Androgen-dependent Activation of Human Cytomegalovirus IE Promoter in Prostate Cancer Cells

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Androgen-dependent Activation of Human Cytomegalovirus IE Promoter in Prostate Cancer Cells

Directed by Professor Kyung-Sup Kim

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ABSTRACT

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Human cytomegalovirus (HCMV) is a member of the β-herpesvirus family, which is sexually transmitted in adults. The HCMV immediate early (IE) promoter is strongly activated by androgen agonist, R1881, in androgen-dependent prostate cancer cells, LNCaP. However, the HCMV IE promoter is not activated at all by R1881 in androgen-independent prostate cancer cells (PC3), human embryonic kidney (HEK-293) cells and cervical cancer cells (HeLa), where androgen receptor (AR) is forced to be expressed. The activation of the HCMV IE promoter by R1881 coincided with the changes of protein kinase A (PKA) activity and could be ablated by treatment with H-89, the inhibitor of PKA, or the mutation of multiple copies of CREB/ATF-binding elements (CRE), indicating that the activation of the HCMV IE promoter by androgen results from the increase of PKA activities. Treatment with R1881 in LNCaP cells strongly suppressed the net production of prostaglandin E_2 (PGE_2) by inhibiting the synthesis and increasing degradation, but enhanced the ability to activate PKA in response to PGE_2 by expressing the EP4R, the receptor for PGE_2. This fact suggests the possibility

that the EP4R expression by androgen enables the cells to react to PGE_2, resulting in activating PKA and the HCMV IE promoter. The expression of PKA catalytic subunit beta transcript variant 2 (PKA-Cβ2) is decreased by serum deprivation and increased by R1881 treatment, in a similar pattern to changes of the HCMV IE promoter activities, suggesting that the regulation of the expression of PKA-Cβ2 might be direct upstream factor in the HCMV IE promoter regulation by androgen in LNCaP cells. Taken together, present study demonstrated that the HCMV IE promoter could be activated in androgen-dependent prostate cancer cells, which directs the expression of IE1/2 proteins containing oncogenic properties, supporting the possibility that HCMV might be involved in oncomodulation in prostate.

Key words : androgen, HCMV, PKA, prostate cancer

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I. INTRODUCTION

Prostate cancer is the most common male cancer and the second leading cause of male cancer death in the United States as well as the western world [1]. Recent epidemiological studies indicate a strong association between the incidence of prostate cancer and the increased exposure to sexually transmitted diseases, implying that the sexually transmissible agents increase the risk of prostate cancer [2, 3]. Human cytomegalovirus (HCMV) is a β-herpesvirus that is universally distributed among the adult population and causes the lesions of multiple organs in immunocompromised patients. Active HCMV infections in adults result mostly from reactivation of latent virus, which persistently resides in granulocyte/monocyte progenitors in the bone marrow [4-6]. During HCMV productive infection, HCMV genes are expressed in a temporal cascade, designated immediate early (IE), early or late [7]. The HCMV IE genes include UL123 (IE1) and UL122 (IE2) as the major IE genes (MIE), and other auxiliary genes [8, 9]. Among these genes, expression of the IE1/2 proteins was shown to be critical for transition from

latency to productive infection [10, 11]. Also, the IE1/2 proteins catalyze oncogenic processes through the interaction with Rb and p53 [12] and play a causative role in the development of various inflammatory diseases and cancers [13]. The IE1/2 gene expression is essential for expression of IE1/2 proteins and efficient viral replication.

Expression of these genes is controlled by the HCMV IE promoter whose activity depends on both viral and cellular factors/proteins [14]. The HCMV IE promoter is highly enriched with a densely packed array of *cis*-elements and is among the strongest known promoters in mammalian cells [15]. The HCMV IE promoter has four NF-kB binding sites, five CREB/ATF binding sites, two AP-1 binding sites, a serum response element (SRE) and multiple SP-1 sites [16]. By clinical studies and *in vitro* experiments the results have demonstrated that systemic inflammation, resulting in TNFα release, may be an important cofactor for HCMV (re)activation in transplant patients as well as nonimmunosuppressed patients with septicemia or other inflammatory diseases [17-19]. TNFα stimulates the HCMV IE promoter by activation of NF-κB which binds to four 18-bp repeats in IE promoter and activates the transcription [20-22].

The 19-bp repeats were also demonstrated to play a critical role in the expression of the IE1/2 genes [23] and their core sequence is identical to consensus of cAMP response element (CRE) [24]. The 19-bp repeats were reported to work as CRE in several lymphoid cell lines [25] and CNS-derived cell lines [26]. Since the CREs are bound *in vitro* by CREB/ATF-1 [25, 27] whose activities were regulated by phsophorylation by cAMP-dependent protein kinase [24, 27, 28], indicating that cAMP-signaling pathway might to play a role in reactivation of HCMV in some tissue.

The DNA, mRNA and/or antigens of HCMV have been frequently detected

in tumor tissues, even if HCMV is not considered as tumor virus. Recently, HCMV has been postulated to play a role in oncomodulation, which might result in a more malignant phenotype in tumor cells. In this context, it is important to elucidate the mechanisms, by which the productive HCMV infection could persist in some tumors.

In the present study, I have investigated the mechanism of the activation of the HCMV IE promoter by androgen in prostate cancer cells. The HCMV IE promoter was strongly activated by androgen in androgen-dependent manner in LNCaP cells. The activation of the HCMV IE promoter was dependent on PKA activity in LNCaP cells. These results indicate that the activation of the HCMV IE promoter by androgen in prostate, which is highly vulnerable to HCMV infection, might contribute oncomodulation in prostate cancer.

