Dietary Chemopreventive Phytochemicals: Too Little or Too Much?

Elena P. Moiseeva and Margaret M. Manson

Abstract There is a large body of evidence that the consumption of fruit and vegetables can decrease the risk of cancer. However, the link between diet and health is extremely complex. Some dietary phytochemicals seem to offer protection in an exposure-related manner and many molecular targets and signaling pathways affected by phytochemicals have been discovered. Although *in vitro* studies have contributed significantly to our understanding, quite a number use concentrations orders of magnitude greater than those achievable in humans or toxic to normal tissues (exemplified by toxic concentrations of indole-3-carbinol, epigallocatechin-3-gallate, curcumin, and genistein for breast cells). Such studies may produce results that are physiologically irrelevant, thus hindering predictions of efficacy. Here, we argue for careful consideration to be given to the *in vitro* experimental conditions under which dietary phytochemicals are investigated. Design features, such as the use of appropriate nontoxic concentrations, extended treatment times, three-dimensional cultures, primary tumor cultures, and comparison of susceptibility of various cancer subtypes, should improve our understanding of their molecular targets. This in turn would facilitate predictions as to their potential usefulness in the clinic.

Chemopreventive Effect of Vegetables and Fruit

Diet is thought to contribute to a significant proportion of cancer cases and about a third of mortalities (1). Several international committees previously concluded that consumption of fruit and vegetables decreases the risk of cancer (2–4). More recently, the second WCRF-AICR report (5) found that overall, the evidence that vegetables and fruit protect against cancer is less impressive. However, among specific groups, Allium vegetables and garlic were considered to offer probable protection against stomach and colorectal cancers, respectively. Other groups, e.g., cruciferous vegetables [source of isothiocyanates (ITC) and indoles] or tea (source of polyphenols), received little attention in this report. The International Agency for Research on Cancer concluded that consumption of cruciferous vegetables is associated with a modest risk reduction for cancers at some sites, although the reductions are no greater than those observed with total vegetable uptake (6).

Because of insufficient evidence, the conclusions of the latest WCRF-AICR report have somewhat undermined the hypothesis that specific phytochemicals, present in vegetables and fruit, may be responsible for chemoprevention. This lack of convincing evidence is partly because important contributing factors were not considered in many of the original studies. Many based on self-reporting are found to be biased. Factors, such as tobacco, gene polymorphisms, and body composition, can modify cancer risk (5). Significant differences in levels of consumed vegetables, and consequently dietary phytochemicals, are found among various populations. For example, the highest levels of cruciferous vegetable uptake in Europe and North America reach just 30% to 50% of the average levels in Japan and China (6). Total isoflavone uptake, mostly from soybeans, in Europe is ~0.44 mg/day in contrast to 33.5 and 46.5 mg/day in China and Japan, respectively (7). Levels of consumed phytochemicals may be below an efficacious threshold in some populations and, thereby, without a chemopreventive effect.

Moreover, availability and circulating levels of phytochemicals are influenced by genotypes of metabolizing genes, frequencies of which vary among populations (8). Hence, benefits can be more prominent in carriers of particular genotypes. High uptake of vegetables reduces the risk of breast cancer for carriers of particular alleles of the CA repeat in the *EGFR* gene (9). The protective effect of cruciferous vegetables and ITC is stronger in individuals null for *GSTM1* and/or *GSTT1* (10–14). Tea polyphenols modify the effect of *CYP19A1* and *COMT* polymorphisms on cancer risk (15, 16). Phytoestrogens interact with polymorphisms in *ESR1* and *NR1I2* genes to modulate estrogens levels (17). Hence, a pooled analysis of diverse populations without consideration of genotype modifiers and frequencies of specific alleles may dilute the cancer-preventive effects of diet.

Accumulating evidence indicates protective effects against specific subtypes and groups of cancers. Soybean-based food

Authors' Affiliation: Cancer Biomarkers and Prevention Group, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester LE1 7RH, United Kingdom
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Requests for reprints: Elena Moiseeva, Cancer Biomarkers and Prevention Group, Biocentre, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom. Phone: 44-0-116-223-1822; Fax: 011-44-116-2231840; E-mail: em9@le.ac.uk.

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has a protective effect against $ER^+/PR^+/HER2^-$ and ER^+/PR^+ breast tumors (18, 19). These data also support, to some extent, previously published data on the protective effect of genistein against ER^+ tumors (20). Similarly, a protective effect of soybean genistein is shown in localized prostate cancer, but not in advanced cancer (21). Differential effects of soybeans also occur with regard to the epidermal growth factor receptor (*EGFR*) mutation status in non–small cell lung cancers (22).

