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Characterization and evaluation of apoptotic potential of double gene construct pVIVO.VP3.NS1

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Viral gene oncotherapy, targeted killing of cancer cells by viral genes, is an emerging non-infectious therapeutic cancer treatment modality. Chemo and radiotherapy in cancer treatment is limited due to their genotoxic side effects on healthy cells and need of functional p53, which is mutated in most of the cancers. VP3 (apoptin) of chicken infectious anaemia (CIA) and NS1 (Non structural protein 1) of Canine Parvovirus-2 (CPV-2) have been proven to have oncolytic potential in our laboratory. To evaluate oncolytic potential of VP3 and NS1 together these genes needed to be cloned in a bicistronic vector. In this study, both these genes were cloned and characterized for expression of their gene products and its apoptotic potential. The expression of VP3 and NS1 was studied by confocal microscopy and flowcytometry. Expression of VP3 and NS1 in pVIVO.VP3.NS1 transfected HeLa cells in comparison to mock transfected cells indicated that the double gene construct expresses both the products. This was further confirmed by flowcytometry where there was increase in cells expressing VP3 and NS1 in pVIVO.VP3.NS1 transfected group in comparison with the mock control group. The apoptotic inducing potential of this characterized pVIVO.VP3.NS1 was evaluated in human cervical cancer cell line (HeLa) by DNA fragmentation assay, TUNEL assay and Hoechst staning. This double construct was observed to induce apoptosis in HeLa cells.

Keywords: Apoptosis, Cancer, Canine Parvovirus-2 (CPV-2), Confocal microscopy, DNA fragmentation, Flowcytometry, Oncotherapy

Cancer, the leading cause of death worldwide, is a challenge to mankind. Use of chemo and radiotherapy for treatment of cancer is limited due to the genotoxic side effects on healthy cells and involvement of anti-apoptotic signal transduction pathways that prevent cell death. Apoptosis is a physiological process that plays an important role in embryonic development and tissue homoeostasis[1]. The deregulation of apoptosis may lead to developmental anomalies, autoimmune diseases and tumorigenesis. Several chemotherapeutic agents act by inducing apoptosis in tumour cells. Deregulation of apoptosis is one of the major causes of chemo-resistance in cancer therapy[2]. Several chemotherapeutic agents fail to induce programmed cell death, because they require functional p53 for induction of apoptosis in cancerous cells[3-5]. Most of the cancers contain mutated p53 gene, and might not undergo apoptosis after the use of chemotherapeutic agents[6]. The use of viruses for treatment of cancer overcomes the bottlenecks of chemo and radiotherapy. Several viruses have been evaluated for oncolytic effect[7]. These include Rabies, Adeno, Herpes-Simplex, Polio, Measles, Vesicular Stomatitis, Hepatitis A, Mumps virus, Newcastle disease virus (NDV) Parvoviruses, etc[8]. But, due to development of antiviral immunity against viruses and risk of re-emergence of virulent viruses, use of viruses as therapeutic agents has been limited. Instead, viral gene therapy for cancer offers novel treatment paradigms that will eventually lead to the destruction of tumor cells. This popular approach does not involve the pathogenic virus as a whole, but a part or few gene(s) is/are manipulated in such a way that after delivering into the cell it/they express(s) to cure the disease or alleviate the symptoms. VP3 (Viral protein 3), apoptin from chicken infectious anaemia (CIA), NS1 (Non structural protein 1) of Canine Parvovirus-2 (CPV-2) and adenovirus protein E40rf4 have been proven to kill tumor cells[9-13]. In our laboratory VP3 gene of CAV (Data under publication) and NS1 gene of CPV-2 have proven to be oncolytic to HeLa cells. The oncolytic potential of both these viral genes together has not been evalauted. The

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present study is taken up to clone both the genes VP3 and NS1 in an expression vector pVIVO2-mcs and characterize the expression of their gene products. This characterized double gene construct was further evaluated for its oncolytic potential by DNA fragmentation assay, Hoechst staining and TUNEL assay.

