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Original Article
Targeting CREB inhibits radiation-induced neuroendocrine differentiation and increases radiation-induced cell death in prostate cancer cells

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Abstract: Neuroendocrine differentiation (NED) is a process by which prostate cancer cells transdifferentiate into neuroendocrine-like (NE-like) cancer cells. Accumulated evidence suggests that NED is associated with disease progression and therapy resistance in prostate cancer patients. We previously reported that by mimicking a clinical radiotherapy protocol, fractionated ionizing radiation (FIR) induces NED in prostate cancer cells. Interestingly, FIR-induced NED constitutes two distinct phases: a radioresistance phase in which a fraction of cells selectively survive during the first two week irradiation, and a neuroendocrine differentiation phase in which surviving cells differentiate into NE-like cancer cells during the second two week irradiation. We have also observed increased activation of the transcription factor cAMP response element binding (CREB) protein during the course of FIR-induced NED. To determine whether targeting NED can be explored as a radiosensitization approach, we employed two CREB targeting strategies, CREB knockdown and overexpression of ACREB, a dominant-negative mutant of CREB, to target both phases. Our results showed that ACREB expression increased FIR-induced cell death and sensitized prostate cancer cells to radiation. Consistent with this, knockdown of CREB also inhibited FIR-induced NED and sensitized prostate cancer cells to radiation. Molecular analysis suggests that CREB targeting primarily increases radiation-induced pre-mitotic apoptosis. Taken together, our results suggest that targeting NED could be developed as a radiosensitization approach for prostate cancer radiotherapy.

Keywords: Prostate cancer, radiosensitization, neuroendocrine differentiation, NED, CREB

Introduction

Prostate cancer is the second-leading cause of cancer death in American men [1]. Approximately 15-20% of prostate cancer patients were diagnosed with high-risk cancer that is either clinical stage T3, a Gleason score of 8-10 or prostate specific antigen > 20 ng/ml [2]. Radiotherapy (RT) plus androgen deprivation therapy (ADT) is the standard treatment for these patients [2-4]. However, 30-60% of patients with high-risk cancer still experience biochemical recurrence within 5 years [5-7]. Thus, high-risk prostate cancer represents a therapeutic challenge for prostate cancer management.

Neuroendocrine differentiation (NED) in prostate cancer is a process by which prostate cancer cells transdifferentiate into neuroendocrine (NE)-like prostate cancer cells [8]. NE cells are one type of prostatic epithelial cells that constitutes less than 1% of total epithelial cells. However, increased numbers of NE-like prostate cancer cells have been observed in prostate cancer patients [9-11]. Accumulated evidence suggests that NED is associated with disease progression, androgen-independent growth and poor prognosis in prostate cancer patients [8, 12-14]. We previously reported that fractionated ionizing radiation (FIR) induces NED in LNCaP prostate cancer cells [19] and this finding has recently been extended to DU-145 and PC-3 cells and in LNCaP xenograft tumors [20]. Importantly, FIR-induced NED is a reversible process and isolated radiation-resistant sublines are cross-resistant to radiation, androgen depletion and docetaxel treatments.
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[19]. Given that our recent pilot clinical study has shown 4 out of 9 patients may undergo NED [20], it is very likely that RT-induced NED may contribute to radioresistance and tumor recurrence in prostate cancer patients.

The mechanisms underlying NED remain to be defined [8]. It appears that distinct mechanisms are involved in NED induced by different stimuli [8, 14, 21]. We found that FIR-induced NED correlates with increased phosphorylation of cAMP response element binding (CREB) protein at Ser133 [19], an activating phosphorylation by many protein kinases [22]. CREB, a member of the ATF-1/CREM/CREB basic region leucine zipper transcription factor family, functions as a homodimer or heterodimer with other ATF-1/CREM/CREB family members to regulate transcription of target genes responsible for a wide range of cellular processes [23]. Studies have established a role for CREB in several human cancers [24-26]. In prostate cancer, increased expression of RGS17 enhances CREB phosphorylation to maintain tumor cell proliferation [27]. CREB activation has also been linked to aberrant expression of vascular endothelial growth factor (VEGF) and the resulting predisposition to bone metastasis [28]. In the present study, we employed a dominant negative CREB and CREB knockdown approaches to inhibiting CREB activity, and demonstrated that targeting FIR-induced NED is an effective approach to sensitizing prostate cancer cells to radiation.