Overall, the link between diet and health seems to be much more complicated than previously anticipated. Further investigations into the potential of phytochemicals, which take account of modifying factors, a potential threshold effect and cancer subgroups, are essential to establish their effective use in chemoprevention.

Exposure-dependent Protection

Laboratory studies indicate that $>10^3$ different phytochemicals possess cancer-preventive activity and about 35 plant-based foods, including garlic, onion, tomatoes, cruciferous vegetables, tea, soybeans, and turmeric have chemopreventive properties (23). The second WCRF-AICR report found evidence for association of lycopene and quercetin with lower risks for some cancers (5).

Where consumption of cruciferous vegetables was estimated by equivalent ITC consumption, there was an association with reduced risk of lung, bladder, and colorectal cancers (10–12, 24). Similarly, urinary ITC levels showed an inverse correlation with risk of lung, colorectal, and breast cancers (13, 14, 25). Urinary tea polyphenols decreased risk of gastric, esophageal, and colon cancers (26, 27).

Several studies found a dose-dependent protective effect of genistein against breast and prostate cancers, mostly in the countries with high levels of soybean uptake in contrast to most of EPIC studies (21, 28–33). However, the role of isoflavones in cancer prevention is controversial: although linked to reduced risk of some cancers, they were also associated with increased risk of advanced prostate cancer and marginally with breast cancer (7, 21, 33). Moreover, soy supplementation increases cell proliferation in breast and colon (34, 35).

Green tea consumption increases the protective effect with the duration of exposure (36–39). Similarly, the protective effects of tea polyphenols and ITC become stronger with increasing follow-up time (25, 26).

Furthermore, translational studies using long-term supplementation (3-82 months), have also shown efficacy. Supplementation with indole-3-carbinol (I3C), derived from cruciferous vegetables, reduces vulval and cervical intraepithelial neoplasia and respiratory papillomatosis (40–42). Green tea catechins, containing epigallocatechin-3-gallate (EGCG) as a major component, prevent development of prostate cancer in patients with high-grade prostate intraepithelial neoplasia (43).

Physiologic Concentrations

Physiologic concentrations of dietary phytochemicals rarely exceed the nanomolar range (44, 45), but vary dramatically in different populations. For example, mean serum concentrations of genistein in the Japanese population (0.5 μmol/L) are significantly higher those in the United Kingdom (33 nmol/L; refs. 30, 46). Supplementation can increase circulating concentrations, such that curcumin, EGCG, and genistein can reach 1.8, 8.7, and 27 μmol/L, respectively (45, 47, 48). Under such conditions, I3C is still undetectable in humans, but generates up to 2.5 μmol/L 3,3′-diindolylmethane (DIM) in serum (49). In mice, I3C supplementation produces plasma concentrations of 28 μmol/L I3C and 4 μmol/L DIM, whereas DIM supplementation produces 24 μmol/L (50, 51). Furthermore, tissue levels can be much higher than in plasma (44). Thus, maximal levels of I3C and DIM found in murine liver exceed circulating levels by six-fold. Curcumin reaches 8 and 13 nmol/g in human colon and colon cancer samples, respectively, and 500 nmol/g in murine colon. Thus far, the highest levels of tea polyphenols have been found in oral epithelial cells, but they should also be high in the colon, the major excretion route (48).

Where phytochemicals (I3C, tea polyphenols, and curcumin) have been investigated in extended trials, they have been associated with very few side effects (41–43, 52). Nonetheless, some adverse effects have been reported, e.g., moderate and significant toxicity for green tea (6 grams/day) and green tea extract, presumably attributed to caffeine (53, 54). In animal models, carcinogen-induced tumorigenicity could be enhanced by high doses of I3C or resveratrol (55–57) and growth and malignancy of tumors was promoted by genistein supplementation (58–65). Moreover, combining EGCG and genistein in the diet enhanced intestinal tumorigenesis in $APC^{-/+}$ mice (66). The tumor-promoting effects of high doses of genistein have been confirmed by the USA National Toxicology Program (67, 68).

Considerations for Mechanistic Studies

Improved *in vitro* models

Numerous studies have identified mechanisms of action for dietary phytochemicals, which include increased detoxifying activity, enhanced steroid hormone catabolism, and inhibition of pathways critical for cancer development and maintenance (23, 69). However, for clarification of relevant molecular mechanisms, consideration should be given to realistic doses, extended treatments, three-dimensional culturing conditions, the use of transformed cells (representing early stages of carcinogenesis), and different cancer subtypes.

