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Original article

Tumor apoptosis in prostate cancer by PGD_2 and its metabolite 15d-PGJ_2 in murine model

Masaki Nakamura [a], Hideyasu Tsumura [b], Takefumi Satoh [b], Kazumasa Matsumoto [b], Hiroko Maruyama [c], Masataka Majima [d,e], Hidero Kitasato [a,f,*]

[a] Department of Environmental Microbiology, Kitasato University Graduate School of Medical Sciences, Kanagawa, Japan
[b] Department of Urology, Kitasato University School of Medicine, Kanagawa, Japan
[c] Department of Pathology, Kitasato University School of Alleid Health Science, Kanagawa, Japan
[d] Department of Pharmacology, Kitasato University School of Medicine, Kanagawa, Japan
[e] Department of Molecular Pharmacology, Kitasato University Graduate School of Medical Sciences, Kanagawa, Japan
[f] Department of Microbiology, Kitasato University School of Alleid Health Science, Kanagawa, Japan

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ABSTRACT

Fifteen-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) is one of non-enzymatically converted metabolite from prostaglandin D_2 (PGD_2). Anti-tumor effects of 15d-PGJ_2 in various tumors are partially known, but the detail of *in vivo* mechanisms of action is still unclear. In this study, we investigated the effects of 15d-PGJ_2 and PGD_2 on murine prostate cancer *in vitro* and *in vivo*. Murine prostate cancer cells RM9 were transfected with murine prostaglandin D_2 synthase (mPGDS) gene by using defective retrovirus vector, designated as RM9-mPGDS. In addition, RM9 was also transfected with only defective retrovirus vector, designated as RM9-EV and used as control in this study. The expression and production of the gene were confirmed by RT-PCR and ELISA, respectively. For *in vivo* study, RM9-mPGDS was injected into the back of C57BL/6 mice, then resulted tumor was used for pathological analysis 14 days after the inoculation. Tumor cell apoptosis in the tissue was detected by TUNEL staining. Retrovirally transfected mPGDS in RM9 significantly induced apoptosis *in vivo* but not *in vitro*, by TUNEL staining and cell death ELISA, respectively. Our results strongly suggested that the apoptosis induced in RM9-mPGDS *in vivo* was probably achieved in tumor environment such as hypoxic condition. The introduction of PGDS gene into cancer cells might be a novel therapy against cancer.

1. Introduction

Prostaglandins (PGs) are lipid compounds derived from arachidonic acid (AA) and have important functions in animal such as vascular contraction, platelet agglutination, and inflammatory mediation [1]. Prostaglandin H_2 (PGH_2) is a common precursor of each PG and derived from AA by the cyclooxygenase-1 and -2 (COX-1, COX-2), and then converted to the major active prostanoids such as PGD_2, PGE_2, PGI_2, or thromboxane A_2 (TXA_2), by each synthase [2]. Among synthases, two PGD_2 synthases (PGDS) have been identified; one is lipocalin-type PGDS expressed in brain and correlates with sleeping, and the other is hematopoietic-type PGDS expressed in mast cells and acts as a mediator of allergy and inflammation [3,4].

PGD_2 is mainly produced from the mast cells and known to have an antiplatelet aggregation activity or induce the sleep [5]. PGD_2 is then non-enzymatically dehydrated to 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), known to act as anti-inflammatory substance via activation of proliferator-activated receptor γ (PPARγ) [6,7]. In addition, 15d-PGJ_2 has anti-tumor effects in human cancers of various organs such as gastric, lung, and colon cancer [8–10]. Some mechanisms of anti-tumor effects of 15d-PGJ_2 have been reported. The activation of PPARγ by 15d-PGJ_2 induces upregulation of the phosphatase and tension homolog (PTEN). PTEN is tumor suppressor gene and modulates tumor cell proliferation [11]. For PPARγ independent pathway, it has also been reported that 15d-PGJ_2 suppressed COX-2 expression via the inhibition of NFκB activity followed by suppression of angiogenesis [12], and induced cancer cell apoptosis [13]. However, these findings are revealed by only *in vitro* studies, and the mechanisms of anti-tumor effects of 15d-PGJ_2 *in vivo* are still unknown.

In this study, we established tumor cell line that produce PGD_2 and 15d-PGJ_2 by introducing PGDS gene by using deficient retroviral vector, and evaluated the anti-tumor effects of PGD_2

* Corresponding author. Department of Microbiology, Kitasato University School of Alleid Health Science, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan. Tel.: +81 42 778 8112; fax: +81 42 778 9400.
E-mail address: hkita@kitasasto-u.ac.jp (H. Kitasato).

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and 15d-PGJ_2 in murine prostate cancer model. Our results strongly indicate that PGD_2 and/or 15d-PGJ_2 induce apoptosis of tumor cells in murine prostate cancer model *in vivo*.

2. Materials and methods

2.1. Cells and mice

The RM9 cells derived from murine prostate cancer were kindly gifted from Dr. Timothy C. Thompson (Department of Genitourinary Medical Oncology-Research, The University of Texas M.D. Anderson Cancer Center, Houston, TX). The RM9 cells were cultured with Dulbecco's modified Eagle's medium (DMEM; SIGMA-ALDRICH, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; GIBCO by Life Technologies, Grand Island, NY), and 100 units/ml penicillin/10 μg/ml streptomycin (GIBCO) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Six-week-old male C57BL/6NJcl mice were purchased from CLEA-Japan (Tokyo, Japan) and were fed with standard chow pellets and water ad libitum. All animal handling and procedures were approved by Institutional Animal Care and Use Committee for Kitasato University (AHS-R 12-22).