Materials and methods

Establishment of stable cell lines for fractionated FIR treatment

Prostate cancer cell lines were maintained and treated with FIR (2 Gy/day, 5 days/week) as previously reported [19, 20]. The tetracycline/doxycycline inducible pcDNA4-TO system (Invitrogen) was used to establish stable cell lines (LNCaP-HA-ACREB#1-4) to express ACREB [19]. The tetracycline/doxycycline inducible lentiviral system to express short hairpin RNA (shRNA) or scrambled control (SC) was utilized to knock down CREB with pLKO.1-Tet-On (Addgene plasmid 21915). The oligonucleotides were selected using validated sequences from Sigma Aldrich and named using the last three digits corresponding to the Sigma TRCN sequence number (TRCN0000007308, TRCN0000226467, TRCN0000226468, TRCN0000226469).

Lentiviral packaging using pLKO.1-CREB shRNA or pLVX-ACREB (Clontech) in HEK293T cells and establishment of prostate cancer stable cell lines expressing ACREB or CREB shRNAs were performed as reported previously [29].

MTT assay

LNCaP-HA-ACREB#1 cells were seeded in triplicate in 48-well plates at a density of 2 × 10^4 cells/ml. Tetracycline (5 µg/ml) was added to induce expression of HA-ACREB for 24 hours before subjecting to FIR. Medium was changed after 3 days and tetracycline was replenished. After achieving the desired dose of FIR, medium was removed from wells and 70 µl of MTT reagent was added. Cells were incubated at 37°C, 5% CO_2 for 4 hours followed by addition of 130 µl of DMSO. Plates were shaken, incubated for an additional 10 min at 37°C, 5% CO_2 and read on Biotek Synergy 4 plate reader at 570 and 700 nm. Results were from three independent experiments, and two-way ANOVA analysis was performed to determine the statistical significance.

Cell cycle analysis via flow cytometry

LNCaP-HA-ACREB#1 cells were treated with tetracycline (5 µg/ml) to induce expression of HA-ACREB for 24 hours, followed by FIR. Medium with fresh tetracycline was changed every 3 days. Cells were harvested, fixed in 70% ethanol and temporarily stored at 4°C, and then resuspended in 500 µl freshly prepared propidium iodide (PI) working solution prior to flow cytometry analysis. Data was collected on Beckman Coulter FC 500 flow cytometer and analysis was completed using FlowJo software (Treestar, Inc., Ashland, OR). Three independent experiments were performed and two-way ANOVA analysis was performed to determine the statistical significance.

Immunoblotting of γH2AX, PARP cleavage, and LC-3 cleavage

LNCaP-HA-ACREB#1 cells were treated with doxycycline or water for 48 hours, and then subjected to FIR (2 Gy/day). Irradiated cells including floating cells were harvested 24 hours after the last IR treatment and total lysate was prepared for immunoblotting analysis using antibodies against γH2AX (Cell Signaling Technology, #9718), cleaved poly ADP ribose polymerase (PARP) (BD Pharmingen, #556494),
and microtubule-associated protein 1A/1B-light chain 3 (LC-3) (Novus Biologicals, NB100-2220) to determine the underlying mechanisms of cell death. To determine whether pre-mitotic or post-mitotic cell death occurred in ACREB expressing cells, cells were induced to express ACREB for 48 hours and then subjected to a single dose of 2 Gy ionizing radiation (IR). The total cell lysate was prepared 4 hours after the irradiation for immunoblotting analysis of PARP cleavage. For preparation of total cell lysate at 24 hours after the irradiation, floating cells were removed by changing the medium at 12 hours, and the total cell lysate was prepared for PARP cleavage analysis at 24 hours after the irradiation treatment.

