Growth Modulation of Diabetic Factors and Antidiabetic Drugs on Prostate Cancer Cell Lines

Shiaw-Wen Chien¹, Dong-Yih Kuo², Jiuan-Miaw Liao², Paulus S. Wang³, ⁴, ⁵, ⁶, ⁷, and Ching-Han Yu², ⁸, *

¹Department of Kidney, Tungs’ Taichung Metroharbor Hospital, Taichung 43503
²Department of Physiology, School of Medicine, Chung Shan Medical University, Taichung 40201
³Graduate Institute of Basic Medical Science, College of Medicine, China Medical University, Taichung 40402
⁴Medical Center of Aging Research, China Medical University Hospital, Taichung 40447
⁵Department of Physiology, School of Medicine, National Yang-Ming University, Taipei 11221
⁶Department of Biotechnology, College of Health Science, Asia University, Taichung 41354
⁷Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 11217
and
⁸Department of Medical Research, Chung Shan Medical University Hospital, Taichung 40201
Taiwan, Republic of China

Abstract

Risk factors for prostate cancer (PCa) include age, hormones, race, family history and diet. Recently, epidemiologic evidence has indicated that history of diabetes mellitus (DM) is inversely associated with risk of PCa. However, epidemiological investigations have yielded inconsistent results. Hence, the exact mechanism of DM-induced reduction in the incidence of PCa has yet to be fully elucidated. The aim of this study was to investigate the effects of DM factors, including glucose, insulin and insulin-like growth factor-1 (IGF-1), on the proliferation of PCa cell lines in vitro. Cell proliferation and expression of hormone receptors was examined in MTT assay and Western blot analysis, respectively. The results showed that DM factors did not affect the viability of androgen receptor (AR)-expressing PCa cell lines. However, cell proliferation increased after treatment with DM factors in androgen-independent PCa cell lines. On PCa tissue arrays, intensities of total AR and nuclear IGF-1R were higher in malignant tissues than in normal prostate glands. In terms of hormonal receptors, androgen-dependent LNCaP cells treated with insulin and IGF-1 in a low-serum medium showed decreased expression of insulin receptor beta (IRβ) and elevated expression of IGF-1 receptor beta (IGF-1Rβ). Moreover, expression of AR was upregulated after insulin and IGF-1 treatment in LNCaP cells, but not in the other PCa cell lines. Most of the studied antidiabetic drugs promoted the viability of PCa cells. However, metformin decreased the viability of AR-expressing PCa cells. These results suggest that diabetic factors modify the expression of AR, IR and IGF-1R to increase cancer cell proliferation. Moreover, the growth suppressing effects of metformin on PCa may be via the regulation of the AR signaling pathway.

Key Words: antidiabetic drugs, diabetes mellitus, IGF-1, insulin, prostate cancer

Introduction

Incidences of prostate cancer (PCa) have been high in recent decades, and it is the second-leading cause of cancer death among men in the US (11). A total of 220,800 new cases of PCa and 27,540 deaths...
due to PCa are estimated in the US in 2015 (28). According to statistical data from the Ministry of Health and Welfare, Taiwan, PCa mortality has increased from 7.1/100,000 persons to 10.4/100,000 persons in the past decade. PCa is currently the fifth-leading cause of cancer death, up from the seventh-leading cause, in Taiwan. Prostate-specific antigen (PSA) assay has allowed early detection of PCa, which is curable by surgical or radiation therapies. Androgen ablation is the major treatment for metastatic PCa. However, 20% to 30% of patients with PCa endure recurrent disease (8, 12). Therefore, chemoprevention and chemical control of PCa have become major concerns.