II. MATERIALS AND METHODS

Materials

The synthetic androgen R1881 (methyltrienolone) was purchased from Dupont-New England Nuclear (Dreiech, Germany), and was dissolved in absolute ethanol and added to the cultures. H-89, a PKA inhibitor was purchased from Sigma-Aldrich (St Louis, MO), and was dissolved in dimethyl sulfoxide (DMSO). Arachidonate (AA) was purchased from Sigma-Aldrich (St Louis, MO), and was dissolved in absolute ethanol.

Cell Culture

All the cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in respective medium containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA.), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA.). The medium for LNCaP and PC3 was RPMI 1640 (Invitrogen), and that for HEK-293 and HeLa was DMEM (Invitrogen). In experiments assessing the effect of androgen, the cells were pre-incubated in the media containing 5% charcoal-treated FBS (CT-FBS) instead of FBS for 48 hrs and then further incubated in the media containing 1% CT-FBS and 10^{-8} M R1881 for 72 hrs [29]. CT-FBS was prepared by incubation of FBS with dextran-coated charcoal at 4 °C overnight and then removal of charcoal by centrifugation [30].

Recombinant Plasmids

The DNA fragment for the HCMV IE promoter was obtained from plasmid pCMV-β-gal (Stratagene) by digestion with EcoRI and BamHI. Resulting 629bp DNA for HCMV IE promoter was inserted into the SmaI and BglII site of pGL3-basic vector (Promgega, Madison, WI), and named as pHCMV-Luc reporter. The CRE mutant construct of pHCMV-Luc was generated, using a QuickChange Site-direct Mutagenesis kit (Stratagene, La Jolla,CA) according to the manufacturer's protocol. The following primers used for mutagenesis were: 5' primer, 5'-CCGCCCATTGACTGCAATAAT GACGTATGT- 3' and 3' primer, 5'-ACATACGTCATTATTGCAGTCAAT GGGCGG- 3' for CRE 1, and 5' primer, 5'-TTTCCATTGACTGCAATGGG TGGAGTATTT- 3' and 3' primer, 5'-AAATACTCCACCCATTGCAGTCA ATGGAAA- 3' for CRE 2, and 5' primer, 5'- CCCCTATTGACTGCAATG ACGGTAAATGGC- 3' and 3' primer, 5'-GCCATTTACCGTCATTGCAGT CAATAGGGG-3' for CRE 3, and 5' primer, 5'-ACCCCATTGACTGCAAT GGGAGTTTGTTTT- 3' and 3' primer, 5'-AAAACAAA CTCCCATTGCAG TCAATGGGGT- 3' for CRE 4, and 5' primer, 5'- CCGCCCCATTGAGCCA AATGGGCGGTAGGCG-3' and 3' primer, 5'-CGCCTACCGCCCATTTGG CTCAATGGGGCGG-3' for CRE 5. The integritiy of the plasmid sequences were confirmed by DNA sequencing. All mutations were confirmed by DNA sequencing using the RV3 primer: 5'-CTAGCAAAATAGGCTGTCCCC-3'. The pSG5-AR plasmid, AR over-expression vector and MMTV-Luc reporter were kindly provided by Dr. Ho-Guen Yoon (University of Yonsei, Korea). Expression vector for PKA-Cβ2 was constructed by subcloning PKA-Cβ2 cDNA amplified using PKA-Cβ2 specific primer, 5' primer, 5'-GGCAGCTTATAGAGAACCACCTTGTAA-3'and 3' primer, 5'-TTTAA

AATTCACCAAATTCTTTTGCA-3', from total RNA of LNCaP cells into SmaI site of pSG5-KHA2M1, which contains HA tag sequence.

Transient Transfections and Reporter Gene Assays

For assays of androgen-dependent transcriptional activation, the cells were seeded in 100-mm dish in media containing 5 % CT-FBS at a density of 3 X 10^6 cells. Two days later, cells were transfected with indicated plasmid DNA using Lipofectamine and the PLUS reagent (Invitrogen) according to the manufacturer's protocol. After 6 hrs incubation, the cells were changed with fresh medium containing 5 % CT-FBS. The next day, cells were trypsinized and transferred to 6-well dishes and further incubated in 1% CT-FBS-containing media with/without 10^{-8} M R1881. After 72 hours, cells were washed with PBS and lysed in 200ul passive lysis buffer (Promega Corp.). Aliquots of 5ul of cleared lysate were used for assay of luciferase activity, using a luciferase reporter assay kit (Promega Corp). Each transfection experiment was performed in triplicate.

Reverse Transcriptase-PCR and Real-Time PCR Analysis (RT-PCR)

Total RNA was isolated from cultured cells using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For quantitative RT-PCR, cDNAs were synthesized from 4 μg of total RNA using random hexamer and SuperScript reverse transcriptase II (Invitrogen) according to the manufacturer's protocol. The resulting cDNA was diluted with 80 μl of Tris-EDTA (pH 8.0) and 2 μl was subsequently mixed with 2 X SYBR Green PCR Master Mix (Applied Biosystems) and various sets of gene-specific primers and then subjected to RT-PCR quantification using the ABI PRISM 7300 Real Time PCR System (Applied Biosystems). The sequences of the

primers used in RT-PCR are as follow: PKA-Cβ2 5' primer, 5'-AGAGAACCACCTTGTAACCAGTATACAGGT-3' and 3' primer, 5'-TTTGGCTTTGGCTAGAAACTCTTTCATT-3'. HPGD 5' primer, 5'-GCACGTGAACGGCAAAGTGG-3' and 3' primer, 5'-GTTCATTGGGTTTTTGCTTGAAATG-3'. EP4R 5' primer, 5'-CAATTCGTCCGCCTCCTTGAG-3' and 3' primer, 5'-TTCACTGGGAAATGTGACTTGCAG-3'. COX1 5' primer, 5'-GAGCCGGAGTCTCTTGCTCTGGTT-3' and 3' primer, 5'-CTCAGAGCTCTGTGGATGGTCGCT-3'. COX2 5' primer, 5'-GCTCAGCCATACAGCAAATCCTT-3' and 3' primer, 5'-TTAGACTTCTACAGTTCAGTCGAACG-3'.

