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Anti-neoplastic effect of avian reovirus σ C protein on Rous sarcoma virus-induced tumors in chicken

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Summary. – This study investigated the anti-neoplastic potential of avian reovirus σC (sigma C) protein on Rous sarcoma virus-induced fibrosarcoma in chicken. The recombinant vector expressing σC protein was injected intra-tumorally into specific pathogen free chicken with fibro-sarcoma at the dose 100μg per bird, while control birds were mock-treated with 100μg of empty vector per bird. Recombinant σC protein induced apoptosis in tumors of treated birds resulting in progressive tumor regression, while similar changes were absent in tumors of mock-treated controls. The σC protein-induced apoptosis in tumors was quantified by flow cytometry and the mean level of apoptosis up to 66% was observed in treated tumors, whereas any significant level of apoptosis was absent in mock-treated controls.

Keywords: avian reovirus; Rous sarcoma virus; tumors; σC protein; apoptosis

Introduction

Oncolytic viral therapy generally involves viruses that have been genetically engineered to infect cancer cells and induce cell death through the propagation of the virus, expression of cytotoxic proteins and resultant cell lysis (Mullen and Tanabe, 2003). Viral proteins capable of inducing tumor-specific apoptosis can target and destroy cancer cells, while remaining innocuous to the rest of the body.

Mammalian reovirus (MRV) is a proven natural oncolytic agent, specifically replicating in cells with activated Ras signaling pathway (Kelly *et al.,* 2009). It is singular among oncolytic viruses entering clinical trials insofar, as it is an un-engineered viral therapeutic agent by virtue of its intrinsically benign nature and endogenous oncolytic property (Hashiro *et al*., 1977; Duncan *et al*., 1978; Strong and Lee, 1996; Strong *et al*., 1998; Egan *et al*., 2003). In normal cells, the activation of the double stranded RNA-activated protein kinase system prevents significant viral replication, whereas in malignant cells with an activated Ras pathway this cellular antiviral response mechanism is perturbed and viral replication occurs, leading to cytolysis of the host cell (Heinemann *et al.,* 2011). About 30% of all human tumors have activated Ras signaling pathway (Bos, 1989), while more than 70% of the human population is seropositive to MRV (Minuk *et al*., 1985, 1987).

Avian reoviruses (ARVs) differ from their mammalian counterparts in their lack of hemagglutination activity (Glass *et al*., 1973), their ability to induce cell fusion (Bodelon *et al*., 2001), and association with naturally occurring pathological conditions in chicken and turkeys (Robertson and Wilcox, 1986). ARVs cause disease conditions like reovirus-induced arthritis, chronic respiratory disease, and malabsorption syndrome in poultry (Fahey and Crawley, 1954; Hieronymus *et al*., 1983; Kibenge and Wilcox, 1983). The σC (*sigma* C) protein of ARV, which is analogous to the σ1 (*sigma* 1) protein of MRV, has been reported to be the virus attachment protein (Martinez-Costas *et al.,* 1997). It is the target for type-specific neutralizing antibodies (Wickramasinghe *et al*., 1993). Since the natural hosts for ARVs are various avian

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Abbreviations: ARV(s) = avian reovirus(es); AO/EtBr = acridine orange/ethidium bromide; CEF = chicken embryonic fibroblast; H&E = hematoxylin and eosin; MRV = mammalian reovirus; MAb = monoclonal antibody; σC = sigma C, σ1 = sigma 1; SPF = specific pathogen free; RSV = Rous sarcoma virus

species (Rosenberger, 2003); mammals including humans and domestic animals could be expected to be seronegative to ARV.

Avian reovirus induces apoptosis in primary chicken embryonic fibroblast (CEF) cells and several mammalian cell lines (Neelima *et al.*, 2003; Labrada *et al.*, 2002). It has been shown that the σC protein of ARV strain S1133 induces apoptosis in both BHK-21 and Vero cells in a dose- and time-dependent manner (Shih *et al.*, 2004).

The present study investigates the *in vivo* anti-neoplastic effect of ARV σC protein on Rous sarcoma virus (RSV)-induced tumors in chicken.

