Phytoestrogens and carcinogenesis—differential effects of genistein in experimental models of normal and malignant rat endometrium

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The phytoestrogen genistein was studied in normal and malignant experimental uterine models in vivo. The action of genistein on the uterus and vagina of ovariectomized DA/Han rats after 3 day oral administration (25, 50 or 100 mg/kg/BW/d) was compared to ethinyl oestradiol (0.1 mg/kg/BW/d). Effects on uterine and vaginal morphology, uterine growth and uterine gene expression were studied. A dose dependent increase of the uterine wet weight and the uterine and vaginal epithelial height, a dose dependent up-regulation of complement C3, down-regulation of clusterin mRNA expression and a stimulation of the vaginal cornification was observed after administration of genistein. Uterine gene expression and vaginal epithelium respond to genistein at doses where no significant effects on uterine wet weight were detectable. In general the vagina was more sensitive to genistein than the uterus. To analyse the action of genistein in malignant uterine tissue, the impact of a 28 d treatment with 50 mg/kg/d of genistein on the in-vivo tumour growth of RUCA I endometrial adenocarcinoma cells, following subcutaneous inoculation into syngeneic DA/Han rats, was assessed. In contrast to ethinyl oestradiol (0.1 mg/kg/BW/d), a dose of 50 mg/kg/BW/d of genistein did not affect tumour growth. Nevertheless C3 and TRPM2 mRNA expression in the tumour were both significantly stimulated by ethinyl oestradiol and genistein. In comparison to ovariectomized animals genistein up-regulated uterine wet weight and uterine dependent gene expression in tumour bearing animals. In conclusion, four independent uterine and vaginal parameters indicate genistein is a weak oestrogen receptor agonist in the uterus and vagina of female DA/Han rats, and evidence is provided for a selective oestrogen receptor modulator (SERM)-like action of genistein in normal and malignant uterine tissue.

Key words: endometrial adenocarcinoma/gene expression/phytoestrogen/rat model/uterus

Introduction

Oestrogen-like effects have been described for a variety of naturally occurring, mainly plant derived substances as well as for numerous synthetic compounds. There is general agreement that high doses of these substances either under laboratory conditions or at extremely polluted sites in the natural environment can affect the endocrine system, causing developmental, reproductive and presumably tumourigenic disorders (Setchell et al., 1984, 1987; McLachlan and Newbold, 1987; Colborn and Clement, 1991; Jones and Hajek, 1995). The relative risks associated with general exposure to oestrogenic-like compounds are controversial. This controversy stems from the uncertainty of how to assess the risk originating from exogenous chemicals interacting with such a complex system as the endocrine system and its even more complex hierarchical organization (Golden et al., 1998, for review).

The biological relevance of xenoestrogens has been questioned as they are found at low concentrations, either in food or as an environmental contaminant. However, considerable concern has been raised in recent publications, which indicate that these substances may cause adverse effects at low, environmentally relevant concentrations (Howdeshell et al., 1999). At present it is difficult to define scientifically sound thresholds (no effect levels) for these compounds. Depending on the model, it has been questioned whether it will be possible to define such a threshold level at all (Sheehan et al., 1999).

Naturally occurring oestrogens (so-called phytoestrogens) are believed to exhibit predominantly beneficial effects in the prevention of breast cancer in animals and humans (Constantinou et al., 1996; Fritz et al., 1998; Lamartiniere et al., 1998a), although it has been known for a long time that plant- and fungi-derived substances can lead to reproductive disturbances in animals (McLachlan et al., 1984), e.g. clover disease in sheep (Shutt, 1976). Depending on dietary habits,
Origin and experimental properties of RUCA-I cells. RUCA-I cells stem from an endometrial adenocarcinoma of the DA/Han rat. These cells are oestrogen sensitive in vitro and produce oestrogen sensitive, metastasizing tumours in vivo if inoculated into syngeneic DA/Han rats. Because of their rapid in-vivo growth those tumours originating from RUCA-I cells are particularly responsive to anti-oestrogen treatment.

In-vitro models are useful to assess the relative potency of environmental compounds with oestrogenic activity (Soto et al., 1995; Gaido et al., 1997) or to elucidate their molecular mode of action (Hopert et al., 1998). However in-vivo models should be chosen carefully with specific endpoints in mind.

