The effects of *Herpes simplex virus* and *Vesicular stomatitis virus* infections on the expression patterns of p63 and Bax in epithelial cell lines

Ph.D. Thesis

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2010

INTRODUCTION

During the course of their replication, viruses perturb many strictly monitored cellular processes, and the profound structural and functional damage eventually kills the infected cells. The demise of virus-infected cells may play a pivotal role in the pathogenesis of diseases by destroying the structural and functional integrity of human tissues. Moreover, the cytopathogenicity of viruses defined as viral oncolytic therapy agents can be exploited in the treatment of malignant tumors. The tissue damage triggered by viruses involves various forms of cell death, including necrosis, apoptosis, anoikis, pyroptosis, necroptosis and autophagy.

The infections of the epithelial tissues by ***Herpes simplex* viruses** (HSVs) cause extensive cell death, the mechanism of which is complex, involving necrosis, apoptosis and autophagy/xenophagy. HSVs invade the human body through the cells of the skin, the mucous membranes and the ocular surface. After the initial virus replication, progeny virions pass through the sensory nerve endings, are transported to sensory ganglia by retrograde axonal flow, and establish lifelong latency within the neuronal cells of the ganglia, brain stem, olfactory bulbs and temporal lobe. Following the establishment of a latent HSV infection in the nervous system, the inhibition of apoptosis predominates and maintains cell survival. However, systemic and local stressors can interrupt the latency and induce viral reactivation, leading to recrudescent infections. HSV-1 and HSV-2 have been identified as causative agents of various mild and even life-threatening diseases, including herpes simplex labialis, herpetic gingivostomatitis, genital herpes and keratitis. The underlying mechanisms that contribute to the development of herpetic diseases are complex, involving tissue damage triggered by HSV multiplication and indirect, immune-mediated events. Although diseases evoked by HSVs are frequent and may lead to serious consequences, the molecular events implicated in the direct cytopathic effect of these viruses remain unclear.

Interesting studies have demonstrated that ***Vesicular stomatitis virus*** (VSV) possesses powerful inherent oncolytic activity that can be exploited in the therapy of malignant tumors. The replication of VSV in immortalized cells is highly efficient, while in normal cells with a functional interferon (IFN) system it is restricted. The finding that the IFN pathway is defective in the majority of transformed cell lines tested indicates that this signaling cascade is important in cell growth control, and it is dysregulated in cancer cells. The mechanism of VSV-mediated oncolysis is linked to apoptotic mechanisms. The infection disrupts the mitochondrial transmembrane potential, leading to the death of infected cells. It has also been established that VSV infection may induce a pro-apoptotic shift in the level of the Bcl-2 family member proteins. A number of cell lines derived from lung, renal, colorectal, conjunctival, ovarian, breast, endometrial, prostate, central nervous system, melanoma and hematologic tumors have been demonstrated to be permissive to VSV. However, the susceptibilities of other cell types have not yet been determined, and the underlying mechanisms involved in the oncolytic effects of this virus have not yet been fully defined.

The isoforms of **p63 transcription factor** regulate a wide array of cellular functions, including cell cycle progression, proliferation, adhesion, senescence and apoptosis. There are different

p63 protein isoforms, which can be expressed from two distinct promoters. Transcription from the first and second promoters gives rise to transactivating (TA) or amino terminally truncated (ΔN) variations of p63, respectively. Both TA and ΔN transcripts can undergo alternative splicing, leading to the formation of three C-terminal variants, denoted α, β and γ, which further increase the diversity of the p63 proteins. The TAp63 proteins have been reported to induce growth arrest and apoptosis. In contrast, the ΔNp63 isoforms may exert dominant-negative activities by antagonizing the target gene induction triggered by TAp63 isoforms and p53. Previous studies have demonstrated that p63 isoforms are involved in the control of the epithelial cell fate and in the regulation of the differentiation program of the skin and the eye. Thereby; these proteins play important roles in embryonic development, tumor progression and certain physiological processes and pathological conditions that affect the epithelial tissues.

The **Bcl-2–associated X protein**, or **Bax**, is a product of the Bcl-2 gene family, and has pro-apoptotic functions. The bax gene encodes multiple splice variants. It is well documented that Bax-α is a central component of apoptosis induction. Death signals trigger conformational change in the structure of Bax-α molecule, thereby lead to its activation. Activated Bax-α translocates to the mitochondrion resulting in the release of cytochrome *c* and caspase-9, which in turn leads to the inevitable execution of the apoptotic process. The Bax-β protein is expressed constitutively in several human cell types, and its level is controlled by proteasomal degradation. Similarly to Bax-α, Bax-β has the capability to trigger apoptosis via the mitochondrial pathway. Moreover, Bax-β facilitates Bax-α activation. Other interesting studies have revealed that proteolytic processing of Bax-α may further increase the diversity of the Bax isoforms. Cleavage of the Bax-α protein at a late stage of apoptosis by cellular enzymes has been shown to result in transition from the p21 Bax to the p18 Bax form. The accumulation of the p18 Bax variant is an important event in the amplification and acceleration of the apoptotic process.

