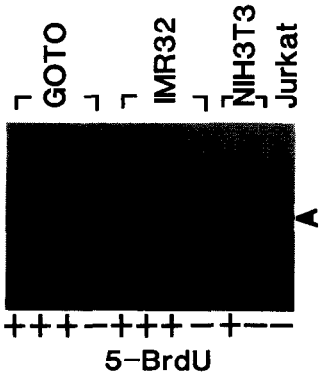

Fig. 3. Binding of the 42mer NF-κB DNA binding motif with nuclear proteins of 5-BrdU-treated cells. A 42mer DNA from Gibco-BRL was end-labeled and used for binding assays. □, with 50-fold excess of unlabeled 42mer DNA added during the band shift assay. ■, with 50-fold excess of unlabeled mutant NF-κB motif added during the band shift assay. The arrowhead indicates the shifted bands.

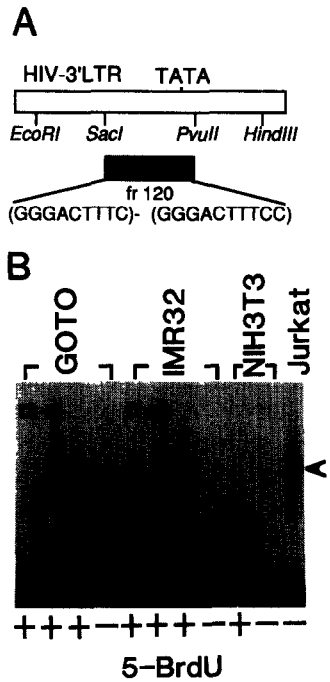

Fig. 4. Binding of HIV-LTR DNA fragments with nuclear proteins of 5-BrdU-treated cells. (A) The used 120 bp DNA fragment cut from HIV-LTR DNA with *SacI* and *PvuII*. The 120 bp DNA fragment was used for binding assays. (B) Band shift assays of nuclear extracts with the 120 bp DNA. □, with 50-fold excess of unlabeled 120 bp DNA added during the band shift assay. ■, with 50-fold excess of unlabeled mutant NF-κB motif added during the band shift assay. The arrowhead indicates the shifted bands.

[γ-^{32}P]ATP, and added to nuclear proteins extracted from GOTO, IMR32, NIH3T3 and PMA-activated Jurkat cells that had been incubated with or without 5-BrdU for 3 days. Then 42mer DNA fragments were separated by electrophoresis and their mobility shifts were examined. No band shift was observed after incubation with the nuclear extract of NIH3T3 incubated with or without 5-BrdU or with those of undifferentiated GOTO and IMR32 cells cultured without 5-BrdU, but nuclear proteins from differentiated GOTO and IMR32 cells (cultured in 5-BrdU) bound with the 42mer DNA fragment (Fig. 3). A shifted band of the same size was also observed after incubation with the nuclear extract of PMA-activated Jurkat cells, which produce NF-κB (Fig. 3). The shifted band disappeared on addition of a 50-fold excess of unlabeled 42mer fragments but it did not disappear on addition of a 50-fold excess of unlabeled mutant NF-κB motif (cf. section 2) (Fig. 3). These results strongly suggested that neuroblastoma cells produce an NF-κB-like transcription-activating factor during differentiation.

3.4. *Binding of the 5-BrdU-induced NF-κB-like factor to HIV-LTR DNA*

To examine the binding activity of the transcription-activating element in HIV-LTR, we separated a 120 bp

fragment containing the NF-κB binding DNA motif [17] of HIV-LTR (Fig. 4A), end-labeled it and incubated it with nuclear protein extracts from GOTO, IMR32, NIH3T3 and PMA-activated Jurkat cells with or without 5-BrdU treatment. The 120 bp DNA fragments were then separated by electrophoresis and mobility shifts were examined. Essentially the same results were obtained for the NF-κB DNA binding motif of HIV-LTR. A shifted band was observed only in nuclear extracts of PMA-activated Jurkat cells and GOTO, IMR32 cells treated with 5-BrdU (Fig. 4B). After band shift assay, shifted bands were removed and eluted from the gel by electrophoresis. The radioactivities of the eluted protein–DNA complexes were 1,783 cpm (GOTO; treated with 5-BrdU), 1,695 cpm (IMR32; treated with 5-BrdU) and 2,345 cpm (Jurkat; treated with PMA). Then, these protein–DNA complexes were treated with anti-NF-κB p65 rabbit serum and immunoprecipitated. The recovered radioactivities were 1,406, 1,361 and 2,023 cpm, respectively. The recovery of radioactivity of these shifted bands was around 80%. These results also indicated that neuroblastoma cells produce an NF-κB or NF-κB-like factor during differentiation and that the transcription-activating factor could bind to the HIV-LTR region.

The presence of an NF-κB or NF-κB-like factor in differentiated human neural cells may be indicative of replication of HIV-1 in neural cells. The CD4 molecule is a receptor of HIV [18], but CD4-negative brain glioma cells have also been shown to be infected with HIV [19]. Recently, GalC (galactosyl ceramide) was found to play a role in HIV entry into neural cells [20]. Differentiated GOTO and IMR32 cells, which also do not express CD4 (data not shown), may be susceptible to infection with HIV and so be able to activate HIV transcription by an NF-κB-like factor. Together with infiltration by macrophages, proliferation of HIV in neuronal cells may be a cause of progressive degeneration of the central nervous system in AIDS patients.

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