Immunoblot Analysis

The cells were harvested and lysed 2 X Laemmli buffer, then briefly sonicated. Total cell lysates were centrifuged at 12000 rpm for 10min at 4°C. Supernatants were collected and determine protein concentration, using a modified Bradford assay kit (BioRad, CA, USA). Each sample was electrophoresed on an 5 or 8% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) gel. Briefly, 20 ug of protein was mixed with appropriate volumes of 5 X Laemmli buffer (0.6M Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 1% bromophenol blue, 0.05% b-mercaptoethanol), boiled at 95°C for 3 min and electrophoresed for 120 min at 120 V. Proteins were transferred to Nitrocellulose transfer membrane (Whatman, Germany) for 90 min at 350mA. Membranes were blocked in 5% skimmed milk powder in Trisbuffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature (RT), and then incubated for 90min at RT with the appropriate primary antibody diluted in TBST. To confirm exogenous androgen receptor (AR) expression in PC3, HEK-293 and HeLa cells, androgen receptor polyclonal antibody was used at 1:5000. In identification of CREB and

CREB phosphorylation expression in LNCaP cells, the nuclear extract lysates of LNCaP cells were used. CREB antibody and phosphorylated CREB antibody (Cell Signaling, Technol., Inc) were used at 1:1000. To confirm exogenous PKA-Cβ2 expression in PC3 cells and endogenous PKA-Cβ2 expression in LNCaP cells, PKA-Cβ2 polyclonal antibody was used at 1:5000. In identification of endogenous PKA-Cβ2 expression in LNCaP cells, the nuclear extract lysates of LNCaP cells were used. The immuno-reactive bands were visualized by horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL) using the SuperSignal West Pico Chemiluminescent System (Pierce, Rockford, IL).

PKA Activity and PGE_2 Assay

The cells in culture flask of 10 cm in diameter were washed with PBS and were lysed by dropping 1ml of lysis buffer (20mM MOPS, 50mM β-glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol(DTT), 1mM benzamidin, 1mM phenylmethanesulphonyfluoride (PMSF), and 10ug/ml leupeptin and aprotinin) into the culture flask. After 10 min incubation period, cell lysate was collected and centrifuged at 13000 rpm for 15 min. Clear supernatant was collected and its protein concentration was determined. PKA activity was determined using a solid phase enzyme-linked immuno-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for PKA and a polyclonal antibody that recognizes the phosphorylated substrate, following the protocols recommended by the company (Assay Designs, Echelon, ICT).

PGE_2 levels in the culture medium samples were determined using a PGE_2 monoclonal enzyme immunoassay kit (Assay Designs, Echelon, ICT), following the protocols recommended by the company.

III. RESULTS

The activation of the HCMV IE promoter by synthetic androgen, R1881, in LNCaP cells

The HCMV IE promoter is strong promoter, generally used in purpose of over-expression of target gene in animal cells. In transfection expreriment, we found that the HCMV IE promoter was drastically activated up to 49 folds by the treatment with synthetic androgen, R1881 in LNCaP cells, of which growth are androgen-dependent (Fig. 1A). The change of the HCMV IE promoter activity according to time course after R1881 treatment is somehow different from those of the MMTV promoter, which can be activated by direct binding of liganded androgen receptor to androgen-responsive elements [31]. Before the androgen treatment, LNCaP cells were pre-incubated in 5% CT-FBS for 48 hrs and then the media was replaced the media, containing 1% CT-FBS in company with 10nM R1881 or not. The HCMV IE promoter activity was gradually repressed until 72 hrs when the serum was depleted, whereas the MMTV promoter activity was not changed (Fig. 1B and 1C). The androgen-mediated activation of the HCMV IE promoter was not marked until 24 hrs and became prominent after 48 hrs with the concomitant decrease of the basal transcription level, while the MMTV promoter was already greatly stimulated 25 folds by androgen at 24 hrs and further increased up to 60 folds until 72 hrs (Fig. 1B and 1C). The late response of the HCMV IE promoter to R1881 suggested that the HCMV IE promoter activation by androgen might be mediated by the indirect action of the activated AR rather than its direct action.

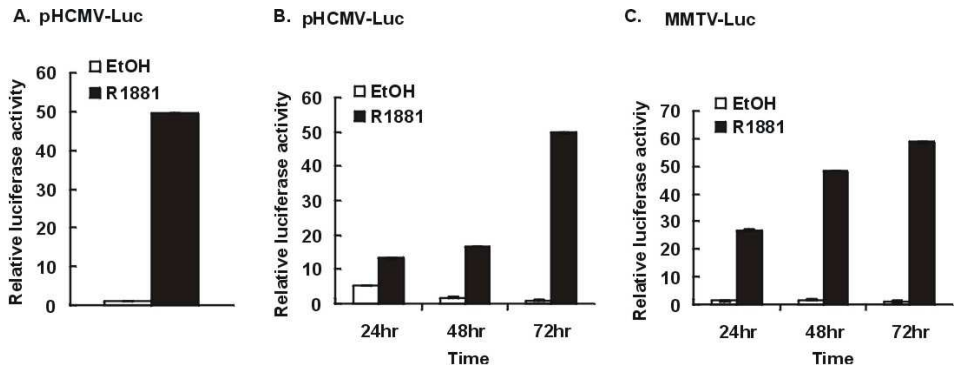

Figure 1. Activation of the HCMV IE promoter by androgen in LNCaP cancer cells. LNCaP cells were transfected with pHCMV-Luc (A), pHCMV-Luc (B), MMTV-Luc (C), and followed by incubation in 1% CT-FBS-containing media without (open column) or with (closed column) 10 nM R1881. The luciferase activities were measured at 72 hrs (A) or at each time denoted in (B) after hormone treatment. The values represent means ± S.D. from triplicate reactions.

