Resveratrol Inhibits Hypoxia-Inducible Factor-1 α -Mediated Androgen Receptor Signaling and Represses Tumor Progression in Castration-Resistant Prostate Cancer

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Summary Androgen-dependent prostate cancer inevitably progresses to incurable castration-resistant prostate cancer (CRPC) after androgen deprivation therapy. Because castration-induced hypoxia-inducible factor (HIF)-1 α enhances the transcriptional activity of androgen receptor (AR) at low androgen levels mimicking the castration-resistant stage, HIF-1 α is expected to be a promising target for suppression of growth of CRPC. We investigated the effect of resveratrol (3,4',5-trihydroxy-*trans*-stilbene) on the growth of human prostate cancer LNCaP xenografts in castrated male BALB/cSlc-*nu/nu* mice (5 wk old). The mice were administered a control diet or a resveratrol diet (4 g/kg diet) for 40 d. The resveratrol diet significantly suppressed tumor growth compared to the control diet. In LNCaP xenografts, dietary resveratrol decreased the protein level of HIF-1 α , but not the AR coactivator β -catenin, and reduced the mRNA levels of androgen-responsive genes. In the control group, β -catenin was predominantly localized in the nucleus with HIF-1 α in LNCaP xenografts, whereas dietary resveratrol inhibited the nuclear accumulation of β -catenin. In hypoxic LNCaP cells at a low androgen level mimicking the castration-resistant stage, hypoxia-induced nuclear accumulation of β -catenin was inhibited by resveratrol. Furthermore, resveratrol repressed the expression level of HIF-1 α even in the presence of a proteasome inhibitor and suppressed hypoxia-enhanced AR transactivation. These results indicate that dietary resveratrol represses nuclear localization of β -catenin by decreasing the HIF-1 α expression, perhaps in a proteasome-independent manner, and inhibits β -catenin-mediated AR signaling; this contributes to suppression of tumor growth of CRPC.

Key Words resveratrol, hypoxia-inducible factor-1 α , androgen receptor, β -catenin, castration-resistant prostate cancer

Prostate cancer is the second most frequently diagnosed cancer of men, with incidence rates being highest in Europe and North America (1). Prostate cancer develops and progresses under the influence of androgens, principally testosterone and its more potent metabolite, 5 α -dihydrotestosterone (DHT). Androgens bind to and activate the androgen receptor (AR). Androgen-bound AR translocates to the nucleus and binds to specific androgen response elements (AREs) in the promoter region of androgen-responsive genes, resulting in activation of their transcription. The transcriptional activity of AR is enhanced by recruitment of coactivators (2, 3). Androgen ablation by medical and surgical castration reduces androgen levels (e.g., 0.1 nM DHT (4))

and concomitantly regresses tumor growth. However, castration-resistant prostate cancer (CRPC), which is referred to as androgen-refractory or androgen-independent prostate cancer, eventually recurs in most patients despite androgen deprivation therapy (5, 6). The inevitable CRPC results in mortality related to prostate cancer.

Even in the low androgen environment, CRPC cells express AR and grow in an AR-dependent manner. Androgen depletion by castration results in hypoxia due to blockade of blood flow in the prostate tissue (7, 8). In hypoxia, hypoxia-inducible factor (HIF)-1 α is stably expressed. HIF-1 α is expressed at a higher level in CRPC than in normal prostate tissues (9). We previously found that HIF-1 α promotes nuclear translocation of β -catenin, which functions as a coactivator of AR, and that HIF-1 α and β -catenin coordinately enhance AR transactivation at low androgen levels mimicking the castration-resistant stage (10, 11). These results suggest that HIF-1 α is a promising target for repressing AR signaling in CRPC.

