**Note**

Detection of Antitumor Promoting Activity in Raji Cells Carrying Epstein-Barr Virus Genome by Immunoblotting Analysis

Akira Kondo, Tetsuhiro Morimoto, and Katsuichiro Okazaki

Department of Bioresource Science, Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795, Japan

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Extract of Raji cells treated with sodium n-butyraté (1 mM) and a tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (TPA, 40 ng/ml), was analyzed by immunoblotting using ten human sera with different antibody titers against Epstein-Barr virus early antigens. Two human sera reacted with one induced polypeptide of 48 kDa and its induction was inhibited by curcumin (4 μg/ml), an antitumor promoter from turmeric. A mouse antiserum against P3HR-1 cells treated with TPA and sodium n-butyraté also detected the 48-kDa polypeptide in Raji cells treated with TPA at concentrations of 2.5 to 80 ng/ml. These results indicate that the immunoblotting analysis can be used in a confirmation test for detection of antitumor promoting activity.

**Key words:** antitumor promoter; Epstein-Barr virus early antigen; Raji cells; P3HR-1 cells; TPA

Raji cells are a human B lymphoblastoid cell line carrying the genome of Epstein-Barr virus (EBV) early antigens (EA), but not a producer of EBV. The EBV EA have been divided into diffuse (EA-D) and restricted (EA-R) components based on their staining patterns in EBV-infected cells by immunofluorescence using human sera of patients with infectious mononucleosis, nasopharyngeal carcinoma, and African Burkitt’s lymphoma. The EBV EA-D staining is detected both in the nucleus and the cytoplasm, and the EBV EA-R staining is restricted to the cytoplasm. EBV EA-D and R are polypeptides of about 50 to 52 kDa and 85 kDa, respectively. Tumor promoters such as 12-0-tetradecanoylphorbol-13-acetate (TPA) and teleocidin B4 induce the synthesis of EBV EA-D and R in Raji cells and an inhibition assay of the induced antigen measured by immunofluorescence using human sera has been used for the first screening of inhibitors of tumor promotion.

In this study, the immunoblotting analysis showed that a curcumin, a well-known antitumor promoter from turmeric, inhibited the TPA-induced polypeptide synthesis of EBV EA-D in Raji cells, indicating that the immunoblotting technique is applicable to a reliable in vitro assay for detection of antitumor promoters.

The sources of the materials used in this study were as follows: TPA from Sigma Chemical Co., St. Louis, Mo, U.S.A., sodium n-butyraté and curcumin from Wako Pure Chemical Industries, Ltd., Osaka, staurosporine from Kyowa, Medex Co. Ltd., Tokyo, biotinylated protein A and goat anti-mouse IgG from Amersham, Buckinghamshire, England, and peroxidase-conjugated streptavidin from Zymed Lab. Inc., San Francisco, CA, U.S.A.. TPA and staurosporine were dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml and diluted in deionized water. Curcumin and sodium n-butyraté were dissolved in methanol and in deionized water, respectively. Stock solutions (4 μg/ml for TPA, 100 mM for sodium n-butyraté, 0.8 mg/ml for curcumin, and 50 μg/ml for staurosporine) were prepared, passed through a 450-nm membrane filter (Millipore Corp., Bedford, MA, U.S.A.).

Raji cells were grown at 37°C in Dulbecco’s modified Eagle’s minimum essential medium (Dainippon Pharmaceutical Co. Ltd., Osaka) supplemented with 20 mM N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES) and 10% fetal calf serum (FCS). EBV producer P3HR-1 cells were maintained at 33°C in RPMI-1640 medium (Dainippon Pharmaceutical Co. Ltd., Osaka) supplemented with 10% FCS by replacement of one-third fresh medium once a week. Ten human sera from EBV-infected patients with IgG antibodies against EBV EA were kindly provided by Dr. M. Nishio and had the following EA-D and R titers (serum dilution) measured by immunofluorescence: 1:40 for one serum (No. 8), 1:160 for 5 sera (No. 2, 3, 5, 6 and 10), 1:320 for 2 sera (No. 4 and 9), and 1:2,500 for 2 sera (No. 1 and 7).

