RESEARCH COMMUNICATION

Enhanced Calreticulin Expression Triggers Apoptosis in the MCF-7 Cell Line

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Abstract

Calreticulin, since its discovery as a ubiquitous protein of endoplasmic reticulum, has become recognized as a multifunctional luminal resident protein affecting many cellular functions ranging from development to death of a cell. However its role in cancer and significance for therapy remains to be clarified. Here to examine its effects, the calreticulin gene was over expressed in MCF-7 human breast cancer cell lines and influence on apoptosis was examined through an MTT assay. Upon culture in a suitable medium, cells transfected with the calreticulin gene were significantly more susceptible to apoptosis compared to controls. These findings therefore render a novel mechanism by which cancerous cells can be triggered to undergo death by upregulation of a protein resident in the lumen of the endoplasmic reticulum.

Keywords: Calreticulin - cancer - apoptosis - MTT assay

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Introduction

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. The multi-functional nature of calreticulin may also be influenced by its distribution. CRT is a ubiquitous calcium-binding protein with wide tissue distribution found in all eukaryotic cells with the exception of yeast (Michalak et al., 1999).

Mature human CRT is a single polypeptide chain of 400 amino acids. CRT is an unusual luminal endoplasmic reticulum (ER) protein with several unique functions including modulation of gene expression (Burns et al., 1994; Dedhar et al., 1994; Michalak et al., 1996), a role in cell adhesion (Coppolino et al., 1995; Opas et al., 1996), and maintenance of intracellular Ca2+ homeostasis (Liu et al., 1994; Bastianutto et al., 1995; Camacho and Lechleiter, 1995; Mery et al., 1996). CRT also has an antithrombotic activity (Kubawara et al., 1995) and it is detected on the cell surface (Gray et al., 1995; White et al., 1995). The protein plays a role in long term "memory" in Aplysia (Kennedy et al., 1992), in cytotoxic T cell function/ activation (Burns et al., 1992; Dupuis et al., 1993), in neutrophils (Stendhal et al., 1994), in viral RNA replication (Singh et al., 1994), in sperm cell function (Nakamura et al., 1993), and in autoimmunity (Sontheimer et al., 1993).

The human CRT gene has been isolated, and the nucleotide sequence analysis of its promoter region has revealed several sites that might play a role in regulation of transcription (McCauliffe et al., 1992).To rationalize the diverse functions of CRT, it is important to identify and understand the mechanisms that regulate its expression. It is apparent that differential expression of CRT will have profound effects on seemingly diverse cellular functions.

Cancer is not, in reality, one disease, but a class of different diseases with the common features of excessive cell proliferation and tissue invasion. It is the combination of these features that makes cancer so dangerous. The most common cause of primary tumors is the genetic mutation of one or more cells, resulting in uncontrolled proliferation. The mutated cells have a proliferative advantage over neighboring, healthy cells and are able to form a growing mass (King, 1996).

The ideal cancer treatment should be able to eradicate systemic tumors at multiple sites in the body while having the specificity to discriminate between neoplastic and nonneoplastic cells. In this regard, apoptosis and antiangiogenesis represent two attractive approaches for cancer treatment. The tumor spread could be influenced by CRT chaperone protein of the endoplasmic reticulum (Erić et al., 2009). CRT has also been found to affect cell susceptibility to apoptosis and to be over expressed in highly apoptotic regions of the mouse embryo. The CRT gene is highly activated in mouse embryo interdigital cells, in the central retina and in lens vesicle suggesting that changes in the expression of CRT, an ER luminal protein, may play a role in apoptosis during embryogenesis (Nakamura et al., 2000).

As a mechanism of cell death, apoptosis is an important

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process for normal development and suppression of oncogenesis. Apoptosis is characterized by a series of typical morphological events, such as cell shrinkage, DNA fragmentation, fragmentation into membrane bound apoptotic bodies and rapid phagocytosis by neighbouring cells. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

The MTT assay is deemed to be a versatile method and accordingly the viability of the cells could be evaluated upon various treatments. The production of resultant formazan appears to be proportional to the level of energy metabolism in the cells. Therefore, it is possible to measure the metabolically activated cells even in the absence of cell proliferation. Besides, having used this assay; very small number of living cells could be detected and the incidence of errors would be minimal since there is no need for washing steps.

In the present study, the effect of human CRT over expression on the proliferation of cancer cells was assessed by MTT assay. As a model, we employed MCF-7 breast cancer cells as reports are lacking on its effect in human breast cancer cells. Moreover the properties of these cells are akin to human breast cancer cells and are successfully used as models for breast cancer research. The role of human CRT gene overexpression on the possible mode of cell death has been examined and the biological relevance is discussed.