Both PCa and diabetes mellitus (DM) are common in elderly men. Previous epidemiologic studies have found that patients with diabetes mellitus type 2 (DM2) have lower risk of PCa (10% to 40% lower than subjects without DM) (2, 16). This finding is consistent across various ethnic populations in the US (25), such as European-American, African-American, Native Hawaiian, and Japanese-American (41), as well as a Taiwanese population (30, 33). There are several possible theories for this phenomenon: [1] The plasma hormone levels of testosterone, insulin and insulin-like growth factor-1 (IGF-1) are lower in patients with diabetes mellitus type 1 (DM1) and uncontrolled DM2. Lower hormone levels may have prohibited PCa cell growth (16). [2] The level of PSA, a marker of androgenic signaling, is lower in DM patients than in subjects without diabetes (22). [3] Antidiabetic drugs have an inhibitory effect on PCa (7). [4] Different stages of DM have different effects on PCa. For example, newly diagnosed DM patients have higher incidence of PCa. However, as DM progresses, PCa risk decreases (25). [5] Patients with DM may seek medical help and, in the process, PCa may be diagnosed (17). [6] In comparison with the lowest quartile of DM2 risk allele count, the highest quartile is negatively correlated with PCa risk (24).

In contrast, a meta-analysis of Asian populations has indicated that diabetes is positively correlated with the risk of PCa (20). A series of research articles by Tseng et al. has suggested that the prevalence (15), incidence (33) and mortality (37) of PCa increase in DM patients. Therefore, population differences might affect the relationship between DM and risk of PCa.

Clinical information provides too many variables for determination of the exact mechanism and relationship between PCa progression and DM in DM patients. In the present study, diabetic factors and antidiabetic drugs were analyzed to determine their associations with possible expression viability of and hormone receptors in PCa cell lines. It is anticipated that the results of this study will provide valuable information for clinical treatment and management of PCa.

Materials and Methods

Cells and Culture Conditions

Androgen receptor (AR)-expressing prostate cancer cell lines, LNCaP and 22RV1, and non-AR-expressing prostate cancer cell lines, PC3 and DU145, were purchased from the Culture Collection and Research Center (CCRC) of the Food Industry Research and Development Institute (FIRDI), Taiwan, R.O.C. Cell lines were maintained in RPMI 1640 (Gibco Laboratories, Buffalo, NY, USA) (LNCaP and 22RV1), in minimum essential medium (Gibco) (DU145) or Kaighn’s modification of F-12K Ham medium (Sigma, St. Louis, MO, USA) (PC3), with 50 IU/ml potassium penicillin G (Sigma), 50 IU/ml streptomycin sulfate (Sigma) and 10% fetal bovine serum (Sigma) in an atmosphere of 5% CO2 at 37°C.

Cell Viability Assessment

The modified colorimetric 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay was employed to quantify cell viability. Briefly, cells were incubated on 96-well microplates (Falcon, Franklin Lakes, NJ, USA) for 24 h at a density of 2,000 cells/well during the pre-incubation process. The culture medium was then removed and replaced with medium containing 1% fetal calf serum (FCS) alone, or with various concentrations of insulin (10, 50 and 100 nM) (Sigma), IGF-1 (10, 50 and 100 ng/ml) (Sigma) or glucose (1, 5 and 10 mM) (Sigma). To determine the anti-proliferative effects of antidiabetic drugs on the PCa cell lines, culture media were replaced with glibenclamide (Sigma), acarbose (Sigma), pioglitazone (Sigma), or repaglinide (Sigma) at a concentration of 0.01 to 10 μM. Metformin (Sigma) was used at a concentration of 1 to 10 mM. Following treatment for 48 h, culture media were removed and replaced with 50 μl 1 mg/ml MTT solution in serum-free medium. After an additional 4-h incubation period, MTT solution was replaced with 50 μl DMSO, and the plates were shaken for 3 min. The optical density of each well was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm with a reference wavelength of 630 nm. Each experimental condition was repeated 3 times.