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a

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Abbreviations: AR, androgen receptor; AREs, androgen response elements; CRPC, castration-resistant prostate cancer; DHT, 5 α -dihydrotestosterone; HIF-1 α , hypoxia-inducible factor-1 α ; PSA, prostate-specific antigen; qRT-PCR, quantitative real-time RT-PCR; VEGF, vascular endothelial growth factor.

polyphenol that is found in several plants such as peanuts, berries and grape skin, and as a consequence, red wine (12). Several lines of evidence show that resveratrol reduces the risk of heart disease and atherosclerosis (13–15). Furthermore, resveratrol inhibits proliferation and induces apoptosis in a variety of tumors including prostate, breast, and colon tumors (16–18). However, the mechanism by which resveratrol exerts potent anti-tumorigenic activity remains unclear. In this study, we demonstrated that in LNCaP xenografts in castrated mice, dietary resveratrol suppresses tumor growth. Furthermore, we report that resveratrol inhibits nuclear localization of β -catenin by decreasing the expression level of HIF-1 α , perhaps in a proteasome-independent manner, and consequently suppresses AR transactivation in hypoxia.

MATERIALS AND METHODS

Cell culture. Human prostate cancer LNCaP cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). LNCaP cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin as described previously (10). For hypoxic exposure, cells were placed in a MCO-5M multi-gas incubator (Sanyo Electric Co., Ltd., Japan) flushed with 1% O₂, 5% CO₂, and 94% N₂ at 37°C at 100% humidity.

Animals. Four-week-old male nude (BALB/cSlc-nu/nu) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were housed under temperature- and light-controlled conditions (23 $\pm$ 2°C; alternating light-dark cycles with 12-h light/12-h darkness) and had free access to water and food (CE-2; CLEA Japan Inc., Tokyo, Japan). All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

Tumor xenograft experiment. Xenograft models of CRPC were generated with minor modification of the method described previously (11). In brief, male BALB/cSlc-nu/nu mice (5 wk old) were castrated under anesthesia. One week later, LNCaP cells (5 $\times$ 10⁶ cells) were suspended in 100 μ L of RPMI 1640 medium with 100 μ L of Matrigel (BD Biosciences, San Jose, CA) and implanted in the flank region of castrated mice under anesthesia. One day after implantation, the mice were divided randomly into two groups, control group and resveratrol group (n =6 per group). The control group was fed a modified AIN-93G diet, in which corn oil was substituted for soybean oil, and the resveratrol group was fed the modified AIN-93G diet containing resveratrol (4 g/kg diet). Implanted tumor size was monitored for 40 d. The long- and short-axis of the tumors was measured using digital calipers, and tumor volume was calculated by the following formula:

$$\text{Tumor volume (mm}^3\text{)} = \text{long-axis (mm)} \times \text{short-axis}^2 \text{ (mm)} \times 0.5$$

The mice were killed 40 d after inoculation of LNCaP cells. Tumors were surgically harvested and stored at

–80°C before use.

Western blot analysis. The LNCaP xenografts were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P-40, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μ g/mL leupeptin, and 1 μ g/mL aprotinin) and centrifuged at 20,000 $\times g$ for 1 h. The supernatant was subjected to SDS-PAGE and analyzed by Western blotting using rabbit polyclonal anti-AR (N20, Santa Cruz Biotechnology, Santa Cruz, CA) and anti- β -catenin (Sigma-Aldrich) antibodies, and mouse monoclonal anti-HIF-1 α (Clone: mgc3, Affinity BioReagents, Golden, CO) and anti- α -tubulin (Clone: DM 1A, Sigma-Aldrich) antibodies. The primary antibodies were immunoreacted with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). The immunoreactive proteins were detected using the Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA). For analyses of the expression levels of AR, β -catenin, and HIF-1 α in LNCaP cells, cells were cultured in phenol red-free RPMI 1640 medium supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum (steroid-free RPMI 1640 medium) for 72 h. The medium was replaced with fresh steroid-free RPMI 1640 medium, and the cells were incubated with 0.1 nM DHT in the presence or absence of resveratrol (1–25 μ M) in hypoxia for 9 h. The cell lysates were subjected to SDS-PAGE and analyzed by Western blotting.

