Up-regulation of insulin-like growth factor binding protein-3 by apigenin leads to growth inhibition and apoptosis of 22Rv1 xenograft in athymic nude mice

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SPECIFIC AIMS

The aim of this study was to determine the chemoprotective potential of apigenin (4′,5,7,-trihydroxyflavone), a flavonoid abundant in fruits and vegetables, in human prostate tumor xenograft and to understand the mechanisms underlying these effects.

PRINCIPAL FINDINGS

1. Apigenin inhibits growth of human prostate cancer 22Rv1 xenograft in nude mice

Studies under two protocols have shown that daily oral intake of apigenin administered to mice bearing human prostate 22Rv1 tumor xenograft implanted in athymic nude mice exhibited dose-dependent inhibition of tumor volume and wet tumor weight. In the first protocol using 20 and 50 μg/mouse/day (w/v) in 0.2 mL of a vehicle containing 0.5% methyl cellulose and 0.025% Tween 20; 2 wk before tumor implantation and continued for 8 wk, tumor volume was inhibited by 44 and 59% ($P<0.002$ and 0.0001) and wet weight of tumor by 41 and 53% ($P<0.05$), respectively. In the second protocol in which mice received apigenin beginning 2 wk after tumor implantation for 8 wk, tumor volumes were reduced by 39 and 53% ($P<0.01$ and 0.002) and wet weights of tumor by 31 and 42% ($P<0.05$), respectively. In neither study did animals exhibit any signs of toxicity or reduced food consumption.

2. Oral intake of apigenin induces IGFBP-3 and reduces IGF-I levels in mouse serum

Oral intake of apigenin at 20 and 50 μg/mouse/day resulted in a significant increase in IGFBP-3 levels ($P<0.0001$) in both treatment protocols that correlated with a concomitant decrease in IGF-I levels ($P<0.001$) after apigenin feeding (**Fig. 1*A, B***). The HPLC profile of serum and tumor from control and apigenin-treated groups showed that the induction of IGFBP-3 levels and inhibition of tumor growth in the apigenin-fed group correlated positively with increased apigenin concentration in mouse serum.

3. Oral intake of apigenin induces IGFBP-3 mRNA and protein expression in tumor xenograft

Apigenin feeding at 20 and 50 μg/mouse/day dose for 8 wk after tumor implantation resulted in 1.6- and 2.8-fold increases in IGFBP-3 mRNA expression in the first protocol. Similar observations were noted in mice in the second protocol, in which apigenin feeding resulted in 1.4- and 1.8-fold increases in IGFBP-3 mRNA expression at doses of 20 and 50 μg/mouse/day compared with expression levels in mice in the corresponding control group. These results were comparable with increases in IGFBP-3 protein expression in the apigenin-fed group in both treatment protocols.

4. Oral intake of apigenin induces apoptosis in 22Rv1 xenograft in nude mice

Compared with control group, 1.7- and 2.9-fold increases ($P<0.0001$) in induction of apoptosis were observed in mice in the first protocol, whereas 1.9- and 3.0-fold increases ($P<0.0001$) in induction of apoptosis were observed in the second protocol after intake of 20 and 50 μg/mouse/day of apigenin.

5. Apigenin treatment results in induction of IGFBP-3 and decrease in IGF-I expression in cell culture medium and lysate of 22Rv1 cells

Apigenin (20 and 40 μM) treatment to 22Rv1 cells for 12–48 h resulted in significant increases in IGFBP-3

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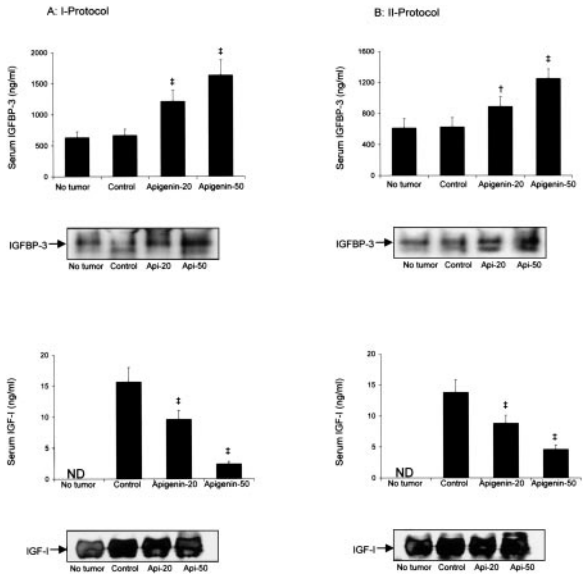

