LYTIC REPLICATION OF HUMAN HERPESVIRUS 8 AND INDUCTION OF APOPTOSIS

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Summary. – Although many viruses can induce apoptosis in infected cells, large DNA viruses, such as poxviruses, herpesviruses and adenoviruses, usually exhibit the ability to suppress the induction of apoptosis in the infected cells. We investigated the ability of Human herpesvirus 8 (HHV-8) to protect cells from apoptosis induced by the virus. HHV-8 has been shown to harbor genes with anti-apoptotic capacity. However, we demonstrate here that a lytic replication of HHV-8 resulted in induction of apoptosis using different techniques to detect apoptosis. Therefore, despite the presence of anti-apoptotic genes in its genome, HHV-8 could complete its cycle of productive infection while inducing apoptosis in infected cells. This finding might have implications for the pathobiology of HHV-8 and other gamma herpesviruses *in vivo*.

Key words: apoptosis; gammaherpesvirus; Human herpesvirus 8

Introduction

Apoptosis is a type of cell death, which is classically defined both morphologically and biochemically as (i) cell shrinkage, (ii) condensation and fragmentation of the cell nuclei and (iii) fragmentation of chromosomal DNA into nucleosomal oligomers (Kerr and Harmon, 1991). Once apoptosis is triggered, a series of programmed events leads to the death of the cell manifested by morphological changes, activation of specific enzymes, and especially by the degradation of cellular DNA (Ellis *et al.*, 1991). Chromosomal fragmentation, possibly caused by cytoplasmic proteases, indicating activation of endonucleases, has become accepted as hallmark for this form of cell death, which is detected as a ladder pattern on gel electrophoresis (Wyllie *et al.*, 1984). Kim *et al.* (2000) have described an *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphates (dUTP)-fluorescein nick end labeling (TUNEL) assay that makes possible demonstration of apoptosis.

Infection with an increasing number of viruses has been reported to induce apoptosis. Apoptosis is a host defense mechanism that the cell uses to limit production of infectious virus. Although many viruses can induce apoptosis in infected cells, large DNA viruses, such as poxviruses, herpesviruses and adenoviruses, usually exhibit the ability to suppress the induction of apoptosis in infected cells (O'Brien, 1998; Tschopp *et al.*, 1998). There is considerable evidence that herpesviruses block apoptosis (Cheng *et al.*, 1997; Koyama and Miwa, 1997; Leopardi and Roizman, 1996; Shen and Shenk, 1995; Sieg *et al.*, 1996; Zhu *et al.*, 1995). HHV-8, a gammaherpesvirus, also known as Kaposi's sarcoma-associated herpesvirus (KSHV), has been shown to harbor genes with anti-apoptotic potential (Katano *et al.*, 2001; Meinl *et al.*, 1998). Two HHV-8-related anti-apoptotic proteins have been identified: vBcl-2 encoded by ORF 16 and vFLIP encoded by ORF K13, which block apoptosis

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Abbreviations: BCIP = 5-bromocresyl-3-indolyl-phosphate; HHV-8 = Human herpesvirus 8; NBT = nitroblue tetrazolium; PBS = phosphate-buffered saline; p.i. = post inoculation; SDS = sodium dodecyl sulfate; TPA = tetradecanoyl phorbol acetate; TUNEL = terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphates-fluorescein nick end labeling

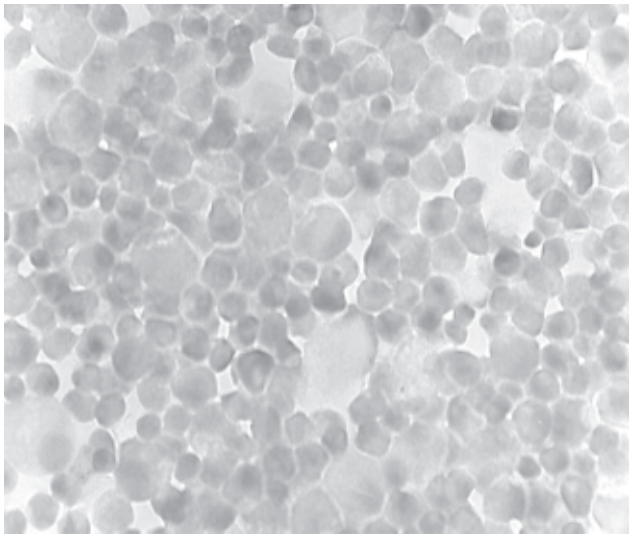

Fig. 1
***In situ* hybridization with TPA-untreated HHV-8-infected BCBL-2 cells at day 4 p.i.**
No detectable HHV-8 DNA. Magnification 400x.

