Growth inhibition of human endothelial cells by the phyto-oestrogen biochanin A, a metabolite of genistein

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This study examined the growth regulatory effects of a phyto-oestrogen, biochanin A, on a transformed human endothelial cell line ECV304 *in vitro*. Biochanin A was found to inhibit cell proliferation in a dose-dependent fashion and this effect was influenced by the concentration of serum present in the culture medium. In the absence of serum, the calculated IC_{50} of biochanin A was 0.18 ± 0.1 μM compared to an IC_{50} of 35 ± 5 μM at 10 % serum. At low cell density, the growth inhibitory effects of biochanin A were more evident than at high cell density. Co-administration of a synthetic oestrogen diethylstilboestrol with biochanin A did not suppress the growth regulatory effects of biochanin A treatment. We conclude that biochanin A inhibits the cell proliferation of human endothelial cells at concentrations that are physiologically achievable *in vivo* and that this effect may play an important role in the cancer-preventing activity of the phyto-oestrogens.

Phyto-oestrogen: Endothelial cells: Cell proliferation

Epidemiological and migrant studies suggest that diet may influence the etiology of certain cancers (Adlercreutz, 1995; Herman *et al.* 1995). High incidence of the so-called Western diseases, including the major hormone-dependent cancers such as breast, prostate and endometrial cancers, colon cancer and coronary heart disease have been associated with a Western diet (Trowell & Burkitt, 1981; Rose *et al.* 1986). Conversely, Asian women and vegetarians are less at risk from these cancers and diseases, apparently because of their high consumption of phyto-oestrogen-rich products such as dietary soy (Adlercreutz *et al.* 1982, 1986). Immigrants from Asia who maintain their traditional diet do not increase their risk of these diseases; however, an increased risk for these diseases accompanies a change toward a Westernized diet (Kolonel, 1988; Lee *et al.* 1991).

Phyto-oestrogens are plant chemicals that are structurally similar to the sex hormone oestrogen (Shutt & Cox, 1972). Since 1931 it has been well known that soybeans contain high amount of phyto-oestrogens, especially daidzein and genistein (Eldridge & Kwolek, 1983). When consumed, the phyto-oestrogens undergo metabolic conversions in the gut, which results in the formation of hormone-like compounds (Setchell & Adlercreutz, 1988). These compounds have been found in the human plasma, urine and faeces (Adlercreutz, 1995). *In vitro* experiments have shown that specific phyto-oestrogens inhibit the growth of several tumour cell types, including mammary (Hsu *et al.* 1999), leukaemia (Honma *et al.* 1990), prostate (Hempstock *et al.* 1998) and pancreatic cancer cells (Lyn-Cook *et al.* 1999). In animals, consumption of phyto-oestrogen-rich soy has been shown to protect rodents from experimentally induced cancers (Barnes *et al.* 1990; Hawrylewicz *et al.* 1995). Several possible mechanisms have been proposed for the antitumour activity of phyto-oestrogens, including agonist and antagonist effects on oestrogen receptors (Adlercreutz *et al.* 1995), stimulation of sex-hormone-binding globulin synthesis (Kurzer & Xu, 1997), inhibition of growth factor-associated tyrosine-kinase signal transduction (Akiyama *et al.* 1987), antioxidant properties (Naim *et al.* 1976), inhibition of DNA topoisomerase (Okura *et al.* 1988) and antiangiogenic effects (Fotsis *et al.* 1993).

