

In vitro and *in vivo* growth inhibition of prostate cancer by the small molecule imiquimod

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Abstract. Prostate cancer is the second leading cause of cancer death in men worldwide. In the present study, we examined *in vitro* and *in vivo* antitumor effect of the small molecule imiquimod, also known as a TLR7 agonist, against prostate cancer. Imiquimod inhibited the growth of mouse (TRAMP-C2) and human (PC-3) prostate cancer cells. Treatment with imiquimod induced cell cycle arrest at the G₂/M phase in TRAMP-C2 cells, confirmed by the changes of G₂/M checkpoint regulators such as reduction of cyclin B1 expression and increase of phospho-CDC2 and p21 in TRAMP-C2 cells treated with imiquimod. Flow cytometry and western blot analysis revealed that imiquimod induced direct apoptosis in TRAMP-C2 cells via a mitochondrial-dependent pathway. Intratumoral injection with imiquimod reduced significantly tumor growth and increased apoptotic cells in mice subcutaneously implanted with TRAMP-C2 cells. Our results indicate that imiquimod can be an alternative therapeutic for locally generated prostate cancer.

Introduction

Prostate cancer is the most commonly diagnosed male malignancy and the second cause of malignant male death

worldwide after lung cancer (1). Infectious agents, physical trauma, hormones and a break of immune tolerance to prostate antigen are considered as the cause of prostate cancer (2). There are several therapeutic options such as radical prostatectomy, radiation and hormonal therapy for prostate cancer (3). However, these protocols have a limitation for metastatic and hormone refractory prostate cancer. Hormonal therapy in the form of medical or surgical castration can induce significant long-term remissions, but androgen-independent patients ultimately develop metastatic prostate cancer resulting in death due to widespread metastases (3-5). Chemotherapy is also effective but long-term use is not feasible due to its toxicity (6). Therefore, the development of alternative therapeutics are required for prostate cancer.

Imiquimod is a low-molecular-weight compound belonging to the imidazoquinolines family. It was first identified as a compound that has anti-viral activity in guinea pigs infected with herpes simplex virus, and has been successfully used for the treatment of genital warts caused by human papilloma virus in the clinic (7,8). In addition, recent studies have attracted considerable interest owing to their profound antitumoral activities (9-11). Imiquimod exerts antitumor effect by activating immune response to suppress tumor growth in a variety of transplantable tumors (9), it also has direct proapoptotic activity against various tumor cell populations *in vitro* and *in vivo* (12,13). The 5% imiquimod cream that is commercially available has been successfully used for the treatment of several cancers including basal cell carcinomas and melanoma (11,14-16).

Antitumor effects of the imidazoquinolines family have been demonstrated in urogenital cancers including bladder cancer and renal cell carcinoma (17-21). Treatment with an imidazoquinoline (3M-011) downregulated c-Myc expression in bladder cancer cells and reduced its transcriptional activity (21). It also significantly suppressed *in vivo* tumor growth in a mouse model for orthotopic bladder cancer (21). Imiquimod also induced apoptosis and cytokines production

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in various bladder cancer cell lines and effectively inhibited *in vivo* tumor growth (17). An imidazoquinoline also enhanced *in vivo* apoptosis and increased lymphocytic infiltration and proinflammatory cytokine production in a mouse model of renal cell carcinoma (22). However, the effect of imidazoquinolines on the growth of prostate cancer has not been studied. Therefore, in this study, we evaluated *in vitro* and *in vivo* antitumor effect of an imidazoquinoline, imiquimod, against prostate cancer.

Materials and methods

Mice. Specific pathogen-free (SPF) C57BL/6 mice were purchased from Koatech (Pyeongtaek, Korea). All animal studies were approved, and followed the regulations of the Institutional Animal Care and Use Committee in Konyang University.

