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Role of p53 in the anti-proliferative effects of Sirt1 inhibition in prostate cancer cells

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Abstract

Prostate Cancer (PCa), next only to skin cancer, is the most commonly occurring malignancy in men in the USA. Aging is recognized as a major risk factor for this neoplasm as a man's chance for developing this disease significantly increases with increasing age. Because aging is inevitable, Americans are living longer, and the existing treatments have not been able to manage this neoplasm, novel mechanism-based approaches are needed. We have recently shown that Sirt1, a sirtuin class III histone deacetylases (HDACs) originally linked to aging and longevity in yeast, was overexpressed in human PCa cells and PCa tissues obtained from patients. We also found that chemical inhibition and/or genetic knockdown of Sirt1 caused a FoxO1-mediated inhibition in the growth and viability of human PCa cells. Since p53 is a target for deacetylation by Sirt1, we wanted to determine the involvement of p53 in Sirt1 inhibition mediated responses in PCa. To achieve our objective, we utilized a pair of isogenic PCa cell lines viz. PC3 and PC3-p53, which differ only in p53 status. Our data demonstrated that Sirt1 inhibition caused a decrease in cell growth, cell viability and the colony formation ability of both cell lines. Further, Sirt1 inhibition resulted in an increase in FoxO1 acetylation and subsequent transcriptional activation in both cell types regardless of p53 status. However, an interesting observation of our study was that Sirt1 inhibition resulted in an increase in senescence in PC3-p53 cells whereas it resulted in an increase in apoptosis in PC3 cells. The results of this study compliment our previous study and suggest that Sirt1 inhibition may have different downstream targets in cells with active p53 *versus* cells where p53 is inactive.

Keywords

Prostate cancer; sirtuins; Sirt1; p53; FoxO1; senescence; apoptosis

Introduction

According to the American Cancer Society, age is a primary risk factor for cancer with about 77% of all cancers being diagnosed in people age 55 and older. An excellent example that connects age with cancer comes from the estimates from the Center for Disease Control (CDC) according to which a man's chances of developing prostate cancer (PCa) by age are: 1 in 2,500 by age 45; 1 in 120 by age 55; 1 in 21 by age 65; and 1 in 9 by age 75. Further, according to

some predictions, by year 2010, the number of annual PCa cases will skyrocket to 330,000. These statistics are striking and this general trend is true for certain other cancers such as breast, ovarian, skin, gastric, lung, oral, and head and neck. Therefore, in the recent past, increasing emphasis has been placed on defining molecular connections between cancer and aging.

Interestingly, it is being appreciated that the sirtuin family of histone deacetylases (HDACs) may be one of the lost links between aging and cancer (1). Sirt1 is a nicotinamide adenine dinucleotide (NAD(+))-dependent deacetylase, which belongs to the silent information regulator 2 (Sir2) family of sirtuin class III HDACs and is the most well-studied member of the sirtuin family (2). Recent studies have demonstrated that Sirt1 plays an important role in the regulation of cell fate, DNA repair, aging, and stress response in mammalian cells (2). Sirt1 promotes cell survival by inhibiting apoptosis or cellular senescence induced by stresses including DNA damage and oxidative stress (2). There have been a number of potential Sirt1 targets identified such as histones, the FoxO factors, p73, NF- κ B, p300, the androgen receptor, p53, etc (reviewed in(2)). The exact role that Sirt1 plays with each of these potential targets is unknown, but a number of studies have focused on the association of Sirt1 with p53.

In a recent study, we found that Sirt1 is significantly overexpressed in human PCa cells (DU145, LNCaP, 22Rv1, and PC3) and PCa specimens compared to normal human prostate epithelial cells and adjacent normal tissues, respectively (3). Further, our data demonstrated that Sirt1 inhibition caused (i) an inhibition in cell growth and viability, (ii) an increase in acetylated FoxO1 protein levels, and (iii) an increase in FoxO1 transcriptional activity of human PCa cells while having no effect on normal prostate epithelial cells (3). A number of other studies by multiple groups have reported similar results in different cancer model systems (4-9). Collectively, these findings have uncovered a pro-proliferative role of Sirt1. It is also important to mention here that a few studies also suggested a tumor suppressor function of Sirt1; the reasoning behind these discrepancies is not clear at present (10-13).