**Immunofluorescence analysis of activated caspase-3**

To quantify the number of cells with activated caspase-3, cells were first induced to express HA-ACREB with or without doxycycline, and then subjected to 2 Gy of IR or without IR treatment, followed by fixation and staining with
anti-cleaved caspase-3 antibody (Cell Signaling Technology, #9664) and a secondary Texas Red-conjugated anti-rabbit antibody and 4',6-diamidino-2-phenylindole (DAPI). The percentage of activated caspase-3 positive cells was calculated by dividing the number of cells stained red by the total number of cells counted (DAPI positive). For each experiment, at least 120 cells were counted, and three independent experiments were conducted. Results were analyzed using student’s t-test.

Clonogenic assays

LNCaP-HA-ACREB#1 or LNCaP-CREB shRNA#468 cells or the control cell lines were first induced with or without doxycycline (1 μg/ml) for 48 hours (for ACREB) or 72 hours (for shRNAs), and then subjected to a single exposure of different doses of IR. Irradiated cells were trypsinized immediately and various numbers of cells were seeded in 6-well plates and cultured in complete medium with or without doxycycline for two weeks. At the end of experiments, the number of colonies was counted and surviving fractions were calculated as described [30]. Student’s t test was used to determine the statistical significance.

Quantification of neurite extension and immunoblotting analysis of chromogranin A and neuron specific enolase

LNCaP-HA-ACREB stable cell lines were subjected to 40 Gy of FIR, and images were captured using a Nikon TE-2000 inverted epifluorescence microscope with CoolSnap CCD camera. Image processing and analysis was completed using ImageJ software modified by the McMaster Biophotonics Facility in Ontario, Canada (revision 1.44k). Neurite extension was quantified using the ImageJ plugin NeuronJ from Erik Meijering [31]. Quantification was performed using 10 image fields per condition. Results presented were from three independent experiments and two-tailed t-test was used to determine the statistical significance. The expression of chromogranin A (CgA) and neuron specific enolase (NSE) was similarly examined as reported previously [19].

Results

CREB knockdown inhibits FIR-induced neurite extension and NSE expression

To dissect the role of CREB in FIR-induced NED in prostate cancer cells (Figure 1A), we employed a lentivirus-based tetracycline-inducible knockdown system to generate four LNCaP cell lines containing stably integrated CREB shRNA expression plasmid. Screening of these four cell lines showed variable knockdown efficiency with CREB #468 achieving approximately 85% knockdown efficiency (data not shown). We then used CREB #468 to conduct three independent transductions to generate stable LNCaP cell lines that had comparable knockdown efficiency (Figure 1B). To determine the effect of CREB knockdown on FIR-induced NED, we performed 40 Gy of FIR and measured the expression of CgA and NSE. While we observed a dramatic inhibition of NSE expression when compared with SC, the expression level of CgA was not altered by CREB knockdown (Figure 1C). To quantify the effect of CREB knockdown on neurite extension and cell viability, we used the established three independent sublines to perform 40 Gy of FIR. Like the expression of a non-phosphorylatable CREB (S133A) [19], we observed that CREB knockdown significantly decreased neurite extension (Figure 1D). However, CREB knockdown failed to increase FIR-induced cell death (Figure 1E). The inability of CREB knockdown to increase FIR-induced cell death is not due to the selection of established stable clones as transient expression of CREB shRNAs also failed to increase FIR-induced cell death after 10 Gy of FIR (unpublished observation) and another CREB knockdown construct targeting a different region of the CREB coding sequence yielded similar results (data not shown).