Raji cells were seeded into 8 ml (2 × 10^7 cells/ml) of medium with or without inducers in 100-mm plastic dishes (Falcon Plastics, Franklin Lakes, NJ, U.S.A.). The cells were cultured at 37°C in a CO2 (5%) incubator for 48 h, harvested, washed three times with Ca2+- and Mg2+-free phosphate-buffered saline (PBS) and then solubilized with an extraction buffer (about 106 cells/25 μl of PBS containing 1% Tween 20, 1% sodium deoxycholate, and 1 mM EDTA) on ice for 60 min. The lysates were centrifuged at 6,000 × g for 10 min and protein in the supernatants was measured by a modification of Lowry method with bovine serum albumin as the standard. The extract (30 μg of protein) of Raji cells (about 10^6 cells) was electrophoresed on a 10% polyacrylamide slab gel containing 0.1% SDS. Proteins in the gel were electrophoretically transferred to a nitrocellulose sheet, which was then immunostained with human sera or mouse antiserum (1:50 dilution), biotinylated protein A or goat anti-mouse IgG (1:500 dilution), and peroxidase conjugated streptavidin (1:1,000 dilution). The immunoreactive bands were developed with a

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1 Corresponding author.
solution containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.01% H₂O₂, and 0.075% cobalt chloride and stopped by rinsing the sheet with cold water.¹⁰

Raji cells were incubated with and without TPA (40 ng/ml) and sodium n-butyrate (1 mM) for 48 h. The number of viable cells in the inducer (TPA and sodium n-butyrate)-treated and sodium n-butyrate-treated Raji cells decreased to 60% and 76%, respectively, of that in untreated Raji cells (4.2 × 10⁵ cells/ml) as measured by trypan blue exclusion. To find whether human sera with different antibody titers against EBV EA can detect the induced viral polypeptide, we examined the reactivities of the inducer-treated Raji cell proteins with ten human sera by immunoblotting. As shown in Fig. 1, one polypeptide of 48 kDa was detected in inducer-treated cells (lane 3) but not in untreated cells (lane 1) and sodium n-butyrate-treated cells (lane 2) by one serum (No. 4). Two non-specific polypeptides (73 kDa and 68 kDa) were observed in the cells treated with and without the inducers. The 48-kDa polypeptide was also detected by serum No. 9 but not by the other 8 sera (data not shown). Furthermore, immunoblotting analysis demonstrated that a mouse monoclonal antibody against EBV EA-D (100 µg/ml, 1:50 dilution, ViroStat, Inc., Portland, OR, U.S.A.) reacted with the 48-kDa polypeptide and that staurosporine (0.5 µg/ml), a protein kinase C inhibitor, completely inhibited the synthesis of the 48-kDa polypeptide (data not shown). These results suggest that the induced polypeptide in Raji cells is EBV EA-D mediated by a protein kinase C.

To confirm the reliability of detection of antitumor promoting activity by the immunoblotting analysis, we examined the effects of curcin on the expression of EBV EA-D in Raji cells treated with TPA and sodium n-butyrate using serum No. 9 (Fig. 2). The induction of EBV EA-D (lane 2) was completely inhibited by 4 µg of curcin per ml (lane 6) but not by 2 µg of curcin per ml (lane 5), suggesting that the concentration required for 50% inhibition of EBV EA-D induction may be a narrow range of 2 to 4 µg of curcin per ml. The number of viable cells in curcin (0.5 to 8 µg/ml)-treated Raji cells decreased to 74% (0.5 µg/ml), 80% (1 µg/ml), 55% (2 µg/ml), 55% (4 µg/ml), 42% (8 µg/ml) of that in the inducer-treated Raji cells (1.5 × 10⁵ cells/ml).

