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Research article

CHICKEN ANEMIA VIRUS VP3 GENE CLONED IN pVAX1 VECTOR CAUSES APOPTIC ACTIVITY IN HeLa CELLS

Raushi Maurya[1] and Anant Rai[2*]

[1]Department of Biotechnology, Mewar University, Chittorgarh, Rajasthan.

[2]Institute of Biotechnology & IT, 197-198, Mudiya Ahmadnagar, Bareilly-2453122, UP.

***Corresponding author: raia48@gmail.com**

ABSTRACT

VP3 protein gene of chicken anemia virus cloned in pVAX1 was analysed for its apoptotic activity using HeLa cells in culture. It was found to produce high level of apoptosis when analysed by DNA fragmentation assay, acridine orange-ethidium bromide staining, caspase assay, annexin V binding assay, Tunel assay and MTT cytotoxic assay.

Keywords: VP3, apoptin, pVAX1, caspase, annexin V binding.

INTRODUCTION

The 2.3 kb chicken anemia virus (CAV) genome encodes three viral proteins VP1, VP2, VP3. VP1 is major capsid protein and VP2 is probably a non-structural protein found in the cells in the early stage of virus replication cycle (Noteborn *et al.*, 1995). VP1 and VP2 are protective proteins that induce neutralising antibodies (Kock *et al.*, 1995). The third viral protein VP3 is a 13 kDa protein that shows apoptic activity and is able to induce apoptosis within infected chicken cells and human tumor cell lines (Noteborn *et al.*, 2004). VP3 has been shown to not only induce apoptosis when introduced into the precursors of chicken thymocytes, but has been found to specifically kill human cancer cells and transformed cells without affecting the proliferation of normal cells (Lee *et al.*, 2012). Apoptin causes apoptosis in various human tumors and transformed cells (Danen Van Oorschot *et al.*, 1997). Apoptin is a promising and ideal agent for cancer gene therapy owing to its intrinsic specificity and the inherent low toxicity (Gueden *et al.*, 2004). Because of its death inducing abilities, the VP3 gene product was renamed apoptin. Interestingly, the apoptic activity of apoptin in not restricted to chicken thymocytes (Danen Van Oorschot *et al.*, 1997). Unlike other tumor suppressor or proapoptic genes, theapoptin does not rely on the function of p53, BCL-2, Bax, FADD, or Caspase-8 (Tavassoli *et al.*, 2005; Liu *et al.*, 2006). Thus apoptin may be used to treat a broad spectrum of tumors that differ in mutational status of p53 and the levels of expressions of apoptotic regulators. The ability of apoptin to induce apoptosis has been demonstrated in more than 70 human cancer cell lines (Backendorf *et al.*, 2008; Noteborn *et al.*,

2009). Apoptin can induce apoptosis in cell lines derived from a great variety of human tumors, eg. hepatoma, lymphoma, leukemia, cholangiocarcinoma, melanoma, breast and lung tumors and colon carcinoma (Noteborn *et al.*, 1991). In contrary, apoptin does not induce apoptosis in normal non-transformed human diploid cells, such as fibroblasts, keratinocytes, smooth muscle cells, T cells or endothelial cells. The possible cause for this phenomenon is that, in tumor cells apoptin is located in the nucleus whereas in normal cells it is present in the cytoplasm. The present work was undertaken to study the apoptin cloned in pVAX1 vector which is approved for rDNA product for use in human.

MATERIALS AND METHODS

Recombinant plasmid

The recombinant plasmid pVAX1.cav.vp3 (Maurya and Rai, 2013) available in Biotechnology Laboratory, IBIT, Bareilly.

HeLa Cells

The HeLa cells used in this study were grown in DMEM (PAA Laboratories GmbH) with 50µg/ml gentamycin (Amresco, USA) and 10% fetal calf serum (Hyclone, Perbio) in 25 cm^2 plastic flasks.

Plasmid DNA Isolation

E.coli DH5α containing pVAX1.cav.vp3 recombinant plasmid was grown in LB broth containing ampicillin 100 µg/ml and plasmid DNA was isolated using TELT method (Ausubel *et al.*, 1990).

Transfection of HeLa cells

The calcium phosphate DNA co-precipitate was prepared by combining 100 µl 2.5 M $CaCl_2$ with 20 µl plasmid DNA in sterile microfuge tube and added 80µl DW in it and kept for 1 min. Immediately transferred 20 µl calcium phosphate DNA suspension in each of 96 well microtiter plate. Then 100 µl HeLa cell suspensions were added into each well. Kept control well without transfection. The plate was incubated at 37ºC in a humidified incubator with an atmosphere of 5% CO_2 tension for 72h (Rai *et al.*, 2014). After incubation, the cells were examined for expression using IPT and IFAT techniques.

DNA fragmentation assay

HeLa cells showing 60% monolayer were transfected as described earlier. After 48h of transfection the monolayer was trypsinised and collected in 1.5 ml microfuge tube. The cells were centrifuged at 3000 rpm for 5 min and the supernatant was removed and the cells were resuspended in 200µl PBS. The genomic DNA was isolated using QIAamp DNA mini kit (Qiagen, Germany) using manufacturer's method.

