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A combined pretreatment of 1,25-dihydroxyvitamin D3 and sodium valproate enhances the damaging effect of ionizing radiation on prostate cancer cells☆

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ABSTRACT

Radiotherapy is one of the curative treatment options for prostate cancer (PCa). However, effective doses of ionizing radiation (IR) have a high risk of side effects. To increase sensitivity of PCa to IR we pretreated human androgen-refractory DU145 PCa cells with a combination of sodium valproate (VPA), a well-tolerated drug with histone deacetylases inhibiting activity, and 1,25-dihydroxyvitamin D3, 1,25(OH)2D3, the active metabolite of vitamin D, a well known anticancer agent. The results show that irradiation (4 Gy) of DU145 PCa cells pretreated with a combination of 1 mM VPA and 100 nM 1,25(OH)2D3 efficiently suppressed (87.9%) PCa cell proliferation. IR after combined pretreatment resulted in increased DNA double-strand breaks expressed as levels of phosphorylated histone H2A.X, compared with non-treated cells the increase was 58.1% in pretreated cells and 11.8% in non-pretreated cells ($p < 0.002$). Combined pretreatment enhanced IR-induced activation of DNA damage checkpoint kinase Chk2, 39.0% in pretreated cells compared to 23.8% in non-pretreated cells ($p < 0.05$). These molecular changes led to DNA replication blockade, S-phase cell-cycle arrest and enhanced apoptosis. Cumulatively, the results indicate that combined pretreatment with VPA and 1,25(OH)2D3 followed by IR is a highly effective treatment for human PCa cells. This observation may have important implications for reducing doses of radiation administered to cancer patients thus limiting the severity of side effects.

1. Introduction

Radiotherapy is one of the curative treatment options for localized androgen-refractory prostate cancer (PCa) [1,2]. The higher the dose of ionizing radiation (IR) delivered the greater the probability of achieving local control [3]. However, higher doses increase the risk of side effects, such as rectal bleeding, erectile dysfunction and urinary incontinence [3]. Therefore, a deliberate search for biological response modifiers that could potentiate the therapeutic effect of IR and limit the occurrence of side effects is an important task in PCa therapy, especially androgen-refractory, since androgen-refractory PCa cells are more resistant than androgen-sensitive PCa cells to radiotherapy [4].

Previous studies reported an increased effectiveness of IR after pre-exposure of cancer cells to 1,25(OH)2D3 [5] or valproic acid [6]. We have observed enhanced suppression of proliferation following exposure of human PCa cells to a combination of 1,25(OH)2D3 and the sodium salt of valproic acid (VPA) (unpublished observation). These findings provided the experimental background to test radiosensitivity of PCa cells pretreated with a combination of 1,25(OH)2D3, the active metabolite of vitamin D3, a well known anticancer agent, and VPA, an antiepileptic drug that inhibits histone deacetylases activity and therefore exerts an anticancer effect. We have studied the effect of the proposed combined pretreatment on the effectiveness of IR by assessing cell proliferation, apoptosis and cell-cycle distribution. Concomitantly, levels of DNA double-strand breaks (DSBs), the most prominent DNA lesion caused by IR [7] and activation of DNA damage checkpoint kinases Chk1 and Chk2 [8] were determined. Cumulatively, our findings indicate that pre-exposure of human prostate cancer cells to a combination of 1,25(OH)2D3 and VPA significantly potentiates the damaging effects of ionizing radiation.

2. Materials and methods

1,25(OH)2D3 was kindly provided by Hoffmann La-Roche, Basel. VPA was purchased from Sigma (St. Louis, MO, USA). Antibodies against phospho H2A.X (Ser139), γ-H2A.X, clone JBW301, a marker of DNA DSBs, were supplied by Upstate Biotechnology, Inc. The anti-phospho-Chk1 (Ser345) and anti-phospho-Chk2 (Thr68) polyclonal

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antibodies were obtained from Cell Signaling Technology. The second antibody (Universal Immuno-peroxidase Polymer, anti-mouse and -rabbit) was supplied by Nichirei Biosciences, Inc.

