The radiation response of androgen-refractory prostate cancer cell line C4-2 derived from androgen-sensitive cell line LNCaP

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Abstract

Radiation therapy is a relatively effective therapeutic method for localized prostate cancer (PCa) patients. However, radioresistance occurs in nearly 30% of patients treated with potentially curative doses. Therapeutic synergy between radiotherapy and androgen ablation treatment provides a promising strategy for improving the clinical outcome. Accordingly, the androgen deprivation-induced signaling pathway may also mediate radiosensitivity in PCa cells. The C4-2 cell line was derived from the androgen-sensitive LNCaP parent line under androgen-depleted condition and had acquired androgen-refractory characteristics. In our study, the response to radiation was evaluated in both LNCaP and C4-2. Results showed that C4-2 cells were more likely to survive from irradiation and appeared more aggressive in their resistance to radiation treatment compared with LNCaP, as measured by clonogenic assays and cell viability and cell cycle analyses. Gene expression analyses revealed that a set of genes involved in cell cycle arrest and DNA repair were differentially regulated in LNCaP and C4-2 in response to radiation, which was also consistent with the radiation-resistant property observed in C4-2 cells. These results strongly suggested that the radiation-resistant property may develop with progression of PCa to androgen-independent status. Not only can the LNCaP and C4-2 PCa progression model be applied for investigating androgen-refractory progression, but it can also be used to explore the development of radiation resistance in PCa.

Keywords: LNCaP, C4-2, prostate cancer, radiation response

1 Introduction

Prostate cancer (PCa) continues to be one of the most prevalent cancers in men of western countries. A total of 218 890 new PCa cases and 27 050 deaths from PCa were projected to occur in the United States in 2007 [1]. In the past 15 years, active screening for PCa has led to a significantly decreased proportion of patients presenting with high-risk, advanced disease [2]. A majority of prostate carcinomas are now detected while the disease is still clinically localized and with intermediate or low risk-factor characteristics. Radiation is a preferred treatment option for localized PCa. However, clinical evidence
reveals that conventional-dose radiation often does not provide complete tumor eradication and a small fraction of tumor cells survive the lethal effects of radiation and eventually repopulate the irradiated site, which results in radiorecurrent PCa and a 5-year distant metastasis-free survival of < 80% [3–5]. The combination of radiation and androgen deprivation therapy provides an effective strategy for preventing treatment failure. Adjunct androgen deprivation therapy has been shown to confer a survival advantage over radiation alone in high-risk localized PCa [6]. Moreover, clinical observation also shows that patients with hormone-resistant (HR) PCa caused by long-term hormone treatment seem to have higher biochemical failure rates after radiation therapy. Previous studies also indicate that the response to radiation treatment is different between HR PCa cells and androgen-sensitive PCa cells [7]. These observations indicate that molecular events mediated by AD may also function in radiosensitization, and that androgen-refractory development may be associated with radiation resistance in PCa.

LNCaP is an androgen-dependent, non-metastasis and marginally tumorigenic PCa cell line [8]. The C4-2 subline was derived from LNCaP through interaction with stromal cells under androgen-depleted condition in castrated hosts. The C4-2 subline is tumorigenic in androgen-depleted environment, which indicates that C4-2 has the acquired characteristics of androgen independence [9, 10]. LNCaP and C4-2 cells have the same genetic background and the unique advantage of remarkably mimicking the phenotypic and genotypic changes that are often observed in clinical human PCa. Even though LNCaP and C4-2 have provided a very useful model for studying the mechanism underlying the progression of PCa from androgen-dependent (AD) to androgen independent (AI) state, the model’s response to radiation has not been studied systematically until now. In this study, the radiation responses of AD LNCaP and AI C4-2 were evaluated. As a result, androgen-refractory C4-2 cells were found to possess radiation-resistant properties compared with androgen-sensitive LNCaP. Taken together, our data strongly suggest that C4-2 also acquires radiation resistance during the process of transition to the androgen-refractory stage. Accordingly, LNCaP and C4-2 PCa cells may provide an ideal cell model for studying the molecular mechanism underlying PCa cells’ progression from the radiation-sensitive state to resistance, and for investigating critical determinants in the efficacy of irradiation and androgen ablation therapy.

