Abstract. Extrarenal synthesis of the active vitamin D metabolite 1,25-dihydroxyvitamin-D₃ (1,25-D) has been observed in cells derived from human organs prone to sporadic cancer incidence. Enhancement of the synthesizing hydroxylase CYP27B1 and reduction of the catabolic CYP24 could support local accumulation of the antimitotic steroid, thus preventing formation of tumors of, e.g., colon and breast. By applying quantitative RT-PCR and HPLC it was observed that in colon-(Caco-2) and breast-(MCF-7) derived cells, 17β-estradiol and genistein induced CYP27B1 but reduced CYP24 activity, while equol was inactive. Mammary cells express both estrogen receptors (ER) α and β, while colon cells express mainly ERβ, possibly explaining why MCF-7 cells were more affected. These results indicate a potential, new approach for cancer prevention by counteraction of the 1,25-D-driven negative feedback, i.e., down-regulation of CYP27B1 and up-regulation of CYP24, which prevents its own local accumulation. However, mammary cells may be more susceptible to this than colonocytes.

Epidemiological studies have suggested that hormone replacement therapy (HRT) reduces the incidence of colorectal cancer (CRC) in postmenopausal women (1). In addition, preservation of bone density is supported. However, there are significant risks associated with taking HRT: the breast and endometrial cancer incidence is increased as well as stroke and coronary heart disease (2). Therefore, alternatives to HRT are urgently required, also in view of the fact that the male population might benefit from substances that could reduce their considerable burden of colorectal tumors.

Epidemiological studies have also demonstrated a low CRC incidence in Asian countries where a soy-rich diet is consumed (3, 4). A major component of soy beans are phytoestrogens which bind to estrogen receptors (ER) and induce transcription of ER-responsive genes. This suggests that mechanisms of CRC prevention could potentially be shared by estrogens and phytoestrogens, though the negative effects of estrogen treatment may be avoided with the plant-derived homolog.

It has been demonstrated that phytoestrogens possess ER-β-selective transcriptional activity (5, 6). Among phytoestrogens, the isoflavones are the most prominent group of compounds. Numerous studies have shown the potential of the isoflavones genistein and daidzein in the regulation of cell growth, apoptosis and angiogenesis. While studies of the chemopreventive effects of genistein in vivo may be impaired by its variable metabolism, such as conjugation and deconjugation that occur in the gastrointestinal tract (7), in vivo studies with daidzein have been hampered by the suggestion that its metabolite equol is the active compound and this metabolism is highly diverse among individuals (see, e.g., (8)). Recently, a study indicated that differences in daidzein metabolism could be related to postmenopausal mammographic density, an intermediate marker of breast cancer risk (9).

A potential role of extra-renal vitamin D synthesis in tumor prevention with respect to the progression of malignancy has been well recognized. Ours was the first group to demonstrate CYP27B1 activity in colonocytes in culture (10) and that in freshly isolated human colon tumor cells a wide spectrum of vitamin D metabolites can be detected (11). While renal hydroxylases regulate systemic 1,25-dihydroxyvitamin-D₃ (1,25-D) levels, extra-renal synthesis 1,25-D could modulate cell proliferation and differentiation in an autocrine and/or paracrine manner. In human colon tissue, semi-quantitative as well as quantitative RT-PCR and immunofluorescence indicated increasing CYP27B1 expression during early CRC progression, which was greatly diminished in late-stage high-grade tumors (12,

Genistein and 17β-estradiol, but not Equol, Regulate Vitamin D Synthesis in Human Colon and Breast Cancer Cells

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Despite its high affinity for ER', expression, whereas equol did not prove to be active at all, than in colon-derived cells commensurate with ER-mediated actions of genistein, equol and 17'-estradiol (17'-E2) on vitamin D metabolism in colon cancer cells (16). In mice fed a low calcium diet (0.04%) resulting in enhanced proliferation of colonocytes, genistein was even more effective and not only increased the expression of CYP27B1, but also significantly reduced that of CYP24, the catabolizing vitamin D hydroxylase (17).

