Calreticulin Overexpression Suppresses Cell Proliferation and Enhances Apoptosis on Human MCF-7 Breast Cancer Cells

Sudha Sellappa*, Sree Jaya Jayachandran, and Vinodhini Jayaraman

***Abstract*—** Apoptosis is the mechanism used by cells to regulate tissue homeostasis through the elimination of surplus or potentially lethal cells. Calreticulin has a potent effect on immunogenic cell death on exposing to high levels that bring forth apoptosis. Since, the molecular mechanisms that direct and execute apoptotic cell death are coming into focus. In this study, we attempt to isolate the Calreticulin gene that drive the calcium-dependent apoptosis from Human MCF-7 mammary gland adenocarcinoma cell line and cDNA synthesized from the CRT was cloned to the XhoI/EcoRI restriction sites of a mammalian expression vector pcDNA 3.1 to construct a recombinant pcDNA3.1/CRT plasmid. Transfection of the MCF-7 cell line with a plasmid coding for CRT leads to cell death by promoting apoptosis. Stably transfected MCF -7 cell lines with the recombinant CRT plasmid showed a considerable increase in the rate of cell death. The transformed cells exhibit chromatin condensation, membrane blebbing, cytoplasmic condensation and nuclear membrane margination that are typical of cells death by apoptosis. The cell proliferation and apoptotic assays of transformed MCF-7 cells revealed that CRT reduced MCF-7 cell proliferation and increased cell death. This report delineates novel mechanism of the action of CRT against MCF-7 cells, suggesting this may have value for development as an anti-breast cancer agent.

***Keywords*—** Apoptosis, MCF-7 cells, transfection, pcDNA3.1, anti-cancer.

I. Introduction

CANCER a global health problem has seen an immense increase in recent times, particularly in developing countries like India, an average of 80,000 women are diagnosed with carcinoma of the breast, and 40,000 women die of this disease every year and it is the second most common cancer among Indian women [1].

Normal breast development and homoeostasis is controlled by a balance between cell proliferation, differentiation and apoptosis of the cells throughout the mammary gland. Situations that can upregulate cell proliferation or downregulate apoptosis may allow accumulation of mutations that result in breast cancer. Dysregulation overrides many of the normal checkpoint pathways and leads to expansion of neoplastic cells [2].

Recently, the relationship between apoptosis and cancer has been emphasized and the recognition that aberrant apoptosis may constitute a major clinical hurdle to overcome the treatment of cancer has spawned a variety of strategies to exploit apoptosis as a therapeutic strategy [3]. Achieving increased apoptosis and decreased proliferation through gene alteration can assist in bringing up a novel therapeutic approach to combat breast cancer. With the decoding of the human genome, it has become a fact of appreciation that the number of genes it contains is smaller than expected. These suggest that many proteins play multiple functional roles to satisfy the complexity of mammalian system. Calreticulin (CRT) is one such multi-functional protein possessing diverse biological activities [4].

Calreticulin is a 46-kDa protein expressed in all cells of higher organisms. CRT was first purified and recognized as a Ca^{2+} binding protein of the skeletal muscle sarcoplasmic reticulum and was later hereditarily recognized in mouse and mammals. CRT a highly conserved protein shows more than 90% amino acid sequence identity among the mammals. The calreticulin gene consists of nine exons and spans approximately 3.6 kb of human genomic DNA which is localized in chromosome 19 [5], [6], [7], [8]. Structural predictions of calreticulin suggest that the protein has three domains [9], [10], [11]; the N-domain, P-domain and C-domain.

The mechanistic pathways involving calreticulin and its role in cancer remains largely unknown, although there have been wide reports about the role of calreticulin in immunogenic cell death of cancerous cells [12], [13], [14]. CRT, as a Ca^{2+} binding protein demonstrates the direct and indirect effect on apoptosis [15]. Ca^{2+} release from the endoplasmic reticulum is essentially implicated in triggering the cytoplasmic and mitochondrial membrane-mediated apoptosis. Cellular response to apoptosis has been occupied by calreticulin. There is an increased sensitivity in calreticulin overexpression that results in to apoptosis [16], [17].

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Although further investigation are made to study the correlation between CRT expression and apoptotic signals, this study has revealed a novel pathway of cellular signaling for apoptosis and its regulation through a change in Ca^{2+} homeostasis [18]. Significantly, CRT/pcDNA3.1 exposure precedes the acquisition of characteristics of apoptosis such as loss of the mitochondrial transmembrane potential and chromatin condensation. However, thus far we have not observed a single example in which CRT exposure could be induced without that the cells would die later, suggesting that CRT exposure is triggered by a level of irreversible cellular damage. In this context, it should be underscored that stimulation of apoptosis is preceded by CRT exposure and that only a few cell death inducers are capable of inducing immunogenic cell death. Hence, CRT exposure depends on the upstream signals that lead to cell death, yet is not an automatic correlate of apoptosis [19].

