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INHIBITORY EFFECT OF EPSTEIN-BARR VIRUS GENE-EBNA1 ON HUMAN TNFRp55 GENE EXPRESSION

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Abstract

The aim of the study was to assess the expression of TNFRp55 mRNA and to examine if the antisense inhibition of Epstein-Barr virus (EBV) encoded EBNA1 gene product alters the expression of gene encoding TNFRp55 in lymphoblastoid cell line (LCL). The experiment was performed on LCL derived from EBV infected human peripheral blood B lymphocytes. The lymphocytes were isolated and cultured. RNA was isolated and examined according to the RNase protection assay. The hybridisation was done with HCR-4 probe. RNA was quantified by densitometry and presented in extinction units. The level of expression was calculated with TotaILab software programme. The results of the study suggest that EBV gene, responsible for the synthesis of EBNA1 protein, has an inhibitory effect on human TNFRp55 gene expression in LCL.

Key words: Epstein-Barr virus, ENBA1, LCL, B lymphocytes, TNFRp55.

Epstein-Barr virus (EBV) is a ubiquitous γ-herpes virus that infects more than 90% of adults worldwide and persists lifelong in the human B lymphocytes. Although most infections are latent and asymptomatic, EBV has been linked to the pathogenesis of a variety of human tumours, including Burkitt's lymphoma, immunosuppression-related lymphomas, Hodgkin's disease, nasal angiocentric T/NK-cell lymphoma, and nasopharyngeal carcinoma. Some of EBV-associated diseases are devastating and life-threatening. EBV has been classified as a group 1 carcinogen by the WHO International Agency for Research on Cancer and it could be an even greater health risk than presently believed (5, 9). The mechanism of EBV-related diseases has yet to be elucidated because of a lack of appropriate animal model. To overcome this limitation and to simulate EBV pathogenesis *in vivo*, several experimental animal models have been established. Recently published data from studies performed on novel animal model provided important clues and seem to be very promising (11). EBV, after an initial lytic phase, establishes a life-long latent infection in resting memory B cells. Latency is associated with immortalisation of B lymphocytes and it is the latent state that is identified in EBV-associated malignancies (9). EBV transforms B-lymphocytes into continuously proliferating lymphoblasts *in vitro*. Latent EBV growth transformation of resting B-cells into indefinitely proliferating cell lines is a successful viral strategy for survival in its host and the basis of several human malignancies. EBV transforms cell growth through viral proteins that modify cell gene expression at the level of transcription or by appropriating signaling pathways. This immortalisation leads to the expression of a limited set of viral genes (4). The EBV nuclear antigen 1 (ENBA1) is expressed in all EBV-associated tumours and EBV-positive proliferating cells in healthy EBV carriers. EBNA1 has been also demonstrated to be oncogenic *in vivo* (13, 8).

Under immunocomponent conditions, EBV infection remains controlled by host defence mechanisms and tumour necrosis factor (TNF α), and its receptors (TNFR) are important mediators of immunological response. TNF α/TNFR are involved in viral pathogenesis and have been considered as the main components of antiviral activity. TNF α was also utilised for the treatment of patients with advanced solid tumours (6).

The aim of our study was to assess the expression of genes coding TNFRp55 in lymphoblastoid cell line (LCL) and also to examine if antisense inhibition of EBV encoded EBNA1 gene product alters the expression of studied genes.

Material and Methods

Blood donors. The experiments were performed on a lymphoblastoid cell line (LCL) derived from EBV infected peripheral blood B lymphocytes obtained from patients with infectious mononucleosis. Primary EBV infection was confirmed by a positive IgM antibody titer for EBV capsid antigen (VCA) assessed by ELISA (Vironostica R Bio-Merieux).

Isolation of lymphocytes. Peripheral blood B lymphocytes were isolated by density gradient centrifugation according to the modified method of Boyuma (1).

Cell culture and obtaining LCL. Isolated lymphocytes were cultured at a concentration of 2-3x10^6 cells per 1 ml of medium in 2.5 ml RMPI 1640 with 10% calf serum (GIBCO), 4 mM of L-glutamine (GIBCO), and antibiotics: crystalline penicillin (100 U/mL) and streptomycin (100 µl/mL). The cultures were divided into three parts after 5-6 weeks when the cell line was established. Forty-eight hours before the end of culturing, antisense and sense EBNA1 (5'- TCC TCA GAC ATG ATT CAC AC-3') oligonucleotides were added in the presence of FuGENE TRANSFECTION REAGENT (ROCHE), enhancing the transfection process. Morphology of the lymphocytes was determined with May-Grünwald staining.

RNA preparation. RNA was isolated from cultured lymphocytes using PharMingen Total RNA Isolation Kit (Cat. No. 4553KC). The isolated RNA was kept in 66% ethanol at -20^{o}C until hybridisation.