Most studies are conducted using monolayer culture, but some have shown that phytochemicals at physiologically achievable concentrations affect behavior of cancer cells when grown in three dimensions. EGCG inhibits spheroid growth of colon cancer and glioblastoma cells (70, 71). Genistein inhibits infiltration of glioblastoma cells into fetal brain aggregates (72). Culturing in collagen gel or as spheroids increases susceptibility of breast cancer cells and normal fibroblasts to I3C (73).

Although use of physiologically achievable concentrations would seem to be the most appropriate option in mechanistic studies, these are unknown for some phytochemicals, or may be increased using improved formulations. For those with known pharmacologic concentrations, some molecular targets identified *in vitro* have been confirmed in models *in vivo* (44). However, low concentrations, given as a single treatment *in vitro*, frequently produce no measurable response, consistent with the requirement for extended treatments to achieve antitumorigenic effects *in vivo*. Such long-term experiments can sometimes prove difficult *in vitro*. The only study, which

has investigated extended treatments of breast tumor cells with physiologically achievable concentrations of five agents, confirmed some reported mechanisms and targets, but failed to confirm reactivation of gene expression by EGCG or modulation of histone acetylation by curcumin (74), raising questions as to the validity of some results obtained with higher concentrations.

Susceptibility of cancer cells in comparison with their nontransformed counterparts

An alternative approach, allowing improved biomarker detection in short-term treatments, would be to use a range of concentrations, which is nontoxic to normal cells and, therefore, selective for susceptible cancer cells. Thus, curcumin induces apoptosis and enhances action of oxaliplatin in colon cancer cells, but not in immortalized normal colon cells (75). Mammary carcinoma cells are more susceptible to curcumin than normal cells (76, 77). Curcumin also induces distinct migratory responses in young adult mouse colon cells compared with immortalized mouse colon cells (78). EGCG causes apoptosis in epidermoid carcinoma cells, but not in normal keratinocytes (79). At high doses, it also induces oxidative stress in oral carcinoma cells, but not in normal cells from salivary gland (80). I3C selectively induces apoptosis in breast cancer MCF10CA cells, but not in nontumorigenic MCF10 cells (81). Conversely, some cancer cells are more resistant to phytochemicals than normal cells, e.g., HBL100 cells are resistant to I3C concentrations, which are toxic to normal primary breast cells (73, 82).

We studied the effect of several compounds on the viability of primary breast cultures generated from reduction mammoplasties in comparison with MDA-MB-231 breast cancer cells. Primary cultures contained epithelial, myoepithelial, and stromal cells, representative of cell populations in the intact breast tissue, as evidenced by expression of E-cadherin, P-cadherin, and cadherin-11 (Fig. 1A). Viability of malignant MDA-MB-231 cells was drastically reduced by curcumin and EGCG at concentrations above 20 and 50 μmol/L, respectively, whereas viability of cells from normal breast was not affected (Fig. 1B). The situation with curcumin was complex, because the normal cells were altered morphologically at concentrations above 20 μmol/L. MDA-MB-231 cells were selectively inhibited by genistein only at 50 μmol/L in comparison with normal breast cells, whereas loss of viability of normal and cancer cells in the presence of I3C was similar. These data suggest that MDA-MB-231 cells are susceptible to curcumin and EGCG, marginally sensitive to genistein, and not more sensitive to I3C, when compared with normal breast cells.

The fact that cell culture conditions influence viability may have particular importance for normal cells, when they are exposed to high concentrations of growth factors and deprived of adhesion to an appropriate extracellular matrix. Therefore, we compared sensitivity of normal breast cells to I3C in different growth conditions. Those grown on plastic in a rich medium were sensitive to ≥100 μmol/L I3C, exhibiting increased caspase 3/7 activity at 200 μmol/L (Fig. 1C), but when grown on Matrigel in less-rich medium, they were resistant to 200 to 250 μmol/L I3C similar to normal fibroblasts (73). In contrast, MDA-MB-231 cells show increased caspase 3/7 activity at 125 to 200 μmol/L (73). These data indicate that concentrations exceeding 20 μmol/L curcumin, 125 μmol/L EGCG, 50 μmol/L genistein, and 200 μmol/L I3C compromise viability of primary cultures from normal breast and should not be used in mechanistic studies relating to breast cancer.

Susceptibility of cancer subtypes

Cancer subtypes are defined for some tissues, e.g., subtypes of breast cancer, colon cancers with different microsatellite stability, androgen receptor–dependent, and androgen receptor–independent prostate cancers. It is feasible that specific subtypes are affected by dietary agents, where others may be resistant. There are very few studies showing preferential protection by vegetables or phytochemicals against particular subtypes.

