行政院國家科學委員會專題研究計畫  成果報告

探討 病毒 基因在其感染細胞中的角色

計畫類別：個別型計畫
計畫編號：
執行期間：年 月 日至 年 月 日
執行單位：中山醫學大學醫學系微生物及免疫學科

計畫主持人：梁熾龍

報告類型：精簡報告

處理方式：本計畫涉及專利或其他智慧財產權，年後可公開查詢

中華民國 年 月 日
中文摘要

EB病毒是人類疱疹病毒 (herpesvirus) 的一員。它能夠持續存活於B淋巴細胞，並透過其潛伏性膜蛋白 I (LMP1) 活化細胞中的Bcl-2蛋白質，導致B細胞轉形 (transformation) 及癌化 (tumorigenesis)。換言之，Bcl-2蛋白在其中扮演非常重要的角色。近來在EB病毒研究中，發現有一種會出現在溶裂期早期的蛋白質，其結構與Bcl-2相似，我們稱它為BALF1。然而我們對BALF1在EB病毒感染細胞中的角色與功能至今仍不清楚。根據我們初步研究結果，已知BALF1具有活化EB病毒Zta的啟動子 (promoter)，但卻對EBNA1的啟動子無明顯影響。此外在無任何病毒活化劑處理下，BALF1 對此二基因的RNA表現完全不受影響。在另一方面，實驗證明ceramide所引起的細胞凋亡 (apoptosis) 會受到BALF1的抑制。然而這些初步實驗並無法告訴我們，BALF1如何抑制細胞凋亡，如何改變病毒生活史以及為什麼活化 Zta 啟動子。這些實驗在在顯示，我們必須找尋新證據來進一步證實。EB病毒是人類腫瘤病毒且與許多癌症有關。本次研究計劃，我們希望從中獲得BALF1基因對其宿主及病毒本身調控機制的資訊，而透過此資訊又可找出宿主與病毒間互動的關係。藉由深入了解彼此關係，可發展出抑制EB病毒的藥物及因病毒所產生疾病的基因治療方法。
Epstein-Barr virus (EBV) is a human herpesvirus, which is persistent in B lymphoid cells and triggers transformation and tumorigenesis of latently infected B cells in vitro via LMP-induced cellular survival protein, Bcl-2. In other words, Bcl-2 protein in EBV-infected B cells plays an important role in prolonging life span of cells. Recently, an early lytic gene, BALF1, which is structurally homologous to Bcl-2 protein, is characterized in EBV infected cells. However the role and function of BALF1 in EBV infected cells still remain to be investigated. According to the present studies, we have known that BALF1 activated Zta promoter activity, but no obvious effect on EBNA1 promoter activity. In addition, the data of RT-PCR also showed BALF1 had no effect on the expression of EBNA1 and Zta transcripts without any treatment of inducing agents. On the other hand, BALF1 was involved in suppressing the ceramide-mediated cell apoptosis. However, these preliminary studies could not allow us to know that how BALF1 inhibited cell apoptosis and changed EBV life cycle and why BALF1 activated EBV immediate early gene. We still need to accumulate more evidences to clarify. EBV is a DNA tumor virus, which is associated with many tumors. Determination of the function of BALF1 in infected cells will further provide us to realize the relationship of EBV and its associated tumors. Moreover, this information also facilitates the development of drugs to inhibit its intrinsic activities and non-immunogenic vectors for use in human gene therapy.
**Introduction**

Epstein-Barr virus (EBV) is a human herpesvirus, which establishes a latent, growth-transforming infection in primary human B-lymphocytes. In the transformed B-lymphocytes, EBV expresses only night latent proteins (six EBNAs, three LMPs) and two abundant small RNAs (EBER1, 2) (reviewed by Kieff and Rickinson, 2001). Upon reactivation by inducing agents, several early and late genes of EBV are activated and induced to enter lytic infection. EBV is also associated with many malignant diseases, including Burkitt’s lymphoma (BL) (de-The et al., 1978), Hodgkin’s disease, T-cell lymphoma and nasopharyngeal carcinoma (NPC) (Henle et al., 1970; Henle et al., 1976; Chang et al., 1990).