Cell lines and reagents. Transgenic adenocarcinoma of the mouse prostate (TRAMP) model-derived prostatic epithelial cell line (TRAMP-C2) (23) and the metastatic human prostate cancer cell line (PC3) were purchased from the American Type Culture Collection (Manassas, VA, USA). TRAMP-C2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, San Diego, CA, USA) with 10% fetal bovine serum, 1% penicillin-streptomycin, 5% Nu-serum IV (Collaborative Biomedical Products, Bedford, MA, USA), 5 μ g/ml insulin (Sigma-Aldrich Co., St. Louis, MO) and 10 nM dihydrotestosterone (Sigma-Aldrich Co.) (24). PC3 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin. Imiquimod was purchased from InvivoGen (San Diego, CA, USA) and dissolved in ultrapure water as a stock solution at a concentration of 5 mg/ml.

MTT assay. The effects of imiquimod (1–10 μ g/ml) on TRAMP-C2 cells growth were determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Amresco®, Solon, OH, USA) assay. TRAMP-C2 cells were plated into 48-well tissue culture plates at concentration of 5×10^4 cells/well. After 12 h, TRAMP-C2 cells were treated with various concentration of imiquimod for 24, 48 and 72 h. The cell culture supernatant was removed and cells were incubated with 5 mg/ml MTT solution for 4 h. At the end of incubation, MTT solution was aspirated and then cells were mixed with dimethyl sulfoxide (DMSO, Amresco). The dye absorption was quantified using an automatic microplate spectrophotometer (Berthold Technologies GmbH, Vienna, Austria) at 540 nm. For TLR7 inhibitor treatment, TRAMP-C2 cells were pretreated with various concentration chloroquine for 2 h and then imiquimod (10 μ g/ml) was added to the medium with chloroquine for 72 h. Cell growth was determined by MTT assay as described above.

Flow cytometry. Imiquimod-treated TRAMP-C2 cells were stained with PI (Sigma-Aldrich Co.) or PI/Annexin V-FITC (BD Bioscience, Franklin Lakes, NJ) and analyzed by flow cytometry (BD LSR Flow cytometer, San Jose, CA, USA).

Western blot analysis. TRAMP-C2 cells were plated into 60-mm culture dish about 2×10^5 cells/well. After overnight incubation, the culture medium was replaced with fresh media and cells were treated with imiquimod (20 μ g/ml). The cells were lysed 0, 12, 24 and 48 h after stimulation using lysis buffer with 1% Nonidet P-40, complete protease inhibitor cocktail (Roche, Basel, Switzerland) and 2 mM dithiothreitol. Cell lysates were incubated on ice for 30 min and centrifuged for 15 min at 13,000 rpm. The protein-containing supernatant was harvested and the total protein amount was quantified using Bradford assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates were added with sample buffer and loaded onto a 10 or 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane and detected with the following antibodies: cleaved-PARP, caspase-3, caspase-7, caspase-9, cyclinB1 and phospho-CDC2 (Abcam, Cambridge, UK), p21 (Cell Signaling, Beverly, MA, USA), and anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology). The blots were developed using ECL substrate (Thermo Scientific, Waltham, MA, USA).

Cytokine production. The culture supernatants of imiquimod-treated TRAMP-C2 cells with or without chloroquine pretreatment at the concentration of 1, 10 and 50 nM were obtained and kept at -20°C until cytokine measurement. The concentration of IL-6 in culture supernatants was determined using a commercial DuoSet ELISA Development kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

***In vivo* antitumor efficacy.** C57BL/6 mice received subcutaneous (s.c.) single injection of TRAMP-C2 cells (1×10^6 cells/mouse in 100 μ l of injectable saline) into the shaven right flank and tumor growth was monitored. On day 10 after tumor implantation, imiquimod was injected intratumorally at 50 μ g daily for 9 days. Tumors were injected in a different site for each treatment day. Tumor length (L) and width (W) were measured and tumor weight (WR) was calculated twice a week as follows: $WR = 1/2 \times L \times W^2$.

Histological analysis. Tumor mass were removed, fixed in 10% formalin for 24 h, and processed in a standard alcohol-xylene series. The tissues were then embedded in paraffin, and 3- μ m sections were prepared, each of which was stained with H&E (Sigma-Aldrich Co.).

TUNEL assay. Sections were stained for apoptotic cells by a modified terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Then sections were counterstained with Methyl Green (Sigma-Aldrich Co.).

