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Original article

Vesicular stomatitis virus infection triggers apoptosis associated with decreased ΔNp63α and increased Bax levels in the immortalized HaCaT keratinocyte cell line

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Abstract

In view of the powerful inherent oncolytic activity exhibited by the vesicular stomatitis virus (VSV) in several tumor types, we set out to investigate the susceptibility of the immortalized HaCaT keratinocyte cell line to VSV, and analyzed the role of apoptosis in the VSV-mediated induction of cell death. Indirect immunofluorescence assays, Western blot analyses and plaque titrations demonstrated that the HaCaT cell line was permissive to VSV replication. The results of ELISA for detection of the enrichment of nucleosomes in the cytoplasm of apoptotic cells revealed that VSV infection elicits the apoptotic death of HaCaT cells. Mock-infected HaCaT cells displayed the endogenous expression of ΔNp63α, p53 mutated on UV hot spots ($p53^{mt}$), Bcl-2 and p21 Bax. The levels of ΔNp63α and $p53^{mt}$ were decreased, Bcl-2 remained unaffected, while the expressions of p21Bax and p18 Bax were increased in VSV-infected HaCaT cells. Together, these data demonstrate 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 responses of infected cells and may also sensitize to other apoptotic stimuli. These findings may stimulate further studies with the goal of developing VSV-based virotherapy into an effective modality for the treatment of epithelial-derived malignant tumors of the skin.

Keywords: *Vesicular stomatitis virus*; Apoptosis; HaCaT cell line

1. Introduction

Skin tumors are the most common malignancies in humans. The major groups comprise the melanocyte-derived, the epithelial-derived and the other-type neoplasms. The epithelial-derived ones occur very frequently and include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [1–4]. These malignancies have multifactorial causes; the etiological factors include ultraviolet radiation (UV), immunosuppression, chronic inflammation, chemical carcinogens and a genetic predisposition [1–4]. A great body of experimental evidence indicates that UV radiation plays a primary role in the pathogenesis. Various investigations have clearly demonstrated that BCC cells carry UV-induced mutations in the p53 tumor suppressor gene ($p53^{mt}$), which is a major cause of cell cycle dysregulation and carcinogenesis [1–4]. These neoplasms are locally invasive, aggressive and destructive. An important difference between BCC and SCC is that BCC has a very limited capacity to metastasize, whereas SCC displays a significant potential to form metastases in the regional lymph nodes. The incidences of BCC and SCC have undergone dramatic increases during the past few

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decades [1–4]. The therapy of these malignancies is complex [5–10]. Surgical excision is the mainstay of the treatment, but alternative therapeutic modalities, such as radiation, cryotherapy, photodynamic therapy and immunomodulatory therapy, have also been used successfully to achieve a cure [5–10]. Treatment of the epithelial-derived malignancies often leads to permanent cosmetic and structural dysfunctions, therefore new approaches are needed to avoid these disadvantages. One such innovative method for the treatment of skin neoplasms is virotherapy.

Oncolytic viruses provide novel modalities for the therapy of malignant tumors. These microorganisms can kill cancer cells selectively, either by serving as replication-defective vectors or by acting as replication-competent biotherapeutic agents [11–14]. *Vesicular stomatitis virus* (VSV), a member of the *Vesiculovirus* genus of the Rhabdoviridae family, has been shown to possess powerful inherent oncolytic activity [15–23]. The VSV genome encodes five proteins: the nucleocapsid (N) protein, the phosphoprotein (P), the large (L) protein, the matrix (M) protein and the glycoprotein (G) [15]. VSV replication in immortalized cells is highly efficient, while in normal cells with a functional interferon (IFN) system it is restricted [16,24,25]. 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 [15–26]. VSV elicits a cytopathic effect (CPE) and apoptosis in cancer cells, whereas normal cells are relatively spared by this virus [16,24,25]. M protein and another viral product (as yet unidentified) are known to be implicated in the apoptotic process triggered by VSV [27,28]. The infection disrupts the mitochondrial transmembrane potential ($\Delta\psi_m$), causing the release of intermembrane proteins, such as cytochrome *c* and apoptosis-inducing factor, into the cytoplasm [29]. Cytochrome *c* then forms a complex, termed apoptosome, with apoptotic protease-activating factor-1 and pro-caspase-9, leading to the activation of caspase-9 and the subsequent cleavage of pro-caspase-3 in the VSV-infected cells [29–31]. It has also been established that VSV infection may induce a pro-apoptotic shift in the level of the Bcl-2 family member proteins, which play pivotal roles in the control of the mitochondria-initiated caspase activation pathway [26,29]. In certain experimental systems, the infected cells display decreased levels of some anti-apoptotic proteins, including Bcl-2 or Bcl-x_L, and increased levels of some pro-apoptotic proteins, including p18 Bax [26,29]. Furthermore, the over-expression of Bcl-2 or Bcl-x_L confers significant protection against the apoptogenic effect of VSV [32]. Further studies have revealed that, besides caspase-9, other upstream caspases, such as caspase-8 and caspase-12, may also become activated in VSV-infected cells, but the extrinsic and the endoplasmic reticulum-initiated apoptotic pathways seem to play only minor roles in the cell demise induced by VSV [33]. Thus, several noteworthy studies have clearly demonstrated that VSV infection triggers primarily the intrinsic pathways of apoptosis in cancer cells. Abundant experimental evidence also indicates that VSV should be considered as a future powerful biotherapeutic agent effective against a broad histological spectrum of cancers [16–33]. However, the potential oncolytic activity of VSV has not yet been evaluated in epithelial-derived skin tumors.

The spontaneously immortalized human keratinocyte cell line HaCaT has been established for the investigation of various diseases that affect the skin [34]. This cell line exhibits UV-induced mutations in both alleles of the p53 gene and further genetic alterations leading to the loss of senescence genes [35]. The phenotypic characteristics of these cells include an increased growth potential, and partial independence from growth factors, while they maintain the ability to differentiate and lack invasive growth behavior [35]. Thus, the HaCaT cell line provides an excellent in vitro model system for studies focusing on the pathogenic mechanisms of various skin diseases and drug discovery [34,35].

With the aim of evaluating the potential oncolytic activity of VSV in epithelial-derived skin cancers, in the present study we have investigated the susceptibility of the immortalized HaCaT keratinocyte cell line to VSV, and analyzed the role of apoptosis in the VSV-mediated induction of cell death. Furthermore, in order to gain insight into the underlying molecular mechanisms implicated in the apoptogenic properties of this virus, we have also determined the effects of VSV infection on the levels of ΔNp63α, mutant p53 ($p53^{mt}$) and certain Bcl-2 family member proteins.

2. Materials and methods

2.1. Cell culture and VSV growth

The HaCaT cell line, kindly provided by Dr N.E. Fusenig (Heidelberg, Germany), was grown in Dulbecco's modified Eagle's minimal essential medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (Gibco/BRL, Grand Island, NY) at 37 °C in a 5% CO_2 atmosphere [34,35].

The Indiana strain of VSV was propagated at a multiplicity of infection (MOI) of 0.001 plaque forming unit (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.

Virus plaque assays were performed on confluent monolayers of Vero cells inoculated with VSV solution for 1 h at 37 °C and overlaid with 0.5% agarose (FMC, Rockland, ME) 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 VSV infection.

For experiments, HaCaT cell cultures were inoculated with VSV at different MOIs. Every experiment was repeated at least three times.

2.2. Indirect immunofluorescence assays

Cytospin cell preparations were fixed in methanol–acetone (1:1) for 15 min at −20 °C. Slides were incubated with a 1:500