Hybridisation. The isolated RNA was analysed in a RNase protection assay (RPA) procedure using PharMingen Kit. The hybridisation was performed with HCR-4 probe that determined the expression level of TNF-α receptor gene (TNFRp55).

Probe synthesis. The samples were quantified in a scintillation counter to measure the probe activity. The activity was accepted between 3 x 10^5 and 3 x 10^6 counts/min for 1 ul of probe. The probe was stored at – 20^{o}C.

RNase treatments. RNA samples were prepared as described above using the PharMingen RPA Kit (Cat.No.4501K). RNase digest were extracted with the use of a pipette and transferred to tubes containing the proteinase K solution.

Gel preparation and electrophoresis. The electrophoresis was done on 5% denaturing polyacrylamide gel containing 40% acrylamide, 2% bis-acrylamide, 10X TBE, and 6 M urea. TEMED and ammonium persulfate (10%) were added as polymerisation activators. The location of autoradiographic bands, corresponding to segments of studied genes, was established according to the PharMingen procedure. RNA was quantified by densitometry. Expression was presented in extinction units (U) and calculated with TotaILab software programme. The values were evaluated in relation to the quantity of mRNA of L32 and GAPDH reference genes.

Results

In the experiment, LCL from the EBV-infected human peripheral blood B lymphocytes were successfully obtained. As a result of RNA electrophoresis and hybridisation with HCR4 probe, an autoradiogram illustrating the expression of gene encoding TNFRp55 at the level of mRNA was received (Fig. 1). Each LCL was numbered from 1 to 3, respectively (lines 1, 2, 3) (Fig. 2).

No sense or antisense EBNA1 oligonucleotides were added to LCL 1, which served as a control line. In this cell line the visible band corresponded to the expression of TNFRp55 gene.

Cell line 2 was the line to which sense EBNA1 oligonucleotides were added. In an autoradiogram, a visible band corresponding to mRNA segments of TNFRp55 gene was noted (Fig. 2). In cell line 3, the influence of antisense oligonucleotides against EBNA-1 on the expression of TNFRp55 gene was examined. After blocking the function of the gene responsible for the synthesis of EBNA1 protein, a band, which corresponded to the expression of the studied TNFRp55 gene was observed.

The next stage of the research included the determination of mRNA quantity in the studied LCL by densitometry. Expression of TNFRp55 gene was presented in extinction units (U) and calculated in relation to the quantity of mRNA of GAPDH reference gene, which is consistently expressed in cells. The results are illustrated in Figs 3 and 4.

Expression of TNFRp55 and GAPDH genes in the control LCL (line 1), measured with the use of densitometry from the autoradiogram (RPA), after having calculated the results in relation with the expression of the reference GAPDH gene was expressed in extinction units. The expression value of TNFRp55 gene was 0.44 U. (Fig. 3)

After using sense EBNA1 oligonucleotides, the expression of TNFRp55 gene was noted to be 0.43 U. Sense EBNA1 oligonucleotides did not affect the expression of the gene. After the use of the antisense EBNA1 oligonucleotides to block the function of EBV gene, which is responsible for the synthesis of EBNA1 protein, the expression of TNFRp 55 gene was 0.69 U, which was a higher value compared to the expression value of this gene in the control LCL (Fig. 4).

Discussion

EBV–transformed lymphoblastoid cell lines are used as a source for human genetic, immunological, and also pharmacogenomic studies. Most of our knowledge regarding gene expression is based on the study of cell lines generated after *in vitro* infection with EBV and the lines are to date a unique cellular model to study establishment and maintenance of viral latency (12, 7). In the study, LCL was successfully isolated from peripheral blood B lymphocytes infected *in vivo* with EBV in patients with infectious mononucleosis.

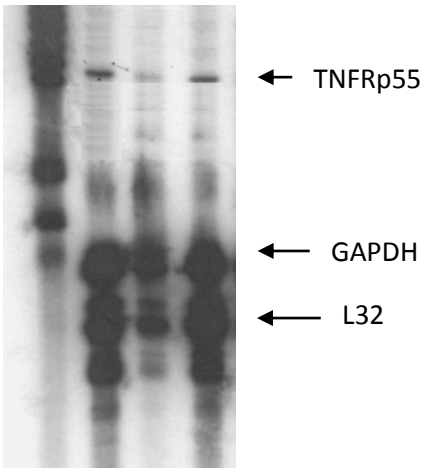


Fig. 1. Autoradiogram showing the expression of TNFRp55, GAPDH, and L32 genes at the level of mRNA in LCL after RNA electrophoresis and hybridisation with HCR4 probe.

