Flavonol-stimulated Efflux of 7,12-Dimethylbenz(a)anthracene in Multidrug-resistant Breast Cancer Cells

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ABSTRACT

We used a series of P-glycoprotein (P-gp) expressing multidrug-resistant (MDR) cells, developed from human breast cancer MCF-7 cells by exposure to Adriamycin, to investigate the effects of flavonoids on P-gp-mediated efflux mechanisms for chemical carcinogens. We previously showed that MDR cells derived from exposure to Adriamycin are cross-resistant to a chemical carcinogen, benz[a]pyrene, due to its cellular efflux by the P-gp-mediated putative drug efflux pump. Our current studies extend this observation to another polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)anthracene, known to induce mammary tumors in animals. In our attempt to find naturally occurring dietary compounds which may stimulate the P-gp-mediated efflux of carcinogens, we found that certain flavonoids, kaempferol, quercetin, and galangin, are potent stimulators of the P-gp-mediated efflux of 7,12-dimethylbenz(a)-anthracene. The increased efflux decreased the cellular burden of 7,12-dimethylbenz(a)anthracene. Since these flavonol compounds are widely distributed in fruits and vegetables, their stimulatory effect on P-gp may be a mechanism relevant to carcinogenesis and the observed lowered cancer risk in humans with higher dietary intake of fruits and vegetables.

INTRODUCTION

Our strategy to elucidate diet-dependent biological mechanisms in carcinogenesis and cancer prevention led us to examine cellular defense mechanisms against carcinogens. In this context, the model for pleiotropic drug resistance offered an attractive model. Many features of the metabolic system for activation and detoxification of pleiotropic anticancer drugs parallel those for chemical carcinogens (1, 2). The central role of P-gp, the putative efflux pump, in drug-resistant tumor cells (3, 4) together with the recent finding that P-gp is widely distributed in normal tissues (5) led us to reason that P-gp may participate in the cellular defenses against carcinogens. We recently reported direct evidence supporting this hypothesis (6). Using a series of MDR cells derived from human breast cancer MCF-7 cells, we showed that (a) MDR cells were cross-resistant to benzo(a)pyrene; (b) MDR cells could efflux BP, whereas WT MCF-7 did not and this efflux was inhibited by verapamil; and (c) BP directly competed with azidopine in photoaffinity labeling of P-gp.

Having shown that P-gp may provide a defense mechanism against a chemical carcinogen (6), we sought to identify dietary factors which modulate P-gp function and thereby serve as a mechanistic link between diet and cancer prevention. Flavonoids were an attractive group of compounds not only because of their abundance and wide distribution in fruits and vegetables (7) but also because of their established chemopreventive activity against tumorigenesis with several carcinogens in animal models (8–10). Of special relevance is the inhibitory effect of flavonoids against BP-induced skin tumors (11) and DMBA-induced mammary tumors (12). Thus, we proposed that the P-gp-mediated efflux of carcinogens is a potential mechanism for the established chemopreventive effects of flavonoids.

We previously established that flavonoids are active in the P-gp-mediated efflux of its established substrate, Adriamycin. In HCT-15 colon cancer cells, we found that a number of flavonoids decreased Adriamycin accumulation and increased Adriamycin efflux in HCT-15 cells; the flavonols, e.g., kaempferol, galangin, and quercetin, were the most potent stimulators of P-gp function (13). However, it remained to be shown whether this effect is demonstrable in established MDR cell lines and applicable to chemical carcinogens. We now report that flavonols markedly activated the P-gp-mediated-efflux of DMBA in a line of defined MDR cells derived from human breast cancer cells, MCF-7. Thus a dietary factor may affect the accumulation of a well-established mammary carcinogen in animals and serve as a possible mechanism for dietary effects on carcinogenesis and cancer prevention.

MATERIALS AND METHODS

Materials. We obtained DMBA, quinine, kaempferol, and quercetin from Sigma Chemical Co. (St. Louis, MO). Galangin was purchased from Aldrich Chemical Co. (Milwaukee, WI), and verapamil was the gift of Knoll Pharmaceuticals, Inc. (Whippany, NJ).

Cells. The MDR cells derived from human breast cancer, MCF-7, have been described (6). In brief, the series of MDR cells derived from continuous exposure to Adriamycin (doxorubicin) showed increasing levels of resistance and increasing expression of P-gp protein on Western immunoblots and mRNA on Northern blots. Cells were maintained in RPMI 1640 (Biofluids, Rockville, MD) with 2 mM glutamine and were supplemented with 10% fetal bovine serum (Biofluids). For the experiments, cells were plated at 2 × 10⁴/chamber in 4-cm² coverglasses of tissue culture chambers (Nunc) in 1 ml RPMI without phenol red (Biofluids) with 10% FBS and 2 mM glutamine. Cells were used after 48–72 h of plating. In some experiments, cells were exposed to 1 μM Adriamycin for 48 h before the experiment.

