LMP-1 induces survivin expression to inhibit cell apoptosis through the NF-κB and PI3K/Akt signaling pathways in nasal NK/T-cell lymphoma

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Abstract. The latent membrane protein-1 (LMP-1) is essential for Epstein-Barr virus (EBV)-induced nasal natural killer/T-cell lymphoma (NKTL). The aim of the present study was to evaluate the role of LMP-1 in NKTL. Two human EBV-positive NKTL cell lines (SNK-6 and SNT-8) were transfected with pcDNA3.1-LMP-1 or LMP-1 siRNA. Compared with the blank control, the cell apoptosis rates were decreased by 10.31 and 12.05% after pcDNA3.1-LMP-1 transfection and increased by 41.48 and 35.63% after lentiviral LMP-1 siRNA infection in the SNK-6 and SNT-8 cells. Survivin expression was induced by LMP-1, and the effect was attenuated by inhibitors of survivin, NF-κB and PI3K/Akt. Reduction in cell apoptosis by LMP-1 was also inhibited by inhibitors of survivin, NF-κB and PI3K/Akt. For the in vivo assay, tumor-bearing mice were established by subcutaneous injection with differentially treated SNT-8 cells into the back of the nude mice, and the tumor growth in the different groups was recorded. The results revealed that tumor formation and growth were also inhibited by treatment with survivin, NF-κB and PI3K/Akt inhibitors. Collectively, LMP-1-induced survivin expression inhibited cell apoptosis through the NF-κB and PI3K/Akt pathways, and survivin may be a new target for the treatment of NKTL induced by EBV.

Introduction

Nasal natural killer/T-cell lymphoma (NKTL) is an uncommon type of lymphoma with a highly aggressive clinical course that mainly occurs in Asian and Latin American countries (1,2). It is usually localized to the upper aerodigestive tract, and exhibits an aggressive disorder with a tendency to invade local tissues and metastasize to the central nervous system. Thus, NKTL is a predominant malignancy, and an effective therapeutic target is needed for the treatment of NKTL. The Epstein-Barr virus (EBV) is a potential oncogenic human herpes virus which can infect lymphoid and epithelial cells. EBV exhibits a latent infection in host cells and is closely associated with many types of cancers including NKTL. Many proteins expressed by EBV, such as EBV nuclear antigens (EBNA-1, -2, -3A, -3B and -3C) and latent membrane proteins (LMP-1, -2A and -2B) are potentially related to carcinogenesis (3).

LMP-1, which is encoded by the BNLF-1 gene, is considered one of the major oncoproteins among the EBV-expressed proteins (4). LMP-1 is a type of transmembrane protein and its cytoplasmic fragment contains three important domains [carboxyl-terminal activating regions 1, 2 and 3 (CTR 1-3)] that activate several pathways including the nuclear factor κB (NF-κB), phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), Notch and mitogen-activated protein kinase (MAPK) signaling pathways (5-7). Although it has been proven that LMP-1 is essential for EBV-induced lymphomas, the signaling pathway involved in the effect has not been fully established (8).

Survivin is a member of the inhibitor of apoptosis protein family (9). It correlates with tumor progression and is overexpressed in lymphoma and cancers including breast and lung cancer, prostate, gastric, colon, bladder and esophageal carcinomas (10,11). It has been shown that survivin overexpression is associated with several EBV-infected lymphomas including NKTL, whereas the relationship between LMP-1 and survivin has not yet been fully evaluated in NKTL (12,13).

The aim of the present study was to investigate the effect of LMP-1 on cell apoptosis in two types of human EBV-positive NKTL cell lines (SNK-6 and SNT-8) and in tumor-bearing mice. The results showed that LMP-1 inhibited cell apoptosis and induced survivin expression in both the in vitro and in vivo assays, which was mediated by the NF-κB and PI3K/Akt signaling pathways. The results indicate that survivin may be a new potential drug target for the treatment of NKTL induced by EBV.
Materials and methods

Animals. In total, 35 female BALB/c nude mice (Vital River Laboratories; 5 weeks of age, 16-18 g) were housed in a temperature-controlled laminar flow cabinet (22-24°C), with a 12-12 h light-dark cycle. Animals were given free access to chow and water. All procedures in the present study were approved by the Animal Care Committee of PLA General Hospital.

Cell lines and cell culture. Two types of EBV-positive NKTL cell lines, SNK-6 and SNT-8, were kindly provided by Professor H. Nagata (Tokyo Medical and Dental University). The cells were cultured in RPMI-1640 (Sigma, St. Louis, MO, USA), supplemented with 10% human serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 700 U/ml recombinant human IL-2 (Novartis, Camberley, UK). All cell lines were incubated at 37°C in an atmosphere containing 5% CO₂.