Androgen-mediated activation of the HCMV IE promoter is specific in LNCaP cells

AR is generally activated by its ligand and then induces the transcription of target genes by its direct binding to ARE. The exogenous expression of AR leads the MMTV promoter to respond to synthetic androgen, R1881 in PC3 prostate cancer cells, where AR is not endogenously expressed (Fig. 2B). However, AR did not activate the HCMV IE promoter at all when it is exogenously expressed in PC3, HEK-293, and HeLa cells (Fig. 2A), even though the forced expression levels of AR in these cells were much higher than in LNCaP cells (Fig. 2A). These results indicate that the HCMV IE promoter is not direct target of AR and also suggest that a signal pathway, which is specifically stimulated in LNCaP cells by androgen, might be involved in its activation.

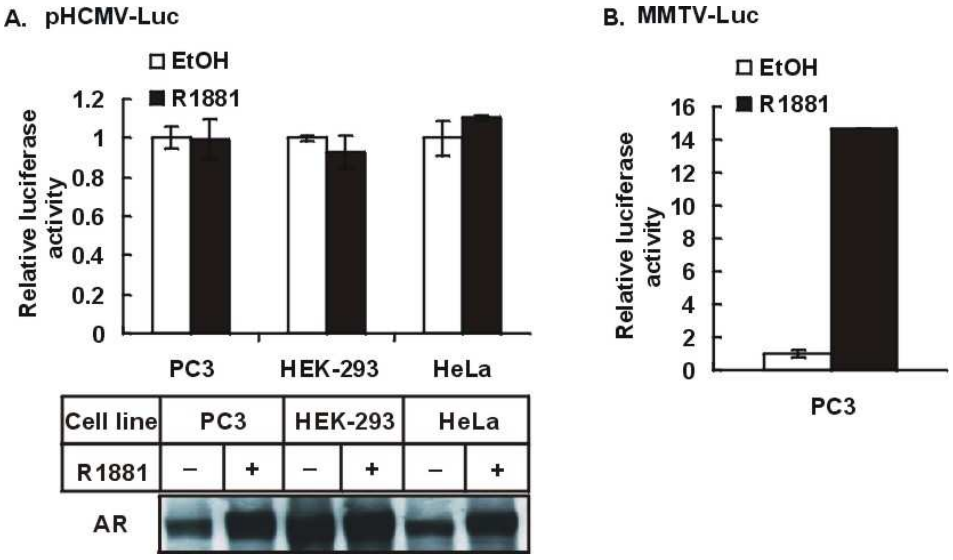

Figure 2. Androgen-responsiveness of the HCMV IE promoter in various cancer cells, exogenously expressing AR. (A) PC3, HEK-293 or HeLa cells were cotransfected with pHCMV-Luc and pSG5-AR. Next day after transfection, media were changed with 1% CT-FBS-containing media without (open column) or with (closed column) 10 nM of R1881. The luciferase activities were measured 72 hrs after hormone treatment. The exogenously over-expressed androgen receptor was confirmed by immunoblot analysis in each group described in (A). (B) MMTV-Luc reporter construct were transfected in PC3 cells and the androgen-dependent activation of the MMTV promoter were measured as same procedures as (A). The values represent means ± S.D. from triplicate reactions.

cAMP-responsive element (CRE) and PKA are involved in androgen-mediated activation of HCMV IE promoter

The 19-bp element, containing the conserved cAMP-responsive element (CRE), repeats five times in the HCMV IE promoter and is known to play an important role in the strong transcriptional activation of the HCMV IE promoter. These elements were reported to mediate the stimulation by cAMP/PKA signaling pathway [25, 26, 32, 33]. To determine whether the androgen-mediated activation of the HCMV IE promoter is dependent on these CRE's, five CRE's in the HCMV IE promoter were mutated as denoted in Fig. 3A and the androgen-dependent activation was compared between wild and mutant constructs (Fig. 3B). The CRE mutations severely suppressed basal activity and almost abolished the androgen-responsiveness of the HCMV IE promoter. This result indicates that CRE is critical in regulation of the HCMV IE promoter by androgen and led us to check the levels of phosphorylated CREBP, which is known to be involved in CRE-mediated activation. Androgen treatment did not affect the total amount of CREBP, but the level of its phosphorylated form was gradually increased until 72 hrs after R1881 treatment (Fig. 3C). Since PKA is the key enzyme catalyzing the phosphorylation of CREBP, the inhibition of PKA was considered to be prevent the HCMV IE promoter from the activation by androgen. As expected, H-89, PKA inhibitor, almost completely inhibits the androgen-mediated activation of the HCMV IE promoter (Fig. 3D). Taken together, these results indicate that the androgen-mediated activation of the HCMV IE promoter depends on the CREBP activation via the PKA signaling pathway.