Materials and Methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Dulbecco's Phosphate Buffer Saline (PBSA), Trypan blue (0.5%) solution, 0.25% Trypsin and 0.02% EDTA, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) were purchased from Hi-media, Mumbai. The human breast cancer cell line MCF-7 was obtained from National Centre for Cell Science, Pune, India.

Plasmid construction

The 496bp length of human CRT cDNA was cloned by reverse transcription polymerase chain reaction (RT-PCR) from healthy human blood obtained after consent of the person. Total RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions and was reverse transcribed into cDNA with SuperScriptTM II reverse transcriptase (Invitrogen) and oligo (dT) primer. Then the cDNA was amplified by PCR with using gene specific primer. FP: 5'-GTTTCGAGCCTTTCAGCAAC-3', RP: 5'-CAGTCCTCAGGCTTGGAGTC-3'. Thirty cycles of PCR were performed under the following conditions: denaturalization at 94°C for 45 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1min. The PCR product was verified by agarose gel electrophoresis and purified by gel extraction kit (Qiagen). The amplified product was ligated into pTZ57R/T vector (2889 bp) (Fermentas) using T4 DNA ligase enzyme and subjected to DNA sequence analysis. The CRT gene was later cloned to pcDNA3 mammalian expression vector (5446 bp) (Invitrogen) with a CMV promoter.

Cell culture and Transfection

MCF-7 cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin in a humid atmosphere with 5% streptomycin in a humid atmosph at 37°C.

The day before transfection, the MCF-7 cells were trypsinized, counted and plated at $2x10^5$cells/well in a 6-well plate (35-mm) containing two milliliter of normally growing DMEM supplemented with 10% FBS for 24 h to reach approximately 60-80% confluence. For transfection, aliquot of 5μg purified plasmid was used.

Calcium phosphate-DNA precipitates were prepared by the method of Graham and van der Eb (1973). Care was taken to prepare very fine precipitates: this could be accomplished reproducibly by mixing the DNA-$CaC1_2$ and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered sodium phosphate solutions under a gentle stream of nitrogen. Precipitates were allowed to stand for 30 min without agitation before being added to the tissue culture cells.

MTT colorimetric survival assay

MCF-7 cells were grown and subcultured as monolayer in DMEM supplemented with 10% FBS and antibiotic antimycotic solution. Cells were seeded in a 96 well microplate (Qiagen) at a concentration of $5x10^3$ cells/well , in a final volume of 100µl culture medium per well and left for 24h at 37°C in a humidified atmosphere of 5% CO_2 /95% air. MTT (25 µl) reagent was added to each well including control and incubated for a further 2h at 37°C to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan crystals. 100 µl of DMSO was then added into each well in order to solubilize the formazan crystals. The plate was incubated overnight at 37°C in order to solubilize the formazan crystals. It has been previously reported that the phenol red possesses estrogen activity which may affect the cell growth pattern within some estrogen responsive cells, ensuing imprecise MTT results. To avoid such impact, we have utilized DMEM without phenol red (Spinner, 2000). Quantification of cell viability was done on a microplate reader at a wavelength of 570nm and the mean absorbance for cells was taken. The number of viable cells was proportional to the extent of formazan crystals produced. The percentage cell viability was calculated using the following formula: Absorbance of the sample / Absorbance of control×100.

Statistical analysis

All experiments were done in triplicate. The data were analyzed by one-way ANOVA followed by Dunnett's post-hoc analysis using the program SPSS 10.0 (SPSS, Chicago, IL) and the SAS system for windows.

Results and Discussion

A successful anticancer therapy should kill or incapacitate cancer cells without causing excessive

damages to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The map of CRT expression vector used in the present study is shown in Figure 1. The expression vector was employed for transient transfection of MCF-7 cells, while controls remain nontransfected.

The effect of CRT over expression on the growth of MCF-7 cells was examined by MTT assay. Percentage viability of cells as evidenced from the MTT assay were estimated as 78% for non transfected MCF-7 cell lines (controls) and 56% for CRT transfected MCF-7 cell lines (Figure 2). Percentage of cell viability was found to be significantly different ($p < 0.05$) compared to the control.

The MTT assay is a colorimetric short term cell culture assay which functions by measuring the metabolic activity of living cells. In aspects of chemo sensitivity, it is considered superior to Trypan Blue Dye exclusion assay. MTT assay is more widely used to distinguish cancer cells from non-cancer cells (Von Hoff et al., 1981). The major advantages of the MTT assay are its speed and simplicity. Because most steps are automated, it is possible to test multiple samples. The automated data analysis is essential in view of the large amounts of data that can be generated. Since results are available within 3 days, such information may be of value in clinical therapy. Furthermore, a short assay duration will minimize the variable effects of cell proliferation and cell death over the assay period (Campling et al., 1991).