Ethidium bromide-acridine orange staining

HeLa cells showing 60% monolayer were transfected as described earlier. After 48h of transfection the staining was performed using following protocol: separately diluted 10 µl each of ethidium bromide and acridine orange from stock (10gm/ml) in 1 ml PBS. Washed the cells with PBS once. To 96 wells plate, 10 µl each of diluted ethiduim bromide and acridine orange were added to each well, and incubated the plate for 5 min at room temperature. The cells were again washed with PBS and fixed with 4% paraformaldehyde. The cells were again washed with PBS and 50% glycerol in PBS was added and observed under UV microscope (Nikon Japan). This staining was done to observe the morphological changes such as fragmentation of nuclei,

nuclear condensation and formation of apoptotic bodies.

Caspase 3 assay

Caspase 3 was detected using CaspGlowTM Fluorescein active caspase-3 staining kit (Biovision, USA) using manufacturer's protocol. Apoptosis was induced by transfecting HeLa cells with pVAX1.cav.vp3 recombinant plasmid as described earlier. After 48h of transfection, the cells were trypsinised and collected in 1.5 ml microfuge tube, cells were washed with PBS and centrifuged at 3000 rpm for 5 min. Resuspended the cells in 300 µl PBS and 1µl of FITC-DEVD-FMK was added into each tube and incubated at 37ºC for 1h. Then centrifuged the cells for 5 min at 3000 rpm and removed the supernatant. Resuspended the cells in 0.5 ml of wash buffer and centrifuged. Repeated this step. Finally the cells were suspended in 50 µl of wash buffer. One drop of cell suspension was put onto a slide and covered with coverslip. The cells were observed under FITC filter.

Annexin V binding assay

This test was performed using Vibrant Apoptosis Assay Kit#2 (Invitrogen, USA). The kit contains recombinant annexin V conjugated to Alexa Flour R 488 dye and a ready to use solution of the red fluorescent propidium Iodide (PI) nucleic acid binding dye. HeLa cells showing 60% monolayer were transfected as described earlier. After 48h of transfection, the cells were collected in 1.5 ml microfuge tube by trypsinization. Further processing was done as per kit protocol. The cells were deposited onto microslide and covered with coverslip and examined under fluorescent microscope (Nikon Japan). This test was mainly done to detect the plasma membrane alteration which occurs during apoptosis.

TUNEL assay

This test was performed using APO–Brdu TUNEL assay kit (Invitrogen, USA) to detect the fragmented DNA in apoptic cells as per kit protocol. Apoptosis was induced in HeLa cells by transfecting with pVAX1.cav.vp3 recombinant plasmid as described earlier. After 48h of transfection the cells were trypsinised and collected in 1.5 ml microfuge tube and further processed as per kit protocol. The samples were analyzed by FACS.

MTT assay

Cell concentration was adjusted to $2x10^6$ cells/ml and 50 µl cells were seeded in 96 well plates in triplicates, and transfected with rplasmid DNA keeping control wells. The groups of wells were VP3 transfected, pVAX1 alone transfected and healthy cells with 4 wells for each group. After 72 h of incubation at 37ºC, 30 µl MTT (5mg/ml in PBS) was added to each well and cells were incubated furthermore for 4h at 37ºC. The supernatant was discarded, plates were dried and 150 µl DMSO was added to each well and MTT crystals were dissolved by pipetting and contents of each well transferred to cuvette and were read immediately at 540 nm with 620 nm as reference wavelength. OD was calculated. The apoptic index was calculated using formula: AI= OD transfected cell /OD control cell.

RESULTS AND DISCUSSION

For evaluation of apoptosis DNA fragmentation assay was done, which revealed nucleosomal laddering on agarose gel electrophoresis, while there was no such laddering pattern in control cells (Figure 1). In ethidium bromide-acridine orange staining, clear changes i.e. nuclear condensation and fragmentation in CAV-VP3 transfected HeLa cells was observed,

whereas no such changes were observed in mock transfected HeLa cells (Figure 2). In caspase detection assay, Caspase-3 was detected. There was green fluorescence in caspase-3 positive cells, while there were no such changes in control (Figure 3). Apoptic cells in Annexin-V-Binding Assay showed bright green fluorescence, and control showed red fluorescence (Figure 4). In TUNEL assay, almost 40% cells showed apoptosis, while there was negligible apoptic activity in mock transfected HeLa cells and vector control cells (Figure 5). MTT analysis revealed that VP3 transfected cells showed apoptin index of 0.1 while mock transfected and healthy cells showed apoptic index of 0.95 confirming the apoptosis in recombinant plasmid transfected HeLa cell.

All these assays which were performed to evaluate apoptic activity of CAV- VP3 apoptin in HeLa cells showed that CAV- VP3, has a great potential to induce apoptosis and showed good apoptic activity in cultured cells.