The epithelia of the skin and eye may be exposed to harmful environmental stimuli, and may also function as entry sites for a wide array of human pathogenic microorganisms. By disturbing the delicate balance between the pro-survival ΔN and the pro-apoptotic TA isoforms, stress signals that alter the expression of p63 may cause profound alterations in the viability of the keratinocytes and ocular cells. However, the effects of microorganisms on the expression patterns of p63 and Bax have not yet been elucidated.

AIMS

I. A. Investigation of the p63, p73 and Bax expression patterns in HSV-infected primary keratinocytes and HaCaT cells

In an effort to gain more insight into the pathogenic mechanisms of skin infections caused by HSV-1 and HSV-2, we set out to investigate the effects of these viruses on the levels of p63, p73 and Bax expression. Our aims were as follows:

a) To investigate the susceptibilities of the HaCaT keratinocyte cell line and primary keratinocytes to HSV-1 and HSV-2.
b) To investigate the role of apoptosis in the cell demise triggered by HSV-1 and HSV-2.
c) To analyze the expression levels of p63, p73 and Bax in HSV-1- or HSV-2-infected HaCaT keratinocytes.

I. B. Investigation of the p63 and Bax expression patterns in HSV-1-infected SIRC corneal cell line

In an effort to gain more insight into the pathogenic mechanism of herpetic ocular surface disease, we set out to investigate the effects of HSV-1 on the levels of p63 and Bax expression. Our aims were as follows:

a) To investigate the susceptibility of the Staatens Seruminstitute Rabbit Cornea cell line (SIRC) to HSV-1.
b) To investigate the role of apoptosis in the cell demise triggered by HSV-1.
c) To analyze the expression levels of p63 and Bax in HSV-1-infected SIRC cells.

II. Investigation of the p63 and Bax expression patterns in VSV-infected HaCaT keratinocyte cell line

In an effort to evaluate the potential oncolytic activity of VSV in epithelial-derived skin cancers, we set out to investigate the cytopathogenicity of this virus in the immortalized HaCaT keratinocyte cell line. Our aims were as follows:

a) To investigate the susceptibility of the HaCaT cell line to VSV.
b) To investigate the role of apoptosis in the cell demise triggered by VSV.
c) To analyze the expression levels of p63 and Bax in VSV-infected HaCaT cells.

MATERIALS AND METHODS

HaCaT cells: The cell line was kindly provided by Dr. Norbert E. Fusenig (Heidelberg, Germany). The line is clonal in origin and has a transformed phenotype *in vitro* but is not tumorigenic, and is noninvasive *in vivo*, however it expresses mutated p53 ($p53^{mt}$). The cells were grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco/BRL, Grand Island, NY, USA).

Primary keratinocytes: The normal human primary keratinocytes, kindly provided by Prof. Dr. Lajos Kemény (Department of Dermatology and Allergology, University of Szeged, Hungary), were cultured at 37 °C in a 5% CO_2 atmosphere in keratinocyte growth medium (Gibco/BRL).

SIRC cell line: The SIRC cell line was obtained from the European Collection of Cell Cultures (Health Protection Agency Culture Collections, Porton Down, UK). Cells were grown in Dulbecco's modified Eagle's minimal essential medium (Sigma) supplemented with 10% fetal bovine serum (Gibco/BRL) at 37 °C in a 5% CO_2 atmosphere.

***Herpes simplex* viruses:** The KOS strain of HSV-1 and the wild-type HSV-2 were propagated at a multiplicity of infection (MOI) of 0.001 plaque forming unit (PFU) per cell in Vero cell cultures for 3 days at 37 °C. The culture fluids of HSV-1- or HSV-2-infected Vero cells were harvested, stored at -70 °C, and used as the infecting stock of the virus.

***Vesicular stomatitis virus*:** The Indiana strain of VSV was propagated at an MOI of 0.001 PFU per cell in L929 cell cultures for 3 days at 37 °C. The culture fluid of VSV-infected L929 cells was harvested, stored at -70 °C, and used as the infecting stock of the virus.

Indirect immunofluorescence assay: Cytospin cell preparations were fixed in methanol-acetone (1:1) for 15 minutes (min) at -20 °C. Slides were incubated with a 1:500 dilution of VSV G protein-specific monoclonal antibody (MAb) (Sigma) or 1:200 dilution of HSV glycoprotein D (gD)-specific MAb (Santa Cruz Biotechnology Inc., Cambridge, MA, USA) for 1 hour (h) at 37 °C. After washing with phosphate-buffered saline (PBS), the samples were reacted with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (1:160) (Sigma) and incubated for 1 h at 37 °C. After washing with PBS, the slides were visualized by confocal microscopy. The ratio of positive to negative cells was determined after counting 1000 cells in random fields.

Quantification of virus replication by plaque titration: Virus plaque assays were performed on confluent monolayers of Vero cells inoculated with HSV or VSV for 1 h at 37 °C and overlaid with 0.5% agarose (FMC, Rockland, ME, USA) in phenol red-free Eagle's minimum essential medium supplemented with 7.5% fetal bovine serum and 2 mM L-glutamine. After 2 days of culturing at 37 °C, a second agarose overlay containing 0.005% neutral red was added. Plaque titers were determined at 3 days after infection.