Materials and Methods

Amplification of VP3 and NS1 Gene in pVIVO2-MCS—VP3 gene of CAV was earlier cloned in pcDNA3.1+ and characterized in our laboratory and was designated as pcDNA3.1+.VP3. This pc.DNA3.1+.VP3 construct is used as a template to amplify the amplicon covering the complete CDS of VP3 gene using forward primer 5′GCATCATG AATGTCTGGCAACCAGTATACTGAG3′ and reverse primer 5′GTTCCTAGGTTAATCCAAGTCG TCTCGAAA ATC3′ with specific restriction sites at 5′ ends (*Bam*HI and *Nhe*I, respectively) for directional cloning in MCS II of bicistronic expression vector pVIVO2-mcs. Similarly, pcDNA3.1+.NS1 gene construct[12-14] available in the laboratory, was used as template to amplify 2.022 Kb amplicon covering the complete CDS of NS1 gene with forward primer 5′GACTCATGATAATGAACGCT CTCCAAGAA G3′ and reverse primer 5′GCCCTAGGCTTACAG TCTTATACACCTT CTT3′ having restriction sites *Bsp*HI and *Avr*II, respectively. The amplifications were carried out in 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 9.0 at 25 °C), 15 mM $(NH4)_2SO4$ and 0.1% Triton-X; 0.4 µM of primers; 200 µM of each dNTPs and 1 unit of Taq polymerase with conditions of an initial denaturation at 94 °C for 45 s, annealing at 54 °C for 45 s for VP3 and 58 °C for 45 s for NS1 and extension at 72 °C for 30 s for VP3 and 2 min for NS1. A final extension step of 10 min at 72 °C ended the reaction. PCR products were resolved in low melting point agarose gel and purified by MDI gel extraction kit. Initially, VP3 PCR product and expression vector pVIVO2-mcs were digested with restriction enzymes *Bam*HI and *Nhe*I, ligated and transformed into *E. coli* DH5α competent cells. This vector pVIVO.VP3 and the amplified NS1 gene were digested with restriction enzymes *Bsp*HI and *Avr*II, ligated and transformed into *E. coli* DH5α competent cells. The double gene construct was designated as pVIVO.VP3.NS1.

Flow Cytometry—The gene construct of pVIVO.VP3.NS1 was functionally assessed and characterized by flowcytometry in *in-vitro* cell culture system. HeLa cells (obtained from the National Centre for Cell Sciences, Pune) overlayed on a 6 well plate (Nunc) for flowcytometry were grown to 70-80 % confluency in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, USA), supplemented with 10% FBS, 100 U /ml penicillin, 100 µg/ml streptomycin (Gibco, USA) and 10 mM HEPES buffer (Duchefa Biocheme, The Netherlands) at 37 °C under 5% CO_2 (95% air). Endotoxin free transfection grade plasmid (pVIVO.VP3.NS1) was extracted using MDI Plasmid Maxi Kit as per the manufacturer's protocol and transfected with transfection reagent Lipofectamine 2000 (Invitrogen). Briefly, 4 µg of the plasmid (pVIVO.VP3.NS1) was mixed in 250 µl OptiMEM medium (Invitrogen) and allowed to stand at room temperature for 5 min. Simultaneously, 10 µl Lipofectamine was mixed with 250 µl OptiMEM medium in another tube and kept at room temperature for 5 min. The contents of both the tubes were then mixed together and kept at 37 °C for 30 min. Meanwhile, the 6-well plate of HeLa cells having the monolayer of the cells was washed with OptiMEM three times. After 30 min, 1 ml of OptiMEM medium was added drop-wise to the Lipofectamine/DNA mixture, mixed well and incubated at 37 °C with 5% CO_2. After 4 h incubation, the medium was replaced with 5% growth medium. The HeLa cells, transfected with pVIVO.VP3.NS1 and incubated for 48 h, were trypsinized and pelleted at 6000 rpm for 5 min. The cells were fixed with 4% PFA, permeabilized, blocked with BSA, washed with cold PBS and incubated with polyclonal antisera of VP3 and NS1 at 1:200 dilution in 2% blocking buffer, independently, and incubated with goat anti-rabbit FITC conjugate secondary antibody at 1:2000 dilution at 37 °C for 1 h. After incubation, cells were pelleted, washed twice with cold PBS and resuspended in 400 µl of 1X wash buffer and analyzed for VP3 and NS1 expression by flow cytometer using FL1 filter.