2 Materials and methods

2.1 Cell culture and reagents

LNCaP and C4-2 cell lines were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 8% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 10 mmol L⁻¹ HEPES and 1.0 mmol L⁻¹ sodium bicarbonate. All cells were cultured at 37°C with 5% CO₂ in a humidified incubator. To study the cell growth in androgen-depleted condition, LNCaP and C4-2 cells were cultured in fresh phenol red-free RPMI-1640 with 5%–10% dextran/charcoal absorbed fetal bovine serum (cFBS; Hyclone). G418 was obtained from GIBCO (Invitrogen), and Casodex was supplied by Sigma (Woodlands, TX, USA).

2.2 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell growth rate was measured using an MTT proliferation assay. Briefly, 4000–5000 cells were seeded in 96-well plates. Cell growth was examined at the indicated time points. Before testing, 20 µL of MTT reagent (2.5 mg mL⁻¹ MTT in PBS, Amresco Inc., Solon, OH, USA) was added and the cells were incubated for a further 4-h at 37°C. Then 150 µL of dissolving reagent DMSO (Amresco Inc.,) was added to dissolve the formazan crystals. The optical density (OD) was measured at wavelength of 490 nm on a microplate reader. Pilot experiments were conducted to determine the optimal cell concentration for the experiments.

2.3 Colony formation in soft agar

A total of 400–10 000 cells were suspended in 0.17% low melting agarose (Difco Laboratories, Detroit, MI, USA) dissolved in 2 mL of RPMI 1640 with 10% FBS or with 10% androgen-free cFBS medium and placed on top of a 2-mL underlayer of 0.5% agarose in the same medium in six-well culture plates. After 3 weeks of incubation at 37°C in a 5% CO₂ incubator in a humidified atmosphere, the colonies with more than 15 cells were counted.

2.4 Cell cycle analysis

LNCaP and C4-2 cells were harvested at several different time points after radiation. Following treatment, the cells were fixed, washed with phosphate-buffered saline, incubated with 5 µg mL⁻¹ DNase-free
RNase for 30 min at 37°C, and stained with 40 µg mL⁻¹ propidium iodide (Sigma-Aldrich, St. Louis, MO, USA). The propidium iodide-stained cells were detected in a FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

2.5 Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from LNCaP and C4-2 cells and the control cells using the RNeasy Minikit (Qiagen, Hilden, Germany). Double-stranded cDNA was synthesized from 1 µg of total RNA with random primer (Promega, San Luis Obispo, CA, USA). Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System (ABI Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Reactions were performed in 50 µL volume with 0.5 µmol L⁻¹ primers and MgCl₂ concentration optimized between 2 and 5 µmol L⁻¹. Nucleotides, Taq DNA polymerase and buffer were included in the SYBR Green PCR Master Mix (ABI Applied Biosystems). A typical protocol included a 5-min denaturation step followed by 40 cycles involving 95°C denaturation for 30 s, 55°C annealing for 30 s and 72°C extension for 30 s. Extension periods varied with specific primers depending on the length of the product (~1 s/25 bp). Detection of the fluorescent product was carried out after an additional 2-s step at 2°C below the product melting temperature. To confirm the amplification specificity of the PCR, products from each primer pair were subjected to a melting curve analysis.

2.6 Western blot assay

Cells were treated with lysis buffer containing 150 mmol L⁻¹ Tris base (pH 7.5), 50 mmol L⁻¹ NaCl, 1 mmol L⁻¹ EDTA (pH 8.0), 1% NP40, 1 mg mL⁻¹ leupeptin and 1 mmol L⁻¹ PMSF. Protein concentrations were determined by BCA protein assay (Pierce Chemical Co., Rockford, IL, USA). Equal amounts of protein denatured in 2 × sodium dodecyl sulfate (SDS) sample buffer (100 mmol L⁻¹ Tris base [pH 6.8], 200 mmol L⁻¹ DTT, 4% SDS, 20% glycerol and 0.005% bromphenol blue) were loaded into 15% SDS-polyacrylamide gel electrophoresis, and gels were transferred onto nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were blocked overnight in Tris-buffered saline containing 5% (w/v) skimmed milk powder, and then were stained by appropriate dilution of primary antibodies against p53, p21, Bcl-2, CHK2 and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) respectively. After a series of washes the blots were further incubated with goat anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China) and detected using the ECL kit (Pierce, Rockford, IL, USA).

2.7 Statistical analysis

Analyses were performed using the statistical software SAS/STAT (SAS Institute Inc., Cary, NC, USA). P < 0.01 was considered the threshold value for statistical significance.