Angiogenesis, the generation of new capillaries from pre-existing vessels, is virtually absent in the healthy adult organism, in which it is restricted to a few conditions such as wound healing and the formation of corpus, luteum, endometrium, and placenta. These conditions of physiological angiogenesis represent ordered, tightly regulated and self-limited process (Klagsbrun & Folkman, 1990). Thus, for example in wound healing, the same endothelial cells

can undergo rapid proliferation during spurts of angiogenesis, followed by long periods of quiescence in which less than 0·1 % of the endothelial cells proliferate (Denekamp, 1990) and the turnover of endothelial cells is measured in thousands of days. However, angiogenesis is dramatically enhanced and loses its self-limiting capacity in certain pathological conditions. For instance, during the development and progression of many diseases, such as rheumatoid arthritis, psoriasis, and diabetic retinopathy, 10 % or more of the endothelial cells actively proliferate (Folkman *et al.* 1989; Denekamp, 1990). Formation of new blood vessels is also critical for the growth of tumours (Folkman, 1985; Blood & Zetter, 1990). Avascular tumours do not grow beyond a diameter of 1–2 mm, whereas well vascularized tumours expand both locally and by metastasis (Folkman & Cotran, 1976; Klagsbrun & Folkman, 1990). Though the mechanisms leading to persistent pathological angiogenesis are still unclear, recent evidence indicates that it may result from an imbalance between angiogenic factors and inhibitors, with the activity of angiogenic factors being too high and/or that of the inhibitors being reduced or completely absent (Liotta *et al.* 1991).

The idea that dietary ingested compounds might be able to modulate pathological angiogenesis clearly merits further investigation. If dietary compounds were indeed to inhibit angiogenesis, this could explain, at least in part, the long-known preventive effects of the phyto-oestrogens on tumourigenesis. In this report, we studied the effects of the phyto-oestrogen biochanin A, which can be converted from genistein in animals (Kurzer & Xu, 1997), on the cell proliferation of transformed human endothelial cells, target cells for angiogenesis. Our results show that biochanin A is able to inhibit cell growth at physiologically achievable concentrations.

Materials and methods

Chemicals

Biochanin A was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The cell line ECV304 (CCRC 60077), originally deposited in the American Type Cell Culture Collection, was obtained from the Culture Collection and Research Center of Taiwan. ECV304 is a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord. Medium 199 (M199), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), and an antibiotic–antimycotic mix were purchased from GIBCO BRL (Gaithersburg, MD, USA). A cell proliferation assay kit (WST-1) was purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). Solutions of biochanin A and diethylstilboestrol (DES), a synthetic oestrogen agonist, were prepared in dimethylsulphoxide (DMSO) and ethanol respectively and stored at $-20°C$ in the dark.

Cell culture conditions

The ECV304 cells were grown in M199 containing 0·6 % N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid, and 0·22 % sodium bicarbonate, and supplemented with 10 % either FBS for routine culture or $3\times$ dextran–charcoal-stripped FBS for all assays. The $3\times$ dextran–charcoal-stripped FBS was prepared by passing the FBS through dextran-coated charcoal three times at $4°C$ and then filter sterilized to deplete the steroids, including oestrogens, from the FBS. The purpose of using charcoal-stripped FBS during assays is to reduce the background of steroid-like reactivity in cells, or to 'de-sensitize' cells that were previously cultured in medium supplemented with FBS. Cultures were maintained at $37°C$ in a humidified atmosphere of 95 % air–5 % CO_2 and fed every 2 days.

Proliferation assays

Cells were seeded in culture flasks in assays that investigated the effects of co-administration of oestrogen and the effects of cell density at indicated cell densities. Cells were grown in 96-well culture plates in the assays that studied the effects of serum concentration. M199 containing 10 % $3\times$ dextran–charcoal-stripped FBS was used to culture the ECV304 cells. After a 24 h preculture period to ensure attachment, the medium was replaced with fresh M199 supplemented with 0–10 % $3\times$ dextran–charcoal-stripped FBS. This replacement medium also contained biochanin A (0–140 μM) and, in the assays that investigated the effects of the co-administration of oestrogen, DES at molar concentrations $1\times$, $10\times$ and $100\times$ higher than that of the biochanin A was also added. DMSO at the same dilutions, was added to parallel cultures as a control. Final concentrations of DMSO in the culture medium were kept below 1 % (v/v), which caused no measurable effects on cell growth or cell morphology. At the end of the incubation period (4 days), the number of viable cells in each sample was determined by the trypan blue dye exclusion method for cells grown in flasks. The trypan blue dye exclusion method has been widely used to determine the number of viable cells since viable cells remain transparent under a microscope while non-viable cells appear blue in the presence of trypan blue dye. Briefly, cells were recovered from the flasks, collected by centrifugation and resuspended in a small amount of culture medium. A portion of cell suspension (90 μl) was mixed with 10 μl of trypan blue dye and the number of apparent cells was counted under the microscope with a haemocytometer.

