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SHORT COMMUNICATION

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The phytoestrogens coumoestrol and genistein induce structural chromosomal aberrations in cultured human peripheral blood lymphocytes

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Abstract The clastogenic potential of the phytoestrogens coumoestrol (COUM), genistein (GEN) and daidzein (DAI) has been studied in human peripheral blood lymphocytes in vitro. After exposure of the cultured lymphocytes to 50 or 75 μM COUM or 25 μM GEN for 6 h, a clear induction of structural chromosomal aberrations was observed by cytogenetic analysis. The major alterations were chromatid breaks, gaps and interchanges. In contrast, DAI did not induce chromosome aberrations even at 100 μM. These results, together with previously published reports on the induction of micronuclei and DNA strand breaks in cultured Chinese hamster V79 cells by COUM and GEN, but not DAI, suggest that some but not all phytoestrogens have the potential for genetic toxicity.

Key words Phytoestrogens · Coumoestrol · Genistein · Daidzein · Human peripheral blood lymphocytes

Introduction

Over 300 plants are known to contain endogenous compounds of diverse chemical classes, which exhibit oestrogenic activity and are therefore called phytoestrogens (Farnsworth et al. 1975). For example, the coumestane-type phytoestrogen coumoestrol (COUM) is commonly found in sprouts of alfalfa and clover, whereas the isoflavone-type phytoestrogens genistein (GEN) and daidzein (DAI) occur at high concentrations in soybeans (see Fig. 1 for chemical formulas).

The consumption of soyfood products is increasing in Western countries, partly because soybean isoflavones are believed to provide several health benefits, e.g. protection against cancer of the breast, prostate and colon, as well as coronary heart disease and osteoporosis (Clarkson et al. 1995). However, very little is known about possible adverse effects of phytoestrogens. Recent studies by Kulling and Metzler (1997) have demonstrated that GEN and COUM, but not DAI, are potent inducers of micronuclei in cultured Chinese hamster V79 cells. The induced micronuclei consist of acentric chromosomal fragments and are therefore indicative for clastogenic activity of GEN and COUM. Moreover, COUM was shown to induce DNA strand breaks and gene mutations at the HPRT locus in V79 cells (Kulling and Metzler 1997). These studies on the genotoxicity of phytoestrogens have now been extended to cultured human peripheral blood lymphocytes.

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Materials and methods

Chemicals

Coumoestrol (purity >98% according to the supplier) was purchased from Eastman Kodak Co., GEN (research grade) from Fluka Chemical Co., DAI (purity >98%) from ICN Pharmaceutical and *m*-amsacrine (AMSA) from Sigma Chemical Co. Analysis by gas chromatography/mass spectrometry (GC/MS) after derivatisation with N,O-bis(trimethylsilyl)acetamide did not reveal any defined impurity. All compounds were dissolved in dimethylsulphoxide (DMSO, American Chemical Society reagent, Sigma Chemical Co.). Other reagents were obtained from either Sigma or Serva Chemical Co. and were of the highest purity available.

Cell culture

Human peripheral blood samples were obtained from a healthy young female (SK, age 30 years) and two healthy young males (TK, age 30, and EJ, age 28 years). Blood was collected by venipuncture directly into a heparinized 15-ml polycarbonate tube. Eight hundred microlitres of the blood was immediately mixed in a sterile plastic vial with 8.0 ml of RPMI 1640 medium (Gibco Life Technologies) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 0.2 ml phytohaemagglutinin M (PHA, Difco Laboratories), and incubated for 66 h at 37 °C under 5% CO_2 prior to incubation with the test compounds for 6 h. The total time of cultivation was 72 h.

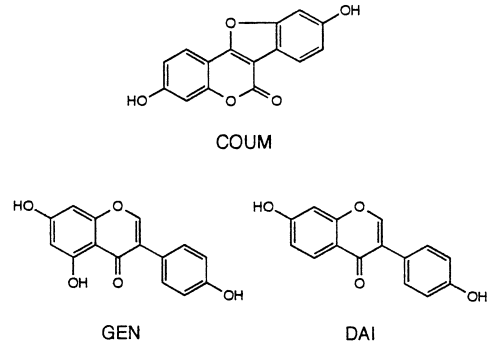

Fig. 1 Chemical structures of coumoestrol (COUM), genistein (GEN) and daidzein (DAI)

Metaphase cells were accumulated by the addition of colcemid (final concentration 0.1 μg/ml) 2 h prior to harvest. Cells were collected by centrifugation at 270 *g* for 10 min. The supernatant was discarded and the pellet resuspended and incubated in 10 ml prewarmed (37 °C) hypotonic KCl (37.5 mM) for 20 min. Following centrifugation cells were fixed with four changes of Carnoy's fixative (freshly prepared mixture of absolute methanol and glacial acetic acid 3:1, v/v) at −20 °C until the cell pellet was colourless. The final pellet was resuspended in fixans and dropped onto clean glass microscope slides. The slides were aged for 12 h at 50 °C, stained for 10 min with a solution of Giemsa [6.5 ml stock solution containing 0.4% (w/v) Giemsa, as obtained from Sigma, mixed with 30 ml 0.1 M Soerensen buffer, pH 7.5, and 63.5 ml distilled water; filtered prior to use], rinsed and air-dried at room temperature. Two or three independent experiments were carried out with each test compound and concentration.