IFAT by Confocal microscope—For IFAT using confocal microscopy, after 48 h p.t., monolayer of the transfected cells on the glass cover slip were washed twice with cold PBS, fixed with 4% paraformaldehyde (PFA) for 20 min at RT, washed twice with ice-cold PBS for 5 min, permeabilized by treating with 0.2% Triton-X 100 in PBS for 5 min and washed again twice with cold PBS and blocked in 2% BSA (in PBS) for 1 h at 37 °C[15]. After washing for

5 min with cold PBS, cells were incubated with polyclonal antisera against VP3[16] and of NS1[17] at 1:200 dilutions in 1% BSA (In PBS) at 37 °C for 2 h, independently. The cells were then washed twice with cold PBS and incubated with goat anti-rabbit FITC conjugate secondary antibody (Santa Cruz) at 1:2000 dilution for 30 min at 37 °C. The cells were again washed thrice with cold PBS and observed the fluorescence under confocal microscope (Olympus).

Study of Apoptosis

DNA Fragmentation Assays—DNA fragmentation is one of the hallmarks in cells undergoing apoptosis detectable by gel electrophoresis, Hoechst staining, and terminal deoxynucleotidyl transferase TdT-mediated dUTP nick end labeling (TUNEL) assay. Here, untransfected HeLa cells acted as control.

Agarose gel electrophoresis—HeLa cells were transfected with pVIVO, pVIVO.VP3, pVIVO.NS1, pVIVO.VP3.NS1 and cells were harvested at 48 h p.t, and DNA was isolated and analyzed by electrophoresis on 1.5 % agarose gel as described by[12].

Hoechst staining—HeLa cells grown to 60-70 % confluency in a 4-well plate were transfected with pVIVO, pVIVO.VP3, pVIVO.NS1, pVIVO.VP3.NS1. After 48 h p.t., cells were processed for Hoechst staining. The cells were washed once with phosphate buffer solution (PBS) and fixed with 3 % PFA for 20 min at RT. Fixing solution was aspirated, and ice cold absolute methanol was added for 15 min to permeabilize the cells. Cells were washed thrice with PBS and incubated with Hoechst 33258 (0.12 µg/ml, final concentration in PBS) solutions for 15 min at RT. Cells were visualized with Nikon fluorescence microscopy under 40X objective[18].

TUNEL Assay—The DNA fragmentation (internucleosomal cleavage) in HeLa cells transfected with pVIVO, pVIVO.VP3, pVIVO.NS1, pVIVO.VP3.NS1 was also examined by APO-BrdU TUNEL Assay Kit (Invitrogen) as described by the manufacturer. Briefly, 2×10^6 cells were fixed with 5 ml of 1 % (w/v) paraformaldehyde for 15 min on ice. After washing, the fixed cells were resuspended in 0.5 ml of PBS and 5 ml of ice-cold 70 % (v/v) ethanol and incubated at −20 °C for 18 h. The cell pellet obtained after centrifugation was resuspended in 50 µl of the DNA labelling solution (10 µl of reaction buffer, 0.75 µl of TdT enzyme, 8.0 µl of BrdUTP, and 31.25 µl of dH2O) for 60 min at 37 °C in a water bath. After washing the cells with rinse buffer, cell pellet was incubated with 100 µl of the diluted antibody solution for 30 min at room temperature in dark. Stained cells were analyzed in a flow cytometer using FL1 filter.