For cells grown in the 96-well culture plates, cell proliferation was determined by the WST-1 method. Adding 10 μl of the WST-1 reagent into each well of 96-well plates and incubated for 4 h at $37°C$ according to manufacturer's instructions, the number of viable cells can be determined. A succinate–tetrazolium reductase system, which belongs to the respiratory chain of the mitochondria and is active only in viable cells, can cleave the WST-1 salt and form formazan dye. Therefore, the resulting amount of formazan dye can then be directly correlated to the number of metabolically active cells in the culture. The formation of formazan dye which absorbed at the wavelength of 420 nm was quantitated using a scanning multiwell spectrophotometer (ELISA reader). The absorbance at 420 nm minus the absorbance at 650 nm, which represented the background, was directly

correlated to the numbers of viable cells in each sample (Hsu *et al.* 1999).

Statistical analysis

The method of least squares ANOVA using the general linear model procedures for a completely randomized design with biochanin A concentration as the treatment factor was used to analyse data (Snedecor & Cochran, 1980; SAS, 1990) The means of treatment were compared, by orthogonal contrast, with the comparisons of each individual biochanin A concentration *v.* control blank.

Results

The effects of biochanin A on growth inhibition of ECV304 cells were examined and the results are shown in Fig. 1. In the presence of 10 % $3\times$ dextran–charcoal-stripped FBS, biochanin A had a cytostatic effect on the cell proliferation at low concentrations and cytotoxic effects at 105 μM and above (Fig. 1a). The calculated IC_{50} (concentration at which 50 %, of the cell growth was inhibited) was 35 μM. In the absence of FBS (Fig. 1b), the growth inhibitory effects of biochanin A were greatly enhanced: at biochanin A, concentration of 35 μM, there was 80 % reduction in the number of cells relative to the control, and the IC_{50} was calculated to be 0·18 μM. To further examine the effects of the presence of serum, ECV304 cells were incubated with biochanin A and various percentages of $3\times$ dextran–charcoal-stripped FBS. As Fig. 2 shows, at all the concentrations of FBS tested, cell proliferation was inhibited in a dose dependent fashion by biochanin A. However, the inhibition sensitivity was greatly enhanced when the FBS was absent. At the lower biochanin A concentrations (0–70 μM), the growth rate of cells in the presence of 7·5 % $3\times$ dextran–charcoal-stripped FBS was unexpectedly slow (Fig. 2).

Cell density was shown to affect the number of viable ECV304 cells treated with biochanin A (Fig. 3). Cells were first seeded in culture plates at different cell densities and the proper attachment of ECV304 cells at all three cell densities was confirmed microscopically. The ECV304 cells were then treated with biochanin A for 4 days and the number of viable cells in each flask was determined (Fig. 3). At low cell density (approximately 40 % confluence), the number of viable cells was reduced by 86 % in the presence of biochanin A at 55 μM, whereas, at higher cell densities (65 % and 90 % confluence), the same concentration of biochanin A reduced the number of viable cells by about 67 % (Fig. 3).

