Critical Review

Lessons from Basic Research in Selenium and Cancer Prevention[1,2]

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ABSTRACT The article reviews the progress in basic research of selenium and cancer prevention during the past decade. Special emphasis is placed on the following four major areas of discussion: *1*) chemical forms of selenium and anticarcinogenic activity; *2*) selenium-enriched food; *3*) in vitro effects of selenite vs. monomethylated selenium; and *4*) aromatic selenium compounds. It is clear that basic research has contributed new knowledge to our understanding of selenium biochemistry, anticancer efficacy and regulation of cell growth. Some of this information could be ready for incorporation into the design of a second-generation selenium trial in humans. J. Nutr. 128: 1845–1854, 1998.

KEY WORDS: • *selenium biochemistry* • *cancer prevention* • *animal models* • *cell growth regulation*

To researchers working in selenium and cancer prevention, the most exciting news in recent years is the finding by Clark et al. (1996) that supplementation of free-living people with selenized brewer's yeast was capable of decreasing the overall cancer morbidity and mortality by nearly 50%. The study was a double-blind, randomized, placebo-controlled trial involving 1312 patients (mostly men) who were recruited initially because of a history of basal cell or squamous cell carcinoma of the skin. Individuals in the treatment arm were given 200 μg Se/d for a mean of 4.5 y (average daily intake in the U.S. is about 100 μg). After a total follow-up of 8271 person-years, selenium treatment did not significantly affect the incidence of these nonmelanoma skin lesions. However, patients receiving the Se-yeast supplement showed a much lower prevalence of developing and dying from lung, colon or prostate cancer. Statistical analyses verified that the relative risk of cancer incidence in lung, colon and prostate was reduced to 0.54 ($P = 0.04$), 0.37 ($P = 0.002$) and 0.42 ($P = 0.03$), respectively. Despite the fact that these are major cancers in the U.S. population, they could be considered only as secondary endpoints because the trial was originally set up to determine whether selenium would decrease the incidence of skin cancer.

A randomized, placebo-controlled intervention trial is the ultimate test to evaluate the efficacy of an anticancer agent. Before Clark's publication, there was already persuasive evidence in the literature suggesting a cancer protective effect of selenium in humans. Geographic correlation data in different regions worldwide and in the U.S. have long noted an inverse association between selenium levels in forage crops or diet and cancer mortality rates (Clark et al. 1991, Schrauzer et al. 1977, Shamberger et al. 1976, Yu et al. 1985). Several prospective and case-control studies also confirmed that people with low blood selenium had an increased risk of cancer (Clark et al. 1984 and 1993, Salonen et al. 1984 and 1985, Willett et al. 1983). Not all selenium and cancer epidemiology investigations produced uniform results because a handful of them failed to find an association (Coates et al. 1988, Knekt et al. 1988, Menkes et al. 1986, Nomura et al. 1987, Ringstad et al. 1988). The discrepancy is not unexpected because epidemiologic designs differ from one another and these diversities are frequently difficult to reconcile. Nonetheless, the potency of selenium is perhaps best exemplified by a meta-analysis of the combined data from a number of studies comparing the significance of serum selenium, retinol, β-carotene and vitamin E in relation to cancer risk (Comstock et al. 1992). Among these micronutrients, selenium emerged as the factor with the most consistent protective effect.

In view of the renewed interest in selenium and cancer, both in the scientific and lay communities, after the publication of Clark's project, it would be timely to examine what has been achieved in basic research during the past decade. The author has been an active participant in the field for many years. A patina of personal perspective is likely to permeate the article. This review is not intended to be all inclusive of every single paper published on the subject. Instead it will focus on four areas that may suggest the direction of our collective effort in the immediate future. In the introductory paragraph of a paper written by Howard Ganther more than 10 years ago (Ganther 1986), he stated that "it is important to keep in mind that the biological activity of selenium is an expression of selenium in a wide variety of chemical compounds, and not the element per se." This message is just as fitting now as ever and could in fact serve as the cornerstone of this review. Incidentally, Ganther has been a long-time collaborator and has contributed in many ways to much of the work in the author's laboratory.