Results and Discussion

VP3 known as apoptin is a serine-threonine rich protein of 121 amino acids. Among the major proteins of CAV, VP2 and VP3 are known to induce apoptosis in infected cells. Apoptin induces p53-independent apoptosis specifically in tumour cells[19]. Canine parvovirus 2, Non-structural 1 gene (NS1) is a novel candidate tumor suppressor gene that induces caspase dependent and p53 independent apoptosis[12]. The apoptotic potential of these two genes together has not been proven. Therefore, to establish the oncolytic potential of VP3 and NS1 genes together, we cloned these genes in pVIVO2-mcs. For this, the 367 bp amplicon of VP3 gene was amplified and cloned in MCS II of bicistronic expression vector pVIVO2-mcs. The desired recombinant plasmid pVIVO.VP3 was confirmed by PCR and restriction enzyme digestion (Fig. 1a). Similarly, the 2.022 Kb

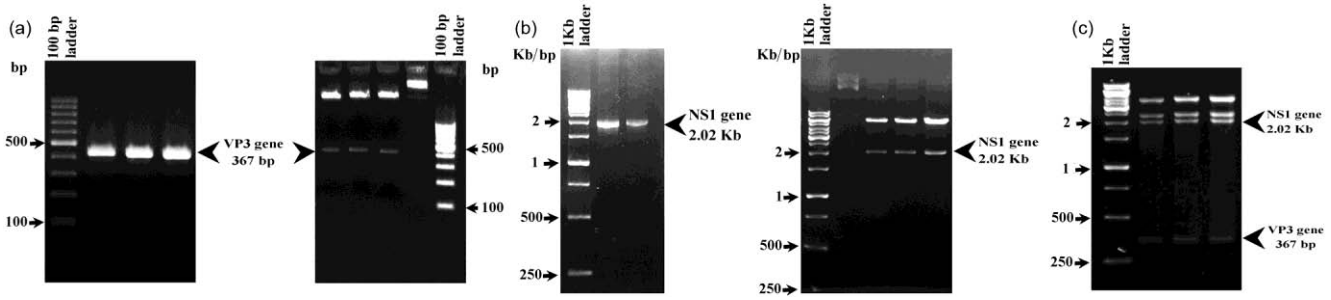

Fig. 1—Cloning of pVIVO.VP3.NS1. (a) PCR amplification of VP3 gene and digestion of pVIVO.VP3. Left image shows PCR amplification of VP3 gene (~367 bp). Right image shows the characteristic release of VP3 gene upon digestion with BamHI & NheI. Undigested DNA was run in the well adjacent to ladder; (b) PCR amplification and digestion of pVIVO.NS1. Left image shows PCR amplification of NS1 gene (2.02Kb). Right image shows the characteristic release of NS1 gene upon digestion with BspHI & AvrII. Undigested DNA was run in the well adjacent to ladder; (c) Digestion of pVIVO.VP3.NS1 for characteristic release of NS1 (2.02Kb) and VP3 (~367 bp).

