EXPRESSION OF CELLULAR PROTEINS BCL-X_L, XIAP AND BAX INVOLVED IN APOPTOSIS IN CELLS INFECTED WITH HERPES SIMPLEX VIRUS 1 AND EFFECT OF PAVINE ALKALOID (-)-THALIMONINE ON VIRUS-INDUCED SUPPRESSION OF APOPTOSIS

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Received March 29, 2003; accepted September 13, 2004

Summary. – The expression of three cellular proteins involved in the modulation of apoptosis, namely antiapoptotic Bcl-X_L and XIAP and proapoptotic Bax, was investigated in cells infected with Herpes simplex virus 1 (HSV-1). To assess whether the regulation of apoptosis in virus-infected cells depends on strain specificity the wild-type (*wt*) strain Victoria and the mutant R-100 resistant to acyclovir (ACV) were used. In addition, the expression of Bcl-X_L, XIAP and Bax was studied in cells infected with HSV-1 and treated with pavine alkaloid (-)-thalimonine. Our previous work has demonstrated that (-)-thalimonine irreversibly inhibits the replication of *wt* HSV-1 in cultured cells. Our data showed that (-)-thalimonine down-regulates the expression of viral proteins U_L17, VP11-12, VP22, VP24 and $\gamma_1$34.5, and affects negatively the posttranslational processing of glycoproteins D (gD) and G (gG). As both $\gamma_1$34.5 and glycoprotein D possess antiapoptotic activity, we investigated whether the antiviral effect of the alkaloid could also be due to its ability to suppress the antiapoptotic activity of the virus. Our results demonstrated that: (i) the virus induced overexpression of antiapoptotic proteins Bcl-X_L and XIAP; (ii) (-)-thalimonine reduced their overexpression, and (iii) this effect was stronger with the acyclovir resistant mutant R-100 than with the *wt* virus. Taken together, these data suggest that: (i) the virus abolishes apoptosis by means of virus-induced up-regulation of cell-specific prosurvival proteins Bcl-X_L and XIAP, and (ii) (-)-thalimonine, apart from affecting essential viral targets, inhibits the infectious progeny production via restoration of apoptosis during viral replication.

Key words: Herpes simplex virus 1; apoptosis; Bax; Bcl-X_L; XIAP; (-)-thalimonine; biological response modifiers

Introduction

The programmed cell death or apoptosis is a tightly regulated process of cell destruction triggered by death signals. Execution of the apoptotic program and the outcome of death receptor signaling are controlled by cellular anti- and proapoptotic proteins. Whereas antiapoptotic proteins such as Bcl-X_L and XIAP promote cell survival, proapoptotic proteins such as Bax promote cell death by apoptosis. Bcl-X_L belongs to the Bcl-2 family of proteins and acts at the level of mitochondria by preventing Bax and other proapoptotic factors from forming a pore in the outer mitochondrial membrane (OMM). XIAP belongs to the IAP (inhibitors of apoptosis) family of proteins and acts by blocking the activation of caspase 3 (Hengartner, 2000). Apoptosis is involved in the regulation of homeostasis, tissue development and elimination of aberrant cells created by DNA damage or infected with pathogenic agents as bacteria and viruses.

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Abbreviations: ACV = acyclovir; BAD = proapoptic Bcl-2 family protein; BRM = biological response modifiers; gD, gG = glycoproteins D, G; HSV-1 = Herpes simplex virus 1; OMM = outer mitochondrial membrane; LAT = latency-associated transcripts; p.i. = post infection; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TBST = Tween-BSA-saline-Tris; *wt* = wild-type

Most animal viruses trigger apoptosis. Host cell killing at the end of the replicative cycle offers several advantages to the virus. The apoptotic way of viral spread is used by viruses which have not evolved mechanisms for apoptosis evasion, e.g. Simian virus 40 (SV40), HIV-1, Epstein-Barr virus, Human herpes viruses 6 and 7 etc. (Fromm *et al*., 1994; Westendorp *et al*., 1995; Parker *et al*., 1996; Inoue *et al*., 1997). However, efficient cell-to-cell spread of infection depends on tightly regulated balance between induction and suppression of apoptosis until sufficient number of progeny particles is produced. Thus, inhibition of apoptosis is a common mechanism used by viruses to prevent the premature death of host cells and to ensure optimal infectious progeny production. The data by Inman *et al*. (2001) show that the latency-associated transcripts (LAT) of HSV-1 effectively block virus-induced apoptosis both *in vitro* and in the trigeminal ganglia of infected rabbits. Ahmed *et al*. (2002) have found that the exon 1 and the 2.0 kb intron of LAT have antiapoptotic functions and ensure a large pool of latently infected neurons, which contribute to efficient virus reactivation. In the context of lytic replication *wt* HSV-1 has also developed a range of strategies to avoid apoptosis by targeting specific stages of the downstream apoptotic pathway (Zhou *et al*., 2000). Several viral products with antiapoptotic activity have been identified (Roulston *et al*., 1999). Thus, the HSV-1 $\gamma_1$34.5 protein prevents the protein kinase R-stimulated inhibition of cellular protein synthesis and induction of apoptosis by redirecting phosphatase 1 to dephosphorylate the eukaryotic transcription factor eIF-2α (He *et al*., 1997). Viral VP16, thymidine kinase, gD and gG also block apoptosis by a mechanism not yet fully elucidated (Zhou and Roizman, 2000; Aubert *et al*., 2001; Medici *et al*., 2003). The data published by Zachos *et al*. (2001) have demonstrated that *wt* HSV-1 suppresses apoptosis by maintaining the Bcl-2 mRNA level and that viral proteins ICP4 and ICP27 are required for this function. Studies by Jerome *et al.* (1999) have shown that two immediate-early genes, U_S5 and U_S3, inhibit apoptosis at early times p. i. in Jurkat cells. Recent studies have demonstrated that U_S3 protein kinase blocks the caspase 3 activation induced by U_S1.5 and UL13 (Hagglund *et al.*, 2002). Moreover, U_S3 prevents apoptosis induced by the proapoptotic Bcl-2 family protein (BAD) (Munger and Roizman, 2001; Cartier *et al.*, 2003), by blocking BAD-induced caspase 3 activation.