Quantitative real-time RT-PCR. Total RNAs were extracted from the LNCaP cells and LNCaP xenografts, and cDNAs were synthesized using reverse transcriptase. The resultant cDNA were subjected to quantitative real-time RT-PCR (qRT-PCR) using the following specific primers: prostate-specific antigen (PSA) (forward primer 5'-ACCCTCAGAAGGTGACCAAGT-3' and reverse primer 5'-TGAAGCACACCATTTACAGACA-3') and vascular endothelial growth factor (VEGF) (forward primer 5'-TGCCTTGCTCTACCTCC-3' and reverse primer 5'-TCACCGCCTCGGCTTGTCAG-3'). Specific primers for β -actin, HIF-1 α , and NKX3.1 were described previously (10). qRT-PCR was performed using GoTaq qPCR Master Mix (Promega, Madison, WI) on a Thermal Cycler Dice real-time system (Takara Bio, Shiga, Japan). The relative amounts of each gene expression were calculated using the comparative Ct method, and the data were normalized to the β -actin as an endogenous control.

Immunofluorescence microscopy. Sections of LNCaP xenograft were blocked in immunohistochemistry solution and incubated with rabbit anti- β -catenin and mouse anti-HIF-1 α antibodies, followed by incubation with Alexa Fluor 488-conjugated secondary anti-rabbit IgG and Alexa Fluor 594-conjugated secondary anti-mouse IgG (Molecular Probes, Eugene, OR), respectively. The nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1 μ g/mL), followed by inspection using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

Subcellular fractionation. LNCaP cells were cultured in steroid-free RPMI 1640 medium for 72 h. The

Fig. 1. Inhibitory effect of resveratrol on the growth of LNCaP xenografts in castrated mice. (A) LNCaP cells were implanted in castrated mice, and mice were fed a control diet or resveratrol-supplemented diet (4 g/kg diet). Tumor volume was recorded for 40 d. The data are presented as means $\pm$ SD ($n=6$) and groups with different letters (a–h) are considered significantly different at $p<0.05$. (B) Tumor (left panel) and body (right panel) weights of control (Cont) and resveratrol (Res) groups were measured. Values are expressed as means $\pm$ SD ($n=6$). * $p<0.05$ vs. control group.

medium was replaced with fresh steroid-free RPMI 1640 medium, and the cells were incubated with 0.1 nM DHT in the presence or absence of resveratrol (1 μ M) in normoxia or hypoxia for 9 h. The cytosolic and nuclear fractions were separated by differential centrifugation as described previously (19). The proteins in each fraction were subjected to SDS-PAGE and analyzed by Western blotting with anti- β -catenin, anti-HIF-1 α , anti-lamin B1 (a nuclear marker) (Clone: L-5, Zymed Laboratories Inc., San Francisco, CA), and anti- α -tubulin antibodies.

Luciferase reporter assay. LNCaP cells were grown on 48-well plates in steroid-free RPMI 1640 medium and transiently transfected with reporter vector (p6xARE-TATA-Luc (10) and pGL4.73[hRluc/SV40] (Promega)) using HilyMax reagent (Dojindo Laboratories, Kumamoto, Japan) for 24 h. The medium was replaced with fresh steroid-free RPMI 1640 medium, and the cells were incubated with or without resveratrol (1 μ M) in the presence or absence of DHT (0.1 nM) in normoxia or hypoxia for 9 h. Luciferase reporter activities were determined using a dual-luciferase reporter assay system (Promega) as described previously (10). Data are expressed as relative light units (RLUs, firefly luciferase activity divided by *Renilla* luciferase activity).

