Abstract

Stress-Induced Premature Senescence (SIPS), a senescence-like state achieved as a result of a variety of stresses, is associated with relative inability to undergo apoptosis. Sirtuins are a family of histone deacetylases known primarily for their implication in the aging process; however their role in cancer, an aging associated disease, is still poorly understood. In the present study, we investigated whether alterations in sirtuins expression may be associated with aggressive tumor behavior. We have compared expression profiles of sirtuins between drug sensitive and resistant cancer cells MCF7, SaOS-2, A2780 and HL-60. Expression levels of Sirtuin-1 to 6 varied among cell lines; however Sirtuin-7 was significantly reduced in all chemoresistant cells tested. Knockdown of Sirt7 expression in human breast MCF7 cell line by RNAi induced senescence-associated β-galactosidase activity, reduced cell proliferation rate, induced drug resistance and increased cell migration, suggesting that this gene may play an active role in regulating cancer cell response to stress. Thus, Sir7 may represent a compelling target for anti-cancer interventions.

Introduction

Development of the multifactorial drug resistance is a major complication in cancer chemotherapy and has been described as “the single most common reason for discontinuation of a drug” [1]. The development of resistance to chemotherapy represents an adaptive biological response by tumor cells that leads to treatment failure and patient relapse. In recent years, it has become obvious that cancer cells can develop resistance not only to classical cytotoxic drugs but also to the newly discovered targeted therapeutics such as histone deacetylase inhibitors that are currently undergoing clinical trials. Therefore, a thorough investigation of the events responsible for this phenomenon is necessary to improve or at least to preserve the efficacy of anticancer therapeutics.

Key mechanisms for the development of drug resistance include: 1) activation of antistress frontline defense such as over-expression of drug efflux pumps P-glycoprotein and MRP [2-4], drug inactivating enzymes cytochrome P450 and GST-pi [5], anti-oxidant molecules SOD and catalase [6,7], 2) activation of repair and/or replacement mechanisms mediated by over-expression of the DNA repair enzymes such as MGMT, DNA-PK, ATM and Rad [8,9], or enzymes implicated in the turnover of damaged proteins such as the proteasome and cathepsins [10,11], and 3) inhibition of proliferation arrest and cell death effectors [12]. This latter possibility is currently under active investigation and the respective roles of apoptosis and senescence in development of cancer resistance to chemotherapy are not yet clearly defined.

Cellular senescence has been identified recently as a tumor-suppressor mechanism and a key determinant of cancer chemotherapy outcome [13-17]. Replicative senescence likely results from the shortening of telomeres to such an extent that the chromosome ends are not fully masked from recognition by the proteins responsible for double strand break repair. Once a critical shortened telomere length is attained, cell senescence is triggered. Recently, Stressinduced Premature Senescence (SIPS) was introduced to define in vitro models with long term growth arrest upon exposure to sublethal stressors, which are characterized by morphological and functional changes common for replicative senescence. A SIP is not prevented by telomere elongation. It is accompanied by intense genetic instability with gross chromosomal abnormalities, possibly due to illegitimate DNA recombination, and is associated with relative inability to undergo apoptosis [18].

Silent information regulator 2 (Sir2) gene family, also known as NAD+-dependent histone deacetylases, were found to function in many cellular process including resistance to stress, metabolism, carcinogenesis, neurodegeneration and even in life span extension [19-21]. In yeast, Sir2 proteins are thought to regulate genome stability by chromatin silencing as a mechanism by which they may control life span in these organisms. Among all the sirtuin family members in mammals, Sir1 has been the most studied mainly. Sir1 has been reported to be overexpressed in various drug resistant cancer cell lines and able to influence expression of stress resistance genes in these cells [22]. This was further confirmed by the findings that cisplatin resistant cancer cells overexpress SIR1, and incubation of their drug-sensitive counterparts in low glucose medium induced the expression of this gene and increased cellular resistance to cisplatin [23].

Earlier studies have shown that Sir7 was overexpressed in human thyroid carcinoma cell lines and tissues but not in adenomas [24]. However, the role of Sir7 in cancer cell response to therapy is not yet fully understood. In the present study, we have compared the...
expression of sirtuin genes in drug sensitive and resistant cells and examined the role of Sirt7 in drug-induced resistance.