The aim of the present study was to evaluate the action of genistein in endometrial models in vivo and to compare these results with those from a corresponding model in vitro. To do this, the relative potency of genistein, the most widely used phytoestrogen, was investigated in the rat endometrial adenocarcinoma cell line RUCA-I in vitro as well as in tumours arising from this cell line after inoculation into syngeneic DA/Han rats in vivo (Figure 1; Vollmer and Schneider, 1996). The effects of test compounds on the normal rat uterus were also evaluated over 28 d. Since this assay has to be regarded as a ‘long-term uterotrophic assay’ with intermediate complexity we included the normal 3 d uterotrophic assay in our analysis for comparative reasons. The strength of this approach is that all experimental manipulations were carried out in cells from a single organ which share a common syngeneic origin (Deerberg et al., 1985; Schütze et al., 1992), which at least limits genetic variability. This experimental procedure allowed the assessment of multiple endpoints, namely the relative binding affinity, uterine wet weight, uterine epithelial height, thickness and cornification of the vaginal epithelium, tumour weight and gene expression in normal uterine tissue and in endometrial adenocarcinoma. This made it possible to compare pre-existing results obtained in vitro with two new sets of data generated in vivo, allowing us to assess the predictive value of the in-vitro and in-vivo models. It was also possible to demonstrate that the uterotrophic assay, if coupled with gene expression analysis, is the most sensitive experimental model and superior to limited in-vitro models or to a corresponding tumour model.

Material and methods

Substances
Ethinyl oestradiol was from Schering AG (Berlin, Germany) and genistein from Sigma (Deissenhofen, Germany).

Animals
DA/Han rats were selected for these experiments since the RUCA-I cell line established from an endometrial adenocarcinoma of DA/Han rats (Vollmer and Schneider, 1996) significantly respond to oestrogen (Vollmer et al., 1995) as well as xenoestrogen treatment by an alteration of gene expression (Hopert et al., 1998). In addition, RUCA-I cells grow as oestrogen-sensitive tumours if inoculated subcutaneously into these syngeneic animals. Juvenile female DA/Han rats (130 g) were obtained from Moellegard (Moellegard breeding and research, Lille Skensved, Denmark) and were maintained under controlled conditions of temperature (20°C ± 1, relative humidity 50–80%) and illumination (12 h light, 12 h dark). All rats had free access to standard rat diet (SSNiff R10-Diet®; SSNiff GmbH, Soest, Germany) and water.

Uterotrophic assay
Animals were ovariectomized. After 14 day of endogenous hormonal decline the animals were treated 3 days p.o. with the respective compounds. The animals were randomly allocated to treatment and vehicle groups (n = 6). Ethinyl oestradiol [100 µg/kg body weight (BW)] was dissolved in ethanol and genistein (100 and 200 mg/kg BW) was dissolved in dimethylsulphoxide (DMSO). Animals were killed by decapitation after light anaesthesia with CO2 inhalation. Uterus wet weight was determined and the uteri were snap frozen in liquid nitrogen for RNA preparation.

Ectopic tumour growth
Animals were ovariectomized. After 14 days of endogenous hormonal decline 10⁶ RUCA-I cells (Vollmer and Schneider, 1996) were subcutaneously inoculated into the right flank of ovariectomized animals. Two days later animals were randomly allocated to treatment and vehicle groups (n = 10). Ethinyl oestradiol (100 µg/kg BW) was dissolved in ethanol and genistein (50 mg/kg BW) was dissolved in DMSO. After 28 days of treatment animals were killed by decapitation after light anaesthesia with CO2 inhalation. Uterus and tumour wet weight was determined and the tissues were snap frozen in liquid nitrogen for RNA preparation.

RNA isolation and complementary DNA synthesis
Total cytoplasmic RNA was extracted from the cells according to the guanidinium-thiocyanate-CsCl method (Chirgwin et al., 1979). DNA-free RNA was obtained by treatment with ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD, USA) in the presence of placental ribonuclease inhibitor for 30 min at 37°C. After phenol–chloroform extraction and ethanol precipitation, reverse transcriptions were performed using the SuperScript pre-amplification system (Life Technologies).
Northern Blotting

Northern Blot analysis was performed as described by Sambrook et al. (1989). Hybridization was carried out according to a protocol for rapid hybridization (Stratagene, Heidelberg, Germany). For reference hybridization a probe coding for 18S RNA was used. Autoradiography was performed by exposing Kodak X-omat AR ® film (Eastman Kodak, Rochester, NY, USA) to the nylon filters at −80°C in presence of an enhancing screen. Autoradiograms were analysed by densitometry.

