Quercetin inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells

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The androgen receptor (AR) is involved in the development and progression of prostate cancer. In order to find new compounds that may present novel mechanisms to attenuate the function of AR, we investigated the effect of a natural flavonoid chemical, quercetin, on androgen action in an androgen-responsive LNCaP prostate cancer cell line. Western blot analysis showed that AR protein expression was inhibited by quercetin in a dose-dependent manner. To demonstrate that the repression effects on AR expression can actually reduce its function, we found that quercetin inhibited the secretion of the prostate-specific, androgen-regulated tumor markers, PSA and hK2. The mRNA levels of androgen-regulated genes such as PSA, NFKX3.1 as well as ornithine decarboxylase (ODC) were down-regulated by quercetin. Transient transfections further showed that quercetin inhibited AR-mediated PSA expression at the transcription level. Finally, it was demonstrated that quercetin could repress the expression of the AR gene at the transcription level. Our result suggests that quercetin can attenuate the function of AR by repressing its expression and has the potential to become a chemopreventive and/or chemotherapeutic agent for prostate cancer.

Introduction

Androgens play a critical role in regulating the growth, differentiation and survival of epithelial cells in the normal prostate. Evidence shows that androgens are also involved in the development and progression of prostate cancer (1). The biological effects of androgens in the prostate are mediated by the androgen receptor (AR), a ligand-activated transcription factor of the nuclear receptor superfamily (2). Endocrine therapy for prostate cancer is aimed at reducing the levels of circulating androgens or blocking agonist activation with antagonists or both. However, endocrine therapy is only palliative. Prostate cancer relapses generally occur within 1–2 years and become hormone refractory with a potentially fatal outcome (3).

Many molecular mechanisms have been postulated to be responsible for the development of recurrent hormone refractory tumors. Most of these mechanisms involve alterations in the function of the AR and its complex signaling pathways (4). Studies of patient tissue show that nearly all cancer tissues retain AR expression regardless of clinical stage or hormone status (5–7). Indeed, that the majority of hormone refractory cancers still express the androgen-responsive prostate-specific antigen (PSA) gene indicates that the AR signaling pathway is functional. Even more intriguingly, amplification of the AR gene was detected in a subgroup of prostate cancer patients who showed tumor progression (8,9). Also, mutations in the AR that enable the receptor to respond to residual androgens, non-androgen steroids or even antagonists have been suggested as potential causative factors for tumor relapse and progression. In addition, the AR could be activated ligand-independently by growth factor/cytokine signalings (4,10). Thus, the AR could still play a role in the development of the hormone refractory disease. Therefore, a more effective strategy in the fight against prostate cancer is to minimize or eliminate the function of the AR.

Quercetin is an abundant, naturally occurring flavonoid compound that can be found in apples, onions, tea, and red wine (11). It has been reported that this compound shows growth inhibitory effects on many different cancer cell lines in vitro and in vivo (12–14). Quercetin exhibits anti-neoplastic activity through various mechanisms. First among them is the antioxidant effect that can inhibit carcinogen activation as well as cellular damage due to radical reactions (15). At relatively high concentrations it inhibits the growth of malignant cells by arresting cell cycle in the late G1 phase (16) or causes apoptosis (17). It also blocks signal transduction pathways by inhibiting protein tyrosine kinase, 1-phosphatidylinositol 4-kinase and 1-phosphatidylinositol 4-phosphate 5-kinase resulting in a reduction of inositol 1,4,5-trisphosphate concentration (17,18). Quercetin can also down-regulate the expression of oncogenes, e.g., c-myc and ki-ras (18) and induce wild-type p53 (19). Recently, it was found that quercetin can down-regulate the estrogen receptor in an estrogen-sensitive breast cancer cell line MCF-7 (20).

The LNCaP cell line is a well-established, androgen-responsive prostate cancer cell line obtained from a lymph node metastasis of a prostate cancer patient (21). LNCaP cells express the AR and a number of androgen-inducible genes such as PSA, human glandular kallikrein (hK2), NFKX3.1 and ornithine decarboxylase (ODC), and their growth is stimulated by androgens. Recently, we have reported that some naturally occurring or synthetic compounds can modulate negatively the expression and function of AR (22–24). In the present study, we found that quercetin can inhibit the expression and function of AR in LNCaP prostate cancer cells.