Cytogenetic analysis

For the analysis of metaphase chromosomes slides were examined under oil immersion at 10 × 100-fold magnification using a green filter for contrast enhancement. One hundred metaphases were analysed for each concentration of the test substance. Only metaphase spreads with a complete number of chromosomes were analysed. Chomosome aberrations were categorized as chromatid gap, chromosome gap, chromatid break, chromosome break, interchanges and ring chromosome (Savage 1976; Scott et al. 1990). The chromatid break was distinguished from the chromatid gap in that the non-stained part of the chromatid is larger than the diameter of the chromatid. The cytotoxicity of the test compounds was assessed by counting the mitotic index in ‰, i.e. number of metaphases per 1000 cells.

Results

In order to study the ability of the phytoestrogens COUM, GEN and DAI to induce chromosome aberrations in cultured human peripheral blood lymphocytes, the lymphocytes were first stimulated with PHA for 66 h and then treated with various concentrations of the test compounds for 6 h. The incubations with the phytoestrogens were carried out in RPMI medium in the absence of fetal calf serum (FCS), because a pronounced binding of all three phytoestrogens to serum proteins was observed in experiments using equilibrium dialysis. For example, COUM at 50 μM concentration in RPMI medium gave rise to 70% binding upon addition of 10% FCS. In contrast, no binding to PHA was observed in analogous studies using PHA at the concentration used for initiating mitosis. The chromosome aberrations observed with COUM, GEN and DAI and the appropriate controls are summarized in Table 1.

Coumoestrol and GEN were clearly positive in this assay: treatment with 50 μM COUM increased the frequency of aberrations three- to fourfold compared with untreated cells and led only to a slight decrease of the mitotic index, which is a parameter for cytotoxicity. The number of aberrant cells was dramatically increased up to 40% with 75 μM COUM. The obtained aberrations were chromatid gaps, chromatid breaks and to a smaller extent chromatid exchanges (interchanges). A representative example of the COUM-induced aberrations is depicted in Fig. 2B. It must be noted that 75 μM COUM caused a marked decline of the mitotic index and is the highest acceptable concentration for COUM in this assay, because 100 μM COUM led to an extreme reduction of the mitotic index. The effect of GEN was similiar to that of COUM: treatment with 25 μM GEN led to an average of 35% aberrant cells (excluding gaps) and a 56% reduction of the mitotic index. An example of the aberrations induced by GEN is shown in Fig. 2C. In contrast to COUM and GEN, no adverse effects on cell division and no indications for the induction of aberrant lymphocytes were obtained after exposure to 100 μM DAI, a concentration which is the limit of solubility. *m*-Amsacrine served as positive control for chromosomal aberrations.

Discussion

In the present study, we have investigated the ability of the phytoestrogens COUM, GEN and DAI to induce chromosomal aberrations in cultured human peripheral blood lymphocytes. COUM and GEN but not DAI clearly increase the number of aberrant metaphases. The present findings are in line with the recently published report that COUM and GEN exhibit clastogenic activity in cultured Chinese hamster V79 cells, as measured by the induction of DNA strand breaks and micronuclei containing acentric chromosomes/chromatids; conversely DAI was negative at these endpoints (Kulling and Metzler 1997). GEN was also reported to induce micronuclei, a low frequency of mutants at the *hprt* gene locus, and apoptosis in human lymphoblastoid cells (Morris et al. 1998).