Immunoblotting Assessment

After culture in the presence of insulin, IGF-1 and glucose for 24 h in 1% FCS medium, cells were lyzed in RIPA buffer (50 mM Triz-Cl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin,
1 mM Na-orthovanadate, 1 mM NaF) on ice for 30 min. Cell lysate was centrifuged at 10,000 × g at 4°C for 15 min, and the supernatant was collected. Equal amounts of cell extract proteins (50 µg) were subjected to 12% SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Waltham, MA, USA). Membranes were incubated in blocking solution (5% dry milk in TBST containing 20 mM Tris-HCl, 135 mM NaCl, 0.1% Tween 20, pH 7.6), followed by incubation with a primary antibody overnight. The following primary antibodies were used at a concentration of 1 µg/ml: phosphor-Ser-81-androgen receptor (AR PSer81) (Millipore, Billerica, MA, USA), insulin receptor β (IRβ) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), insulin-like growth factor-1 receptor β (IGF-1Rβ) (Santa Cruz) and β-actin (Sigma). After washing three times with TBST, the blot was incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:5,000) (Santa Cruz), and proteins were visualized using enhanced chemiluminescence detection (ECL) (Perkin Elmer). Protein expression was detected by chemiluminescence/fluorescence imaging analyzer (Fujifilm, Tokyo, Japan). Each experimental condition was repeated three times.

**Immunohistochemical Detection**

Human prostate tissue arrays (Biomax, Rockville, MD, USA) were purchased to detect protein expression in normal and cancerous tissues. Paraffin-embedded tissue arrays were deparaffinized and hydrated to visualize expression of AR, IR and IGF-1R. Antigen retrieval was done by incubating the sections in boiling 10 mM citrate buffer, pH 6.0, for 20 min. Endogenous peroxidase activity was inhibited by 3% (v/v) H2O2 in methanol. Sections were then incubated with rabbit polyclonal anti-AR, anti-IR or anti-IGF-1R antibodies (Santa Cruz) at 1:50 dilutions. Finally, the sections were stained with ultravision quanto detection system (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer’s instructions. Graded alcohol and xylene were applied to the sections, which were then coverslipped with a mounting medium. Sections were observed at 40× or 100× magnification.

**Statistical Analyses**

All values are presented as the mean ± standard error of the mean (SEM). Means were tested for homogeneity by one-way analysis of variance (ANOVA), and the difference between specific means was tested for significance by Duncan’s multiple-range test. The difference between two means was considered statistically significant when \( P < 0.05 \).

**Results**

![Fig. 1. Effects of insulin, IGF-1 and glucose on the viabilities of PCa cell lines. Cells were cultured in 1% FCS medium (1%S) and then challenged with insulin, glucose or IGF-1 for 48 h. The levels of insulin (Ins), IGF-1 and glucose (Glc) were 10, 50, 100 nM; 10, 50, 100 ng/ml and 1, 5, 10 mM, respectively. Cell viability was measured in MTT assays. Each value represents mean ± SEM. Control value (1%S group) = 100%; *P < 0.05 versus 1%S group.](image-url)
the viabilities of both AR-expressing and non-AR-expressing PCa cell lines were examined after treatment with insulin, IGF-1 and glucose (Fig. 1). Treatment concentrations used in this study were based on those of previous DM studies (21, 27, 46). Cell viability was examined in MTT assays. In LNCaP cells, insulin and glucose did not show significant growth-promoting effects. IGF-1 at a concentration of 100 ng/ml elevated the viability in comparison to the control group. In 22RV1 cells, none of the 3 factors revealed significant changes in terms of viability. All 3 factors exhibited pro-proliferative effects on DU145 cells. Viability of PC3 cells increased only after treatment at the highest concentration.

Effects of Modulation of Protein Expression of Hormone Receptors by Insulin, IGF-1, and Glucose