Quantification of cell viability by MTT assay: The viability of virus-infected cells was measured with the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] assay Tox-1 kit (Sigma). The cells were seeded in 96-well plates at a density of $1x10^4$/well, and were infected with HSV or VSV at different MOIs. At 24 or 48 hours postinfection (hpi) at 37 ^oC, 10 µl MTT reagent (5 mg/ml) was added to each well. After 2 h incubation, MTT solvent containing 0.1 M HCl and isopropanol was added for 15 h. Absorbance was measured at 545 and 630 nm. The ratio of living cells was calculated via the following formula: percentage viability = [(absorbance of infected cells – blank) / (absorbance of corresponding mock-infected control cells – blank)] x 100.

Inhibition of viral DNA replication: To inhibit the DNA replication of HSV-1 and HSV-2, 9-[(2-Hydroxyethoxy)methyl]guanine [(ACG) (Sigma)] was used at various concentrations when indicated.

Quantification of apoptosis by enzyme-linked immunosorbent assay (ELISA): The cells were washed in PBS and the cell pellet was processed in a cell death detection ELISA kit (Roche Diagnostics GmbH, Penzberg, Germany) based on the measurement of histones complexed with mono- and oligonucleosome fragments formed during cell death. The cells were incubated in lysis buffer for 30 min and centrifuged at 12,000 rpm for 10 min. The supernatants were transferred into a

streptavidin-coated microplate and incubated with biotin-conjugated anti-histone and peroxidase-conjugated anti-DNA monoclonal antibodies for 2 h. After washing, substrate solution 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was added to each well for 15 min. Absorbance was measured at 405 and 490 nm. The specific enrichment of mono- and oligonucleosomes was calculated as the enrichment factor (EF) = absorbance of infected cells/absorbance of corresponding non-infected control cells.

Quantification of apoptosis by annexin V staining: The cells were stained with FITC-labeled annexin V and propidium iodide (PI) (Bender MedSystems Inc., Burlingame, CA, USA) according to the manufacturer's instructions. The fluorescence intensities of annexin-FITC and PI were determined with a FACStar Plus flow cytometer (BD Biosciences, San Diego, CA, USA) by using the WinMDI software. The percentages of apoptotic cells were calculated by sorting the cells that were positive only for annexin V (early apoptotic stage) or positive for both annexin V and PI (late apoptotic and necrotic stages).

Western blot assays: Cells (1×10^7) were homogenized in ice-cold lysis buffer containing 150 mM NaCl, 10 mM Tris·HCl, pH 7.6, 5 mM EDTA, 1% (v/v) Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate and protease inhibitor cocktail (Sigma), and the mixture was then centrifuged at 10,000 g for 10 min to remove cell debris. Protein concentrations of cell lysates were determined by using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Supernatants were mixed with Laemmli's sample buffer and boiled for 3 min. Aliquots of the supernatants, containing 50 μg of total protein to detect HSV gD, VSV G protein, p53, p63, p73, Bax and β-actin, were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto nitrocellulose filters (Amersham, Buckinghamshire, UK). Preblocked blots were reacted with specific antibodies to VSV G protein (Sigma), HSV gD (Sigma), p63 (clone 4A4) detecting all of the various p63 isoforms (Santa Cruz), p40 detecting the ΔNp63 isoforms (Merck KGaA, Darmstadt, Germany), p53 (Serotec Inc. Raleigh, NC), p73 (clone H-79) detecting all of the various p73 isoforms (Santa Cruz), β-actin (Sigma), and Bax (PharMingen, San Diego, CA) for 4 h in PBS containing 0.05% (v/v) Tween 20, 1% (w/v) dried non-fat milk (Difco Laboratories, Detroit, MI) and 1% (w/v) BSA (fraction V; Sigma). Blots were then incubated for 2 h with species-specific secondary antibodies coupled to peroxidase [peroxidase-conjugated anti-mouse antibody (DakoCytomation, Carpinteria, CA, USA), or peroxidase-conjugated anti-rabbit antibody (DakoCytomation)]. Filters were washed five times in PBS–Tween for 5 min after each step and were developed by using a chemiluminescence detection system (Amersham). The autoradiographs were scanned with a GS-800 densitometer (Bio-Rad), and the relative band intensities were quantified by use of the ImageQuant software (Amersham).

Gene silencing by small interfering RNA (siRNA): Chemically synthetized siRNA targeting TAp63 (Silencer siRNA 4798) and non-silencing control siRNA (Silencer negative control #2 siRNA 4613) were obtained from Ambion Inc. (Austin, TX, USA). Transient transfections were performed by using the siPORT amine reagent (Ambion) according to the manufacturer's protocol, with a final

siRNA concentration of 50 nM. The transfected HaCaT cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. The effect of silencing was analyzed at the protein level by Western blot assay.

Statistical analysis: All values are expressed as means ± standard deviation (SD). Student's unpaired t test was used for comparisons and P values < 0.05 were considered statistically significant. The one-way ANOVA test with the Bonferroni post-test was used for pairwise multiple comparisons, and P values < 0.05 were considered statistically significant.