**Materials and Methods**

**Establishment and characterization of chemoresistant cells**

Human breast cancer MCF7, osteosarcoma SaOS-2, acute promyelocytic leukemia HL-60 and A2780 cell lines were generous gift from Dr. Abdelhadi Rebaa (Children’s Memorial Research Center, Chicago, IL). Drug-resistant cells were generated by continuous incubation of parental cell lines with stepwise increases in drug concentration over a period of 3 to 6 months [22]. The MCF7/ADR-RES resistant cell line was a gift from Dr. Michael E. Bromberg (Temple University School of Medicine, Philadelphia, PA). Cells were grown in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle’s BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. HL-60 cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, USA), 2 mM L-glutamine, and 50 μg/ml gentamycin at 37°C in a fully humidified atmosphere of 95% air and 5% CO2. All cell lines were grown in drug free media for at least 3 months.

**Cell transfection**

MCF7 cells were transfected with pSilencer 4.1-CMV neo vector (Ambion, Inc., Austin, TX) containing the cloned hairpin RNAi target for Sirt7 using the Amaxa GmbH transfection system (Amaxa, Inc., Gaithersburg, MD). Three constructs were generated targeting three different regions of Sirt7. Control cells were transfected with pSilencer 4.1-CMV neo-vector that expresses a hairpin RNAi with limited homology to any known sequences in the human. This method has 80% transfection efficiency in MCF7. mRNA Quantification by Real-Time RT-PCR Total RNA was isolated using the Ambion Aqueous mRNA isolation kit (Ambion, Inc., Austin, TX) containing the cloned hairpin RNAi template for Sirt7 using the Amaxa GmbH transfection system (Hercules, CA). Then, 1 μg of total RNA was reverse transcribed using Advantage RT-for-PCR Kit (Clontech; Mountain View, CA). Real time RT-PCR was performed using Cepheid Smart Cycler (Sunnyvale, CA), which has a 2 μL cDNA, 10 μL Sybergreen Master mix (Qiagen; Valencia, CA) along with 0.5 μL of 20 μM gene-specific primers were used. The specificity and the size of the PCR products were tested by adding a melt curve at the end of the amplifications and by running it on a 2% agarose gel and sequencing the bands. All values were normalized to GAPDH.

**Cell proliferation assay**

Cells were incubated with either doxorubicin or cisplatin at concentrations varying between 1 × 10^{-8} and 1 × 10^{-4} mol/L for 96 hours in 96-well plate. Viable cells were quantitatively estimated by a fluorometric assay using CyQUANT cell proliferation assay (Molecular Probes; Eugene, OR). Cells were washed with PBS and frozen at -70°C until being analyzed. Plates were thawed at room temperature, and then 200 μL of the CyQUANT GR dye/cell lysis buffer were added to each well. The fluorescence of each well was determined in a fluorescent plate reader using an excitation wavelength of 480 nm and emission wavelength of 520 nm. The percentage of viable cells was determined by comparison with untreated control cells.

**Senescence analysis**

Senescence of drug-resistant cell lines and the parental cells was compared by histochemical staining of both cell types for expression of SA-β-Gal activity. Cells were cultured in DMEM + 10% FBS. Cells were trypsinized, counted, and plated at the same cell numbers and incubated for 48 hours. The media was then removed and the cells were gently rinsed once with PBS. After removing the PBS, fixative (0.2% glutaraldehyde and 2% formaldehyde) was carefully added to the culture plate so as not to disturb the cells. The cells were incubated in fixative at room temperature for 15 minutes. The fixative was then slowly removed and the cells were rinsed three times in PBS. The stain solution (β-Gal Staining kit [Roche, Indianapolis, IN]), adjusted to pH 6.0 (for SA-β-Gal), was added to each culture plate and incubated for 16 hours at 37°C. The plates were then removed from the incubator, staining substrate was removed from the plates, and the plates were rinsed with PBS. Plates were then viewed and photographed.