Oligonucleotide primers for polymerase chain reactions

Based on the cDNA sequences available at the EMBL databank, the following specific primer pairs were designed: C3 sense primer 5′-CACTCCCATATTCCATCAGCTA-3′, C3 antisense primer 5′-CTGTTCATCCTGTTCCAGCTC-3′, CLU sense primer 5′-CTCCCTTCACTTCTGGATGAA-3′, CLU antisense primer 5′-GACCAGTCCTCCAGGACAGAT-3′, Cytochrome c oxidase subunit I sense primer, 5′-CTGTCCAGCCTGACCTGCTG-3′; antisense primer 5′-CAGCCCGAAGAGTGCCAGTAGTC-3′, Cytochrome c oxidase subunit I (1A) was used as a reference gene. Primers were synthesized by MWG Biotech AG (Ebersbach, Germany). Polymerase chain reaction (PCR)-products were sequenced to verify their identity and homology to corresponding cDNA sequences in the EMBL databank.

Semiquantitative PCR

Semiquantitative PCR was performed according to the method described by Murphy et al. (1990) and modified by Knauthe et al. (1996). To normalize signals from different RNA samples c oxidase subunit I was co-amplified as an internal standard. Amplification reactions were stopped before leaving the exponential phase. Amplification was performed using a Perkin–Elmer Cetus 9600® thermal cycler (Norwalk, CT, USA). Thermus flavus polymerase (0.5 U; Life Technologies); dNTPs (dATP, dGTP, dCTP and dTTP, 200 µmol/l each); and the respective oligonucleotid primers (500 ng each; Life Technologies) were added to an amount of first strand cDNA equivalent to 200 ng total RNA. The reaction volume was adjusted to 50 µl using 1×PCR buffer (50 mmol/l Tris–HCl (pH 9.0), 20 mmol/l (NH₄)₂SO₄ and 2.5 mmol/l MgCl₂. Amplification cycles comprised a 1 min step at 94°C for denaturation, a 1 min step at 58°C for annealing and a 1 min step at 72°C for elongation. Reaction products were separated on 1×Tris Borate EDTA 6% polyacrylamide gels and detected by ethidium bromide staining.

Statistical analysis

We used two-way analysis of variance followed by pair-wise comparison of selected means using the pooled within-group variance comparisons, Mann–Whitney U-test. The criterion for significance was set at P ≤ 0.05.

Results

Three day uterotrophic assay

Endometrial parameters were studied in a 3 day uterotrophic assay as described recently (Diel et al., 2000). The potential influence of genistein on immediate tumour growth was studied following inoculation s.c. of RUCA-I cells into syngeneic DA/Han rats. These cells form to a solid tumour at the ectopic site within 28 days (Horn et al., 1994). The model is particularly sensitive to any anti-oestrogenic activity, both in terms of tumour growth and tumour dependent gene expression (Wunsche et al., 1998).

Figure 2. Uterine wet weight following genistein treatment of ovariectomized DA/Han rats. Ovariectomized DA/Han rats were treated orally for 3 days with either ethinyl oestradiol or genistein using the above concentrations. Shown are uterine wet weights depending on the respective treatment. Note that dosage for ethinyl oestradiol is given in µg/kg/d, whereas doses for genistein represent mg/kg/d. * Means significant against control group, P ≤ 0.05, Mann–Whitney U-test. GEN = genistein; EE = ethinyl oestradiol.

The 3 day uterotrophic assay was evaluated on the basis of the following four independent experimental parameters: uterine weight, uterine epithelial height, uterine gene expression and vaginal epithelial height. The results demonstrated that oral administration of genistein had a profound oestrogenic effect in this assay. Genistein weakly stimulated uterine wet weight in a dose dependent manner ranging from 25 mg/kg/d to 100 mg/kg/d. The 29% and 36% increases in tissue weight detectable for the 50 mg/kg/d and 100 mg/kg/d doses were statistically significant, although much lower than that detectable for 0.1 mg/kg/d of ethinyl oestradiol (311% increase, Figure 2).