II. Procedure

A. Cell lines and cell culture

Human MCF-7 mammary gland adenocarcinoma cell line obtained from the National Centre for Cell Science (NCCS), Pune, India and was routinely maintained in Minimal Essential Medium (Gibco) supplemented with 10% FBS and antibiotic solution (Penicillin and Streptomycin) used in the study were grown in the presence of 5% CO_2 in a humidified incubator at 37ºC [20], [21].

B. Isolation and construction of recombinant plasmid

The full-length RNA was isolated using RNeasy Mini kit (Qiagen) and cDNA of CRT was constructed in cDNA synthesis kit (Axygen) using 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'-GTTGCTGAA AGGCTCGAAAC-3' respectively. The polymerase chain reaction was performed at 95℃ 3min, then 95℃ 30sec, 58℃ 30sec and 72℃ 45sec for 35 cycles, and 72℃ for 5min. The product was finally held at 4℃. 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) [22],[23].

C. Transfection and selection of cells

The mock and CRT/pcDNA 3.1 recombinant plasmid was transfected to MCF-7 cells using Lipofectamine (Invitrogen) according to manufacturer's protocol. Stable transfectants were selected by treating the cells with 500μg/ml G418. The cloned G418-resistant lines were then screened for expression of CRT.

D. Cytotoxicity assay

The viability of the cells was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl) -2 -5-diphenyltetrazoliumbromide) assay which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. At 24 h and 48 h after the cells were exposed to the plasmids, 50μl of MTT (5 mg/ml) was added to each well. After incubation at 37°C for additional 4 h, the formazan crystals were dissolved in 150 μl of DMSO and the absorbance (OD) was measured at 570 nm using Hybrid Reader (Synergy H4). Each condition was tested in triplicate. Eight independent experiments were performed, and the results were analyzed for statistical significance.

E. Trypan Blue Exclusion Assay

For cytotoxicity assessment, trypan blue exclusion assay was performed [24]. Briefly, the cells were seeded, at a density of 0.5×10^5 cells/ml in complete medium. At 24h and 48h after the cells were exposed to the plasmids, cells were trypsinized, washed and resuspended in PBS containing 0.4% trypan blue and 100 cells were counted at various fields in haemocytometer for each experiment. The counting was done in triplicate. The cell growth was determined by plotting the graph of percentage of cell viability [25].

F. DNA Fragmentation assay

DNA fragmentation was analyzed by agarose gel electrophoresis as previously described [26] with slight modifications. At 48 h after transfection, cells were gently scraped and harvested by centrifugation. The cell pellets were incubated for 60 min at 50 ^oC in 100 ml lysis buffer (100mM Tris–HCl pH 8, 100mM NaCl and 10mM EDTA). Proteinase K (10 μl of 20 mg/ml) was added and further incubated for 30 min at 50 ^oC. RNase (3μl of 10 mg/ml) was then added and incubated for 2 h at 50 ^oC. The DNA was extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and 2.5 volume of absolute ethanol, then subjected to 2.0% of agarose gel electrophoresis, stained with ethidium bromide was visualized under UV light transilluminator [27], [28].

G. Morphological studies

Morphological changes of MCF-7 cells detected at 24 h post transfection using inverted microscope. Briefly MCF-7cells were plated at 5×10^4 cells/well into a six-well chamber plate. At >80% confluence, the cells were transfected with plasmid, for 48 hr. The cells were washed with PBS fixed in methanol: acetic acid (3:1v/v) for 10 min and stained with 50 μg/ml of propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed or fragmented nuclei was examined and counted to assess apoptotic cell death.

H. Statistical Analysis

All experiments were repeated three times and then data were shown as means ± standard deviation (S.D) of three

assays. Student t- test was applied, and $p<0.05$ was considered as statistically significant.

III. Results and Discussion

Anticancer effects of diverse chemotherapeutic agents are mediated through an ultimate common pathway via activation of caspases and subsequent DNA fragmentation.

For determining the apoptotic effect of CRT, human breast cancer cell line MCF-7 was analyzed for their viability using the trypan blue dye exclusion method. 48 h after transfection showed a significant increase in cell death than 24h after transfection. These results suggest that the overexpression of the CRT gene induces cytotoxicity in human breast cancer cells in time dependent manner (Figure 1and 2).

Successful insertion of recombinant CRT plasmid into MCF-7 is most likely to repair the genome of the mutant MCF-7 cells. Therefore, the morphology of the CRT/pcDNA3.1 treated MCF-7 cells were examined using Axiovert-25 inverted microscope (Soft-ware: Axiovision 4.0).