Fig. 2
TUNEL assay on TPA-untreated HHV-8-infected BCBL-2 cells at day 4 p.i.
No apoptotic DNA. Magnification 400x.

(Wang *et al*., 2002). However, no information is available on real ability of these proteins to regulate apoptosis following productive infection. Since an increasing evidence would indicate that several viruses could simultaneously carry genes able to both block and induce apoptosis (Teodoro and Branton, 1997), we have used the availability of an efficient experimental model of infection *in vitro*, namely HHV-8 to investigate whether the presence of anti-apoptotic genes in the genome of a gammaherpesvirus effectively and invariably leads to the inhibition of apoptosis during lytic replication.

Materials and Methods

Preparation of infected cells. BCBL-2 cells infected latently with HHV-8 were induced by treatment with 20 ng/ml tetradecanoyl phorbol acetate (TPA, Sigma) as described earlier (Renne *et al.*, 1996). At time 0, the cells were collected by centrifugation at 1,500 rpm for 5 mins and suspended in fresh RPMI 1640 medium supplemented with 10% of fetal calf serum, 2 mol/l L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin to a density of 5 x 10^5/ml. Then, four equal aliquots were incubated with either the standard medium or the medium supplemented with TPA. After 2 days and 4 days each, the cultured cells were centrifuged onto Silane-coated glass slides (Sigma) in a Cytospin 2 centrifuge (Shandon, USA) for 5 mins at 1,000 rpm and fixed in 4% paraformaldehyde for 10 mins for *in situ* hybridization and for the TUNEL assay. Also, for DNA laddering analysis, viral DNAs were extracted from virus-infected cells using the Apoptotic DNA Ladder Kit (Roche).

In situ hybridization. For identification of viral replication, we used an *in situ* hybridization technique for the detection of viral nucleic acids in infected cells. A 233-bp DNA fragment from $KS330_{233}$ was used as a probe. The PCR was carried out as described by Chang *et al.* (1994). The PCR products were purified with the Wizard PCR Preps (Promega) and were labeled by random priming with digoxigenin-dUTP using a commercial kit (Boehringer Mannheim) according to the manufacturer's instructions. The *in situ* hybridization was carried out as described by Kim and Chae (2000). Briefly, after washing with phosphate-buffered saline (PBS), a deproteinization step was performed by treatment with 10 µg/ml proteinase K (Roche) at 37°C for 30 mins. The hybridization solution contained 5 x SSC, 50% deionized formamide, 2% buffered blocking solution (Roche), 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulfate (SDS). The hybridization proceeded overnight at 45°C. The digoxigenin-labeled probe (0.1 ng/µl) was diluted in 300 µl of the standard hybridization buffer and approximately 50 ng of the digoxigenin-labeled probe was added to the standard hybridization buffer (50 µl), which was then layered over the slide. To detect hybridization, slides were incubated with the digoxigenin antibody conjugated with alkaline phosphatase (Roche) and stained with nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolyl-phosphate (BCIP). Then, the slides were counterstained with 0.5% methylene green. Specific dark blue signals were examined with light microscope.

Detection of chromosomal DNA fragmentation was done using the Apoptotic DNA Ladder Kit (Roche). Briefly, The infected cells were harvested with trypsin, resuspended in 200 µl of a lysis buffer (6 mol/l guanidine-HCl, 10 mmol/l urea, 10 mmol/l Tris-HCl, 20% Triton X-100 pH 4.4) for 10 mins at 25°C. Then the lysate (400 µl) was mixed with 100 µl of isopropanol, applied to a filter tube with