2.1. Cell culture

Human PCa androgen-resistant cell line DU145 (American Type Culture Collection) was cultured at 37 °C and 5% CO_2 in humidified air in RPMI-1640 medium supplemented with 10% FCS.

2.2. Cell proliferation test

One thousand DU145 cells/well were seeded simultaneously into two 96-well tissue culture plates. One plate was used for the IR experiments and the other served as non-irradiated control. Cells were grown overnight and treated with drugs either alone or in combination. The treatments included: (a) 100 nM 1,25(OH)2D3, (b) 1 mM VPA, (c) a combination of 100 nM 1,25(OH)2D3 and 1 mM VPA, and (d) control (untreated). After 72 h, the cells were irradiated with a dose of 4 Gy and incubated for additional 96 h. Cell proliferation was measured using crystal violet assay [9,10]. Briefly, cells were fixed, stained with crystal violet, the incorporated dye was solubilized in 0.1 M sodium citrate in 50% ethanol and absorbance was measured at 550 nm.

2.3. Cell-cycle distribution analysis and apoptosis measurement

Two hundred fifty thousand DU145 cells were seeded into 75-cm^2 flasks. Cells were treated as described above for the cell proliferation test. Cell-cycle distribution was assessed by flow cytometric analysis of DNA content using propidium iodide [11]. Apoptosis was assessed by measurement of the sub-G1 (<2N ploidy) fraction in cell-cycle histograms.

2.4. Cell-based ELISA (CB-ELISA)

Expression of specific proteins (γ-H2A.X, phospho-Chk1 and phospho-Chk2) was assessed by CB-ELISA [12]. PCa cells were pretreated and irradiated as outlined under cell proliferation test. Three hours after IR cells were fixed, permeabilized and exposed to primary and the secondary antibodies. Immunostaining was detected using TMB peroxidase substrate. The absorbance was measured at 450 nm. Results of CB-ELISA were corrected for cell number by dividing OD of CB-ELISA for a given well by OD of crystal violet.

2.5. Statistical analysis

Data were analyzed using paired *t*-test. A value of $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. DU145 cell proliferation inhibition

IR decreased proliferation of DU145 cells by 30.4% ($p < 0.0001$) (Fig. 1). 1,25(OH)2D3 or VPA alone or in combination reduced proliferation of PCa cells by 19.2%, 61.3% and 81.1%, respectively ($p < 0.0001$). Pretreatment of cancer cells with either 1,25(OH)2D3 or VPA or their combination followed by IR resulted in suppression of DU145 cells proliferation by 46.4%, 83.0% and 87.9%, respectively ($p < 0.0001$). While IR decreased proliferation of the non-pretreated DU145 cells only by 30.4%, in cells pretreated with 1,25(OH)2D3, VPA or their combination IR decreased cell proliferation by 33.8%, 56.1% and 36.0%, respectively ($p < 0.0001$).

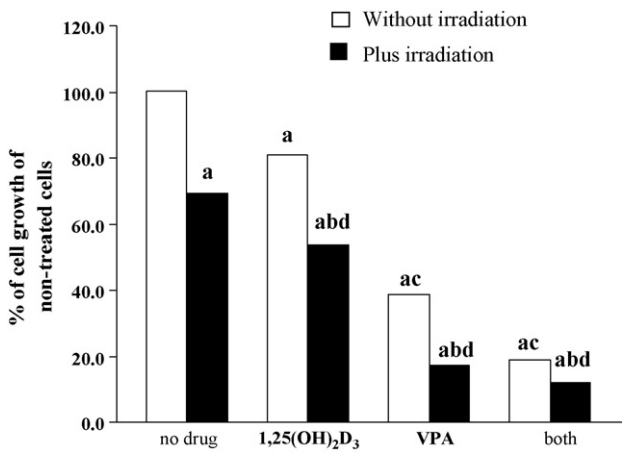

Fig. 1. Effects of IR on DU145 cell proliferation, with and without pretreatment with 1,25(OH)2D3 and VPA. a—$p < 0.0001$ compared to control (without IR), b—$p < 0.0001$ compared to analogues treatment without IR, c—$p < 0.0001$ compared to previous treatment without IR, and d—$p < 0.0001$ compared to previous treatment with IR.