RESULTS

I. A. The effects of HSV infection on the expression patterns of p63, p73 and Bax in HaCaT cells and primary keratinocytes

The HaCaT cells were infected with the KOS strain of HSV-1 at various multiplicities and maintained for different periods of time. The production of progeny virus was determined by plaque titration of the culture supernatants taken from HaCaT cells at 6, 12, 24 or 48 hpi. Depending on the infectious dose, the level of HSV-1 production varied between $<5\times10^2$ and 1×10^4 PFU/ml at 6 hpi. The virus titers thereafter increased, and ranged from 6×10^6 and 3.3×10^8 PFU/ml at 48 h after inoculation. Accordingly, the maximum yield at 0.001, 0.01, 0.1, 1 and 10 MOI corresponded to 30, 550, 600, 1650 and 1500 PFU/cell, respectively. These data demonstrate that HSV-1 replicates efficiently in the HaCaT keratinocyte cell line.

The cytopathogenicity of HSV-1 and HSV-2 was determined by the MTT assay. HSV-1-infected cells displayed decreased viability at 24 hpi. HSV-2-infected cells likewise exhibited decreased viability at 24 hpi. To examine the ability of HSV-1 and HSV-2 to induce apoptosis in HaCaT cells, the extent of apoptosis was measured by annexin V binding assay at 24 hpi. The proportions of annexin V-single-positive (early apoptotic) and double-positive (early apoptotic and necrotic) cells in cultures infected with HSV-1 at an MOI of 10 were 21 and 16%, respectively. In contrast, the proportions of annexin V-single-positive and double-positive cells in cultures infected with HSV-2 at an MOI of 10 were 12 and 25%, respectively. These results indicate that HSV-1 and HSV-2 trigger different types of cell death in HaCaT cultures.

To determine whether HSV-1 and HSV-2 can alter the expressions of Bax, p63 and p73 in the HaCat cell line, the steady-state levels of these proteins were determined by Western blot analysis. Experiments to investigate the kinetics of HSV-1 replication revealed the presence of gD in cultures infected with HSV-1 as early as 6 hpi. The level of gD thereafter increased, and its expression was highly upregulated in every culture infected with HSV-1 at 48 h after inoculation. Mock-infected HaCaT cells displayed the endogenous expression of Bax-α, which remained constant during the 48 h of culturing. Interestingly, the analysis revealed the presence of a Bax isoform corresponding to Bax-β in HSV-1-infected cultures as early as 6 hpi. The level of Bax-β thereafter increased, and its expression was highly upregulated in every culture infected with HSV-1 by 48 hpi. The expression pattern of p63 was determined by using an antibody preparation, which recognizes all of the various

p63 isoforms. The analysis demonstrated that the predominant isoform in the HaCaT cell line is a p63 protein migrating near 68 kDa. HSV-1 triggered an impressive reduction in the level of this 68 kDa p63 isoform. The HSV-1-infected cells exhibited decreased levels of this protein as early as 12 hpi. The expression of the 68 kDa p63 isoform was downregulated in cells infected at MOIs of 0.1, 1 and 10 at 48 h after inoculation. Interestingly, a p63 isoform migrating between 51 and 62 kDa was also detected in HSV-1-infected cells as early as 6 hpi. The level of the 51-62 kDa p63 isoform thereafter increased, and the expression of this protein was highly upregulated in every culture infected with HSV-1 by 48 hpi. To identify the different p63 isoforms, the steady-state levels of these proteins were also determined by Western blot analysis, using a polyclonal antiserum which reacts only with the ΔN forms. The ΔNp63-specific antibody preparation detected the 68 kDa p63 isoform, but failed to recognize the 51-62 kDa p63 isoform in HSV-1-infected cultures. This result indicates that the 68 kDa isoform belongs in the ΔN subclass and might be identical with ΔNp63α, while the 51-62 kDa isoform is a member of the TA subclass and corresponds to TAp63γ. Furthermore, these experiments confirmed that the level of ΔNp63α was decreased, while the expression of TAp63γ was highly increased following HSV-1 infection. The expression pattern of p73 was determined by using an antibody preparation which recognizes all of the various p73 isoforms. Mock-infected HaCaT cells expressed two p73 isoforms, migrating near 50 and 44.5 kDa, the levels of which remained constant during the 48 h of culturing. The HSV-1-infected cells exhibited a decreased level of the 50 kDa p73 isoform at 24 h after virus inoculation. In every infected culture, the expression of this protein was likewise downregulated by 48 hpi. The HSV-1-infected cells displayed an increased level of the 44.5 kDa p73 isoform at 24 hpi. Similarly, the level of this protein was upregulated by 48 hpi in cultures infected with HSV-1 at MOIs of 0.01, 0.1, 1 and 10. Experiments to investigate the replication of HSV-2 revealed the presence of gD in cultures infected with HSV-2 at 24 hpi. The levels of the 50 and 44.5 kDa p73 isoforms and ΔNp63α were decreased; Bax-α and TAp63γ remained unaffected, while the expression of Bax-β was slightly increased at 24 h after inoculation in HSV-2-infected HaCaT cells. These findings suggest that HSV-1 and HSV-2 alter the levels p63, p73 and Bax in a type-specific manner in HaCaT epithelial cell cultures.

