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Radiosensitivity of human prostate cancer cells can be modulated by inhibition of 12-lipoxygenase

J. Lövey [a], D. Nie [b], J. Tóvári [c], I. Kenessey [d], J. Tímár [d], M. Kandouz [e,f,g], K.V. Honn [e,f,g,*]

[a] Departments of Radiotherapy and Experimental Therapeutics, National Institute of Oncology, Budapest, Hungary
[b] Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine, Springfield, IL, United States
[c] Department of Experimental Pharmacology, National Institute of Oncology, Budapest, Hungary
[d] 2nd Department of Pathology, Semmelweis University, Budapest, Hungary
[e] Department of Pathology, Wayne State University School of Medicine, Detroit, MI 48202, United States
[f] Karmanos Cancer Institute, Detroit, MI 48202, United States
[g] Bioactive Lipids Research Program, Detroit, MI 48202, United States

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ABSTRACT

Nearly 30% of prostate cancer (PCa) patients treated with potentially curative doses relapse at the sites of irradiation. How some tumor cells acquire radioresistance is poorly understood. The platelet-type 12-lipoxygenases (12-LOX)-mediated arachidonic acid metabolism is important in PCa progression. Here we show that 12-LOX confers radioresistance upon PCa cells. Treatment with 12-LOX inhibitors baicalein or BMD122 sensitizes PCa cells to radiation, without radiosensitizing normal cells. 12-LOX inhibitors and radiation, when combined, have super additive or synergistic inhibitory effects on the colony formation of both androgen-dependent LNCaP and androgen-independent PC-3 PCa cells. *In vivo*, the combination therapy significantly reduced tumor growth.

1. Introduction

Arachidonic acid is metabolized by cyclooxygenases (COX) and lipoxygenases (LOX) into various bioactive lipids which were shown to regulate cell survival, cell cycle control, invasion and the angiogenic phenotype [1]. The mammalian LOX family contains a number of lipid peroxidizing enzymes, which are distinguished by their regional specificity of arachidonic acid oxygenation. Accordingly 5-, 8-, 12-, and 15-LOX isoforms are known, which produce 5(S)-, 8(S)-, 12(S)- and 15(S)-hydroxyeicosatetraenoic acid (HETE) metabolites respectively [2]. 12-LOX has two isoforms, the platelet- and the leukocyte-types sharing 65% DNA homology [3,4]. In prostate cancer a number of studies outlined the pattern of changes in the expression of AA-metabolizing enzymes. Based on preclinical and clinicopathological studies it is evident that in prostate cancer COX-2, TxS, platelet-type 12-LOX and 15-LO1 (leukocyte-type 12-LOX) are upregulated, while the 15-LOX2 is downregulated, resulting in a characteristic pattern of AA metabolites rich in PGE2, TX, 12-S-HETE, 15-S-HETE and 13-S-HODE [1]. It is interesting, that the ectopic expression of 12-lipoxygenases can be considered as the manifestation of stem cell characteristrics since platelet- or leukocyte-type 12-LOX (15-LO1) are constitutively expressed in bone marrow progenitors [5].

Previous data indicated that 12-S-HETE activates antiapoptotic-, motility- and angiogenic signaling cascades [6–8]. 12-S-HETE also regulates gene expression including VEGF [8]. 12-LOX levels positively correlated with the stage and grade of prostate cancer [9]. Overexpression of 12-LOX also enhances tumor growth through the induction of angiogenesis [10]. Inhibition of 12-LOX activity with specific inhibitors induced apoptosis and decreased the metastatic potential of DU-145 prostate cancer cells in a lung colonization assay [11].

As screening methods for prostate cancer improve, more patients with localized disease are diagnosed. The curative treatment choice of localized prostate cancer can be both surgery and radiotherapy [12]. Recently, due to patients' preference, the lower morbidity and the comparable result achieved with radical radiotherapy, the number of prostate radiotherapy treatments both with external irradiation and brachytherapy is increasing [13]. However, radiosensitivity of prostate cancer is variable due to mostly unknown factors among which the geno- and phenotype of the tumor must be important [14]. Therefore, potentiation of the effect of radiotherapy may have clinical relevance in this tumor

* Corresponding author. Address: Department of Pathology, 431 Chemistry Bldg., Wayne State University, Detroit, MI 48202, United States. Tel.: +1 313 577 1018; fax: +1 313 577 0798.
E-mail address: k.v.honn@wayne.edu (K.V. Honn).

