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EXPRESSION PROFILE OF NUCLEAR RECEPTORS UPON EPSTEIN — BARR VIRUS INDUCED B CELL TRANSFORMATION

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Background: **Infection of human B cells with Epstein — Barr virus (EBV) induces metabolic activation, morphological transformation, cell proliferation and eventual immortalization.** ***Aim:*** **To identify the nuclear receptors, which are the cellular interaction partners of EBNAs, that will help to elucidate the mechanism of B cell transformation.** ***Methods:*** **We have compared the nuclear receptor profile in the naïve and EBV-transformed B-lymphocytes, using TaqMan LDA microfluidic card technology.** ***Results:*** **Out of 48 nuclear receptor, 17 showed differential expression at the mRNA level. The expression of 5 genes was elevated in EBV-transformed cells, whereas 12 genes were down-regulated in lymphoblastoid cells (LCLs). 7 genes were studied at the protein level; 2 genes were up regulated (Nr2F2 and RARA) and 4 genes were down regulated (ERB, NUR77, PPARG, and VDR) in LCLs.** ***Conclusion:*** **The nuclear receptor profiling on EBV infected B cells showed alterations of nuclear receptors expression at both mRNA and protein levels compared with non infected peripheral blood cells. Further analysis on a possible role of each nuclear receptor in EBV induced cell transformation should be performed.**

Key Words: **EBV, cell transformation, nuclear receptors, expression pattern, microarrays.**

Infection of human B cells with Epstein — Barr virus (EBV) induces metabolic activation, morphological transformation, cell proliferation and eventual immortalization [1, 2]. Six of the 9 EBV-encoded proteins (EBNA -1, -2, -3, -5, -6, and LMP-1) are necessary for the efficient transformation [3]. During transformation no genetic aberrations were detected. However, signal transduction pathways were ultimately changed — either blocked, either activated. For example, LMP1 activates TNFα/CD40 downstream signaling pathways that can stimulate cell growth and survival through activation of NFκB, jun and p38/map kinase. LMP2A activates constitutively B-cell receptor (BCR) (reviewed in [1]). Latency III genes expression leads to the change of gene expression profile. EBNA-2 activates and regulates the transcription of Notch and PU.1 responsive promoters of the cellular genes due to a binding to RBP-Jκ. A more detailed description of some cellular pathways and, moreover, nuclear receptors that may be implicated in the EBV-induced B-cell transformation, are reviewed in [4].

Anyway, many questions about the mechanism of B cell transformation into lymphoblasts are unanswered yet. The identification of the cellular partners of EBNAs and determination of the intervening cellular pathways will help to elucidate the mechanism of B cell transformation. The aim of the present paper was to compare the nuclear receptor profile in the naïve and EBV-transformed B-lymphocytes (freshly infected B cells and long-term cultured LCLs).

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Abbreviations used: EBV – Epstein – Barr Virus; EBNA – EBV-encoded nuclear antigen; LCL – lymphoblastoid cell line; LMP – latent membrane protein ; PBC – peripheral blood cells; TBC – tonsil B cells.

MATERIALS AND METHODS

Western blotting. We prepared whole cell lysates using NP40 lysis buffer (1% NP40, 150 mM NaCl, 50mM Tris, pH = 8) with a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Lysates were cleared by centrifugation. Proteins were separated using the sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). After transfer the membrane was probed with the specific antibodies: mouse monoclonal anti-actin (Sigma-Aldrich), anti-Nr2F2 (Abnova Corp., Taipei, Taiwan), anti-RARA (Abnova Corp.), anti-VDR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and polyclonal mouse serum against PPARG (Abnova Corp.); rabbit polyclonal serum against NUR77 (Santa Cruz Biotechnology Inc.). Secondary antibodies (anti-rabbit and anti-mouse IgG Horseradish-conjugated) were purchased from GE Healthcare Bio-Sciences AB, Uppsala, Sweden.

Low density array. TaqMan LDA microfluidic card technology from Applied Biosystems (Foster City, CA, USA) allows the simultaneous assay of mRNA gene expression of up to 384 targets on a single card. The LDA used in this study was custom designed to consist of 48 TaqMan Gene Expression Assays (Applied Biosystems) per loading port (48 genes × 5 samples run three times for statistical significance). Each reaction well contained all reagents specific for a given assay. Each target assay consisted of a forward primer and a reverse primer.