The aim of the present study was to investigate whether additional strategies involving the Bcl-2 and IAP families of proteins are used by HSV-1 in order to escape the host self-death functions. For this purpose we examined the expression of two antiapoptotic cellular proteins, Bcl-X_L and XIAP, in cells infected with either *wt* or ACV-resistant HSV-1. We present the first data showing that HSV-1 activates host cell antiapoptotic machinery via overexpression of Bcl-X_L and XIAP.

As alkaloids possess a wide spectrum of activities they are considered biological response modifiers (BRM). Previously we have found that pavine alkaloid (-)-thalimonine inhibits irreversibly the replication of HSV-1 in cultured cells (Varadinova *et al*., 1996). The strong anti-HSV-1 effect of (-)-thalimonine is due to its ability to affect negatively several key viral proteins (Angelova A., unpublished data). Two of them, the tegument-associated protein kinase $\gamma_1$34.5 and gD exert antiapoptotic functions. That was why we hypothesized that (-)-thalimonine could inhibit HSV-1 replication by two mechanisms, that is, by affecting specific viral targets and by stimulating host cell defense mechanisms, such as apoptosis. Indeed, our results suggest that (-)-thalimonine, allowing apoptosis to proceed during HSV-1 infection, ensures cell death and suppresses the production of virus progeny.

Materials and Methods

Cells and viruses. Madine-Darby bovine kidney (MDBK) cells were cultured in DMEM medium (Gibco BRL) with 10% of newborn calf serum. The cells were infected with either *wt* HSV-1 strain Victoria or the ACV resistant mutant R-100. Infected cells were either treated with (-)-thalimonine or left untreated. The alkaloid was added to the culture medium in its maximal tolerated concentration (MTC) – 10 μmol/l. The following controls were used: (i) uninfected cells cultured in an alkaloid-free medium; (ii) uninfected cells cultured in medium with 10 μmol/l (-)-thalimonine. Samples were collected at 15 hrs post infection (p.i.) with the Victoria strain and at 20 hrs p.i. with R-100.

Western blot analysis. Samples were centrifuged at 1,500 rpm for 3 mins. The pellets were resuspended in a cold lysis buffer. Cell lysates were clarified by centrifugation at 10,000 rpm for 5 mins and the supernatants were mixed with a loading buffer. Proteins were resolved by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) with a 4–20% gradient gel (Bio-Rad) and blotted to a PVDF membrane in a semi-dry manner. The blots were blocked with a Tween-BSA-saline-Tris (TBST) overnight and incubated with primary antibodies against the protein of interest. After washing with TBST the blots were incubated with a secondary antibody and developed with the Enhanced Chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). Quantitation was done with the ImageQuant TL software (Amersham Pharmacia Biotech).

Primary antibodies. A polyclonal rabbit antibody against Bcl-X_L (Oncogene), a monoclonal mouse antibody against XIAP (Transduction Laboratories), a polyclonal rabbit antibody against Bax (Santa Cruz), a polyclonal goat antibody against β-actin (Santa Cruz), and a polyclonal rabbit antibody against viral VP16 (Clontech) were used.

Secondary antibodies. Horseradish peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse antibodies (Santa Cruz) were employed. Primary and secondary antibodies were equally diluted (1:2,000 in TBST).