Apoptosis is important in the elimination of malignant or virally infected cells through a genetic program of enzymatic and morphologic events (Cheng et al., 1996, Fadeel et al., 1999, Kawanishi 1997, Chau et al, 2000). In organism, a famous superfamily, Bcl-2 family, controls cell homeostasis and programmed cell death (apoptosis) (Kawanishi 1997). Bcl-2, which is a prototype of Bcl-2 family, is first identified as a proto-oncogene in follicular lymphoma (Kirsch et al., 1999). It is known to cooperate with its family members to maintain lymphoid cells by heterodimers or homodimers. Cell transomation or apoptosis may be dependent on this interaction of Bcl-2 family members in which the caspase cascade is trigged or inhibited. Therefore, Bcl-2 family members are pivotal in determining the destiny of cells and central to lymphoid homeostasis. Previous studies have showed that EBV encoded a special gene, which was homologous to Bcl-2. This protein was named as BHRF1. Due to the similarity of Bcl-2, scientists are highly interested in the function of BHRF1 and speculated that it may be involved in inhibiting cell apoptosis induced by a various stimuli (Pearson et al., 1987, Henderson et al., 1993, Foghsgaard and Jaattela, 1997, Fanidi et al., 1998). Recently other scientists discovered another Bcl-2-like EBV gene, which was thought to be the second v-Bcl-2 of EBV. It is named as BALF1, which is 0.7 kb in size and shows a predicted 220-amino acid protein in a region of early EBV transcripts, indicating that it inhibits apoptosis through the association of Bax and Bak (Marshall et al., 1999). Analysis of BALF1 in amino acid sequences reveals the structure features in functionally important BH domains, BH1 to BH4 are similar to that of Bcl-2 (Kroemer, 1997). The predicted amino acid sequence of BALF1 shows three unique features for a v-Bcl-2. First, a glycine within BH1 domain of BALF1 is replaced...
by a serine where in virtually all Bcl-2 family members there is a critical glycine (Yin et al., 1994). Mutation of this glycine to alanine in BH1 domain abolishes anti-apoptotic function. Secondly, all other gamma-herpesvirus Bcl-2-like members possess hydrophobic C-termini capable of integrated into organellar membranes. However, EBV BALF1 lacks the C-termini of hydrophobic domain, as do EB1 (Lakshmi et al., 1992, Brun et al., 1996, Cheng et al., 1997). Finally, in contrast to the divergence in the BH4 domain of other Bcl-2 members compared to Bcl-2, BALF1 is very similar to Bcl-2 and Bcl-xl, which region is conserved. Thus the feature unique to BALF1 probably provides its distinct functions compared to those cellular Bcl-2 family members in some respects (Lakshmi et al., 1992, Brun et al., 1996, Cheng et al., 1997, Nava et al., 1997, Sarid et al., 1997). Interestingly, there is closer similarity between BALF1 and Bcl-2 than between BHRF1 and Bcl-2. It seems to mean that BALF1 could be more potential than BHRF1 in EBV growth transformation of primary B cells and EBV mediated tumor diseases. However Bellows group provided the opposite results in the function of BALF1. They found that EBV BALF1 lacks anti-apoptotic function of Bcl-2 and impairs the ability of BHRF1 to inhibit apoptosis (Bellows et al., 2002). This phenomenon is just like cellular counterpart where anti-apoptotic Bcl-2 proteins modulate the function of pro-apoptotic Bcl-2 members. On the other hand, previous report showed that BALF1 was not only expressed during the very early lytic infection but also expressed during latency (Marshall et al., 1999). This raises the possibility that BALF1 modulates the cell transformation and apoptosis during latency by playing an additional role in EBV pathogenesis. Taken together, these previous data leave many questions and allow us to be in confusion and be controversial. In other words, there needs to provide other substantial evidences on the study of BALF1 function to unravel the role of BALF1 in infected cells. To date, the latent genes, LMP1 and EBNA1 are thought to be two potential oncogenes for EBV induced transformation (Kilger et al., 1998, Leight and Sugden, 2000). However, whether BALF1, which is an early lytic gene and similar to Bcl-2 structure, is potentially to be the third EBV oncogene in formation of cell tumors needs to further investigated.

In this paper, we first generated a Myc-tagged BALF1 expression plasmid, which encoded a BALF1 protein with a predicted molecular weight of 27 kDa. Overexpression of BALF1 induced Zta promoter activity, but less effect on EBNA1 promoter. However, BALF1 protein in P3HR1 cells had no clear change of EBNA1 and Zta transcripts. In
addition, the BALF1 protein obviously had the ability to decrease ceramide-mediated apoptosis 24 hr post-treatment. This preliminary data had implicated that BALF1 plays an important role of BALF1 in EBV life cycle.