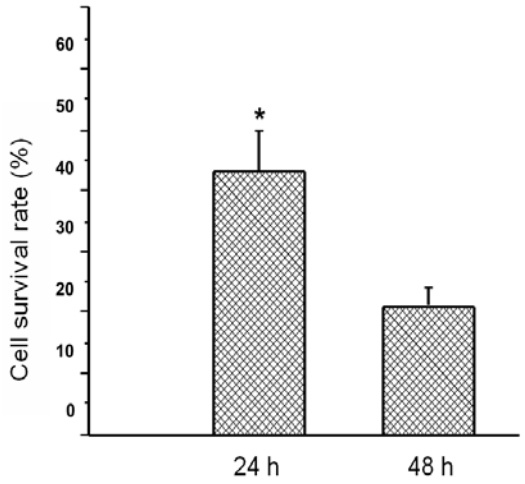

Fig. 1 Percentage of cell death on MCF- 7 cells treated with CRT/pcDNA3.1, at 24 h and 48 h post transfection (MTT assay)

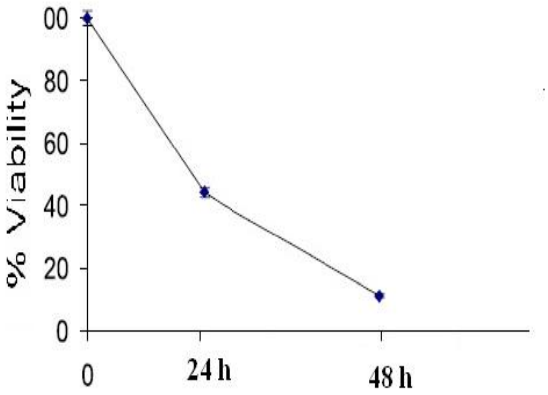

Fig. 2 Percentage of cell death on MCF -7 cells treated with CRT/pcDNA3.1, at 24 h and 48 h post transfection (Trypan blue exclusion assay)

Our data revealed that the untreated MCF-7 cells were smaller than those of transformed MCF-7. Both the transformed MCF-7 cells and non transformed cells were monolayered whereas the untreated MCF-7 cells were multilayered. The effect of CRT in cell morphology and differentiation as reported above led to the suspicion that this polymorphic marker could induce apoptosis in MCF-7 cells.

Extensive genomic DNA fragmentation a striking biochemical event in apoptosis is the inter-nucleosomal cleavage of DNA, initially producing 50- to 200-kb segments and fragments in multiples of approximately 185 bp [30], [31] as seen by agarose gel electrophoresis [32].

The DNA fragmentation of MCF-7 cells were detected on a 2.0% agarose gel electrophoresis after transfection of PCDNA3.1-CRT plasmid, after 48 h fragmented DNA was clearly observed in MCF-7 cells whereas control cells did not provide ladders. This results in ladder-like pattern of DNA fragments consisting of multiples of approximately 180–200 base pairs of the chromosomal DNA (Figure 3). Thereby, it showed a possible apoptosis-inducing activity.

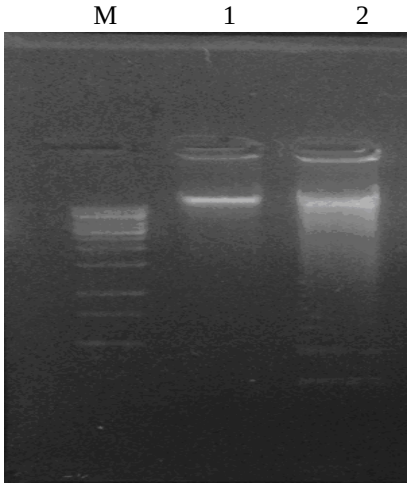

Fig. 3 DNA fragmentation in MCF-7 cells treated with CRT/pcDNA3.1, at 24 h and 48 h post transfection (Lane 1: Marker, Lane2: 24 h, Lane3: 48 h)

When treated with recombinant CRT plasmid, the MCF-7 cells expose apoptotic morphological changes. Untreated or control MCF-7 cells were exhibited typical growth patterns and a smooth, flattened morphology with normal nuclei. MCF- 7 cells at after 48 h plasmid exposure led to retraction, rounding, shrinking, lose of contact with neighbouring cell and some sensitive cells were detached from the surface. Nontypical apoptotic features, including highly condensed membrane blebbing, occurred during the middle apoptotic stage. At the pre-late apoptotic stage, membrane vesicles quickly formed, blebbed, and were finally worn out from the

cell membrane. These observations provide evidence that an apoptotic pathway is triggered with the overexpression of CRT.

Nuclear and chromatin fragmentation occurs during apoptosis, apoptotic and necrotic nuclei display different morphology. During necrotic cell death, mild clumping of nuclear chromatin can occur [34] but necrotic chromatin clumps do not significantly redistribute and the aggregates that form during necrosis do not bud to form discrete, membrane bound fragments. At the molecular level, ample numbers of genes are involved in the cell death pathways resulting in apoptosis.

IV. Conclusion

In conclusion, our data support the hypothesis that calreticulin inhibits growth or metastasis of breast cancer cells through extraordinary increase in cell apoptosis. In the coming years, the effect of overexpression of calreticulin seems likely to manipulate new cogent strategies for cancer diagnosis, prognosis and therapy.

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