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APOPTIC ACTIVITY OF CHICKEN ANEMIA VIRUS VP3 GENE CLONED IN REPLICASE BASED EUKARYOTIC VECTOR

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ABSTRACT

CAV is one of the smallest avian viruses; it is 23–25 nm in size, icosahedral in shape and non-enveloped, having a 2.3 kb, circular, single-stranded, negative sense DNA genome. The genome encodes three viral proteins VP1, VP2 and VP3, that are transcribed from a single major transcript of 2.0 kb. The apoptosis inducing potential of CAV-VP3 (apoptin) was studied in HeLa cell line. The study confirmed that VP3 induced apoptosis in HeLa cells, which was confirmed by demonstrating the characteristics changes of apoptosis which include nuclear condensation, DNA fragmentation by DNA laddering assay, plasma membrane alteration by annexin-V binding assay and Caspase 3. The objective of our work was to study the apoptic activity of apoptin in HeLa cell line.

Keywords: VP3 gene, apoptin, apoptosis, replicase vector, antitumor

INTRODUCTION

The apoptotic demise of a cell results in the formation of small membrane bound entities known as apoptotic bodies. These bodies pinch off from the dying cell and are consumed by the phagocytic action of neighboring cells. This engulfment provides a means for the dissemination of the virus without initiating a concomitant host response, which would follow the release of progeny into the extracellular fluid **(Teodoro and Branton, 1997)**.

Chicken anaemia virus, (CAV), is a virus that affects poultry. CAV causes anaemia, bone marrow atrophy, and severe immunosuppression. Clinical signs of infection of CAV is predominantly found in young chicks due to maternal antibodies present in most adult chickens **(Sommer and Cardona, 2003).**

Apoptin, a small protein derived from chicken anemia virus, can specifically induce apoptosis in transformed cells or tumor cells, but not in normal cells. The tumor specificity of apoptin relates to its subcellular localization. In transformed cells or tumor cells, apoptin migrates to the nuclei, whereas in non-transformed cells, it remains mainly within the cytoplasm. Phosphorylation is responsible for the nuclear localization of apoptin. In tumor cells, apoptin is phosphorylated, then translocates into the nuclei, and induces cell apoptosis. Apoptin-induced apoptosis does not depend on functional p53, and can't be inhibited by overexpression of Bcl-2 and Bcl-xL, but caspase-3 activation is necessary for apoptin-induced rapid apoptosis. Apoptin has a strong tendency to aggregate, and exists as aggregates in living cells, but in vivo formation and dissociation of the aggregates are not required for apoptosis-inducing activity of apoptin. It is possible that apoptin's ability to bind DNA closely relates to its ability to induce apoptosis **(Wang and He, 2005).**

Apoptosis is a physiological form of cell death characterized by nuclear chromatin condensation, cytoplasmic shrinking and memberane blebbing **(Schmitz *et al.*, 2000; Lauber *et al.*, 2004)**. This form of programmed cell death is predominantly induced by cancer therapy **(Kawanishi and Hiraku, 2004; Wesselborg and Lauber, 2005)**.

In the present study, the vp3 gene of chicken anemia virus was cloned in pSin mammalian expression vector and the resultant recombinant plasmid was designated as pSin.cav.vp3. The apoptosis inducing potential of cav.vp3 (apoptin) was studied in cultured HeLa cell line. The findings of present study show that vp3 gene cloned in pSin expression vector showed potent apoptotic activity in HeLa cell line and further in vivo trials may be undertaken to know its therapeutic potential.

MATERIAL AND METHODS

Vector

The pSin vector is derived from an alpha virus (Sindbis virus). The subgenomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it. In comparative studies of conventional (nonreplicating) plasmid DNA vectors and alphavirus DNA-based replicon vectors, the latter generally produces larger quantity of DNA concentrations than does conventional vectors.

Gene

The VP3 gene of CAV used in this study was available in Biotechnology laboratory of IBIT, Bareilly. The nucleotide sequence of VP3 gene of CAV was downloaded from NCBI GenBank.

Cell Culture

HeLa cell line was obtained from National Centre for Cell Science (NCCS). This was used in the study for apoptotic activity of recombinant plasmid and was maintained in DMEM (Gibco, NY) supplemented with 50 µg/ml gentamycin (Amresco, USA) and 10% fetal calf serum (Hyclone, USA).

