The pure anti-androgen bicalutamide inhibits cyclin A expression both in androgen-dependent and -independent cell lines

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Abstract. We investigated the effects of testosterone and the pure anti-androgen, bicalutamide, on DNA synthesis and cell cycle in androgen-sensitive or -insensitive human and mouse cell lines by ^{3}H-thymidine incorporation, flow cytometry, RT-PCR and Western blotting. In androgen-dependent mouse SC-3 cells, testosterone induced DNA synthesis, shift of cell cycle distribution from G0/G1 to S/G2/M and expression of cyclin A. The induction was preceded by that of fibroblast growth factor 8 (FGF-8), and completely blocked by monoclonal antibody to FGF-8. Dihydrotestosterone (DHT) induced cyclin A expression in androgen-sensitive human prostate cancer cells, but not in androgen-independent cell lines. Bicalutamide almost completely inhibited these androgen-dependent effects both in LNCaP and SC-3 cells, but had no or limited effect on androgen-independent or FGF-8-induced DNA synthesis, and FGF-8 induced cyclin A expression. Interestingly, bicalutamide inhibited both DNA synthesis and the cyclin A expression in androgen-independent human cell lines in serum-free condition. A MEK1/2 inhibitor U0126 blocked both androgen- and rFGF-8-induced DNA synthesis. Overall, bicalutamide inhibits the cyclin A expression possibly by inhibiting FGF-8 mRNA expression and FGF-8 protein secretion but not by inhibiting FGF receptor (FGFR) signalling in androgen-dependent cell lines, and by other mechanisms in androgen-independent cell lines. The results suggest that combination with compounds such as FGFR signalling inhibitors may provide additional benefits to anti-androgens. It is also suggested that cyclin A could be a sensitive marker for androgen-induced cancer growth and for the growth inhibitory effects of anti-androgen.

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Introduction

Steroid hormones, such as androgens or estrogens, induce proliferation of various tumor cells. Typical examples are breast, endometrial and prostate cancer. It has been suggested that steroid hormone-dependent cell growth is mediated by growth factors, such as fibroblast growth factor (FGF), epidermal growth factor (EGF) and interleukin-6 (IL-6), induced by steroid hormone-steroid hormone receptor complexes. For example, the crosstalk between androgen receptor (AR) and EGF receptor (EGFR)/human epidermal growth factor receptor type 2 (HER2) signalling pathways has been disrupted with a combination of bicalutamide (casodex), a pure anti-androgen, and an EGFR tyrosine kinase inhibitor gefitinib (1). In addition, ligand-independent transactivation of AR by growth factors has been well-documented and could be one of the mechanisms of androgen-dependent and androgen-resistant cell proliferation (2).

Although there are some reports on molecular mechanism of androgen-regulated tumor cell growth (2,3), it has not been fully investigated. Aberrations in cell cycle progression are thought to be one of the most common features of human malignancies. Cell cycle progression is regulated by cyclin-dependent kinases (CDKs), and their activities are regulated by cyclins and CDK inhibitors (CKIs). It has also been reported that androgen regulates the expression of key cell cycle regulators (4). To investigate the androgen-dependent cell growth including the relationship between androgen and the expression of key cell cycle regulators, we used several androgen-sensitive and -insensitive cell lines; androgen-sensitive human prostate cancer cell line LNCaP (AR-positive), mouse androgen-dependent cell line SC-3 (AR-positive), and androgen-insensitive human prostate cancer cell lines DU145 and PC-3 (AR-negative).

LNCaP has been widely used as a standard cell line to investigate androgen-dependent cell growth. DU145 and PC-3 are well-known as androgen-insensitive cell lines and also used as control cell lines for the investigation of the

androgen-dependent cell growth. Although there is no doubt that LNCaP is androgen-sensitive, it is not completely androgen-dependent (5). If the androgen-dependent effects are investigated in LNCaP cells, this should be taken into account, and additional investigation may be required to claim that the effect is androgen-specific, e.g. by using other androgen-sensitive cell lines, or by testing in serum-free condition.

The androgen-dependent mouse mammary carcinoma, Shionogi carcinoma 115 (SC115), was established by Minesita and Yamaguchi (6). SC-3 is a highly androgen-dependent cell line isolated from SC115 (7), and it has been shown that androgen-dependent growth of SC-3 cells is mediated in an autocrine manner by an androgen-induced growth factor (AIGF), FGF-8 (8). In SC-3 cells, androgen induces FGF-8 mRNA expression. There is clinical evidence suggesting that FGF-8 has an important role in hormone-responsive cancers, such as prostate cancer (9) or breast cancer (10). Therefore, SC-3 cells could provide a clue to elucidate the mechanism of tumor cell growth induced by steroid hormone activity. In addition, it is suitable to investigate the androgen-specific effects with SC-3 cells together with LNCaP cells because of its highly androgen-dependent nature.

Here we report the effects of androgen on the expression of cell cycle regulators and the inhibition of these effects by bicalutamide both in the androgen-dependent and -independent cell lines in serum-free condition.

Materials and methods

Materials. Human prostate cancer cell lines were obtained from Dainippon Pharmaceuticals (for LNCaP.FGC and PC-3, Osaka, Japan) and American Type Culture Collection (for DU145, Manassas, USA). The non-steroidal pure anti-androgen bicalutamide (casodex) was from AstraZeneca Pharmaceuticals (Macclesfield, UK). Anti-cyclin A monoclonal antibody (C-4710), CelLytic-M mammalian cell lysis/ extraction reagent, bovine serum albumin (BSA, fatty acid free), mitogen-activated protein kinase-extracellular signal-regulated kinase kinase (MEK) 1/2 inhibitor U0126, RPMI-1640, trypsin and activated charcoal were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant FGF-8b (rFGF-8) and anti-FGF-8b monocloncal antibody were from R&D Systems (Minneapolis, MN, USA). Anti-GAPDH monoclonal antibody was from Novus Biologicals (Littleton, CO, USA). Envision+ was from Dako Japan (Tokyo, Japan). Dextran T-70 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Eagle's minimum essential medium (MEM) and Ham's F-12 were from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Biosciences. TRIzol reagent was from Gibco BRL (Grand Island, NY, USA). Agarose SFR gel was from Amresco (Solon, OH, USA). BioRad Protein Assay kit was from BioRad (Hercules, CA, USA). NuPAGE Pre-Cast gel system was from Invitrogen (Carlsbad, CA, USA). [methyl-^{3}H]thymidine, Ready-To-Go beads, PCR primers, polyvinylidene difluoride (PVDF) membrane (Hybond-P) and ECL Western blotting system were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK).

Cell culture. SC-3 cells, established from an androgen-dependent mouse mammary tumor SC115, were maintained in MEM containing 2% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) and 10 nM testosterone at 37˚C, and human prostate cancer cell lines were maintained in the RPMI-1640 (LNCaP and DU145) or Ham's F-12 (PC-3) medium supplemented with 10% FBS at 37˚C.