Materials and methods

Cell cultures and treatments

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Rockville, MD). Another human prostate cancer cell line LAPC-4 was a gift from Dr Charles L. Sawyers (University of California Los Angeles, Los Angeles, CA). Both cell lines were propagated in 24-well, 60 or 100 mm culture dishes at the desired density in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 5% fetal calf serum (FCS) (Biofluids, Rockville, MD) at 37°C and 5% CO2 until reaching 50–70% confluence. The
Fig. 1. Western blot analysis of AR or SP1 expression levels in LNCaP cells or LAPC-4 cells treated with quercetin. (a) Whole-cell lysates from LNCaP cells treated with varying amount of quercetin with or without Mib for 24 h were used for gel electrophoresis and western blotting with primary antibody of anti-AR. (b) Nuclear extracts from LNCaP cells treated with varying amount of quercetin with or without Mib for 24 h were used for gel electrophoresis and western blotting with primary antibody of anti-AR. (c) Whole-cell lysates from LNCaP cells treated with varying amount of quercetin with or without Mib for 24 h were used for gel electrophoresis and western blotting with primary antibody of anti-SP1. (d) Whole-cell lysates from LAPC-4 cells treated with varying amount of quercetin with or without Mib for 24 h were used for gel electrophoresis and western blot with primary antibody of anti-AR. The Ponceau S-stained protein band was used for normalization. The graphs show the normalized densitometric results as a percentage of control (no treatment).

cells were treated with quercetin at designated concentrations with or without 1 nM mibolerone (Mib) (NEN, Boston, MA). Mib is a synthetic androgen that is not metabolized in cell culture. Quercetin was purchased from Sigma (St Louis, MO) and dissolved in DMSO.

Western blot analysis
LNCaP or LAPC-4 cells were plated in 10 cm dishes at 9×10^5 cells per dish in RPMI 1640 and 5% FCS. After 48 h the cells were treated with 1.0 nM Mib and varying concentrations of quercetin. Cells were harvested at designated times, and the whole cell lysate was prepared according to Santa Cruz research applications. Nuclear extraction was performed using the method described by Andrews and Faller (25). Protein levels were measured with a DC protein assay (Bio-Rad, Hercules, CA). Protein samples (20 µg/sample) were loaded into precast 4–12% NuPage gels, run with MOPS buffer and transferred onto a nitrocellulose membrane. The membranes were blocked overnight at 4°C in TBST (20 mM Tris–HCl pH 8.0, 137 mM NaCl and 0.1% Tween 20) and 5% dry milk. The membranes were washed three times for 10 min each with TBST. Primary antibody for AR (1:2000 dilution) (Pharmingen, San Diego, CA), Sp1 (1:2000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or heat shock protein 70 (Hsp70) (1:2000) (StressGen Biotechnologies Corp., Canada) was incubated at room temperature for 1 h.

The membranes were washed three times for 10 min each with TBST. Antibody HRP secondary antibody (Amersham, Piscataway, NJ) used at a 1:10 000 dilution was also incubated for 1 h at room temperature. The membranes were washed again and Renaissance chemiluminescence (NEN) was used according to the manufacturer’s instructions.

PSA and hK2 protein expression
LNCaP or LAPC-4 cells were seeded at 2×10^4 cells per well in 24-well plates. After 2 days they were treated with varying amounts of quercetin with or without 1 nM Mib. After a 5 day incubation, the spent media were harvested, and the levels of PSA and hK2 in spent media were quantified by an immunoassay as described previously (26). An MTS assay was performed (Promega, Madison, WI) as per the manufacturer’s instructions. The protein levels of PSA and hK2 were normalized by cell density measurements with the MTS assay.