**Cell cycle analysis by flow cytometry**

The measurement of DNA content was performed by flow cytometric analyses. Cells were harvested by treatment with 1% trypsin/0.05% EDTA and fixed overnight in 70% ice cold ethanol. After washing with PBS aliquots of 1x10^6 cells were stained with PI/RNase staining buffer (BD Pharmingen). Fluorescence was measured using the Becton Dickinson FACScan (Bedford, MA). Distribution of cells in distinct cell cycle phases was determined using ModFIT cell cycle.

**p53, p21, caspase-3, GAPDH and Sirt7 Western blotting**

Western blotting was carried out by standard Western blotting techniques. Total cellular homogenates were prepared using Pierce mammalian extraction reagent (Pierce, Rockford, IL), according to the instructions provided by the manufacturer. Protein quantitation was performed by BCA method (Pierce Biotechnology, Inc., Rockford, IL). A total of 60 μg of cell lysate were boiled in 2X SDS buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 0.06% bromophenol blue, and 200 mM DTT), proteins separated by SDS-PAGE and then transferred to PVDF membrane.

Membranes were blocked in blocking buffer (5% dried milk powder, 1%TBS-T) after which the primary antibodies against p53, p21, caspase-3, GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and Sirt7 ( Sigma, St. Louis, MO) were added. The washed blots were then incubated in secondary antibody (goat anti-rabbit; Bio-Rad) diluted to 1:10000 and proteins were visualized using SuperSignal West Dura Extended Duration Substrate as per manufacturer’s specifications. (Pierce Chemical, IL). Images were captured using ChemiDoc XRS system (Bio- Rad, Hercules, CA).

**Tumor Formation of MCF7 cells transfected with Sirt7 RNAi in the chick Embryo chorioallantoic membrane (CAM) model**

The chick Chorioallantoic Membrane (CAM) model was used to study the effect of Sirt7 inhibition on angiogenesis by implanting MCF7 cells transfected with Sirt7 RNAi or mock transfection onto the CAM and by comparing tumor growth and vascularization. Fertilized White Leghorn chicken eggs were placed in an incubator and were kept under constant humidity at 37°C. A square window was opened in the shell. Cells (0.75 × 10^6) were drawn up into a sterile pipette and layered onto the CAM of 7-day-old embryos, avoiding contact between the CAM and the pipette. Grafted cells were allowed to grow on the CAM for 7 days.

**MCF7 Xenografts in NCr Nude Mice**

Female NCr nude homozygous mice aged 5-6 weeks and body...
weight of 20 gm were purchased from Taconic Farms (Hudson, NY). Animals were maintained under specific pathogen-free conditions and housed 4-5 per cage, under controlled conditions of temperature (20-24°C) and humidity (60-70%) and a 12 h light/dark cycle. Water and food were provided ad libitum. Experimental protocol was approved by the VA IACUC. Mice were allowed to acclimate for 5 days prior to the start of treatments. Approximately 10 x 10^6 cells in 100 μl of medium were injected left 4th mamillary fat pad of each mouse. Tumors were measured daily by calipers and tumor volume was calculated according to a standard formula (L x W^2 / 2). Doxorubicin (0.6 mg/kg) group was administered intraperitoneally (I.P.) daily for 8 days. Mice were weighed daily and tumor size was measured daily. At the conclusion of experiments, all animals were euthanized in a CO_2 chamber and tumor masses were collected and weighed.

Migration assay

Tumor cell invasion assays were performed using polycarbonate bottomed 96-well plates. MCF7 cells, a non-invasive breast cancer cell line, transfected with Sirt7 RNAi or mock were cultured in RPMI containing 0.1% BSA, penicillin, and streptomycin. Membranes were coated with human collagen type IV prior to the addition of cells (50,000 cells/well) to the upper wells. Chemotactic factors (IL-8 [10 ng/mL], MCP-1 [10 ng/mL], C5a [10 ng/mL], fibronectin [25 μg/mL], VEGF [100 ng/mL], vitronectin [25 μg/mL], FGF [100 ng/mL], and 10% FBS) were added to the lower wells. Cells in the lower chamber were counted using the CyQuant assay.