3.2. Cell-cycle distribution

The results (Fig. 2) show that the cell growth-inhibiting effect of IR is to a large extent the result of both enhanced apoptosis (increased sub-G1 peak) ($p < 0.05$) and arrest of cell-cycle mainly in S-phase ($p \leq 0.02$). Interestingly, VPA alone altered cell-cycle distribution in a manner similar to that induced by IR. The most significant S-phase arrest and apoptosis were in the irradiated cells pretreated with VPA and its combination with 1,25(OH)2D3.

3.3. CB-ELISA of γ-H2A.X, phospho-Chk1 and phospho-Chk2

IR alone enhanced γ-H2A.X expression by 11.8%. ($p < 0.01$). VPA on its own or in combination with 1,25(OH)2D3 increased γ-H2A.X level by 20.3% ($p < 0.02$) and 17.4% ($p < 0.04$), respectively. The expression of γ-H2A.X in DU145 cells irradiated after pretreatment with VPA or 1,25(OH)2D3 or their combination was increased by 42.7%, 24.8% and 58.1%, respectively ($p < 0.04$, compared to cells exposed to IR only). We observed no effect of IR on Chk1 phosphorylation. However, IR enhanced Chk2 phosphorylation by 23.8% ($p < 0.001$). Chk2 phosphorylation in pretreated cancer cells followed by IR increased by 33–39% ($p < 0.05$, compared to cells exposed to IR only).

4. Discussion

The results of the present study show that irradiation of DU145 PCa cells pre-exposed to a combination of VPA and 1,25(OH)2D3 markedly decreased prostate cancer cell proliferation compared to irradiated control cells not previously exposed to these biological modifiers. It would seem that the combination of VPA and 1,25(OH)2D3 enhances IR-induced cell-cycle S-phase arrest and cell apoptotic death. Unexpectedly, a more prominent S-phase arrest and apoptotic death were observed in irradiated cells pretreated with VPA alone, a finding seemingly in contradiction to results pertaining to VPA effect on DU145 PCa cells proliferation (Fig. 1). Interestingly, pretreatment with 1,25(OH)2D3 alone did not alter radiation-induced changes in cell-cycle distribution pattern (Fig. 2), despite a more pronounced inhibition of cell proliferation (Fig. 1). We surmise that 1,25(OH)2D3 alone or in combination with VPA lowers transition rates of pivotal phases of the cell-cycle ultimately restraining the proliferation rate of cancer cells despite no overt evidence of changes in cell-cycle.

