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Determination of cell survival rate in MCF-7 cells via calreticulin overexpression

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ABSTRACT

Calreticulin (CRT) protein has multifaceted role in apoptosis. Since the molecular mechanism that express and perform apoptotic cell death are coming into focus, we attempt to isolate the human calreticulin gene from blood, cDNA synthesized from the CRT was cloned to mammalian expression vector pcDNA to construct a recombinant CRT plasmid. Transfection of the MCF -7 cell line pcDNA3-CRT plasmid leads to cell death by promoting apoptosis. The transformed cells exhibited nuclear margination, membrane blebbing and chromatin condensation that are typical for cells death by apoptosis. The clonogenic survival assay of transformed MCF-7 cells revealed that CRT overexpression reduced MCF-7 cell proliferation and increased cell death.

Keywords: Apoptosis, MCF-7 cells, transfection, cytotoxicity, anti-cancer.

INTRODUCTION

Calreticulin is a protein with diverse functions that has been identified as a possible biomarker for varied diseases [1, 2]. Moreover, calreticulin concerned in variety of cellular roles, Ca^{2+} storage, including modulation of Ca^{2+} signals, cell adhesion, chaperone in protein folding, regulation of steroid-sensitive gene expression, autoimmune response and neuromodulations [3, 4].

The function of CRT in cancer and the mechanistic pathway involved in it remains unidentified; while there are extensive reports about the role of calreticulin in immunogenic cell death of cancerous cells [5, 6]. Structural predictions of calreticulin suggest that the protein has three domains [7, 8, 9], the N-domain, P-domain and C-domain. The N-domain of calreticulin also inhibits proliferation of endothelial cells and suppresses angiogenesis [10]. There are many reports on the suppression of cell proliferation in higher organisms *in vitro* by calreticulin [11, 12, 13, 14] and the over expression of CRT gene have contributed to diminution of cell viability when compared with its control. CRT holds promise as novel therapeutics for the treatment of cancer [15].

As apoptotic fudging is a central aspect of cancer, there is an urgent need for increased understanding of the key regulatory mechanism that controls the birth or loss of cancer cells. Functional expression cloning permits the isolation of CRT gene to control the rate of cell proliferation or death and offers an achievable way out to this problem. Therefore, the objective was to study the effect of CRT overexpression on MCF-7 cells by clonogenic survival assay and morphological determination.

MATERIALS AND METHODS

Cell lines and culture

Human MCF-7 cell line was obtained from the National Centre for Cell Science, Pune, India. It was routinely maintained in MEM (Gibco) supplemented with 10% FBS with antibiotic solution (Penicillin and Streptomycin) used in the study were grown in the presence of 5% CO_2 in a humidified incubator at 37 ^{0}C [16, 17].

Transfection

The total RNA was isolated using RNeasy Mini kit (Qiagen) and cDNA of CRT was constructed by using cDNA synthesis kit (Axygen) with Super-Script II RNase H reverse transcriptase (Invitrogen). Two 20 meroligonucleotides as PCR primers were designed according to the DNA sequence of CRT (GenBank). The forward and reverse primers were 5'-TCTCAGTTCCGGCAAGTTCT-3' and 5'-GTTGCTGAAAGGCTCGAAAC-3' respectively. The polymerase chain reaction was performed at 95 ^{o}C 3minutes, then 95 ^{o}C 30seconds, 58 ^{o}C 30seconds and 72 ^{o}C 45seconds for 35 cycles, and 72 ^{o}C for 5minutes. The product was finally held at 4^{o}C. An agarose gel electrophoresis was carried out to confirm the amplified 1.2kb product. The amplified target gene was inserted at XhoI and EcoRI restriction sites of mammalian expression vector pcDNA 3.1 (Invitrogen). The mock and CRT/pcDNA 3.1 recombinant plasmid was transfected to MCF-7 cells using Lipofectamine (Invitrogen) according to manufacturer's protocol.

Clonogenic assay

Colony formation assay was performed as delineated previously [18, 19, 20]. MCF-7-control and MCF-7-transfected (3ng/ml of CRT) cells were cultured in MEM with 10% FBS. Cells were then plated at a density of 3,000 cells per well in six-well plates and allowed to adhere overnight at 37 ^{o}C, 5% CO_2. Cells were allowed to grow until control treatment colonies reached >50 cells per colony (approximately 10 to 12 days). Colonies were then fixed with glutaraldehyde for 30 minutes, stained with crystal violet (0.1% in 20% methanol) for 30 minutes and washed. Colony number was determined manually.

