ABSTRACT Soybean products are highly represented in the traditional Asian diet. Major components of soy proteins are phytoestrogens, such as isoflavones. They may be responsible for the extremely low incidence of prostate and mammary tumors and possibly also of colon cancer in countries such as China and Japan. Serum 1,25-dihydroxyvitamin D3 level is inversely related to incidence of some cancers. Levels are determined by skin exposure to ultraviolet light or, to a minor extent, nutritional uptake and by subsequent conversion of the precursor vitamin D to the active hormone by the cytochrome P450 hydroxylases CYP27A1, CYP27B1 (responsible for synthesis) and CYP24 (responsible for catabolism) in liver and kidney. However, vitamin D synthesis is also found in colonocytes and is enhanced during incipient malignancy. This may indicate an autocrine/paracrine role for this differentiation-inducing hormone in defense against progression. We were able to demonstrate that either a single large oral dose of genistein or feeding soy protein for 4 mo elevated CYP27B1 and decreased CYP24 expression in the mouse colon. Our data therefore suggest that an inverse correlation of soy product consumption with colon cancer incidence may be consequent to enhanced colonic synthesis of the antimitotic hormone 1,25-dihydroxyvitamin D3.

KEY WORDS: • soy protein feeding • genistein • 1,25-dihydroxyvitamin D3 synthesis • 25-hydroxyvitamin D3-1α-hydroxylase (CYP27B1) • 25-hydroxyvitamin D3-24-hydroxylase (CYP24)
and some of its synthetic analogs (5) may act as antimitotic and prodifferentiating agents in, for example, colon cancer cells, the treatment of tumor patients with the active metabolite is still not feasible with most vitamin D analogs because of their hypercalcemic effect at the large pharmacologic doses necessary to achieve antimitotic action.

A completely different and unexpected physiological link between vitamin D and colon cancer treatment and prevention was discovered only recently. We were the first to demonstrate that colon cells in culture can synthesize 1,25-D$_3$ from the precursor 25-D$_3$ (6). Our further studies showed that levels of CYP27B1 and vitamin D receptor mRNA increased very early during human colon tumor progression, whereas expression is greatly diminished in late-stage high-grade cancer tissue. In contrast, expression is exceedingly low in colon crypts of people without cancer (7–9). This suggested the existence of an autocrine protective action of 1,25-D$_3$ synthesized during incipient malignancy in colon cells that could slow down or even stop disease progression. In late-stage high-grade cancer, this protective system apparently fails, conceivably because of further somatic mutations. However, at the same time high or aberrant expression of the 25-hydroxvitamin D$_2$-24-hydroxylase (CYP24) at the tumor site (9) could cause rapid metabolism of 1,25-D$_3$ into less active vitamin D compounds and thus could counteract any of its inhibitory actions on tumor growth. In this respect it is of interest that CYP24 was recently suggested to be a potential oncogene (10).

These data suggested to us a new concept for colon cancer prevention and potentially also for therapy: increasing the availability of 1,25-D$_3$ either in serum or produced endogenously could influence tumor growth. In this respect it is of interest that CYP24 at the tumor site (9) could cause rapid metabolism of 1,25-D$_3$ into less active vitamin D compounds and thus could counteract any of its inhibitory actions on tumor growth. In this respect it is of interest that CYP24 was recently suggested to be a potential oncogene (10).

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A significantly lower prevalence of hormone-dependent cancers, such as prostatic and mammary cancers, occurs in Asian compared with Western industrialized countries (11), but a reduced incidence of colorectal cancer may occur as well. The reduced incidence has been linked to the consumption of the typical Asian diet, which contains high amounts of soy products and thus is rich in phytoestrogens, particularly in isoflavonoids. One major isoflavonoid is genistein, which has negative effects on tyrosine kinases (12) and topoisomerases (13). Through their potential to act as selective estrogen receptor modulators, genistein and other phytoestrogens could conceivably affect vitamin D–related inhibition of tumor growth. Interactions between vitamin D and estrogen have been observed in human breast cancer cells (14) and in murine colon carcinomas (15). Because genistein is an inhibitor of several members of the cytochrome P450 enzyme family (16–18), a likely site of interaction with the vitamin D system is at the regulation of expression and activity of the vitamin D–metabolizing cytochrome P450 enzymes, CYP27B1 and CYP24. We therefore initiated a study to address the questions of whether soy protein fed to mice as a normal food constituent for several months would alter CYP27B1 and CYP24 expression and whether a single gavage with 250 µg genistein would provide a similar result.

