NEOPLASIA
www.neoplasia.com

Volume 16 Number 9 September 2014 pp. 679–693 679

# Human-Gyrovirus-Apoptin Triggers Mitochondrial Death Pathway—Nur77 is Required for Apoptosis Triggering

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## Abstract

The human gyrovirus derived protein Apoptin (HGV-Apoptin) a homologue of the chicken anemia virus Apoptin (CAV-Apoptin), a protein with high cancer cells selective toxicity, triggers apoptosis selectively in cancer cells. In this paper, we show that HGV-Apoptin acts independently from the death receptor pathway as it induces apoptosis in similar rates in Jurkat cells deficient in either FADD (fas-associated death domain) function or caspase-8 (key players of the extrinsic pathway) and their parental clones. HGV-Apoptin induces apoptosis via the activation of the mitochondrial intrinsic pathway. It induces both mitochondrial inner and outer membrane permebilization, characterized by the loss of the mitochondrial potential and the release into cytoplasm of the pro-apoptotic molecules including apoptosis inducing factor and cytochrome *c*. HGV-Apoptin acts via the apoptosome, as lack of expression of apoptotic protease-activating factor 1 in murine embryonic fibroblast strongly protected the cells from HGV-Apoptin–induced apoptosis. Moreover, QVD-oph a broad-spectrum caspase inhibitor delayed HGV-Apoptin–induced death. On the other hand, overexpression of the anti-apoptotic BCL-XL confers resistance to HGV-Apoptin–induced cell death. In contrast, cells that lack the expression of the pro-apoptotic BAX and BAK are protected from HGV-Apoptin induced apoptosis. Furthermore, HGV-Apoptin acts independently from p53 signal but triggers the cytoplasmic translocation of Nur77. Taking together these data indicate that HGV-Apoptin acts through the mitochondrial pathway, in a caspase-dependent manner but independently from the death receptor pathway.

*Neoplasia (2014) 16, 679–693*

## Introduction

Apoptosis is the process whereby individual cells of multicellular organisms undergo systematic self-destruction in response to a wide variety of stimuli [1]. Apoptosis is a genetically encoded program that is involved in normal development and homeostasis and in diverse patho-physiological processes [2]. Apoptosis functions to eliminate cells during development when they become redundant or as an emergency response after radiation damage, viral infection, or aberrant growth induced by the activation of oncogenes [1]. The morphology of apoptosis is orchestrated by the proteolytic activity of

Abbreviations: 7AAD, 7-amino-actinomycin D; AIF, Apoptosis inducing factor; BCL-XL, B-cell lymphoma extra-large; CAV-Apoptin, Chicken anemia virus apoptin; cyt *c*, cytochrome *c*; DISC, Death-inducing signal complex; FADD, Fas-associated death domain; HGV-Apoptin, Human gyrovirus apoptin; MEF, Mouse embryonic fibroblast; MOMP, Mitochondrial outer membrane permeabilization; TMRM, Tetramethylrhodamine methyl ester perchlorate

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Received 4 July 2014; Revised 31 July 2014; Accepted 5 August 2014



1476-5586/14
http://dx.doi.org/10.1016/j.neo.2014.08.001

the caspase proteases [3–5] through which the cleavage of many proteins largely orchestrate apoptotic process [6]. In vertebrate cells, apoptosis typically occurs through one of two the major signaling pathways termed the extrinsic/cell death receptor pathway and the intrinsic/mitochondrial-initiator pathway [7]. In the extrinsic pathway, the ligation of the death receptors leads to the recruitment of the adaptor molecule FADD (fas-associated death domain) that bind, trimerize, and activate an initiator caspase (caspase-8), that in turn directly cleaves and activates the apoptosis executioner caspases (caspase-3 and -7) [2,7,8]. In the intrinsic pathway, the mitochondria responds to apoptotic stimuli through mitochondrial outer membrane permeabilization (MOMP). MOMP leads to the release of pro-apoptotic proteins from the mitochondrial intermembrane space. Following its release, cytochrome *c* (cyt *c*) binds apoptotic protease-activating factor 1 (APAF1), inducing its conformational change and oligomerization and leading to the formation of a caspase activation platform termed apoptosome. The apoptosome recruits, dimerizes, and activates an initiator caspase, caspase-9, which, in turn, cleaves and activates caspase-3 and -7 [2,7,8]. Thus the caspase cascade activation result from the remarkable MOMP and its subsequent intermembrane space mitochondrial proteins release. MOMP is highly regulated by the B cell lymphoma 2 (BCL-2) family members [2] which have been classified into 3 classes [9,10]. One class inhibits apoptosis (BCL-2, BCL-XL, MCL-1, etc), the second class promotes apoptosis (BAX, BAK), and a third class termed the *BH3-only proteins* (BAD, BIK, BID, BIM, BOK, etc.) binds and regulates the anti-apoptotic BCL-2 proteins to promote apoptosis [4]. While the pro-apoptotic family members BAX and BAK are crucial for the induction of MOMP and the release of the pro-apoptotic molecules, the anti-apoptotic family members BCL-2 and BCL-XL inhibit BAX and BAK [4,11]. Following MOMP, the mitochondrial transmembrane potential is dissipated through caspase-dependent and caspase-independent means [2,12,13]. The intrinsic death pathway is induced by many different stress signals including DNA-damaging agents, viral and cellular oncogenes, and transcriptional blockade [12,14]. The stimuli are transmitted from the nucleus to the mitochondria by two main molecules: the tumor suppressor gene p53 and the orphan steroid receptor Nur77 [15].