Expression of a dominant negative CREB increases FIR-induced cell death

Our observation that CREB knockdown did not increase FIR-induced cell death is surprising, given that CREB phosphorylation was induced even after 10 Gy of FIR [19]. Because there are at least 3 members in the CREB/CREM/ATF-1 family that can form dimers with CREB to regulate target gene transcription [22], we reasoned that these family members might compensate for the reduction of CREB to regulate expression of target genes essential for cell survival. Alternatively, the residual amount of CREB might be sufficient to regulate expression of these target genes. To circumvent this potential problem, we used ACREB, a dominant negative CREB, in which the leucine zipper region of CREB is used and the basic region is replaced with acidic amino acid residues [32], to evalu-
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The role of CREB in FIR-induced NED. Because ACREB retains the ability to dimerize with endogenous CREB and other CREB dimerization partners but cannot bind DNA, overexpressed ACREB can efficiently inhibit transcription of CREB target genes [32, 33]. For this purpose, we established stable, tetracycline-inducible, LNCaP cell lines expressing HA-ACREB as a hemagglutinin (HA) fusion protein. Four individual clones were isolated, and these clones exhibited variable expression of HA-ACREB. Because CREB can autoregulate its own transcription [34], these clones also demonstrated unique effects on CREB expression (Figure 2A). Notably, induction of ACREB in clones #1 and #4 reduced CREB by 90%. Consistent with the expression level of ACREB and the down-regulation of CREB in these clones, induction of ACREB in clone #1 increased FIR-induced cell killing after 10 Gy of FIR (Figure 2B) whereas induction of ACREB in clones #2 and #3 had little effect on FIR-induced cell killing (unpublished observation). These results not only demonstrate that ACREB is a potent inhibitor of CREB activity but also suggest that CREB plays a role in conferring radioresistance even during the first week of irradiation.

**Long-term expression of ACREB dramatically increases FIR-induced cell killing**

To determine the effect of long-term expression of ACREB on FIR-induced cell death, we performed long-term FIR treatment. While attempt-
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During these experiments, using clones derived from the Invitrogen pcDNA6/TR/pcDNA4/TO expression system, there was excessive cell death under both induced and non-induced conditions, which is likely due to the effect of radiation-induced damage to the DNA encoding the tetracycline-resistance operon [35]. To overcome this problem, we utilized the Clontech pLVX-Tet-On lentiviral expression system that does not rely on the dissociation of the Tet repressor protein from the tetracycline-resistance operon [36]. Stable clones were prepared using three independent transductions and induction of ACREB sufficiently down-regulated the expression of CREB in each cell line (Figure 2C). To separate the role of CREB in both phases, we specifically induced ACREB expression during the NED phase only (weeks 3 and 4, post-20 Gy induction) and during the entire 4 weeks (pre-induction) to assess the impact of ACREB expression on the total number of viable cells at the end of 40 Gy FIR (Figure 2D). Induction of ACREB during the entire FIR treatment period resulted in a 7.6-fold reduction in cell number, and induction of ACREB during the NED phase also resulted in a 2.5-fold reduction (Figure 2E). These results suggest that CREB plays a critical role in the acquisition of radiore- sistance and the acquisition of NED during the process of FIR-induced NED.

ACREB expression increases radiation-induced apoptosis

The transcriptional activity of CREB is required for regulation of many cellular processes includ-
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...ing cell cycle, apoptosis, cell proliferation and differentiation [23]. To uncover the molecular mechanism by which ACREB expression increases IR-induced cell death, we examined the effect of ACREB expression on cell cycle, apoptosis, autophagy and DNA damage. Flow cytometry analysis revealed that FIR treatment in ACREB expressing cells exhibited increased granularity after 10 Gy of FIR. This granular population of cells increased by 2.3 fold when compared with FIR treated LNCaP not expressing ACREB (Figure 3A). Flow cytometry analysis using PI showed a 4-fold increase in the sub-G1 population in ACREB expressing cells treated with 10 Gy of FIR (Figure 3B). No significant difference in other phases of cell cycle was observed (data not shown). These results suggest that ACREB expression increases FIR-induced cell death. Because increased granularity can be associated with events such as autophagy [37] and apoptosis [38], we examined their involvement in ACREB-induced radiosensitivity. We harvested all floating and adherent cells after 10 Gy of FIR to measure PARP cleavage, and confirmed that ACREB expression indeed increased PARP cleavage (Figure 3C). We also performed immunoblotting analysis of LC3. Conversion of the cytosolic LC3I into autophagosome-associated LC3II allows assessment of autophagy via immunoblotting. However, ACREB induction did not increase the amount of FIR-induced LC3II nor the ratio of LC3II/LC3I (Figure 3D). However, ACREB expression slightly increased FIR-induced γH2AX level (Figure 3D).