LMP-1 expression vector transfection. The pcDNA3.1-LMP-1 plasmid was constructed by inserting the cDNA fragment retrotranscribed from the full-length of LMP-1 mRNA (14). Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used for the transfection of the LMP-1 plasmid (pcDNA3.1-LMP-1) and empty plasmid (pcDNA3.1) according to the manufacturer’s protocol. Stable expression clones were selected by G418 (neomycin sulfate, 800 µg/ml). The cell culture medium was replaced with fresh G418-containing medium every 2-3 days until resistant colonies were identified. The inhibitors of survivin [Terameprocol (TP), 10 µmol/l], PI3kAkt [LY294002 (LY), 20 µmol/l] NF-κB [pyrrolidine dithiocarbamate (PDTC), 10 µmol/l], Notch [γ-secretase inhibitor (GSI) 10 µmol/l], and MAPK [SP600125 (SP), 10 µmol/l] (Sigma) were added to the medium of LMP-1 stably expressing cells for 48 h. The cells were collected for further analysis.

Infection of SNK-6 and SNT-8 cells with the lentiviral LMP-1 siRNA expression vector. The lentiviral LMP-1 siRNA and negative control siRNA (NC siRNA) were constructed by Hanheng Biotech (Shanghai, China). SNK-6 and SNT-8 cells in the logarithmic growth phase were cultured with lentiviral vector solution for 6 h, and supplemented with the lentiviral vector for another 6 h. After 72 h, the cells were selected by hygromycin B until positive cells were identified.

Establishment of the lymphoma model in nude mice. The BALB/c nude mice were randomly separated into 7 groups: i) blank control (normal SNT-8 cell line); ii) LMP-1 (pcDNA3.1-LMP-1 SNT-8); iii) LMP-1 siRNA (LMP-1 siRNA SNT-8); iv) LMP-1+TP (pcDNA3.1-LMP-1 SNT-8+TP); v) LMP-1+PDTC (pcDNA3.1-LMP-1 SNT-8+PDTC); vi) LMP-1+LY (pcDNA3.1-LMP-1 SNT-8+LY); and vii) LMP-1+PDTC+LY (pcDNA3.1-LMP-1 SNT-8+PDTC+LY). The different groups of cells (0.1 ml, 5x10⁶) in the logarithmic growth phase were subcutaneously injected into the back of the nude mice. For the TP (35 mg/kg), PDTC (50 mg/kg) or LY (50 mg/kg) treatment groups, the mice received daily subcutaneous injection (same position for each injection). The time of the tumor formation was recorded. The volume of the tumor was measured by the formula (V = 1/2 length x width x height) every week (15). After six weeks, the mice were sacrificed and tumors were collected for further RT-PCR and western blot analysis.

Quantitative RT-PCR analysis. Total RNA of the cells and tumors was extracted using TRizol reagent (Invitrogen). The cDNA was synthesized from 5 µg of the total RNA using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA). The obtained cDNA was then used as template for qRT-PCR analysis. Quantitative RT-PCR was carried out on an ABI Prism 7500 System (Applied Biosystems Inc., USA) with the Tli RNaseH Plus kit (Takara Biotechnology, Dalian, China). The qRT-PCR was performed as follows: 95°C for 2 min for initial denaturation; 94°C for 15 sec, 58°C for 15 sec and 72°C for 20 sec; 2 sec for plate reading for 40 cycles; and melt curve from 65 to 95°C. The fold-changes were calculated using the formula: R = 2ΔΔCt. Primer sequences are shown in Table I.

Western blot analysis. The transfected cells were homogenized and lysed with RIPA lysis buffer. A micro-BCA protein assay kit (Pierce Chemical, Rockford, IL, USA) was used for protein concentration analysis. Proteins (20-30 µg/lane) were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Pharmacia, Germany). After a 1-h incubation in blocking solution (5% non-fat milk), the membranes were incubated with a 1:1000 dilution of primary antibodies against LMP-1, survivin, β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. The membranes were washed five times with PBST buffer and incubated with HRP-conjugated secondary antibodies (1:5,000) for 1 h at room temperature. Chemiluminescent detection was performed using an ECL kit (Pierce Chemical). The gray value of the bands was analyzed by ImageJ 2X software.