To determine whether HSV-1 can dysregulate the expressions of Bax and p63 in primary keratinocytes, the steady-state levels of these proteins were determined by Western blot analysis. Primary keratinocytes were infected at an MOI of 1, and the kinetics of HSV-1 replication was investigated. The experiments revealed the presence of gD in cultures infected with HSV-1 as early as 6 hpi, and its level was highly increased at 12 and 24 h after inoculation. The mock-infected cells displayed the endogenous expression of the wild-type p53 protein ($p53^{wt}$), ΔNp63α and Bax-α. The level of ΔNp63α was decreased, $p53^{wt}$ and Bax-α remained unaffected, while the expressions of the Bax-β and TAp63γ were highly increased by 12 hpi in HSV-1-infected primary keratinocytes. These data indicate that HSV-1 alters the levels of Bax and p63 in primary keratinocytes.

To investigate the basis of the HSV-1-induced accumulation of TAp63γ, HaCaT cells were infected in the presence or absence of the viral DNA replication inhibitor ACG. The cells were analyzed for the presence of p63 and Bax by Western blot analysis. The lack of the late protein gD in samples treated with 100, 50 or 10 μg/ml ACG indicated that the drug treatment inhibited viral DNA replication efficiently. In HSV-1-infected cells treated with 100, 50 or 10 μg/ml ACG, the levels of Bax-β and TAp63γ were decreased; Bax-α remained constant, while the expression of ΔNp63α was increased, as compared with HSV-1-infected cultures maintained in the absence of ACG. These findings demonstrate that the HSV-1-mediated TAp63γ expression requires viral DNA replication.

To evaluate the biological effects of the accumulation of TAp63γ in HSV-1-infected cells, siRNA technology was used. The delivery of TAp63-specific siRNA resulted in an 85% reduction in HSV-1-induced TAp63γ expression as compared with cultures treated with a negative control siRNA preparation. In the presence of the TAp63-specific siRNA, the viability of HSV-1-infected cells was increased by about 15% at 24 hpi. These results confirm that the 51-62 kDa p63 isoform corresponds to the TA subclass, and suggest that TAp63γ may play some role in the cytopathogenicity of HSV-1.

I. B. The effects of HSV infection on the expression patterns of p63 and Bax in SIRC cells

The SIRC cell line was infected with the KOS strain of HSV-1 at various multiplicities and maintained for different periods of time. Indirect immunofluorescence assays to evaluate HSV-1 replication revealed positive staining for gD at 48 hpi in ≥99% of SIRC cells infected at an MOI of 1.

MTT assays to evaluate the cytopathogenicity of HSV-1 revealed decreased viability at 48 hpi. ELISA to evaluate the extent of apoptosis revealed increased apoptotic rates in HSV-1-infected SIRC cells at 48 hpi. These results reveal that HSV-1 elicits a strong cytopathic effect in the SIRC cell line, and apoptosis plays an important role in the demise of the infected cells.

To determine whether HSV-1 can alter the expressions of Bax and p63, the steady-state levels of these proteins were determined by Western blot analysis. First, the kinetics of HSV-1 gD expression was investigated. The presence of gD was observed in the SIRC cell cultures infected with HSV-1 at an MOI of 10 at 12 hpi. The gD protein accumulated in the cultures infected with HSV-1 at MOIs of 0.1, 1 and 10 at 24 hpi. High-level expression of the gD protein was also revealed in every culture infected with HSV-1 by 48 hpi. Together, these data demonstrate the expression of HSV-1 gD protein that is consistent with efficient viral replication. The analysis revealed the presence of a Bax isoform corresponding to Bax-β in HSV-1-infected SIRC cultures at 12 hpi. At the 24-h time point, the expression of the Bax-β protein in the HSV-1-infected SIRC cultures was upregulated. At the 48-h time point, the HSV-1-infected SIRC cultures displayed elevated levels of Bax-β. The expression pattern of p63 was determined by using an antibody preparation which recognizes all of the various p63 isoforms. The analysis revealed the constitutive expression of a p63 protein migrating near 68 kDa in the mock-infected SIRC cells. Previously published data demonstrated that the 68 kDa protein possibly corresponds to ΔNp63α. At 12 hpi, the expression of ΔNp63α in the HSV-1-infected SIRC cultures was downregulated. At the 24-h time point, HSV-1 triggered an impressive reduction in the

level of ΔNp63α in the SIRC cells. At the 48-h time point, the HSV-1-infected SIRC cultures exhibited decreased levels of ΔNp63α. The experiments also revealed the presence of a 51-62 kDa protein in HSV-1-infected SIRC cultures. Previously published data demonstrated that the 51-62 kDa protein possibly corresponds to TAp63γ. At 12 hpi, HSV-1-infected SIRC cells exhibited increased levels of TAp63γ. At the 24-h time point, the expression of TAp63γ in the HSV-1-infected SIRC cultures was highly upregulated. At 48 hpi, the HSV-1-infected SIRC cultures displayed elevated levels of TAp63γ. To identify the p63 isoforms, the steady-state levels of these proteins were determined by Western blot analysis, using a polyclonal antiserum which reacts only with the ΔN forms. The ΔNp63-specific antibody preparation detected the 68 kDa p63 isoform in the mock-infected SIRC cells, but failed to recognize the 51-62 kDa p63 isoform in the cultures infected with HSV-1 at an MOI of 10 for 24 hpi. These results clearly reveal that the 68 kDa p63 protein detected in the mock-infected SIRC cells is ΔNp63α, while the 51-62 kDa p63 isoform detected in HSV-1-infected cultures is TAp63γ. Together, these results indicate that HSV-1 modulates the expression patterns of Bax and p63. The level of ΔNp63α was decreased, while the expressions of Bax-β and TAp63γ were highly increased in the HSV-1-infected SIRC cells.