Since we observed increased cell death with increased doses of FIR, we next determined whether this correlates with the extent of apoptosis by measuring PARP cleavage after various doses of FIR. Although ACREB expression increased the amount of cleaved PARP in all doses, there was no significant increase in cleaved PARP in higher doses (Figure 4A). Because we prepared total cell lysate for immunoblotting analysis of PARP cleavage 24 hours after the last irradiation of the indicated doses, these results suggest that apoptosis likely occurs within 24 hours.

Radiation-induced cell death can occur as pre-mitotic and post-mitotic cell death [39]. The former usually occurs within 4-5 hours whereas the latter occurs after 24 hours. To know whether ACREB expression increases radiation-induced pre-mitotic cell death, we performed a single dose IR and harvested cells at 4 and 24 hours to examine the level of PARP cleavage. We observed increased cell death at 4 hours after 2 Gy of irradiation, and some cells showed membrane blebbing, a typical feature of apoptotic cells (unpublished observations). Consistent with this, increased PARP cleavage in irradiated ACREB-expressing cells was observed (Figure 4B). However, we observed less cell death and PARP cleavage at 24 hours (Figure 4B). No increase in cell death or PARP cleavage was observed after 48 hours. These results suggest that ACREB induction may primarily induce pre-mitotic cell death. Because radiation-induced pre-mitotic cell death usually results from activation of pre-existing apoptotic machinery [39], we next examined the activation of caspase-3 by immunostaining, and observed that ACREB induction by itself appeared to slightly activate caspase-3. However, ACREB
expression dramatically increased IR-induced caspase-3 activation (Figure 4C). These results collectively suggest that ACREB expression primarily increases radiation-induced pre-mitotic apoptosis via activation of caspase-3.

CREB targeting sensitizes prostate cancer cells to radiation

Our above results strongly suggest that targeting CREB signaling is an effective approach to
sensitizing prostate cancer cells to radiation. To further determine this, we used the ACREB stable cell lines to perform clonogenic assays, a standard assay for determination of radiosensitivity [30]. As shown in Figure 5A, induction of ACREB expression significantly sensitized LNCaP cells to radiation at all doses examined. Because the clonogenic assay utilizes a single dose treatment to assess the impact of DNA damage on cell reproduction, this is different from FIR, during which damaged DNA may be repaired by compensation for the reduction of CREB. Thus, we sought to determine whether CREB knockdown can sensitize LNCaP cells to radiation. Using the same stable cell line (#468), we observed that knockdown of CREB also sensitized LNCaP cells to radiation when compared with the scrambled control (Figure 5B). A similar result was observed in DU-145 (Figure 5C). Consistent with the lack of significant CREB activation by FIR in PC-3 cells [20], knockdown of CREB did not sensitize PC-3 to radiation (Figure 5D). Note that CREB expression was comparably knocked down in LNCaP (Figure 5E), DU-145 (Figure 5F) and PC-3 (Figure 5G) stable cell lines. Taken together, our results suggest that targeting CREB can sensitize a subset of prostate cancer cells to radiation.

Discussion

Numerous studies have demonstrated that NED is associated with disease progression and poor clinical outcome in prostate cancer patients [12]. The clinical significance of NED is further supported by the fact that ADT- and chemotherapy-induced NED correlates with poor therapeutic responses and clinical outcomes [15-18, 40]. Because NE-like cells are highly resistant to apoptosis [41] and cAMP and androgen depletion-induced NED are reversible [8, 42], it has been hypothesized that therapy-induced NED allows prostate cancer cells to survive treatment and contribute to tumor recurrence [8, 13, 14]. However, it remains unclear whether targeting therapy-induced NED can be explored to sensitize prostate cancer cells to treatments such as ADT, radiotherapy or chemotherapy. Using LNCaP cells as a model, we have demonstrated that FIR-induced NED constitutes two distinct phases: selection of radioresistant cells and NED onset (Figure 1A). Using two CREB targeting approaches, we provide evidence in the present study that CREB is involved in both phases and targeting CREB can increase FIR-induced cell death. In particular, expression of ACREB, a potent dominant negative CREB, increased FIR-induced cell death and sensitized LNCaP cells to FIR. Consistent with FIR-induced activation of CREB in LNCaP and DU-145 cells [20], knockdown of CREB also sensitized LNCaP and DU-145 cells to radiation. Our results suggest that inhibition of RT-induced NED may be explored to sensitize prostate cancer cells to radiotherapy. Further investigation of CREB targeting strategies [24] or identification of CREB upstream regulators will likely lead to development of novel radiosensitizers.