Flow cytometric assay. Cell apoptosis was analyzed by the flow cytometric method (FCM) using an Annexin V-PI apoptosis detection kit (Abcam, Cambridge, UK). Briefly, the cells were collected 48 h after transfection, washed with

<table>
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<th>Genes</th>
<th>Primers</th>
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<tr>
<td>LMP-1</td>
<td>S: 5'-AACCGAGCGAGCCACTCTACG-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-CACGAGCAACTGAGTGTGTCAC-3'</td>
</tr>
<tr>
<td>Survivin</td>
<td>S: 5'-AAAGAGCCCAAGACAAATTGC-3'</td>
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<tr>
<td></td>
<td>A: 5'-GAGAGAGAGACGCGACCTGTTAC-3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>S: 5'-TTTGTGTGTGnCAACGGGA-3'</td>
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<tr>
<td></td>
<td>A: 5'-GGCTGGGACACATTCTGTT-3'</td>
</tr>
<tr>
<td>Bax</td>
<td>S: 5'-AGGTCTTTTTTCCCGATGTCACG-3'</td>
</tr>
<tr>
<td></td>
<td>A: 5'-CCCGG AGGAATCTCAATGTGCC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>S: 5'-CATCAGCTTGGCAATGAGC-3'</td>
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<tr>
<td></td>
<td>A: 5'-GACAGCACTGTGTTGGGACATA-3'</td>
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LMP-1, latent membrane protein-1; S, sense; A, antisense.

Table I. Primer sequences.
phosphate-buffered saline (PBS) and suspended in 500 µl binding buffer. The cells were incubated with Annexin V at room temperature for 10 min and stained by propidium iodide (PI), and then analyzed by FCM for relative quantitative apoptosis.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All data are presented as mean ± SD. The statistical significance of differences among groups was evaluated with Dunnett's test subsequent to analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant result.

Results

Expression of LMP-1 in the transfected SNK-6 and SNT-8 cells. The SNK-6 and SNT-8 cells were transfected with pcDNA3.1, pcDNA3.1-LMP-1 or LMP-1 siRNA. After the selection of positive cells, the LMP-1 expression was detected by western blotting. As shown in Fig. 1a and b, compared with the empty vector-transfected cells, the expression of LMP-1 was increased by 6- and 5-fold in the pcDNA3.1-LMP-1-transfected SNK-6 and SNT-8 cells. IFN-α, which is an inducer of LMP-1, induced the expression of LMP-1 in both the SNK-6 and SNT-8 cells (P<0.05). The expression of LMP-1 in the IFN-α-induced cells was less than that in the pcDNA3.1-LMP-1-transfected cells (P<0.05). After LMP-1 siRNA transfection, the LMP-1 expression in the SNK-6 and SNT-8 cells was significantly reduced compared with that in the control groups (P<0.05).

LMP-1 inhibits the cell apoptosis of SNK-6 and SNT-8 cells. The cell apoptosis of the differentially treated SNK-6 and SNT-8 cells was measured by FCM. The results showed that the apoptosis rates of the pcDNA3.1-LMP-1-transfected and IFN-α-treated SNK-6 and SNT-8 cells were significantly reduced (Fig. 2). However, in the LMP-1 siRNA-infected cells, the apoptosis rates were increased when compared with the control groups. The results indicate that LMP-1 inhibited cellular apoptosis in NKTL.

LMP-1 inhibits cell apoptosis by promoting survivin expression. To evaluate the effect of LMP-1 on survivin expression in the SNK-6 and SNT-8 cells, the protein and mRNA levels of survivin were detected by western blotting and RT-PCR. As shown in Fig. 3a-d, TP (an inhibitor of survivin) inhibited the expression of survivin in both the SNK-6 and SNT-8 cells, with SNT-8 cells being more sensitive to TP than SNK-6 cells. The protein and mRNA levels of survivin were enhanced by LMP-1 overexpression and IFN-α induction in the SNK-6 and SNT-8 cells (P<0.05). Compared with the blank control group, the survivin expression in the cells with LMP-1 siRNA transfection was significantly decreased, suggesting that survivin expression was induced by LMP-1 in NKTL. To further confirm whether survivin is essential in LMP-1-induced cell survival, TP was added to the medium of the pcDNA3.1-LMP-1-transfected SNK-6 and SNT-8 cells; the cell apoptosis rates were significantly increased by 39 and 47% in the SNK-6 and SNT-8 cells, respectively, compared to the TP blank cells (Fig. 3e and f). The results of FCM also showed that SNT-8 cells were more sensitive to TP than SNK-6. The results suggested that LMP-1 inhibited cell apoptosis by promoting survivin expression.