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CHEMICAL FORMS OF SELENIUM AND ANTICARCINOGENIC ACTIVITY

One fascinating aspect of selenium biology is related to its extreme potency. Selenium, in the form of selenite or selenomethionine, functions as an essential micronutrient at levels of ~0.1 ppm (mg/kg) in the animal diet, but it becomes a toxin at levels of 8–10 ppm (Jacobs and Frost 1981). At the other extreme, selenium deficiency is customarily induced in laboratory animals by the feeding of a specially formulated diet which contains <0.01 ppm Se. It should be clarified at the outset that we will not deal with the effect of selenium deficiency on carcinogenesis. The information in this particular topic is not only sketchy but also inconsistent. For this reason, the review is limited to a discussion of the effect of selenium at levels above dietary requirement, usually in the range of 1–5 ppm Se. More than 90% of the selenium cancer chemoprevention experiments have used either sodium selenite or selenomethionine as the test reagent because they are commercially available. Both of these compounds are known to suppress carcinogenesis in many animal models (Combs 1997, El-Bayoumy 1991, Ip 1986, Medina and Morrison 1988). The effect is not organ specific, because tumor inhibition has been reported in mammary gland, liver, skin, pancreas, esophagus, colon and a few other sites. In general, there is a dose-dependent response, and selenium chemoprevention can be realized in the absence of toxicity.

On the basis of a large number of experiments that used a rat chemical-induced mammary tumor model, we showed that selenomethionine was not as active as selenite in cancer inhibition (Ip and Hayes 1989). Tissue selenium concentrations in blood, liver, kidney and skeletal muscle, on the other hand, were always higher in rats given selenomethionine compared with those given selenite. Therefore the greater total body burden of selenium in selenomethionine-treated rats did not appear to confer a better protection against tumorigenesis. The question that came to mind was whether selenium metabolism is necessary for its anticarcinogenic activity.

The above postulate was supported by additional indirect evidence from our laboratory. We found that a low methionine diet significantly reduced the protective effect of selenomethionine, even though tissue selenium was actually higher in these rats compared with those given an adequate amount of methionine (Ip 1988). When methionine is limiting, a greater percentage of selenomethionine is incorporated nonspecifically into body proteins in place of methionine (see **Fig. 1**) because met-tRNA cannot distinguish between methionine and selenomethionine. In other words, the anticarcinogenic activity of selenomethionine is severely compromised in a situation in which it is preferentially compartmentalized into tissue proteins instead of entering the metabolic pathway.

The schematic diagram in Figure 1 shows that methylation is a well-known fate of selenium metabolism (Ganther 1986). With a high intake of selenite or selenomethionine, the levels of methylated metabolites, including methylselenol, dimethyl selenide (expired in breath) and trimethylselenonium (excreted in urine), are expected to rise. Through the support of a collaborative research program with Ganther, we conducted a series of studies that were aimed at addressing the following questions: *1*) Does selenium have to flow through the intermediary inorganic hydrogen selenide pool for the cancer protective effect to be manifested? *2*) Does methylation of selenium enhance or diminish its chemopreventive efficacy? *3*) Is the degree of methylation important? Our strategy was to select precursor compounds that were capable of delivering selenium to specific locations along the methylation pathway