Oestrogens are naturally present in animals and the effects of oestrogens must be taken into account if phyto-oestrogens are to be considered as potential tumour-preventing agents. When the synthetic oestrogen DES was added in molar excess ($1\times$, $10\times$, $100\times$) simultaneously with biochanin A in the presence or absence of 10 % FBS, the effects of biochanin A were modified only slightly (Fig. 4). In the absence of FBS, 0·18 μM of biochanin A reduced the number of viable cells to the same degree as when the DES was added (Fig. 4a). A similar observation was obtained when cells were treated with
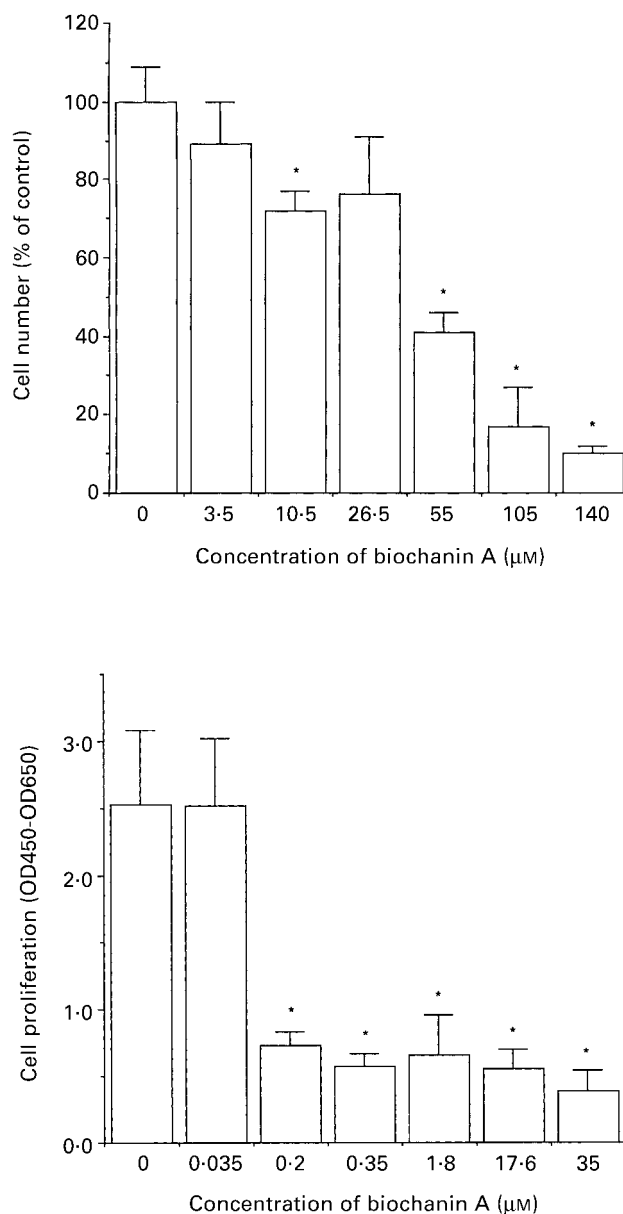

Fig. 1. The effects of biochanin A on the cell proliferation of ECV304 cells. (a) Biochanin A was added at the indicated concentrations to ECV304 cells (initially $9\cdot2\times10^4$ cells/T-25 flask) in the presence of M199 and 10 % $3\times$ dextran–charcoal-stripped FBS. After the cells had been incubated for 4 days at 37°C with the indicated concentration of biochanin A, the number of cells was determined by the trypan blue dye exclusion method. (b) The ECV304 cells were seeded in 96-well culture plates at $3\cdot4\times10^4$ per well in M199 without FBS. After 24 h at 37°C, fresh M199 medium alone or with biochanin A as indicated was added into each well. Following incubation for 4 days, the number of cells per well was determined by WST-1 assay. Experiments with similar designs were performed four times and the value (mean and SD) with a * indicates significant difference ($P < 0\cdot01$) compared with the DMSO-treated cells (0 μg/ml biochanin A).