To investigate the basis of the HSV-1-induced increase of the TAp63γ level, SIRC cells were infected in the presence or absence of the viral DNA replication inhibitor ACG. The cells were analyzed for the presence of HSV gD, ΔNp63α, TAp63γ and Bax-β. The low level of the late protein gD expression in SIRC samples treated with 50 or 10 μg/ml ACG indicated that the drug treatment efficiently inhibited viral DNA replication. The Bax-β protein levels in the HSV-1-infected SIRC cells treated with 50, 10 and 1 μg/ml ACG were greatly decreased. The TAp63γ protein levels in the HSV-1-infected SIRC cells treated with 50 and 10 μg/ml ACG were greatly decreased. The expression of the TAp63γ isoform in the HSV-1-infected cultures treated with 1 μg/ml ACG was downregulated.

II. The effects of VSV infection on the expression patterns of p63 and Bax in HaCaT cells

To determine whether VSV multiplicates in the HaCaT cell line, different methods were used. Indirect immunofluorescence assays revealed positive staining for VSV G protein at 48 h after virus inoculation in ≥98% of the cells infected at an MOI of 1. Western blot analyses revealed the presence of the G protein in cultures infected at MOIs of 0.1 and 1 at 24 h after VSV inoculation. By 48 h, the G protein had accumulated in every culture infected with VSV. The progeny virus production was determined by plaque titration of the culture supernatants taken from HaCaT cells at 12, 24, 48 and 72 hpi. Depending on the infectious dose, the level of VSV production varied between 3.0×10^3 and 1.9×10^6 PFU/ml at 12 hpi. The virus titers thereafter increased, and ranged from 4.5×10^5 to 4.6×10^7 PFU/ml at 24 h after virus inoculation. The level of virus production varied between 2.6×10^7 and 6.4×10^7 PFU/ml at 48 hpi. The VSV production of cells infected with various MOIs rose to titers of about 2×10^8 PFU/ml after 72 h of culturing. Accordingly, the maximum yield at 0.001, 0.01, 0.1 and

1 MOI corresponded to 1300, 1400, 1200, and 1000 PFU/cell, respectively. Together, these data clearly demonstrate that VSV replicates efficiently in the HaCaT keratinocyte cell line.

ELISA to evaluate the extent of apoptosis revealed increased apoptotic rates in HSV-1-infected SIRC cells at 24 and 48 hpi. These data indicate that VSV elicits apoptosis in HaCaT cells.

To determine whether VSV infection can alter the expressions of proteins involved in apoptosis, the steady-state levels of ΔNp63α, $p53^{mt}$ and Bax were measured by Western blot assay. The analysis revealed the endogenous expression of ΔNp63α, $p53^{mt}$ and p21 Bax in mock-infected HaCaT cells. Previously published data demonstrated that the 21 kDa Bax isoform is Bax-α. The endogenous expression of ΔNp63α in mock-infected cells remained constant throughout the 48 h of culturing. The VSV-infected cells exhibited decreased levels of ΔNp63α at 24 h after virus inoculation. The expression of ΔNp63α protein in the VSV-infected cultures at the 48-h time point was downregulated. The endogenous expression of $p53^{mt}$ in mock-infected cells similarly remained constant throughout 48 h of culturing. No quantitative differences between the VSV-infected and control cultures were observed in the level of expression of $p53^{mt}$ protein at the 24-h time point. The expression of $p53^{mt}$ in VSV-infected cultures at 48 h after VSV inoculation was downregulated. The endogenous expression of Bax-α in mock-infected cells likewise remained constant in the course of the 48 h of culturing. No quantitative differences between the VSV-infected and control cultures were displayed in the level of expression of Bax-α at the 24-h time point. The expression of Bax-α in VSV-infected cells at 48 h after inoculation was upregulated. Furthermore, VSV-infected cells exhibited increased levels of p18 Bax at 48 h after inoculation. Together, these data indicate that the expressions of ΔNp63α, $p53^{mt}$, Bax-α and p18 Bax are differentially modulated by VSV.

DISCUSSION

The effects of HSV infection on the expression patterns of p63, p73 and Bax in primary keratinocytes, HaCaT and SIRC cells

Our data revealed that HSV-1 replicated to high titers and triggered a strong cytopathic effect in the HaCaT and SIRC cell lines. Furthermore, apoptosis played an important role in the demise of the infected keratinocytes and cornea epithelial cells.