**Results**

A approximate 27 kDa of BALF1 protein expressed in cells

To test the function of BALF1 on the EBV genes and cellular genes, we first generated a Myc-tagged BALF1 expression plasmid and examine whether BALF1 expressed after transfection of BALF1 expression plasmid into either 293 or P3HR-1 cells. The result indicated that the molecular mass of the BALF1 protein detected by western blotting was in agreement with the predicted molecular mass of 27kDa (Fig.1).

**Expression of BALF1 results in activation of Zta promoter, but less effect on EBNA1 promoter**

Expression of EBV key genes, such as EBNA1, LMP1 and Zta will determine the fate of EBV life cycle. Therefore, the fluctuation of expression of these EBV genes will determine to either maintain EBV latent stage or promote to lytic cycle. Because BALF1 expressed in both EBV latent and lytic stage, we intended to see if BALF1 leads to the change of EBV life cycle through its regulation of the expression of EBV latent and lytic genes during EBV infection. To determine the role of BALF1 in EBV life cycle, we transfected BALF1 into 293 cells with either Zta promoter reporter gene (Zp236) or EBNA1 promoter reporter gene (Qp125) for 24hr. The promoter activity of the transfected cells were detected by luciferase assay post 24hr transfection. As shown in Fig 2, the expression of BALF1 indeed activated about 4-5 fold of Z promoter activity (panel A), but had less effect on EBNA1 promoter activity (panel B). The results indicated BALF1 could be associated with EBV lytic infection.

**Expression of BALF1 in EBV positive cells had no effect on EBNA1 and Zta expression with no treatment of inducing agents**

To further illustrate the effect of BALF1 on EBNA1 and Zta, we introduced BALF1 into P3HR-1 cells (a Burkitt’s lymphoma cell line) by electroporation and detected the gene expression of EBNA1 and Zta by RT-PCR. The result of RT-PCR showed that overexpression of BALF1 in P3HR-1 cells has a less effect on the expression of EBNA1 and Zta gene with no treatment of inducing agents (Fig.3). That means that BALF1 had no effect on EBV latent infection and lytic cycle under no treatment of inducing agents.

**Expression of BALF1 in EBV positive cells results in inhibition of**
ceramide-induced apoptosis

Bcl-2 is important for inhibition of cell apoptosis and maintenance of homeostasis. Owing to the similarity of BALF1 to Bcl-2 in structure, we wanted to see whether the function of BALF1 was similar to that of Bcl-2, which inhibiting the cell apoptosis. To prove the possibility, the P3HR-1 cells with or without the ectopic BALF1 were treated with ceramide (an apoptosis-inducing agent) for various time-course, such as 0, 6, 12 and 24 hr. As shown in Fig.4, the BALF1 protein obviously had the ability to decrease ceramide-mediated apoptosis 24 hr post-treatment. The result indicated that BALF1 could interfere with signal pathway of ceramide-mediated apoptosis via unknown mechanism.

Discussion

EBV is thought to be a tumor virus because of its association with several human cancers, such as B cell lymphoma, Hodgkin’s disease, and nasopharyngeal carcinoma (Kieff and Rickinson, 2001). Despite studying for many years, the precise mechanisms by which the EBV drives primary B cells from quiescence into the cell cycle and develops into the EBV-associated tumorigenesis are still only partially understood. The studies of the recombinant EBV have demonstrated that only six latent genes, EBNA1, 2, 3A, 3C, LP, and LMP1 are absolutely crucial to promote cell transformation (Dirmeier et al., 2003; Humme et al., 2003). In addition to these six latent genes, two Bcl-2 –like EBV proteins, BHRF1 and BALF1 can be the best candidates to cause EBV-induced tumors. BHRF1 has an anti-apoptotic activity, but is rarely expressed in nasopharyngeal carcinoma. However the function of BALF1 is not yet characterized.

