ORIGINAL ARTICLE

Green Fluorescent Proteins (GFP) Induce Apoptosis in Normal Nasopharyngeal Cells Expression

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ABSTRACT

Objective: **To evaluate the effect of Green fluorescent proteins (GFP) expression on apoptosis in normal nasopharyngeal cells.**

Material and Methods: **pTracer vector that contained GFP expression and pcDNA transfected into normal nasopharyngeal cell line. Apoptosis was accessed by measuring Caspase activities using Caspase-Glo ® 3/7 Assay kit following the manufacturer's protocol. The luminescence intensity was measured by microplate reader. Association of GFP expression with caspase activation was analysed by independent Sample T test.**

Results: **The association between caspase activity and GFP expression are statistically significant compared to pcDNA vector control cells.**

Conclusion: **Our results show that there is an association of GFP expression in pTracer vector with caspase activity in normal nasopharyngeal cell thus support that GFP expression induce apoptosis in normal nasopharyngeal cell.**

KEY WORDS

GFP, pTracer, pcDNA, Caspase Activity Nasopharyngeal Cell

INTRODUCTION

Apoptosis is a naturally occurring cell death process that is important in various biological systems[1]. It is characterised by a series of typical morphological features, such as shrinkage of the cell, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighbouring cells. Most of these morphological changes result from the activity of a class of cysteine proteases, called caspases[2]. A number of caspases have been identified, where about two-thirds of them function in apoptosis[3]. Caspases normally exist as inactive pro-enzymes. When apoptosis is triggered, caspases are converted into active enzyme to cleave a subset of proteins[4].

One of the key enzymes in apoptosis is caspase-activated DNase (CAD)[5]. Normally CAD exists as an inactive complex with its inhibitor, the Inhibitor of CAD (ICAD). During apoptosis induction, ICAD is cleaved by caspase-3, thus releasing the activated CAD, allowing it to cleave the genomic DNA into HMW DNA as well as internucleosomal DNA ladder[6]. Caspase-activated DNase is one of the enzymes involved in apoptosis and play an important role in chromosome rearrangement mostly found in leukaemia[7]. The finding also suggested that CAD may play an important role in chromosomal cleavages mediated by oxidative stress-induced apoptosis. Thus, a potential model for oxidative stress-induced apoptosis mediating chromosomal rearrangements in nasopharyngeal carcinoma (NPC) was proposed[8].

Apoptotic DNA fragmentation can be induced by a range of stimuli including virus infection and oxidative stress[9]. Oxidative stress induces apoptotic DNA fragmentation in endothelial cells under an ATP rich environment as well as in skeletal muscle myoblasts[10,11]. It was also found to induce the formation of High Molecular Weight (HMW) DNA fragmentation in leukemic cells[12]. Reactive oxygen species (e.g., H_2O_2, hydroxyl radical and superoxide), cause injuries on various cellular macromolecules. These damages have been proposed to contribute to the development of cancer[13]. Data indicated that NPC cells under stress undergo apoptosis-induced chromosome breaks[10]. Although apoptosis is a cell death process, it has also been implicated in chromosome rearrangement. Chemotherapeutic drug-induced apoptosis has been implicated in the introduction of chromosome break within the Mixed Lineage Leukaemia (MLL) gene, a gene frequently involved in chromosome translocations[7].

Green fluorescent protein (GFP) was used for protein tracking and enzyme activity detection, enhance fusion protein folding and is relatively small[14]. The GFP ability to quantitatively monitor gene expression has been demonstrated in different organisms[15]. Green fluorescent protein (GFP) was the first and has been the most used fluorescent protein[16]. The fluorescent chromophore of fluorescent proteins is formed through an intramolecular reaction between the side chains of certain amino acids localized inside the barrel structure of the protein[17]. It has been calculated that one molecule of hydrogen peroxide (H_2O_2) is generated independently of NAD(P)H during the maturation of each chromophore[18]. Current study design to evaluate the effect of GFP expression on oxidative stress generation in normal nasopharyngeal cell.