We produced mouse antiserum against P3HR-1 cells treated with the inducers because of difficulty of supply of the human sera with antibodies to EBV. P3HR-1 cells (about 7 × 10⁵ cells) were treated with TPA (40 ng/ml) and sodium n-butyrate (1 mM) in the RPMI-1640 medium at 37°C for 48 h to increase EBV production.¹⁵,¹⁶ The cells (about 2 × 10⁷ cells) in complete Freund's adjuvant injected subcutaneously into a BALB/c mouse (4 weeks old). Ten days and 5 weeks later, the mouse was inoculated subcutaneously with the same cell number in the adjuvant. The animal was bled 2 weeks after the final injection. The reactivity of the antiserum with the Raji cells treated with various concentrations of TPA (1.25 to 80 ng/ml) was measured by the immunoblotting analysis (Fig. 3). The induction of 48-kDa polypeptide in Raji cells was detected by treatment with TPA at concentrations more than 2.5 ng/ml, indicating that the antiserum is useful for detection of antitumor promoting activity and that TPA is active in EBV EA-D-induction at the lowest concentration used (2.5 ng/ml).

This study demonstrated that among ten human sera with different antibody titers against EBV EA-D and R measured by immunofluorescence, only two sera with antibody titer of 1:320 recognized the 48-kDa polypeptide (EBV EA-D) of inducer-treated Raji cells by im-

Fig. 1. Immunoblotting Analysis of Extracted Raji Cell Proteins using Human Serum with Antibody against EBV EA.

The detergent-solubilized extracts (30 µg of protein) of untreated Raji cells (lane 1), Raji cells treated with sodium n-butyrate (1 mM) (lane 2), and Raji cells treated with TPA (40 ng/ml) and sodium n-butyrate (1 mM) (lane 3) were electrophoresed on a 10% polyacrylamide slab gel. The proteins in the gel were electrophoretically transferred to a nitrocellulose sheet and then the sheet was immunostained with human serum (No. 4, 1:50 dilution), biotinylated protein A (1:500 dilution) and peroxidase conjugated streptavidin (1:1000 dilution). Lane M, prestained marker proteins from Bio-Rad. Immunoblotting analysis was the same as in Fig. 1 except of use of human serum No. 9.

Fig. 2. Effects of Curcin on the Synthesis of EBV EA in Raji Cells Treated with TPA and Sodium n-Butyrate using Immunoblotting Analysis.

Lane 1, untreated Raji cells; lane 2, TPA (40 ng/ml) and sodium n-butyrate-treated (1 mM)-treated Raji cells; lane 3 to 7, TPA and sodium n-butyrate-treated Raji cells with curcin at a concentration of 0.5 µg/ml (lane 3), 1 µg/ml (lane 4), 2 µg/ml (lane 5), 4 µg/ml (lane 6) and 8 µg/ml (lane 7); lane M, prestained marker proteins from Bio-Rad. Immunoblotting analysis was the same as in Fig. 1 except of use of human serum No. 9.
Fig. 3. Effects of TPA on the Synthesis of EBV EA-D in Raji Cells by Immunoblotting Analysis using Mouse Antiserum against P3HR-1 Cells.

Raji cells were treated with sodium n-butyrate (1 mmol) and with various concentrations of TPA. Immunoblotting analysis was the same as in Fig. 1 except that the mouse antiserum (1:50 dilution) and biotinylated anti-mouse IgG (1:500 dilution) were used instead of human serum and biotinylated protein A.

Immunoblotting analysis. This finding suggested that the non-reactive sera could recognize only the folded, native conformation of the EBV EA-R. Therefore, no EBV EA-R could be detected in the immunoblotting analysis. Nakamura et al. examined the antitumor activity of curcumin in Raji cells by immunofluorescence using human serum of patients with nasopharyngeal carcinoma. They found that the induced expression of EBV EA is completely inhibited by curcumin at a concentration of 9.2 μg/ml, but its inhibitory effect is insufficient at concentrations below 1.84 μg/ml. Their results are consistent with the antitumor activity of curcumin by the immunoblotting analysis done in this study. As for judgment of antitumor promoting activity, immunoblotting is easier than immunofluorescence method, in which the percentage of EBV EA-positive cells must be calculated by counting at least 500 cells in each sample. Immunoblotting can detect antibodies responding to individual polypeptides and the effect of non-specific binding can be avoided by comparing the Raji cells treated with and without the inducers. Therefore, the immunoblotting analysis described in this study should be a useful confirmation test for detecting antitumor promoters.

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