DNA fragmentation assay by agarose gel electrophoresis

HeLa cells showing 60% monolayer were transfected as described earlier. After 48 h. of transfection, the monolayer was trypsinized and collected in a 1.5 ml tube. The cells were centrifuged at 3000 rpm for 5 min. After centrifugation, the media was removed and the cells were resuspended in 200 µl PBS and the genomic DNA was isolated using protocol of **Sambrook and Russell (2001).** Transferred the cell suspension to a microfuge tube containing 600 µl of ice-cold cell lysis buffer. Added 3 µl of proteinase solution to the lysate to increase the yield of genomic DNA. Incubate it for 15-60 minutes at 37^0C. Allowed it to cool to room temperature and then added 3ml RNase, Incubate it for 15-60 minutes at 37^0C. Allowed the sample to cool to room temperature. Added 200 ml of potassium acetate solution and mixed the contents of the tube by vortexing vigorously for 20 seconds. Pellet the precipitated protein by centrifugation at 10000 rpm for 3 minutes. Transferred the supernatant to a fresh microfuge tube containing 600 µl of isopropanol. Mixed the solution well and then recovered the

precipitate of DNA by centrifuging the tube at 10000 rpm for 1 minute in a microfuge tube. Removed the supernatant and added 600 ml of 70 & ethanol to the DNA pellet. Inverted the tube several times and centrifuged the tube at 10000 rpm for 1 minute in a microfuge tube. Carefully removed the supernatant and allowed the DNA pellet to dry in air for 15 minutes. Redissolved the pellet of DNA in 10 µl of TE (pH -7.6). The eluted DNA was stored at -2^0C**.** The genomic DNA collected by above procedure was subjected to agarose gel electrophoresis.

Caspase 3 detection assay

Activation of caspases plays an important role in apoptosis. Caspase 3 was detected using CaspGLOW Fluoroscein Active caspase -3 Staining kit (Biovision, USA). The assay utilizes the caspase 3 inhibitor, DEVD-FMK, conjugated to FITC (FITC-DEVD- FMK) as a marker. FITC-DEVD- FMK is cell permeable, nontoxic and irreversibly binds to activated caspase 3 in apoptotic cells. The FITC label allows for direct detection of activated caspases in apoptotic cells by fluorescence microscopy.

Annexin V binding assay

This test was mainly done to detect plasma membrane alteration, which occur during apoptosis. In normal live cells, phosphatidylserine (PS) is located on cytoplasmic surface of cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet to the plasma membrane thus exposing PS to the external cellular environment. The human anticoagulant, annexin V, is a 35-36-kDCa dependent phospholipid binding protein that has a high affinity for PS. Labeled Annexin V can identify apoptotic cells by binding to PS exposed on the outer leaflet. This test was performed using Vybrant Apoptosis Assay Kit # 2 (Invitrogen, USA). The kit contains recombinant annexinV conjugated to Alexa Flour 488 dye. The kit also contains a ready-to-use solution of the red fluorescent propidium iodide (PI) nucleic acid binding dye. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids cells in the cell. After staining a cell population with Alexa Flour 488 annexin V and PI in the provided buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence and live cells show little or no fluorescence.

RESULTS AND DISCUSSION

The apoptosis inducing potential of CAV-VP3 (apoptin) was evaluated in HeLa cells by the following three assays: DNA laddering assay, Caspase detection assay and Annexin-V-binding assay

DNA laddering assay: In this the cleavage of chromosomal DNA occurs into the oligonucleosomal size fragments, which is an integral part of apoptosis. Following this property, the bands merged and nucleosomal laddering was detected on agarose gel electrophoresis as shown in figure 1, while control showed no such laddering pattern.

Caspase detection assay: The caspases are a family that are one of the main executers of apoptotic process. These proteins breakdown or cleave key cellular components that are required for normal; cellular function including structural proteins in cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degrative enzymes such as DNAses which begin to cleave the DNA in the nucleus. In caspase detection assay, caspase 3 positive cells showed green fluorescence as shown in figure 2. However, control cells showed no such fluorescence.