Effect of LMP-1 on survivin expression is mediated by the NF-κB and PI3k/Akt pathways. LMP-1 has been proven to
activate several pathways, while the pathway involved in the induction of survivin expression was unknown. To investigate the role of the NF-κB, PI3K/Akt, Notch and MAPK pathways in the induction of survivin expression by LMP-1, inhibitors of the pathways were added to the LMP-1-transfected cells, and the expression of survivin was measured by western blotting. As shown in Fig. 4a and b, the expression of survivin was significantly decreased in cells with PDTC and LY treatment, while SP and GSI had no obvious effect on survivin (P>0.05). To further illustrate the role of the NF-κB and PI3K/Akt pathways in the induction of survivin by LMP-1, the effects of LMP-1 on Akt phosphorylation and IκBα expression were measured. The results showed that LMP-1 overexpression and IFN-α treatment increased Akt phosphorylation and decreased IκBα expression; the effect of siRNA was controversial in both SNK-6 and SNT-8 cells (Fig. 4c and d), indicating that LMP-1 promoted NF-κB and PI3K/Akt pathway activation. To evaluate whether the two pathways acted together in the effect of LMP-1 on cell apoptosis, PTTD and/or LY were added to the LMP-1-transfected cells, and cell apoptosis was detected by FCM. As shown in Fig. 4e and f, the cell apoptosis rate was increased by 20 and 18% after PDTC or LY treatment, respectively, in the SNK-6 cells, and was increased by 45% in the cells with PDTC and LY joint treatment. The results noted in the SNT-8 cells were coincident with those in the SNK-6 cells. All of the data indicated that LMP-1-induced survivin
expression inhibited cell apoptosis through the NF-κB and PI3K/Akt pathways.

**LMP-1 promotes the growth of transplanted tumors in nude mice.** To evaluate the effect of LMP-1 on cell survival in vivo, the SNT-8 cells with different treatments were injected into nude mice. The data of tumor formation and growth are shown in Fig. 5. The average time of tumor formation in the control group was 10.60±0.51 days, and the average size of the tumors on the 42 day was 1.21±0.23 cm³. LMP-1 significantly promoted tumor formation and growth (P<0.05). After treatment with TP/PDTC/LY, the time of tumor formation and tumor growth were inhibited compared to that of the LMP-1 overexpression group. The joint treatment of PDTC and LY exhibited a greater effect than a single treatment with either of the two inhibitors, indicating that the effect of LMP-1 on tumor formation was associated with the NF-κB and PI3K/Akt pathways.

**LMP-1 inhibits the cell apoptosis of transplanted tumors by inducing survivin expression.** The tumors in each group of mice were collected, and the protein and mRNA levels of LMP-1 and survivin were detected by western blotting and RT-PCR. As shown in Fig. 6, LMP-1 was highly expressed in the LMP-1 transfected groups, and expressed at a lower level in the LMP-1 siRNA-SNT-8 group. Survivin expression in the LMP-1 group was higher than that in the control group, whereas in the LMP-1 siRNA group, the expression was lower. In the TP, PDTC, LY and PDTC+LY injection groups, survivin expression was significantly decreased compared with the LMP-1 group. In addition, PDTC and LY joint treatment showed stronger inhibition than noted for the PDTC/LY group. The results suggested that LMP-1 induced survivin expression through the NF-κB and PI3K/Akt pathways in vivo.

To evaluate the effect of LMP-1 on cellular apoptosis in vivo, the mRNA and protein levels of bax and bcl-2 were detected by RT-PCR and western blotting, respectively. The results showed that LMP-1 inhibited bax expression and promoted bcl-2 expression, while treatment with TP, PDTC, LY or PDTC+LY attenuated the effect (Fig. 6). Moreover, treatment with both PDTC and LY was more effective than the single treatment. Therefore, the inhibitory effect of LMP-1 on cell apoptosis is associated with the NF-κB and PI3K/Akt pathways in vivo.