Recently, it was reported that, in a mouse model, there was a concentrative effect for genistein in the colon and that genistein increased the expression of CYP27B1 in the gut mucosa (16). In mice fed a low calcium diet (0.04%) resulting in enhanced proliferation of colonocytes, genistein was even more effective and not only increased the expression of CYP27B1, but also significantly reduced that of CYP24, the catabolizing vitamin D hydroxylase (17).

In the present study, a mechanism of action for these in vivo observations was investigated by comparing the ER-mediated actions of genistein, equol and 17β-estradiol (17β-E2) on vitamin D metabolism in colon cancer cells with that found in mammary cancer cells in vitro. Our results demonstrated regulation of both CYP27B1 and of CYP24 by genistein and by 17β-E2, in mammary cells more than in colon-derived cells commensurate with ER expression, whereas equol did not prove to be active at all, despite its high affinity for ERβ.

Materials and Methods

Cell cultures. A panel of cell lines, including colon-, prostate- and breast-derived human cells, were initially studied for expression of ERs (Figure 1). For further experiments, the well-differentiated colon cancer cell line Caco-2, as well as the breast cancer cell line MCF-7, which is an inhomogeneous mixture of primarily undifferentiated, blast-like cells and a minority of cells showing a more differentiated phenotype, were selected.

At 37°C in a humidified atmosphere of 95% air and 5% CO2, the cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM; colon cell lines and MCF-7) and RPMI-1640 medium (prostate cells), respectively. The media were supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate (Sigma, Vienna, Austria; only RPMI-1640), 10 mM Hepes (only DMEM), 50 IU penicillin, 50 mg/ml streptomycin and 4 mM glutamine (all from Gibco Life Technologies, Gaithersburg, MD, USA). The media were changed every second day. Two days after confluency, the FCS was replaced with 10 mg/ml transferrin (Sigma) and 5 ng/ml sodium selenite (Merck, Darmstadt, Germany). After a period of 48 h, incubations were performed in fresh medium for the indicated durations.

Treatments. 17β-E2, 25-hydroxyvitamin-D3 (25-D) and genistein were purchased from Sigma (Vienna, Austria), equol (racemic mixture) was kindly donated by Dr. Herman Adlercreutz (University of Helsinki, Finland). 1,25-D was kindly provided by Hoffmann-La Roche (Basel, Switzerland). Stock solutions were prepared in ethanol (1,25-D, 17β-E2) and DMSO (genistein, equol), respectively, and were stored at -20°C until used for experiments.

High pressure liquid chromatography (HPLC). 0.5 µCi/ml 25-OH-[26,27-methyl-3H]-D3 (from now on referred to as [3H]-25-D; specific activity 30 Ci/mmol; purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK) as a tracer was added to each well containing serum-free medium at a final concentration of 16.6 nM 25-D. The experiments were terminated with methanol. For lipid extraction, the cells were scraped off and – together with the medium and methanol – transferred into glass tubes; each well was rinsed with H2O and methanol. One nM each of 25-D and 1,25-D were added as internal standards to evaluate the efficiency of the extraction procedures. Following the addition of dichloromethane (CH2Cl2) and centrifugation, the lower phase was collected and the supernatant was re-extracted twice with CH2Cl2. The extracts were dried under a gentle stream of nitrogen at 55°C. After reconstitution in the mobile phase (94% n-hexane: 6% isopropanol), the extracts were directly subjected to HPLC analysis.

HPLC was performed on a system encompassing a pump (Model 515, Waters, Vienna, Austria) operating with an isocratic flow rate of 2 ml/min, an UltraspHERE Silica column (5 mm, 4.6 x 250 mm; Beckman Instruments, Fullerton, CA, USA) for separation of vitamin D metabolites, a photodiode array detector (model SPD-M10Avp, Shimadzu, Japan) for monitoring UV absorption of added standards and a flow radiochromatography detector for detection of tritium-labelled metabolites (model CS05TR from Packard Bioscience, Groningen, The Netherlands) after addition of Ultima Flow™ M scintillation fluid (Packard...
Bioassays at a flow rate of 2 ml/min. The eluted metabolites of \( ^{3}H \)-25-D were identified by comigration with known standards. The identity of vitamin D metabolites had been verified previously by GC/MS (18). Counts per minute (cpm) were measured and areas under the curve (AUC) were used for evaluation of enzymatic activities.