DMBA Accumulation and Efflux. To start the experiment, we removed the growth medium and washed the cell monolayer 3 times with 1 ml fresh medium. For accumulation studies, DMBA (1 μM, final concentration) was added in dimethyl sulfoxide (0.1%, final concentration) to 1 ml fresh medium, and fluorescence was monitored. For DMBA efflux studies, a 10-min preincubation with or without inhibitors was followed by a 20-min loading period with 1 μM DMBA. The monolayer was then washed 3 times and incubated with fresh medium without DMBA and with or without inhibitors while efflux was monitored. In experiments when flavonols were added, they were present only during the efflux period. Possible toxicity with flavonols at the concentrations used was ruled out by separate studies using trypan blue exclusion as the end point.

In the aforementioned studies in intact cells, real time DMBA dynamics was determined by fluorescence cytometry on the ACAS 570 (Meridian Instruments, Okemos, MI) fitted with a 37°C incubator stage. Laser excitation was at 320 nm. A brief description of the methodology for interactive laser cytometry of attached cells follows. After a field was selected, it was scanned in 180 × 1 μm steps in both X and Y axes. The stored data was processed to form a digitalized computer image of fluorescence intensity (see Fig. 3 from Ref. 6). For studies of accumulation, the field was scanned repeatedly every 3–5 min over a period of up to 60 min; and for efflux studies, the field was scanned every 75 sec for 15 or 20 min. The ACAS kinetics computer program calculated the average fluorescence over multiple, specified areas comprised of groups of cells (6). To quantitate efflux, the values obtained for each scan were...
normalized to their respective initial average fluorescence, and the rate of change was calculated.

We routinely checked for photobleaching by examining an adjacent, unscanned field. There was no detectible photobleaching with the level of laser energy used for excitation. Quenching by reagents, i.e., flavonols, verapamil, and quinine, was ruled out using wild-type cells loaded with DMBA in which fluorescence was measured immediately before and after the addition of the aforementioned reagents to the medium. Quenching was not a problem presumably because the inverted optical design of ACAS excluded the medium from the light path. Another factor may be the optical depth-of-field along the Z axis which was limited to about 10 µm.

RESULTS

Although we showed that BP is a substrate for P-gp, the demonstration of this effect with a related polycyclic aromatic hydrocarbon, DMBA, was important because it is a well-established experimental carcinogen for mammary tissue in animals (14). When cells were exposed to 1 µM DMBA, the accumulation of DMBA was lower in MDR cells compared to WT MCF-7 cells (Fig. 1). The differential pattern of DMBA accumulation in MDR and WT cells was similar to that seen with other P-gp substrates; DMBA in WT cells showed a curvilinear rise whereas in MDR cells DMBA accumulation rapidly reached a plateau. By 60 min, the level of DMBA in WT was about twice that in MDR cells. When DMBA efflux was monitored, the rate in MDR cells was markedly higher than in WT cells (Fig. 2). Although this was true for all stages of MDR MCF-7 cells tested, the level of efflux was not directly correlated with the fold resistance to Adriamycin, i.e., 200-fold resistant cells, R200, had a rate similar to that of 65-fold resistant cells, R65 (Fig. 3). Importantly, the efflux of DMBA in MDR cells was blocked by verapamil and by quinine, two known inhibitors of P-gp function. Normalized values for DMBA efflux in MDR controls with 40 µM verapamil and 40 µM quinine were 0.073 ± 0.005 (SE)/min, 0.039 ± 0.004/min, and 0.041 ± 0.004/, respectively. The inhibitory effects of verapamil and quinine essentially abolished P-gp-mediated efflux since the rates were indistinguishable from that in WT cells (0.038 ± 0.003/min). Importantly, DMBA efflux in WT was unaffected by either verapamil (0.034 ± 0.004) or by quinine (0.037 ± 0.002). These findings clearly demonstrated that DMBA, like BP, was a substrate for the P-gp-mediated efflux mechanism.