Consistently with previous findings, we found that several p63 isoforms can be detected both in the HaCaT cell line and in primary keratinocytes, and that ΔNp63α is the predominant isoform, migrating as a doublet due to its post-translational modification by phosphorylation. For the first time, our experiments have also revealed the constitutive expression of ΔNp63α in the rabbit corneal SIRC cell line. Interestingly, HSV-1 triggered an impressive reduction in the level of the ΔNp63α doublet and a dramatic increase in the expression of TAp63γ. The kinetics of HSV-1 replication and the alteration in the stoichiometric ratio of the p63 isoforms correlated strictly. Our experiments revealed that the knockdown of TAp63 expression increases the viability of infected HaCaT cells, suggesting

that TAp63γ may play some role in the complex mechanisms involved in the cytopathogenicity of HSV-1. Interesting recent studies have revealed that HSV genome synthesis activates the cellular DNA damage response (DDR). Other observations indicate that genotoxic stress induces the accelerated degradation of ΔNp63 and the accumulation of TAp63 isoforms; in turn, these function as downstream mediators of the DDR, to provide time for repair or to kill cells bearing irreparable DNA damage and unstable genome by inducing apoptotic demise. Our experiments have demonstrated that the viral DNA replication inhibitor ACG completely abolished the HSV-1-mediated induction of TAp63γ in both HaCaT and SIRC cells indicating that replication of viral DNA is necessary for the accumulation of TAp63γ. This observation strongly supports the view that the dysregulation of p63 expression depends on the cellular DDR, but does not exclude the role of HSV-1-encoded proteins.

In line with these data, we next investigated the expression of the p53 family member p73. Similar to p63, the p73 gene has two transcription start sites, producing two p73 subclasses: the TA and ΔN isoforms. In addition to these amino-terminal differences, alternative splicing generates seven carboxy-terminal variants, denoted α, β, γ, δ, ε, ζ and η. The TAp73 isoforms transactivate a variety of p53 and p73 target genes and induce apoptosis, while the ΔNp73 isoforms possess little transcriptional activity, display dominant negative behavior and inhibit apoptosis. Our studies have shown that the level of a 50 kDa p73 isoform was decreased, while the expression of a 44.5 kDa p73 protein was increased in HaCaT cells following HSV-1 infection. On the basis of previously published data we suggest that the p73 isoforms migrating near 50 and 44.5 kDa may correspond to ΔNp73β and TAp73δ, respectively. However, further studies are required for the clear-cut identification of the p73 isoforms detected in HaCaT keratinocytes. Together, these data demonstrate that HSV-1 dysregulates the expression pattern of p73 in the HaCaT cell line.

In order to gain more insight into the stress response triggered by HSV-1, we also studied the expression of Bax, which is known to be upregulated by TAp63α and TAp63γ. Our experiments revealed no alterations in the expression of Bax-α. Interestingly, we observed a dramatic rise in the level of Bax-β in HSV-1-infected HaCaT and SIRC cultures. Following the demonstration of an altered Bax expression pattern in the HaCaT and SIRC cells, we postulate an important role for Bax-β in the apoptotic responsiveness of keratinocytes and corneal epithelial cells infected with HSV-1. Other interesting recent data have proved that HSVs encode ubiquitinating and deubiquitinating enzymes, which can modify the ubiquitination status of both viral and host cell proteins. In view of these observations, it is reasonable to infer that the Bax-β protein may be a novel target of HSV-1-mediated deubiquitinating events. However, the precise molecular mechanisms responsible for stabilization of the Bax-β protein in HSV-1-infected cells remain to be elucidated.

It has been clearly proved that HSV-1 and HSV-2 differ in their nucleotide sequences and rates of reactivation, and also in the cellular transcriptional responses and spectrum of diseases they evoke. Interesting previous studies have also revealed fundamental differences between HSV-1 and HSV-2 in their apoptosis-modulating effect. Accordingly, we examined the expression patterns of the

p63, p73 and Bax isoforms and determined the proportion of apoptotic HaCaT keratinocytes after infection with HSV-2. Although the proportions of dead cells were comparable in the HSV-1- and HSV-2-infected cultures, the early apoptotic population was larger in the cultures infected with HSV-1 than in those infected with HSV-2. These data raise the possibility that HSV-1-infected keratinocytes, displaying highly elevated TAp63γ levels, may be prone to commit apoptosis, while HSV-2-infected cells may rather be disposed to die by way of necrosis or autophagy. Similarly as in the case of HSV-1, HSV-2-infected HaCaT cultures exhibited impressive reductions in the expressions of ΔNp63α and the 50 kDa p73 isoform; unexpectedly, however, the level of TAp63γ remained unaffected and the expression of a 44.5 kDa p73 isoform was decreased in HSV-2-infected HaCaT keratinocytes. HSV-2 infection further resulted in a very slight increase in the expression of Bax-β, the magnitude of which proved to be much lower than that observed after HSV-1 infection.

Taken together, our results indicate that both HSV-1 and HSV-2 replicate efficiently and elicit powerful cytopathogenicity, and apoptosis plays an important role in the demise of the infected keratinocytes and corneal epithelial cells. For the first time, our data also demonstrate that HSV-1 and HSV-2 modulate the patterns of p63, p73 and Bax expression in a serotype-specific manner. The dysregulated pattern of p63 expression observed in HSV-infected HaCaT and SIRC cultures may comprise part of a mechanism by which these viruses perturb the functions of epithelial cells and lead to their demise. These data may bear on the pathogenic mechanisms of diseases caused by HSV-1 and HSV-2, as p63 isoforms play a pivotal role in the epithelial homeostasis.

The effects of VSV infection on the expression patterns of p63 and Bax in HaCaT cells

Our data revealed that VSV was able to establish an infection, affecting virtually all of the cells and yielding high titers of progeny virus. VSV infection elicited a strong cytopathic effect and apoptosis, leading to the demise of cultures within 72 h. For the first time, our data clearly demonstrated the susceptibility of immortalized keratinocytes to the deadly infection caused by VSV.