biochanin A and DES simultaneously in the presence of 10 % FBS (Fig. 4b). The inhibitory effect of biochanin A alone or of the combined effects of biochanin A and DES were not different. Administration of DES alone at concentrations starting from a physiological concentration
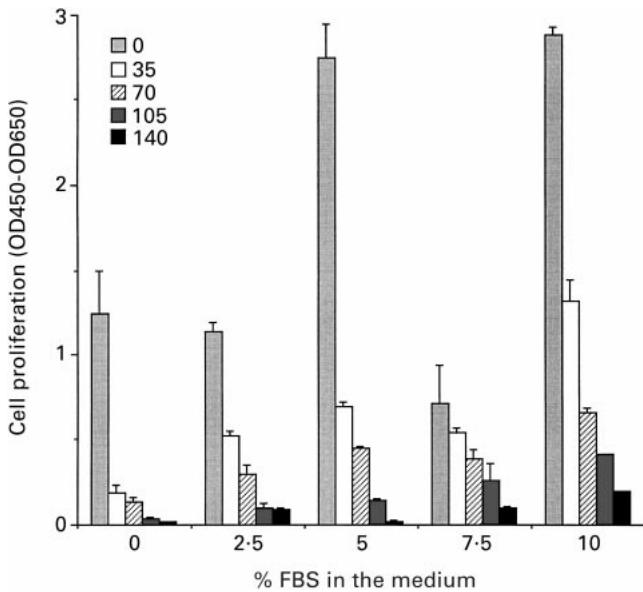
Fig. 2. Serum concentration affects the cell proliferation by biochanin A. ECV304 cells seeded at $2 \cdot 0 \times 10^4$/well were treated with M199 containing the indicated percentage of $3 \times$ dextran–charcoal-stripped FBS (0–10 %) alone or with the indicated concentration of biochanin A (35–140 μM) for 4 days. The number of cells was determined by WST-1 assay. Experiments were repeated three times and the values are mean and SD.
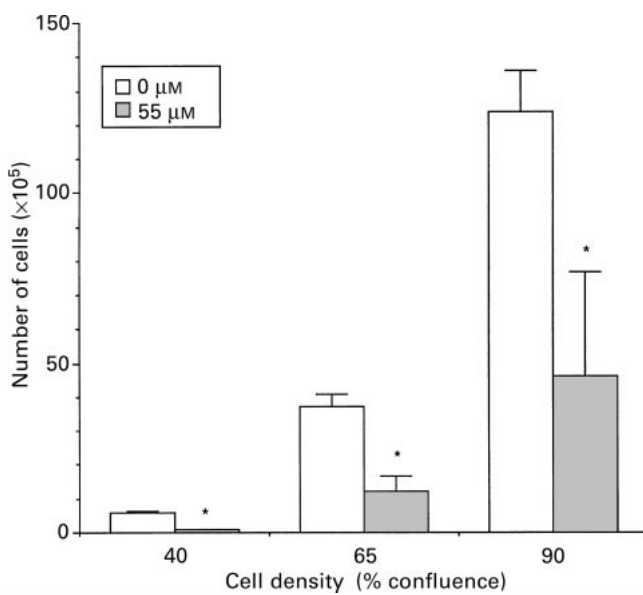
Fig. 3. The growth regulatory effect of biochanin A is influenced by cell density. The ECV304 cells were seeded in T-25 flasks at 40, 65 or 90 % cell density respectively. The cells were then incubated with ethanol (indicated as 0 μM) or 55 μM biochanin A for 4 days in medium containing 10 % $3 \times$ dextran–charcoal-stripped FBS, after which the number of viable cells in each flask was determined