Cell growth experiments. For experiments, firstly maintenance medium was changed to testosterone (T) or dihydrotestosterone (DHT)-free MEM (SC-3), RPMI-1640 (LNCaP and DU145) or Ham's F-12 (PC-3) containing 2% DCC-FBS. Cells were seeded into 96-well plates (0.3-3x10^4 cells/well, for DNA synthesis experiment), 60-mm dishes (5x10^5 cells/dish) or 100-mm dishes (2x10^6 cells/dish, for RNA or cell lysate preparation). The following day (day 0), the medium was replaced with the growth medium, HMB0.1 medium [HAM F-12-MEM (1:1, v/v) containing 0.1% BSA (essential fatty acid-free)] with or without T or DHT (0.1-1000 nM), rFGF-8 (3-100 ng/ml) and various concentrations of inhibitors (bicalutamide and U0126). Androgens and inhibitors were dissolved in ethanol (androgens and bicalutamide) or DMSO (U0126). Final concentration of vehicle was up to 0.4% in total. We confirmed that the vehicles did not affect DNA synthesis of the cell lines tested.

For DNA synthesis experiment in all cell lines, after 3-day culture with the growth medium as described above, cells were pulsed with ^{3}H-thymidine (0.33 μCi/well) for 2 h (5 h for LNCaP cells) at 37˚C and the radioactivity incorporated into the cells were measured. For RNA or cell lysate preparation, cells were harvested on days 1-3 except for time course experiments.

For time course experiments, SC-3 cells were seeded into 100-mm dishes (2x10^6 cells/dish) containing T-free MEM with 2% DCC-FBS. The following day (-48 h), the medium was replaced with HMB0.1 medium without T. After 2 days of testosterone deprivation (time 0), medium was changed again to HMB0.1 medium with or without T (1 nM) and bicalutamide (1 μM). Cells were harvested at indicated hours of incubation.

Flow cytometry analysis. Exponentially growing SC-3 cells were cultured with or without T and bicalutamide as described above. For cell cycle distribution analysis, cells were fixed for >4 h at 4˚C with 70% ethanol and incubated with RNase A (final 100 μg/ml) for 30 min at 37˚C and the DNA intercalating dye, propidium iodide (final 50 μg/ml) for 30 min at room temperature. Flow cytometry analysis was performed using FACSCalibur (Becton-Dickinson Japan, Tokyo) comparing vehicle control, T-treated and T and bicalutamide-treated cells.

RNA isolation. After aspiration of the medium, cells were harvested with a cell scraper into TRIzol reagent and stored at -80˚C until use. RNA isolation was done according to the manufacturer's instruction.

Cell lysate preparation. Cells were washed once with phosphate-buffered saline (PBS) and incubate the cells with CelLytic-M reagent (1 ml/dish) at 4˚C for 15 min. Then cells

Table I. RT-PCR conditions.

Molecules	Primer sequences	PCR condition			No. of cycles	Length of PCR product (bp)
		Denaturation	Annealing	Extension		
Mouse cyclin A	(F) 5'-CACTCACACACTTAGTGTCTCTGGTGGG-3' (R) 5'-GCCGCGATGCCGGGCACCTCGAGGCATTCG-3'	95˚C, 30 sec	56˚C, 1 min	72˚C, 2 min	30	1277
Human cyclin A	(F) 5'-GTCTTCCATGTCAGTGCTGA-3' (R) 5'-CAACTTTGCAGTTTGCAGGC-3'	94˚C, 1 min	55˚C, 1 min	72˚C, 2 min	25	260
Mouse cyclin D1	(F) 5'-CGCCTTCCGTTTCTTACTTCA-3' (R) 5'-AACTTCTCGGCAGTCAGGGGA-3'	94˚C, 1 min	60˚C, 1 min	72˚C, 2 min	30	250
Mouse cyclin E	(F) 5'-CTGGCTGAATGTTTATGTCC-3' (R) 5'-TCTTTGCTTGGGCTTTGTCC-3'	94˚C, 45 sec	58˚C, 45 sec	72˚C, 1.5 min	30	386
Mouse $p21^{Waf1/Cip1}$	(F) 5'-AGCCTGAAGACTGTGATGGG-3' (R) 5'-AAAGTTCCACCGTTCTCGG-3'	94˚C, 45 sec	62˚C, 45 sec	72˚C, 1 min	27	228
Mouse $p27^{Kip1}$	(F) 5'-CCCGCCCGAGGAGGAAGATGTCAAAC-3' (R) 5'-CCCTTTTGTTTTGCGAAGAAGAATCT-3'	94˚C, 1 min	59˚C, 1 min	72˚C, 1 min	30	516
FGF-8	(F) 5'-TTTACACAGCATGTGAGGGAG-3' (R) 5'-GTAGTTGAGGAACTCGAAGCG-3'	95˚C, 1 min	55˚C, 1 min	72˚C, 1 min	30	494
AR	(F) 5'-TCTCAAGAGTTTGGATGGCTCC-3' (R) 5' -TCACTGGGTGTGGAAATAGATG-3'	94˚C, 45 sec	58˚C, 45 sec	72˚C, 1 min	20	390
Mouse GAPDH	(F) 5'-ATCTTCCAGGAGCGAGACCCC-3' (R) 5'-TCCACAATGCCAAAGTTGTCATGG-3'	94˚C, 45 sec	55˚C, 45 sec	72˚C, 1 min	25	289
Human GAPDH	(F) 5'-CCACCCATGGCAAATTCCATGGCA-3' (R) 5'-TCTAGACGGCAGGTCAGGTCCACC-3'	94˚C, 1 min	55˚C, 1 min	72˚C, 1 min	32	598

were removed from plates by cell scraper. The lysed cells were centrifuged for 15 min at 12,000 x g and the supernatant was used for Western blot analysis. Protein concentration was determined using BioRad Protein Assay kit.

RT-PCR. RT-PCR (semi-quantitative) was performed using Ready-To-Go beads. First strand cDNA was synthesized at 42˚C for 30 min using 1 μg of total RNA and $pd(T)_{12-18}$ primer (except for FGF-8 and mouse glyceraldehyde-3-phosphate dehydrogenase, mGAPDH, using specific primers). PCR reaction was performed using sequence specific primers. Sequences of each set of primers and detailed PCR conditions are described in Table I (11-19). Final extension was at 72˚C for 10 min. PCR products were confirmed in 2% agarose SFR gel with ethidium bromide except 1% for mouse cyclin A. A house-keeping gene GAPDH was used to normalize the target gene expression.

Western blot analysis. Cell lysates were fractionated by electrophoresis with NuPAGE 4-12% Bis-Tris gel, and electrotransfered to PVDF membrane (Hybond-P). Human and mouse cyclin A and FGF-8 protein levels were determined with primary antibodies, Envision+ as a secondary antibody and ECL system. GAPDH was used as a loading control.

Data analysis. Results of the effects on androgen-induced DNA synthesis were expressed as the mean ± standard errors of mean (SEM) of four replicates per group. Values of 50% inhibitory concentration (IC_{50}) were calculated using JMP version 6 (SAS Institute Japan, Tokyo). Cell cycle distribution analysis was done with ModFit LT version 3.0.