Materials and Methods

Virus. An Indian field isolate of ARV (VA-1) used in the present study was obtained from the Section of Avian Diseases, Indian Veterinary Research Institute. The ARV isolate VA-1 was propagated in CEF primary cultures prepared from 10–11-day-old SPF (specific pathogen free) chicken embryos as per the method described by Merchant *et al.* (1960). The isolate was serially passaged five times in CEF cell cultures and total RNA was extracted from infected CEF cells by TRIzol (Sigma, USA) method. The concentration and purity of extracted viral RNA was determined by A_{260} and A_{280} in a spectrophotometer (UV-1201 Spectrophotometer, Shimadzu) before further use.

RT-PCR. The whole σC-encoding gene of the ARV isolate VA-1 was amplified from the total RNA extracted from infected CEF culture by RT-PCR using self-designed σC-specific primer pair ARVσCF (5′-TAGTTCATTGGGATGGCGGGTCTCAATCCAT-3′) and ARVσCR (5′-ACGGCGCCRC-ACCTTAGGTRTCGATGCC-3′). The protocol described by Zhixun *et al.* (1997) was used for cDNA synthesis by reverse transcription with random primers and PCR using σC-specific primer pair to amplify the whole σC-encoding gene of 1006 bp. The thermal cycling profile consisted of denaturation at 94°C for 1 min, annealing at 69°C for 1 min and extension at 72°C for 1.5 min for 35 cycles, followed by a final extension at 72°C for 10 min.

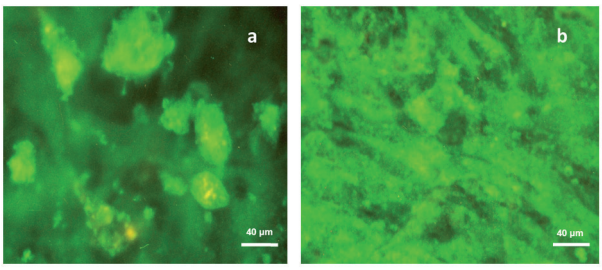

Fig. 1

Expression of the ARV σC protein in CEF

Cells at 72 hrs post transfection with the recombinant vector expressing σC protein (a) and the empty vector (b, negative control), respectively.

Cloning. The 1006 bp PCR product comprising the whole σC-encoding gene of the VA-1 isolate was purified using QIAquick gel extraction kit (Qiagen, Germany) and cloned into InsT/Aclone vector (Fermentas, USA). The recombinant clones obtained were screened by PCR and restriction analysis for confirmation of the presence of the desired gene insert. The gene insert was sub-cloned into pVAX1 (Invitrogen, USA) expression vector using Rapid Ligation and Transformation kit (Fermentas, USA). The recombinant clones were selected in LB-Kanamycin agar plates and further screened by PCR and restriction analysis for the presence of the desired gene insert.

Expression of σC protein in CEFs. A single recombinant clone was propagated in LB-Kanamycin broth and the recombinant pVAX1 plasmid DNA containing the whole VA-1 σC gene was extracted using Endo Free Plasmid Mega Kit (Qiagen, Germany) following the recommended protocol. The concentration and purity of the extracted plasmid DNA was determined using a spectrophotometer (UV-1201 Spectrophotometer, Shimadzu). The CEF monolayers seeded in 6-well tissue culture plates showing 60% confluence were transfected with the recombinant plasmid using Lipofectamine plus reagent (Invitrogen, USA) following the protocol recommended by manufacturer. The monolayers were screened 48 hours after transfection by indirect immuno-fluorescent technique with anti-ARV σC monoclonal antibody (MAb) and anti-mouse IgG FITC conjugate (Upstate, USA) to confirm the *in-vitro* expression of σC protein (Fig. 1).

Assessment of the oncolytic activity of σC protein. Fibrosarcomas were experimentally induced in thirty one-day-old SPF chicken of either sex by injecting RSV (freeze-dried tumor suspension dissolved in sterile PBS) subcutaneously in the wing-web region. Birds started developing tumors by 7 days of age and tumors of perceptible size were observed in all birds by 10 days of age. The birds were randomly divided into two experimental groups at 10 days of age and treatments given as follows.

1. σC protein-treated birds: Intra-tumoral injection of recombinant pVAX1 plasmid containing the whole VA-1 σC-encoding gene of ARV was given at the dose of 100 µg per bird.
2. Control birds: Intra-tumoral injection of pVAX1 plasmid was given at the dose of 100 µg per bird.