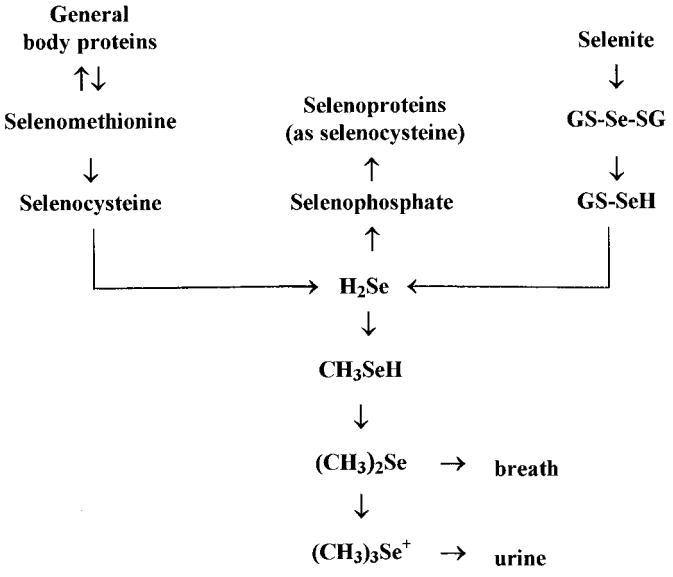

FIGURE 1 Selenium metabolic pathway. Selenomethionine can be incorporated into proteins in place of methionine because it readily acylates Met-tRNA. Alternatively it can be converted through the transsulfuration mechanism to selenocysteine, which in turn is degraded to hydrogen selenide (H_2Se) by the enzyme β-lyase. In contrast, selenite is metabolized to H_2Se via selenodiglutathione and glutathione selenopersulfide. Hydrogen selenide is generally regarded as the precursor for supplying selenium in an active form for the synthesis of selenoproteins. The further metabolism of H_2Se involves sequential methylation by S-adenosylmethionine to methylselenol, dimethylselenide and trimethylselenonium ion.

(**Fig. 2**). By this approach, we hoped to be able to pinpoint more closely the active intermediate that is involved in cancer protection (Ip and Ganther 1992). For a more detailed discussion of the biochemistry of selenium metabolism and the generation of potential chemopreventive metabolites, readers are urged to refer to a recent review by Ganther and Lawrence (1997).

Selenobetaine and Se-methylselenocysteine are good precursors for generating monomethylated selenium. As shown in Figure 2, selenobetaine tends to lose a methyl group first before scission of the Se-methylene carbon bond to form methylselenol (Foster et al. 1986a). Se-methylselenocysteine, on the other hand, is converted to methylselenol directly via a β-lyase reaction (Foster et al. 1986b), and unlike selenomethionine, it cannot be incorporated nonspecifically into proteins. We found that both selenobetaine and Se-methylselenocysteine were more efficacious than either selenite or selenomethionine in cancer chemoprevention in the range of 1–3 ppm Se (Ip and Ganther 1990 and 1992, Ip et al. 1991).

In contrast to the above two compounds, dimethylselenoxide undergoes rapid reduction to dimethylselenide. It had very low chemopreventive activity even at a level of 10 ppm Se (Ip et al. 1991). After a single oral dose of dimethylselenoxide, ~90% was recovered as exhalable dimethylselenide within a 24-h period (Vadhanavikit et al. 1993). Its facile conversion to dimethylselenide, which was then rapidly eliminated via the breath, could provide a plausible explanation for the low anticancer activity.

Selenobetaine methyl ester is known to undergo breakage of the Se-methylene carbon bond to form dimethylselenide directly (Foster et al. 1986a). However, the rate of conversion to dimethylselenide might not be as fast as that with dimethylselenoxide. Interestingly, the anticarcinogenic activity of

selenobetaine methyl ester was found to be comparable to that of selenobetaine (Ip and Ganther 1990). The metabolic profile studies also provided evidence that di- and trimethylated metabolites were capable of undergoing demethylation (Vadhanavikit et al. 1993). Because of the slower metabolism of selenobetaine methyl ester to dimethylselenide, some reverse traffic of dimethylselenide demethylation might occur, thereby attaining a critical level of methylselenol in this situation. The above explanation was supported by additional data indicating that there was considerably more back conversion to the inorganic H_2Se pool from selenobetaine methyl ester than from dimethylselenoxide (Ip and Ganther 1992).

In summary, our studies indicated that the formation of H_2Se is not essential for the expression of anticarcinogenic activity. Precursor selenium compounds that are able to produce a steady stream of monomethylated metabolite are likely to have good chemopreventive activity. On the other hand, selenium compounds that are rapidly metabolized to exhalable dimethylselenide are likely to be poor candidates. The degree of methylation is also an important factor. Our results showed that the fully methylated form, trimethylselenonium, was totally ineffective (Ip and Ganther 1988), probably because it was quantitatively excreted in urine (Vadhanavikit et al. 1993). The poor tissue retention of this compound might account for its low biological activity.