Previous studies have found that in addition to AR, other hormone receptors have growth promoting or inhibitory effects on PCa. In the present study, AR, and the insulin-related receptors, IRβ and IGF-1Rβ, were examined on tissue arrays of normal and malignant prostate sections. Representative images are shown in Fig. 2. From the results of H&E staining (Figs. 2A to 2D), malignant tissues were less differentiated and had higher nuclear-cytoplasmic ratio in comparison to normal prostate. Total AR (Figs. 2E to 2H) was expressed in both the cytoplasm and the nucleus of normal prostate gland and malignant tissue. The intensity of AR staining was stronger in stage IV prostate cancer tissues. IRβ (Figs. 2I to 2L) was expressed in the cytoplasm of normal prostate gland cells. However, expression was lower in cancerous tissues. In addition, expression of IGF-1Rβ (Figs. 2I to 2L) was primarily in the cell membrane and cytoplasm of the stroma in normal prostate tissues. This phenomenon was previously reported to be observed in rapidly proliferating non-malignant cells (1). There was minor nuclear staining in gland cells. Significantly higher

Fig. 2. Protein expression levels of AR, IGF-1R and IR in normal and malignant human prostate tissues. Tissue arrays were examined with H&E stain and IHC stain as described in Materials and Methods. A-D: H&E staining observed at 40× magnification; E-H: IHC of AR; I-L: IHC of IGF-1R, and M-P: IHC staining of IR observed at 400× magnification.
nuclear staining of IGF-1R was observed in stage IV adenocarcinoma (Figs. 2K and 2L).

AR, IRβ and IGF-1Rβ were expressed in malignant PCa tissues. Thus, these hormone receptors were further investigated in the PCa cell lines (Fig. 3). Cells were divided into five groups: control (10% FCS medium), 1%S (1% FCS medium), Ins (100 nM insulin), IGF-1 (100 ng/ml IGF-1) and Glc (10 mM glucose). Insulin, IGF-1 and glucose used in this study were prepared in 1% FCS medium. After incubation in 1% FCS medium, AR phosphorylation at serine 81 (ARPSer81) decreased in LNCaP and 22RV1 cells. Insulin, IGF-1 and glucose treatments led to the recovery of ARPSer81 expression in LNCaP cells. Moreover, insulin and IGF-1 decreased IR expression and increased IGF-1R expression in comparison to the 1% FCS group (Fig. 3A). However, these phenomena were not observed in AR-expressing 22RV1 cells. In 22RV1 cells, insulin and IGF-1 had no effects on ARPSer81 expression, but insulin downregulated the expression of IRβ and IGF-1Rβ. Similar results were found in two other androgen-independent PCa cell lines, DU145 and PC3 (Fig. 3B).

Promoting and Inhibitory Effects of Antidiabetic Drugs on the Viability of PCa Cell Lines

One of the theories about the inverse correlation of DM history and PCa risk is that antidiabetic drugs inhibit PCa growth (7). The categories of antidiabetic drugs used in this study included sulfonylurea (glybenclamide), megtinlidi (repaglinide), biguanide (metformin), alpha-glucosidase inhibitor (acarbose) and thiazolidinedione (pioglitazone). The ranges of drug concentrations applied to the cells matched those of plasma dosages in DM patients. Glybenclamide, repaglinide, acarbose and pioglitazone showed pro-proliferative effects on both AR-expressing (LNCaP and 22RV1) and non-AR-expressing (DU145 and PC3) cells, with the most significant effects produced by pioglitazone and acarbose (Fig. 4). Metformin, the antidiabetic drug most often used for inhibiting glycogenolysis, revealed anti-proliferative effects on AR-expressing PCa cell lines, especially on LNCaP cells (Fig. 5).

Discussion

In the present study, we demonstrated the following: [1] Insulin, IGF-1 and glucose do not significantly affect the viability of AR-expressing PCa cells in comparison to control group (1% FCS medium) (Fig. 1). [2] Insulin, IGF-1 and glucose have pro-proliferative effects on androgen-independent PCa cells (Fig. 1). [3] IGF-1Rβ is elevated in the nucleus of PCa tissues (Fig. 2). [4] Insulin and IGF-1 increase ARPSer81 expression in the LNCaP cell line but not in the other PCa cell lines (Fig. 3). [5] Both insulin and IGF-1 show inverse results for the expressions of IRβ versus IGF-1Rβ in LNCaP cells (Fig. 3). [6] IRβ and IGF-1Rβ expressions are downregulated in 22RV1, DU145, and PC3 cell lines after treatment with insulin and IGF-1 (Fig. 3). [7] Most of the antidiabetic drugs used in this study have pro-proliferative effects on PCa cells (Fig. 4). [8] Metformin exhibits anti-proliferative effect on AR-expressing PCa cells, especially LNCaP cells (Fig. 5).