3 Results

3.1 Cell growth in vitro in response to radiation treatment

The effects of radiation treatment on cell growth in vitro were determined by MTT assay. Androgen-sensitive LNCaP and androgen-refractory C4-2 cells were exposed to radiation doses of 0, 2.5, 5 and 10 Gy and the values of OD 490 were measured at 0, 6, 12, 24, 48 and 72 h post-radiation. The survival curve showed that the growth rates of LNCaP and C4-2 were inhibited by irradiation in dose-dependent manner, whereas C4-2 acquired greater radioresistance compared with LNCaP control (P < 0.01, Figure 1). The growth ratio of LNCaP exposed to 5-Gy radiation was 1.098 at 72 h post-radiation, while it was 5.886 in the non-treatment control LNCaP at the same time point. Hence, the growth of LNCaP was inhibited by 81.34% as a result of the 5-Gy radiation. The growth ratio of C4-2 exposed to 5-Gy radiation was 4.224 at 72 h post-radiation, while it was 6.379 in the control group. Therefore, the growth inhibition mediated by 5-Gy radiation was 33.79% in C4-2. When exposed to 10-Gy radiation, the growth ratio was reduced by 88.32% in LNCaP at 72 h post-radiation, while it decreased by 35.88% in C4-2 in the same condition. These results indicate that the growth-inhibitory effect caused by radiation is obviously reduced in C4-2, and that the growth ability of C4-2 exhibited increased radiosensitivity compared with LNCaP.

3.2 Clonogenicity in response to radiation treatment

Colony formation assays were performed in plates and soft agar to examine the anchorage-dependent
The radiation response of LNCaP/C4-2 cell

Bang-Xiang Xie et al.

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408

and anchorage-independent clonogenicity of LNCaP and C4-2 in response to radiation treatment (5 Gy). Clonogenic assays in plates revealed that C4-2 retained a low level of clonogenicity after 5-Gy radiation treatment, whereas LNCaP cells almost lost their clonogenicity under the same condition (Figure 2). Moreover, anchorage-independent clonogenic assays in soft agar showed that the clonogenicities of LNCaP and C4-2 were obviously inhibited by the treatment with 5-Gy radiation. When seeded at 4 000 cells/well and exposed to 5-Gy radiation, the colony formation ratio of LNCaP was 0.325%, while the ratio was 2.275% in the non-treatment control. Hence, the colony formation ratio of LNCaP was reduced by 85.71% when exposed to 5-Gy radiation. Under the same condition, the colony formation ratio of C4-2 was 2.60% when exposed to 5-Gy radiation, whereas it was 5.40% in the non-treatment control. The colony formation ratio was lowered by 51.82% as a result of radiation exposure. Similarly, when seeded at 6 000 cells per well and exposed to 5-Gy radiation, the colony formation ratio of LNCaP was decreased by 86.70% compared with the control, while the ratio of C4-2 was reduced by 47.52% in the same environment (P < 0.01, Figure 3). From the results described above, it can be seen that the radiation-mediated inhibitory effect on clonogenicity is obviously decreased in C4-2, and that C4-2 possesses evidently increased radioresistance compared with radiosensitive LNCaP as measured by clonogenic assays.

3.3 G2/M arrest of LNCaP and C4-2 induced by radiation treatment

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The radiation response of LNCaP/C4-2 cell
Bang-Xiang Xie et al.

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3.4 DNA repair and cell cycle-associated genes expression

Real-time PCR was performed to examine the expressions of p53, p21, ATR, CHK1 and CHK2 under normal condition. Results indicated that the expressions of the respective genes were down-regulated in C4-2 compared with LNCaP. Besides p21, which was observed to be down-regulated only by 1.28-fold, the other genes were found to be down-regulated more evidently in C4-2. For example, ATR, CHK1 and CHK2 were down-regulated in C4-2 by 5.21-, 6.02- and 1.93-fold, respectively (Figure 5). Then we examined the changes induced by radiation in the respective gene expressions. Results showed that the pattern of gene expression changes mediated by radiation were completely different in LNCaP and C4-2. As shown in Figures 5B–G, the expressions of p53, ATR, MRE11, CHK1 and CHK2 were obviously increased after radiation in C4-2. In contrast, almost all of the genes described above were down-regulated in LNCaP. For instance, the expression of ATR was reduced by 2.03-fold in LNCaP after 5-Gy radiation treatment, whereas it increased by 31.56-fold in C4-2 in response to 5-Gy radiation. The expression of CHK1 was decreased by 6.92-fold in LNCaP after treatment with 5-Gy radiation, while it increased by 6.28-fold in C4-2 under the same condition. The mRNA expression of p21 was found to increase in response to 5- and 10-Gy radiation both in LNCaP and in C4-2. We also evaluated the protein expression changes mediated by radiation in p53, p21, Bcl-2 and Chk2. As a result, we found that the p53 protein expression was slightly decreased in response to 5-Gy radiation in LNCaP, whereas it was obviously up-regulated in C4-2 after 5- and 10-Gy radiation. The expression of Bcl-2 before and after 5-Gy radiation was increased in C4-2 compared with LNCaP (data...
Figure 4. G2 checkpoint activation and G2/M arrest induced by irradiation treatment in LNCaP and C4-2. LNCaP and C4-2 were exposed to 5-Gy radiation. Cell cycle distribution was detected by flow cytometry at 6, 12, 24, 48 and 72 h post-treatment.
The radiation response of LNCaP/C4-2 cell
Bang-Xiang Xie et al.