Results

To test the involvement of sirtuins in chemoresistance, the mRNA expression levels of Sirt1 through Sirt7 were measured in the drug-resistant cells (MCF7, SaOS-2, A2780 and HL-60). The data indicate that expression of Sirt1 to Sirt6 varied among cell lines, however, Sirt7 inhibition was predominant in all chemoresistant cells tested (Figures 1A-D). Similarly, the protein expression of Sirt7 was significantly inhibited in all drug-resistant cells compared to parental cells (Figure 1E). These findings suggest that the lack of Sirt7 is associated with the
development of drug resistance and raise the possibility that this gene may repress the function of molecules implicated in this process.

To establish a relationship between Sirt7 and drug resistance, MCF7 cells were transfected with pSilencer 4.1-CMV neo-vector containing the cloned hairpin RNAi for Sirt7 using the Amaza GmbH transfection system (Amaza, Inc., Gaithersburg, MD). Three specific constructs targeting 3 different regions were established. Control cells were transfected with pSilencer 4.1-CMV neo-vector that expresses a hairpin RNAi with limited homology to any known sequences in the human. G418-resistant cells were selected over 2-week period. Sirt7 mRNA and protein levels were significantly inhibited in the stably transfected Sirt7 RNAi MCF7 cells (Figures 2A and 2B). Sirt7 RNAi transfected MCF7 cells displayed slower growth rate when compared to mock transfected cells. They also became smaller and refractile with thin cytoplasmic projections and increased anchorage-independent growth and challenging these cells with different concentrations of doxorubicin and cisplatin resulted in resistance to doxorubicin and cisplatin (Figures 2C and 2D). Mock transfected MCF7 cells were negative for senescence associated β-galactosidase (SA-β-Gal) stain and were positive with Sirt7 knock down, even in the absence of doxorubicin. The intensity and the percentage of positive cells increased significantly in the presence of doxorubicin (Figure 2E). To study cell proliferation of MCF7 transfected with Sirt7 RNAi, the cells were synchronized by serum withdrawal for 24h and then the re-entry time into cell cycle in the presence of complete medium was determined. Inhibition of Sirt7 resulted in slower proliferation rate of MCF7 when compared to mock transfected cells (Figure 3A). On the other hand, 0.1 μM doxorubicin had no effect on Sirt7 RNAi transfected MCF7 cell cycle and caused cell cycle arrest at the G2/M phase in the mock transfected cells.

To further investigate the consequence of gain and loss of Sirt7 function in resistance to drugs, we first determined whether a correlation exists between decreased Sirt7 expression and reduced expression of p53 and p21/WAFI. Secondly we compared the expression of p53 and p21 in non-transfected cells and those transfected with either Sirt7 cDNA or Sirt7 RNAi. As shown in Figure 4A, the mRNA levels of both p53 and p21 are decreased in the drug resistant cells tested, further supporting the notion that reduced expression of these genes is associated with cancer aggressivity and that their levels correlate with that of Sirt7. Inhibition of Sirt7 in MCF7 cells resulted in increased p53 and p21 protein expression following addition of doxorubicin to the cells at higher concentrations (Figure 4B). Since MCF-7 cells express mainly a truncated isoform of caspase-3 transcript and to a lesser extent a full-length caspase-3 transcript while doxorubicin resistant MCF7 sublines express the full length functional caspase-3, we investigated the effect of Sirt7 inhibition on caspase-3 expression. Pro-caspase-3 protein expression was significantly higher in Sirt7 RNAi transfected MCF7 cells (Figure 4B). There was no change in pro-caspase-3 following incubation with high doses of doxorubicin indicative of impaired apoptotic pathway.

Since the metastatic potential is tightly associated with cancer cell invasion and that the latter is known to be induced by a variety of factors produced either by cancer cells or the host tissue, we analyzed the effect of Sirt7 knockdown on the ability of some of these factors to enhance invasion of a non-metastatic cell line MCF7. As shown in Figure 5, the chemoattractants IL-8, MCP1, C5A, fibronectin, VEGF,
**Figure 3:** Inhibition of Sirt7 in MCF7 cells caused S phase cell cycle arrest. A) Effect of knocking down Sirt7 on cell growth. Cells were seeded at 2000 cells/well in complete media and then counted at 3 and 5 days; B) Cell cycle analysis by flow cytometry of Sirt7 RNAi-transfected MCF7 cells following 0.1 µM doxorubicin. Doxorubicin at 0.1 µM had no effect on Sirt7 RNAi transfected MCF7 cell cycle and caused cell cycle arrest at the G2/M phase in the mock transfected cells. Results are presented as mean ± SE; *: P>0.05; n=4.