Under clinical conditions, standard treatments for prostate cancer are surgery and medical castration to depress the proliferative function of AR (5). Recurrence of PCa after androgen-deprivation therapy (ADT) is referred to as castration-resistant prostate cancer (CRPC) (45). Most CRPCs demonstrate high AR expression. Approximately 30% of CRPCs show AR gene amplification and 15% show an increase in AR co-activator (44). In our study, insulin revealed no effects on cell viability in AR-expressing PCa cells, but increased proliferation of non-AR-expressing cells (Fig. 1). The androgen-independent cell lines, which responded to insulin stimulation, did not express AR or PSA. Insulin did not increase the proliferation of AR-expressing PCa cell lines. This may explain the lack of clinical effectiveness of insulin use on the risk and mortality of PCa (34, 35).

Insulin, IGF-1 and glucose generally promote cancer cell growth (21, 27). Epidemiologic studies have indicated that hyperinsulinemia and hyperglycemia are related to increased risk of several cancers, such as melanoma and of the colon, pancreas, breast, lung, endometrium and liver (10, 13, 14, 40). In addition, high IGF-1 and low IGF-binding protein (IGFBP)-1 in plasma contribute to the occurrence of PCa. IGF-1R and IR may have the potential to form hybrid receptors which enhance tumorigenesis and tumor vascularization (29). Activated IGF-1R targets many signaling pathways, including Ras/Raf/MAPK, PI3K/Akt, STAT3 and Twist1, promoting cell growth and viability (31, 47). Due to different properties of the PCa cell lines used in this study, insulin, IGF-1 and glucose showed distinct growth-promoting effects after 48-h challenge (Fig. 1).

AR is important for the growth of PCa. Higher levels of total AR were found to be expressed in cancerous tissues than in normal prostate gland in the present study (Fig. 2). AR may be activated via ligand-dependent mechanism [e.g. testosterone or dihydrotestosterone (DHT)] (4) or ligand-independent pathway (38). Activated AR (ARPSer81) binds to androgen response element (ARE) and regulates expression of downstream genes, as well as influences PCa cell growth and PSA secretion (8). IGF-1R, IR and their downstream factors are involved in androgen-inde-
Fig. 3. Protein expression levels of AR$^{\text{Pse81}}$, IR$^\beta$ and IGF-1R$^\beta$ after treatment with insulin, IGF-1 and glucose in both AR-expressing and non-AR-expressing PCa cell lines. Cells were incubated with insulin (100 nM), IGF-1 (100 ng/ml) or glucose (10 mM) for 24 h. Whole-cell lysates were subjected to 8% SDS-PAGE. Each lane was loaded with 50 µg protein. Similar results were obtained from three other experiments. B. Quantitative data of protein expression including AR$^{\text{Pse81}}$, IR$^\beta$, or IGF-1R$^\beta$.

$P < 0.05$. Each value represents mean ± SEM. Cont, control; 1%S, medium with 1% FCS; Ins, insulin; Glc, glucose.
Diabetic Factors and Medication on PCa Growth

The relationship between AR and IGF-1R is one of two-way regulation. DHT not only stimulates the growth of CRPC, but also elevates the protein and mRNA levels of IGF-1R via a genomic pathway (26). In addition, androgen upregulates IGF-1R by phosphorylating cyclic AMP response element binding protein (CREB) binding protein (CBP), in AR wild-type and mutant PCa cells via a non-genomic pathway (9). Blocking the activation of IGF-1R down-regulates androgen-mediated gene expression (43) and nuclear localization of AR. Moreover, IGF-1R and AR engage in crosstalk to activate the downstream factors that synchronically accelerate tumor growth in PCa.