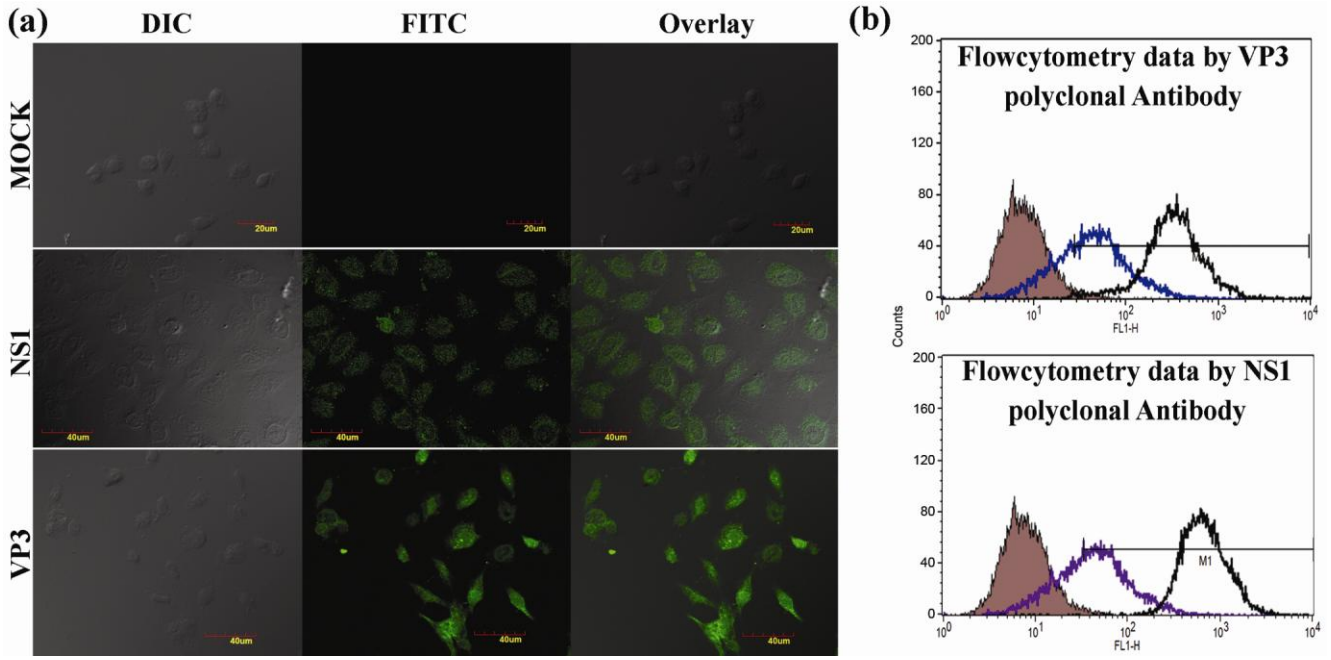

Fig. 2—Functional characterization of VP3 and NS1 expression by IFAT with confocal microscopy and Flow cytometry. (a) Confocal Microscopic images of HeLa cells transfected with pVIVO.VP3.NS1. Top upper panel showing the images of mock transfected HeLa cells; Middle panel showing HeLa cells transfected with pVIVO.VP3.NS1 and immunostained with NS1 specific antibody followed by FITC conjugated secondary antibody. The green bottom panel showing pVIVO.VP3.NS1 transfected HeLa cells immunostained with VP3 specific antibody followed by FITC conjugated secondary antibody. The fluorescent cells show expression of NS1 and VP3 expression in respective panels; (b) Flowcytometric quantification of VP3 and NS1 expression. There is a marked shift to right in pVIVO.VP3.NS1 transfected HeLa cells after staining with VP3 specific antibody (Top image) and NS1 specific antibody (Bottom image) followed by FITC conjugated secondary antibody independently.

amplicon of NS1 gene was amplified and cloned in MCS I of bicistronic expression vector pVIVO2-mcs. The recombinant clones containing the gene construct with VP3 and NS1 were confirmed by PCR for both the genes with their respective primers and by restriction enzyme digestion, *BamHI* and *NheI* for release of VP3 insert; and *BspHI* and *AvrII* for the release of NS1 insert (Fig. 1b & c). Confocal microscopy was performed to check the expression of NS1 and VP3 in HeLa cells using individual polyclonal antibodies of VP3 and NS1[16,17]. Fluorescence in the HeLa cells transfected with pVIVO.VP3.NS1 indicated the expression of NS1 and VP3[20] (Fig. 2a). The expression of VP3 and NS1 was further quantified by flow cytometry. The pVIVO.VP3.NS1 transfected cells showed marked difference as compared to control due to the binding of VP3 and NS1 specific polyclonal antibodies with the expressed VP3 and NS1 in HeLa cells following the binding of FITC labelled secondary antibody (Fig. 2b). The gene construct pVIVO.VP3.NS1, characterized for expression of VP3 and NS1 was then evaluated for its apoptotic potential. Cells undergoing apoptosis are characterized by distinct morphological and biochemical changes including chromatin condensation, membrane blebbing, nuclear breakdown, internucleosomal DNA fragmentation and TUNEL assay. The cell death due to apoptosis is marked by landmark changes such as nuclear condensation, its break down and DNA fragmentation. These changes form the basis of Hoechst staining, formation of DNA ladder and TUNEL assays[21]. In the cells undergoing apoptosis, activated cellular nucleases eventually digest cellular DNA into fragments consisting of multimers of approximately 200 base pairs which can be detected qualitatively by agarose gel electrophoresis and Hoechst staining, and quantitatively by TUNEL assay. The characteristic DNA ladder pattern noticed on agarose gel electrophoresis and presence of fragmented nuclei upon Hoechst 33342 staining, at 48 h p.t. in the present study indicated DNA fragmentation. This DNA fragmentation was further evaluated by TUNEL assay which relies on the presence of nicks in the DNA that are identified by terminal deoxynucleotidyl transferase, enzyme that