Figure 1. Effect of oral intake of apigenin on serum IGFBP-3 and IGF-I levels in nude mice 22Rv1 tumor xenograft study. *A)* First and *B)* second experimental protocol based on prevention and therapeutic regimens. At the termination of the study serum concentration of human IGFBP-3 and IGF-I were estimated using active IGFBP-3 and IGF-I ELISA kits and by Western blot. IGFBP-3 and IGF-I concentration (ng/mL) in serum samples were calculated by logistic curve fit generated standard curve and are represented as mean of 5 or 6 serum samples from individual mouse in each treatment group. †$P < 0.05$, ‡$P < 0.001$; bars ± SE, ND, nondetectable

levels along with a concomitant decrease in IGF-I expression in 22Rv1 cell culture medium. Similar results were observed in cell lysate in which 20 and 40 μM apigenin treatment resulted in a significant increase in IGFBP-3 and reduction in IGF-I expression at 12–48 h, respectively.

6. Apigenin treatment inhibits IGF-I stimulated cell cycle progression and IRS-1 tyrosine phosphorylation in 22Rv1 cells

In earlier studies we showed that treatment of human prostate carcinoma cells with apigenin resulted in cell growth inhibition and induction of apoptosis. To understand the mechanism(s) of action, we determined the effect of apigenin on IGF-I induced cell cycle progression. 22Rv1 cells were grown under serum-deprived medium for 36 h and stimulated with 100 ng/mL IGF-I for 15 min, washed three times with serum and phenol-free RPMI 1640, then incubated for 24 h with vehicle only or 40 μM apigenin, 5 μg/mL IGFBP-3 antisense oligonucleotide alone, or in combination. Compared with serum-deprived cells, IGF-I stimulation significantly increased the proportion of 22Rv1 cells in S phase (10.5 vs. 29.1%) of the cell cycle. Treatment of 22Rv1 cells with apigenin resulted in a marked increase of cells in G0/G1 phase (51.8 vs. 68.8%) and sub-G1 phase (10.2 vs. 18.8%) of the cell cycle, which may be an indication for apoptosis. Treatment of cells with IGFBP-3 antisense oligonucleotide led to increased accumulation of cells in G0/G1 phase (68.8 vs. 79.2%) along with significant reduction of cells in sub-G1 phase (18.8 vs. 3.1%) of the cell cycle. These results suggest that antisense IGFBP-3 partially rescues tumor cells from apigenin-mediated apoptosis (**Fig. 2*A***).

Next we evaluated the influence of apigenin on the effects of exogenously added IGFBP-3 on IGF-I-induced IRS tyrosine phosphorylation. 22Rv1 cells were grown under serum-depleted medium for 36 h, then exposed to vehicle or 40 μM apigenin or 500 ng/mL rh IGFBP-3 for 3 h. Later cells were incubated for 15 min with or without 100 ng/mL IGF-I. As shown in Fig. 2*B*, lane1, cells grown under serum-depleted conditions exhibit very low or undetected levels of IRS-1 tyrosine phosphorylation, as evidenced by the absence of reactivity of immunoprecipitated IRS-1 with antiphosphotyrosine antibody by Western blot. In contrast, treatment of IGF-I for 15 min resulted in a significant activation of IRS-1 (lane 2). A 3 h pretreatment with apigenin resulted in a modest decrease in IGF-I-induced IRS-1 tyrosine phosphorylation (lane 3 vs. lane 2) whereas significant inhibition (75%) of IGFI-induced IRS-1