Cell culture, immunostaining and imaging. All cells were cultured at 37 °C, in Iscove's medium containing 10% fetal bovine serum and appropriate

antibiotics. Tonsil B cells (TBC) were isolated from human tonsils obtained from routine tonsillectomy (Karolinska Hospital, Stockholm). The tonsils were cut into the fragments and passed through a metal mesh. Peripheral blood B cells were isolated from buffy coat blood (Karolinska Hospital) on Lymphoprep gradients. An ethical permission was received for both procedures of B cell isolation. Two subsequent rounds of E-rosetting removed the T-cells. The B95.8 EBV strain was used for B cell infection. Control B cells were activated by anti-CD40 mouse monoclonal antibody (Nordic Biosite AB, Täby, Sweden, 1 μg/ml) and IL4 (ImmunoTools GmbH, Friesoythe, Germany) 25 ng/ml for 48 h. Prior to immunostaining experiments, the cells were spun on glass slides, using Cytospin centrifuge. Immunostaining and digital image capturing was performed as described earlier [5]. Briefly, cells on slides after cytospin were fixed in a 1 : 1 mixture of cold methanol and acetone (–20 °C). After rehydration in phosphate buffer saline, cells were stained with antibodies. Hoechst 33258 (Sigma-Aldrich) was added at a concentration of 0.4 μg/ml to the secondary antibody for DNA staining when necessary. The images were captured using DAS microscope Leitz DM RB with a dual mode cooled charged coupled device (CCD) camera C4880 (Hamamatsu, Japan) or Zeiss LSM 510 laser scanning confocal microscope with ORCA-ER CCD camera (Hamamatsu).

RESULTS

Nuclear receptor expression profile on the microfluidic cards. We have run Nuclear Receptor profiling in freshly EBV-infected B cells (48 h) and lymphoblastoid cell lines (LCLs) versus primary B cells. We have used two different RNA samples of B cells: peripheral blood B cells (PBC) and TBC, and three different RNA samples of EBV-infected cells: 48 h post infection (EBV 48 h), and LCL that were cultured for 2 month and 1.5 year. The set of 48 nuclear receptors and GAPD were on the cards. Among them 12 receptors were not expressed, and 33 nuclear receptors were expressed differentially in normal PBC and EBV-infected cells (level of expression differed more than 2-fold). Standard deviation did not exceed 5.0%.

Only 17 genes showed consistent differences in expression at the mRNA level — decreased in B cells, upregulated in EBV-infected cells and LCLs, or vice versa. The expression of 5 genes was elevated in EBV-transformed cells, whereas 12 genes were downregulated in LCLs (Table).

We have studied expression of 7 genes at the protein levels; 2 genes were upregulated and 4 genes were downregulated, ER-α protein was not detected.

Genes upregulated upon EBV-infection. The five genes: *Nr2F2* (COUP2, NP_066285), *Nr4A3* (MINOR, NP_775290), *Nr6A1* (GCNF, NP_201591), *RARA* (NP_000955), and *RXRA* (NP_002948) were induced in LCLs at the mRNA level (Fig. 1, *a*). Slight elevation of mRNA was observed in TBC, probably, due to the activation by infection. Strong correlation was observed at mRNA and protein level for Nr2F2 (transcriptional factor COUP2) and RARA (retinoic acid receptor α), when primary PBC and TBC were compared with freshly EBV infected cells and LCLs. Nr2F2 and RARA proteins were elevated in LCLs, compared with primary B cells (Fig. 1, *b*, *c*, respectively).

Table. Differently expressing genes in EBV-infected compared to primary B-cells

№	Name of the gene	Accession number, OMIM link
	Upregulated receptors	
1	Nr2F2, Nuclear receptor subfamily 2, group F, member 2; TF COUP2; COUPTFII	NP_066285 *107773
2	Nr4A3, Nuclear receptor subfamily 4, group A, member 3; Neuron-derived orphan receptor 1, NOR1; Mitogen-induced orphan receptor, MINOR	NP_775290 +600542
3	Nr6A1, Nuclear receptor subfamily 6, group A, member 1; Germ cell nuclear factor, GCNF	NP_201591 *602778
4	RARA, Retinoid acid receptor alpha	NP_000955 *180240
5	RXRA, Retinoid X receptor alpha	NP_002948 *180245
	Downregulated receptors	
1	PPAR-gamma, Peroxisome proliferator-acivated receptor gamma; PPARG	NP_619725 *601487
2	ER-alpha, Estrogen receptor alpha; ESR; ESR1; ER1	NP_000116 *133430
3	ER-beta, Estrogen receptor beta; ESR2; ER2	Q92731 *601663
4	Nr1H3, Nuclear receptor subfamily 1, group H, member 3; Liver X receptor alpha; LXRA	NP_005684 *602423
5	Nr2F1, Nuclear receptor subfamily 2, group F, member 1; Transcription factor COUP1; TFCOUP1	NP_005645 *132890
6	Nr3C1, Nuclear receptor subfamily 3, group C, member 1; Glucoccorticoid receptor; GCCR	NP_001018087 +138040
7	Nr4A1, Nuclear receptor subfamily 4, group A, member 1; NAK1; Nuclear hormone receptor TR3; TR3; NUR 77 (homolog of mouse NUR77)	NP_775180 *139139
8	RARB, Retinoic acid receptor beta	NP_000956 *180220
9	RORC, RAR-related orphan receptor gamma	NP_005051 *602943
10	RXRB, Retinoid X receptor beta	NP_068811 *180246
11	THRB, Thyroid hormone receptor beta	NP_000452 +190160
12	VDR, Vitamin D3 receptor	NP_000367 *601769