by trypan blue dye exclusion assay. Similar experiments were performed three times and values are shown as mean and SD. *Indicates significant difference ($P < 0 \cdot 01$) compared with its respective DMSO-treated cells (0 μg/ml of biochanin A).
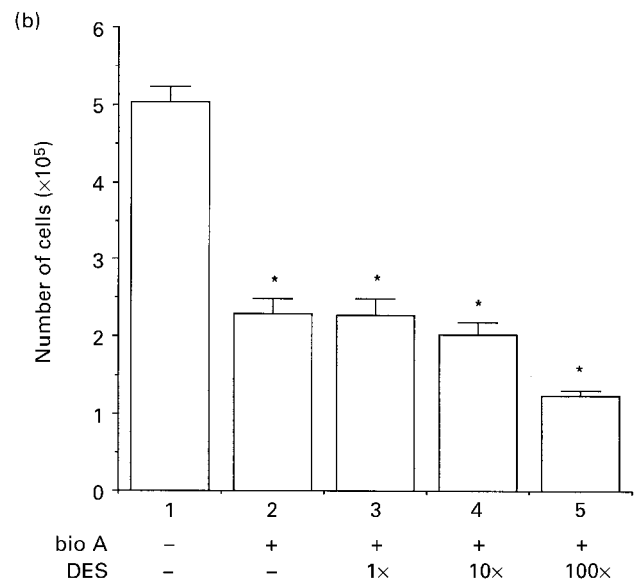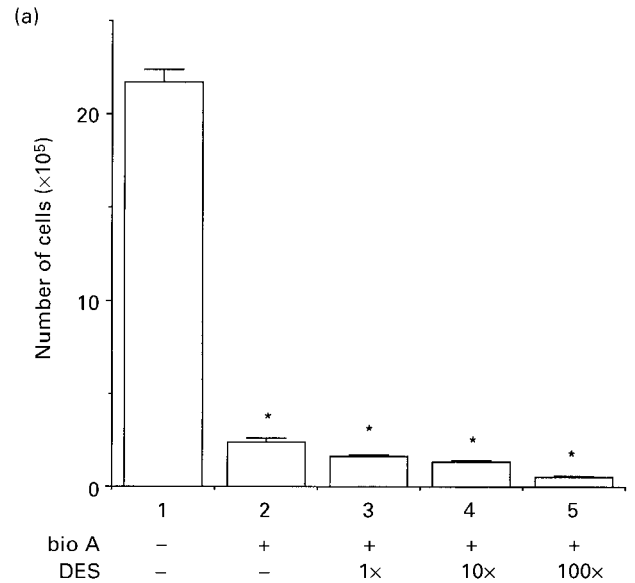
Fig. 4. The effects of co-administration of oestrogen on ECV304 cells treated with biochanin A. (a) Cells were treated with $0 \cdot 18$ μM of biochanin A alone (column 2) or with indicated molar excess of DES (columns 3–5) in the absence of FBS. (b) The ECV304 cells were incubated with 55 μM of the biochanin A alone (column 2) or with indicated molar excess of DES (column 3–5) in the presence of 10 % $3 \times$ dextran–charcoal-stripped FBS. DES concentrations were at 1, 10 or $100 \times$ molar excess of the treated biochanin A concentration. Viable cells were counted by trypan blue dye exclusion assays after incubation for 4 days. Column 1 represents the control. Experiments with similar designs were performed twice. Values are mean and SD. *Indicates significant difference ($P < 0 \cdot 01$) compared with control cells (0 μg/ml of biochanin A and DES).