Reverse transcriptase – polymerase chain reaction (RT-PCR). Total RNA was prepared with TRIZol™ reagent (Invitrogen, Lofer, Austria) according to the manufacturer’s instructions. Two micrograms of RNA were reverse-transcribed (SuperScript™ II; Invitrogen) using oligo-dT primer (for semi-quantitative RT-PCR) and random hexamer primer (for real-time RT-PCR), respectively. cDNA strands were used for PCR (Taq PCR Core Kit™, Qiagen, West Sussex, UK). The expression of ER\( \alpha \) mRNA was evaluated by amplification of a 279-bp fragment with the following primers: 5'-AGA CAT GAG AGC TGC CAA CC-3' (sense) and 5'-GCC AGG CAC ATT CTA GAA GG-3' (antisense). The PCR conditions were: 15 sec at 94°C, 30 sec at 59°C and 1 min at 72°C for 40 cycles. To identify a 346-bp segment of ER\( \beta \), the following primers were used: 5'-TCA CAT CTG TAT GCG GAA CC-3' (sense) and 5'-CGT AAC ACT TCC GAA GTC GG-3' (antisense). The PCR conditions were: 15 sec at 94°C, 30 sec at 58°C and 1 min at 72°C for 40 cycles. The \( \beta \)-actin primers used were: 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT-3' (sense) and 5'-CTA GAA GG-3' (antisense). The PCR products were loaded onto a 2% agarose gel containing ethidium bromide and were separated at 80 V and 250 mA. The bands were analyzed using a video camera imaging system under UV light (Herolab, Wiesloch, Germany).

Quantitative real-time RT-PCR. In order to assess mRNA levels accurately, the CYP27B1 mRNA levels were the quantified by the comparative \( \Delta \Delta C_{T} \) method. The reliability of the data was improved by including an invariant endogenous control, 18S rRNA, to correct for sample-to-sample variations in RT-PCR efficiency and errors in sample quantification. Relative abundance values were then calculated for 18S rRNA as well as for the experimental CYP27B1 sequence. For each experimental sample, the relative abundance value obtained was normalized to the value derived from the control sequence (18S rRNA) in the corresponding sample. The normalized values for different samples were directly compared. The real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Triplicates were set up for each sample and transcript under investigation. PCR conditions were: 50°C for 2 min (UDG [uracil DNA glycosylase] decontamination step), 94°C for 2 min, which was followed by 40 cycles of 94°C for 15 sec and 60°C for 30 sec. Real-time PCR analysis was performed with FAM-labeled "Assays-on-Demand™ Gene Expression" products for 18S rRNA and CYP27B1 (Applied Biosystems).

Results

Expression of estrogen receptors \( \alpha \) and \( \beta \). The expressions of ER\( \alpha \) and ER\( \beta \) mRNA in six different human cell lines are illustrated in Figure 1. Three of the cell lines used originate from human colon tumors at different levels of malignancy (Caco-2 cells are highly-differentiated, COGA-1 cells are derived from a G2 tumor and undifferentiated, COGA-13 cells are derived from a tumor graded G3). The other three are well-established cell lines from the prostate (the immortalized normal PNT-2 and the tumor-derived DU-145) and the mammary gland (MCF-7). While ER\( \alpha \) mRNA expression (upper panel) was shown to be comparatively low in all of the tested colon-derived cell lines, whether in the proliferative (subconfluent) or the quiescent (confluent) state, very high levels of ER\( \alpha \) were found in MCF-7 and PNT-2. Subconfluent DU-145 cells showed only intermediate levels, which further decreased in the quiescent state. In contrast, ER\( \beta \) mRNA expression (middle panel), in most cases, was well represented in the confluent colonic cell lines, even in the undifferentiated COGA-13. Among the cells derived from classic hormone-responsive organs, DU-145 and MCF-7 strongly expressed ER\( \beta \), although confluence seemed to be associated with a weak down-regulation of ER\( \beta \). The lower panel shows evenly distributed amounts of cytoskeleton-associated \( \beta \)-actin mRNA, which served as the internal control in this experiment.