Genistein, which influences the levels of estrogens and pathways regulated by estrogen and androgen receptors at physiologic concentrations (83–85), is expected to affect hormone-dependent and hormone-independent breast and prostate cancers differently. Indeed, soybeans and genistein, in particular, have a protective effect against ER^+ breast tumors (18–20). Importantly, this effect is likely to be caused by reduced estradiol levels due to genistein exposure (83), rather than by a direct effect of genistein on ER^+ cancer cells. In contrast, an ER^+ tumor–promoting effect of genistein is shown in animal and/or *in vitro* models (59, 60, 86). This contradiction questions suitability of rodent xenograft ER^+ models.

I3C represses ERα signaling and increases 2-hydroxylation of estrogen, regarded as favorable in cancer prevention (87), and may selectively prevent development of ER^+ breast cancer. When a panel of breast cancer cell lines has been compared with primary cultures from Tag mouse mammary tumors with a basal-like subtype, expressing EGFR and high levels of activated Src, for susceptibility to I3C and DIM (88), the primary cultures were susceptible to I3C, but not DIM. Among the established cell lines, those with basal-like and HER2 subtypes, which overexpress EGFR and HER2, are the most susceptible to I3C (82, 88). In both primary cultures and breast cancer lines, overexpression of Src and EGFR/HER2 has been implicated in the mechanism of action of I3C, suggesting I3C may prevent or delay growth of the basal-like and HER2 subtypes, which are dependent on Src, EGFR, and HER2. It is tempting to speculate that high consumption of dietary indoles, including I3C, originating from cruciferous vegetable, may contribute to the preventive effect of vegetables on breast cancer in carriers of various *EGFR* genes (9).

Use of inappropriate doses may obscure the pathways involved in cell response

Investigation of specific pathways shows that response to phytochemicals is related to dose; e.g., hemeoxygenase-1 and the Nrf-2 pathway are induced at low doses of curcumin and EGCG, but not at high doses (89). Although fairly high concentrations of some phytochemicals are appropriate in cells from tissues with high bioavailability (e.g., liver for I3C and DIM or colon for curcumin and EGCG), lower concentrations should be used for cells from tissues dependent on systemic bioavailability. Physiologically unrealistic concentrations have been used in many studies, including some of our own, to increase or hasten detection of end points (73, 82, 90). When the most susceptible breast cancer MDA-MB-468 cells were compared with several other lines, a role for EGFR and Src in apoptosis induced by I3C (125-250 μmol/L) was elucidated, and this was later confirmed for EGFR in MDA-

