

## Apoptosis and Tumour Cell Death in Response to Pro-apoptotic Gene

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### ABSTRACT

The process of cell death, or apoptosis, is commonly defined by its distinct morphological characteristic. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, the cellular antiviral response pathway, embryonic development and chemical-induced cell death. In this study, retroviral vector was utilized as the gene delivery vehicles and the tumour-specific viral death effector VP3 as pro-apoptotic agent for malignant colon cancer cells treatment. Here, pro-apoptotic gene inducing apoptosis in CT26 colon tumour cells is reported. In addition, the activation of a typical apoptotic programme was observed in response to this therapeutic agent. An increased number of colon tumour cells suffered apoptosis upon treatment with pro-apoptotic agent. As a result, these tumour cells displayed loss of membrane asymmetry and impermeability, cell shrinkage, nuclear condensation and DNA fragmentation under H&E images. In the presence of VP3, TEM also confirmed that VP3-treated CT26 tumour cells showed apoptotic features. These results suggested that VP3 was a potential pro-apoptotic agent and the apoptosis induced by VP3 was a key anti-tumour mechanism.

**Keywords:** CT26, mouse models, pro-apoptotic agent, retroviral vector, tumour, VP3

### INTRODUCTION

Apoptosis is generally characterized by its distinct morphological characteristics such as shrinkage of cells, segmentation of nucleus, condensation, and cleavage of DNA into domain-sized fragment in most cells followed by nucleolus degradation (Wagstaff and Jans, 2009). These characteristics of cell death may be identified easily in histological sections stained with haematoxylin and eosin (H&E) reagents. The identification and counting of apoptotic cells in the H&E staining have been extensively used to study apoptosis induced by radiation or other cytotoxic agents for many years (Matsuu-Matsuyama *et al.*, 2008). This stain is also

excellent for displaying tissue morphology, but insufficient to distinguish between the apoptotic and necrotic cells. Thus, powerful tools, such as transmission electron microscopy (TEM), are needed to solve this matter. In addition, TEM is especially used to distinguish between apoptotic and necrosis in oncolytic cell population, as these processes may share common features (Miller *et al.*, 2002). Normally, apoptotic cells have intact membranes and organelles, as well as condensed nuclear membrane fragments. Therefore, many references found on the subject of apoptosis were appropriately observed and recorded by using the TEM equipment (Wyllie *et al.*, 1999; Blankenberg *et al.*, 2000).

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In the present study, a recombinant of retroviral vector containing pro-apoptotic gene (VP3 gene) was used. VP3 which is also known as apoptin is a 14-kDa basic protein derived from the chicken anemia virus (CAV) that can induce apoptosis in transformed and malignant cell lines (De Smit and Noteborn, 2009). This pro-apoptotic gene was predominantly found in the cytoplasm, whereas in transformed and tumour cells, it was located in the nucleus, suggesting that the location of apoptin is related to its activity (Los *et al.*, 2009).

The present study focused on determining VP3 induced apoptosis in light microscopic assessment and ultrastructure of tumour cells *in vivo* level. This particular study also concentrated on our studies with CT26 tumour-bearing mice. Apoptosis induced by VP3 in the treated tumour was analyzed using the H&E staining and TEM analysis. This combination of H&E staining and TEM application would provide us with a powerful approach to obtain useful information about the image of tumour cells, and to explain the results at the ultrastructural level.

## MATERIALS AND METHODS

### *Cell Culture and Reagents*

CT26 cells obtained from the ATCC were grown in high-glucose RPMI6410 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), penicillin (Gibco, USA) and streptomycin (Gibco, USA). Meanwhile, retrovirus packaging cell lines, PT67 cells from ATCC, were maintained in high-glucose DMEM medium supplemented with 10% FBS.

### *Transfection and Virus Production*

The recombinant retrovirus of pMSCV-VP3 was transfected into PT67 packaging cells by Calcium Phosphate Transfection System (BD Biosciences Clontech, USA). The culture media were replaced after 12 h to remove the calcium phosphate and residual DNA. Stably transfected cells were selected in a medium containing 50 mg/mL neomycin (Sigma, USA). Pooled colonies of transfected cells were allowed

to grow to confluency, and viral supernatant harvested, following overnight incubation in serum-free DMEM for infections. All the supernatants were filtered (0.45 µm) for further step.

### *In vivo Experiments in Mice Model*

Balb/c mice at five weeks of age were kept at constant temperature in 12 h circadian cycles. They were provided with standard rodent chow and water *ad libitum*. For induction of tumour,  $1.0 \times 10^6$  CT26 mouse colon carcinoma cells in 200 µl of phosphate buffered-saline were subcutaneously injected into the right flank of each mouse. The appearance time of palpable tumour was recorded, and the longest perpendicular diameters were measured twice weekly using callipers. Tumours were allowed to grow for 7-10 days prior to administration of recombinant retrovirus particles. A total of 250 µl ( $3 \times 10^4$ ) of the retroviral supernatants, produced as described above, were administered to the tumour-bearing mice. Mice were sacrificed 48 h post-treatment, at which time small pieces of tumour tissues were then removed for histopathological processing and TEM analysis.

### *Histopathology*

Subcutaneous tumour tissues were fixed overnight in fresh buffered 4% paraformaldehyde, and subsequently processed through a serial of alcohol dehydration, chlorate, and paraffinization. All of the tissues were embedded in paraffin and sectioned at 3 µm. All slides were stained with hematoxylin-eosin and examined microscopically.