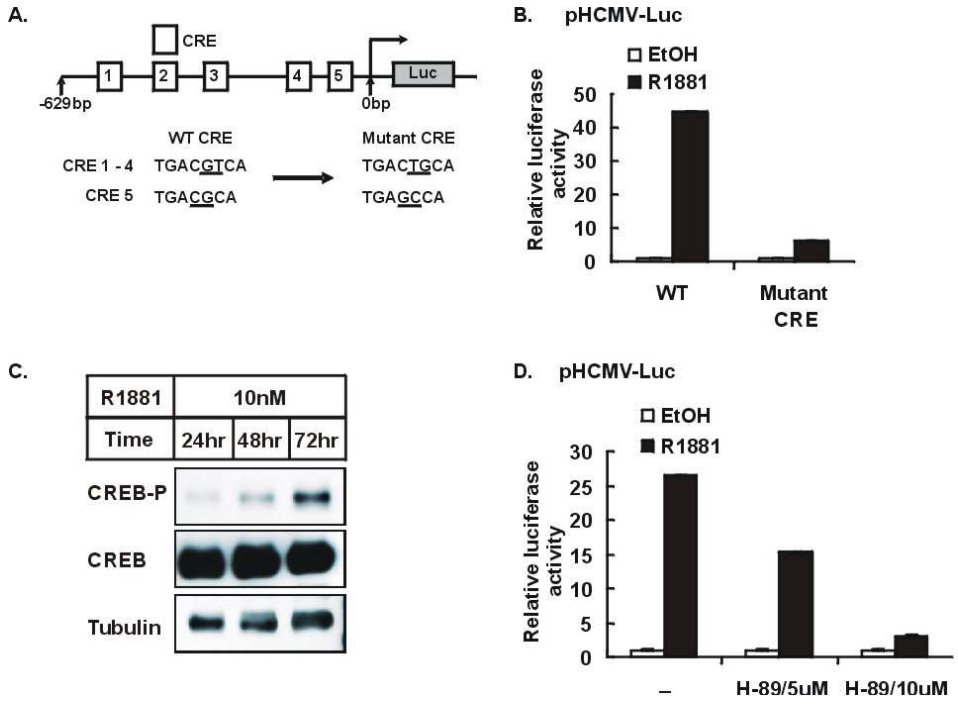

Figure 3. Effect of CRE mutation and PKA inhibitor on the HCMV IE promoter activity in LNCaP cells. (A) Diagram of CRE sites in the HCMV IE promoter. (B) LNCaP cells were transfected with wild and mutant CRE of HCMV-Luc construct. The cells were treated with (closed column) or without (open column) 10 nM of R1881 for 72 hrs. (C) Phosphorylation of CREB by androgen was detected in LNCaP cells by immunoblot analysis. The nuclear extract lysates of LNCaP cells were used. Antibody against CREB antibody, phosphorylated CREB antibody used for immunoblot analysis. (D) LNCaP cells were transfected with pHCMV-Luc construct. The cells were then treated with or without 10 nM of R1881 for 72 hrs and 5 or 10 uM of H-89 24 hrs before harvest. Luciferase activity in cell lysates were determined. The values represent means ± S.D. from triplicate reactions.

The role of PGE_2 and PGE_2 receptors on the HCMV IE promoter activity through PKA activity in LNCaP cells

First candidate for signal molecules involved in PKA activation by androgen was prostaglandins, because prostate is easily exposed to semen containing high level of the prostaglandin (PG). The expression levels of the gene involved in prostaglandin metabolism were checked by microarray analysis of RNA isolated from LNCaP cells treated with R1881 or not (Fig. 4A).

Cyclooxygenase-2 (COX2), synthetic enzyme, was suppressed and 15-hydroxyprostaglandin dehydrogenase (HPGD), catabolic enzyme, was markedly elevated up to 33 folds by R1881 treatment. The levels of PGE_2 were not changed until 72 hrs after R1881 treatment, whereas they were significantly accumulated in media in absence of R1881 (Fig. 4B). Moreover, the addition of arachidonate (AA) induced the production of PGE_2 in absence of R1881, while AA-induced PGE_2 production was strongly suppressed by R1881 treatment (Fig. 4C). These results indicated that androgen suppress the prostaglandin levels. However, the activity of PKA was not affected at all by addition of AA, even if PGE_2 was markedly increased in absence of R1881. R1881 treatment significantly activates the PKA, suggesting that PG-signaling pathway is not likely to be related to androgen-dependent PKA activation.

Interestingly, PKA activity was activated by AA in R1881-treated group, indicating that R1881 treatment enable the cell to respond PGE_2 (Fig. 4D). This might probably result from androgen-dependent induction of the EP4R, which escalates cAMP levels in response to PGE_2 [34]. Even if androgen markedly suppresses the levels of PGs in cancer cells, it might enable the

cancer cells to response to the PGs, plentifully supplied from neighboring tissues, resulting in stimulating the PKA signal pathway.