In our published study, we found that Sirt1 inhibition imparts an anti-proliferative response in multiple human PCa cells irrespective of their p53 status (3). This is an interesting and somewhat unexpected observation because several studies have shown that Sirt1 inactivates the tumor suppressor p53 via its deacetylation (14-18). Therefore, to further ascertain if p53 status determines the response of Sirt1 inhibition in PCa cells, employing a pair of isogenic PCa cell lines viz. PC3 and PC3-p53, which differ only in p53 status, we determined the response of Sirt1 inhibition in these cells. We found that Sirt1 inhibition by sirtinol caused (i) an increase in FoxO1 acetylation, (ii) an increase in FoxO1 transcriptional activation, (iii) a decrease in cell growth and viability, and (iv) a decrease in the colony formation ability of PCa cells regardless of p53 status. However, in PC3-p53 cells (with wild-type p53), shRNA-mediated knockdown of Sirt1 resulted in an increase in senescence; whereas, in PC3 cells (which lack p53), Sirt1 knockdown resulted in an induction of apoptosis.

Results and Discussion

This study was designed to define the differential mechanism of anti-proliferative response of Sirt1 inhibition in human PCa cells differing in p53 status. Mutations in the tumor suppressor p53 gene are known to occur in several cancer types including PCa. Earlier studies have suggested that p53 mutations occur as a late event during the progression of PCa and were associated with androgen-independence, increased angiogenesis, metastasis, recurrence, and a worse prognosis (19). Interestingly, recently p53 mutations have been reported to occur in approximately one third of early stage PCa (19). Further, p53 has been shown to be a downstream target of Sirt1 (14-18). Vaziri *et al.* found that Sirt1 bound to and deacetylated the p53 protein with a specificity for its C-terminal Lys382, reducing the transcriptional activity of p53 (16). Furthermore, Luo and colleagues reported that mammalian Sir2 α physically

interacted with and attenuated p53-mediated function. In addition, Sir2 α was found to repress p53-dependent apoptosis in response to DNA damage and oxidative stress whereas expression of a Sir2 α point mutant increased the sensitivity of cells to the stress response (15). While it is generally accepted that Sirt1 does indeed interact and deacetylate p53, the biological outcome of this regulation is under debate. A number of studies have shown that Sirt1 does not affect many of the p53-mediated biological activities despite the fact that acetylated p53 has shown to be a target of Sirt1. For example, Kamel *et al.* found that while Sirt1 does interact with p53, the Sirt1 protein had little effect on p53-dependent transcription of transfected or endogenous genes and did not affect the sensitivity of thymocytes and splenocytes to radiation induced apoptosis (20). Solomon and colleagues reported that treatment of cells with EX-527 (a specific small-molecule inhibitor of Sirt1) dramatically increased the acetylation of p53 at lysine 382 following DNA damage, but had no effect on cell growth, viability, or p53-controlled gene expression in cells treated with etoposide (21). Conversely, Cheng *et al.* reported that Sirt1-deficient cells from mice with Sirt1 gene-targeted mutations, exhibited p53 hyperacetylation following DNA damage and increased ionizing radiation-induced thymocyte apoptosis (22). Another study showed that inhibition of Sirt1 expression with microRNA 34a (miR-34a) resulted in an increase in acetylated p53 and ultimately an increase in apoptosis in colon cancer cells with wild type p53, but not in colon cancer cells lacking p53 (23). In addition, Stunkel and colleagues found that Sirt1 mRNA knockdown lead to a p53-independent decrease of cell proliferation and induction of apoptosis (24). These studies suggest that Sirt1 plays a role in the p53 pathway, albeit possibly in a cell-type specific fashion.

In this study, to dissect the role of p53 in Sirt1 inhibition-mediated responses in PCa cells, we employed two isogenic PCa cell lines differing in p53 status *viz.* PC3 and PC3-p53 cells. PC3 cells are null for both p53 as well as the androgen receptor (AR) whereas PC3-p53 cells (kind gift from Dr. Munna L. Agarwal at Case Western Reserve University) are PC3 cells stably transfected with wild-type p53 (25). Thus, the only difference between these two cell lines is their p53 status. We confirmed the status of p53 in these cell lines. As shown by the Western blot analysis, PC3 cells lacked p53 protein whereas PC3-p53 cells were found to have a significant level of p53 protein (data not shown).