**Figure 4:** Expression of p53 and p21 (cip-1/waf-1) in drug-resistant cells (MCF7 and A2780) and cells transfected with Sirt7 or mock measured by PCR primers amplifying exon 5; B) Western blot analysis of p53, p21 and caspase-3 in control and Sirt7 knockdown MCF7 cells in the absence or presence of doxorubicin. RNAi-mediated inhibition of Sirt7 expression resulted in increased procaspase-3 expression. Doxorubicin increased the protein expression levels of p53 and p21 in a dose-dependent manner in Sirt7 knockdown cells. The mRNA and protein expression levels of p53 and p21 were quantified, and the results are presented in graphical form below each panel in A and B, respectively. Results represent the means ± SE of least 4 experiments; *: P<0.05.
vitronectin, and FGF, stimulated Sirt7-RNAi transfected cell invasion at a higher rate than mock transfected cells.

Like for metastasis, increased angiogenesis is associated with tumor aggressiveness and enhanced resistance to therapy. Neovascular outgrowths from the CAM toward the implant were more predominant in implants containing MCF7 cells transfected with Sirt7 RNAi (Figure 6). Cells transfected with Sirt7 RNAi showed evidence of central necrosis and thrombosis of some of the newly formed vessels. Inhibition of Sirt7 resulted in accelerated tumor growth in the chick model (Figure 6C). On the other hand, implantation of MCF7 cells transfected with Sirt7 RNAi in female athymic nude mice resulted in slower growth rate when compared to mock transfected cells (Figure 7), possibly due to slower growth rate. Doxorubicin (0.6 mg/kg) treatment, administered I.P. daily for 8 days, inhibited tumor growth in mock transfected cells and had no significant effect on MCF7 cells transfected with Sirt7 RNAi. Comparable results were obtained relative to tumor weights (Figure 7C).

Discussion

This study describes Sirt7 as a player in cancer aggressiveness and provides evidence that targeting this gene may affect cancer cell response to drugs and therefore their metastatic and angiogenic abilities. Early studies have shown that Sirt7 is associated with active rRNA genes (rDNA) and histones [25]. Overexpression of Sirt7 increased RNA polymerase I (Pol I)-mediated transcription, whereas knockdown of Sirt7 or inhibition of its catalytic activity resulted in decreased association of pol I with rDNA and reduced pol I transcription. The association of Sirt7 with resistance to stress in normal tissues has been addressed and this gene was found to play a protective role and may even have anti-aging functions. However, its implication in cancer cell response to stress has not yet been addressed. Our data provide evidence that this protective action of Sirt7 may not apply to cancer tissues as we show that expression of this gene consistently decreases at the message as well as at the protein levels in cancer cells that have developed resistance to chemotherapy. Although this decrease seems to correlates with enhanced expression of the MDR1 gene [22], the fact that cancer cells deficient in Sirt7 become resistant to cisplatin, a non p-gp substrate, suggests that other drug resistance mechanisms maybe regulated by this gene.

Cell death signaling pathways may also be affected in relation to alterations in Sirt7 expression. Although SIPS is not associated with cell replication or dysfunctional telomeres, it resembles replicative senescence in many components in their signaling pathways and displays similar cellular phenotypes. DNA damage-induced senescence in human cells is characterized by p53 activation and subsequent accumulation of the cyclin-dependent kinase inhibitor, p21WAF1/CIP1, which leads to growth arrest. To test if this pathway is a key player in Sirt7 inhibition-induced drug resistance, we examined p53 expression in drug resistant and their counterparts. p53 and p21 expressions were inhibited in MCF7 and A2780 drug resistant cells. However, since the osteosarcoma SaOS-2 [26] and acute promyelocytic leukemia HL-60 [27] have p53 gene rearrangements and deletions and both have decreased Sirt7 expression, this suggests that Sirt7 inhibition-induced drug resistance is a non-p53 mediated mechanism.