Results

Growth stimulatory effects of androgen in SC-3 and human prostate cancer cells. The effects of the androgen-induced cell growth were examined as DNA synthesis measured by ^{3}H-thymidine incorporation in SC-3 and human prostate cancer cell lines.

As shown in Fig. 1A, testosterone stimulated the uptake of ^{3}H-thymidine in a concentration-dependent manner in SC-3 cells, as reported previously (5). A non-steroidal pure anti-androgen, bicalutamide caused right hand shift of the growth stimulatory curve of the testosterone in SC-3 cells, suggesting its competitive inhibition on testosterone-induced DNA synthesis.

Likewise, DHT stimulated the DNA synthesis in human prostate cancer LNCaP cells dose-dependently, but not in androgen-independent human prostate cancer DU145 and PC-3 cells, as expected (Fig. 1B). However, the growth of

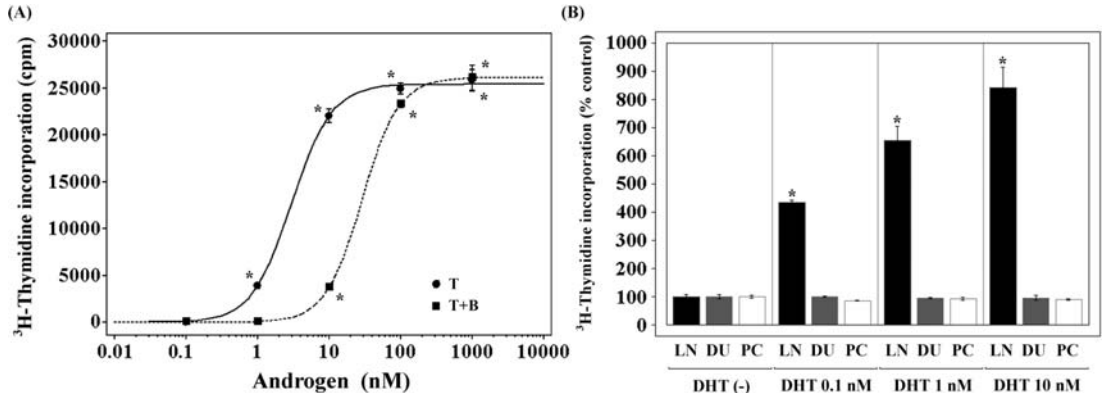

Figure 1. Growth stimulatory effect of androgen in SC-3 and human prostate cancer cells (n=4, mean ± SEM). (A) SC-3 cells were seeded into a 96-well plate ($8x10^3$ cells/well) and medium was changed to HMB0.1 with testosterone alone (0.1-1000 nM), or in combination with bicalutamide (1 μM) the following day (day 0). Cells were pulsed with ^{3}H-thymidine on day 3 for 2 h at 37˚C and the radioactivity incorporated into the cells was measured. (B) Human prostate cancer cells were seeded into a 96-well plate ($0.3-3x10^4$ cells/well) and medium was changed to HMB0.1 with dihydrotestosterone (0.1-10 nM) the following day (day 0). Cells were pulsed with ^{3}H-thymidine on day 3 for 2 h (5 h for LNCaP cells) at 37˚C and the radioactivity incorporated into the cells was measured. T, testosterone; DHT, dihydrotestosterone; BIC, bicalutamide; LN, LNCaP; DU, DU145; PC, PC-3.

LNCaP cells were not fully androgen-dependent both in serum-supplemented and serum-free conditions based on the fact that ^{3}H-thymidine incorporation without DHT were ~10-15% of those at physiological concentrations of DHT, i.e. 1 and 10 nM.

Growth inhibitory effects of bicalutamide in SC-3 and human prostate cancer cells. Growth inhibitory effects of bicalutamide on the androgen-induced and androgen-independent cell growth were examined as DNA synthesis measured by ^{3}H-thymidine incorporation.

Bicalutamide inhibited androgen-induced DNA synthesis in a concentration-dependent manner in SC-3 cells (Fig. 2A). IC_{50} values of bicalutamide on the DNA synthesis induced by testosterone 1 nM, DHT 1 or 10 nM were 82.2 (69.1-96.1) nM, 5.06 (3.62-7.43) and 20.9 (18.8-23.3) μM (95% confidence limits), respectively. No growth stimulatory effect was observed with bicalutamide alone up to 30 μM (data not shown). The concentration ratio between testosterone or DHT and bicalutamide required for full inhibition was needed to be 1:1,000 (Fig. 2A) and 1:10,000-30,000 (Fig. 2A), respectively. The results were consistent with those reported by Darbre *et al* with S115 cells (20).

Bicalutamide also showed dose-dependent inhibition on the DHT-induced growth of LNCaP cells, but the effects were limited to androgen-dependent component, i.e., no effect on the growth without DHT up to 30 μM (Fig. 2B). IC_{50} values of bicalutamide at DHT 1 and 10 nM were 124 (47-302) and 660 (352-1,197) nM, respectively. Interestingly, bicalutamide inhibited the DNA synthesis in androgen-independent prostate cancer cells, PC-3 and DU145 at 30 μM (Fig. 2C and D) in androgen-independent manner.

In the time course experiments using SC-3 cells, statistically significant effects were observed on day 2 and afterwards (data not shown). It is anticipated that the early changes in cell cycle distribution and mRNA expression of cell cycle regulator genes would be observed within three days of culture.

For further investigation, testosterone and dihydrotestosterone were selected for SC-3 and LNCaP cells to investigate androgen-dependent effects, respectively, based on the findings above and the fact that these androgens have been widely used for investigations in each cell line; testosterone (1 nM) was selected for SC-3 cells to investigate very early, sensitive events induced by testosterone, whereas physiological concentration of dihydrotestosterone (10 nM) was selected for human prostate cancer cells to investigate the clinical relevancy of the effects.

Effect of testosterone and bicalutamide on cell cycle distribution. The effects on testosterone-induced changes in cell cycle distribution in SC-3 cells was analysed using flow cytometry. Results are shown in Fig. 3A. Testosterone (1 nM) induced shift of cell cycle distribution from G0/G1 to S/G2/M. This shift of the cell cycle distribution was almost completely blocked by bicalutamide at 1 μM, whereas bicalutamide itself did not induce a change in cell cycle distribution.

Androgen-induced changes in cell cycle regulator mRNA levels and the inhibitory effects of bicalutamide. The effects on androgen-induced changes in cell cycle regulator mRNA levels were examined by RT-PCR. The effects of bicalutamide on these changes were also examined.

A typical example of the results is shown in Fig. 3B for SC-3 cells. As previously reported (8), FGF-8 (AIGF) mRNA was upregulated by testosterone in SC-3 cells. In the cell cycle regulators examined, mRNA levels of cyclin A were markedly upregulated by testosterone. The effect was almost completely inhibited by bicalutamide. Although there were tendencies of increase in transcription of cyclin D_1 and E by testosterone, no clear change was observed in the cell cycle regulators examined other than cyclin A. There was no consistent change in $p16^{INK4a}$ expression (data not shown). Bicalutamide, at 1 μM, almost completely inhibited testosterone-induced upregulation of FGF-8 and cyclin A mRNA.