not shown). The immunoblotting results also showed that Chk2 protein expression was down-regulated in response to 5- and 10-Gy radiation in LNCaP (Figure 6), while it slightly increased in response to 5-Gy radiation in C4-2. The protein expression of p21 was revealed to be evidently elevated after radiation in LNCaP and C4-2. The Western blot results were consistent with the quantitative RT-PCR results.

4 Discussion

Besides radical prostatectomy, radiation therapy has been proven to be a relatively effective treatment for localized PCa, with local tumor control and improvement of patient survival rates. However, PCa cells vary in their sensitivity to ionizing radiation and nearly 30% of patients treated with potentially curative doses relapsed at the sites of irradiated tumors [11–13]. AD through surgery or chemical methods is the most effective therapeutic approach for advanced PCa. Unfortunately, despite the effectivity of such procedures during the initial period, PCa eventually progresses to the androgen-independent stage and resists additional androgen withdrawal [14]. It is known that hormone-independent PCa cells are only modestly sensitive to chemotherapy and to radiation therapy [15]. No curative and durable therapeutic methods are available for AI and metastatic PCa.

Therapeutic synergy between radiotherapy and androgen ablation in patients with advanced metastatic PCa has been documented with promising clinical outcome. Neoadjuvant AD improves intro prostatic local control and reduces distant metastasis. Neoadjuvant androgen withdrawal achieves this endpoint through sensitization of the tumor to radiation and through improved oxygenation [16]. In addition, HR PCa cells appeared to be more aggressive in their resistance to radiation treatment in comparison with

Figure 5. Real-time PCR analysis of gene expression associated with DNA repair and cell cycle arrest in LNCaP and C4-2. (A): p53, p21, ATR, CHK1 and CHK2 mRNA expression were examined using real-time PCR assays in LNCaP and C4-2. (B) –(G): Gene expression changes induced by 5- or 10-Gy radiation in LNCaP and C4-2 were examined in p53, p21, ATR, MRE11, CHK1 and CHK2 at 24 h post-radiation. Data shown are mean ± SD of triplicate independent measurements. The relative amounts of mRNA of target genes to β-actin were calculated using the equation 2^(-ΔΔCt(target/β-actin)).

Figure 6. Immunoblotting analysis of gene expression changes in response to radiation in LNCaP and C4-2. p53, p21 and Chk2 protein expression changes in response to radiation were evaluated in LNCaP and C4-2 at 24 h post-radiation using Western blot assays, with β-actin used as normalizing control.
androgen-sensitive PCa cells [7]. Accordingly, androgen ablation-mediated molecular events may have important roles in the radiosensitization of PCa cells. Conversely, gene expression alterations mediating androgen-refractory progression of PCa may also have an impact on the development of radiation resistance.