**Discussion**

LMP-1 is considered a major viral oncoprotein of the EBV carcinogenic process, and its effect in several diseases has been evaluated. LMP-1 is important for the progression of EBV-related nasopharyngeal carcinoma (NPC) since it suppresses microRNA-204 expression thereby facilitating the
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Figure 4. LMP-1 induces survivin expression to inhibit cell apoptosis through the NF-κB and PI3K/Akt pathways in SNK-6 and SNT-8 cells. Protein levels of survivin in the (a) SNK-6 and (b) SNT-8 cells following differential treatments. Protein levels of pAkt and IκBα in the (c) SNK-6 and (d) SNT-8 cells following different treatments. Cell apoptosis in the (e) SNK-6 and (f) SNT-8 cells following differential treatments. *P<0.05 vs. the pcDNA3.1-LMP-1-transfected cells, †P<0.05 vs. the blank control, aP<0.05 vs. the pcDNA3.1-LMP-1-transfected cells with PDTC treatment, bP<0.05 vs. the pcDNA3.1-LMP-1-transfected cells with LY treatment. Data are represented as mean ± SD. LMP-1, latent membrane protein-1.

invasion of NPC cells (16). Another study showed that LMP-1 stimulates the transcription of eukaryotic translation initiation factor 4E to induce the proliferation and invasion of human NPC cells (17). Thus, it is necessary to confirm whether LMP-1
possesses an essential role in NKTL induced by EBV (18). SNK-6 and SNT-8 cells are two types of EBV-positive human NK cell lines which were established in 2001. They were obtained from two Japanese patients with NKTL, and have been used in NKTL relative studies (19). It has been proven that LMP-1 is stably expressed in SNK-6 and SNT-8 cells (20). The two cell lines were used in the present study to investigate the role of LMP-1 in NKTL.

Overexpression of survivin has been widely demonstrated to occur in various types of cancer. It has been revealed in previous studies that patients with survivin overexpression present with a more aggressive and metastatic colorectal carcinoma (21). It has been proven that LMP-1 increases the survivin expression in human nasopharyngeal cells and nasopharyngeal cancer (22). LMP-1 also promotes cell proliferation and inhibits cell apoptosis by regulating survivin expression in nasopharyngeal carcinoma (23,24). A previous study selected 33 cases of NKTL, and found that overexpression of survivin was observed in 97% of the cases (12). To evaluate the relationship between LMP-1 and survivin in NKTL, LMP-1 was overexpressed and knocked down in the SNK-6 and SNT-8 cell lines in the present study; we found that survivin expression induced by LMP-1 overexpression was inhibited by LMP-1 siRNA transfection. The inhibitor of survivin (TP) inhibited cell survival induced by LMP-1 in both SNK-6 and SNT-8 cells. A previous study found that SNK-6 cells were not sensitive to TP (12), while the results of the present study showed that SNK-6 cells were sensitive to TP, yet less than SNT-8. This may be due to the different experimental conditions and operations. Thus, SNT-8 cells with different treatments were used in the in vivo studies. All of the results indicated that survivin plays an important role in LMP-1-induced cell survival in NKTL.

A previous study proved that LMP-1 increased the development of lymphoma in LMP-1 transgenic mice through the NF-κB pathway (25). In addition, LMP-2A upregulated the gene expression of survivin to inhibit cell apoptosis in gastric carcinoma cell lines; this upregulation was mediated by the NF-κB pathway (26). The role of NF-κB in the induction of survivin expression by LMP-1 was evaluated in the present study, and we found that survivin expression induced by LMP-1 overexpression was inhibited by an inhibitor of NF-κB. In addition, LMP-1 has the ability to activate the PI3K/Akt signaling pathway (27). In addition, survivin has been shown to be upregulated by the PI3K/Akt pathway in several studies (28,29). In the present study, the PI3K/Akt signaling pathway was shown to be involved in the induction of survivin expression by LMP-1. Although the Notch and MAPK pathways are activated by LMP-1 in other diseases (30,31), we found that the roles of the Notch and MAPK pathways in NKTL were not obvious. Thus, we can infer that LMP-1 induced survivin expression by activating the NF-κB and PI3K/Akt pathways in NKTL. However, the regulation of survivin expression is quite complex; therefore, its exact molecular regulation and functions in NKTL need to be further investigated.

In summary, LMP-1 is essential in NKTL induced by EBV. LMP-1 inhibited the cell apoptosis of SNK-6 and SNT-8 by regulating survivin expression, while the inhibitors of survivin,
NF-xB and PI3K/Akt weakened the inhibition. Moreover, the results showed that NF-xB and PI3K/Akt acted together to inhibit the effect of LMP-1. In the transplantation tumor experiment in nude mice, LMP-1 promoted tumor formation and growth in nude mice, which was inhibited by inhibitors of survivin, NF-xB and PI3K/Akt. The results revealed that LMP-1 induced survivin expression, thus inhibiting cell apoptosis through the NF-xB and PI3K/Akt signaling pathways, and that survivin may be a new medicinal target for the treatment of NKTL induced by EBV.

References