As the androgen-induced effects on mRNA expression of cell cycle regulators were limited to that of cyclin A, and the effects were possibly related to FGF-8, the effects were also investigated in human prostate cancer cell lines. As shown in Fig. 3C, 10 nM of DHT induced the mRNA expression of

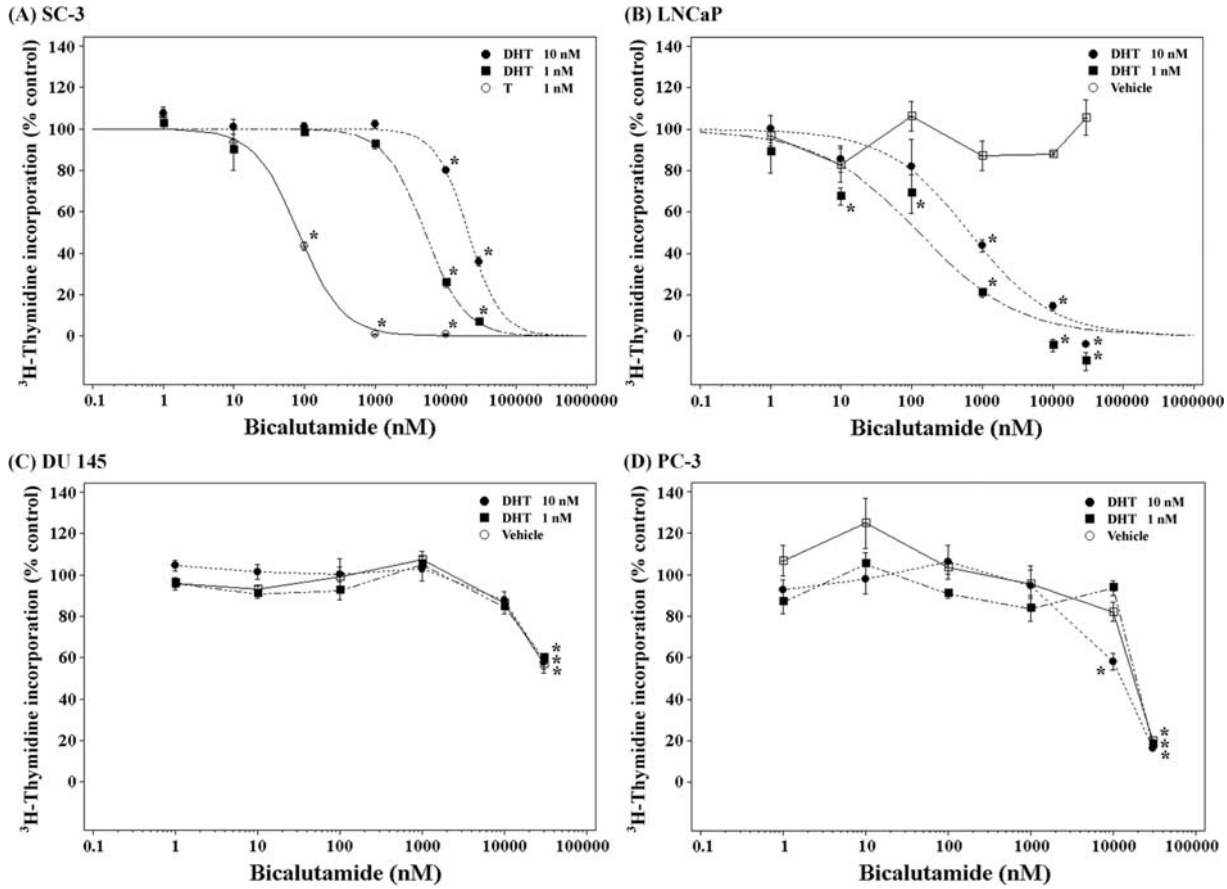

Figure 2. Concentration-dependent growth inhibitory effects of bicalutamide on androgen induced growth (n=4, mean ± SEM). (A) SC-3 cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with testosterone (1 nM) or dihydrotestosterone (1 or 10 nM) and various concentrations of bicalutamide (1 nM-30 μM). (B)-(D) Human prostate cancer cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with DHT (0, 1 or 10 nM) and various concentrations of bicalutamide (1 nM-30 μM). $^*p<0.05$ (comparison with vehicle control, Dunnett's t-test).

cyclin A in LNCaP cells, but the induction of FGF-8 mRNA, which is induced by testosterone in SC-3, was relatively low. Bicalutamide inhibited the cyclin A mRNA expression at 30 μM, but not for FGF-8. Interestingly, bicalutamide also inhibited the cyclin A mRNA expression in PC-3, but not in DU145 cells, at 30 μM. DHT had no effect on the inhibition by bicalutamide in PC-3 cells.

To investigate the time course of cyclin A mRNA induction, RT-PCR analysis was conducted with total RNA samples from the SC-3 cells at various time points from 4 to 24 h after the start of testosterone stimulation. In this experiment, DCC-FBS and testosterone were deprived for 48 h prior to testosterone stimulation to minimize possible effects of DCC-FBS and carry over effects of testosterone. As shown in Fig. 3D, serum deprivation for 48 h causes almost complete reduction in cyclin A and FGF-8 mRNA levels (-48h vs 0), indicating that effects of DCC-FBS and carry over effects of testosterone were negligible. Cyclin A mRNA induction was only seen after 24-h stimulation with testosterone. On the other hand, FGF-8 mRNA induction was observed at 8 h and afterwards. Bicalutamide almost completely inhibited expression of these mRNAs by testosterone.

Western blot analysis of androgen-induced changes in cyclin A. As cyclin A mRNA level was upregulated by testosterone, Western blot analysis was performed using anti-cyclin A monoclonal antibody to investigate whether or not cyclin A protein levels are affected by androgen and bicalutamide. It has been reported that approximately half of the growth promoting activity in the total extract of SC-3 cells was found to be extracellular matrix associated when stimulated with testosterone (21). Cyclin A protein expression was induced by 1 nM of testosterone in SC-3 cells as observed in mRNA level. Bicalutamide (1 μM) alone did not induce cyclin A protein expression, and completely inhibited the cyclin A protein expression induced by testosterone. Changes in FGF-8 protein levels in the cell lysates were not detected by testosterone or bicalutamide alone, but a slight increase was observed in the combination (Fig. 4A).