CRT was recently reported to be the second general marker, the membrane phospholipid phosphatidylserine being the first, for mammalian apoptotic cells to be recognized by phagocytes (Kuraishi et al., 2007). It has been reported that when CRT is exposed on the cell surface

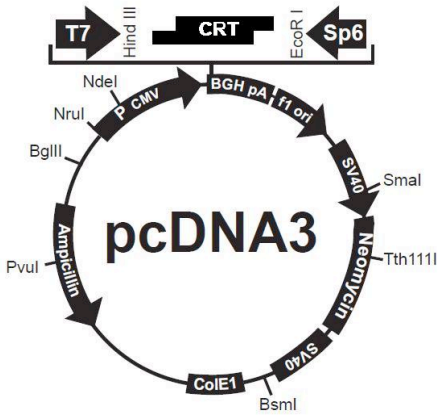

Figure 1. CRT Expression Vector Map.

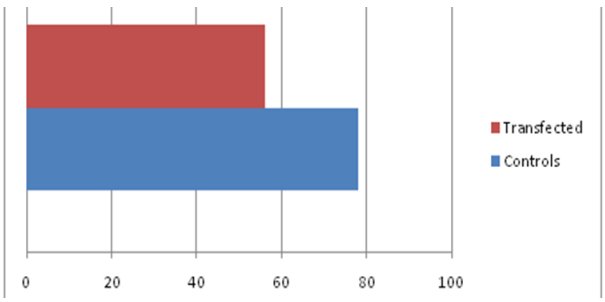

Figure 2. Percentage Cell Viability of Non-transfected and Transfected MCF-7 cells

it serves as an 'eat me' signal that allows a dying cell to be recognized, ingested and processed by specialized phagocytic cells (Clarke et al., 2007). Expression of CRT is associated with infiltration of T-cells, which implies that a low expression level of molecular marker may represent a new mechanism underlying immune escape in colon cancer (Peng et al., 2010).

Obeid et al., (2007) have shown that apoptotic cell may be immunogenic or non immunogenic depending on whether CRT is exposed on the plasma membrane of the dying cell. High CRT expression levels have been observed in several cancer tissues including hepatoma, neuroblastoma, mammary gland cancer, bladder cancer and colon cancer. These observations imply that high CRT expression levels might correlate with carcinogenesis processes in these cancers (Chen et al., 2007).

CRT is abundantly expressed and regulated by androgens in prostate epithelial cells. A study has reported that prostate cancer cells over expressing exogenous CRT produced fewer colonies in both monolayer culture and soft agar in support of the hypothesis that CRT inhibits growth and/or metastasis of prostate cancer cells (Alur et al., 2009). Studies based on proteomic approaches on whole tissue samples containing both neoplastic and non-neoplastic cells, have shown alterations of CRT expression in colon carcinomas, albeit with divergent results. CRT expression is significantly associated with the mucinous differentiation of colon tumor (Toquet et al., 2007).

Experiments with CRT-over expressing transgenic mice suggested the possibility that CRT is related to congenital heart block in humans (Nakamura et al., 2001; Kageyama et al., 2002; Salameh et al., 2004), but the exact physiological function of CRT remains obscure. Studies with CRT-deficient cells suggest that this protein participates in apoptosis. Cells deficient in CRT are relatively resistant to apoptosis (Nakamura et al., 2000; Pinton et al., 2001) and mice with a targeted disruption of the CRT gene die in utero with decreased ventricular cell mass due to increased apoptosis of cardiac myocytes (Rauch et al., 2000). Over expression of CRT suppresses Akt signaling and causes differentiation induced apoptosis in H9c2 cells (Kageyama et al., 2002).

Signaling between CRT in the lumen of the endoplasmic reticulum and calcineurin in the cytoplasm may play a role in the modulation of cell sensitivity to apoptosis and the regulation of Ca^{2+}-dependent apoptotic pathways (Groenendyk et al., 2004). Cells over expressing CRT have altered Ca^{2+} homeostasis and undergo increased apoptosis, confirming that enhanced CRT is related to cell death in postnatal cardiomyocytes (Groenendyk et al., 2004). Over expression of CRT modulates the activity of PKC (Protein Kinase C) and SERCA2a (Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} ATPase) and that intracellular calcium levels change in the over expressing cells. CRT over expression causes apoptosis of mature cardiomyocytes (Kageyama et al., 2002).

Hence from the results of the present study it is hypothesized that over expression of CRT gene could have contributed to diminution of cell viability when compared with its control. CRT holds promise as novel therapeutics for the treatment of cancer.

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