We previously showed in HCT-15 colon cancer cells that certain flavonols stimulated the P-gp-mediated efflux of Adriamycin (13). Since we hypothesized that P-gp may play a role in the cellular defense against carcinogens (6), the possibility that these stimulators of P-gp may affect the accumulation and efflux of DMBA was of great interest. First, we studied the effect of flavonols on the cellular accumulation of DMBA in MDR cells and found that the accumulation of DMBA at a concentration of 1 µM was markedly decreased by kaempferol, galangin, and quercetin (Fig. 4). We further demonstrated with kaempferol that the effect on DMBA accumulation was concentration dependent. Compared to control levels after 30 min of accumulation with 1 µM DMBA, the levels with added kaempferol at 5 µM,
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Fig. 4. Effect of flavonols on the accumulation of DMBA in MDR MCF-7 cells (R12). Kaempferol (KF), quercetin (QCT), or galangin (GAL) were added to the incubation medium at a concentration of 100 µM during the incubation with 1 µM DMBA. The cellular accumulation of DMBA was monitored as described in "Materials and Methods" and in the legend for Fig. 1.

20 µM, and 100 µM were 69, 62 and 26%, respectively, of control. Furthermore, the time course with kaempferol treatment showed that DMBA accumulation (Fig. 4) followed a pattern similar to that for Adriamycin accumulation. This finding suggested that kaempferol decreased accumulation primarily through a stimulatory effect on efflux.

Direct estimates of the stimulatory effect of flavonols on DMBA efflux was performed in MDR and WT cells loaded with 1 µM DMBA for 20 min. Efflux in MDR cells was markedly increased with 5 µM kaempferol. Rates in controls and with 5 µM kaempferol were 0.073 ± 0.005/min and 0.149 ± 0.008/min, respectively. In contrast, kaempferol had no effect on DMBA efflux in WT cells. The control DMBA efflux in WT cells was 0.038 ± 0.010/min, a level significantly lower than the control rate in MDR cells. Importantly, the presence of 5 µM kaempferol did not increase DMBA efflux in WT cells (0.033 ± 0.006/min). We further showed that the magnitude of stimulation with kaempferol increased with increasing concentrations up to 25 µM (Fig. 5), but at concentrations greater than 5 µM, efflux was so rapid that precise determinations were difficult. Therefore, most of the experiments on DMBA efflux with kaempferol were performed with 5 µM kaempferol. The observed effect of the flavonols was generalized to quercetin and galangin which at 5 µM yielded efflux values of 0.146 ± 0.020/min and 0.145 ± 0.014/min, respectively.

The finding that flavonols markedly stimulated DMBA efflux in MDR cells but was without effect in WT cells suggested that flavonols exerted their effect through a mechanism mediated by P-gp. However, additional evidence was needed to support this interpretation. We found that P-gp reversal agents, verapamil and quinine, inhibited DMBA efflux in MDR cells from kaempferol-stimulated levels down to the level found in WT cells. However, higher concentrations of these P-gp reversal agents were necessary to abolish P-gp-mediated efflux of DMBA in the presence of kaempferol than in controls. In the presence of 5 µM kaempferol, 160 µM verapamil and 80 µM quinine were necessary to abolish DMBA efflux to the basal levels of WT cells which do not express P-gp (Fig. 6).

DISCUSSION

These studies clearly showed that DMBA, like benzo(a)pyrene (6), is a substrate for P-gp in MDR cells derived from human breast cancer, MCF-7. The differential accumulation and efflux of DMBA in MDR versus WT cells as well as the abrogation of these differences by P-gp reversal agents strongly supported this interpretation. The relevance of DMBA to carcinogenesis in mammary tissue has been well established in animals (14), but polycyclic aromatic hydrocarbons as an etiological agent for human breast cancer remains unproven (15). Because of their hydrophobic nature, these carcinogens are usually thought confined to the sites of primary exposure, the gastrointestinal tract and the lung, due to exposure from the diet and tobacco smoke, respectively. However, recent studies showed that ingested hydrophobic carcinogens, e.g., benzo(a)pyrene, are absorbed
cells were washed 3 times with fresh medium. Adriamycin does not fluoresce when excited at 320 nm. MDR cells first were preincubated for 10 min with or without reversal agents. The preincubation medium was replaced with loading medium containing 1 μM DMBA with or without the indicated reversal agents, and incubation was continued for 20 min. Following this period of DMBA accumulation, the medium was removed, and the monolayer was washed 3 times with 1 ml of fresh medium. The efflux period was monitored in the presence or absence of kaempferol (KF) at a concentration of 5 μM. The indicated reversal agents were also present during the efflux period. Efflux was calculated as described in the legend for Fig. 2 and was expressed as the fractional decrease in normalized fluorescence/min. Mean, ± SE of at least 5 determinations.