Uterine and vaginal epithelial height

Uterine epithelial height was assessed from 5 µm sections of paraffin embedded uterine tissue (Figure 3A). Following azan staining uterine epithelial height was assessed by semi-quantitative morphometry and determined using the KS 300 morphometry software (Carl, Zeiss, Oberkochen, Germany). A 3 day treatment of animals with 100 mg/kg/d of genistein resulted in a statistically significant increase in the uterine epithelial height of ~80% if compared with uteri of ovariectomized control animals (Figure 3B). Epithelial height in animals treated with 0.1 mg/kg/d of ethinyl oestradiol was threefold when compared with untreated controls (Figure 3B).

Measurement of the vaginal epithelial height proved to be a more sensitive parameter. 100 mg/kg/d genistein given orally doubled vaginal epithelial height when compared with untreated ovariectomized control animals but was roughly half as effective as an oral administration of 0.1 mg/kg/d of ethinyl oestradiol (Figure 4A). In line with these data on vaginal
growth was the finding that vaginal cornification in the presence of genistein was more advanced than in ovariectomized animals although not as prominent following ethinyl oestradiol treatment of animals (Figure 4B).

**Uterine dependent gene expression**

In a previous study we demonstrated that up-regulation of complement C3 gene expression and down-regulation of clusterin gene expression were the most sensitive markers of uterine dependent gene expression (Diel et al., 2000). Here we investigated the expression of these two genes in response to genistein. We found a dose dependent up-regulation of complement C3 gene expression and a dose dependent down-regulation of clusterin mRNA levels in response to genistein from 25–100 mg/kg/d (Figure 5). Up-regulation of complement C3 mRNA in response to 100 mg/kg/d of genistein was only 75% as effective as 0.1 mg/kg/d of ethinyl oestradiol and the down-regulation of clusterin was found to be half maximal (Figure 5).

**Genistein and endometrial tumour growth**

Data in the literature suggest that genistein’s oestrogenic and non-estrogenic activities may have a chemopreventive potential especially in the mammary gland (Ingram et al., 1997; Barnes, 1997). To test whether genistein would have an immediate effect on endometrial tumour growth in vivo we used RUCA-I endometrial adenocarcinoma cells and inoculated them into ovariectomized syngeneic DA/Han animals (Figure 1). Following a treatment period of 28 days with either 0.1 mg/kg/d of ethinyl oestradiol or 50 mg/kg/d of genistein, tumour weight and tumour specific gene expression were evaluated. To determine whether the experimental model responded to the oestrogenic treatment procedures, the response of the uterine wet weight, the height of the uterine epithelium and uterine C3 and CLU gene expression were measured.

This 28 day uterotrophic assay provided additional evidence for the oestrogenic potential of genistein. Genistein was administered orally at a concentration of 50 mg/kg/d over a period of 28 days. This increased uterine wet weight by 58% (Figure 6A), which was still far below the value seen for 0.1mg/kg/d of ethinyl oestradiol (375%). Genistein marginally increased uterine epithelial height too (13%; Figure 6B). We also tested whether treatment with genistein would slow down the growth of ectopic tumours originating following subcutaneous inoculation of RUCA-I cells into the flank of syngeneic DA/Han animals. The RUCA-I model, because of
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Figure 4. Vaginal epithelial height and cornification following genistein treatment of ovariectomized DA/Han rats. Ovariectomized DA/Han rats were treated orally for 3 days with either ethinyl oestradiol or genistein using the above concentrations. Shown are vaginal morphology (A) and semiquantitative evaluation of vaginal epithelial height by morphometry (B). Note that dosage for ethinyl oestradiol is given in µg/kg/d, whereas doses for genistein represent mg/kg/d. *Means significant against control group, P < 0.05, Mann–Whitney U-test.

its rapid growth rate in vivo, is particularly sensitive to anti-oestrogenic or antiproliferative activities that inhibit tumour growth (Wünsche et al., 1998). However, 50 mg/kg/d treatment with genistein marginally decreased tumour wet weight, although this decrease as not statistically significant (Figure 6C), whereas ethinyl oestradiol treatment showed a statistically significant increase in tumour wet weight (Figure 6C). Whereas complement C3 mRNA expression in response to genistein, in the 28 day uterotrophic assay, was significantly increased to 75% of the levels detectable for 0.1 mg/kg/d of ethinyl oestradiol (Figure 7), the dose of 50 mg/kg/d of genistein failed to down-regulate clusterin gene expression. In contrast, clusterin mRNA concentrations were increased in response to a 28 day treatment with genistein, whereas treatment with ethinyl oestradiol down regulated clusterin mRNA expression—as expected (Figure 7).