To confirm the effects in SC-3 cells, Western blot analysis was also conducted with the cell lysate from human prostate cancer cells (Fig. 4B). Cyclin A protein expression was downregulated by the replacement of the maintenance medium with serum-free medium in LNCaP cells, but upregulated in DU145 cells. In PC-3 cells, the replacement with serum-free medium did not affect cyclin A protein expression. In serum-free medium, DHT upregulated the cyclin A protein expression in LNCaP cells compared to the vehicle control, but had no effect in DU145 and PC-3 cells. Bicalutamide inhibited DHT-induced cyclin A protein expression in LNCaP cells,

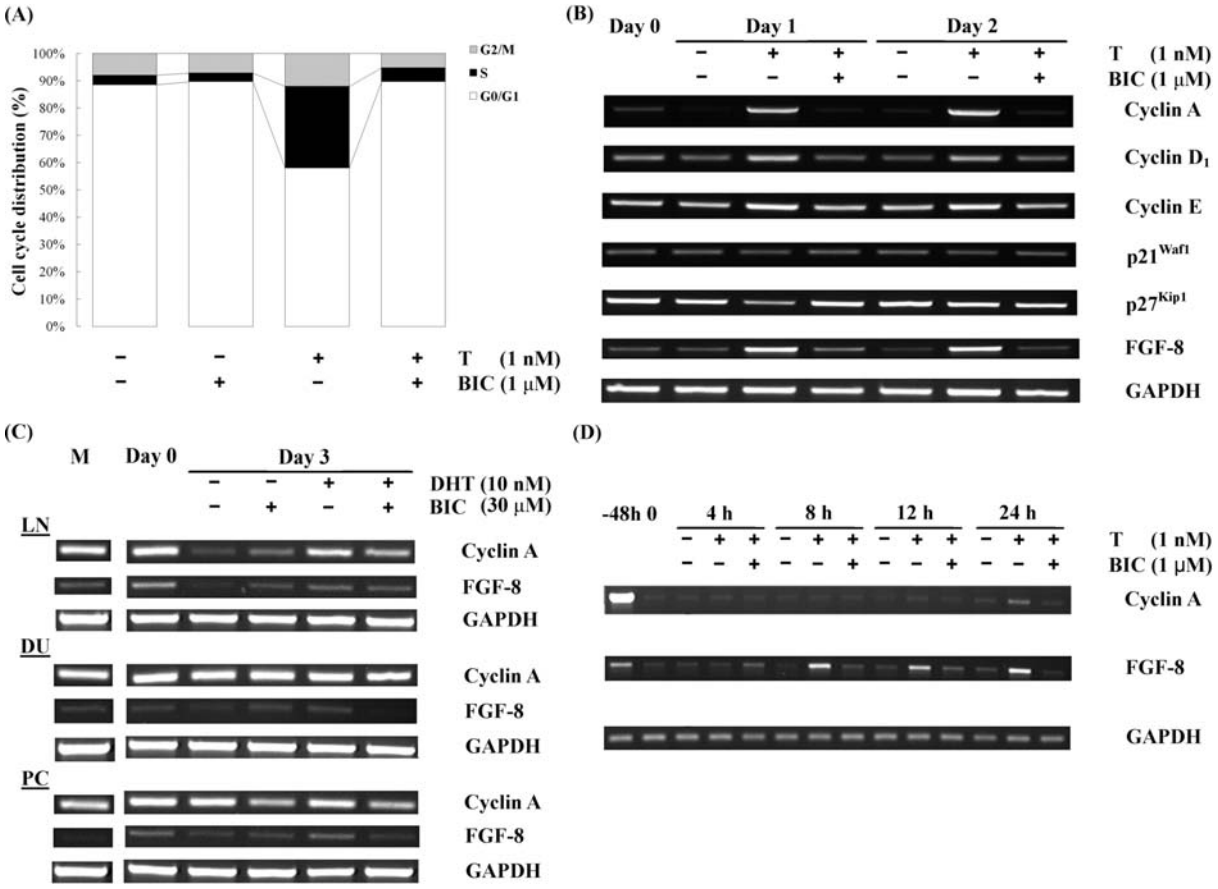

Figure 3. (A). Effect of testosterone and bicalutamide on cell cycle distribution. SC-3 cells were seeded into 100-mm dishes ($2x10^6$ cells/dish) and medium was changed to HMB0.1 with or without testosterone (1 nM) and bicalutamide (1 μM) the following day (day 0), and harvested on day 2. Cells were fixed for >4 h at 4˚C with 70% ethanol and incubated with RNase A (final 100 μg/ml) for 30 min at 37˚C and the DNA intercalating dye, propidium iodide (final 50 μg/ml) for 30 min at room temperature just before analysis by flow cytometry. T, testosterone; BIC, bicalutamide. Effects of androgen and bicalutamide on mRNA expression of cell cycle regulators and FGF-8 in SC-3 (B) and human PC (C) cells. (B) SC-3 cells were seeded into 100-mm dishes ($2x10^6$ cells/dish) and medium was changed to HMB0.1 with or without testosterone (1 nM) and bicalutamide (1 μM) the following day (day 0), and harvested on days 1 or 2. Total RNA isolation and RT-PCR was done as described in Materials and methods. (C) Cells were seeded into 60-mm dishes ($5x10^5$ cells/dish) and medium was changed to HMB0.1 with or without dihydrotestosterone (10 nM) and bicalutamide (30 μM) the following day (day 0), and harvested on day 3. Total RNA isolation and RT-PCR was done as described in Materials and methods. T, testosterone; DHT, dihydrotestosterone; BIC, bicalutamide; LN, LNCaP; DU, DU145; PC, PC-3. (D) Time course of testosterone induced mRNA expression of cyclin A and FGF-8. SC-3 cells were seeded into 100-mm dishes ($2x10^6$ cells/dish) containing T-free MEM with 2% DCC-treated FBS. The following day (-48 h), the medium was replaced with HMB0.1 medium without T. After two days of testosterone deprivation, medium was changed again to HMB0.1 medium with or without T (1 nM) and bicalutamide (1 μM) (time 0). Cells were harvested at indicated hours of incubation. Total RNA isolation and RT-PCR was done as described in Materials and methods. T, testosterone; BIC, bicalutamide.

and also inhibited cyclin A protein expression regardless of the presence or absence of DHT both in DU145 and PC-3 cells. FGF-8 protein levels were variable but no significant effects of DHT and/or bicalutamide were observed in human prostate cancer cells. Bicalutamide had no effect on the FGF-8 protein expression in the cell lines tested.

Effects of anti-FGF-8 antibody and U0126 on the androgen-induced DNA synthesis and cyclin A mRNA expression. Since FGF-8 has shown to be critical for androgen-induced growth of SC-3 cells (22), anti-FGF8 monoclonal antibody and a MEK1/2 inhibitor, known to inhibit FGFR signalling, U0126 were used to investigate whether or not FGF-8 has an intermediate role in the growth and the induction of cyclin A mRNA expression by androgen. As shown in Fig. 5A, anti-FGF-8 antibody partially inhibited testosterone-induced (1 nM) DNA synthesis. U0126 showed concentration-dependent inhibition on androgen-induced growth in SC-3 and LNCaP cells (Fig. 5B). Furthermore, anti-FGF8 antibody inhibited cyclin A mRNA expression induced by testosterone (1 nM) in SC-3 cells (Fig. 5C), suggesting that FGF-8 may be involved in cyclin A expression by androgen.