Statistical analysis. Data for comparisons between

Fig. 2. Expression patterns of HIF-1 α and AR target genes in LNCaP xenografts. (A) Tumor xenografts were homogenized and analyzed by Western blotting ($n=3$) (upper panels). Intensities of each band were quantified by ImageJ 1.44 (National Institutes of Health, Bethesda, MD), and the ratio of each protein level was normalized to the α -tubulin level. Values are expressed as means $\pm$ SD ($n=3$). * $p<0.05$ vs. control group (lower panel). (B) The mRNA levels derived from tumor xenografts of control (Cont) and resveratrol (Res) groups were determined by qRT-PCR. The data were normalized to the β -actin mRNA level. Values are expressed as means $\pm$ SD ($n=6$). * $p<0.05$ vs. control group.

two groups were analyzed by Student's *t* test. Other data were assessed by two-way analyses of variance with Turkey's post hoc testing. Statistical analysis was performed using JMP statistical software version 8.0.1. (SAS Institute, Cary, NC). Data are expressed as mean $\pm$ SD, and differences were considered statistically significant at p values of <0.05 .

RESULTS

In castrated mice with LNCaP xenografts, the tumor volume was not significantly different between the control group and the resveratrol group until 30 d after the start of feeding (Fig. 1A). However, dietary resveratrol

Fig. 3. Subcellular distribution of β -catenin in the presence or absence of resveratrol. (A) Sections of LNCaP xenografts were visualized with primary antibodies (anti- β -catenin and anti-HIF-1 α) and fluorescence-labeled secondary antibodies. The nuclei were stained with DAPI. (B) LNCaP cells were incubated in the presence or absence of resveratrol at 0.1 nM DHT in normoxia or hypoxia for 9 h. The cell homogenates were fractionated by differential centrifugation. The proteins in each fraction were analyzed by Western blotting. The result is representative of three independent experiments.

markedly suppressed tumor volume at 32–40 d after the start of feeding ($p < 0.05$). At 40 d, the final tumor volume was smaller in the resveratrol group ($138 \pm 48 \text{ mm}^3$) than in the control group ($319 \pm 110 \text{ mm}^3$) ($p < 0.05$). The tumor weights of the resveratrol group and the control group were $0.14 \pm 0.03 \text{ g}$ and $0.27 \pm 0.08 \text{ g}$ ($p < 0.05$), respectively (Fig. 1B, left panel), although the body weight in mice was not different between the two groups (Fig. 1B, right panel).

In LNCaP xenografts, dietary resveratrol significantly reduced the HIF-1 α protein level (Fig. 2A). However, dietary resveratrol had no influence on the β -catenin protein level. On the other hand, because the AR protein levels were different among xenografts in both groups, they were not statistically analyzed. qRT-PCR analysis showed that dietary resveratrol decreased the expression levels of hypoxia-responsive genes (*VEGF* and *PSA*) and androgen-responsive genes (*NKX3.1* and *PSA*) (Fig. 2B). In contrast, the expression level of *HIF-1 α* mRNA was not suppressed by dietary resveratrol.

In LNCaP xenografts, β -catenin was co-localized with HIF-1 α in the nucleus in the control group (Fig. 3A, top panels). However, in the resveratrol group, β -catenin

Fig. 4. Effect of resveratrol on the expression of HIF-1 α in hypoxic prostate cancer cells. (A) LNCaP cells were exposed to hypoxia in the presence of various concentrations (0–25 μM) of resveratrol (Res) at 0.1 nM DHT for 9 h. The cell lysates were prepared and analyzed by Western blotting. (B) LNCaP cells were incubated in the presence of various concentrations (0–25 μM) of resveratrol (Res) in hypoxia at 0.1 nM DHT for 9 h. The mRNA levels were semi-quantitatively measured by RT-PCR. (C) LNCaP cells were pre-incubated with vehicle or MG132 (10 μM) for 30 min, followed by incubation in the presence or absence of 1 μM resveratrol (Res) in hypoxia for 9 h. The cell lysates were analyzed by Western blotting. In all experiments, the result is representative of three independent experiments.