LNCaP is an androgen-responsive, non-metastatic and marginally tumorigenic PCa cell. The C4-2 subline was derived from LNCaP through interaction with stromal cells under androgen-depleted conditions in vivo and has acquired the phenotypes of androgen independence and osseous metastases. In our study, C4-2 was found to possess increased radioresistance and decreased radiosensitivity compared with the parent cell line LNCaP. C4-2 sustained the ability of clonogenicity after radiation, whereas LNCaP lost the ability. The survival and proliferation rates of C4-2 after exposure to radiation are increased compared with LNCaP. The increased expression of Bcl-2 both in non-irradiated and in irradiated C4-2 indicates the enhanced anti-apoptosis characteristics in C4-2, because Bcl-2 expression is required for tumor maintenance and prevents cells from apoptosis [17, 18]. The basal expression levels of p53 and p21 were found to be suppressed in C4-2 in comparison with LNCaP. The expressions of ATR, Chk1 and Chk2, which have been implicated to have important roles in cell cycle arrest, were also shown to exist in lower levels in C4-2. Decreased basal expressions of the genes described above indicate that C4-2 is able to pass the cell cycle checkpoints more easily under normal conditions, which is consistent with the increased percentage of S phase C4-2 cells observed in cell cycle analysis. We also found that the G2/M checkpoint of C4-2 was activated more quickly and intensively than that of LNCaP after radiation exposure, which can be explained by the increased expression of ATR, MRE11, CHK1 and CHK2 as well as p53 in C4-2 after radiation treatment. The increased expression of these genes may be transient, but they still have important roles in initiating the G2/M checkpoints and in responding to DNA damage. ATR is one of the major regulators of DNA damage response (DDR) and targets a set of substrates that promote cell cycle arrest and DNA repair. As a downstream substrate of ATR, Chk1 functions to signal DNA damage to the rest of the nucleus. Chk1 phosphorylation of the CDC25 proteins inhibits their activity and prevents CDK activations, which is a major checkpoint mechanism that prevents entry into mitosis. The ATR/Chk1/CDC25 signaling pathway has an essential role in the checkpoint response to radiation. Even the initiation of the G2/M checkpoint is impaired in the absence of ATR. ATR signaling also promotes the repairing of a variety of DNA lesions by regulating recombination at stalled and collapsed replication forks. Moreover, the ATM/Chk2 signaling pathway has an essential role in G2 checkpoint activation and the ATM-dependent repair pathway (presumably homologous recombination) functions to restore chromosomal integrity after radiation exposure [19–21]. As for p53, it has been well established that the p53 protein contributes to both G1 and G2 arrest through transcriptional regulation, which includes the induction of CDKN1/p21 [22, 23]. The cell cycle arrest maintenance role of p53 in response to radiation-induced damage may be protective and allows sufficient time for DNA damage repair. Transient increase in p53 expression in response to radiation contributes to cell cycle checkpoint activation and increases cell survival. Functional p53 expression significantly increased PC-3 cell clonogenic survival after exposure to daily doses of 2-Gy of IR [24]. This result is consistent with our data showing that p53 gene expression is up-regulated in C4-2 after 5- and 10-Gy radiation. A normal function of cell cycle checkpoints is to protect cells from the deleterious consequences of replicating or segregating damaged chromosomes. Abrogation of the G2 checkpoint often leads to a marked increase in the sensitivity to ionizing radiation. The sensitizing actions of caffeine and UCN-01 have raised the probability that adjunctive therapy with G2 checkpoint inhibitors will increase the therapeutic efficacies of radiation [25, 26]. Cell cycle analysis and molecular examination after radiation revealed that C4-2 had acquired an enhanced G2 checkpoint in comparison with LNCaP, which is consistent with the decreased radiation sensitivity in C4-2. More interestingly, we found that the cell cycle checkpoint-associated gene expressions maintained a relatively low level in C4-2 under normal condition, but were elevated by radiation. Conversely, in LNCaP, these gene expressions were at a higher level under normal condition, but were inhibited or unchanged by radiation. It is intriguing to propose that C4-2 is able to pass the checkpoints more easily under normal conditions, while acquiring more effective cell cycle checkpoints to allow sufficient time for DNA repair when DNA damage occurs. Furthermore, as observed in cell cycle analysis, C4-2 recovered from G2/M phase arrest caused by radiation more easily...
than LNCaP. The MRE11/RAD50/NBS complex is a central player in most aspects of cellular response to DNA double-strand breaks, including homologous recombination, non-homologous end joining, telomere maintenance and DNA damage checkpoint activation [27, 28]. The increased ATR and MRE11 expressions in C4-2 in response to radiation indicated that C4-2 had acquired increased potential of DNA repair after IR exposure, which may contribute to the ability of C4-2 to recover from G2/M arrest more easily and quickly. The molecular examinations are consistent with results observed in the cell viability and cell cycle analysis described above. All of these results revealed that the radiation-resistant property may correlate with the androgen-independent status in PCa development. Accordingly, LNCaP and C4-2, which, respectively, represent the androgen-dependent and independent status of PCa, can also be applied to explore new therapeutic strategies for increasing the radiosensitivity of PCa cells. The gene expression differentiation between LNCaP and C4-2 may also shed novel insights into the molecular determinants in the efficacy of androgen depletion and radiation therapeutic strategies.

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