In an attempt to improve the anticarcinogenic activity of the monomethylated selenium derivative, we had also examined a series of aliphatic selenocyanates with increasing length of the carbon side chain, CH_3-$(CH_2)_n$-SeCN, in which $n = 0$, 2, 4 or 6. Selenocyanates (RSeCN) were used as the carrier of selenium because they are known to be efficiently metabolized to selenols (RSeH) and therefore represent a convenient precursor compound. Our bioassay data showed that the order of chemopreventive potency for these aliphatic selenocyanates was as follows: heptyl = pentyl > propyl > methyl (Ip et al. 1995). Thus it appeared that the longer alkyl chain homologs might be superior to methyl selenocyanate. This was a novel finding and could offer further clues to the design of more powerful anticancer selenium compounds.

Selenized yeast was the supplement given to people in Clark's study (Clark et al. 1996). Contrary to previous reports in which less sophisticated methods were used in determining that selenomethionine was the major constituent in yeast, recent analysis by a state-of-the-art technique of high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS)[3] demonstrated that selenomethionine accounted for no more than 20% of all selenium-containing materials (Bird et al. 1997). In addition to selenomethionine, the other compounds that had been identified included selenocystine, Se-methylselenocysteine and selenoethionine (representing ~20%). On top of that, there were several unidentified peaks that combined to represent 40–50% of the total. Thus the selenized yeast actually contains a cocktail of selenium in a variety of chemical forms. Among these, we have some understanding only of selenomethionine and Se-methylselenocysteine. At this time, there are no data regarding whether these different compounds exert distinctive effects on cell biology or how they might differentially affect the multistep process of carcinogenesis. Translational research generally involves the flow of applied learning from laboratories to clinics. In selenium cancer prevention, we have an unusual scenario in which a human trial ironically magnifies the paucity of knowledge in basic science.

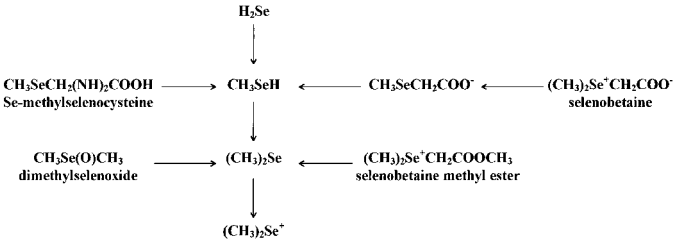

FIGURE 2 This schematic flow chart shows the main sites at which selenobetaine, Se-methylselenocysteine, selenobetaine methyl ester and dimethylselenoxide enter the selenium metabolic pathway below the H_2Se step.

RESEARCH ON SELENIUM-ENRICHED GARLIC

The intervention trial of Clark et al. (1996) is a classic example of "targeted chemoprevention" in which a particular substance is given to high risk individuals for the purpose of reducing cancer morbidity. There is a second concept of chemoprevention that is aimed at providing cancer protective chemicals to large segments of the population that are not at an increased risk because of known exposure to carcinogens, genetic predisposition or prior diagnosis of malignancy. Because of the intrinsic requirement of this plan for a wide distribution method, an expeditious way of delivering these protective agents is through the food system. Incidentally, a driving force for general population chemoprevention can be traced to the mounting epidemiologic and experimental data that strongly suggest the beneficial effects of various plant constituents present in our diet.

It is almost impossible to increase selenium intake by eating certain types of food because most common foods have a very low selenium content (Morris and Levander 1970). In the early 1990s, Ip and Lisk started a project in which they tried to enrich garlic with selenium by fertilizing the crop with water-soluble selenite salt. The idea was stimulated by the fact that plants are known to convert inorganic selenium in soil to organic selenium compounds following the sulfur assimilatory pathway (Shrift 1973). Because garlic contains an abundance of sulfur derivatives, it might be able to accumulate high levels of selenium. Initially, our goal was to see whether the idea could be put into practice and if so, to characterize the biological activities of this Se-garlic.