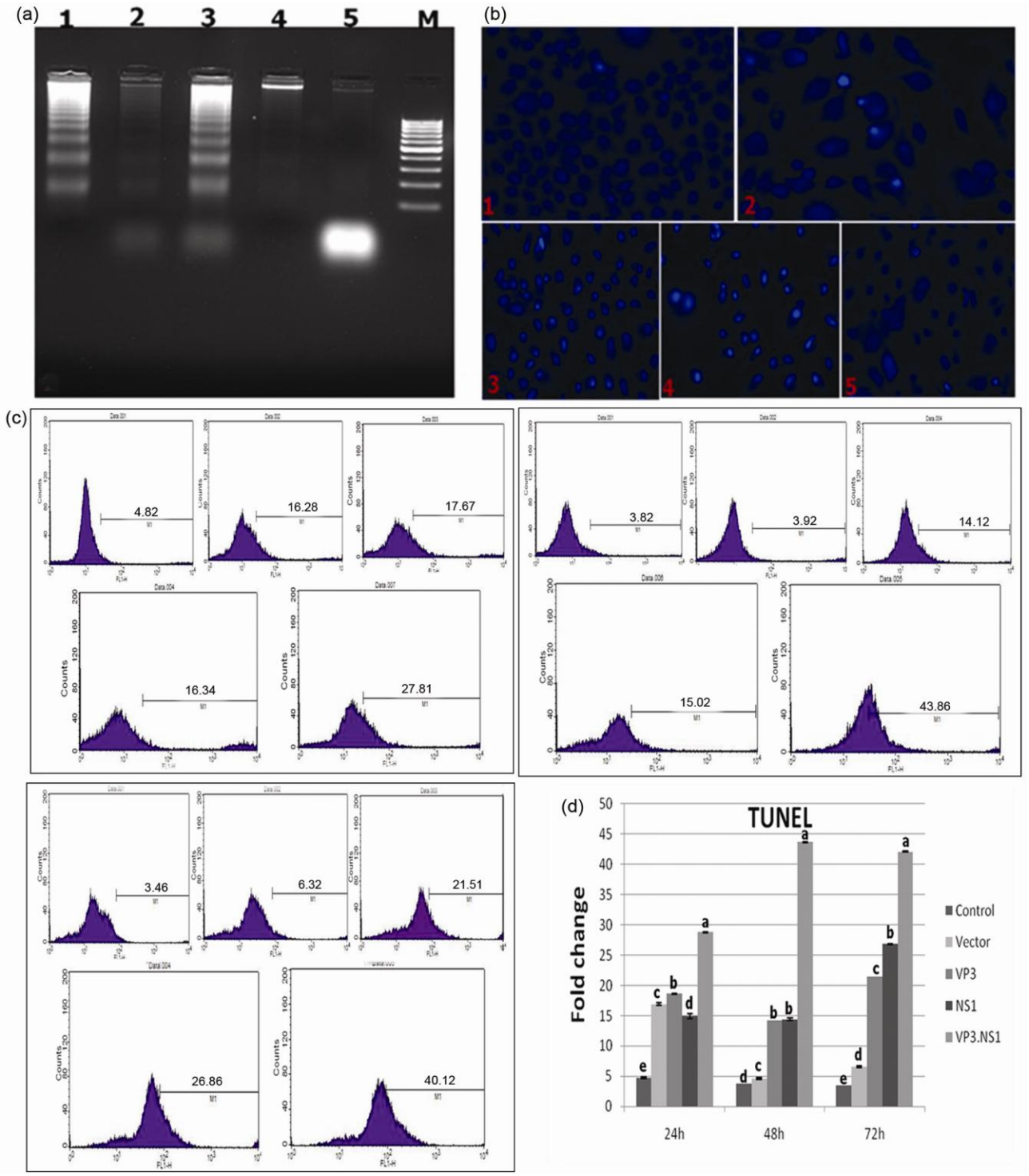

Fig. 3—(a) DNA Fragmentation in double gene construct induced apoptosis in HeLa cells after 48 h p.t. [Lane M: 100 bp marker, Lane 1: pVIVO (vector control) transfected HeLa cells, Lane 2: pVIVO.VP3 transfected HeLa cells, Lane 3: pVIVO.NS1, Lane 4: pVIVO.VP3.NS1 transfected HeLa cells and Lane 5: control cells]; (b) Hoechst staining of 48 h p.t, HeLa cells (1) Control cells, (2) pVIVO.VP3 transfected HeLa cells, (3) pVIVO.NS1 transfected HeLa cells, (4) pVIVO.NS1 and (5) pVIVO.VP3.NS1 transfected HeLa cells. No condensation of chromatin was found in figs 3b-1 and 3b-2. Figs 3b-3 to 3b-5 show condensation of chromatin/ fragmented nuclei; (c) Percentage of TUNEL positive cells in double gene construct transfected HeLa cells by flow cytometry at 24, 48 and 72 h p.t.; (d) SAS analysis of Tunel positive cells showing significant increase in double gene constructs transfected HeLa cells by flow cytometry.

catalyzes the addition of dUTPs that are secondarily labelled with a marker. The increase in TUNEL positive cells indicated DNA nicking and fragmentation in transfected cells. The significant difference in TUNEL positive cells between the pVIVO, pVIVO.VP3, pVIVO.NS1, pVIVO.VP3.NS1 transfected HeLa cells and mock control cells at all time intervals suggested apoptosis. Hoechst dye which binds to the minor groove of DNA has been extensively used by many workers for studying nuclear changes occurring during apoptosis[22]. DNA isolated from HeLa cells transfected with pVIVO, pVIVO.VP3, pVIVO.NS1, pVIVO.VP3.NS1 was analyzed in 1.5 % agarose gel. Presence of DNA ladder at 48 h p.i. suggested fragmentation of DNA and induction of apoptosis in HeLa cells after 48 h of transfection (Fig. 3a). No characteristic DNA ladder pattern was seen in mock control cells. The HeLa cells transfected with pVIVO, pVIVO.VP3, pVIVO.NS1, pVIVO.VP3.NS1 also exhibited highly condensed chromatin/fragmented nuclei upon staining with Hoechst 33342, 48 h p.t. (Fig. 3b). This DNA fragmentation was further confirmed by TUNEL assay. At 24, 48 and 72 h p.t., 4.75±0.129, 14.93±0.42, 16.80±0.199, 28.81±0.04, 18.59±0.043 percent cells; 3.76±0.03 4.63±0.13, 14.18±0.02, 14.40±0.22, 43.68±0.10 percent cells and 3.47±0.05, 6.35±0.12, 21.42±0.04, 26.82±0.04, 43.68±0.10 percent cells were TUNEL positive in mock control, pVIVO, pVIVO.VP3, pVIVO.NS1, pVIVO.VP3.NS1 transfected HeLa cells, respectively (Fig. 3c). A significant increase ($P \leq 0.05$) in double construct transfected HeLa cells (Fig. 3d) suggested a synergestic apoptotic effect of both the genes NS1 and VP3.

Conclusion

Viral gene oncotherapy is a promising tool to treat cancer. In order to meet stringent criteria for safety and efficacy for successful application, instead of viruses, use of viral genes for cancer therapy is fast emerging. In this report we cloned viral genes NS1 and VP3 in a bicistronic vector pVIVO2-mcs. The laser scanning confocal microscopy and flowcytometry results have confirmed the expression of NS1 and VP3 in HeLa cells. This bicistronic construct was found to induce apoptosis in HeLa cells.

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