Effect of bicalutamide and U0126 on the FGF-8-induced DNA synthesis and cyclin A expression. The effect of bicalutamide on FGFR signalling was investigated indirectly by ^{3}H-thymidine incorporation induced by recombinant FGF-8 (rFGF-8). rFGF-8 induced DNA synthesis both in LNCaP cells (Fig. 6A) and SC-3 cells (Fig. 6D). Bicalutamide partially inhibited the FGF-8-induced DNA synthesis in LNCaP cells (Fig. 6B), but not in SC-3 cells (Fig. 6C). U0126 showed concentration-dependent inhibition on rFGF-8-induced DNA synthesis in SC-3 cells with IC_{50} value of 1.18 (0.86-1.60) μM (95% confidence limits) (Fig. 6C). When DNA synthesis was co-stimulated with testosterone and rFGF-8 in SC-3 cells, bicalutamide inhibited DNA synthesis only partially, almost

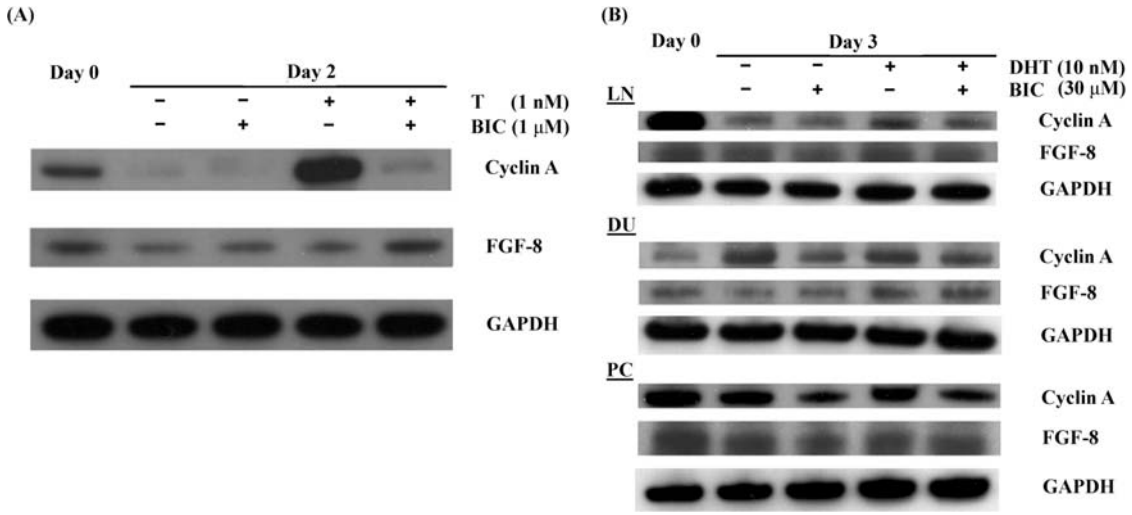

Figure 4. Androgen-induced cyclin A protein expression and inhibitory effect of bicalutamide. (A) SC-3 cells were seeded into 60-mm dishes ($5x10^5$ cells/dish) and medium was changed to HMB0.1 with or without testosterone (1 nM) and bicalutamide (1 μM) the following day (day 0), and harvested on day 2. Cell lysate preparation and Western blotting analysis was done as described in Materials and methods. (B) Human prostate cancer cells were seeded into 60-mm dishes ($5x10^5$ cells/dish) and medium was changed to HMB0.1 with or without dhydrotestosterone (10 nM) and bicalutamide (30 μM) the following day (day 0), and harvested on day 3. Cell lysate preparation and Western blotting analysis was performed as described in Materials and methods. T, testosterone; DHT, dihydrotestosterone; BIC, bicalutamide; LN, LNCaP; DU, DU145; PC, PC-3.