In the preliminary study, we have obtained that a BALF1 protein with a predicted molecular weight of 27 kDa had a less effect on the expression of EBV EBNA1 and Zta genes in P3HR1 cells without the treatment of inducing agents. On the other hand, the BALF1 protein obviously had the ability to decrease ceramide-mediated apoptosis 24 hr post-treatment. Though the data of RT-PCR assay indicated BALF1 has no effect on the transcription of EBNA1 and Zta in the latently infected cells, it could possibly affect on their transcripts during the early lytic cycle. Therefore, we have to determine the fluctuation of transcription of EBNA1 and Zta after EBV reactivation by using RT-PCR and luciferase assay. The results will help us to further understand the functions of BALF1 in the EBV life cycle. It has previously been proposed that the inhibition of EBV reactivation by cell cycle-promoting factors, c-Myc and E2F1 occurs through
inhibition of the transactivation function of Zta protein (Young et al., 2000; DeGregori 2002; Lin et al., 2004). That means that c-Myc or E2F1 proteins may play an important role in inhibition of EBV reactivation and promote cell proliferation. In the current studies, the data of flow cytometer assay showed the ceramide-induced apoptosis can be inhibited by BALF1. That will be very interesting to see for us whether the viral Bcl-2 like protein, BALF1 could function as c-Myc or E2F1, which promoted cell cycle progression through inactivation of Zta protein, to induce cell proliferation. In the future we will continue to demonstrate whether the BALF1 are associated with the maintenance of EBV latency via induction of Zta inhibitory factors (ZIF) or try to gain insight into the role of BALF1 in the switch from latency to lytic infection. The new information of BALF1 function may allow us to change the old concept of EBV life cycle and provide a new definition of BALF1 protein. In addition, we also will continue to explore the association of BALF1 with EBV-related tumorigenesis or see if the formation of tumors is due to change the pathways of apoptosis or genomic instability. Though EBV induced tumorigenesis is still debating, all the experimental results will at least help us to find the most important way to understanding of how EBV cause a tumor.

Materials and Methods

Cell culture
EBV-positive Burkitt’s lymphoma (BL) cell lines, Rael (type I) and P3HR-1 or EBV-negative burkitt’s lymphoma cells, CA46 and Akata, are maintained in RPMI 1640 (GIBCO, USA) supplemented with 15% (v/v) fetal bovine serum (FBS). 293T cells is cultured in Delbecco’s modified Eagle medium (DMEM) with 10% FBS.

Plasmid construction
The fragment covering the full-length BALF1 are generated by polymerase chain reaction (PCR) with DNA isolated from Rael cells and one set of primers. The BALF1 fragment is inserted into EcoRI site of pCMV-Myc expression vector (Clontech), which contains the CMV promoter and BALF1 open reading fragment named as pCMV-Myc-BALF1. For BALF1 stable plasmid, the genomic fragment of BALF1 is
obtained by PCR technique, which is inserted into the pTriEx-2 neo, which drives the BALF1 under β-actin promoter and contains a neomycin resistant gene as a selection marker. The resulting stable plasmid will be used to establish the stable cell line of BALF1. The pBALF1-siRNA plasmid is constructed according to the instruction described by Brummelkamp group (Brummelkamp et al., 2002). Briefly, the synthetic 19 nt located at initiation site region is inserted into pSuper vector downstream of H1-RNA promoter (Hannon et al., 1991), which is separated by a short spacer from the reverse complement of the same sequence. The siRNA cassette containing H1-RNA promoter, 19 nt of BALF1 gene is named as pBALF1-siRNA. The 19 nt BALF1 sequence should be flanked in the mRNA with AA at the 5’ and TT at 3’. Region at the mRNA to select the 19 nt from, preferably in the coding region: 100 bp from start and termination of translation.

**RNA extraction and RT-PCR**

Total RNA will be extracted from the cells according to the instructions (Qiagen) and then the extracted RNA will be used to carry out RT-PCR. Briefly, the five ug of RNA is added into the 20ul of reaction, which contains 1X reverse transcriptase (RT) buffer and 1u of RT to generate those cDNAs. One hr after incubation at 42°C, the one fourth of cDNA reaction is used to perform the PCR analysis with three sets of the specific primers (GAPDH, EBNA1 and Zta). Finally the products of PCR are subjected to be separated by using 2% agarose gel and analyzed under UV after staining.

**Detection of apoptotic cells by flow cytometry**

The presence of apoptotic cells was detected by propidium iodide staining followed by flow cytometric analysis of hypodiploid cells. Cells (1 x 106) were fixed in 2 ml of 70% ethanol/PBS and placed at 4°C for at least 30 min. After centrifugation, cells were resuspended in 800 µl of PBS containing 40 µg/ml of propidium iodide (Sigma-Aldrich) and 100 µg/ml of RNase (Calbiochem), and incubated at room temperature for 30 min. The DNA content of cells was analyzed on a FACScan (Becton Dickinson, Mountain...
View, CA) with excitation set at 488 nm.