Fig. 5. Effect of resveratrol on transcriptional activity of AR in hypoxia. LNCaP cells were transiently transfected with reporter vectors, followed by incubation in the presence or absence of DHT with or without resveratrol (Res) in normoxia or hypoxia for 9 h. Luciferase activities were determined. The data are presented as means $\pm$ SD ($n = 3$) and groups with different letters (a–c) are considered significantly different at $p < 0.05$. In all experiments, the result is representative of three independent experiments.

was mainly localized in the cytoplasm, and HIF-1 α was not detected in the cytoplasm or nucleus (Fig. 3A, bottom panels). Furthermore, in LNCaP cells, resveratrol inhibited hypoxia-induced accumulation of β -catenin and HIF-1 α in the nucleus (Fig. 3B) but had no effect on the nuclear β -catenin level in normoxia.

Resveratrol at concentrations above 1 μ M decreased the expression of HIF-1 α protein in hypoxic LNCaP cells under low androgen conditions that are characteristic of the castration-resistant stage (Fig. 4A). However, 1 μ M resveratrol had no influence on the expression levels of AR and β -catenin. In contrast, resveratrol had no influence on HIF-1 α mRNA level at the concentrations tested (Fig. 4B). Although the proteasome inhibitor MG132 increased the HIF-1 α protein level in hypoxia LNCaP cells, it did not recover the decrease of HIF-1 α protein level by resveratrol (Fig. 4C).

Hypoxia enhanced the transcriptional activity of AR in LNCaP cells under low androgen conditions (in the presence of 0.1 nM DHT) ($p < 0.05$), although hypoxia had no influence on AR transactivation in the absence of DHT (Fig. 5). Resveratrol at 1 μ M inhibited hypoxia-enhanced AR transactivation under the low androgen condition.

DISCUSSION

Our previous studies demonstrated that HIF-1 α enhances β -catenin-activated AR transactivation at low androgen levels mimicking the castration-resistant stage, indicating that HIF-1 α supports AR function and up-regulates the androgen-responsive genes (e.g., *NKX3.1* and *PSA*) in hypoxic CRPC (10). On the other hand, HIF-1 α dimerizes with HIF-1 β , and the resultant HIF-1 up-regulates the expression of hypoxia-responsive genes (e.g., *VEGF* and *PSA*) in tumor cells (20). Thus, HIF-1 signaling also plays a central role in the progression of tumors. Therefore, we expect that HIF-1 α could be a target molecule not only in the AR signaling pathway, but also in the HIF-1 signaling pathway in CRPC. Here, we demonstrated that dietary resveratrol suppressed the growth of LNCaP xenografts in a CRPC model and reduced the protein levels of HIF-1 α in vivo and in vitro. Furthermore, resveratrol down-regulated the gene expression of *VEGF*, *PSA*, and *NKX3.1*. These results indicate that resveratrol inhibits both the AR and HIF-1 signaling pathways and is an effective chemopreventive compound against the development of CRPC.

Resveratrol decreased the nuclear β -catenin level in LNCaP xenografts. β -Catenin is a structural component in adherence-mediated cell-cell junctions, but it has also been implicated as an oncogenic factor in several tumors including prostate cancer (21). Although β -catenin is mostly located on the cell membrane and in the cytosol, it is highly expressed in the nucleus in CRPC (22). Increased nuclear β -catenin is associated with the acquisition of an apoptosis-resistant cell phenotype and therapeutic resistance in prostate cancer (23). β -Catenin functions as an AR coactivator that enhances the transcriptional activity of AR. HIF-1 α forms a complex with β -catenin in the cytoplasm and promotes

nuclear translocation of β -catenin in hypoxia (11). HIF-1 α alone has no influence on AR function, and HIF-1 α enhances β -catenin-mediated AR transactivation. Furthermore, in hypoxia, β -catenin forms a complex with HIF-1 α , leading to activation of the HIF-1 signaling pathway (24). Because a decreased level of β -catenin in the nucleus inhibits the AR and HIF-1 signaling pathways, β -catenin also is expected to be a promising target molecule for CRPC prevention. These results indicate that resveratrol suppresses β -catenin-mediated AR transactivation by inhibiting nuclear localization of β -catenin in CRPC.