The birds of the two experimental groups were reared under strict isolation and observed for signs of tumor regression. In both groups, half of the birds were sacrificed humanely at 4 days post treatment and the rest at 7 days post treatment to study the anti-neoplastic effect. Tumors were surgically excised and weights recorded as to compare tumor weight: body weight ratio of birds in either experimental group. The data obtained was analyzed statistically according to Snedecor and Cochran (1980). The collected tumor tissues were analyzed for apoptotic changes using fluorescence microscopy, light microscopy and flow cytometry.

Fluorescence microscopy. Fine cryosections of 2–3 µm thickness were made from tumor tissues of sacrificed birds from both experimental groups in a cryotome. The sections were mounted on clean microscope slides and fixed for 15 min using chilled acetone.

The fixed sections were washed three times in PBS (pH 7.5) and the acridine orange/ethidium bromide (AO/EtBr) dye mixture containing acridine orange (1 mg/ml) and ethidium bromide (1 mg/ml) in the ratio 1:1 (v/v) was added to cover the entire tissue section. After 30 min incubation at room temperature, the slides were washed thrice in PBS and air-dried. A drop of PBS was added to cover the stained section and the slides were viewed under a UV microscope (Nikon, Japan).

Light microscopy. The tumor tissues from all sacrificed birds of both experimental groups were collected in 10% buffered formalin and fine sections made with a microtome after proper fixing and embedding. The tissue sections were stained with hematoxylin and eosin (H&E) stain and screened for cytomorphological changes associated with apoptosis under the light microscope as described by Lee (1993).

Flow cytometry. Single cell suspensions of the collected tumor samples from all sacrificed birds of various experimental groups were prepared according to the method described by Nicolleti *et al.* (1991) with some modifications. These were then analyzed by flow cytometry for quantitation of apoptosis using Fluorescence Activated Cell Sorter (FACS) Calibur (Becton Dickinson, San Jose, USA). Two hundred µl of PBS and 2 µl Merocyanine 540 dye (Sigma, USA) at a final concentration of 30 µmol/l was added to 200µl of cell suspension and mixed well. Cells were incubated in dark at room temperature for 10 min and immediately analyzed in flow cytometer for DNA content and hypodiploid or sub-G1 DNA peaks of apoptotic cells. Forward scatter (FSC for size) and side scatter (SSC for granularity) counts were recorded using excitation wavelength of 488 nm by an argon laser with detection at 575 ± 26 nm. The data obtained was analyzed statistically according to Snedecor and Cochran (1980).

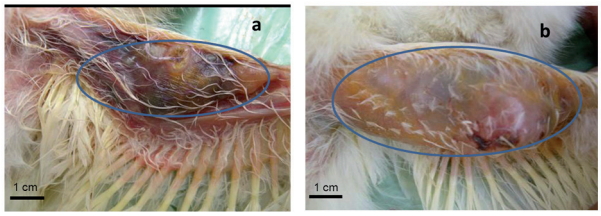

Fig. 2

Tumor regression caused by the ARV σC protein

Tumors treated with the recombinant vector expressing σC protein (a) and empty vector (b, negative control), respectively.

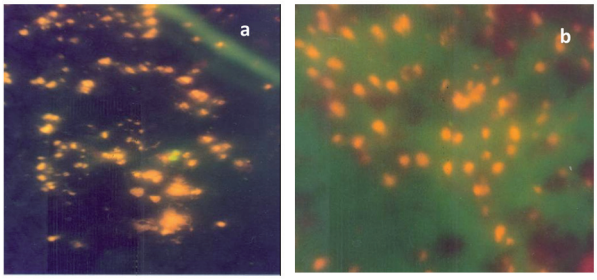

Fig. 3

Apoptotic subcellular changes in tumors caused by the ARV σC protein

AO/EtBr staining, fluorescence microscopy. Tumors treated with the recombinant vector expressing σC protein (a) and empty vector (b, negative control), respectively.

Results

The present study investigated the anti-neoplastic potential of ARV σC protein by *in vivo* experimental inoculation with the recombinant eukaryotic expression plasmid vector expressing the whole σC gene of the ARV isolate in RSV-induced tumors of SPF chicks.