In contrast to the growth parameters using the above dosages in ectopic tumours, both genistein and ethinyl oestradiol stimulated an increase in complement C3 and clusterin gene expression (Figure 7). The latter is not surprising because clusterin gene expression in endometrial adenocarcinoma is inverse to that of normal endometrial tissue (Wünsche et al., 1998).

Discussion

The potential adverse or beneficial impact of phytoestrogens on human health is hotly debated. Phytoestrogens, particularly genistein, are discussed as potential chemopreventive agents for breast cancer (Fritz et al., 1998; Lamartiniere et al., 1998), for the improvement of endothelial dysfunction (Squadrito et al., 2000), against bone loss (Fanti et al., 1998) and more generally for the management of menopause (Anderson et al., 1999; Ramsey et al., 1999). For humans a case control study, that is currently under debate, suggests a strong link between a high excretion of phytoestrogens or their metabolites and the substantial reduction in breast cancer risk (Ingram et al., 1997).

Comparatively little is known about the action of genistein in the uterus, except its effect on uterotrophic activity and endometrial adenocarcinoma. An association between soy and fibre consumption and the risk for endometrial cancer has been discussed (Goodman et al., 1997; Cline and Hughes, 1998). A thorough cell and molecular evaluation of uterine or molecular and cellular parameters is available for Coumestrol, which clearly demonstrates an agonistic activity of this phytoestrogen in the reproductive tract, brain and hypothalamic/hypophysal/gonadal axis (Whitten et al., 1995). Coumestrol in combination with oestradiol resulted in no detectable antagonistic activity,
However pretreatment with Coumestrol dampened oestradiol action, e.g. up-regulation of the progesterone receptor (Whitten et al., 1994). The molecular and cellular effects of genistein have been investigated in the uterus of ovariectomized Sprague–Dawley rats. A total of 750 μg/g genistein added to the diet induced uterine wet weight and c-fos expression (Santell et al., 1997). Antagonistic activities for genistein were not detectable in the above mentioned rat model in macaque monkeys (Foth et al., 2000).

Because the molecular mechanism of action of genistein in the uterus and endometrial adenocarcinoma have not been well characterized we decided to evaluate thoroughly the action of genistein in normal and malignant gynaecological in-vivo models. The major findings were that 100 mg/kg/d of genistein significantly stimulated oestrogen dependent uterine and vaginal parameters in a 3 day uterotrophic assay with ovariectomized DA/Han rats. Genistein behaved as an agonist, with no apparent anti-oestrogenic effects detected in this assay. Up-regulation of uterine specific parameters were already detectable at the lower concentrations of 25 and 50 mg/kg/d but were not statistically significant.

These findings are in line with previous observations using soy diet (Makela et al., 1995) or pure genistein. Recently Makela et al. (1999) reported no indication of any uterotrophic activity when Wistar rats were fed 2.5 mg/kg/d of genistein (Makela et al., 1999). This dose has been proven to be too low to observe uterotrophic activity of genistein in vivo. In experiments with B6D2F1 mice (Farmakalidis et al., 1985) doses of ~200 mg/kg/d were required to observe uterotrophic effects, with the aglycone being twice as potent as the glycosylated genistein. More recently, using Sprague–Dawley rats Santell et al. (1997) reported a significant uterotrophic activity of 750 μg genistein added per g of rat chow, which should be equivalent to a daily intake of 38 mg/kg/d of genistein (Santell et al., 1997). Apparently Sprague–Dawley rats are more sensitive than DA/Han rats, as shown here by an increase in the uterine wet weight at doses of 50 mg/kg/d and 100 mg/kg/d, with the latter being statistically significant. Santell et al. (1997) reported on gene expression studies with c-fos expression as molecular marker for oestrogenicity. They were not able to detect an alteration of gene expression in all of the specimens analysed, whereas we detected a dose-dependent statistically significant up-regulation of complement C3 and down-regulation of clusterin expression. From these findings we conclude that complement C3 is one of the most sensitive markers if not the most sensitive to measure the oestrogenic activities of phytoestrogens in uterine tissue.