In this study, we first determined if p53 status had any effect on Sirt1 inhibition mediated anti-proliferative effects in PC3-p53 cells *versus* PC3 cells. As shown in Fig. 1A and Fig. 1B, treatment of cells with the small molecule Sirt1 inhibitor sirtinol (30 and 120 μ M; for 24 hours) resulted in a decrease in cell growth and cell viability of both cell types compared to the vehicle (DMSO) control as assessed by Trypan blue assay. Thus, we did not observe a differential response in cell growth and viability in these cells differing in p53 status. We next determined the effect of sirtinol on FoxO1, since we and others have shown that Sirt1 can regulate mammalian FoxO transcription factors through direct binding and/or deacetylation. Our data demonstrated that sirtinol-mediated inhibition of Sirt1 caused an increase in the protein levels of nuclear and cytoplasmic acetylated FoxO1 while having no effect on total FoxO1 protein as shown by the Western blot analysis (Figure 1C). Further, the observed increase in acetylated FoxO1 protein was found to be accompanied by an increase in FoxO1 transcription as determined by a luciferase reporter assay (Figure 1D). Thus our data showed that Sirt1 inhibition-mediated activation of FoxO1 occurs in both PC3 and PC3-p53 cells, irrespective of their p53 status.

Because sirtinol, at higher concentrations, has been reported to inhibit other sirtuin proteins (i.e. Sirt2), we utilized Sirt1 specific short hairpin RNA (shRNA) to knockdown Sirt1 gene to determine response of Sirt1 specific inhibition in PC3 and PC3-p53 cells. We found that Sirt1 shRNA-mediated inhibition of Sirt1 resulted in a decrease in growth and viability of both the cell types ((3) and *data not shown*). In addition, we also found that shRNA-mediated inhibition

of Sirt1 caused a decrease in the clonogenic survival of both PC3-p53 and PC3 cells, irrespective of p53 association (Figure 2).

Our next aim was to determine the reason of decreased cell growth by Sirt1 inhibition in these isogenic PCa cell lines. Studies have shown that Sirt1 regulates senescence, a permanent state of cell growth arrest after a limited number of cell divisions (8;26;27). Specifically, Huang *et al.* demonstrated that enforced Sirt1 expression promoted cell proliferation and antagonized cellular senescence in human diploid fibroblasts, possibly via the activation of ERK/S6K1 signaling (27). Senescence is associated with a number of specific cellular alterations such as growth inhibition, morphological changes (an increase in cell size and granularity), and the appearance of senescence-associated beta-galactosidase (SA- β -gal) activity. We determined if the observed anti-proliferative response of Sirt1 inhibition was associated with a senescence induction in PC3-p53 and PC3 cells. We found that shRNA-mediated Sirt1 knockdown resulted in a marked increase in SA- β -gal staining (Figure 3A) in PC3-p53 cells suggestive of senescence induction. Interestingly, Sirt1 knockdown did not cause an increase in SA- β -gal stain in PC3 cells which lack p53. The induction of senescence was further confirmed by an increase in both forward-angle light scatter (FSC), roughly equivalent to particle size, and side-angle light scatter (SSC), roughly equivalent to granularity (Figure 3B), again only in PC3-p53 cells. The induction of senescence was also confirmed by concurrently using doxorubicin, a positive control for senescence induction (28) (data not shown).

The observed lack of senescence by Sirt1 knockdown in PC3 cells prompted us to question other possible modes of cell growth inhibition. Since apoptosis, another process of inhibiting cell growth, has also been linked to the functionality of Sirt1 (4;5;9), we assessed if shRNA-mediated knockdown of Sirt1 resulted in apoptosis in PC3-p53 and PC3 cells. For this purpose, we determined the extent of poly(ADP-ribose) polymerase (PARP) cleavage, an established indicator of apoptosis in mammalian cells (29;30). As shown by Western blot analysis, we found that shRNA-mediated knockdown of Sirt1 resulted in a marked decrease in full length PARP accompanied with an increase in the cleaved PARP in PC3 cells (which lack p53), but not in PC3-p53 cells (Figure 4A). The differential induction of apoptosis in PC3 cells was further confirmed by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay that detects DNA fragmentation by labeling the terminal end of nucleic acids (Figure 4B) (31).