Results and Discussion

Bcl-X_L and XIAP are key cellular survival factors, which block apoptosis by inhibiting insertion of proapoptotic proteins into OMM and by inhibiting procaspase 3 activation, respectively. Up to now there are no published data on overexpression of Bcl-X_L and/or XIAP in cells infected with HSV-1. That was why we decided to investigate whether these key cellular antiapoptotic proteins could play a role in successful viral replication. For this purpose we studied the expression of Bcl-X_L, XIAP and Bax in HSV-1-infected cultured cells. Moreover, in order to investigate whether the effect of HSV-1 on the expression of these proteins depends on strain specificity we compared the *wt* strain Victoria and ACV-resistant mutant R-100. As shown in Fig. 1, equal intensity of β-actin bands was indicative for equal protein amount in both virus infected and control samples. Viral protein VP16 was used as a marker of viral infection and was identified in infected cells only. Virus infection did not affect the expression of proapoptotic Bax protein, as it was demonstrated by equal intensity of corresponding bands. On the contrary, the virus induced overexpression of antiapoptotic proteins Bcl-X_L and XIAP. This was demonstrated by an increased intensity of the corresponding bands as compared to the uninfected control samples. Moreover, our data suggested that the antiapoptotic activity of HSV-1 did not depend on the viral strain/mutant used. This was proved by the observation that the overexpression of Bcl-X_L and XIAP took place in cells infected with either the *wt* strain Victoria or the ACV-resistant mutant R-100.

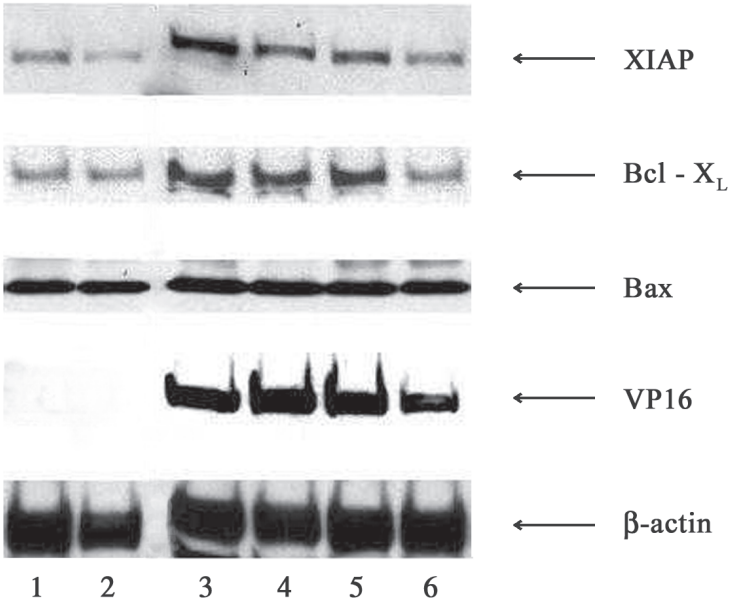

Fig. 1

Western blot of analysis of cellular and HSV-1 specific proteins

MDBK cells infected with *wt* Victoria strain were analyzed at 15 hrs p.i., while the cells infected with the R-100 mutant were analyzed at 20 hrs p.i. TM = (-)-thalimonine. Uninfected untreated control (lane 1), uninfected TM-treated control (lane 2), Victoria strain (lane 3), Victoria strain + TM (lane 4), R-100 mutant (lane 5), and R-100 mutant + TM (lane 6).

Interference with cellular apoptotic pathway is an important adaptive strategy of HSV-1, which allows viral progeny to be produced even under the pressure of proapoptotic stimuli (Aubert *et al*., 2001). HSV-1 can protect cells from apoptosis by several mechanisms including reduction of caspase 3 and caspase 8 activity (Jerome *et al*., 1999), dephosphorylation of eIF-2a (He *et al*., 1997) and stabilization of Bcl-2 mRNA by ICP4 and ICP27 (Zachos *et al*., 2001). In the present study we proved for the first time that yet another strategy could be used by HSV-1 to prevent host cells from undergoing apoptosis – a strain independent overexpresssion of Bcl-X_L and XIAP.

Next we tested the effect of (-)-thalimonine on the expression of Bcl-X_L and XIAP in HSV-1-infected cells. The results demonstrated that the alkaloid down-regulated the expression of Bcl-X_L and XIAP in virus-infected cells as compared to untreated virus-infected ones. Moreover, a strain-specific response was observed. In the cells infected with the *wt* Victoria strain (-)-thalimonine down-regulated the XIAP expression only, while in the cells infected with the R-100 mutant the expression of both XIAP and Bcl-X_L was suppressed It has to be noted that (-)-thalimonine by itself did not affect the expression of either Bcl-X_L, XIAP or Bax. This suggested that the alkaloid exerted its proapoptotic effect only in the context of HSV-1 infection.

In conclusion, our data demonstrated that (i) HSV-1 infection results in overexpression of antiapoptotic proteins Bcl-X_L and XIAP and (ii) (-)-thalimonine interferes with this virus-specific antiapoptotic strategy, thus supporting the host cell defense against HSV-1.

Acknowledgements. The authors thank Dr. M.Velcheva, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, for providing (-)-thalimonine. This work was supported by the National Science Fund project L-810.

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