Genes downregulated in EBV-infected B cells. Eleven genes were downregulated in LCLs compared with primary B cells (Fig. 2, *a*, *b*). We have run Western blotting for the 5 of them: *ER-α* and *-β* (Estrogen receptor α (NP_000116) and -β (Q92731)), *Nr4A1* (Nur77, NP_775180), *PPARG* (peroxisome proliferator-activated receptor γ, NP_619725), and *VDR* (vitamin D receptor, NP_000367). ER-α protein was not detected by Western blotting. ER-β protein level was very low in primary B cells and LCLs. Nur77 and PPARG protein levels in LCLs does not differ much from the protein level in the primary B cells (Fig. 2, *c*). Different trends in mRNA and protein levels (compare Fig. 2, *a*, *c*) could be due to the protein stability. However, VDR protein expression (Fig. 2, *d*) followed a pattern of mRNA expression (compare Fig. 2, *a*, *d*). Moreover, after a brief increase, all 11 receptors were expressed at lower level in LCLs, compared with primary B cells.

We have to mention, that not only the level of expression, but also a cellular distribution of the nuclear receptors were changed after EBV infection. For

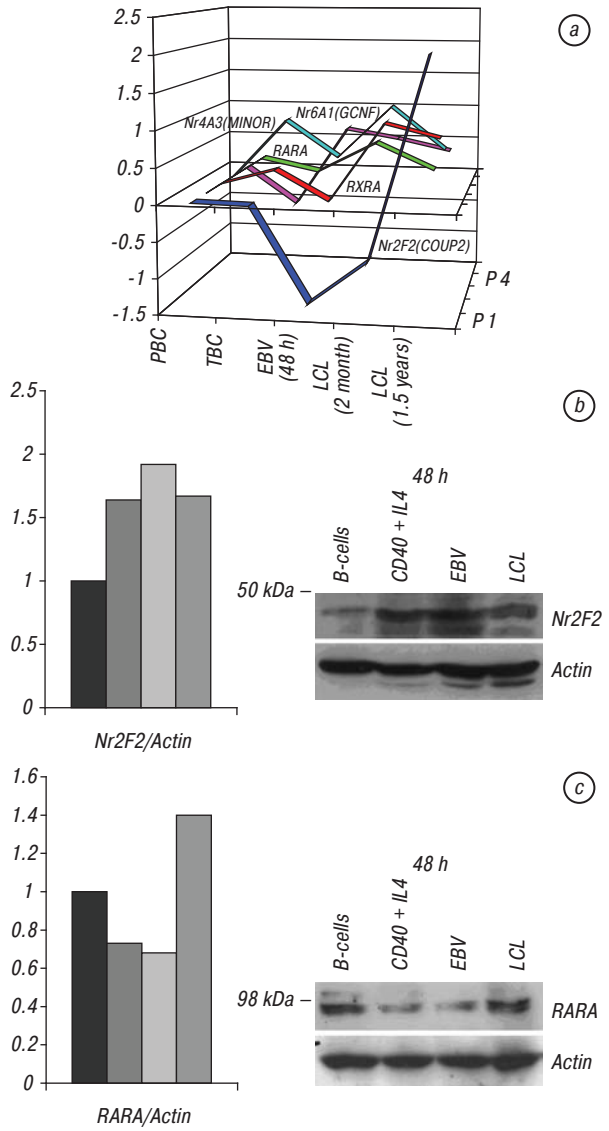

Fig. 1. *a*, mRNA expression values (log) of upregulated nuclear receptors. Names of receptors are indicated in corresponding colors. Notice increased expression in LCLs. *b*, Western blotting of the Nr2F2 in primary, CD40+Il4 activated, freshly EBV infected B cells, and LCLs (cultured for 1.5 years). Lower panel — relative ratio of protein signal to actin. *c*, Similar to *b*, for RARA protein