If uterotrophic activity is assessed over a period of 28 days, as in our tumour treatment experiment, a dosage of 50 mg/kg/d of genistein significantly stimulates oestrogen dependent uterine parameters, to a different extent. Whereas the uterine wet weight and complement C3 dependent gene expression...
Figure 6. Uterine wet weight, uterine epithelial height and tumour weight. Ovariectomized DA/Han rats were subcutaneously inoculated with RUCA-I cells and treated orally with either 100 µg/kg/d of ethinyl oestradiol or with 50 mg/kg/d of genistein. Untreated animals were used as control. After 28 days the uterine wet weight (A), the uterine epithelial height (B) and the tumour weights were evaluated. *Means significant against control group, \( P \leq 0.05 \), Mann–Whitney \( U \)-test.

Figure 7. Uterine and tumour dependent gene expression in response to genistein treatment of ovariectomized DA/Han rats. Ovariectomized DA/Han rats were subcutaneously inoculated with RUCA-I cells and treated orally with either 100 µg/kg/d of ethinyl oestradiol or with 50 mg/kg/d of genistein. Untreated animals were used as control. The semiquantitive densitometric evaluation of mRNA expression following semiquantitative rtPCR and densitometric analysis of PCR products is shown above. The results are shown as mean ± SD. *Means significant different to untreated group, \( P \leq 0.05 \), Mann–Whitney \( U \)-test.
was increased, the effect on the uterine epithelial height was only marginally increased. An oestrogenic effect at this dosage is not surprising in view of a recent publication in which the pharmacokinetics of \(^{14}\)C-genistein has been investigated. In the case of female animals an accumulation of radioactivity in the liver and in genital tract organs was clearly apparent (Coldham and Sauer, 2000).

If the results of the 3 day uterotrophic assay and the uterine specific effects in the 28 d assay are compared, one controversial result becomes apparent. In the 3 day assay *clusterin* expression is down-regulated by both genistein and ethynyl oestradiol treatment. In the 28 day assay oestradiol down-regulates *clusterin* expression and genistein up-regulates *clusterin* expression. The up-regulation of *clusterin* is described either in response to the apoptotic regression of hormone dependent organs, e.g. in the prostate following androgen withdrawal (Guenette et al., 1994a) or in the lactating breast following weaning (Guenette et al., 1994b) or following the malignant transformation of the endometrium in the presence of oestradiol which up-regulates *clusterin* expression (Wünsche et al., 1998). An oncogenic event within a 28 d treatment period is very unlikely, which makes the second hypothesis of apoptosis in the uterus in response to a 28 d treatment with 50 mg/kg/d of genistein more likely. So far we do not have any experimental evidence supporting apoptotic events, but at least in cell culture using MDA–MB human breast cancer cells genistein induces apoptosis at a concentration of 10 μmol/l and above (Balabhadrathruni et al., 2000).

Genistein has been described as a chemopreventive agent for breast cancer. In a series of experiments it had been shown that genistein treatment during perinatal, neonatal, or prepubertal periods may prevent breast cancer. This means the preventive agent has been given long before the carcinogen (Barnes, 1997; Fritz et al., 1998; Lamartiniere et al., 1998a,b). This situation is completely different from the RUCA-I cell endometrial adenocarcinoma tumour model, where we face an acute situation, where highly metastatic endometrial adenocarcinoma cells are inoculated into syngeneic animals resulting in tumour formation at the ectopic site. This experimental set up represents an acute situation apparently not in tumour formation at the ectopic site. This experimental set up represents an acute situation apparently not influenced by genistein treatment. In a nitrosomethylurea-induced mammary carcinoma model genistein reduced tumour multiplicity but failed to reduce tumour incidence (Constantinou et al., 1996) or had no effect on mammary tumourgenesis (Yang et al., 2000) which again is indicative of the rather low potency of genistein in an acute situation. RUCA-I cells were isolated from an endometrial adenocarcinoma of DA/Han rats, >60% of which die naturally from endometrial adenocarcinoma (Deerberg et al., 1985). It would be interesting to test whether genistein would be chemopreventive using this spontaneous tumour model. It might be valuable to test whether genistein pretreatment of RUCA-I cells would result in a reduction in tumour growth, because a 6 d maturation of MCF-7 breast cancer cells in the presence of genistein results in the prevention of tumour growth of these cells in nude mice (Constantinou et al., 1998).