MATERIALS AND METHODS

Plasmid DNA

pTracer plasmid DNA was sub-cloning into pTracerTM-EF/V5-His B (pTracer). Recombination of pTracer then transformed by One Shot ®

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Table 1: Mean and Standard Deviation of Caspace Activity in Transfected NP69

Transfection Plasmid DNA	N	Mean	Std. Deviation	Std. Error Mean
pcDNA	100	271.52	95.827	9.583
pTracer	100	529.99	322.257	32.226

Table 2: Association of Caspase Activity with Vector Control Transfected Cells

Plasmid DNA		t value	Df	Sig. (2-tailed)	95% Confidence Interval of the Difference	
					Lower	Upper
pcDNA & pTracer	Equal variances assumed	-7.688	198	.000	-324.770	-192.170
	Equal variances not assumed	-7.688	116.372	.000	-325.057	-191.883

Electrocomp™ *E.coli* by electroporation. Subsequently, the restriction of the pTracer was done prior to the glycerol deposition into the -80℃ freezer. Recombinant plasmid with the GFP expression and Zeocin resistance gene was used in the respective transfection efficiency determination and stable transfectant selection experiments.

The plasmid pcDNA was then isolated by using the High Pure Plasmid Isolation Kit (Roche). Subsequently, restriction mapping of the pcDNA was done prior to the glycerol stock deposition into the -80℃ freezer.

Cell Culture

Cell line NP69 is an immortalized nasopharyngeal epithelial cell line was use in current study. The cell line was provided by Prof. Tsao Sai Wah and Prof. Lo Kwok Wai from University of Hong Kong and The Chinese University of Hong Kong. This cell line was established by transfection with SV40 large T oncogene. This cell line retains its normal nasopharyngeal epithelial cells and is non-tumourigenic. The cell line was culture in Keratinocyte-SFM medium supplemented with recombinant Epidermal Growth Factor (rEGF) (4-5 ng/ml), Bovine Pituitary Extract (BPE) (40-50 ug/mL), 2% (v/v) heat-inactivation fetal bovine serum and 1% penicillin-streptomycine (GIBCO, Invitrogen, USA). Then, the medium contained cell were incubated in a humidified 5% CO_2 incubator at 37℃.

Transfection

In this study, transfection using Lipofectamine ® 3000 Reagent. The transfection of all cell line consisted of pTracer and pcDNA confirmed by Western Blot to ensure the transfection contained the vectors before proceed to analysis. After third times passage, 5 x 10^2 cells of NP69 cell was added into 100 µl seeding medium and incubate 37?C for overnight. Then, replaced seeding medium with 100 ul transfection/ plain medium. Added 0.2 µg plasmid DNA into 10 µl optimum, 0.2 µl p3000 reagent and 0.3 µl 3000 reagent in 0.2 ml centrifuge tube. Then the mixture incubated at room temperature for 10 minutes. After incubation, the mixture was added into 96 well plate that already contain with NP69 cell with transfection medium and incubate for 4 hours. After 4 hours, transfection medium was replaced with 100 µl culture medium. The transfection completed after 48 hours.

Measurement of Caspases Activity in pTracer and pcDNA Transfection

NP69 cells of 70% confluency in a T-25 flask were with Trypsinized with TrypLE express, incubated for 4 minutes, and add with 4 ml of culture medium. Transfection of pTracer and pcDNA into NP69 was carried out for 48 hours in 100 µl growth medium per well in a white-walled 96-well plate with clear bottom (Corning). Caspase activity was measured by using Caspase-Glo ® 3/7 Assay kit according to manufacturer's manual. Caspase-Glo ® 3/7 Buffer and the lyophilized Caspase-Glo ® 3/7 substrate was equilibrated to room temperature prior to use. Caspase-Glo ® 3/7 Buffer was transferred into an amber bottle containing the Caspase-Glo ® 3/7 substrate. Solution was mixed by inverting the bottle until dissolved to form Caspase-Glo ® 3/7 reagent. Seeding medium from the plate and cells were washed with 1x PBS buffer (room temperature) once. A mixture of 100 µl of 1x PBS buffer and 100 µl of Caspase-Glo ® 3/7 reagent was added to the cells in the plate. The mixture was incubated at room temperature for 30 minutes. The plate was shake at amplitude of 1 mm (~174 rpm) for 30 seconds before and after incubation. The luminescence was measured by microplate reader.