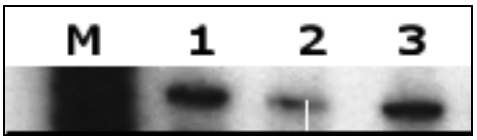


Fig. 2.
M - marker
1 - line 1
2 - line 2
3 - line 3

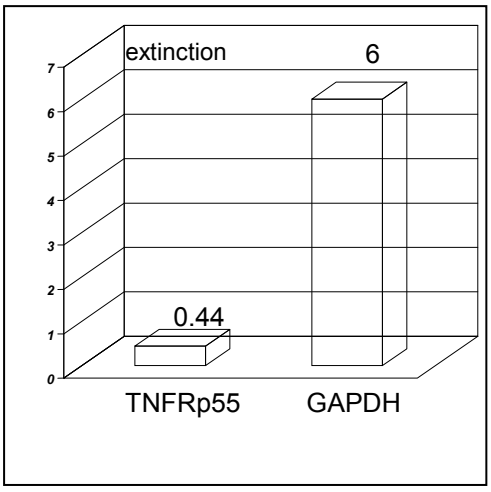


Fig. 3. Expression of TNFRp55, GAPDH genes, in the control LCL measured with the use of densitometry from the autoradiogram.

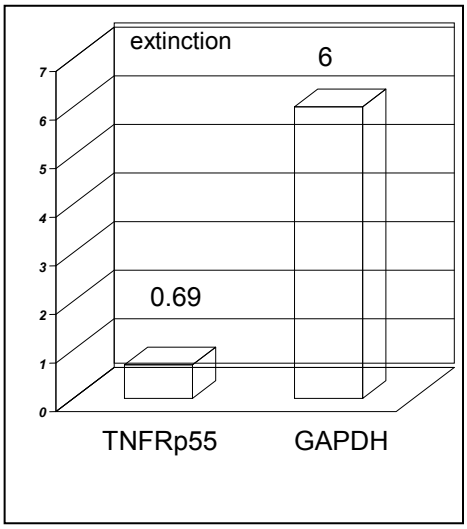


Fig. 4. Expression of TNFRp55, GAPDH genes, in LCL with added antisense EBNA1 oligonucleotides measured with the use of densitometry from the autoradiogram.

The mechanisms of EBV-related diseases and malignancies has still remained unclear. It is well accepted that Epstein-Barr virus interacts intimately with the immune system, and tumour necrosis factor-α (TNF α) is involved in viral pathogenesis. TNF α binds to two distinct receptors (TNFRp55 and TNFRp75) and it has been generally believed that TNFRp55 (TNFR1) is responsible for the majority of biological activities of TNF α. TNF α is one of the cytokines that is expressed by activated human B cells and LCL expresses high level of this cytokine (6, 10). It is well accepted that TNFRp55 is expressed in many mammalian cells but it is not known whether TNFRp55 mRNA is expressed in LCL derived from human peripheral B lymphocytes *in vivo* infected with EBV during primary infection with this virus. To resolve this question we have performed the experiment and as a result of RNA electrophoresis and hybridisation with HCR4 probe, the mRNA expression on TNFRp55 was detected in studied LCL. TNF α enhances or inhibits viral replication depending on the virus involved and type of infected cells. The binding of TNF α to the TNFR can activate, differentiate, or kill target cells thereby interfering with the viral life cycle. In contrast, viruses have evolved to appropriate the TNF/TNFR pathway to evade immune response and favour viral dissemination. In recent years, growing evidence has shown that both DNA and RNA viruses can interfere with the TNF/TNFR pathway to escape the immune surveillance (6). There are data suggesting that Epstein-Barr virus inhibits expression of TNFR 1 (2). Gosselin *et al.* (4) reported that EBV exerts a strong inhibitory effect on TNF α production. To determine the influence of EBV proteins on mRNA TNFRp55 expression in LCL, the antisense oligonucleotides against EBNA1 protein were used. Recent publications regarding the antiviral action of antisense technology against herpes viruses have mainly focused on Epstein-Barr virus and EBV-associated malignancies. In the past decade, many antisense molecules were used in clinical trials up to phase III (3). We have decided to use antisense oligonucleotides against EBNA1 protein, which functions both in replication and in maintenance of the EBV episome in latency, and is consistently detected in latently infected B cells and EBV associated disease tissues. EBNA1 influences the expression of a range of cellular genes, including those involved in transcription, translation, and cell signaling (8). The results of the presented experiment indicate that EBV encoding EBNA1 protein has an influence on the expression of TNFRp55 mRNA in LCL derived from peripheral human blood B lymphocytes *in vivo* infected with EBV. Inhibition of EBNA1 protein synthesis resulted in increased expression of TNFRp55 gene. The obtained results confirm previously published data on EBV inhibitory effect on TNFR1 gene expression (2).

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