By 4 days post treatment, tumors in σC protein-treated birds showed gross signs of tumor regression, in contrast to the progressive tumor morphology observed in mock-treated tumors of control birds (Fig. 2). The oncolytic effects of the recombinant σC protein of ARV on RSV-induced tumors were analyzed by measuring the relative reduction in tumor weights in birds of the two experimental groups. The mean percentage tumor weight: body weight ratio of birds in σC protein-treated group was significantly lower than that of control birds at 4 and 7 days post treatment. Mean percentage tumor weight: body weight ratio of σC protein-treated birds at 4 and 7 days post treatment were 4.1333 ± 0.367 and 5.467 ± 0.669, respectively, compared to 20.233 ± 1.775 and 21.667 ± 1.637 of control birds. Gross tumor regression could be detected at the site of administration in birds treated with the recombinant plasmid. The regression level was significantly different from the control group as evidenced by the tumor weight to body weight ratio, which indicates the oncolytic potential of ARV σC protein in RSV-induced fibro-sarcomatous tumor.

Apoptosis was detected in tumor tissues of birds subjected to oncolytic treatment by staining cryosections of tumor samples from birds of the two experimental groups with AO/EtBr stain mix. Screening of stained cryosections by fluorescence microscopy detected the characteristic sequential nuclear changes of apoptosis (Fig. 3) i.e. condensation, fragmentation and formation of apoptotic bodies in tumors from σC protein-treated birds, whereas apoptosis-associated nuclear changes could not be detected in any of the screened tumor sections from control birds.

Histopathological analysis of tumor tissues obtained from birds treated with σC protein revealed progressive changes of cell death due to apoptosis (Fig. 4). The cells showed characteristic nuclear changes of apoptotic cell death, like condensation of nuclei, fragmentation of chromatin and formation of apoptotic bodies. Such apoptotic changes were

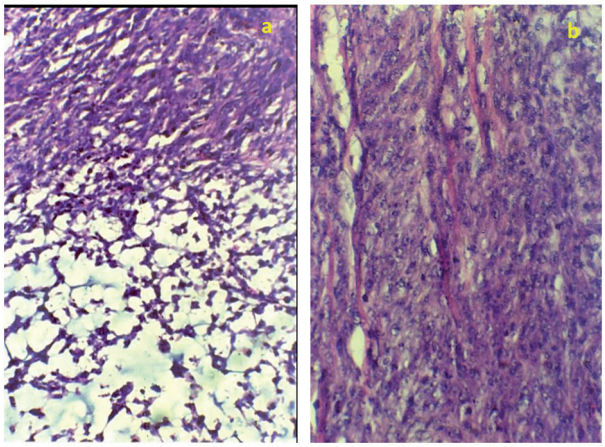

Fig. 4

Apoptotic subcellular changes in tumors caused by the ARV σC protein

H&E staining, light microscopy. Tumors four days post treatment with the recombinant vector expressing σC protein (a) and empty vector (b, negative control), respectively.

progressive towards the healthy portion of tumor tissue, spreading from the central zone towards the periphery. Cells that underwent apoptosis exhibited loss of nuclear material and dissolution of cell structure adjacent to normal cells in the tumor.

Natesan and co-workers (2006) reported similar nuclear morphological changes in RSV-induced tumors of SPF chicks after intra-tumoral administration of recombinant pVAX-1 plasmid vector expressing the chicken anemia virus whole viral protein 3 (VP3). Such characteristic morphological changes of apoptosis have also been reported in *in-vitro* studies involving other viral agents like Newcastle disease virus (Lam and Vasconcelos, 1994) and infectious bursal disease virus (Vasconcelos and Lam, 1994) by using similar techniques.

Apoptosis contributing to oncolysis of tumors in birds of both the groups was quantitated by flow cytometry analysis of single cell suspensions prepared from tumor samples stained with merocyanine 540 fluorescent dye. The mean percentage of hypodiploid nuclei as measured by flow cytometry was significantly higher in tumors of σC protein-treated group than the basal level of apoptosis observed in tumors of control birds, at both post-treatment intervals of 4 and 7 days. Up to 66% mean apoptosis was observed in σC protein-treated tumors (Fig. 5). This indicated that the oncolytic effect of ARV σC protein resulted from the protein-induced apoptosis of tumor cells. Avian reoviruses, like other fusogenic reoviruses, cause fusion-associated small transmembrane (FAST) protein-mediated cell fusion and syncytium formation, leading to diminished membrane integrity, which in turn activates DNA fragmentation, chromatin condensation, and eventually apoptosis (Salsman *et al.*, 2005). The absence of apoptosis in control birds ruled out the possibility of any *in-vivo* pVAX1 vector-induced anti-tumoral response.