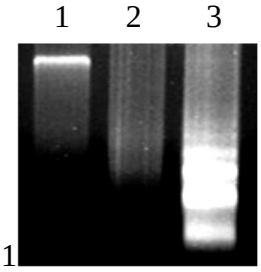


Figure1.DNA laddering assay.
Lane1: healthy control,
Lane 2: Vector control,
Lane 3: pVAX1.cav-VP3.

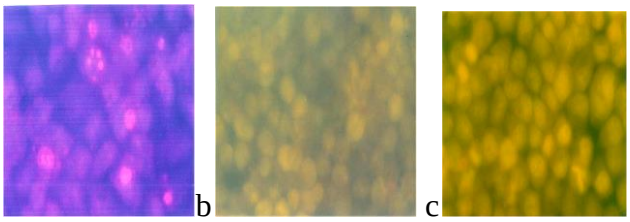


Figure 2.Ethidium bromide/acridine orange staining of pVAXI.cav-VP3 transfected HeLa cells. a. Apoptotic cell b. Vector control HeLa cells c. Healthy control Hela cells.

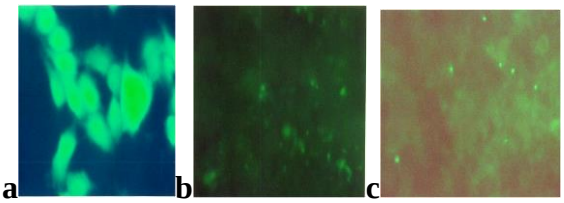


Figure 3. Caspase 3 detection assay of pVAXI.cav-VP3 in HeLa cells (a) Caspase 3 +ve cell (b) Vector control HeLa Cells (c) Healthy control HeLa cells.

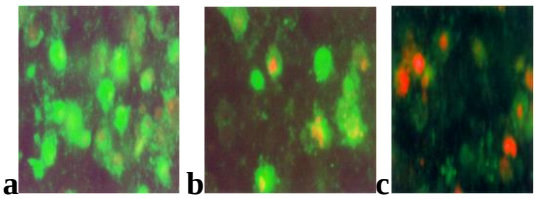
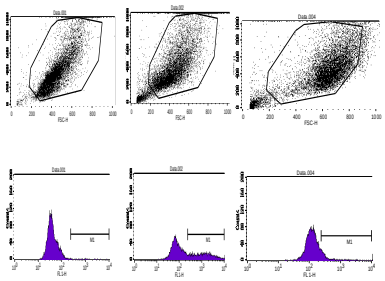


Figure 4.Annexin-V-binding assay of pVAXI.cav--VP3 in HeLa cells (a) Apoptotic cell (b) Vector control (c) Healthy control.



(a) (b) (c)

Figure 5.TUNEL assay in HeLa cells.
(a) Mock transfected; (b) pVAX1Cav-VP3 transfected; (c)Vector control.

Apoptin is a strong inducer of apoptosis in precursor chicken thymocytes and various human transformed and tumor cell lines but not in normal cells (Noteborn *et al.,* 1994). It has been demonstrated to have apoptic activity and to be able to specifically kill several types of tumor cells including cell lines HeLa, Saos-2 lung cancer cells H1299 and HepG2 (Gueden *et al*., 2004; Li *et al.,* 2006; Han *et al.,* 2008; Flinterman *et al*., 2009; Los *et al*., 2009). Lee *et al.* (2012), also studied the efficient production of engineered apoptin in recombinant *E. coli* for tumor therapeutic applications. In ethidium bromide acridine orange staining, we found cytomorphological changes of nuclear contention and fragmentation. Natesan *et al*., (2006) also used this technique to study the anti-neoplastic effects of VP3 protein (apoptin) in Rous sarcoma virus induced tumor in chickens. In their *in vitro* study, it was confirmed that apoptin induces apoptosis in RSV-transformed CEF-cells which was demonstrated by observation of characteristic changes of apoptosis using acridine-ethidium bromide staining. Apoptosis was also confirmed by TUNEL assay which confirmed that apoptin induced more obvious apoptic cell in tumors as compared to control. The results of tunel assay are in agreement with other workers (Lam *et al.,* 2008; Zhang *et al.,* 2012; Zhan *et al.,* 2012). We used annexin-V-Binding assay for confirmation of apoptic cells in HeLa cell and the analysis showed bright green fluorescence in apoptic cells whereas red fluorescence in control. It confirms plasma membrane alteration in apoptic cells. Lam *et al.,* (2008) used this technique to study avian influenza virus A/HK/483/97 (H5NI) protein induced apoptosis in human airways epithelial cells. They found positive results of apoptosis in NCL-H292 cells transfected with pcDNA4-NSI.

Caspase detection assay showed green fluorescence in caspase-3 positive cells. Adams (2003) showed that both caspase-8 and caspase-9 activate caspase-3, which along with other effector caspases, cleaves critical cellular proteins resulting in apoptosis. Caspases are initiators and effectors arm proteins of apoptosis and once the caspase cascade is activated, the process of cell death is irreversible (Adams, 2003; Kumar, 2007). Our data suggest that apoptin is a promising candidate and can be exploited as a powerful agent to evaluate for its antitumor activity and can be used in future for therapeutic purposes.

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