Apoptosis plays an important role in the treatment of cancer as it is induced by many treatments [16]. While the most used strategies aim at targeting the apoptotic defects [16], some of the emerging strategies aim at the development of cancer selective therapies by molecules that target and kill preferentially cancer cells. One of the potential tools for cancer selective therapy is CAV-Apoptin as it induces apoptosis selectively in cancer cells [17,18]. CAV-Apoptin is a viral protein of 14 kDa derived from the chicken anemia virus [19,20]. The selective toxicity of CAV-Apoptin is associated at least in part to its tumor specific nuclear localization and its tumor specific phosphorylation at Theorine-108, which are essential for its nuclear accumulation and its induction of apoptosis [21,22]. Recently, the human homolog of the CAV named the human gyrovirus (HGV) has been identified [23]. Its genome presents an overall organization similar to that of CAV [23,24], it consists of a single negative-strand circular DNA of 2315 nucleotides. HGV has a similar organization of the promoter region and the encoded proteins as the CAV as revealed by both virus sequence alignment. It encodes a 125 amino-acid homologue of the CAV-Apoptin VP3 protein that despite a low overall identity has conserved important sites including nuclear localization and export signals and phosphorylation sites [23,25]. HGV-Apoptin has the same subcellular distribution as the CAV-Apoptin, it localizes in the nuclei of cancer cells where it shows a granular distribution that later clusters to form aggregates while it remains in the cytoplasm of normal human cells [25]. Like CAV-Apoptin, HGV-Apoptin induces apoptosis selectively in cancer cells but not in normal cells [25] and is therefore a potential biologics anti-tumor candidate.

In this paper, we focus on the molecular mechanisms of HGV-Apoptin selective toxicity. Using cells with defective FADD or caspase-8 (key players in death receptor signaling), APAF1 deficient cells, BAK/BAX-deficient cells, and other molecular tools, we demonstrate that HGV-Apoptin induces apoptosis independently of the death receptor pathway. Hence, it triggers the activation of the mitochondrial death pathway via MOMP and the release of cyt *c*, and apoptosis-inducing factor (AIF) from mitochondria, in a caspase dependent manner. HGV-Apoptin induced apoptosis is modulated by the BCL-2 family members, is independent from p53 signal and causes the cytoplasmic translocation of Nur77.

## Material and Methods

### *Chemotherapeutics Inhibitors*

Staurosporine (2.5 μM) was purchased from Roche Diagnostics, Mannheim, Germany. The broad-range caspase inhibitor QVD-OPh was from Enzyme Systems (Dublin, CA, USA) and was added to the cells at a concentration of 25 μM immediately after transfection.

### *Antibodies*

The following primary antibodies were used: mouse anti-active caspase-8 (Cell Signaling Technology, Beverly, USA), rabbit anti FAS, and anti FADD antibodies (Santa Cruz Biotechnology), murine activating anti-CD95/FAS (50 ng/ml) from upstate signaling, rabbit anti-AIF IgG (Sigma-Aldrich), mouse anti-p53 antibody (Millipore), Rabbit anti-Flag (Thermo/Fisher Scientific), rabbit anti-nur77 IgG (Santa Cruz), mouse anti–β-actin (Abcam). The following secondary antibodies were used: Infrared dye 800cw goat, anti-rabbit antibody, Infrared dye 680cw goat, anti-mouse antibody (Licor), Rhodamine redX anti-rabbit antibody (Life Technologies), cy5 anti-murine antibody (Abcam).

### *Cell Culture and Reagents*

Jurkat (T-cell leukaemia) cells, Jurkat clones stably transfected with FADD-DN (a dominant-negative FADD mutant lacking the N-terminal death-effector domain), caspase-8 deficient Jurkat cells, Jurkat cells overexpressing BCL-XL and MCF7 (breast adenocarcinoma), MCF7 expressing caspase-3, MCF7 overexpressing BCL-XL cells, and MCF7 expressing GFP–cyt *c* were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (Hyclone), 100 μg/ml penicillin and 0.1 μg/ml streptomycin (Gibco BRL). HCT116 (colon carcinoma), MEF (mouse embryonic fibroblasts) immortalized by retroviral transduction with a temperature-sensitive simian virus 40 large T antigen as described in [26], MEF-APAF1$^{-/-}$, and MEF-BAX-BAK$^{-/-}$ were grown in DMEM medium supplemented with 10% fetal calf serum (Hyclone), 100 μg/ml penicillin and 0.1 μg/ml streptomycin (Gibco BRL). Human primary fibroblasts were grown in FibroGRO media for culture of human fibroblast (Millipore). Cells were grown at 37 °C with 5% $CO_2$ in a humidified incubator.

### *Plasmids and Transient Transfections*

The expression vectors of HGV-Apoptin GFP-HGV-APT and FLAG- HGV-APT were provided by Dr M. Tavassoli [25]. The empty vector pEGFPC1 was used as negative control. Cells were