Annexin-V-binding assay: Annexin V was used as a probe in this assay, to detect the cells that have expressed phosphatidylserine on the cell surface, a feature found in apoptosis. Phosphatidyl serine at the outer membrane surface of a cell is a universal process occurring during early apoptosis. Using the Annexin-V affinity assay, the apoptic cells in suspension can be determined in a fast, simple and sensitive way. Annexin V staining was specific to apoptic cells and background staining was low in unaffected cells. In Annexin-V-binding assay, apoptic cells showed bright green fluorescence while control showed yellow fluorescence as shown in figure. 3.

All these three assays revealed that CAV-VP3 showed good apoptic activity in cultured cells. It was also observed that majority of the cells showed apoptic activity.

DISCUSSION

The present study was undertaken to observe the apoptotic effect of pSin.cav.vp3 gene in HeLa cells. The objective was to study of apoptotic activity of apoptin (VP3 protein) in cell line.

The VP3 gene of CAV was successfully cloned into pSin vector. pSin is derived from an alpha virus (Sindbis virus). The subgenomic promoter of alpha virus is very strong so that it makes large number of target mRNA from the sequence downstream to it and less amount of DNA is needed to be delivered for therapeutic purpose. We have used pSin vector due to the following reasons. There are many

properties which make alphavirus vectors a desirable alternative to other virus-derived vector systems being developed. These properties include potential high-level expression of up to 10^8 molecules of heterologous protein per cell **(Xiong *et al.*, 1989).** Infection of nondividing cells and a broad host range **(Strauss and Strauss 1994)**. **Frulov *et al.* (1996)** explained the advantages of alphavirus, which include a broad range of susceptible host cells, high levels of cytoplasmic RNA and protein expression and manipulation of recombinant RNA molecules using full-length cDNA clones from which infectious RNA transcripts can be generated by *in vitro* transcription.

In our study, in DNA laddering assay, nucleosomal laddering was detected on 2% agarose gel electrophoresis. The bands were found to be merged and a laddering pattern was observed. The nucleosomal laddering is a hallmark of apoptosis.. **Ahmed *et al.* (2008)** investigated whether endosulfan, an organochlorine pesticide was able to deplete glutathione (GSH) and induce apoptosis in human peripheral blood mononuclear cells (PBMC) *in vitro*. Apoptotic cell death was determined by DNA fragmentation assays. Significant ladder formation was observed at higher concentration, which was indicative of apoptotic cell death. **Jiang *et al.* (2012)** showed tha**t** HepG2 cells were incubated with tectorigenin at different concentrations, and their viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was detected by morphological observation of nuclear change, agarose gel electrophoresis of DNA ladder. Tectorigenin at a concentration of 20 mg/L greatly inhibited the viability of HepG2 cells and induced the condensation of chromatin and fragmentation of nuclei. **Steigerova *et al.* (2012)** showed that the effects of brassinosteroids (BR) on prostate cancer cells were surveyed using DNA ladder assays. They observed BRs inhibited cell growth. **Mohamed (2012)** detected DNA ladder assay with 0.9% agarose. He observed DNA laddering pattern with multiple bands opf 180-200 bp and its multiplication that is considered a characteristic feature of apoptosis.

Wang and He (2005) showed that apoptin-induced apoptosis does not depend on functional p53, and can't be inhibited by overexpression of Bcl-2 and Bcl-xL, but caspase-3 activation was necessary for apoptin-induced rapid apoptosis. **Schoop *et al.* (2008)** observed that the active form of caspase 3 was present only in apoptin positive cells having an apoptic morphology. **Tumane *et al.* (2010)** observed that silica-induced apoptosis of the alveolar macrophages could potentially favor a proinflammatory state, occurring in the lungs of silicotic patients, resulting in the activation of caspase prior to induction of the intrinsic and extrinsic apoptosis pathways. **Han *et al.* (2011)** did combination therapy with radiation and apoptin which dramatically induced mitochondrial cytochrome c release and the cleavage of caspases -9, -3 and -7 and showed that apoptin treatment represented a potential method for enhancing the effectiveness of radiotherapy in poorly responding hepatocellular carcinoma. It is evident that the caspases are a family that are one of the main executers of apoptotic process. It was seen that our work is in conformity with other workers.