**MATERIALS AND METHODS**

**Animals and diets.** C57BL/6 mice were housed in the Centre for Laboratory Animal Care at the University of Vienna in a contained environment. Mice were weaned at age 2–3 wk and then fed ad libitum either the standard diet (basic AIN 76A) (19) or a soy diet (AIN 76A containing 200 g extracted soybean meal/kg instead of casein). Mice were anesthetized with ether and killed by cervical dislocation at age 16–17 wk. Another group was fed the standard diet and 250 µg genistein (Sigma-Aldrich, Vienna, Austria) in 70 µL H$_2$O/5% ethanol or vehicle only was administered via gavage. Blood was collected from the tail vein, and again, 24 h after gavage, when animals were killed and tissue samples were collected. Treatment groups consisted of at least 8 animals. The study protocols were reviewed and approved by the institutional committee of animal experimentation of the University of Vienna Medical School and by the Austrian Ministry of Science and Education.

**Determination of tissue concentrations of genistein.** Colon was extirpated from mice 24 h after oral administration of 250 µg genistein, freeze-dried, and stored at −70°C. For the experiments, 50 µg of samples was dissolved in 300 µL H$_2$O and sonicated for 10 min; 700 µL methanol was added for precipitation overnight at −20°C. After centrifugation and reprecipitation, methanol was evaporated and the water phase was extracted with hexane to remove lipids. The delipidated water phase was incubated with the hydrolysis mixture (ascorbic acid, charcoal-stripped Helix Pomatia extract; Bisefra, France) for 2 h at 60°C. Isoflavonoids were extracted twice with diethyl ether. The ether phase was evaporated in a stream of air, dissolved in 300 µL of assay buffer (0.5% bovine serum albumin-Tris, pH 7.76). Samples were analyzed by time-resolved fluorescence immunoassay.

**Determination of plasma concentrations of genistein.** Blood was collected from mice 6 and 24 h after oral administration of 250 µg genistein. Plasma was prepared by conventional methods and freeze-dried. For analysis, samples were resolated in the same volume of distilled H$_2$O as the original plasma volume. Enzymatic hydrolysis was performed as described (20). Briefly, 0.2 U β-glucuronidase/mL (Boehringer Mannheim, Mannheim, Germany) and 1 U sulfatase/mL (Sigma) were added, and samples were incubated overnight at 37°C. Isoflavonoids were extracted from plasma twice with diethyl ether. After evaporation of the ether phase, samples were suspended in 300 µL assay buffer (0.5% bovine serum albumin–Tris pH 7.76). Levels of genistein were analyzed by time-resolved fluorescence immunoassay.

**Time-resolved fluorescence immunoassay.** Time-resolved fluorescence immunoassay was performed as indicated (20). Briefly, 15 µL of [4]-estradiol glucuronide (NEN Life Science Products, Wallac Oy, Turku, Finland) was added to tubes to measure recovery; 20 µL of hydrolyzed plasma was pipetted into prewashed goat anti-rabbit immunoglobulin G microtiterplate strips (Wallac Oy). To each was added 100 µL antiserum and 100 µL europium-labeled genistein. After incubation on a plate shaker at room temperature for 90 min, the strips were washed and 200 µL of dissociation-enhanced lanthanide fluorescence immunoassay enhancement solution (Wallac Oy) was added; after agitation for 5 min, analysis was performed in a VICTOR 1420 multilabel counter (Wallac Oy).

**Western blot analysis.** Western blot analysis was performed as described previously (4). Briefly, total protein, extracted from snap-frozen ascending and descending mouse colon, was separated by 12% SDS–polyacrylamide gel electrophoresis and subsequently blotted to a nitrocellulose membrane. The membranes were incubated overnight with sheep anti-CYP27B1 (The Binding Site, Heidelberg, Germany), sheep anti-CYP24 (kind gift from Dr. Moray Campbell, University of Birmingham, U.K.) and mouse anti-cytokeratin 8 (CK 8) (Cymbus Biotech, Charders Fort, Hant, U.K.) antibodies. Horseradish peroxidase–conjugated secondary antibodies (Amersham Life Sciences, Buckinghamshire, U.K.) were used. Subsequent detection was performed with the SuperSignal CL-HRP substrate system (Pierce, Rockford, IL). Bands were evaluated by densitometry with a video camera imaging system (Herolab, Wiesloch, Germany).

**Semiquantitative reverse transcription–polymerase chain reaction.** For analysis of CYP27B1 and CYP24 mRNA in relation to CK 8 mRNA expression by reverse transcription–polymerase chain reaction (RT-PCR), total RNA was extracted from snap-frozen mouse colon ascends and descends with TRIzol reagent (Invitrogen, Paisley, U.K.); 2 µg total RNA was used for synthesis of single-stranded cDNA (SUPERScript II kit, Invitrogen).

Multiplex RT-PCR (i.e., simultaneous amplification of transcripts...
specific for either CYP27B1 or CYP24 and a transcript specific for the epithelial cell marker CK 8) was used for semiquantitative evaluation of mRNA expression levels.