Although CREB signaling has been explored for its role in oncogenesis [43], the impact of CREB in cancer cell signaling has recently attracted attention. CREB targeting CRE-decoy oligonucleotides induce apoptosis in ovarian cancer cells [25] and CREB is involved in prostate cancer bone metastasis through regulation of VEGF [28]. In several studies, the dominant negative ACREB has been utilized to target CREB. One such study reported the mechanism of ACREB-induced apoptosis in rat thyroid cells [44]. It was demonstrated that S phase delay led to activation of ATR and the S-phase checkpoint without altering the regulation of pro- or anti-survival genes. These findings are consistent with the role of CREB in regulating expression of several target genes involved in the cell cycle [23]. In the present study, we demonstrate that ACREB expression efficiently sensitized LNCaP cells to FIR by increasing FIR-induced apoptosis. However, we did not see any significant S phase delay in ACREB expressing cells. It is worth noting that CREB knockdown only inhibited FIR-induced neurite outgrowth and the expression of NSE without significant effect on FIR-induced CgA expression and cell death during FIR treatment. Paradoxically, CREB knockdown was sufficient to inhibit colony formation in clonogenic assays in LNCaP and DU-145 cells. Given that CREB/CREM/ATF-1 family members can form both homodimers and heterodimers and that some target genes are regulated by these dimeric complexes [22], it is likely that the loss of CREB may be functionally compensated for by other dimeric complexes during FIR [45]. This is supported by the observation that CREB knockdown did not inhibit FIR-induced CgA expression, though CREB is a transcriptional activator
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of CgA [46]. Thus, it is likely that expression of some CREB target genes critical for cell survival are not affected by CREB knockdown, but are suppressed by ACREB expression during the course of FIR treatment. Alternatively, a residual amount of CREB (e.g., 10-20%) is enough to activate the expression of target genes that confer the resistance and cell survival to FIR treatment. In agreement with this, we indeed observed that induction of ACREB in clones #1 and 4, in which CREB expression was decreased by more than 90%, efficiently increased IR-induced cell death. Conversely, induction of ACREB in clones #2 and #3, in which CREB expression was only decreased by 60% and 13% respectively, was ineffective.

Radiation-induced cell death can be a result of induction of apoptosis or autophagy [47]. It has been reported that IR-induced apoptosis and autophagy can occur in prostate cancer cells such as LNCaP [37, 48]. However, induction of ACREB did not significantly increase FIR-induced autophagy. Thus, it is unlikely that CREB is involved in the regulation of FIR-induced autophagy in LNCaP cells. Instead, we observed increased PARP cleavage and caspase-3 activation as early as 4 hours after a single exposure to IR. Interestingly, this effect appears to last for at least 24 hours. However, we failed to observe any further increase in apoptosis after 48 hours. These results collectively suggest that ACREB induction primarily increases IR-induced pre-mitotic apoptosis, and to a lesser extent post-mitotic apoptosis. Future identification of CREB target genes involved in IR-induced apoptosis and FIR-induced NED will provide new insight into the role of CREB in radioresistance and FIR-induced NED.

In conclusion, we have employed two CREB targeting approaches and demonstrated that CREB is involved in both the acquisition of radioresistance and the acquisition of NED during FIR-induced NED. In particular, expression of ACREB potently increased FIR-induced apoptosis and sensitized prostate cancer cells to radiation. Our results suggest that targeting FIR-induced NED is an effective approach to sensitizing prostate cancer cells to radiation.

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Disclosure of conflict of interest

None to declare.

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