In our study, the Annexin-V-binding assay revealed apoptic cells showing bright green fluorescence while control cells showed faint green fluorescence. In double labeling experiments, using Annexin V binding and counterstaining with the supervital DNA dye Hoechst 33342, **Koopman *et al.* (1994)** showed that cells showing chromatin condensation were annexin v positive. **Martin *et al.* (1995)** showed that annexin-v has been shown to bind specifically to phosphatidyl serine in a calcium-dependent manner and has been used to stain dying cells that expose phosphatidyl serine on their cell surface. **Van Engeland *et al.* (1998)** demonstrated that Annexin V was shown to interact strongly and specifically with phosphatidyl serine (PS) and can be used to detect apoptosis by targeting for the loss of plasma membrane asymmetry**. (Kekre *et al.*, 2005)** showed that Annexin-V assay was carried out at several time-points in order to monitor phosphatidyl serine flipping to the outer leaflet of the plasma membrane, which is a characteristic apoptotic event. Annexin-V staining is specific to apoptotic cells, and background staining is low in unaffected cells **Jiang *et al.* (2012)** showed that HepG2 cells were incubated with tectorigenin at different concentrations, and their viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Apoptosis was detected by Annexin V-EGFP and propidium iodide staining. Tectorigenin induced apoptosis of HepG2 cells mainly via mitochondrial-mediated pathway, and produces a slight cytotoxicity to L02 cells. It was seen that our work is in conformity with other workers reports.

The present study clearly showed the apoptotic activity in neoplastic HeLa cell line and further studies are required to exploit its therapeutic potential.

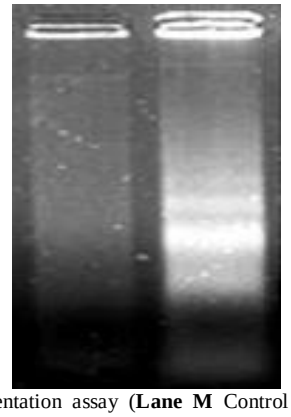

Figure 1 DNA fragmentation assay (**Lane M** Control showing no laddering pattern, **Lane 1** DNA laddering pattern of DNA from pSin.cav.vp3 transfected HeLa cells)

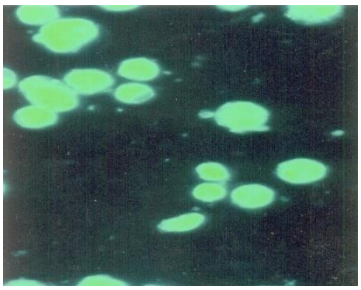

Figure 2 Caspase positive HeLa cells showing green fluorescence, indicating positive apoptic reaction, magnification 100x.

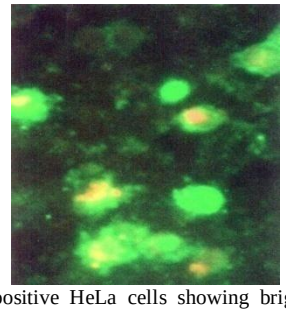

Figure 3 Annexin-V positive HeLa cells showing bright green fluorescence, indicating positive apoptic reaction, magnification 100x

CONCLUSION

Tumors are commonly prevalent in Indian population. Apoptin induces apoptosis/ destruction of tumor cells in human transformed and malignant cells but not in normal cells. The apoptin (VP3 protein) derived from chicken anemia virus is a potential agent for the treatment of a large number of tumors. The VP3 protein (apoptin) induces apoptosis in chicken mononuclear cells. It has attracted great attention, because it specifically kills tumor cells while leaving normal cells unharmed. Other tumorogenic agents show resistance when injected in body but apoptin does not show any such type of resistance. In normal cells, apoptin resides in the cytoplasm, whereas in cancerous cells it translocates into the nucleus. Animal tumor models have revealed apoptin as a safe and efficient antitumor agent, resulting in significant tumor regression. Future antitumor therapies could use apoptin as a potential therapeutic drug. The constructed recombinant plasmid has replicase gene which produces large amount of apoptin protein and thus only small amount of DNA will be needed to be injected for therapeutic agent.

For improved treatments to be developed, the ability to target tumor cells selectively is essential, allowing a greater dose of therapeutic agent to be delivered without adversely affecting non-malignant tissue and further studies are required to exploit its therapeutic potential.

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