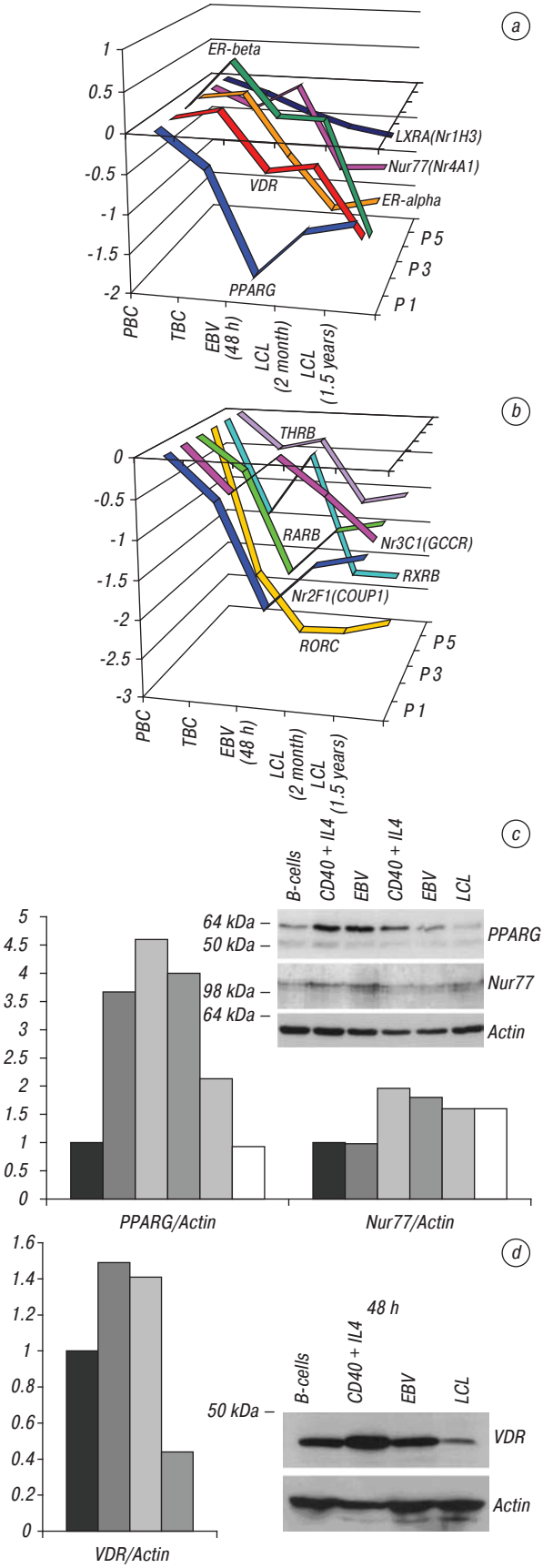

Fig. 2. *a*, *b*, mRNA expression values (log) of downregulated nuclear receptors. Names of receptors are indicated in corresponding colors. Notice decreased expression in LCLs. *c*, Western blotting of the Nur77 and PPARG in primary, CD40+Il4 activated, freshly EBV infected B cells, and LCLs (cultured for 1,5 years). Lower panels — relative ratio of protein signal to actin. *d,* Similar to *c*, for VDR protein

example, in primary TBC the PPARG was expressed as small cytoplasmic patches (Fig. 3, *a*). Upon activation, pattern of expression of PPARG was changed. Upon LPS (lipopolysaccharide) stimulation of B cells PPARG showed perinuclear localization, which could also be found in the patches (Fig. 3, *b*). In B cells stimulated by anti-CD40 and IL-4, PPARG expressed not only in patches, but also at the membrane of the joined cells (Fig. 3, *c*). Upon EBV-infection PPARG was observed in the cellular membrane, and great portion of protein was distributed in both, a nucleus and the cytoplasm (Fig. 3, *d*). We may hypothesize that PPARG would function differently depending on distribution pattern in cells. However, this should be further elucidated.