Northern blots
LNCaP cells were treated with designated concentrations of quercetin with or without Mib. Cells were harvested 24 h later and RNA was isolated by the guanidine/isoctoate method (27). Total RNA (15 µg) was run in a denatured gel and transferred onto a nylon membrane according to the GeneScreen protocol by New England Nuclear, cDNAs for PSA, NKX3.1, ODC and AR were used as probes labeled with [32P]dCTP by random priming.
Quercetin inhibits androgen receptor

Fig. 2. Quercetin inhibits the secretion of PSA and hK2 proteins in both LNCaP and LAPC-4 cells. LNCaP or LAPC-4 cells were treated with varying amounts of quercetin for 5 days. Spent media were collected and used for (a) PSA assay for LNCaP cells, (b) hK2 assay for LNCaP cells, (c) PSA assay for LAPC-4 cells and (d) hK2 assay for LAPC-4 cells. These protein levels were normalized to cell density measured by MTS assay. Error bars indicate the standard error of three separate experiments.

The hybridization was performed according to Clontech's protocols with ExpressHyb hybridization solution. The films were autoradiographed at −70°C.

Transient transfections
LNCaP cells were plated in 60 mm dishes until they reached a confluence of 50–70%. Cells were co-transfected with CMV-β-galactosidase (β-gal) expression vector and pGL3 containing a 6 kb PSA promoter or 2 kb AR promoter using lipofectin (Life Technologies, Grand Island, NY), while the parental vector, pGL3, was used as a control. Twenty-four hours after the transfection, cells were treated with different concentrations of quercetin with or without Mib for an additional 24 h. Whole-cell extracts were prepared and a luciferase assay was performed according to manufacturer’s instructions (Promega) for the PSA promoter/luciferase or AR promoter/luciferase transfection. β-gal activity was assayed for normalization purposes (28). Each transfection was done three times and standard deviations were calculated.

Results
Quercetin inhibits the expression of AR protein
To determine whether AR protein levels are changed with quercetin treatment, western blots were performed. Figure 1a shows that AR protein levels are decreased in a dose-dependent manner with quercetin in the presence or absence of Mib. Since AR is a nuclear protein and functions in the nucleus, we prepared the nuclear extracts for western blot after quercetin
Quercetin inhibits the expression of PSA gene at the transcription level. A transient transfection was performed in LNCaP cells using a 6 kb PSA promoter or the control vector (pGL3) and treated with 50 µM quercetin with or without Mib. The resulting luciferase activities were normalized with β-gal activities. The transfections were performed three times and presented as an average; bars denote one standard deviation.

In Figure 1b, nuclear AR protein levels were, as anticipated, decreased by quercetin treatment. On the other hand, we showed that a general transcription factor SP1 (Figure 1c) and Hsp70 (data are not shown) were not affected by quercetin treatment. This means that quercetin probably does not have a broad inhibitory effect on gene expression.

The AR in the LNCaP cell line contains a functional mutation in its ligand binding domain (29). Therefore, we also used another androgen-responsive prostate cancer cell line, LAPC-4, expressing a wild-type AR (30), to demonstrate that the quercetin effect on the expression of AR is not due to the mutation. Indeed, a similar effect on AR in LAPC-4 by quercetin was found as shown in Figure 1d.

**Quercetin inhibits PSA and hK2 secretion**

In order to ascertain if quercetin can actually block androgen action, the androgen-dependent secretion of PSA and hK2 was measured. Both PSA and hK2 are prostate-specific, androgen-regulated tumor markers (31,32). LNCaP or LAPC-4 cells were treated with different concentrations of quercetin with or without Mib for 5 days, spent media were harvested for assays of total PSA and hK2 proteins. The normalized data in Figure 2 show that quercetin inhibits both PSA and hK2 protein levels in both LNCaP and LAPC-4 cells and in a dose-dependent manner.

**Quercetin inhibits the expression of other classes of androgen-regulated genes**

In addition to PSA and hK2, NKX3.1 is also a prostate-specific, androgen-regulated gene coding for a homeodomain transcription factor, which may play a role in the development and differentiation of the prostate (33,34). Ornithine decarboxylase (ODC) catalyzes the first and rate-limiting step on polyamine synthesis (35) and is regulated by androgen in prostate cells (36). Northern blot analysis showed quercetin greatly reduced PSA, NKX3.1 and ODC mRNA levels in the presence of androgen (Figure 3). This result demonstrates that quercetin has a general inhibition effect on androgen-stimulated gene expression.