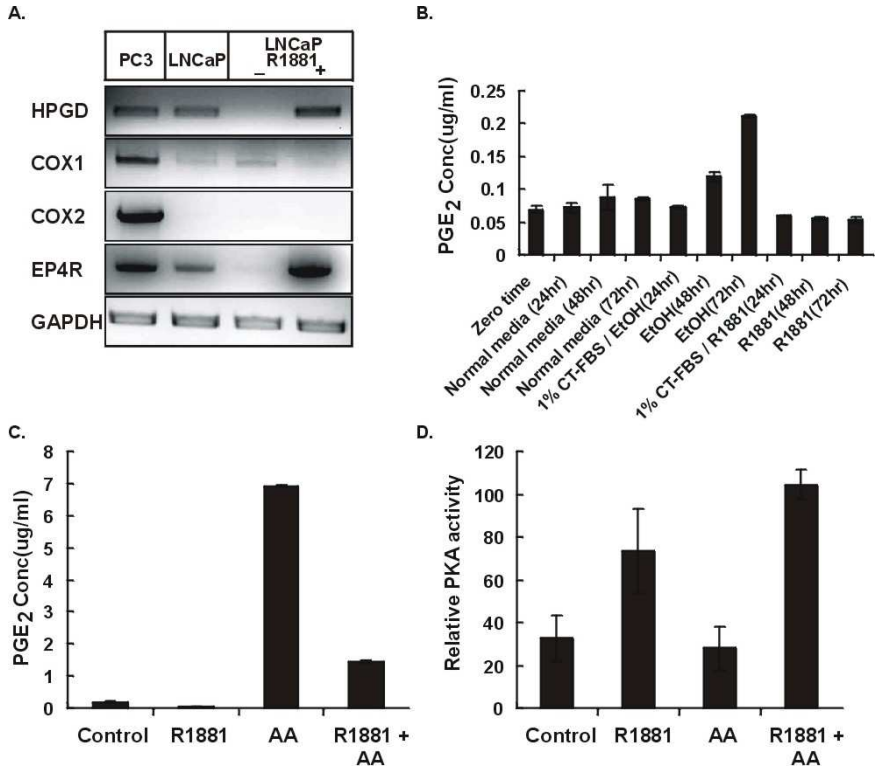

Figure 4. The role of PGE_2 and EP4R on the HCMV IE promoter activity in LNCaP cells. (A) The expression levels of the genes related to PGE_2 metabloism, such as HPGD, COX1, COX2 and EP4R in PC3 and LNCaP cells grown in 10% FBS-containing media or LNCaP cells grown in 1% CT-FBS-containing media with/without 10 nM of R1881 for 72 hrs. (B) PGE_2 levels were measured in the media where LNCaP cells were grown as denoted in figure. (C and D) PGE_2 levels and PKA activity were measured in media or cell lysates, respectively, after treatment with R1881 and/or AA. R1881 treatment (10 nM) was performed for 72 hrs and AA treatment (20 μM) was 24 hrs before harvest. The values represent means ± S.D. from duplicate reactions.

Androgen increased the expression of PKA-Cβ2, resulting in the HCMV IE promoter activation

Even though androgen enables the cell to activate PKA in response to PGE_2 by elevating the expression of EP4R, prostaglandin signaling pathway is not the key regulating system in androgen-dependent stimulation of the PKA activity and the HCMV IE promoter in LNCaP cells. In this reason, the expression levels of genes were compared between LNCaP cells and PC3 cells by microarray analysis in order to find out LNCaP-specific genes involved in cAMP signaling pathway, identifying PKA showing LNCaP-specific expression. The RNA level of PKA-Cβ2 gene was highly expressed 35-folds in LNCaP cells when it was compared to those of PC3 cells (Fig. 5A third and fourth bars) and was increased 12-folds by androgen (Fig. 5A, first and second bars). The change of media containing 10% FBS to 1% CT-FBS-containing media markedly repressed the PKA-Cβ2 expression (Fig. 5A, first and third bars). The protein expression of PKA-Cβ2 is markedly increased from 48 to 72 hrs after R1881 treatment in LNCaP cells (Fig. 5B). The changes of PKA-Cβ2 gene expression are almost completely coincide with those of PKA activities (Fig. 5C), and are similarly regulated as phosphorylation of CREB and activity of HCMV IE promoter. To confirm the PKA-Cβ2 is able to activate the HCMV IE promoter, the changes of the HCMV IE promoter activity were measured in PC3 cells when PKA-Cβ2 was exogenously over-expressed. As expected, the over-expression of PKA-Cβ2 sufficiently activated the HCMV IE promoter (Fig. 5D). Taken together, it could be concluded that the regulation of the HCMV IE promoter activities in LNCaP cells might be due to the levels of PKA activities, resulted from the levels of PKA-Cβ2 expression.