For further evaluation of the potential effects of the two isoflavones, genistein and equol, as well as 17\( \beta \)-E2, on vitamin D metabolizing hydroxylases, Caco-2 colon and MCF-7 breast cancer cells were selected.

Regulation of the vitamin D synthesizing hydroxylase CYP27B1 mRNA expression. The influence of genistein and equol (both at 1 \( \mu \)M) and the effect of 10 nM 17\( \beta \)-E2 on the expression of CYP27B1 mRNA in MCF-7 (Figure 2A) and Caco-2 cells (Figure 2B) was evaluated by real-time RT-PCR. As demonstrated in Figure 2A, treatment of MCF-7 cells with 17\( \beta \)-E2 tripled the CYP27B1 expression after 4 h, whereas genistein, while elevating the expression significantly, did not even double it. Both compounds, however, exerted maximum effects at 4 h and no significant differences in CYP27B1 could be found after administration for 8 h. Interestingly, equol was found to have no effects on CYP27B1 mRNA expression at all.

Though the response of Caco-2 cells to 17\( \beta \)-E2 was weaker than that found in the mammary cell line (expression was maximally doubled), the induction of CYP27B1 expression after 4 h was still significant. Genistein, in the colon cells as well, had less effect on stimulation of CYP27B1 expression than 17\( \beta \)-E2, since it maximally raised the mRNA expression by 60% over the control values. Again, equol had, if any, only marginal activity.

Regulation of CYP27B1 activity by 17\( \beta \)-estradiol and isoflavones. The results illustrated in Figure 3, obtained by HPLC, show the typical response of the Caco-2 cells to the control (A) and 1 \( \mu \)M genistein (B) treatments for 24 h. Under control conditions, Caco-2 cells were exposed to the radioactively-labelled precursor molecule \( ^{3}H \)-25-D for 6 h
and the 1,25-D peak which appeared after an elution time of approximately 27 min identified this cell line as having constitutively highly active CYP27B1. A smaller peak shortly before 25 min represented conversion of the hormonally-active metabolite to its epimer, 1,25-dihydroxy-vitamin-epi-D₃ (1,25-epi-D). As shown in the relevant histogram (C), treatment with genistein elevated 1α-hydroxylase activity in Caco-2 cells by 37%.

The data from a series of HPLC experiments investigating the effects of genistein (B), equol (trace not shown) and 17β-E2 (C) on CYP27B1 activity in MCF-7 cells when compared with control treatments (A) are presented in Figure 4. In comparison to Caco-2 cells, a rather low basal CYP27B1 activity was observed for MCF-7 cells, while genistein stimulated synthesis of 1α-hydroxylated metabolites by more than 70% and 17β-E2 more than doubled the synthesis (see also histogram; D). In contrast and in accord with real-time RT-PCR data, equol did not affect CYP27B1 activity at all.

Effect of isoflavones and 17β-estradiol on CYP24 activity. We have previously shown in colon cells that 1,25-D down-regulated the expression and activity of its synthesizing enzyme CYP27B1 and causes up-regulation of its own degradative pathway (19). In order to optimize and maintain high levels of 1,25-D generated by colon or mammary cells, this degradative pathway should be inhibited. A strong inhibitory effect of genistein on CYP24 mRNA expression and activity was demonstrated in prostate cells (20). When treating MCF-7 cells with 10 nM 1,25-D for 12 h, the typical induction of CYP24 and reduction of CYP27B1 metabolites were observed (Figure 5A). Both of these effects can be reversed, to different degrees, by exposure to genistein or 17β-E2 for 24 h. Whereas the phytoestrogen almost completely restored the production of 1α-hydroxylated metabolites, treatment with 17β-E2 caused 1,25-D and 1,25-epi-D to reach levels which even exceeded the control levels. Importantly, this was made feasible by the combined
stimulation of CYP27B1 and inhibition of CYP24. However, the metabolite of daidzein, equol, did not alter the activity of either CYP27B1 or CYP24 in the MCF-7 cells.