Although the parental MCF-7 cells lack caspase-3 expression because of a 47-base-pair deletion in exon 3 of the CASP-3 gene [28], MCF-7/ADR and MCF-7 TH cells, and several established MCF-7/DOX cells strongly express the full-length functional caspase-3 protein [29,30]. MCF-7 cells express mainly a truncated isoform of caspase-3 transcript and to a lesser extent a full-length caspase-3 transcript, whereas drug resistant sublines express mainly full-length caspase-3

Figure 5: Tumor cell invasion assay of MCF7 transfected with Sirt7 RNAi or mock at 24 hours. Chemotacttractant and growth factors were added to the lower chambers. Results are presented as mean ± SE; *: P<0.05.

Figure 6: Nodule formation and simultaneous angiogenic response in the CAM after implantation of MCF7 cells transfected with A) Sirt7 RNAi or B) mock transfection. A dense network of newly formed capillaries from the chick chorioallantoic membrane directed toward a 72-h implant was predominant in MCF7 cells transfected with Sirt7 RNAi when compared to the control cells. Mock transfected MCF7 remained on the ectodermal surface whereas MCF7 cells transfected with Sirt7 RNAi formed deeper and larger masses with evidence of central necrosis and thrombosis of some of the newly formed vessels. Both nodules are seen from above. C) Tumor weights 7 days after implantation on the CAM. Results are presented as mean ± SE; n=6; *: P<0.005.
transcript [29]. Inhibition of Sirt7 in MCF7 cells increased caspase-3 proenzyme protein expression. These results are consistent with the previous findings that doxorubicin-resistant cells express full-length caspase-3 transcript and suggest a role of Sirt7 in regulating the expression of caspase-3.

An elevated Sirt7 expression has been detected in several human cancers [31,23]. Expression of Sirt3 and Sirt7 increased in primary mammary cells approaching senescence and in a node-positive malignant breast cancer. In addition, Sirt7 expression was also associated with an increase in nodal metastasis, bad prognosis and poorer survival [31]. Thus, Sirt7 would be expected to increase in chemoresistance. However, our results are not compatible with the proposed role of Sirt7 being an anti-senescence protein. These conflicting results may be explained by the different cellular and experimental settings in which Sirt7 was measured. Cells in our study were stressed with chemotherapeutic agents for 3-6 months. Interestingly, a recent study by which Sir2 was over expressed in drosophila flies resulted in promotion of caspase-dependent but p53-independent apoptosis. Furthermore, loss-of Sir2 function prevented apoptosis induced by UV irradiation [32]. Our results of Sirt7, a Sir2 homologue, are compatible with these findings.

The fact that inhibition of Sirt7 in MCF7 cells results in slower proliferation rate, implanting these cells into the immunocompromised athymic nude mice resulted in smaller tumor volumes when compared to the control cells. On the other hand, implanting MCF7 cells transfected with Sirt7 RNAi into the CAM resulted in larger tumors when compared to the mock transfected cells. These cells were implanted at Embryo Development Day (EDD) 7 while the chick embryo immune system is not completely developed. It lacks both B and T cell-mediated immune functions. Consequently, the young embryos are not fully immunocompetent. The presence of T cells can be first detected at EDD 11 and of B cells at EDD 12. After EDD 15, the B cell begins to diversify, and by EDD 18 chicken embryos become immunocompetent [33]. In our study, tumors were collected at EDD 20. Our data could be explained simply by the fact that MCF7 cells transfected with Sirt7 RNAi resulted in resistance to the chicken embryo immune system whereas the mock transfected MCF7 cells were rejected by the chicken embryo.

**Conclusion**

Chronic stress by chemotherapeutic agents leads to inhibition of Sirt7. This induces premature senescence-like phenotype, which results in aggressive tumor behavior and apoptosis inhibition, in this setting, Sirt7 inhibition constitutes pro-survival signaling. Thus, Sirt7 may represent a unique target for the treatment of aggressive cancers. Further investigations are warranted to define the underlying mechanism of its action and the efficacy of its targeting in various tumor types.

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**References**