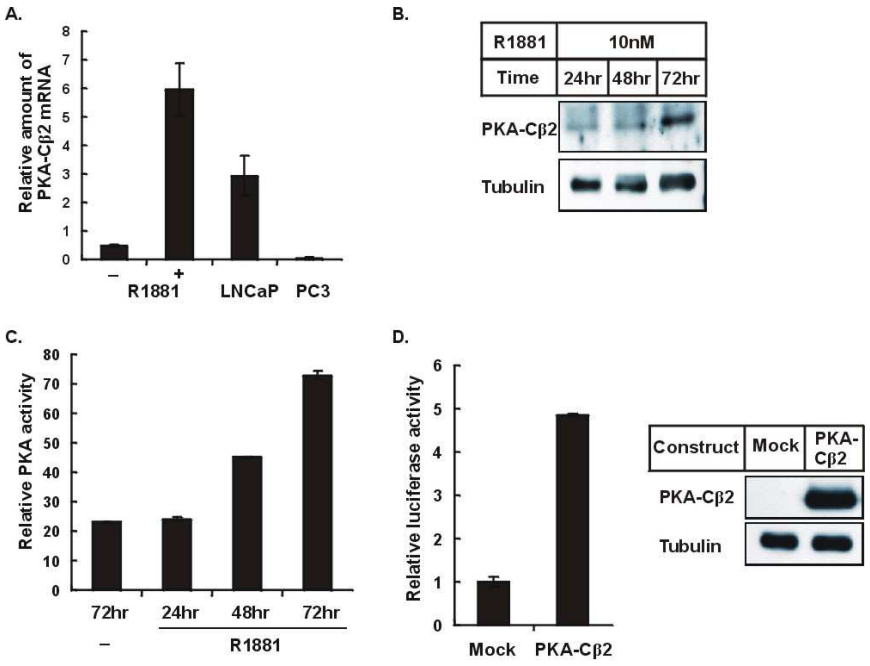

Figure 5. PKA-Cβ2 expression is up-regulated by androgen. (A) Total RNAs were isolated from LNCaP cells cultured in 1% CT-FBS-containing media in absence or presence of 10 nM of R1881 for 72 hrs, or LNCaP and PC3 cells cultured in 10% FBS-containing media. Total RNAs were subject to RT-PCR to quantitate the levels of PKA-Cβ2 and GAPDH mRNA and the values were shown as the ratio of PKA-Cβ2 to GAPDH. (B) PKA-Cβ proteins were detected in nuclear extracts isolated from LNCaP cells treated with R1881 in 1% CT-FCS-containing media for each time by immunoblot analysis. (C) Activities of PKA were measured in the cell lysates from LNCaP cells incubatd in 10% FBS-containing media (normal media) or 1% CT-FBS-containing media with or without 10 nM of R1881 for each time. (D) PC3 cells were transfected with pSG5 or pSG5-PKA-Cβ2 in company with pHCMV-Luc reporter. The luciferase activities were measured 48 hrs after transfection. The values represent means ± S.D. from duplicate reactions. The expression of exogenous PKA-Cβ2 was confirmed in each experimental group for immunoblot analysis.

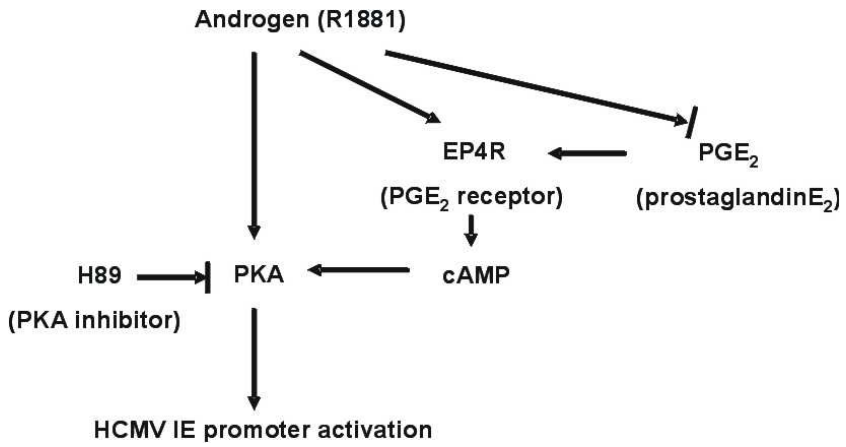

Figure 6. Schematic diagram to explain the mechanism involved in androgen-dependent activation of the HCMV IE promoter in LNCaP cells.

IV. DISCUSSION

In LNCaP cells, the HCMV IE promoter was suppressed by serum-deprivation and drastically activated by androgen. The HCMV IE promoter contains highly enriched array of *cis*-elements for a large number of transcription factors [15, 35, 36], including CREB/ATF binding sites (CRE). The five 19-bp repeats, containing CRE, contribute to strong basal promoter activity [24] and induction by cAMP. Basal and androgen-induced transcriptional activities of the HCMV IE promoter in LNCaP cells were inhibited by site-direct mutagenesis of these CREs. PKA inhibitor, H-89, completely blocked the androgen-mediated activation of the HCMV IE promoter. PKA activity is really increased by androgen treatment in LNCaP cells and its time-course changes are similar to those of the HCMV IE promoter activities. These data support the notion that the regulation of the HCMV IE promoter activity by androgen in LNCaP cells depends on the activation of PKA signaling.

The PKA signaling pathway is a multiple component system that requires the production of cAMP and induces the activation of PKA, the phosphorylation of CREB, and then gene expression [37]. The cAMP-PKA pathway is one of the most common and versatile signal transduction pathway in eukaryotic cells and is involved in regulation of diverse cellular processes such as cell cycle, proliferation, differentiation and so on [37].

The first candidate to be able to activate PKA in prostate cancer is PGs, because biosynthesis of PGs is highly active in the prostate cancer and it is known to plays an important role in tumor growth in prostate cancer [38, 39]. The representative signaling pathways from PGs is the cAMP/PKA system via mainly the EP4R [34]. Interestingly, when cells were cultured in

androgen-depleted state, the AA did not activate PKA activity at all even though PGE_2 levels were markedly increased. In culture system, PGE_2 is not likely to be primary factor for PKA activation by androgen because androgen rather decreases the PGE_2 levels by activation of HPGD, PG-catabolizing enzyme. However, in vivo system, androgen may reinforce the responsiveness to PGE_2 derived from neighboring tissues or organs by increasing the EP4R expression, and probably enables prostate cells to activate the HCMV IE promoter via PG signal pathway.