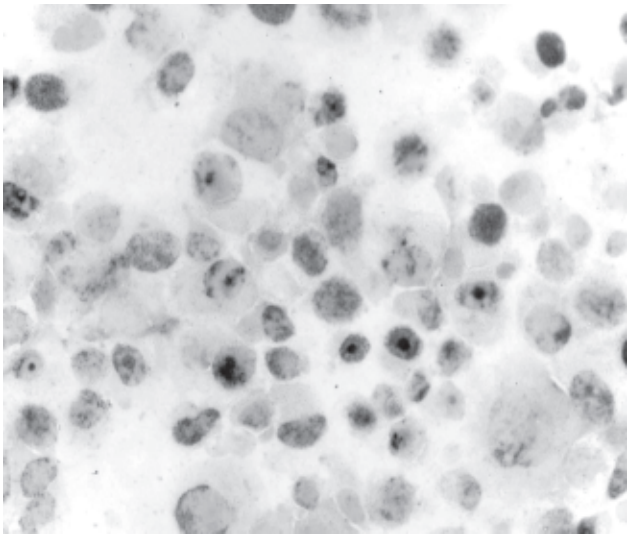

Fig. 3
***In situ* hybridization with TPA-treated HHV-8-infected BCBL-2 cells at day 4 p.i.**

Strong HHV-8 positive signal. Magnification 400x.

glass fiber (Roche) and filtered through the glass fiber by centrifugation at 8,000 rpm for 1 min. Residual impurities were removed with a washing buffer (20 mmol/l NaCl and 2 mmol/l Tris-HCl, pH 7.5). Nucleic acids binding specifically to the surface of the glass fiber in the presence of chaotropic salts were extracted with 400 µl an elution buffer (10 mmol/l Tris pH 8.5). The eluate was subjected to agarose (1.5%) gel electrophoresis. Chromosomal DNA fragmentation was demonstrated by a laddering pattern.

In situ TUNEL assay. Briefly, after washing in PBS, the prepared slides were covered with 5 µl of the TUNEL reaction mixture (Roche) and a coverslip and incubated in a humidified chamber for 1 hour at 37°C. The reaction was stopped by washing the slides in PBS for 15 mins at room temperature. The slides were then incubated with a fluorescein-labeled antibody conjugate (Roche) diluted 1:3 with a mixture of 100 mmol/l Tris-HCl, 150 mmol/l NaCl pH 7.5 and 1% blocking agent room temperature for 1 hr. After three washes in PBS, the slides were mounted using the Fluoromount G (Fisher Scientific, USA). The fluorescence was observed under a fluorescence microscope.

Table 1. Results of *in situ* hybridization and TUNEL assay in BCBL-2 cells treated or untreated with TPA

TPA	Day 2 p.i		Day 4 p.i.	
	ISH	TUNEL	ISH	TUNEL
Treated	11	6	34	21
Untreated	0	0	0	0

The results represent percentage of positive cells.

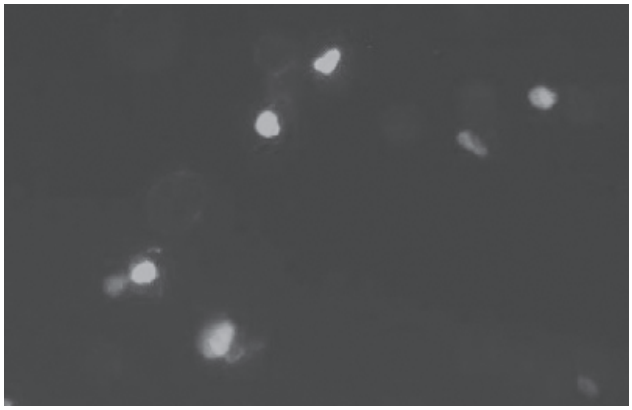

Fig. 4
TUNEL assay on TPA-treated BCBL-2 cells infected with HHV-8

The assay performed at day 4 p.i. Apoptotic DNA detected. Magnification 400x.

Results

In situ hybridization of the cells treated with TPA resulted in a strong dark brown signal in distinct areas in the nucleus and cytoplasm of HHV-8 replicating cells (Fig. 3). Also, no positive signal was seen in the cells not treated with TPA (Fig. 1).

To assess that the lytic infection with HHV-8 could be associated with the ability of the virus to interact with apoptotic signals, we characterized the HHV-8-infected BCBL-2 cells treated with TPA as a model of lytic infection of cells with HHV-8. By using 1.5% agarose gel electrophoresis, DNA laddering pattern was observed in the cells treated with TPA at day 4 post inoculation (p.i.) (Fig. 5). The cells incubated with standard medium did not induce DNA fragmentation.