Interestingly, we observed an impressive reduction of the ΔNp63α level of VSV-infected cells. It is important that the kinetics of VSV replication, apoptosis and suppression of ΔNp63α expression correlated strictly. This suggests that the VSV-induced decrease in ΔNp63α levels is a key event in the apoptotic response of the infected keratinocytes.

Consistent with previous results, our experiments have shown that the HaCaT cell line accumulates high amounts of $p53^{mt}$. Our further experiments revealed that VSV infection decreases the level of $p53^{mt}$. Since apoptosis was detected in infected cultures displaying unaffected levels of $p53^{mt}$, the downregulation of $p53^{mt}$ expression does not seem to be involved in the induction of apoptosis; it may rather operate in the executional phase and contribute to the inevitable death of heavily infected cells.

In line with these data, we investigated the expression of Bax isoforms. Our experiments revealed the endogenous expression of Bax-α in the HaCaT cell line. Interestingly, we observed high increases in the levels of Bax-α and p18 Bax in VSV-infected HaCaT cultures. Cleavage of Bax-α

was shown to yield a p18 Bax product, which behaves like a sensitizer type of BH3-only proteins. The p18 truncated form of Bax-α is more potent in disrupting mitochondrial integrity and inducing apoptosis. Thus, the dramatic rises detected in the levels of Bax-α and p18 Bax in HaCaT cells following VSV infection are indicative of a pro-apoptotic shift and may be of importance in the amplification of the apoptotic process, and contribute to the powerful cytopathogenicity of this virus.

Taken together, our results demonstrate for the first time that VSV replicates efficiently and triggers apoptosis in the immortalized HaCaT keratinocyte cell line. The VSV-mediated alterations in the expressions of ΔNp63α and Bax may be implicated in the apoptotic demise of infected cells and may also sensitize to other apoptotic stimuli. Our findings extend the known spectrum of cell types susceptible for the powerful oncolytic activity of VSV to immortalized keratinocytes. These observations may stimulate further studies aimed at the development of VSV-based virotherapy into an effective modality for the treatment of epithelial-derived tumors of the skin.

SUMMARY

The major new findings of our experiments are as follows:

1. Both HSV-1 and HSV-2 elicit powerful cytopathogenicity in primary keratinocytes, HaCaT and SIRC cells. However, there are fundamental differences between the two HSV serotypes in their apoptosis-modulating effect.
2. In primary keratinocytes, HaCaT or SIRC cells, HSV-1 and HSV-2 modulate the patterns of p63, p73 and Bax expression in a serotype-specific manner. By disturbing the delicate balance between the pro-survival and the pro-apoptotic p63, p73 and Bax isoforms, HSV-1 and HSV-2 may cause profound alterations in the tissue homeostasis of the skin and the ocular surface.
3. Viral DNA replication is necessary for the decrease of ΔNp63α, for the accumulation of TAp63γ and for the increase of Bax-β in both the HSV-1-infected HaCaT keratinocytes and SIRC cornea epithelial cells. This observation suggests that the HSV-1-mediated dysregulation of p63 and Bax expression operates in the cellular DNA damage response pathway triggered by replication stress during HSV-1 genome replication, and may comprise part of the cellular stress response.
4. VSV has a powerful oncolytic activity on immortalized keratinocytes that is linked to apoptotic mechanisms. This observation suggests that VSV could be developed into an effective modality for the treatment of epithelial-derived squamous tumors of the skin.
5. VSV modulates the expression patterns of p63 and Bax in immortalized keratinocytes. The VSV-mediated dysregulation of p63 and Bax expression is a key event in the apoptotic response of the infected keratinocytes and may also sensitize to other apoptotic stimuli.

ACKNOWLEDGEMENTS

This work has been carried out at the Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged.

I am deeply indebted to my supervisor, **Associate Professor Klára Megyeri**, who has helped me with good sense, unfailing efficiency and friendly encouragement. She has made the enterprise of research work a challenge, as well as an education for me. I am also very grateful to her for useful advice and for critical reading of the manuscript.

I greatly acknowledge **Professor Yvette Mándi**, Head of the Department of Medical Microbiology and Immunobiology for providing working facilities and for her support and advice.

My warmest thanks are due to **Professor Lajos Kemény** for the significant help.

I would like to acknowledge the support of **Professor Norbert E. Fusenig**.

I also thank **Gyöngyi Ábrahám** for her excellent technical assistance and advice.

I owe much to my colleagues, especially **Dr. Éva Gallyas, Dr. Andrea Facskó, Prof. Zsuzsanna Bata-Csörgő, Dr. György Seprényi, Katalin Pásztor, Dr. Béla Taródi, Dr. Imre Ocsovszki** and **Bernadett Kormos** for pleasant cooperation.

I thank **all my colleagues** at the Department of Medical Microbiology and Immunobiology for their support and for creating a pleasant work-environment.

I record my gratitude to **my family** for their love, support and understanding.

The financial support received from grants OTKA/T043144 by the Hungarian Scientific Research Fund and ETT/398/2003 by the Hungarian Ministry of Health, Social and Family Affairs is gratefully acknowledged.

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Total impact factor: 6.182

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Total impact factor: 2.206

Cumulative impact factor: 8.388