of 10 nM to concentrations tested in Fig. 4 did not show any significant effect on the number of viable cells among different treatments, however there is a significant difference between the treatment and control groups ($P <$ 0·01). This therefore suggests that the growth regulatory effects on ECV304 cells by biochanin A were not suppressed, and nor were any synergistic effects observed.

Discussion

Previous reports have shown that in rats a single oral dose of the phyto-oestrogen genistein in pure form (20 mg/kg body weight) or a similar dose of genistein in the form of phyto-oestrogen-rich soy extract resulted in genistein plasma concentrations of 11 μM and 4·9 μM, respectively (Xu *et al.* 1994; Peterson, 1995). Similarly, after receiving a single dose of phyto-oestrogens of 2·0 mg/kg of body weight in soy milk, phyto-oestrogen levels in human plasma have been measured at 2·2 μM (Xu *et al.* 1994). However, the vast majority of publications that describe the effects of phyto-oestrogens *in vitro* have reported significant results mostly at concentrations higher than those that are achievable *in vivo* after oral consumption of soy-based foods or products enriched in phyto-oestrogens. Wang & Kurzer (1998), for instance showed that biochanin A inhibited DNA synthesis at 20–90 μM, and Fotsis *et al.* (1997) showed that the phyto-oestrogens genistein inhibited *in vitro* angiogenesis at a concentration of 10 μM. The present study extends those observations; the lowest concentration which was found to exhibit significant growth inhibitory effects in the presence of 10 % serum was 3·5 μM, which is within the physiologically achievable concentration ranges. Our results therefore show that biochanin A was able to inhibit growth in human endothelial cells at concentrations that are readily achievable in plasma.

In the absence of FBS, the IC_{50} for biochanin A was more than 200 times lower (0·18 μM *v.* 35 μM) than in the presence of 10 % FBS. Zava & Duwe (1997) have suggested that the bioavailability of phyto-oestrogens at growth-inhibitory concentrations might be substantially lower in the plasma due to binding to factors present in the serum. Our results are consistent with the idea that the effects of biochanin A on cell proliferation might be partially masked or inactivated by factors in the serum. Cell density also appears to influence the effectiveness of the growth regulatory effects caused by biochanin A (Fig. 3). At the concentration tested here (55 μM), biochanin A exhibits cytostatic rather than cytotoxic effects, and it was found that the number of viable cells was most strongly reduced in the cells at low (~40 %) cell density. This presumably reflects the fact that at low cell density, cells were in a more active growth phase; the growth regulatory effects induced by biochanin A would thus have a proportionally greater impact. Wang & Kurzer (1998) have pointed out that the *in vitro* growth inhibitory effects of phyto-oestrogen might not be reproducible *in vivo* because of interference by naturally occurring oestrogen, which is present in serum at concentrations of approximately 0·1–1 nM. The results shown in Fig. 4, however, suggest that it is likely that the growth regulatory effects of

biochanin A would also be evident *in vivo* because even at the highest concentration of DES tested ($5·5 \times 10^{-3}$ M), the growth regulatory effects of biochanin A were not blocked. No blocking of the inhibitory effects of biochanin A by DES was found either in the presence or absence of serum.

Recently, much interest has been focused on the possible cancer-protective role of the hormone-like phyto-oestrogens; *in vitro* inhibition of cancer incidence or progression by soy products or pure phyto-oestrogens has been reported for gastric cancer, leukaemia cells, breast cancer and other cancers (Matsukawa *et al.* 1993; Hempstock *et al.* 1998; Wang & Kurzer, 1998; Lyn-Cook *et al.* 1999). Histological examination has shown that the consumption of soy products significantly reduced tumour cell proliferation, increased apoptosis and reduced microvessel density (Zhou *et al.* 1999); and a possible mechanism has also been suggested by the interesting observation that the angiogenic protein insulin-like growth factor-I was reduced in the circulation of mice fed soy protein and phytochemical concentrate (Zhou *et al.* 1999). However, several studies have found that soy-based dietary treatments had tumour-promoting effects (Makela *et al.* 1994; Wang & Kurzer, 1998; Hsu *et al.* 1999). Under some conditions, oestrogenic properties of the phyto-oestrogens stimulated the growth of human breast cancer cell *in vitro*. Also increased colon tumour incidence and tumour burden in rats treated by chemical carcinogens were observed when a soybean-based diet or pure phyto-oestrogens were given to these animals (McIntosh *et al.* 1995; Rao *et al.* 1997). In the present study on transformed human endothelial cells, no growth stimulatory effects induced by biochanin A were detected at any of the concentrations tested. Despite the encouraging results presented here, however, much more work clearly remains to be done before soy supplements enriched in phyto-oestrogen can be universally recommended for cancer prevention.

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