This study unraveled the anti-neoplastic potential of the ARV σC protein. A gene therapy-based approach, utilizing the anti-neoplastic potential of the σC protein, which is the counterpart of the apoptotic σ1 protein of MRV, offers an alternative to the use of wild MRVs, which may be pathogenic to immuno-compromised cancer patients. Mammalian

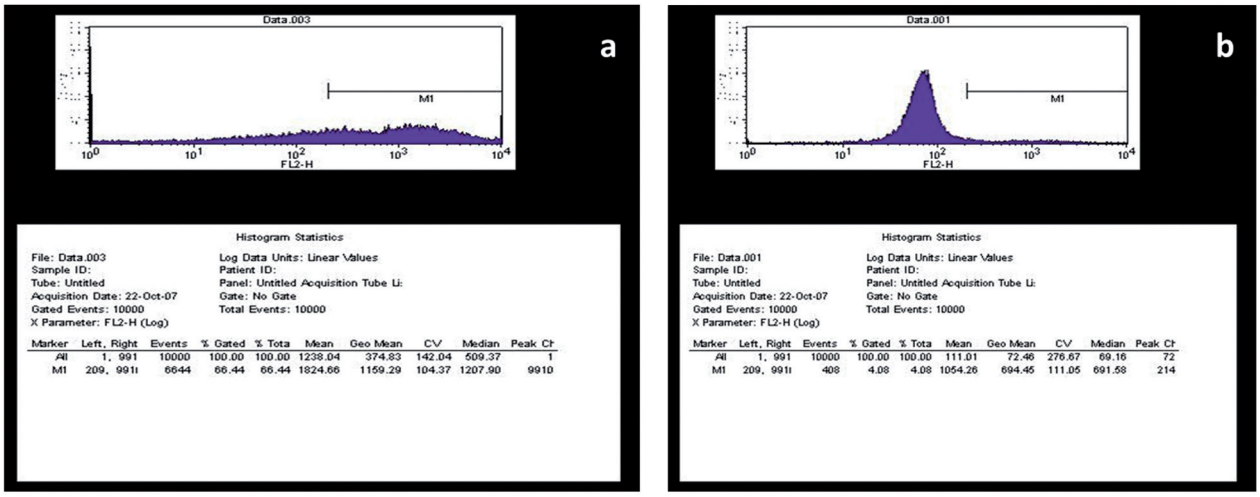

Fig. 5

Effect of the ARV σC protein on the percentage of apoptotic cells in tumors

Flow cytometry. Tumors 4 days post treatment with the recombinant vector expressing σC protein (a) and empty vector (b, negative control), respectively.

reovirus is a proven natural oncolytic agent with inherent tumor selectivity, which specifically replicates in cells with an activated Ras signaling pathway (Coffey *et al.*, 1998; Norman and Lee, 2005). Ras-activating mutations promote angiogenesis, metastasis and loss of growth control, and are present in 30 to 40% of all human tumors (Bos, 1989). The outcome of our study is tempting us to speculate that the σ1 protein of MRV, which is the functional analogue of ARV σC protein, mediates MRV-induced oncolysis and could be used for oncolytic gene therapy of tumors that do not have an activated Ras signaling pathway and hence are non-permissible to MRV replication and lytic infection.

The gene therapy-based approach, which exploits the anti-neoplastic potential of ARV σC protein, could be applied for treatment of human and animal cancers without any inherent derangement of the Ras signaling pathway. Such tumor cells may not be permissible to the wild reovirus replication and hence not amenable to the natural oncolytic potential of reoviruses. In contrast, such tumors could be treated with recombinant plasmids capable of expressing σC protein of ARVs or possibly σ1 protein of MRVs. Though, in this case, the anti-neoplastic potential of an ARV protein was tested in an avian species, which is the natural host for the virus, the capacity of avian reovirus to replicate and adapt to mammalian cell lines like Vero (Rosenberger, 2003) indicates the possibility of using ARV σC protein for oncolytic therapy of mammalian tumors as well.

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