In summary, our data demonstrated that Sirt1 inhibition resulted in a decrease in cell growth and viability, a decrease in colony formation, an increase in acetylated FoxO1 protein levels as well as an increase in subsequent FoxO1 transcriptional activity in PCa cells regardless of p53 status. The most interesting and novel finding of this study is that Sirt1 inhibition resulted in an increase in senescence (but not apoptosis) in PC3-p53 which possess wild-type p53. On the other hand, Sirt1 inhibition resulted in an induction of apoptosis (but not senescence) in PC3 cells which lack p53. In addition, the results presented here further support the pro-proliferative action of Sirt1 in PCa cells.

Based on our data, it is tempting to suggest that Sirt1 inhibition may have different downstream targets in cells with active p53 *versus* cells where p53 is inactive (mutated or deleted). In cells with active p53, two scenarios are possible. *First*, Sirt1 inhibition may inhibit p53 binding and deacetylation either directly or via inhibition of p300/CBP binding and deacetylation thereby activating p53 mediated responses (such as induction of p21/Waf1) leading to cell cycle arrest or apoptosis or senescence in cancer cells. *Second*, Sirt1 inhibition may inhibit FoxO binding and deacetylation either directly or via inhibition of p300/CBP binding and deacetylation thereby activating FoxO mediated responses (such activation of caspases) leading to apoptosis of cancer cells. In cells with active p53 both events may happen; whereas, in cells without active p53, only the second option is possible. These possible scenarios are depicted in Fig. 5.

However, further in-depth studies are needed to dissect the molecular mechanism(s) of the observed differential responses of Sirt1 inhibition in PCa cells, especially in connection with p53.

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Abbreviations

PCa, prostate cancer
 AR, androgen receptor
 Sirt1, sirtuin 1
 HDAC, histone deacetylase
 shRNA, short hairpin RNA
 microRNA 34a, miR-34a
 SA- β -gal, senescence-associated beta-galactosidase
 FSC, forward-angle light scatter
 SSC, side-angle light scatter
 PARP, poly(ADP-ribose) polymerase
 TBP, TATA-binding protein
 TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

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Figure 1. Effects of sirtinol treatment on PC3 and PC3-p53 cells

A) Effect of sirtinol on cell growth. PC3-p53 and PC3 cells were treated with sirtinol (30μM or 120μM) or vehicle (DMSO) for 24 hours and analyzed by Trypan blue assay to assess cell growth. Briefly, following treatments, cells were trypsinized and collected in a 1.5-ml Eppendorf tube. The cells were pelleted by centrifugation and resuspended in PBS. Trypan blue (0.4% in PBS) was added to a smaller aliquot of cell suspension, and cell number was counted. The data are expressed as the mean $\pm$ S.E. of three experiments; * p <0.01. **B) Effect of sirtinol on cell viability.** PC3-p53 and PC3 cells were treated with sirtinol (30μM or 120μM) or vehicle (DMSO) for 24 hours and analyzed by Trypan blue assay, as described above, to assess cell viability. Cell viability is expressed as the percent viable cells of the total number of cells. The data are expressed as the mean $\pm$ S.E. of three experiments; * p <0.01. **C)** Effect of sirtinol on protein levels of Sirt1, FoxO1 and Ac-FoxO1. PC3-p53 and PC3 were treated with sirtinol (30μM or 120μM) or vehicle (DMSO) for 24 hours, and then nuclear and cytosolic protein lysates were prepared. Sirt1, FoxO1, and Ac-FoxO1 protein levels were detected by Western blot analysis. Briefly, 30μg of protein was subjected to SDS-PAGE and immunoblot analysis using the appropriate primary and secondary antibodies followed by chemiluminescent detection. Equal loading was confirmed by re-probing the blots for β -actin (cytosolic) or TATA-binding protein (*TBP*) (nuclear). The data represent three experiments with similar results. **D)** Effect of sirtinol on the transcriptional activity of FoxO1. PC3-p53 and PC3 cells were transfected with phRL-TK and 3XIRS Luc-FoxO1 using Lipofectamine 2000 (Invitrogen, CA) following the vendor's protocol. After 48 hours, cells were re-plated at equal density and allowed to adhere overnight. Twenty-four hours later, transfected cells were treated with sirtinol (30 or 120μM in DMSO) for 24 hours. Then cell lysates were prepared, and luciferase activity was determined using the dual luciferase assay system (Promega, WI). Luciferase activity was normalized to *Renilla* luciferase activity (*RLU*). The data represent mean $\pm$ S.E. of three experiments; * p <0.01.