**Quercetin inhibits the PSA gene at the transcription level**

It has been well documented that the PSA gene is mainly regulated by androgens at the transcription level via interaction of the AR with androgen-responsive elements in its promoter (37,38). To test whether the inhibitory effect of quercetin on PSA expression occurs at the transcription level, we transfected a construct containing a 6 kb PSA promoter fragment in front of a luciferase reporter gene into LNCaP cells with or without Mib. As seen in Mib-treated cells, the PSA promoter gave a strong androgenic induction of the luciferase activity, while transfection with the control vector, pGL3 basic, showed no induction. Treatment with quercetin abolishes the androgenic induction of the 6 kb PSA promoter (P < 0.01) (Figure 4).

**Quercetin inhibits the expression of the AR gene at the transcription level**

To shed further light on the mechanism by which quercetin inhibits the expression of AR, northern blot analysis was done.
As shown in Figure 5a, AR mRNA was down-regulated by quercetin treatment. To further ascertain that quercetin can affect AR at the transcription level, gene transfer assays were performed with a 2 kb AR promoter. Figure 5b shows that the 2 kb AR promoter activity was decreased by quercetin (P < 0.01) regardless of the presence or absence of androgens. This experiment strongly demonstrates that quercetin blocks the expression of the AR gene at transcription level.

Discussion

Quercetin exhibits its inhibitory effects on various stages of tumor development in animal studies. There is an inverse association between intake of the main food sources of the quercetin and the risk of lung cancer (39). After quercetin was administered at dose levels that delivered 40–1900 mg/kg/day to male and female rats, there were no treatment-related effects on survival and no treatment-related clinical signs of toxicity (40). This indicates that quercetin is a safe chemical for the potential clinical usage.

The scientific basis for the treatment of prostate cancer by androgen ablation or endocrine therapy was established by Huggins and Hodges in 1941 (41). It has dominated much of the research and clinical practice fields for prostate cancer these days. The initial effective results of the endocrine therapy is followed in virtually all cases by relapse of the disease. In many recurrent or advanced prostate cancers, AR is still expressed, either mutated or amplified. The AR in these cancers can be activated by lower concentrations of androgens, other non-androgenic ligands or antagonists as well as growth factors (4). This implies that AR itself could still mediate androgen-independent progression. Therefore, only aiming at reducing the circulating levels of androgen or the use of anti-androgens could be a major reason for the overall failure of endocrine therapy. Thus, the novel property of quercetin to inhibit the expression and function of AR, apart from its other anti-cancer properties, could have a great potential for preventing or even treating relapsed prostate cancers expressing AR.

PSA and hK2 are serum proteases that can process and specifically activate certain biologically important proteins. It has been suggested that PSA and hK2 can regulate indirectly tumor cell growth, tumor invasion and osteoblastic metastasis (32,42,43). As shown in Figures 2 and 3, PSA and hK2 expression was significantly decreased by quercetin treatment, as a result of AR down-regulation by quercetin. This further implicates the potential utility of quercetin for prostate cancer prevention.

Quercetin can also significantly down-regulate the expression of another prostate-specific gene, NKX3.1, whose expression was found to be associated with a more aggressive phenotype of prostate cancer recently (44). In addition, the androgen up-regulated level of ODC mRNA was found to be inhibited by quercetin. ODC is the key regulator of the synthesis of polyamines, which are essential for cell proliferation. ODC is critical in cell transformation and suggested to be a proto-oncogene (45). It was found that ODC levels are higher in prostate cancer compared to benign tissue (46). Together, our study strongly suggests that quercetin has an inhibitory effect on androgen-regulated genes that can directly or indirectly affect cell growth.

In summary, the natural polyphenolic quercetin can inhibit the AR expression at the transcriptional level, and thereby down-regulate the androgen-inducible genes including PSA, hK2, NKX3.1 and ODC, which play roles in development and progression of prostate cancer. Quercetin has the potential to become a chemopreventive and/or chemotherapeutic agent for prostate cancer.

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References


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