The genotoxic effects of GEN and COUM are examples for modes of action of phytoestrogens that are not mediated by oestrogen receptors (ER) and are therefore independent of the hormonal activity. Several such non-ER mediated effects of phytoestrogens are known (Cassidy 1996). For example, GEN is a non-intercalative inhibitor of topoisomerase II, an enzyme involved in DNA replication, recombination, chromatid segregation and transcription by catalysing the breakage and rejoining of both DNA strands (Yamashita et al. 1990). The main lesions induced by non-intercalative as

Table 1 Induction of chromosomal aberrations in human lymphocyte cultures treated with the phytoestrogens coumoestrol genistein and daidzein. (*COUM* coumoestrol, *GEN* genistein, *DAI* daidzein, *DMSO* dimethyl sulphoxide, *AMSA m*-amsacrine)

Treatment	Conc. (μM)	Donor	Ctg[a]	Ctb[b]	Int[c]	Chg[d]	Chb[e]	RC[f]	% Aber. cells includ. gaps	% Aber. cells exclud. gaps	∑ Lesions includ. gaps	∑ Lesions exclud. gaps	Mitotic index [‰]
None	–	SK	5	0	0	0	0	0	4	0	5	0	47.6
	–	TK	2	1	0	0	0	0	3	1	3	1	46.1
	–	EJ	3	1	0	0	0	0	4	1	4	1	43.2
DMSO	0.1%	SK	4	3	0	0	0	0	7	3	7	3	41.4
	0.1%	TK	2	2	0	0	1	0	4	2	5	3	50.2
	0.1%	EJ	5	0	0	0	0	0	4	0	5	0	47.0
COUM	50	SK	14	9	0	0	0	0	19	8	23	9	37.8
	50	TK	7	8	3	1	2	0	16	11	21	13	41.3
	75	SK	42	62	15	3	1	1	45	41	124	79	15.8
	75	EJ	10	14	7	2	0	0	19	16	33	21	31.4
	100	SK						cytotoxic concentration					2.0
GEN	25	TK	18	36	23	1	5	0	43	36	83	64	17.4
	25	SK	29	43	29	2	6	0	46	38	109	78	14.4
	25	EJ	22	40	6	2	2	0	42	30	72	48	28.0
DAI	100	SK	3	3	0	0	0	0	6	3	6	3	39.6
	100	EJ	4	0	0	0	0	1	5	1	5	1	46.6
AMSA	0.5	EJ	8	8	0	1	0	0	14	7	17	8	51.0
	2	SK	7	8	3	1	1	0	17	11	20	12	44.8

[a] Chromatid gap
[b] Chromatid break
[c] Interchange
[d] Chromosome gap
[e] Chromosome break
[f] Ring chromosome