Morphological Analysis

MCF 7 cells after treatment with recombinant CRT vector for 48 hours, were fixed with methanol (75%) and acetic acid (25%) for 10 min at room temperature. The fixed cells are stained with Giemsa (diluted with phosphate buffer), visualized under trinocular microscope (40x) and analyzed using MagnusPro detection software [21]. Also, morphological changes of MCF-7 cells were detected at 24, 48, 72 and 96 hours post transfection using phase contrast inverted microscope. Briefly MCF-7cells were plated at 3×10^4 cells/well into a six-well chamber plate. At >80% confluence, the cells were transfected with plasmid. Morphological changes occurring in the cells were observed under inverted microscope. At least 1×10^3 cells were counted to assess apoptotic cell death.

Statistical Analysis

All experiments were repeated three times and then data were shown as means ± standard deviation (SD) of three assays. Student's t-test was applied, and $p<0.05$ was considered as statistically significant.

RESULTS

The rate of MCF-7 cells proliferation on treatment with CRT plasmid was determined using clonogenic assay. The proliferation rate of 48h post transfected cells was significantly inhibited in the presence CRT when compared with the untreated control. After normalization with the numbers of the cells seeded in individual groups, the percentages of surviving colonies of MCF -7 treated and non treated cells were plotted against the time of exposure received by the cells (Figure 1).

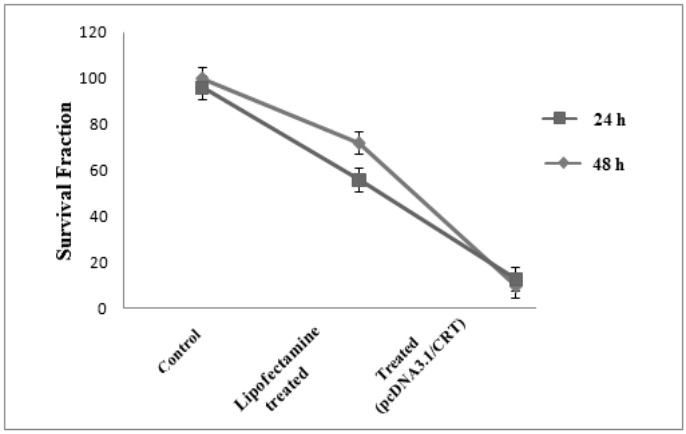

Figure 1. The percentage of surviving colonies of MCF-7 cells treated with pcDNA3.1/ CRT and untreated control cells were plotted against the duration of exposure

MCF-7 cells on treatment with CRT recombinant vector showed an increased level of apoptosis which was confirmed by Giemsa staining. The control cell does not show any variations in the cell morphology (Figure 2).

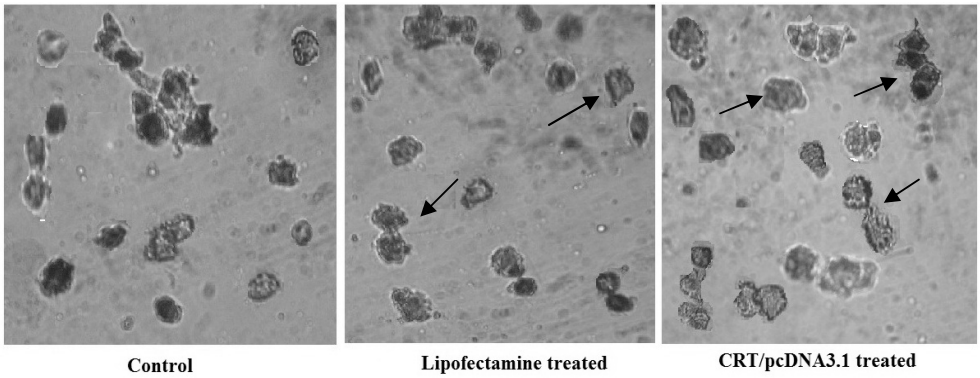

Figure 2. CRT induced morphological changes in MCF-7 cells (48 h post transfection with CRT/pcDNA3.1). The cells were treated with 6 ng CRT/pcDNA3.1 for 48 h and stained with Giemsa. Arrow mark shows the apoptotic cells with membrane disruption and nuclear condensation