**DNA extraction and agarose gel electrophoresis**

Cells (1 x 10^6) were suspended in 500 μl of cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.2% Triton X-100 for 10 min on ice. After centrifugation at 12,000 rpm for 10 min, the supernatant that contained fragmented DNA was incubated with 200μg/ml proteinase K (Merck, Darmstadt, Germany) in a 50°C water bath overnight and digested with 100 μg/ml RNase for an additional 6 h at 37°C. The DNA was extracted by standard phenol/chloroform method and then precipitated in 50% isopropanol and 20μg/ml glycogen at -20°C overnight. After centrifugation, the resulting pellet was dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and the DNA sample was electrophoresed on 2% agarose gel in Tris-acetate EDTA buffer (pH 8.0). After electrophoresis, the gel was stained with ethidium bromide at 1 μg/ml and the DNA was visualized with UV light.

**DNA transfection and Luciferase assays**

The indicated amount of expression plasmids are introduced into the suspension cells (Rael, LCL) by electroporation at 960 F and 0.22 kV using the Gene Pulser (Bio-Rad, USA) and the transfected cells are incubated for 24 hr. The cells are then harvested and lysed in 50 l of lysis buffer (Promega, USA). The protein concentration of the cell lysate is measured by using a Bio-Rad protein assay reagent (Bio-Rad, USA). A 50μg-sample of protein extract is used to measure the luciferase activity in an illuminometer (Berthold autolumat model LB953, Germany). The epithelial cells are transfected with the indicated plasmids through Lipofectamine (Gibco, UK). 48hr after incubation, the steps of luciferase assay in epithelial cells are similar to that in the suspension cells as described above.

**Western blotting**

Western blot analysis is carried out by incubation of cell lysates with the indicated antibody. Briefly, the cells are lysed for 30 min. on ice in 0.5% NP-40 lysis buffer.
containing 50mM HEPES (pH 7.4) 250mM NaCl, 10% glycerol, 2mM EDTA, 1mM PMSF, 2µg / ml aprotinin, 2µg / ml pepstatin and 2µg / ml leupeptin. Cell debris is removed by centrifugation at 10,000Xg for 10 min. at 4°C. The protein concentration of cell lysate will be measured by Bradford method (Bio-Red, Richmond CA). The lysate will be applied to 10% SDS-PAGE gel and then transferred to the nitrocellulose membrane. The membrane is incubated with polyclonal antibody of anti-Myc (Santa Cruz Biotechnology Inc.) or anti-S antibody and reacted with the horseradish peroxidase-conjugated anti-rabbit antibodies as secondary antibody.

References
de-The, G., Geser, A., Day, N. E., Tukey, P. M., Williams, E. H., Beri, D. P., Smith, P.


**Fig. 1** The expression of BALF1 in P3HR-1 and 293 cells. The cells (P3HR-1 or 293) were transfected with c-Myc-BALF1 plasmid by using electroporation. 24hr post transfection, the expression of BALF1 protein was analyzed by western blotting with anti-Myc antibody.
**Fig 2 The effect of BALF1 on the Q promoter and Z promoter** The BALF1 expression plasmid was transfected into 293 cells with Q promoter, Qp125(panel A) or Z promoter, Zp236(panel B). 24hr post transfection, the promoter activity was determined by luciferase assay. myc serves as vector control. the number of Y axis is the RLU normalized by internal control(Renilla).The data shown in panel A and B are representative results of two independent experiments.
Fig. 3 The expression of EBV EBNA1 and Zta transcripts regulated by BALF1. The P3HR-1 cells were transfected with either c-Myc-BALF1 expression plasmid or vector by electroporation. 24 hr after transfection, the transfected cells were used to detect the transcripts of EBNA1 and Zta by RT-PCR. The number of 1 and 2 represents two independent experiments.
Fig. 4 Flow cytometry analysis of sub-G1 fraction in P3HR-1 cells transfected with BALF1. The EBV positive P3HR-1 cells were transfected with c-Myc-BALF1 or vector. 24hr post transfection, the cells were collected at indicated time points to analyze the sub-G1 cell percentage by Flow cytometry with propidium iodide staining. Representative results of two independent experiments.
1. Demonstration of the transformation ability of EBV to primary B-lymphocytes is always our major goal. In this project, we did not change the original ideas. The content of this project very similar to that of the original project. The progress of this project is just up to about 30 percent, because of the lack of human and financial resources. If NSC continues to support more human and financial resources without any condition, this project will be finished soon.

2. There are some experiments, which need to be improved in this project, such as the detection system of ChIP assay. If these experimental blocks are breakthrough, it will obviously increase the chance to success.

3. If this project is completely finished, we will not only publish our project on the science Journal but also establish a set of new therapeutic methods for EBV induced diseases.