The activity of genistein in the ovariectomized rat uterus *in vivo* is in a dosage range which would not have been predicted from the in-vitro data. The relative binding affinity of genistein in RUCA-I cells was measured as 1% of oestradiol and 100-fold higher concentrations of genistein stimulated *C3* gene expression to the same extent as oestradiol (Hopert et al., 1998). *In vivo*, if genistein was administered in 1000-fold higher concentrations than oestradiol, it induced statistically significant effects, which, however, were still far below the levels detectable for ethinyl oestradiol. This finding is clearly indicative of the necessity of in-vivo experiments, because effectiveness may not necessarily be predicted from in-vitro data. In-vitro approaches represent excellent screening tools.

In summary, our studies clearly indicated that genistein is an oestrogen agonist in the uterus of rats. However, oestrogenic effects were found to be statistically significant at concentrations of 50 mg/kg/d of an oral dose, if molecular parameters are accepted. This dose is 10- to 100-fold higher than the estimated human exposure. Widespread estimates of human exposure are described in the literature. Setchell and Cassidy (1999) estimated exposure to isoflavones of <1 mg per day in Western countries and 20–50 mg/d in Asian populations. Conversely, Cassidy et al. (1994) published an exposure of 150–200 mg/d of isoflavones for people eating a traditional Asian diet. According to our study the effective dose (50 mg/kg/d), however, is rather close to the estimated exposure in infants who are fed with a soy-based diet. Daily exposure in these infants has been calculated as 6–9 mg/kg/d.

Despite some restrictions, which may apply, our finding clearly supports the possibility that approaches to protect blood vessels or approaches to protect for loss of bone mass by phytoestrogen (genistein) treatment may be feasible. Genistein is at least capable of protecting the arterial wall against post-denudation intimal dysplasia in the same dosage range as oestradiol does (Makela et al., 1999), probably due to its specific selectivity for the oestrogen receptor-β.

Our data further indicates that genistein does not inhibit tumour growth in an established tumour. However, the compound is able to stimulate the expression of the oestrogen sensitive genes *C3* and *CLU* in RUCA I tumours and is comparable to ethinyl oestradiol. This result demonstrates that genistein acts like an oestrogen in regard to the expression of oestrogen sensitive genes in the tumour but nevertheless is unable to promote tumour growth. This finding is in agreement with the observed action of genistein in the non-malignant uterine tissue. Genistein is able to stimulate uterine weight but even at high doses is unable to stimulate the uterine weight to the same extent as ethinyl oestradiol. These findings characterize genistein as an oestrogen receptor agonist, which, for some unknown molecular reasons, is not able to stimulate proliferation of uterine tissue and in this regard shares similarities with the synthetic SERMRaloxifene.

For these reasons it may be valuable to test whether genistein will protect against endometrial carcinogenesis if administered neonatally or prepuber tally, the time points when genistein treatment is most effective in the prevention of mammary cancer.

Finally, we evaluated an array of several independent markers for oestrogenicity. Our results suggest that the measurement of complement *C3* gene expression is the most
sensitive marker for oestrogen action and superior to uterine wet weight, uterine epithelial height, vaginal epithelial height or cornification. Expression of complement C3 mRNA is even detectable in an experimental situation where no uterotrophic activity is measurable, as recently shown for Daidzein in the uterus of ovariec-tomized DA/Han rats (Diel et al., 2000).

In conclusion, the 3 day uterotropic assay in combination with gene expression studies represents a very sensitive tool to measure and to assess oestrogenic activities in vivo. In our study a comparatively low proliferative activity of genistein in normal and the malignant uterine tissue was observed. However genistein acts like an agonist on the vagina and on the expression of some oestrogen sensitive genes in the uterus. These findings suggest that the development of new phytoestrogens may be worthwhile.

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