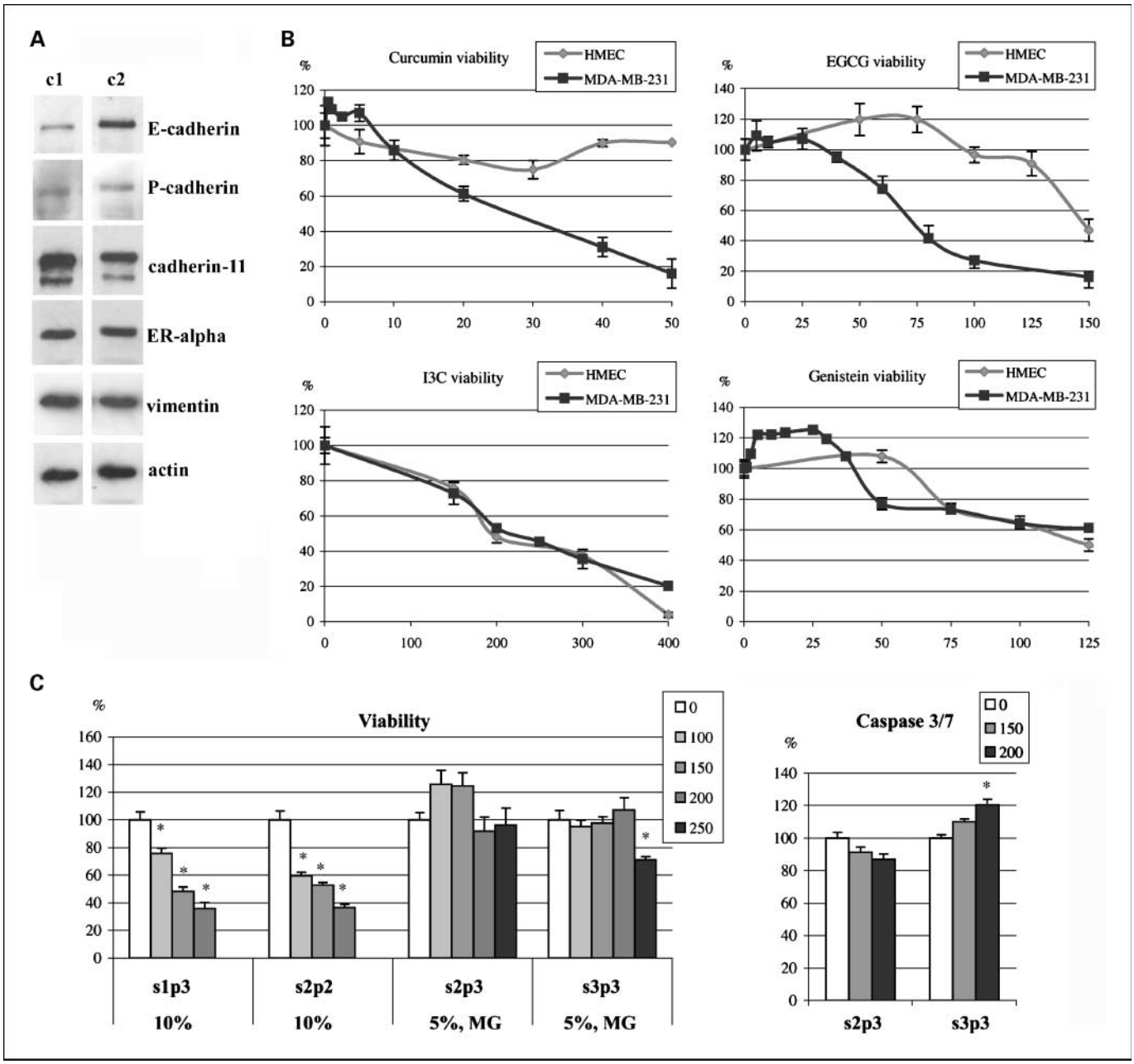

Fig. 1. *A*, cultures 1 and 2 (*passages 2-3*) from reduction mammoplasties were analyzed for biomarker expression using Western blotting with the antibodies indicated. EGFR and HER2 were not detectable. ERα had molecular weight about 55 kDa probably representing a short isoform. *B*, effect of curcumin, EGCG, I3C, DIM, and genistein (μmol/L) on viability of breast cancer MDA-MB-231 cells and primary cell culture from normal breast (HMEC) after 48 h of treatment, as measured by Moiseeva et al. (82). *C*, effect of treatment with μmol/L I3C on viability and activity of caspase-3/7 in primary cultures from normal breast after 48 h. Viability was measured in cells grown in normal conditions, e.g., medium with 10% FCS, and in cells grown on Matrigel (MG) in the medium with 5% FCS. Caspase activity was measured in the normal medium. The numbers for specimens (*s*) and passages (*p*) are indicated.

MB-231 cells following extended treatments with pharmacologic concentrations (30 μmol/L; ref. 74). Conversely, experiments with high concentrations can indicate pathways that are irrelevant to loss of viability in cancer cells. For example, down-regulation of Akt, observed at high I3C concentrations, was subsequently shown not to be responsible for I3C-induced apoptosis (90, 91). Recent studies in breast cancer cells using EGCG (40-160 μg/mL = 87-351 μmol/L, which is borderline or toxic for normal breast cells) indicate that FOXO3 is involved in EGCG-induced cell death, reduced invasiveness, increased expression of E-cadherin and ERα, whereas further work from the same group suggests that EGCG may modulate E-cadherin expression via down-regulation of CK2 and c-Rel signaling (92–94).

Genistein at 200 μmol/L reduced IL-6 levels in breast cancer MDA-MB-231 cells, whereas extended treatment with 2.5 μmol/L increased IL-6 mRNA levels in these cells (74, 95). Similar results were obtained with curcumin (74, 96). Phytochemicals, such as curcumin and EGCG, can increase oxidative stress and cause DNA damage at high concentrations (see

references in ref. 74), whereas acting as antioxidants to reduce DNA damage at dietary or pharmacologic concentrations (97).

Conclusions

Use of inappropriate concentrations of dietary phytochemicals in mechanistic studies may generate artifactual results, which can be misleading and physiologically irrelevant. These studies require careful consideration and realistic approaches with respect to dose, growth conditions, and choice of cell types to provide meaningful translational information. In particular, comparing susceptibility of cancer cells of various subtypes is likely to highlight certain oncogenes as specific targets for phytochemicals. Such studies, combined with those in animal models of various cancer subtypes, will contribute to an in-depth understanding of molecular mechanisms of action of dietary components. Likewise, population studies on protective effects of vegetables and individual phytochemicals can benefit from analysis of specific cancer subtypes, converging with mechanistic studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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