PCR conditions were established for each individual primer pair and subsequently adapted for multiplex PCR with the CK 8 primers: 15 s at 94°C, 30 s at 66°C and 1 min at 72°C for 34 cycles using the GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, CA). Primers used were the following: CYP27B1 sense, 5’-CAA GCA GCC GCG GGC TAT GCT GG-3’; CYP27B1 antisense, 5’-TGT CGT CTT GGA GGG AAT TCC-3’; CYP24 sense, 5’-AAG GAC ACA GAG GAA GCC -3’; CYP24 antisense, 5’-GAA TGG CAC ACT TGG GTG AA-3’; CK 8 sense, 5’-GTG CCC AGT ACG AGG ACA TTG-3’; and CK 8 antisense, 5’-TGT TGC GGT TCA TCT CGG AG-3’. PCR products were checked for correct size and fragment length through multiple digestions with restriction enzymes. Gels were scanned and analyzed with a video camera imaging system (Herolab), and band density was measured under ultraviolet light. The levels of CYP27B1 and CYP24 expression were correlated with that of the epithelial cell marker CK 8. For quantification of immunoblotting, densitometric units were referred to a Caco-2 cell homogenate as 100% control. Data are mean ± sd (n = 8 mice). *P < 0.05 vs. vehicle.

CYP24 protein is about equally distributed in different parts of the mouse colon and is approximately equally expressed by soy feeding (Fig. 2B). CYP27B1 levels were enhanced by the soy diet (data not shown).

**RESULTS**

**Effect of gavage with genistein**

**Plasma and colon tissue accumulation.** When 250 μg genistein was administered via gavage and blood was drawn from the tail vein after 6 h, we found a >3000-fold accumulation of genistein in plasma compared with control animals. The second blood sample after 24 h showed a steep decline of genistein concentration to a 40-fold accumulation. Interest-

**CYP24 mRNA and protein expression.** There is significant variation of CYP24 mRNA expression in colon segments. Although CYP24 mRNA is more highly expressed in the ascending colon of control animals and is suppressed by soy feeding, control values are much lower in colon descendents and soy does not affect its expression (Fig. 2A). In contrast,
genistein and glycine. In Japanese who consume their traditional diet, plasma concentrations of genistein of up to 2960 nmol/L are measured (21). This far exceeds human normal plasma estradiol concentrations, which range between 147 and 294 nmol/L. Relative molar binding affinities of different estrogenic compounds revealed that phytoestrogens have significantly higher affinities for estrogen receptor β than for estrogen receptor α (22). Recently it was suggested that in women the major type of estrogen receptor may be estrogen receptor β (23).

Although genistein, the major isoflavonoid present in soybeans, was shown to inhibit protein tyrosine kinase activity, which links its action to growth factor signaling pathways, a definite mechanism for cancer prevention in vivo has not been determined. One mode of preventive action may be via the regulation of cytochrome P450 enzymes involved in the synthesis of 25-D₃. This secosteroid has consistently been shown in many laboratories to have general antimitotic pro-differentiating action on cancer cells (24). However, elevation of its serum levels would result in hypercalcaemia. Thus the concept of extrarenal production of 1,25-D₃ with localized differentiating action on cancer cells (24) is supported by findings that phytoestrogens may decrease CYP24 in proximal and distal mouse colon, which again would lead to optimal autocrine/paracrine action (25). We were able to demonstrate increased expression of CYP27B1 and decreased expression of CYP24 in proximal and distal mouse colon after a single high oral dose of genistein. CYP27B1 mediates 1α-hydroxylation of the precursor 25-D₃, whereas CYP24 could, when highly expressed, conceivably be achieved by nutritional means, we fed mice the basic AIN-76A diet with 20% soy as the protein source. This resulted in consistent downregulation of CYP24 in the mouse colon, which again would lead to optimal autocrine/paracrine production of the secosteroid in colon cells. Interestingly, this hydroxylase regulation coincides with measurable accumulation of genistein in mouse colon tissue, which is not as substantial as that reported by us in the mouse prostate, where 50-fold tissue concentrations of genistein are reached via some unknown concentrative mechanism (25).

During human food consumption, even under optimal conditions, high concentrations of genistein such as we used for gavage would never be reached. Because we wanted to ascertain whether regulation of vitamin D hydroxylases could conceivably be achieved by nutritional means, we fed mice the basic AIN-76A diet with 20% soy as the protein source. This resulted in consistent downregulation of CYP27B2 in the mouse colon, which again would lead to optimal autocrine/paracrine production of the antimitotic secosteroid 1,25-D₃. Although a conclusive link among colonic 1,25-D₃ synthesis, phytoestrogen consumption and reduced human colon cancer incidence still needs to be established, it is interesting in this respect that colon cancer morbidity and mortality rates are lower in women than in men (26) and that hormone replacement therapy has consistently been implicated in reduced risk of colon cancer occurrence (27).

LITERATURE CITED