Statistical analysis. All assays were derived from at least three independent experiments. Statistical comparisons among the different values were performed using the Prism 5 GraphPad software (San Diego, CA, USA). Data are presented as mean \pm SD.

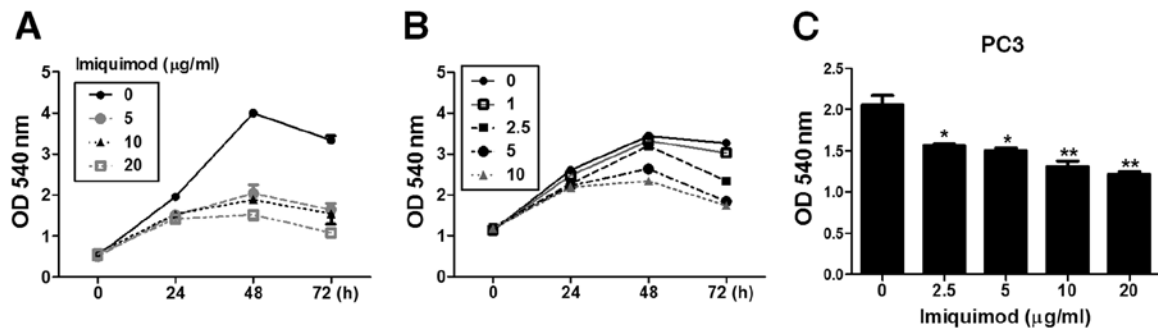


Figure 1. Imiquimod inhibits cell proliferation in prostate cancer cell lines. (A and B) TRAMP-C2 or (C) PC3 cells were treated with the indicated concentration of imiquimod for 24, 48 and 72 h. Cell growth inhibition was assessed by MTT assay at (A) high dose and (B) low dose of imiquimod treatment. The results presented here are from one representative experiment of three independent experiments and are presented as mean \pm SD. * P <0.05, ** P <0.01.

Results

Imiquimod inhibits the growth of TRAMP-C2 cells. Inhibitory effect of imiquimod on the growth of TRAMP-C2 cells was examined by MTT assay. The growth curve of untreated cells increased rapidly by 48 h and slightly decreased at 72 h. Treatment at doses over 5 μ g/ml of imiquimod delayed the cell growth by 48 h (Fig. 1A). To determine more definite dose-dependent effect of imiquimod on the cell growth, the experiment was repeated with a narrow dose range of imiquimod, 1 and 2.5 μ g/ml of imiquimod did not affect the cell growth by 48 h post-treatment (Fig. 1B). However, the cell growth was more reduced by a treatment with 2.5 μ g/ml of imiquimod at 72 h post-treatment, as compared with that in untreated cells (Fig. 1B). The growth of PC3 cells, a human prostate cancer cell line, was inhibited by imiquimod at 72 h post-treatment in a dose-dependent manner (Fig. 1C).

Imiquimod leads to G_2/M cell cycle arrest in TRAMP-C2 cells. We next investigated the effect of imiquimod on the cell cycle arrest in TRAMP-C2 cells. The cells were treated with various doses of imiquimod for 48 h and DNA contents for cell cycle arrest were determined by FACS analysis. The cell percentage in G_1 phase was reduced in imiquimod-treated TRAMP-C2 cells (Fig. 2A and B). In contrast, imiquimod increased the cell percentage in G_2/M phase dose-dependently, as compared with untreated cells (Fig. 2A and B). In addition, western blot analysis was performed to determine the changes of expression or activation of G_2/M cell cycle-specific markers. Results showed that the expression of cyclin B1, which promotes nuclear accumulation and initiation of mitosis (25), was decreased in TRAMP-C2 cells by imiquimod at 48 h post-treatment (Fig. 2C). Moreover, imiquimod enhanced the phosphorylation of CDC2, which is a master regulatory kinase on the control of the G_2/M transition (26), from 6 h after treatment (Fig. 2C). The expression of cell cycle regulator p21 was also increased by imiquimod from 12 h after treatment (Fig. 2C). These findings suggest that imiquimod may suppress the growth of prostate cancer cells via G_2/M cell cycle arrest.