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type as well. Since signaling pathways involving 12-LOX play an important role in the progression of various cancers including prostate cancer [1], we have postulated that 12-LOX derived bioactive lipids may modulate the cytotoxic effect of ionizing radiation as well.

2. Materials and methods

2.1. Cell lines

Human prostate cancer cell lines PC-3, DU 145 and LNCaP were obtained from ATCC (Manassas, VA) and maintained in humidified atmosphere of 5% CO_2 at 37 °C and cultured in RPMI 1640 or DMEM supplemented with 10% FBS and 100 μg/ml penicillin–streptomycin.

2.2. In vitro treatments

Prior to treatment, prostate cancer cells were plated in 10 cm diameter tissue culture plates in serum containing medium and allowed 12 h to attach. Cells were treated with irradiation, 12-LOX inhibitors Baicalein (Biomol, Plymouth, PA) [15] or N-benzyl-N-hydroxy-5-phenylpentamide (BMD122) (Biomide Corp., Grosse Pointe Farms, MI) [11], 5-LOX inhibitor Rev-5901 (Cayman Chemicals, Ann Arbor, MI) and with irradiation in combination with the above mentioned LOX inhibitors. Add-back experiments were carried out using 12(S)-, 5(S) - and 15(S)-HETE (Cayman Chemicals). Irradiation was carried out with a Cs-137 scientific gamma irradiator with a dose rate of 85 cGy/min. LOX inhibitors and different HETEs were diluted in serum-free medium. Treatment schedules were as follows. Single treatment with BMD122 and Rev-5901 was carried out with concentrations of 10–50 μM, and of Baicalein with 3–15 μM. Doses of irradiation as single treatment were 25–500 cGy. During combined treatment 25 μM BMD122, 25 μM Rev-5901 or 7.5 μM Baicalein was used 1 h prior irradiation. Doses of irradiation were the same as above described. During the add-back experiments, 100 ng/ml of 5(S)-, 12(S)- or 15(S)-HETE was applied just before administering BMD122 or Baicalein or delivering irradiation.

2.3. Clonogenic survival

Prostate carcinoma cells were placed in 6-well culture plates and were allowed to attach for 24 h in the presence of serum. The number of cells per well was set to produce around 100–200 surviving colonies after treatment. Plating efficiency ranged 45–70%. Cells were treated according to the schedules described above. Plates were incubated for 14 d (except fast growing LNCaP for 7 d) at 37 °C in 10% FBS (Gibco) containing medium. Cells then were fixed in 4% buffered formaldehyde and stained with crystal violet. Colonies containing at least 50 cells were counted under a light microscope and survival was normalized to untreated controls.

2.4. Apoptosis

Apoptosis was assessed by the incorporation of fluorescein-12-dUTP mediated by terminal deoxynucleotidyl transferase into fragmented DNA (TUNEL-assay Roche Diagnostics GmbH). Cells were seeded onto 10 cm diameter Petri-dishes and treated as described previously in the treatment section. Throughout the experiment cells were maintained in the presence of serum. Following treatment 24 h, cells were digested by trypsin–EDTA, washed in PBS and fixed in ice cold ethanol for 1 h. Permeabilization was carried out applying ice-cold 0.1% Triton-X100 and 0.1% sodium-citrate for 3 min, then cells were washed in PBS and working solution of the TUNEL reaction was added. Samples were incubated for 1 h at 37 °C, washed and resuspended in PBS. The apoptotic fraction of the cells was quantified by laser flow cytometry.

2.5. Western blot analysis

Forty-eight hours following the above described treatments, total cell lysates were prepared. Proteins (20 μg) were fractionated on precast SDS–PAGE and transferred onto nitrocellulose membranes. Following incubation in blocking buffer containing 5% skimmed milk dissolved in 1 mM TRIS–HCl, 100 mM NaCl and 0.1% Tween 20 for 1 h, blots were probed overnight at 4 °C with primary antibodies against Bcl-2, Bax, and survivin (R&D Systems, MN, USA) with a dilution of 1:1000.