Fig. 6. Inhibition of the kaempferol stimulation by MDR reversal agents. In these experiments, MDR cells (R12) were plated as described in “Materials and Methods” but were treated with 1 μM Adriamycin for 48 h. Before the experiment, the cells were washed 3 times with fresh medium. Adriamycin does not fluoresce when excited at 320 nm. MDR cells first were preincubated for 10 min with or without reversal agents. The preincubation medium was replaced with loading medium containing 1 μM DMBA with or without the indicated reversal agents, and incubation was continued for 20 min. Following this period of DMBA accumulation, the medium was removed, and the monolayer was washed 3 times with 1 ml of fresh medium. The efflux period was monitored in the presence or absence of kaempferol (KF) at a concentration of 5 μM. The indicated reversal agents were also present during the efflux period. Efflux was calculated as described in the legend for Fig. 2 and was expressed as the fractional decrease in normalized fluorescence/min. Mean, ± SE of at least 5 determinations.

readily into the circulation through the lymphatics bound to lipoproteins (16), and thereby can be delivered to a variety of tissues including presumably the breast.

Of special interest to us, flavonols, widely distributed dietary factors known to have chemopreventive activity against a variety of tumors (8–12), decreased the accumulation of DMBA by stimulating its efflux through the P-gp-mediated efflux pump. The effect of three flavonols, kaempferol, galangin, and quercetin, on DMBA accumulation and efflux has been clearly demonstrated in MDR cells. Previous work from our laboratory showed that these three flavonols were potent in stimulating the efflux of Adriamycin in HCT-15 colon cancer cells through a P-gp-dependent mechanism (13). In our current studies, the involvement of P-gp in stimulating DMBA efflux in MDR MCF-7 cells was supported by the inability of flavonols to stimulate DMBA efflux in WT cells and by the finding that verapamil and quinine, two P-gp reversal agents, completely abolished P-gp-mediated DMBA efflux in MDR cells even when stimulated by flavonols.

Although it is well established that flavonoids can inhibit tumorogenesis in animals, their specific mechanisms of action are not fully understood. An interaction with both activation and detoxification mechanisms have been implicated. Our studies showed that in cells exposed to both flavonols and carcinogens, flavonols reduced the cellular burden of carcinogens by increasing their active efflux through the P-gp-mediated efflux pump. Whether this effect of flavonols identified in vitro is relevant to human carcinogenesis depends, to a large part, on their absorption, metabolism, and delivery to target tissues. To our knowledge, there are few studies of flavonol absorption, circulation, tissue uptake, and urinary excretion. However, in fruits and vegetables, flavonols, e.g., quercetin and kaempferol, are present as glycosides (17), a form which should be easily absorbed from the gastrointestinal tract. Genistein, an isoflavone found in soybeans primarily as conjugates, is readily absorbed and excreted in the urine of humans ingesting soymilk (18).

Just how flavonols produce their rapid activation of the P-gp-mediated efflux pump remains unknown. Elucidation of these mechanisms will be important not only in understanding the flavonol effect but also in the understanding of mechanisms by which P-gp apparently mediates the efflux of a variety of substrates (19). Interestingly, P-gp also may function as a membrane channel for chloride (20) and adenosine triphosphate (21). Some of the known effects of flavonols, i.e., regulation of phosphorylation mechanisms (22) and mediation of redox interactions (23), are attractive possibilities. Our laboratory is currently investigating this question actively.

In the context of mechanisms inhibiting tumor initiation, P-gp and perhaps other multifunctional efflux pumps may be important because they limit the cellular accumulation of carcinogens. Chemoprevention studies have targeted detoxification enzymes (phase II) as an inducible mechanism affording protection against carcinogens (24). However, a caveat is that activation enzymes (phase I), e.g., p450, which generate genotoxic metabolites from precursors, also may be induced (25). Thus, the net effect resulting from the complex interaction between activation and detoxification mechanisms is not easily predictable. To establish the relative importance of P-gp (or other efflux mechanisms) in mitigating the cellular burden of carcinogens in the context of tumor initiation requires additional studies. Nevertheless, P-gp may serve as an alternative or complementary mechanism to that provided by phase II detoxification enzymes.

Finally, in the general context of diet and cancer prevention, the interaction between flavonols and chemical carcinogens suggests that cellular efflux pumps may be a previously unrecognized diet-dependent mechanism relevant to carcinogenesis. Although carcinogen exposure may occur from the diet, other dietary factors may mitigate the carcinogenic effect. Previous work by others has shown interactions occurring at the organ level, e.g., binding of carcinogens to dietary fiber in the lumen of the intestine. Importantly, interaction also occurs at the cellular level as demonstrated in the current studies. We believe that the elucidation of these putative interactions may be important in revealing paradigms of novel cellular defenses against exposure to dietary carcinogens and xenobiotics.

REFERENCES


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