### *Transmission Electron Microscopic (TEM)*

The tissues were also particularly observed by the TEM in following procedure. They were cut into small pieces (2-4 mm) and fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2) at 4°C for 4 h. The tissues were then rinsed with cold PBS and post-fixed in 1% osmium tetroxide at room temperature for another 2 h. After dehydration with gradient ethanol and twice with

acetone for 15 min each time, the samples were embedded in resin and acetone (1:1) for 30 min, followed by 100% resin for 1 h. Finally, resin was solidified at 37°C for 24 h and at 60°C for another 48 h. Ultrathin sections were obtained and subsequently stained with uranyl acetate and lead citrate for examination by TEM.

## RESULTS AND DISCUSSION

### Histopathology

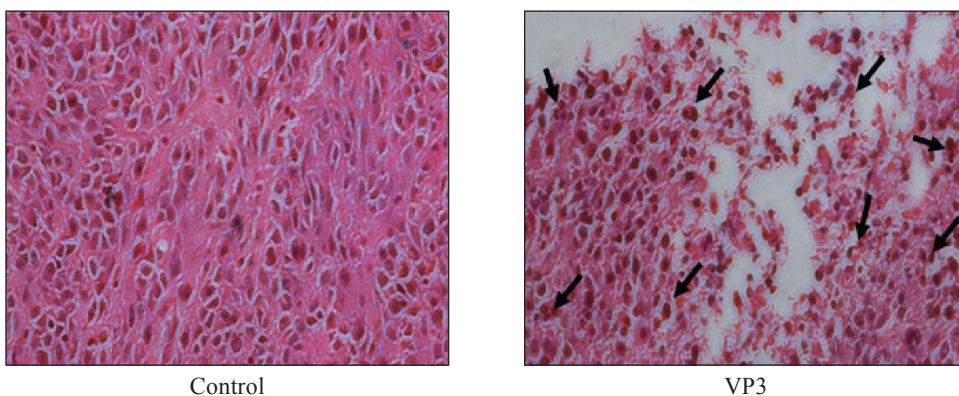
Apoptosis and tumour cell death in response to cytotoxic drug i.e., pro-apoptotic gene was normally assessed by monitoring the appearance of typical nuclear morphological changes and internucleosomal DNA cleavage. In this study, Balb/c mice were sacrificed after 48 h of the last injection of recombinant retroviral supernatants. The anti-tumour effect of VP3-expressing tissues in cellular biology was histopathologically observed using the H&E staining. In VP3-treated tumours, the induction of apoptosis by VP3 was confirmed by the cytomorphological features, which include nuclear condensation, rounded dark nucleic, presence of interstitial space and formation of apoptotic bodies (*Fig. 1*). Similar characteristics of the morphological changes of cells undergoing apoptosis have been reported (Pulkkanen *et al.*, 2002; Natesan *et al.*, 2006; Pietersen *et al.*, 1999).

### TEM

For further determination of apoptosis response in subcutaneous tumour, the samples were evaluated using the TEM analysis, which is the best method for morphological observation through clearly differentiating nucleic and organelle. It is important to note that this approach is normally used to distinguish and confirm the presence of different alterations affecting tumour cells due to apoptosis or necrosis (Reid *et al.*, 2003). The ultrastructures of the morphological changes during apoptosis were also seen, including the reduction in volume of the nucleus, condensed nuclear material and nuclear membrane blebbing, all of which could be found in VP3-treated tumours (*Fig. 2*). Supporting this was the presence of some intact organelles in the cytoplasm. In addition, these data showed that the administration of retroviral-mediated VP3 gene was able to induce apoptosis in tumour cells.

## CONCLUSIONS

In conclusion, these data clearly demonstrated that cancer cells response against VP3 was predominantly found in nuclear membrane with distinctive morphological features. This investigation is also worthy for further consideration as a move approach in the treatment of solid tumours.



*Fig. 1: Apoptotic effect of pro-apoptotic gene; VP3 on subcutaneous tumour at the cellular level (arrows). CT26 tumour tissue was isolated from mice treated with pro-apoptotic gene (right panel) or control (left-panel) (H&E, x 200)*

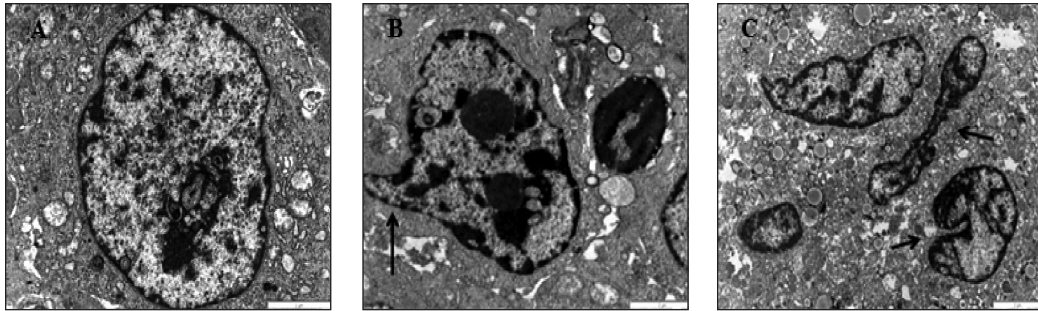


Fig. 2: TEM profiles showed control (A) and VP3-infected cancer cells (B&C).  
 A: Ultrastructure of control tumour cells ( $\times 5500$ ); B: Note the demonstrated nuclear blebs (arrow) ( $\times 12500$ ); C: The features of apoptosis showed the shrinkage of nuclear membrane which had become smaller (arrows) ( $\times 7700$ )

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