Because the AR protein levels were different among xenografts in the resveratrol group, we did not define whether dietary resveratrol (4 g/kg diet) influences the AR protein level. In transgenic rats developed as an androgen-dependent prostate cancer model, oral resveratrol (50 μ g/mL of drinking) suppressed growth of prostate cancer and decreased AR expression in the ventral prostate, suggesting that resveratrol induces apoptosis of prostate cancer cells (25). On the other hand, in transgenic mice developed as an androgen-dependent prostate cancer model, oral resveratrol (625 mg/kg diet) suppressed the progression of prostate cancer, but increased AR expression (26). Thus, AR expression behaved differently in the rat and mouse models. Taken together, the AR protein level seems not to be involved in the mechanism by which resveratrol is effective in suppressing tumor growth in either androgen-dependent prostate cancer or in CRPC.

Dietary resveratrol is rapidly metabolized to its 3- or 4'-O-glucuronide, or 3- or 4'-O-sulfate conjugates in the small intestine and liver and mainly exists as glucuronide and sulfate conjugates both in plasma and in urine (27). The conjugated derivatives exhibit poor biological activities for various diseases (28). However, the derivatives might be regenerated to the parent resveratrol by deconjugation of glucuronides or sulfates and consequently restore numerous health benefits. In this study, plasma was prepared from mice at 40 d after xenografts and treated with β -glucuronidase (type H-2 from *Helix pomatia*, Sigma-Aldrich), and plasma resveratrol aglycone concentration was determined. However, resveratrol aglycon could not be detected (data not shown). Several polyphenols are accumulated within human cells in a tissue-specific manner (29, 30). Like resveratrol, quercetin also acts as an anti-inflammatory polyphenol in cardiovascular diseases (31, 32). Quercetin-3-glucuronide is accumulated in atherosclerotic lesions, but not in the normal aorta, and macrophage cells are deconjugated into the aglycon, suggesting that the level of quercetin aglycon is higher in inflammatory tissues than in the normal tissues (32). Hypoxia activates the inflammation signaling, and activated inflammation signal enhances the progression and development of CRPC (33, 34). Therefore, resveratrol derivatives also might be converted to the parent resveratrol and exert anti-tumor activity in CRPC.

Resveratrol suppressed the expression level of HIF-1 α protein. Some flavonoids also inhibit the expression level

of HIF-1 α protein. These include the flavone apigenin in prostate cancer PC3-M cells in vitro (35) and soy isoflavones (e.g., genistein, daidzein, and glycitein) in prostate cancer PC-3 cells (36). In LNCaP cells, the resveratrol-induced decrease in the HIF-1 α protein level was not restored by the proteasome inhibitor MG132. Likewise, the quercetin-induced decrease in the HIF-1 α protein level was not prevented by MG132 in hypoxic LNCaP cells (37). Thus, resveratrol and quercetin down-regulate the expression of HIF-1 α protein in LNCaP cells in a ubiquitin-proteasome-independent manner. In contrast, in human tongue squamous cell carcinoma (SCC-9) and ovarian cancer cells (OVCAR-3), resveratrol decreases the expression of HIF-1 α protein in a ubiquitin-proteasome dependent manner (38, 39). Furthermore, in hepatoma HepG2 cells, incubation with red wine polyphenol (resveratrol, piceatannol, quercetin, or myricetin) prior to hypoxic exposure increases the expression of HIF-1 α protein (40). Thus, these results suggest that the mechanism by which resveratrol regulates the expression of HIF-1 α protein in hypoxia depends on the cell types. We are presently attempting to characterize the mechanism by which resveratrol decreases the HIF-1 α protein level in hypoxic LNCaP cells.

In conclusion, dietary resveratrol inhibits β -catenin-mediated AR function by decreasing the expression of HIF-1 α protein in hypoxic LNCaP and consequently suppresses CRPC growth in vivo. Our findings suggest that resveratrol is a promising candidate for suppressing the progression or development of CRPC in humans.

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