Imiquimod induces mitochondria-dependent apoptosis in prostate cancer cells. Previous studies showed that

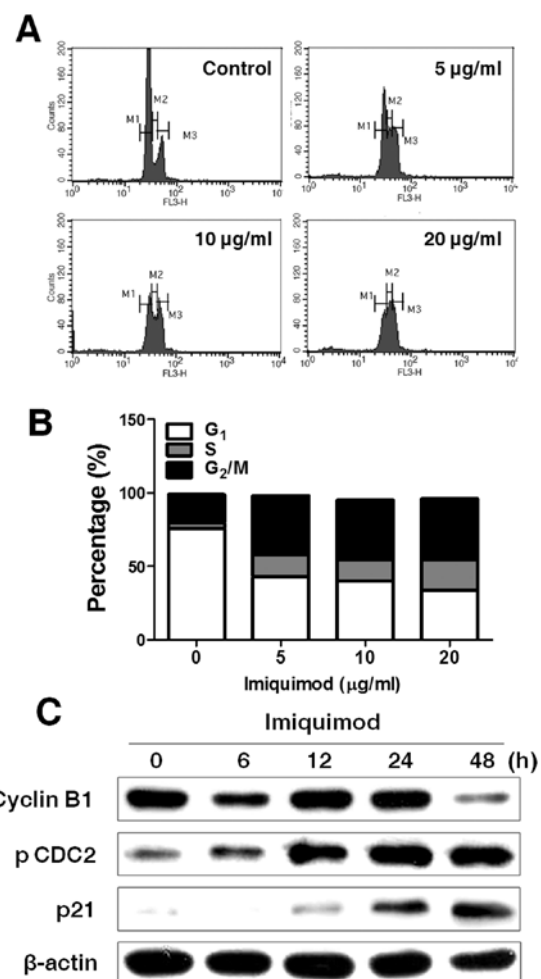


Figure 2. Imiquimod leads to G_2/M cell cycle arrest in TRAMP-C2 cells. (A) TRAMP-C2 cells with or without imiquimod treatment were stained with PI and DNA contents were analyzed by flow cytometry. (B) The percentage of cells in each phase of the cell cycle is shown. (C) The protein level of cell cycle-related genes in the imiquimod-treated TRAMP-C2 cells was evaluated by western blot analysis.

imiquimod can induce direct apoptosis in various cancer cells (13,17,18,20). To investigate whether imiquimod induces apoptosis in prostate cancer cells, TRAMP-C2 cells were treated with 10 or 20 μ g/ml of imiquimod for 48 h, stained

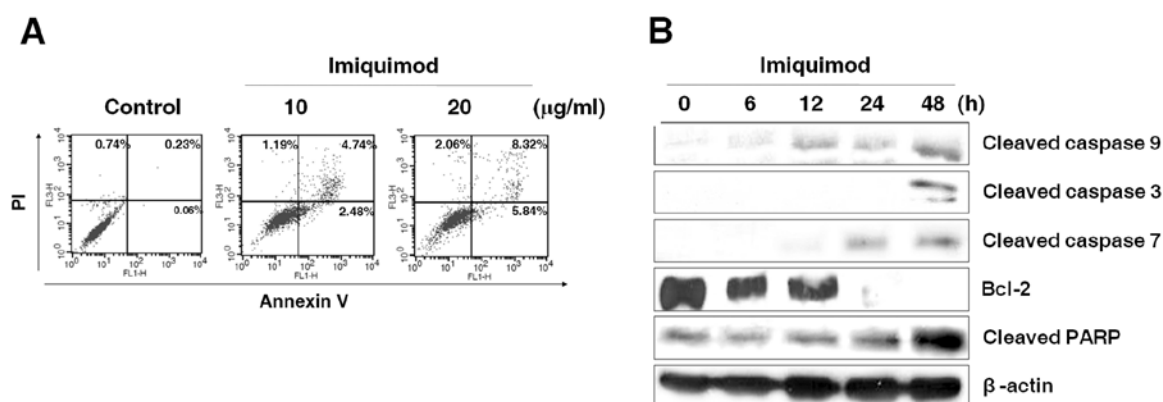


Figure 3. Imiquimod induces apoptosis in TRAMP-C2 cells. (A) TRAMP-C2 cells with or without imiquimod treatment were stained with PI and Annexin V-FITC and analyzed by flow cytometry. (B) The protein level of cell apoptosis-related genes in the imiquimod-treated TRAMP-C2 cells was evaluated by western blot analysis.