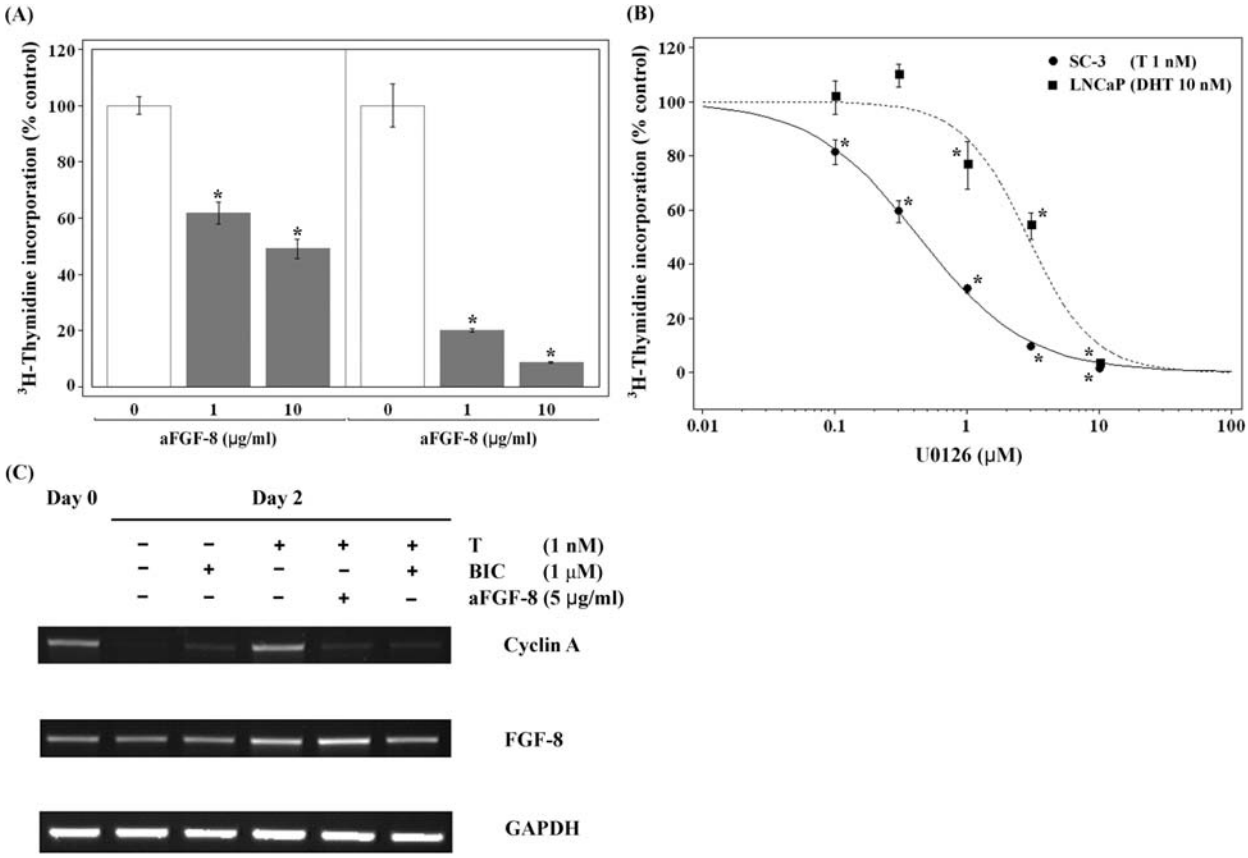

Figure 5. (A) Effect of anti-FGF8 monoclonal antibody on the testosterone-induced growth. Cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with testosterone (1 nM) or rFGF-8 (40 ng/ml) and anti-FGF8 antibody at indicated concentrations. $^*p<0.05$ (comparison with vehicle control, Dunnett's t-test). (B) Concentration-dependent growth inhibitory effects of U0126 on androgen-induced growth (n=4, mean ± SEM). Cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with testosterone (1 nM) for SC-3 cells or DHT (10 nM) for LNCaP cells, respectively, and various concentrations of U0126 (0.1-10 μM). (C) Effect of anti-FGF8 monoclonal antibody on the testosterone-induced mRNA expression of cyclin A. SC-3 cells were seeded into 60-mm dishes ($5x10^5$ cells/dish) and medium was changed to HMB0.1 with or without testosterone (1 nM), bicalutamide (1 μM) and anti-FGF8 antibody (5 μg/ml) the following day (day 0), and harvested on day 2. Total RNA isolation and RT-PCR was done as described in Materials and methods. T, testosterone; BIC, bicalutamide; aFGF-8, anti-FGF-8 monoclonal antibody.

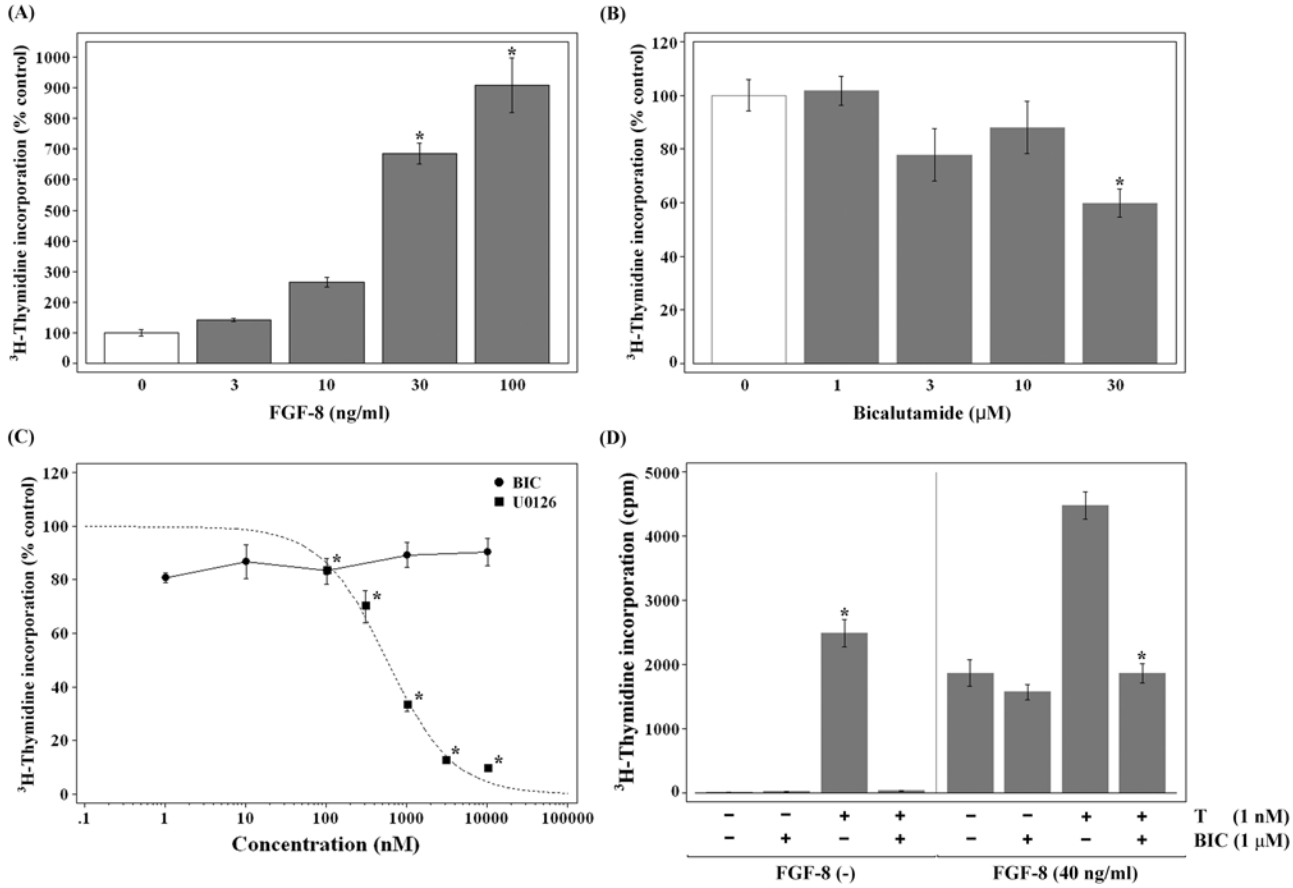

Figure 6. Growth inhibitory effects of bicalutamide and U0126 on androgen and/or rFGF-8 induced growth (mean ± SEM, n=4). (A) LNCaP cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with various concentrations of rFGF-8 (0-100 ng/ml). $^*p<0.05$ (comparison with vehicle control, Dunnett's t-test). (B) LNCaP cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with rFGF-8 (30 ng/ml) and various concentrations of bicalutamide (0-30 μM). $^*p<0.05$ (comparison with vehicle control, Dunnett's t-test). (C) SC-3 cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with rFGF-8 (40 ng/ml) and various concentrations of bicalutamide (1 nM-10 μM) or U0126 (0.1-10 μM). $^*p<0.05$ (comparison with vehicle control, Dunnett's t-test). (D) SC-3 cells were seeded into a 96-well plate and pulsed with ^{3}H-thymidine as described in Fig. 1, except for medium (HMB0.1) with or without testosterone (1 nM), rFGF-8 (40 ng/ml) and bicalutamide (1 μM). T, testosterone; BIC, bicalutamide. $^*p<0.05$ (comparison with vehicle control, Dunnett's t-test), $^\#p<0.05$ (comparison with rFGF-8 alone, Dunnett's t-test).

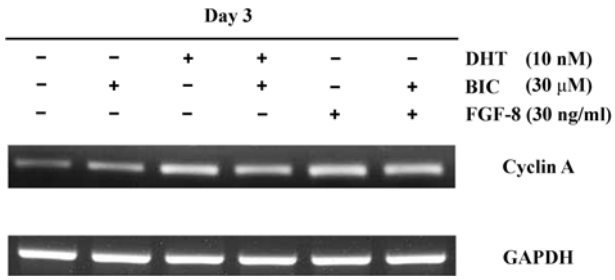

Figure 7. FGF-8-induced cyclin A mRNA expression and inhibitory effect of bicalutamide in LNCaP cells. Cells were seeded into 60-mm dishes ($5x10^5$ cells/dish) and medium was changed to HMB0.1 with or without dihydrotestosterone (10 nM), rFGF-8 (30 ng/ml) and bicalutamide (30 μM) on day 3. Total RNA isolation and RT-PCR was done as described in Materials and methods. DHT, dihydrotestosterone; BIC, bicalutamide.

identically to the effects derived from testosterone (Fig. 6D). rFGF-8 induced cyclin A mRNA expression in LNCaP cells, but the effect of bicalutamide on the cyclin A expression induced by rFGF-8 was limited compared to the one induced by DHT (Fig. 7).