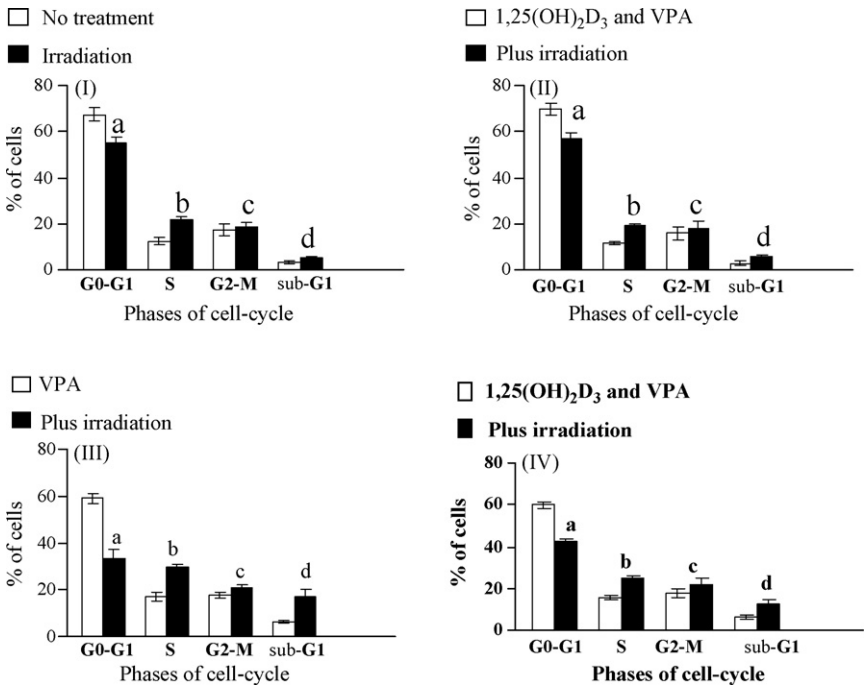

Fig. 2. Effect of IR on cell-cycle distribution of DU145 cells, with and without pretreatment with 1,25(OH)2D3 and VPA. *Statistical analysis*: All comparisons performed between the same peaks in irradiated and non-irradiated cells. (I) Non-pretreated cells: IR decreased G0–G1 fraction (a—$p < 0.001$), induced S-phase delay (b—$p < 0.002$) and enhanced apoptosis (sub-G1 peak, d—$p < 0.003$), non-significant (NS) difference (c) was found for G2–M phase. (II) Cells pretreated with 1,25(OH)2D3: IR decreased G0–G1 fraction (a—$p < 0.001$), induced S-phase delay (b—$p < 0.01$) and enhanced apoptosis (d—$p < 0.02$), NS difference (c) was found for G2–M phase; (III) cells pretreated with VPA: IR decreased G0–G1 fraction (a—$p < 0.02$), induced S-phase delay (b—$p = 0.02$), increased G2–M phase (c—$p < 0.02$) and enhanced apoptosis (d—$p < 0.05$); (IV) cells pretreated with 1,25(OH)2D3 and VPA: IR decreased G0–G1 fraction (a—$p < 0.002$), induced S-phase delay (b—$p < 0.003$) and enhanced apoptosis (d—$p < 0.04$), NS difference (c) was found for G2–M phase.

Measurement of γ-H2A.X, a well recognized marker of DNA DSBs [13], revealed a significantly increased level of radiation-induced DSBs in DU145 cells pretreated with VPA, 1,25(OH)2D3 or with their combination ($p < 0.0001$, $p < 0.006$ and $p < 0.0001$, respectively). The highest level of radiation-induced γ-H2A.X was detected in the cells pretreated with a combination of VPA and 1,25(OH)2D3. We suggest that VPA has a prominent role in impeding DNA DSBs repair, a view consonant with the known ability of HDAC inhibitors, including VPA, to interfere with DNA repair [14]. Of note, treatment with VPA alone or in combination with 1,25(OH)2D3 led to increased level of DSBs in non-irradiated DU145 PCa cells. A plausible explanation for the DNA damaging action of VPA may be sought in the ability of HDAC inhibitors to induce the generation of reactive oxygen species [15] known to induce severe DNA damage.

Determination of the DNA damage checkpoint kinase Chk2 showed a significant increase in Chk2 activation ($p < 0.05$) in pretreated PCa cells compared to IR alone. Chk2 kinase is a key component of the ATM pathway. Activation of this pathway and phosphorylation of Chk2 induces a transient blockade of DNA replication and S-phase cell-cycle arrest [16]. However, the increase in Chk2 activation was similar in all pretreatments and therefore cell-cycle arrest at this key DNA damage checkpoint may be only part of molecular events leading to inhibition of PCa cells growth.

In conclusion, the results of the present study indicate that the combination of VPA and 1,25(OH)2D3 enhances PCa cell radiosensitivity. This observation may have important implications for reducing the doses of IR administered to cancer patients thus limiting the severity of side effects. In addition, the study demonstrated high effectiveness of the combination of 1,25(OH)2D3 and VPA, even without IR, in suppression of DU145 cell proliferation. Therefore, this combination alone or combined with other anticancer drugs may serve as effective treatment of metastatic androgen-refractory PCa which is chemotherapy resistant [17].

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