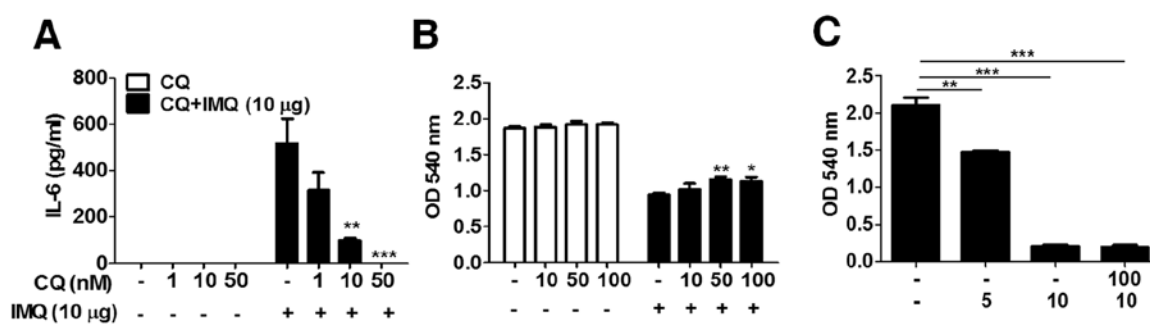


Figure 4. TLR7 is not required for the growth inhibition by imiquimod in TRAMP-C2 cells. (A) Imiquimod-induced IL-6 production and (B and C) cell growth inhibition were evaluated with or without chloroquine pretreatment. (A) Culture supernatants from imiquimod-treated TRAMP-C2 cells were collected and IL-6 levels were measured by ELISA. Growth inhibition was measured (B) 3 days or (C) 9 days after imiquimod treatment by MTT assay (B). The results presented here are from one representative experiment of two independent experiments and are presented as mean \pm SD. * P <0.05, ** P <0.01, *** P <0.001.

with PI and Annexin V, and analyzed by flow cytometry. The percentage of Annexin V-positive cells was increased by the treatment of imiquimod dose-dependently (Fig. 3A). In addition, we examined the change of molecules associating with mitochondrial-dependent apoptosis pathway by western blot analysis. The anti-apoptotic molecule bcl-2 expression in TRAMP-C2 cells was reduced by imiquimod in a time-dependent manner (Fig. 3B). Imiquimod also led to cleavage of caspase-9, caspase-3 and caspase-7 by 48 h (Fig. 3B). Moreover, cleaved form of PARP was also increased by imiquimod in TRAMP-C2 cells (Fig. 3B). These findings indicate that imiquimod may lead to apoptosis in prostate cancer cells via the intrinsic pathway.

A TLR7 inhibitor chloroquine does not restore the growth inhibition by imiquimod in prostate cancer cells. Although imiquimod has been described as a TLR7 agonist, it is still unclear whether TLR7 is required for its antitumor effect. To clarify this, we performed an inhibitor assay using chloroquine, which is an anti-malarial drug to block activation of endosomal TLRs such as TLR3, 7 and 9 by inhibiting endosomal acidification (27). Our preliminary experiment showed that imiquimod enhanced the gene expression of

TLR7 and induced IL-6 production in TRAMP-C2 cells (data not shown). As shown in Fig. 4A, chloroquine inhibited imiquimod-induced production of IL-6 in the cells in a dose-dependent manner. Although statistically significant, the restorative effect of chloroquine on imiquimod-induced inhibition of cell growth was minor (Fig. 4B). In an experiment with longer incubation time (9 days), chloroquine did not restore the growth inhibition by imiquimod in TRAMP-C2 cells (Fig. 4C).