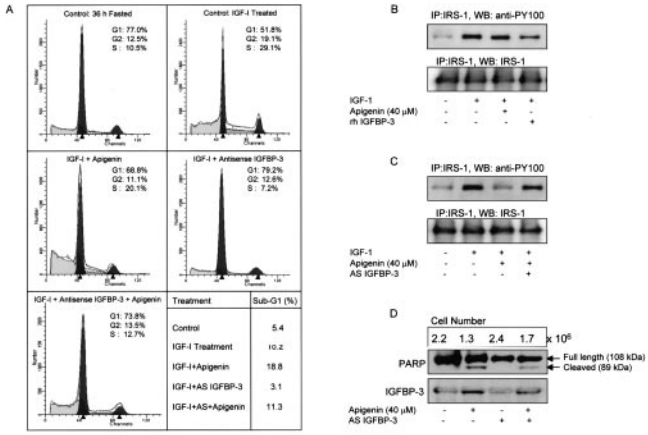

Figure 2. Effect of apigenin on IGF-I induced cell cycle progression and IRS-1 tyrosine phosphorylation in 22Rv1 cells. *A)* After reaching 70% confluence, cells were washed and grown under serum-deprived conditions for 36 h. Cells were incubated with PBS or 100 ng/mL IGF-I for 15 min at 37°C, washed three times with serum and phenol-free RPMI 1640, then incubated in this media for 24 h with *1)* vehicle only; *2)* 40 μM apigenin; *3)* 5 μg/mL IGFBP-3 antisense oligonucleotide alone or combination, then subjected to cell cycle analysis. *B)* During last 3 h of serum starvation, 22Rv1 cells were treated with *1)* vehicle only; *2)* 40 μM apigenin; *3)* 500 ng/mL rh IGFBP-3 alone or a combination. After treatments, cell lysates were prepared and IRS-1 was immunoprecipitated using antiIRS-1 antibody, then SDS-PAGE and Western blot was performed. *C)* Cells were incubated for 48 h in serum and phenol-free medium with 5 μg/mL IGFBP-3-antisense (AS) oligonucleotides in the presence or absence of 40 μM apigenin, then stimulated for 15 min with 100 ng/mL IGF-I. *D)* Log phase growing cells were treated with *1)* vehicle only; *2)* 40 μM apigenin; *3)* 5 μg/mL AS IGFBP-3 alone or combination for 24 h. After treatments, cell number was determined; cell lysates were prepared and subjected to SDS-PAGE and Western blot.

phosphorylation was observed with a 3 h pretreatment with 500 ng/mL rh-IGFBP-3 (lane 4 vs. lane 2).

We next conducted an experiment to determine whether prolonged exposure to apigenin could reduce IRS-1 tyrosine phosphorylation and whether this was dependent on IGFBP-3 induction. As shown in Fig. 2*C*, incubation of 22Rv1 cells with 100 ng/mL IGF-I for 15 min led to a significant activation of IRS-1 compared with vehicle-treated control (lane 2 vs. lane 1). In cells pretreated with 40 μM apigenin for 48 h, the 15 min IGF-1 incubation resulted in a significant decrease (82%) in IRS-1 activation (lane 3 vs. lane 2). However, in cells pretreated with both 40 μM apigenin and IGFBP-3 antisense oligonucleotide for 48 h, the reduction in IRS-1 tyrosine phosphorylation was attenuated (lane 4 vs. lane 3). None of these treatments affected IRS-1 protein levels (Fig. 2*A, B*, bottom). These data provide evidence that the antiproliferative effects of apigenin in 22Rv1 cells involve inhibition of IGF-I-induced IRS-1 phosphorylation.

7. IGFBP-3 antisense oligonucleotide treatment reverses the apoptotic effects of apigenin in 22Rv1 cells

Next we determined whether apigenin-mediated cell growth inhibition and apoptosis was mediated by increased IGFBP-3 secretion. Log phase growing cells were treated with 40 μM apigenin or 5 μg/mL IGFBP-3 antisense oligonucleotide alone or in combination for 24 h. As shown in Fig. 2*D*, treatment of 22Rv1 cells with 40 μM concentration of apigenin for 24 h resulted in a significant decrease (41%) in number of viable cells; IGFBP-3 antisense oligonucleotide exhibited an increase (9%) in number of viable cells compared with vehicle-treated control. A combination of apigenin and IGFBP-3 antisense oligonucleotide reversed cell growth inhibitory effects of apigenin by 31%. Treatment of 22Rv1 cells with 40 μM concentration of apigenin for 24 h resulted in increased apoptosis as evidenced by 89 kDa fragment, a large fragment of cleaved product of PARP (lane 2 vs. 1). In cells treated with IGFBP-3 antisense oligonucleotide, no evidence of PARP cleavage was ascertained (lane 3 vs. 2). These results correlated with IGFBP-3 protein expression (Fig. 2*D*, bottom panel) and provide evidence that the cell growth inhibitory and apoptotic effects of apigenin in 22Rv1 cells are associated with increased IGFBP-3 expression.