DISCUSSION

EBV-encoded proteins expressed in latently infected B cells are known to interact with a cellular signaling pathways to establish latency and ensure the growth of

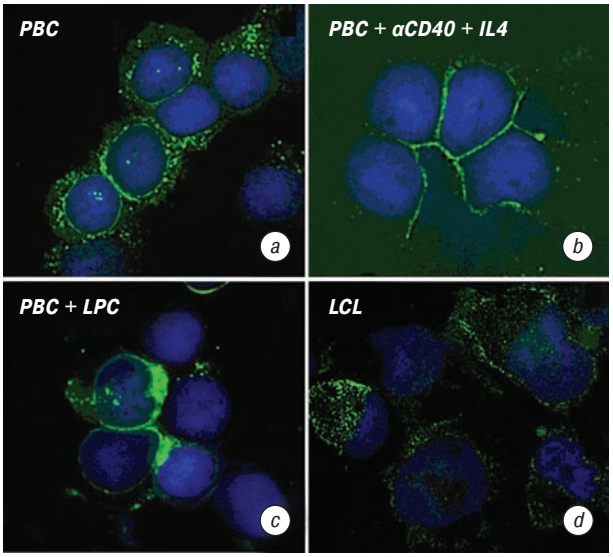

Fig. 3. PPARG immunostaining (green signal). Secondary antibody was FITC-conjugated. DNA is stained with Hoechst (blue signal). *a*, peripheral B cells (PBC). *b*, PBC, activated with lipopolysaccharide (LPS) for 48 h. *c*, PBC activated with anti-CD40 mAbs and IL-4 cytokine for 48 h. *d*, lymphoblastoid cell line (LCL), long-term cultured (1.5 years)

the transformed cells (reviewed in [6]). Beside latent membrane proteins (LMPs), EBV-encoded nuclear antigens (EBNAs) are implicated in this process as well. For example, the EBNA-2 can bind to the nuclear receptor Nur77 and block NUR77 mediated apoptosis [7, 8]. EBNA-2 can also inhibit the pro-apoptotic and anti-proliferative functions of the transforming growth factor β1 (TGFβ1, NP_000651) cytokine, as shown in the EBNA-2 inducible EREB system [9]. We have previously shown that EBNA-3 can bind to and regulate the transactivation function of the cellular nuclear receptor AhR [10]. Importantly, EBNA-3 influenced the transcription of AhR dependent genes at the basal receptor level and after ligands (xenobiotics) activation, including 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD). The physiological role of the EBNA-3 — AhR interaction was illuminated by treating B cell lines with TCDD. EBNA-3 was found to protect cells from TCDD-induced growth arrest and/or apoptosis.

However, the question about regulation of the nuclear receptors upon EBV-infection of B cells is not completely elucidated yet. In current study we aimed to get an overview about relationship between EBV-induced transformation of B cells and the expression profile of 48 nuclear receptors.

Summarizing, we have found that 17 nuclear receptors were expressed differently in primary and EBV-infected B cells; 12 of them were downregulated and 5 were upregulated in LCIs (see Table).

Some of these receptors previously were shown to be implicated in the regulation of the EBV-infected cell fate. For example, it was demonstrated that EBV lytic cycle activation was inhibited upon retinoic acid treatment due to direct binding between RARA and the EBV-encoded lytic BZLF1 protein [11, 12]. It was also found that retinoids inhibited naïve B cell proliferation, but promoted cell survival [13]. Noteworthy, here we show that RARA is upregulated in LCLs (see Fig. 1, *a*, *c*).

Earlier, it was reported that in B cells (PBCs and LCLs) the VDR-dependent gene regulation was blocked [14]. Moreover, the active VDR pathway could inhibit proliferation and enhance differentiation of leukemic cells [15]. Interestingly, the level of VDR expression (at the mRNA and protein levels) was found to be lower in the EBV transformed cells compared with primary B cells (see Fig. 2, *a*, *d*). Recently, an anti-tumor effect was proposed for vitamin D and VDR [16] (for review see [17, 18]).

Expression of functional PPARG was shown in lymphocytes, and its activation led to apoptosis, or growth arrest [19, 20], or differentiation [21]. PPARG can have both, transactivating and transrepressing activity (reviewed in [22]). PPARG can repress some interferon-gamma and LPS-inducible genes, such, as IL-12 and IP10, for example. In their turn, cytokines can repress PPARG by inhibiting of DNA binding [23]. Here we showed that PPRG was downregulated in LCLs compared with primary B cells. Moreover, cellular distribution of this nuclear receptor was changed in LCLs.

Levels of nuclear receptors mRNA and protein expression vary from maximum to minimum before they get stabilized, as it is seen from the Fig. 1 and 2. Noteworthy, it was shown earlier that EBV infection often results in temporary (0–72 h) up- or downregulation of many cellular and viral genes (reviewed in [1]).

The nuclear receptor profiling on EBV infected B cells showed alterations of nuclear receptors expression at both mRNA and protein levels compared with non infected peripheral blood cells. In most of the cases, the mRNA levels observed via LDA are strongly corroborated by expression at protein level. Further analysis on a possible role of each nuclear receptor in EBV induced cell transformation should be carried out.

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