2.2. Retrovirally transfection of mPGDS gene

Murine PGDS (mPGDS) cDNA was cloned into a deficient retroviral vector pDON-5 Neo (Takara, Tokyo, Japan), then transfected into PT67 cells (Clontech by Takara, Tokyo, Japan), followed by G418 (Roche, Basel, Schweiz) selection. Resulted temporary infectious recombinant virus containing mPGDS was infected into NIH/3T3 cells, followed by G418 selection in order to evaluate the infectious titer of those viruses and resulted titer was approximately 1×10^3 cfu/ml. RM9 cells were infected with these temporarily infectious retroviruses and selected with G418, then designated as RM9-mPGDS. Similarly the virus only containing pDON-5 Neo was infected to RM9, then designed as RM9-EV.

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

To ensure the expression of transfected mPGDS gene, total RNA was extracted from RM9-mPGDS using an RNeasy Plus Mini Kit (QIAGEN, Tokyo, Japan), then treated with DNase using TURBO DNA-free Kit (Ambion by Life Technologies), and those 5 μg were used for cDNA synthesis in a total volume of 50 μl using Omniscript RT Kit (QIAGEN). Five microlitres out of 50 μl were submitted for RT-PCR in a total volume of 50 μl, followed by in one cycle of initial activation at 94 °C for 15 min, 30 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 1 min in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems by Life Technologies). The oligonucleotide primers used for the RT-PCR are listed in Table 1.

2.4. Production of PGD_2 and 15d-PGJ_2

RM9-mPGDS and RM9-EV cells were incubated with 10 μM AA (Cayman Chemical, Ann Arbor, MI) for 6 hours, then those supernatants were used for measurement of PGD_2 and 15d-PGJ_2, by prostaglandin D_2-MOX EIA kit (Cayman Chemical) and 15-deoxy-$\Delta^{12,14}$-PGJ_2 ELISA kit (Enzo Life Sciences, Farmingdale, NY) according to the instruction manual, respectively.

Table 1
PCR primers for mGAPDH and mPGDS.

Target gene	Product length (bp)	Sequence (5'-3')
mGAPDH	370	Forward: GACGGCCGCATCTTCTTGTG Backward: GCCCCGGCCTTCTCCAT
mPGDS	385	Forward: ACCAGAGCCTCGCAATAGCAAGAT Backward: CAGGCAGAAATGGCAGGGATAGC

2.5. Cell proliferation assay

For the MTT assay, RM9-mPGDS and RM9-EV cells were seeded at a density of 1.0×10^4 cells/well in 96-well culture plates. After 48 hours incubation, cell viability were evaluated with Cell Proliferation Kit® [MTT] (Roche) according to the manufacturer's instruction.

2.6. Detection of apoptosis in vitro

For the detection of apoptosis *in vitro*, RM9-mPGDS and RM9-EV cells were seeded at a density of 2.0×10^4 cells/well in 96-well culture plates. After 24 hours incubation, cellular apoptosis was detected with Cell Death Detection ELISA Plus Kit (Roche) according to the manufacturer's instruction.

2.7. Induction of cellular apoptosis by 15d-PGJ_2

Cellular apoptosis by 15d-PGJ_2 in RM9 were assessed with cellular proliferation and apoptosis detection assays. RM9 cell were seeded at a density of 1.0×10^5 cells/well in 6-well culture plate for cell count or 3.0×10^3 cells/well in 96-well for MTT and Cell Death Detection ELISA. After incubation for 24 hours, 15d-PGJ_2 was added into each well at the concentration of 0, 0.01, 0.1, 1, 10, and 100 μM, respectively. After 24 hours, cells were harvested and the number of cells in each well were counted by trypan blue staining. MTT assay was also performed after 24 hours incubation with different concentration of 15d-PGJ_2. RM9 cells were assessed with Cell Death Detection ELISA Plus Kit, and evaluated the rate of cellular apoptosis by 15d-PGJ_2.

2.8. Murine prostate cancer model

RM9-mPGDS or RM9-EV cells were harvested and suspended in phosphate-buffered saline (PBS) at a density of 1.0×10^6 cells/100 μl. The resulted suspension was injected into the subcutaneous of the back of the C57 mouse (1.0×10^6 cells/mouse). On the day 14, mice were sacrificed with an excess dose of ether and the tumor tissues were surgically resected.

The resected tumor tissues were fixed with paraformaldehyde, then embedded in paraffin. Paraffin-embedded tissues were sectioned to 3 μm thickness, then stained with hematoxyline-eosin (HE). Images were captured by film scanner, Coolscan IV (Nikon, Tokyo, Japan), then necrotic areas in the tumor tissues were calculated by image analyzing software, ImageJ (NIH, Bethesda, MD).

2.9. Evaluation of role of PGD_2 and 15d-PGJ_2 in murine prostate cancer model

2.9.1. Cellular apoptosis assay by TUNEL staining

For apoptotic cell labeling, the sections were stained with TUNEL using in situ Apoptosis Detection Kit (Takara) according to the manufacturer's instruction, then the sections were counter-stained with methyl green. The percentage of the number of TUNEL positive cells were calculated with ImageJ.

2.9.2. Cell proliferation assay by Ki67 immunofluorescence staining

Paraffin-embedded sections were used for anti-Ki67 immunofluorescence staining. The primary antibody was anti-mouse Ki67 rabbit antibody (1: 100, Novus Biologicals, Littleton, CO) and the