Accumulation of nuclear IGF-1R (nIGF-1R) relates inversely to the survival of cancer patients, including those with renal cancer or rhabdomyosarcoma.
In normal cells, there is little or no nIGF-1R expression. However, the ratio of nuclear/membrane IGF-1R is 13-fold higher in breast cancer cells than in breast epithelial cells (6). Over-accumulation of nIGF-1R may downregulate gene expression, contributing to the growth of cancer cells (6). In this work, IGF-1R expression was higher in cancerous cells than in normal prostate tissue, especially in the nucleus (Fig. 2K). Although, there are currently no published reports on the expression of nIGF-1R in PCa, the strong nuclear staining of IGF-1R in stage IV PCa tissues reveals a possible role for nIGF-1R in the pathophysiology of PCa.

AR<sup>Pser81</sup> decreased after incubation in 1% FCS medium in AR-expressing PCa cell lines (Fig. 3). This might have resulted from lower levels of androgen in the medium (32). LNCaP cell line requires AR signaling for proliferation. AR<sup>Pser81</sup> and IGF-1R expression was recovered to maintain cell growth after insulin or IGF-1 treatment in 1% FCS medium. IGF-1R elevates AR transcriptional activities, including AR phosphorylation and translocation to the nucleus (42). Thus, insulin and IGF-1 may increase AR<sup>Pser81</sup> expression via IGF-1R signaling (Fig. 3A). Although IR and IGF-1R can be activated by the same ligand, IR and IGF-1R do not play equal roles. IR is more metabolic, whereas IGF-1R is more mitogenic. The ratio of these two receptors alters when cells encounter different environments (3). Therefore, IGF-1R showed higher expression than IR after insulin and IGF-1 treatment in LNCaP cells under low androgen conditions (Fig. 3A). In addition, IR expression was lower in malignant tissue than in normal prostate gland (Fig. 2M-2P). In the PCa cell lines, insulin downregulated IR expression in parallel with hyperinsulinemia, consistent with decreased IR in DM2 patients (23).

Meta-analyses have demonstrated an inverse correlation between DM and PCa risk. This may be the result of medical treatment of DM patients (6, 17). The classification of antidiabetic drugs includes insulin stimulator (sulfonylurea and megnitilide), glucose uptake stimulator (biguanide and thiazolidine-dione), and glucosidase inhibitor. One drug of each type was selected to challenge PCa cell lines in our work. As glucose can be used as a fuel for cancer cells, these medications either directly facilitate the utilization of glucose, or indirectly promote cancer cell growth by increasing insulin concentration. Not surprisingly, the five drugs used in this study showed pro-proliferative effects on the androgen-independent PCa cell lines DU145 and PC3 (Fig. 4). However, a single drug from each class cannot be considered representative of the function of all the drugs in that class. Our data indicated the effects of only these five drugs on the proliferation of PCa cells.

In AR-expressing PCa cells, only metformin revealed anti-growth effects at higher concentrations (Fig. 5). Metformin exerts impacts on mitochondrial respiration and activates AMP-activated protein kinase (AMPK), which modulates energy homeostasis of cells. AMPK also reduces gluconeogenesis, which results in decline in insulin and its downstream pathway (19). Hence, metformin has an anti-growth or even pro-apoptotic effect on cancer cells. In addition, metformin inhibits AR functions leading to growth suppression of AR-expressing PCa cells (18). This may have resulted in the specific inhibitory effect of metformin on AR-expressing PCa cell lines in this study (Fig. 5).

There is currently no precise treatment for DM2 in patients with cancer. Our data have indicated that most antidiabetic medications and plasma factors of DM2 patients, such as insulin, IGF-1 and glucose, are associated with PCa tumor growth. Alterations of the expression of AR, IR and IGF-1R are involved in tumor progression. However, metformin suppressed cell viability especially in AR-expressing PCa cells. These findings revealed that it is not DM2, but rather the medication used to treat it, that has the potential for PCa treatment. Future studies are needed to translate the application of these findings to clinical management of PCa patients with DM2.

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