CONCLUSIONS AND SIGNIFICANCE

Prospective studies have suggested that regular consumption of fruits and vegetables protects against various types of human cancers. Asian men who consume low-fat, high-fiber, plant-based diets rich in flavonoids have the lowest prostate cancer incidence in the world. Asian men who migrate to the U.S. and adopt a Western diet acquire a risk of developing prostate cancer that approaches the average U.S. incidence within one generation. Therefore, there is an increased impetus to provide scientific support for the use of dietary agents in chemoprevention strategies.

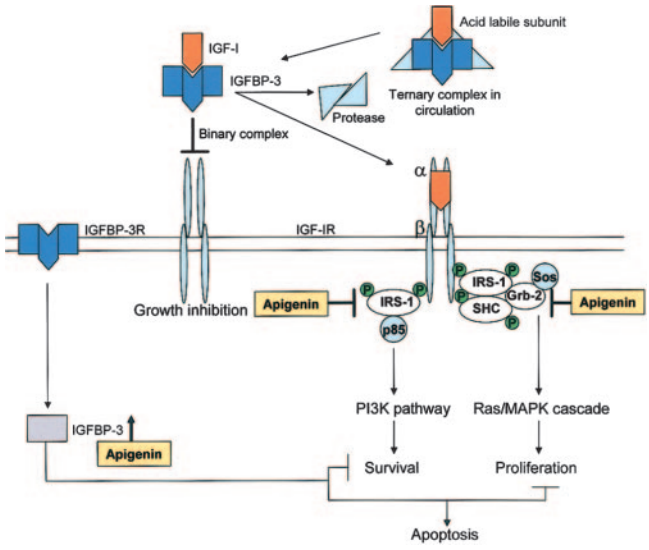

Figure 3. Schematic representation of the effect of apigenin on IGF axis signaling in prostate cancer. IGF-I is sequestered as ternary complex with IGFBP-3 and acid-labile subunit in the intravascular compartment or as binary complex with IGFBP-3 (or other IGFBPs) in the cellular environment. Modification of IGFBP-3 due to cleavage or phosphorylation releases free IGF to bind to the IGF-IR, which phosphorylates itself and several downstream targets including the adaptor proteins IRS-1 and SHC, which link the IGF-IR to the PI3K and Ras/MAPK signaling pathways leading to cell survival and proliferation. IGFBP-3 also has IGF-independent antiproliferative and proapoptotic effects. Apigenin has been shown to modulate this process (↑ activation; ⊤ inhibition).

The importance of IGF-I signaling in deregulated cellular growth has been established in prostate cancer-cells and transgenic mice. Increased IGF signaling stimulates proliferation and inhibits apoptosis in prostate cancer cells. In contrast, IGFBP-3 has been shown to induce apoptosis in prostate cancer cells through IGF-I-independent pathways. Further evidence for these effects was provided by studies showing that IGFBP-3 mutants that do not bind IGFs stimulate apoptosis in human prostate cancer cells. Together, these studies suggested that mitogenic signaling as well as cell survival signaling via the IGF/IGFBP-3 pathway is constitutively activated in human prostate cancer cells. In the present study, apigenin was able to induce elevated IGFBP-3 levels with a simultaneous decrease in IGF-I levels both in cell culture and in vivo environments correlated with cell growth inhibition and apoptosis of cancer cells. These results suggest that prostate cancer inhibition by apigenin may be due in part to modulation in IGF axis signaling **(Fig. 3)**.

In conclusion, our findings present the possibility that apigenin has the potential to target IGF signaling, a strategy that may be useful in the prevention or treatment of prostate cancer.