Discussion

Cyclin A is a key cell cycle regulator in the S phase of the cell cycle through its interaction with CDK1 and 2, key kinases in both S phase and mitosis (23). Many investigations on the link between cancer and aberration in cell cycle regulation have been done, suggesting the importance of cyclin A over-expression in hormone responsive cancers, such as breast cancer and prostate cancer (23-25). The results of the present study were consistent with these clinical findings. In addition, the results also suggest that cyclin A may be one of the key molecules in androgen-induced tumor cell growth since the growth of SC-3 cells are highly-dependent on androgen (7,8). The induction by androgen was also observed in human androgen-sensitive LNCaP cells at serum-free condition, but not in androgen-insensitive human prostate cancer cell lines, suggesting that the induction was purely an androgen-dependent effect.

Menjo *et al* reported that a CKI, $p27^{kip1}$ plays a critical role in cell cycle arrest after androgen depletion in SC-3 cells, and that no apparent changes were seen in cyclin A protein levels (26). The difference between the results of Menjo *et al* and

ours may be due to the difference in the methodology used. Menjo *et al* focused on the effects of androgen depletion from maintenance medium containing 100 nM of testosterone. If the SC-3 cells were maintained with high concentration of testosterone, they might be less sensitive to testosterone; in fact significant growth stimulation was seen only after 3 days of culture in their study whereas significant stimulation of DNA synthesis was seen after 2-day culture with physiological concentration, i.e., 1-10 nM testosterone in ours. Therefore, the effects of testosterone on cyclin A expression could be masked in their study.

As previously mentioned, FGF-8 is critical for androgen-dependent growth of SC-3 cells. In this experiment, we have shown that FGF-8 gene expression was reduced by deprivation of DCC-FBS and testosterone and that it was induced by only 8 h of testosterone stimulation. Thus, FGF-8 gene expression was highly dependent on testosterone and seen at a very early stage of the induction of the growth. However, a stable transfectant of SC-3 cells expressing FGF-8 gene did not grow in serum-free medium unless stimulated with androgen (27), suggesting that other molecules may also be essential for androgen-dependent growth. Although the detailed mechanism of cyclin A induction remains to be elucidated, inhibitory effects of a pure anti-androgen, bicalutamide, on cyclin A and FGF-8 gene expression, and the fact that induction of cyclin A mRNA was preceded by that of FGF-8 suggest that testosterone induces cyclin A expression via FGF-8 in SC-3 cells. This hypothesis is supported by the fact that a MEK1/2 inhibitor U0126 completely inhibited DNA synthesis induced by androgen (Fig. 5) and that rFGF-8 induced cyclin mRNA expression (Fig. 7). Induction of cyclin A has been reported in other FGF-FGFR systems (28,29), but not for FGF-8. Anti-FGF-8 monoclonal antibody inhibited the cyclin A mRNA expression but did not completely inhibit the testosterone (1 nM)-induced DNA synthesis in SC-3 cells at the concentration where cyclin A mRNA expression was almost completely inhibited (Fig. 5) (30). Although FGF-8 is the most important and critical growth factor of androgen-induced growth of SC-3 cells (21), it could not substitute androgen by itself to maintain the cell line, suggesting that another factor(s) is also involved in testosterone-induced effects in SC-3 cells (27).

Interestingly, bicalutamide partially inhibited both DNA synthesis and cyclin A expression in androgen-insensitive human prostate cancer cell lines. These findings suggest bicalutamide may have another growth inhibitory mechanism of action on the human prostate cancer cells because the effects were seen at 30 μM, which is comparable to the plasma concentration in man after chronic oral administration of bicalutamide (31), but it needs to be investigated further, e.g investigation using the AR-positive but androgen-insensitive cell lines.

It is well known that extracellular matrix proteins are essential for the interaction between FGF and FGF receptors (32). We investigated the FGF-8 protein expression in human prostate cancer cells and SC-3 cells by Western blotting. In human prostate cancer cell lines, there was no obvious difference in FGF-8 protein level between the cell lysates derived from androgen-treated and controls both in androgen-sensitive and -insensitive cell lines by Western blotting. However, FGF-8 protein slightly increased in SC-3 cells treated with testosterone and bicalutamide compared to the vehicle control. The mechanism of this increase is yet to be investigated. Given that Western blotting has been performed with whole cell lysates, one hypothesis would be that bicalutamide might block FGF-8 protein secretion and/or folding of the protein by extracellular matrix protein such as heparin sulphate.

Bicalutamide showed inhibitory effects on testosterone-induced FGF-8 mRNA expression, but had not effect on FGF-8-induced DNA synthesis in SC-3 cells. In the human prostate cancer cell line LNCaP, bicalutamide showed concentration-dependent inhibition on the DHT-induced DNA synthesis, but had no or limited effect on androgen-independent or FGF-8-induced DNA synthesis, and FGF-8-induced cyclin A expression. Although crosstalk between androgen receptor and growth factor receptor signalling pathways has already been reported widely, there may be no or little interaction with FGFR. Androgen may affect not only the transcription of the FGF-8 gene but also the FGF-8 protein secretion and/or folding of the protein by extracellular matrix protein such as heparin sulphate. Furthermore, the MEK1/2 inhibitor U0126, known to inhibit FGFR signalling, did inhibit androgen-induced DNA synthesis both in SC-3 and LNCaP cells in our experiment and it has been reported that stable transfection of the dominant negative FGFRs inhibited colony formation of LNCaP cells (33). These findings suggest that androgen-sensitive cells are highly dependent on FGFR signal transduction. Taken together, it is considered that by inducing both the mRNA expression of FGF-8 gene and FGF-8 protein secretion, androgen can trigger DNA synthesis and cyclin A expression in SC-3 cells, which may also contribute to the growth-promoting effects in human prostate cancer cells. Complete inhibition of bicalutamide on the androgen-induced DNA synthesis may also be achieved via its effects on both pathways.

Overall, bicalutamide inhibited androgen-induced cyclin A mRNA and protein expression in androgen-sensitive cells by inhibiting the FGF-8 mRNA expression and possibly by FGF-8 protein secretion. Effects of bicalutamide on FGF-8 signalling is limited, so an inhibitor of FGFR signalling may provide additional effects to anti-androgens with regards to androgen-induced cell growth. Bicalutamide also inhibited cyclin A mRNA and protein expression in androgen-independent cells, suggesting that other mechanisms may also be involved in the inhibitory effects of bicalutamide. It is also suggested that cyclin A could be a sensitive marker for androgen-induced cancer growth and for the growth inhibitory effect of anti-androgen. The significance of our findings should be confirmed in further experiments, e.g. the effects of androgens and anti-androgens on the cell-associated FGF-8 levels in relation to the cell cycle, or experiment with tumor tissues derived from patients with human prostate cancer.

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