Apoptosis in the cells treated with TPA was also evaluated using the TUNEL assay, able to detect directly the DNA fragmentation at the single-cell level. The results showed a marked increase in the number of apoptotic cells in the TPA-treated cells as compared with the TPA-untreated ones (Fig. 4). The level of apoptosis reached maximum at day 4 p.i. in the TPA-treated cells and correlated with the results of *in situ* hybridization (Figs. 2 and 4). A less pronounced effect was observed at day 2 p.i. in the TPA-treated cells (Table 1).

Discussion

Apoptosis or programmed cell death is a cell suicide programme characterized by chromatin condensation, DNA fragmentation, membrane asymmetry and membrane blebbing (Trump and Berezesky, 1998). A variety of viruses

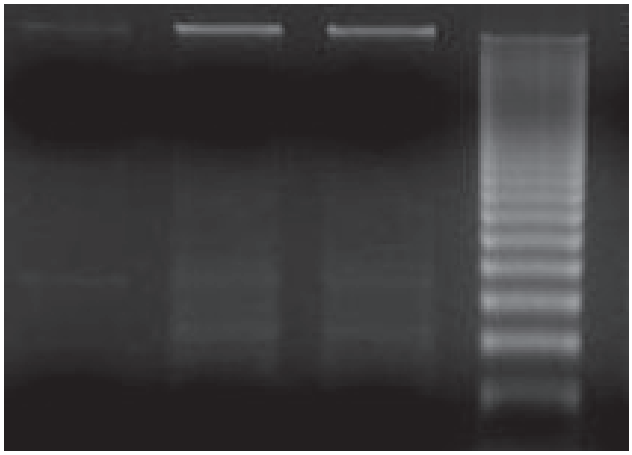

Fig. 5
Agarose gel electrophoresis of DNA extracted from TPA-treated BCBL-2 cells infected with HHV-8

The extraction performed at days 2 and 4 p.i. The DNA laddering pattern was detected with DNA extracted at day 4 p.i. TPA-untreated cells, extraction at day 2 p.i. (lane 1); TPA-untreated cells, extraction at day 4 p.i. (lane 2); TPA-treated cells, extraction at day 2 p.i. (lane 3); TPA-treated cells, extraction at day 4 p.i. (lane 4).

such as HIV, VZV, rotaviruses, and influenza virus have been found to induce apoptosis in infected cells (Ameisen and Capron, 1991; Sadzot-Delvaux *et al*., 1995; Superti *et al*., 1996). Likewise, HSV has been shown to induce apoptosis in cultured lymphocytes (Ito *et al*., 1997), human peripheral blood mononuclear cells (Tropea *et al*., 1995), and Hep-2 cells (Koyama and Adachi, 1997). However, in some kinds of viruses, control of apoptosis by viruses may be critical to produce adequate levels of progeny virus, to spread virus in tissues or to facilitate virus persistence (Shen and Shenk, 1995; Tropea *et al*., 1995). HHV-8 is a gammaherpesvirus that contains genes with anti-apoptotic potentialities (Katano *et al*., 2001; Meinl *et al*., 1998) and plays a role in the persistent infection and lymphoproliferation. Two HHV-8-related anti-apoptotic proteins have been identified: the vBcl-2 encoded by ORF 16 and the vFLIP encoded by ORF K13 (Wang *et al*., 2002). However, the results reported in the present study demonstrate that apoptosis was present in the lytic infection of HHV-8. These results suggested that the switch from latent to lytic infection may affect the expression of those anti-apoptosis-related genes. Our results do not allow us to fully understand the pathways involved in the HHV-8-induced apoptosis and further studies are necessary. Nevertheless, whatever the exact mechanism of apoptosis, our results extend information on the gammaherpesvirus-induced apoptosis and lead to an observation that even though gammaherpesviruses have anti-apoptotic ability they induce the apoptosis in permissive cells in the case of their productive lytic infection.

In conclusion, our results demonstrate a viral replication-dependent induction of apoptosis in cells productively infected with HHV-8 *in vitro*.

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