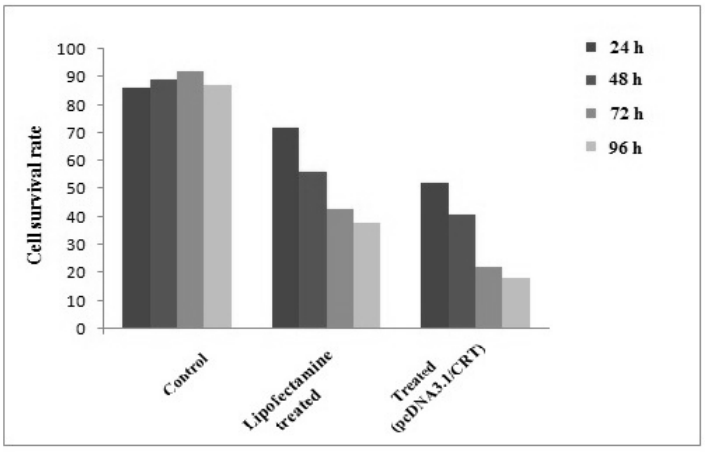

Figure 3. Cell survival rate occurred on treatment with CRT and its overexpression at 24, 48, 72 and 96 hours

The changes in the cell morphology of MCF-7 cells treated with CRT vector at 24 hours and 48 hours post transfection was observed using Zeiss Axiovert-25 inverted microscope (Soft-ware: Axiovision 4.0).

The results exposed a distinctive apoptosis with morphological changes such as chromatin condensation, membrane blebbing and cell shrinkage. These changes were typical at 24 hours, and became 60% or more after 72 hours, further reduced. The greater part of cells at 96 hours post transfection lead to shrink, round up and detach from the culture dish. The survival rate of the cells after transfection was plotted in graphically against the time dependence (Figure 3). Thereby, these results showed a possible way for detection of apoptosis induced by the overexpression of CRT.

DISCUSSION

A number of genes are responsible for the maintenance of cell homeostasis, among which CRT is involved in many biological processes together with regulation of cell homeostasis and programmed cell death [12]. The cell proliferation and apoptosis examined by MTT and trypan blue exclusion assay showed an increased cell death after CRT transfection were indicative of apoptosis. These findings demonstrated that overexpression CRT gene in MCF-7 cells significantly inhibited the proliferation of breast cancer cells *in vitro* by inducing apoptosis compared to untreated cells. These results are reliable with few of our earlier observations in MCF-7 cells [15, 22].

These results put forth the further confirmation of apoptosis by CRT in MCF-7 cells on clonogenic assay which is first to report by our findings. When the cells were measured for clonogenic assays, we found that a 24h post transfection of the cells to CRT was not sufficient to produce noticeable difference between CRT treated cells and untreated control cells in their clonogenic survival. Thus, we conducted experiments for 48h post transfection with the same concentration in the presence CRT which reduced the clonogenic survival cells from 100% in untreated cells to 80% in the 14-day culture period. Many studies conducted on tumors exhibit resistance to radiation [23] and chemotherapeutic drugs and have enhanced colony-forming efficiency [24]. The mechanism leading to the colony forming efficiency was incompletely understood. Our observation indicates that a continued inhibition of CRT mediated signal transduction is important in suppressing the colony- formation after CRT plasmid treatment.

In order to determine the action of CRT mediated changes in the morphological character of MCF-7 cells, we observed and analyzed the cells under inverted microscopy to determine the induction of apoptosis and also cells were stained with Giemsa to substantiate the apoptotic changes. CRT plasmid treated cells showed apoptotic bodies with cytoplasmic condensation indicating apoptosis resemblances. These observations provide evidence that an apoptotic pathway is triggered with the CRT overexpression in breast cancer cell line. Vector alone and controls failed to produce significant morphological changes.

Further, an obliging anticancer drug that can destroy cancer cells devoid of causing less damage to normal cells can be evolved from calreticulin. The ultimate aim of eradicating breast cancer is achieved by inducing apoptosis in cancer cells in the upcoming future through these findings.

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