Imiquimod inhibits in vivo growth of prostate cancer in mice. *In vivo* antitumor efficacy of imiquimod was evaluated in a mouse model s.c. implanted with TRAMP-C2 cells. On day 10 after tumor implantation, mice were daily treated with PBS or imiquimod (50 µg) by intratumoral injection for 9 days. Tumor size was significantly reduced in imiquimod-treated mice, as compared with PBS-treated group (Fig. 5A and B). Histologically, live tumor cells were compactly grown in PBS-treated mice (Fig. 5C). In contrast, in imiquimod-treated mice, necrotic area was broadly observed and cellular components were mostly dead cells (Fig. 5C). TUNEL staining revealed that apoptotic cells were increased by intratumoral injection of imiquimod in TRAMP-C2 cell-implanted mice (Fig. 5D).

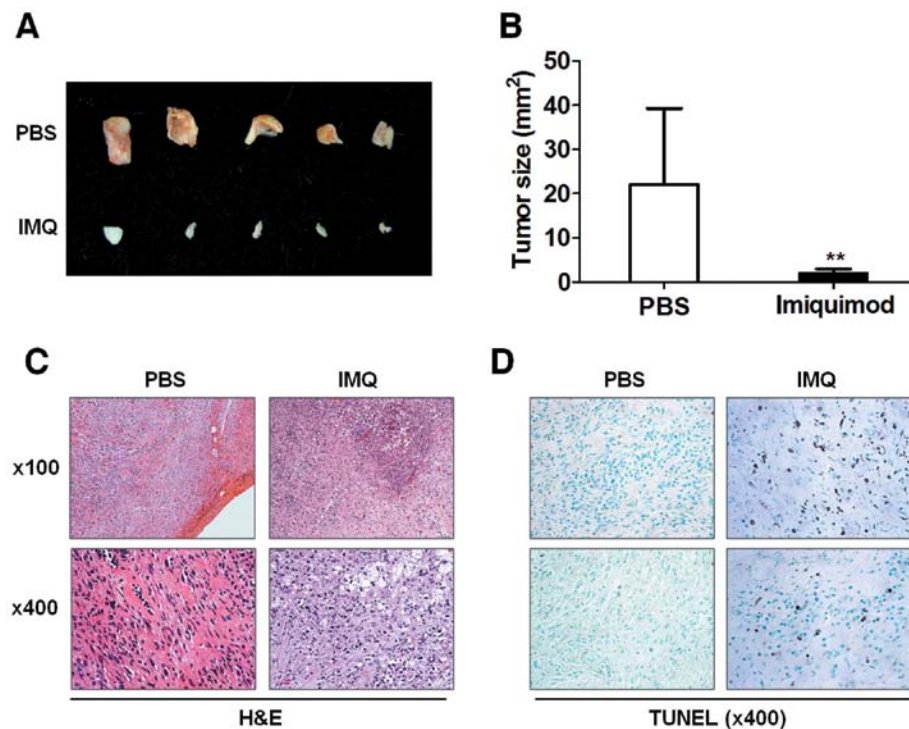


Figure 5. Imiquimod inhibits *in vivo* growth of prostate cancer in mice. (A) After cancer cell implantation, mice were intratumorally injected with PBS (upper) or imiquimod (lower). (B) The mean size of prostate tumor in each group is shown. Tumor mass section was evaluated histologically by (C) H&E staining or (D) TUNEL staining. Statistical comparisons are made between the treated groups and the control groups injected with the same volume of the saline: **P<0.01.

Discussion

Although imiquimod is known as a TLR7 agonist, it can also induce cellular signaling TLR7-independently. Imiquimod can induce transcriptional activation of proinflammatory factors through adenosine receptor signaling (28). In addition, it activates p38, ERK and JNK MAPKs and induces apoptosis in primary keratinocytes independently of TLR7 and MyD88 (29). Schön *et al* suggest that imiquimod triggers inflammatory response via TLR7/8 or adenosine receptor signaling, whereas it induces direct apoptosis independently (28). In this study, an endosomal TLR inhibitor chloroquine inhibited imiquimod-induced production of IL-6 in TRAMP-C2 cells, whereas it did not affect cell growth inhibition by imiquimod. These results suggest that imiquimod may primarily induce growth inhibition in prostate cancer cells through a TLR7-independent mechanism.