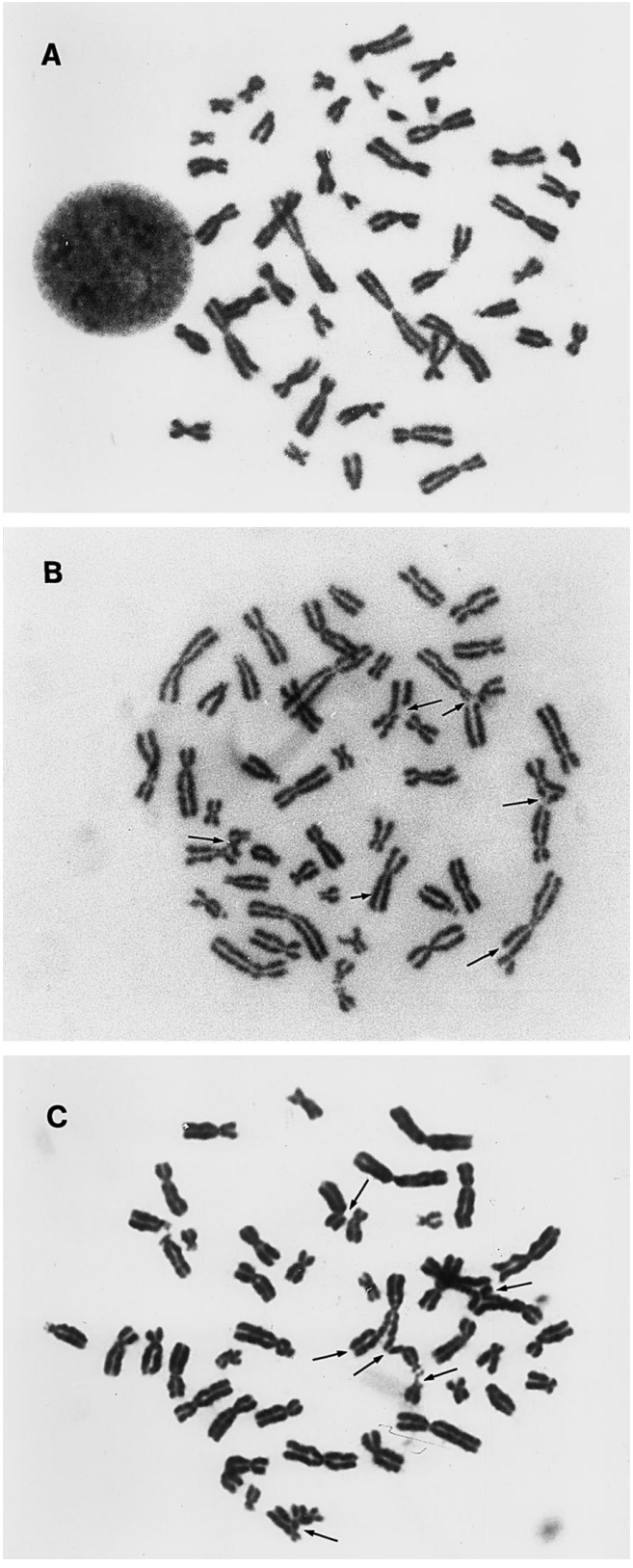

Fig. 2 Metaphase spreads of human peripheral blood lymphocytes stained with Giemsa: **A** untreated cell; **B** cell exposed to 75 μM COUM for 6 h; **C** cell exposed to 25 μM GEN for 6 h. *Arrows* indicate chromosomal damage

well as intercalative inhibitors of topoisomerase II are double-strand breaks, which are considered to be the ultimate DNA lesions leading to chromosome aberrations (Liu 1989). Treatment of cells in S- and G2-phases of the cell cycle with inhibitors of topoisomerase II causes chromatid-type aberrations as shown by Andersson and Kihlmann (1989) and Palitti et al. (1990). In our study, the time point and the length of exposure, i.e. the last 6 h of the 72 h cultivation period, mean that only the first post-treatment metaphases were analysed (which is essential for the detection of primary aberrations); moreover these metaphases were derived from cells which had been in the very late S- or early G2-phases at the beginning of the exposure period, because the average duration of G2-phase of cultured lymphocytes is 3.5 to 4 h (Mindek and Ziehmann 1994). As expected from topoisomerase II inhibitors and the time point of exposure, the observed chromosomal aberrations were predominately chromatid gaps, chromatid breaks and chromatid-type interchanges (Table 1). Likewise, the intercalative topoisomerase II inhibitor *m*-amsacrine (AMSA), which served as a positive control in our study, gave rise to this type of aberration, as did the phytoestrogen COUM (Table 1). The behaviour of COUM in lymphocytes reported in this communication and in various assays for genotoxicity (Kulling and Metzler 1997), as well as molecular modelling studies by Lehner et al. (1987) suggest that COUM may inhibit topoisomerase II by DNA intercalation. In this context it is interesting to note that topoisomerase II inhibitors used for cancer chemotherapy have recently been reported to induce leukaemias as a consequence of various chromosome aberrations (Felix 1998).

Genistein is also a potent inhibitor of tyrosine protein kinase activity (Akiyama et al. 1987). The inhibition of this enzymes is often associated with an anticarcinogenic activity of GEN, because several growth factor receptors and oncogenes are regulated by tyrosine phosphorylation. On the other hand it has been reported that the inhibition of tyrosine phosphorylation also plays an important role in the response of cell cycle checkpoints to DNA damage. For example, progression from G2- into M-phase is controlled partly by a cyclin-dependent kinase (cyclin B/Cdk1) and regulated by tyrosine phosphorylation. Inhibition of this tyrosine kinase may inactivate the checkpoint and hence damaged cells enter mitosis without delay (Kaufmann 1998; Baguley and Ferguson 1998). If such damaged cells survive, cells with permanent genetic alterations are the result.

In conclusion, this study provides further evidence for the proposition that COUM and GEN are clastogens. The implications of this adverse potential for the health of individuals with high dietary intake of these phytoestrogens are unknown. In our present study, 25 μmol/l of the isoflavone genistein caused a clear increase of aberrant lymphocyte metaphases. The population with the highest exposure appear to be infants on soy-based formulas, which contain isoflavone levels ranging from 17 to 30 mg/l (Murphy et al. 1997; Knight

et al. 1998). The mean concentration of isoflavones measured recently in the plasma of 4-month-old infants fed exclusively soy-based infant formulas was ~4 μmol/l, of which 60% was GEN; in comparison, plasma levels of isoflavones in adults consuming soy-based diets range from 0.2 to 1 μmol/l (Setchell et al. 1997). Thus the active concentration of GEN used in our study is only about tenfold higher than that observed in humans on soy diets.

It is presently unknown which proportion of the isoflavones in plasma is conjugated with glucuronic acid and sulphate, and whether these conjugates retain biological activity. With respect to COUM, no data are available on plasma levels in humans. It may be assumed that such levels are low, because the concentration of COUM in edible plants is relatively small, with the exception of alfalfa, soy and clover sprouts which may reach 500 ppm (Francis and Millington 1971). More research on both the adverse and the beneficial biological activities of phytoestrogens and their metabolites as well as on human exposure is clearly needed in order to properly evaluate the health risks and benefits of these important dietary compounds.

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