2.6. In vivo growth

PC3 cells (10^6) were injected s.c. into SCID mice. Tumors were grown till 5 mm diameter for 2 weeks. BMD122 was administered i.p. (100 mg/kg/day) for 4 consecutive days. Irradiation was completed on day 16, with a single dose of 5 Gy. Animals were followed afterwards for another 2 weeks. Tumor was measured each third day by measuring 2 diameters to calculate tumor volume. Control animals lost more than 20% of their body weight on day 28 therefore they were terminated with Nembutal overdose.

2.7. Statistical analysis

For the analysis of potential treatment interactions (synergism or antagonism), a combination index (CI) was calculated with the Calcusyn software, which uses the Chou–Talalay equation, based on the median effect principle [16]. First, clonogenic survival curves are created. For the analysis of additivity of the combined treatments, additive curves were created by multiplying the survival fractions of the groups treated with the compounds alone with the survival fractions of the different doses of radiation alone. This theoretical additive curve represents the maximal possible additive effect of the two modalities. Therefore if survival curves of combined modality treatments result in a lower survival than the theoretical additive curve, then combined treatment are super-additive or synergistic. For the analysis of the different curves, linear regression of all curves was performed. When the survival curves of treatments containing radiation are non-linear, curves of logarithms of survival are used for regression and compared to each other pairwise. Curve (line) comparisons were carried out by Graphpad Prism software (GraphPad Software, Inc. San Diego, CA). When comparing two lines using Prism, slopes are compared first. It calculates a P value testing the null hypothesis that the slopes are all identical in the overall populations. If the P value is less than 0.05, Prism concludes that the lines are significantly different. In that case, there is no point in comparing the intercepts. The P value is two-sided. If the P value for comparing slopes is greater than 0.05, Prism concludes that the slopes are not significantly different and calculates an overall (pooled) slope. Since the slopes are assumed to be identical, there are two possibilities. Either the lines are identical, or they are different but parallel. Prism calculates a second P value testing the null hypothesis that the lines are identical. If this P value is low, it can be concluded that the lines are not identical, but rather that they are distinct but parallel. If this second P value is high, we can conclude that there is no evidence that the lines are not identical.

For the animal experiments we used the non-parametric Kruskal–Wallis test with post hoc analysis. Statistical analysis was performed by Statistica 9.0 software (StatSoft, Tulsa, OK).

3. Results

3.1. Effect of 12-LOX inhibitors and irradiation on the clonogenic survival of prostate cancer cells

Treatment of PC-3, LNCaP and DU145 human prostate cancer cells with different concentrations of 12-LOX inhibitors (Baicalein and BMD122, Fig. 1A and B) or different doses of radiation (Fig. 1C) resulted in a dose dependent decrease in the clonogenic survival of the cells. The less sensitive prostate cancer cell line for 12-LOX inhibition was DU145, while the three cell lines were similarly sensitive to irradiation, although at lower doses PC3 seemed to be moderately resistant.

Human prostate cancer cell lines were treated with the combination of radiation (0–500 cGy doses) and 25 μM BMD122 and clonogenic survival was analyzed. The synergism between radiation and 12-LOX inhibition was evaluated by combination indexes calculated employing mutually exclusive assumption and median effect principle based on Chou–Talalay equation using the Calcusyn software. In PC-3 cells the combined effect of radiation and 12-LOX inhibitor proved to be supra-additive as shown by the combination indexes calculated employing the median effect principle (Table 1). In the case of LNCaP cells the radiation enhancing effect of 12-LOX inhibitor was apparent only from higher – over 100 cGy – radiation doses. In contrast to PC-3 and LNCaP cells, the combined radiation and 12-LOX inhibitor treatment of DU145 showed only a simple additive effect (Table 1). We also examined the effect of baicalein, a selective inhibitor of 12-LOX, on radioresponse of LNCaP and PC3 cells. These cells were treated with 7.5 μM baicalein for 2 h before initiation of radiation. As shown in Fig. 2A, baicalein and radiation, when combined, have super additive or synergistic inhibitory effect on the colony formation of LNCaP cells. Regression analysis indicates that combined treatment of LNCaP cells with radiation and baicalein has significant super-additive or synergistic effect ($P < 0.05$). Similarly, as shown in Fig. 2B, baicalein and radiation, when combined, have super additive or synergistic inhibition on the colony formation of PC3 cells ($P < 0.01$). Taken together, these data suggest that 12-LOX