Regulation of cell cycle and apoptosis can be important targets for cancer chemotherapy (30). The anti-proliferative effects of imidazoquinolines family are mediated by cell cycle arrest and/or apoptosis in various cancer cells (31,32). However, the pattern of cell cycle arrest caused by imidazoquinolines is different between the studies. An imidazoquinoline derivative NVP-BEZ235 inhibits the growth of T cell acute lymphoblastic leukemia in the G₀/G₁ phase of the cell cycle (33). In addition, imiquimod and resiquimod treatment of cancer cells decreased cell proliferation through G₁/S phase arrest of the cell cycle through opioid growth factor receptor (OGFr) pathway (32). In contrast, treatment of EAPB0203, a member of the imidazo[1,2-a]quinoxalines, inhibited cell growth through

G₂/M cell cycle arrest and apoptosis in HTLV-I-transformed and HTLV-I-negative malignant T cells and fresh ATL cells (31). In the present study, flow cytometry and western blot analysis revealed that imiquimod induces cell cycle arrest at G₂/M phase in TRAMP-C2 cells. It remains to be clarified whether different pattern of imidazoquinolines-induced cell cycle arrest is due to difference of cancer cell types or structural difference of imidazoquinolines.

It is well known that imiquimod induces direct apoptosis in various cancer cells (12,13,16,34). The mechanism of imiquimod-induced apoptosis in tumor cells seems to be mitochondria-dependent. In melanoma cells, the pro-apoptotic activity of imiquimod was independent of cell surface death receptors including CD95, TNF receptors or TRAIL (TNF-related apoptosis-inducing ligand) receptors (12,13). Rather, it was depended on Bcl-2 degradation because melanoma cells overexpressing Bcl-2 were relatively resistant against imiquimod-induced apoptosis as compared with their sham-transfected control cells (13). Apoptosis of tumor cells was abrogated by inhibition of caspase activation (13). Moreover, blocking the functions of membrane-bound death receptors did not affect the pro-apoptotic activity of imiquimod (12). Imiquimod resulted in release of mitochondrial cytochrome *c* into the cytosol, which is a process that eventually leads to activation of caspase-9 and caspase-3 by proteolytic cleavage (35). In this study, imiquimod led to Bcl-2 degradation and cleavage of caspase-9, which are critical for intrinsic apoptosis. Therefore, as in other types of cancer, imiquimod seems to induce apoptosis in prostate cancer cells via mitochondria-dependent pathway.

In this study, intratumoral injection of imiquimod effectively inhibited *in vivo* tumor growth in mice s.c. implanted with TRAMP-C2 cells. Although the number of apoptotic cells was increased in imiquimod-treated mice, *in vivo* antitumor action mechanism of imiquimod seems to be more complex. Several studies have demonstrated that cytokines such as type I IFN or cytotoxic T cells could participate in tumor destruction *in vivo* induced by imiquimod. Imiquimod can induce a profound tumor-directed cellular immune response (17,36). Sullivan *et al* revealed that antitumorigenic effect of imiquimod is mediated by upregulation of local IFN- α levels (16). Moreover, a recent *in vivo* study showed that imiquimod strongly enhances antigen-specific activation of antitumoral CD8⁺ T cells (37). Collectively, it is likely that *in vivo* antitumor effect of imiquimod is achieved by direct apoptosis and enhancement of immune responses.

To our knowledge, this study is the first attempt to demonstrate that a direct treatment of imiquimod can significantly inhibit the growth of prostate cancer. Our current study revealed that imiquimod suppressed the proliferation of both mouse (TRAMP-C2) and human (PC3) prostate cancer cells in a TLR7-independent manner. Treatment of imiquimod resulted in G₂/M phase cell cycle arrest and intrinsic apoptosis. Finally, we showed that imiquimod effectively inhibited tumor growth in mice s.c. implanted with TRAMP-C2 cells. These results suggest that imiquimod can be an effective therapeutic against locally generated prostate cancer.

Acknowledgements

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