Statistical Analysis

Association of GFP expression with caspase activity were derived using Statistical Package for Social Sciences (SPSS) version 23 by performed Independent Sample T test.

RESULTS

To measure the role of green fluorescence protein (GFP) with caspase activity that express together in pTracer expression by compare vector control of pTracer and pcDNA transfected cell. Table 1 shows the mean and standard deviation of caspase activity in cells transfected with pTracer and pcDNA. For pcDNA vector control transfected cells, the mean is 271.52 with standard deviation of 95.827. Mean of 529.99 and standard deviation of 322.257 were observed in pTracer vector transfected cell.

Table 2 shows the association of caspase activity with pTracer and pcDNA transfected cells. The association between caspase activity and pTracer expression are statistically significant with value of t (-7.688), p value less than 0.05 compared to pcDNA vector control cells.

DISCUSSION

Caspase-activated DNase (CAD) seems to be playing multiple roles. CAD is the apoptotic nuclease that found to play a role in chromosome rearrangement commonly found in leukaemia[19]. In addition, CAD was also shown to promote cell differentiation by inducing DNA strand breaks[20]. The finding also suggested that, CAD may play an important role in chromosomal cleavages mediated by oxidative stress-induced apoptosis as well as bile-acid induced apoptosis[21-23]. CAD is activated by caspase-dependent signal transduction, a proteolytic signalling cascade that alters the activity of numerous substrate proteins[24]. The CAD is associated with an inhibitor of CAD (ICAD) and possibly, the ICAD serves as a chaperone during the synthesis of CAD[5]. To promote DNA fragmentation, Caspase 3 activates CAD by proteolytic inactivation of the inhibitor of CAD[24]. While, Caspase 3 inactivates ICAD by cleaving at two aspartic acid residues, D117 and D224, destabilizing its interaction with CAD and allowing CAD dimerization and subsequent DNA fragmentation[6]. In a study of mouse ICAD and human CAD, expression of ICAD was reported to enhance the expression of endogenous and exogenous CAD[21]. In the study also reported that, ICAD expression induced endogenous CAD expression, it has also extensively reduced H_2O_2-induced *MLL* gene cleavage.

In current study, comparing vector control pTracer and pcDNA, pTracer shows induce apoptosis compared to pcDNA with statistically significant differences value. Our study supported by a study showing that initiation of the apoptosis cascade has been postulated as a possible mechanism for the toxicity of GFP and cellular death[25]. They also find out that, GFP caused redistribution of phosphatidylserine, an indicator of the apoptosis signalling cascade initiation, to the cell surface. Study shows that, in initiation apoptosis cascade, reactive oxygen production induced by GFP has been linked to cellular toxicity and eventual death in GFP expressing cells[26]. In addition, neuroblastoma cell lines, showed increased sensitivity to cytotoxic agents when transduced with GFP, eGFP, and YFP[27]. In another study, liver cells transfected with GFP plasmid have been shown to be increased cellular permeability following the initiation of cellular death[28].

Association study is the most applicable tool to access the gene sus-

ceptibility to complex diseases that involve high interaction between genetic and environmental factors. Many complex diseases have a variety of genetic variants that affect the disease risk even though with minimal effects. In current study, GFP expression shows contribution in apoptosis in normal nasopharyngeal cells. Thus, vectors that contain GFP expression are not suitable to use in study of apoptosis measurement.

CONCLUSION

Our results show that there is an association of GFP expression and apoptosis in normal nasopharyngeal cell, thus support that GFP expression involve in induced apoptosis process.

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