PKA is a heterotrimeric enzyme, containing regulatory (R) subunit dimer and two catalytic (C) subunits. In human PKA, four different C subunits (Cα, Cβ, Cγ and PrKX) have been identified [40]. In present study, we demonstrated that Cβ2, a splicing variant of Cβ, was down-regulated by incubation in serum/androgen-depleted media, compared to 10% FBS-containing media and was markedly up-regulated by androgen. The changes of Cβ2 levels coincide to those of PKA activity in LNCaP cells, suggesting that Cβ2 might be primary element to determine PKA activity in LNCaP cells. Cβ2 is a splicing variant, which is highly expressed in tissues involved in immune system, whereas other PKA-Cβ variants are most abundant in brain [41]. Kviissel et al [42] observed that PKA-Cβ2 is up-regulated by androgen in LNCaP cells and is highly expressed in prostate cancer tissues compared to normal tissues. The PKA holoenzyme containing catalytic subunit PKA-Cβ was reported to exhibit much low Ka for cAMP and to activate cAMP-responsive luciferase reporter at even low concentration of cAMP [43]. These previous reports and present data suggest that the up-regulation of PKA-Cβ2 is primary factor to increase PKA activity by androgen, causing the activation of the HCMV IE promoter.

The HCMV IE promoter controls the expressions of IE1 and IE2 proteins,

which are generated by alternative splicing and are inevitable factors for viral replication. IE1 interacts with p107 of Rb protein family, resulting in relieving the suppression of E2F activity [44, 45]. IE2 also interacts with pRB to alleviate the repression of E2F target genes. IE2 has been shown inhibits the transactivation function of p53. These findings indicates that these IE1/2 proteins have the abilities to modulate the cell cycle of an infected cells and show the similarities of the HCMV IE proteins to oncogenic proteins of other small DNA tumor viruses, such as SV40 large T antigen of SV40 virus and E6/E7 proteins of human papilloma virus. The modulation of the HCMV IE promoter in infected cells has important meanings in viral replication and the oncogenic effects of HCMV. Even though several reports have provided the evidences that HCMV infection is related with prostatitis and prostatic cancers [46, 47], HCMV genomic functions in prostate gland was not studied.

The present study revealed that the activation of the HCMV IE promoter by androgen occurred in androgen-dependent prostatic cancer cells and PKA is involved in this activation. These results might explain why prostate is the organ to produce active viruses of HCMV persistently even if the titer of its antibodies were high in blood [46, 48] and support the possibility that this persistent infection could potentially promote oncogenic pathways.

V. CONCLUSION

1. The activation of HCMV IE promoter by synthetic androgen, R1881, in LNCaP cells.

2. Androgen-mediated activation of the HCMV IE promoter is specific in LNCaP cells.

3. cAMP-responsive element (CRE) and PKA are involved in androgen -mediated activation of the HCMV IE promoter.

4. The role of PGE_2 and PGE_2 receptor on the HCMV IE promoter activity through PKA activity in LNCaP cells.

5. Androgen increased the expression of PKA-Cβ2, resulting in the HCMV IE promoter activation.

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selected populations. J Infect Dis 1975 Oct;132(4):472-3.

ABSTRACT(IN KOREAN)

전립선 암세포에서 Androgen 에 의한 Human Cytomegalovirus IE promoter 의 활성화

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Human cytomegalovirus (HCMV) 는 β-herpesvirus family 중의 하나로, 성적 접촉이 주된 감염 경로이다. 전립선 암세포 중 androgen dependent 세포인 LNCaP 세포에서 합성 androgen 인 R1881 에 의해 HCMV IE promoter 활성이 매우 증가하였다. 그러나 androgen receptor (AR) 이 없는 PC3 전립선 암세포주, HEK-293 세포주, HeLa 세포주 등에서 AR 을 인위적으로 과 발현 하여도 HCMV IE promoter 활성이 androgen 에 의해 영향을 받지 않았다. R1881 에 의한 HCMV IE promoter 활성은 protein kinase A (PKA) 의 활성의 변화와 동일하였고, 또한 PKA 활성 억제제인 H-89 에 의해서 HCMV IE promoter 활성이 거의 나타나지 않았고, HCMV IE promoter 내의 5 개의 CREB/ATF-binding elements (CRE) 의 변형에 의해서도 HCMV

IE promoter 활성이 사라지는 것을 확인하였다. 이 결과는 androgen 에 의한 HCMV IE promoter 의 활성이 PKA 활성 증가에 의해서 나타난다는 것을 알 수 있었다. Androgen dependent 세포인 LNCap 세포에 R1881 의 처리는 prostaglandin E_2 (PGE_2) 의 생성 감소와 분해 유도를 통하여 PGE_2 의 합성을 억제시키지만, PGE_2 와 반응하여 PKA 의 활성을 유도하는 PGE_2 수용체인 EP4R 는 발현 증가를 유도하였다. 이 사실은 R1881 에 의해 증가된 EP4R 가 PGE_2 와 작용하여 PKA 의 활성을 유도하고, 이에 따라 HCMV IE promoter 의 활성을 유도 할 수 있음을 제안한다. Protein kinase A catalytic subunit beta transcript variant 2 (PKA-Cβ2) 의 발현은 serum 이 없는 조건에서 감소하고, R1881 의 처리에 의해서는 증가하는 것을 확인하였고, 이러한 변화는 HCMV IE promoter 활성의 변화 양상과 유사하였다. 따라서 LNCaP 세포에서 androgen 에 의한 HCMV IE promoter 의 조절에 PKA-Cβ2 의 발현조절이 direct upstream factor 라는 것을 암시한다.

이 연구결과는 HCMV IE promoter 의 활성이 androgen 의존적인 전립선 암세포에서 유도될 수 있으며, HCMV IE promoter 에 의해서 직접적으로 발암적인 요소를 가진 IE1/2 단백질의 발현이 유도 될 수 있고, 따라서 전립선에서 HCMV 가 전립선에서 암의 유도인자로서 작용 할 수 있다는 것을 제시하였다.

핵심되는 말 : Androgen, HCMV, PKA, 전립선 암