When treating Caco-2 cells with 10 nM 1,25-D, the typical reduction of CYP27B1 activity by almost 40% was observed. In contrast to MCF-7 and several other confluent cell lines tested, however, 1,25-D was unable to induce CYP24 activity in the highly-differentiated Caco-2 cells. Hence, in these colon cancer cells, the augmentation of 1,25-D production with 1 µM genistein can solely be explained by a direct influence on the 1,25-D-synthesizing enzyme (Figure 5B).

Discussion

A number of epidemiological studies found that certain Asian populations, known for their high soy consumption, have a clearly manifold lower risk of developing cancer of hormone-
responsive organs. This has been suggested for colon cancer as well. The primary goal of this in vitro study was to investigate whether extra-renal vitamin D metabolism might be involved. Our results demonstrated that genistein, a prominent phytoestrogen contained in soy, induced CYP27B1 and reduced CYP24 expression and activity, whereas equol was found to have no effects on vitamin D hydroxylases. In particular, genistein is known to exert a number of different biological effects not related to estrogenic pathways (4). However, we found that similar, although clearly stronger, effects on extra-renal vitamin D metabolism could be evoked by the administration of the natural endogenous hormone 17β-E2, thus corroborating a mechanism of action mediated by the cellular estrogenic system. Further support for this hypothesis comes from the distinct distribution of ERs in the two cell lines chosen: comparably high levels of ERβ were found in both confluent Caco-2 and MCF-7 cells, whereas ERα mRNA was essentially absent in Caco-2, but MCF-7 were highly ERα-positive. Although the effectiveness of the isoflavone and 17β-E2 are much higher in MCF-7 than in the Caco-2 cells, these results point to a mechanism primarily involving ERβ. An increasing amount of data point to a predominant role of ERβ as mediator of (phyto-)estrogenic action, especially in the colon. It has been previously suggested that genistein binds preferentially to ERβ (21). In transactivation assays for ERα and ERβ in MCF-7 mammary cells, genistein showed robust transactivation of ERα-and ERβ-induced transcription, however with an up to 100-fold stronger activation of ERβ (22). Against a background of 17β-E2, genistein addition resulted in superstimulation of transcription. The IC50 values for estrogenic properties in MCF-7 cells were determined at 1x10^-11 M for 17β-E2, 4x10^-8 M for genistein and 1x10^-7 M for equol (23).

It has been claimed that the biological effects of soy foods are mainly due to the equol produced by fecal bacteria (24). Equol is bacterially derived from daidzein as two distinct diastereoisomers. The S-equol enantiomer is apparently the active metabolite since it has a high affinity for ER-β, whereas R-equol is rather inactive (25). Since only about 30–50% of the human population are equol producers this could potentially account for discrepancies in human nutritional studies with soy (8). In previous in vitro studies with prostate cells, we found that daidzein did not affect vitamin D hydroxylase expression at all. However, the racemic mixture of equol used in this study was not effective either. At 1 μM, equol did not alter the expression of CYP27B1 mRNA; neither did it affect the activity of CYP27B1, nor that of the degrading enzyme CYP24. While there is some evidence for a relationship between daidzein-metabolizing phenotypes and human health, such as reduced risk of breast and prostate cancer, this must follow another mechanism of action than that concerning the extra-renal synthesis of 1,25-D.

Our results clearly demonstrate that genistein as well as 17β-E2, at physiological doses of 1 μM and 10 nM, respectively, up-regulated the expression and synthesizing activity of CYP27B1 and down-regulated the catabolic vitamin D hydroxylase, both in mammary and colon cells. While HRT is considered to have potentially serious health hazards, phytoestrogens might serve as an alternative. In this respect, genistein could attenuate the negative feedback mechanism which prevents the antiproliferative 1,25-D from reaching tissue concentrations sufficient to counteract tumor formation and/or progression. A number of studies have identified colonic tissue as being especially susceptible to the cancer-preventive action of vitamin D and its metabolites. Thus, maintaining high tissue levels of 1,25-D could help to prevent tumor formation and progression particularly in the colon, although other organs, such as breast and prostate, may be positively influenced as well.

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