Figure 2. Effect of shRNA mediated Sirt1 inhibition on clonogenic survival of PC3-p53 and PC3 cells

PC3-p53 and PC3 cells were transfected with short hairpin *Sirt1* clone or non-sense clone in a pSHAG-MAGIC2 vector using Arrest-In (Open Biosystems, AL) protocol provided by the vendor. Briefly, plasmid DNA was diluted in serum-free medium and mixed with Arrest-In diluted in serum-free medium and incubated for 10 min at room temperature. The DNA Arrest-In complex mixture was added to 60–80% confluent cells and incubated for 6 hours at 37°C in 5% CO₂ in a humidified incubator. After 6 hours, transfection medium was removed, and medium with serum was added and incubated in a humidified chamber at 37°C in 5% CO₂ for 48 hours. After 48 hours, the cells were treated with complete growth media supplemented with 2µg/ml Puromycin for 48 hours. This allowed for selection of transfected cells, which were then collected with trypsin and re-dispersed into 6-well tissue culture plates at 3,000 cells per well. The cells were cultured for 10–14 days with growth media being replaced every 3 days. The cells were stained with 0.5% crystal violet and pictures were captured with a digital camera. Representative pictures from three experiments with similar results are shown. The colonies were counted manually and are represented graphically. The data are expressed as the mean ±S.E. of three experiments; * $p < 0.01$.

Figure 3. Effect of shRNA mediated Sirt1 inhibition on senescence in PC3-p53 and PC3 cells

A) Effect of Sirt1 knockdown on β -galactosidase staining. PC3-p53 and PC3 cells were plated on a 6-well tissue culture plates and transfected by shRNA constructs as described earlier. Forty-eight hours post transfection, cells were washed with PBS and fixed with 2% Formaldehyde/0.2% Glutaraldehyde for 5 minutes at room temperature. Cells were then washed again with PBS and incubated with β -gal staining solution at pH 6.0 overnight at 37°C followed by capturing pictures. Blue staining represents senescence induction. Representative pictures from three experiments with similar results are shown. B) Effect of Sirt1 knockdown on forward (FSC) and side (SSC) scatter. Cells were transfected as described above and then collected and resuspended in PBS. Five minutes before analysis, 1 μ g/mL of PI stain was added to the cell suspension. Cells were analyzed for side and forward scatter measurements using a FACScan benchtop cytometer at the UWCCC Flow Cytometry Core Facility. The average FSC and SSC signal was quantified as an average channel number using Cell Quest software. The data represent three experiments with similar results.

Figure 4. Effect of shRNA mediated Sirt1 inhibition on apoptosis in PC3-p53 and PC3 cells
A) Effect of Sirt1 inhibition on PARP cleavage. PC3-p53 and PC3 cells were transfected with Sirt1 shRNA construct as described previously. After 48 hours, protein lysates were prepared and protein levels of total PARP and cleaved PARP were assessed by Western blot analyses as described previously. Equal loading was confirmed by re-probing the blot for β -actin. PARP and cleaved PARP protein levels were quantitated by densitometric analysis of protein bands. The quantification of protein was performed by a digital analyses of protein bands (TIFF images) using UN-SCAN-IT software. The data (relative density of protein band normalized to β -actin) are expressed as mean $\pm$ S.E. of three experiments; * p <0.01. **B) Effect of Sirt1 inhibition on apoptosis.** The induction and extent of apoptosis was assessed employing the

APO-BrdU TUNEL Apoptosis Assay kit (Invitrogen, CA). Briefly, following the previously described transfection protocol, PC3-p53 and PC3 cells were collected and fixed in ethanol. The cells were then washed and labeled with UTP-BrdU overnight, and then washed, and incubated with Alexa488 Anti-BrdU antibody followed by counterstaining with PI. BrdU incorporation was analyzed using a FACScan benchtop cytometer at the UWCCC Flow Cytometry Core Facility. When analyzing the cell population with ModFit LT software, the sub-G₁ population (cell debris) was excluded. The data are expressed as the mean $\pm$ S.E. of three experiments; * p <0.01.

Figure 5. Proposed model of Sirt1 inhibition-mediated responses in human PCa cells differing in p53 status