By controlling the intensity and frequency of selenite fertilization, Lisk was successful in cultivating Se-garlic enriched with a low of 100 ppm to a high of 1300 ppm Se dry weight. As a point of reference, natural garlic sold in the grocery stores contains <0.05 ppm Se. After harvest and processing, the Se-garlic was usually lyophilized and milled to a powder for feeding in animal research (Ip et al. 1992). We have published a series of papers with this material. Selected findings from these studies are summarized below.

A dose-dependent cancer protective effect was expressed in the range of 1–3 ppm Se in the diet (Ip and Lisk 1994a and 1994b). Total tumor yield was consistently reduced by 50–60% with 2 ppm Se supplementation. To ascertain that the efficacy of Se-garlic in cancer protection was primarily dependent on the action of selenium, we compared the effects of two batches of garlic powder with different levels of selenium enrichment, 112 vs. 1355 ppm Se dry weight. To achieve 2

[3]Abbreviations used: DMBA, dimethylbenz(a)anthracene; HPLC-ICP-MS, high performance liquid chromatography-inductively coupled plasma-mass spectrometry; IDP, intraductal proliferations; LD_{50}, lethal dose (the dose age that will cause 50% mortality); MNU, methylnitrosourea; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; UDP, uridine diphosphate.

ppm Se in the diet with these two batches of garlic powder, the amount needed was 1.8% for the 112 ppm Se-garlic vs. 0.15% for the 1355 ppm Se-garlic. In this way, we could vary the intake of garlic powder by more than 10-fold but keep the intake of total selenium constant. The results from several experiments led to the conclusion that the anticancer activity of Se-garlic was primarily accounted for by the effect of selenium, rather than the effect of garlic per se (Ip and Lisk 1995).

With the use of the rat dimethylbenz(a)anthracene (DMBA) model, we reported that supplementation of Se-garlic was capable of inhibiting both the initiation and postinitiation stages of mammary carcinogenesis (Ip and Lisk 1994b). DMBA is a procarcinogen requiring metabolic conversion to the ultimate carcinogen, DMBA-3,4-diol-1,2-epoxide, which then reacts with DNA to form adducts (Dipple et al. 1983, Liu and Milner 1992). Adduct formation is therefore the first manifestation of genotoxicity by the initiated cells. After absorption from the intestinal tract, DMBA undergoes first-pass metabolism in the liver. Although the liver is not a target site for DMBA-induced carcinogenesis, DMBA adducts are known to be present in liver DNA. After leaving the liver, some of the activated DMBA metabolites travel via the circulation to the mammary gland. Thus an analysis of DMBA adducts in both mammary cells and liver would provide confirmatory information of changes in DMBA metabolism. Our research showed that three types of adducts, *anti*-dG, *anti*-dA and *syn*-dA, were detected in mammary gland, whereas only the first two adducts were found in liver. Prior treatment with Se-garlic resulted in a consistent reduction of all DMBA-DNA adducts in both tissues (Ip and Lisk 1995 and 1997), suggesting that Se-garlic interfered with DMBA in causing genotoxic damage to DNA.

The decrease in DMBA adducts could be due to modulation of phase I and/or phase II xenobiotic metabolizing enzymes. Phase I enzymes are members of the cytochrome P450 system, which is responsible for converting chemical carcinogens to both electrophilic and nonelectrophilic products. The enzyme P450 1A1 is believed to play a key role in the formation of DMBA-3,4-diol-1,2-epoxide (Morrison et al. 1991). Thus a reduction in the activity of P450 1A1 would be expected to cause a decrease in adduct levels. Defenses against carcinogenic injury, on the other hand, are provided by phase II enzymes [such as glutathione-S-transferase and uridine diphosphate (UDP)-glucuronyltransferase], which are involved in the removal of metabolites through conjugation with glutathione or glucuronic acid (Talalay 1992). An increase in the activity of these phase II detoxifying enzymes could diminish the availability of DMBA metabolites in interacting with DNA.

In addition to 1A1, we also examined four other liver P450 enzymes (1A2, 2B1, 2E1 and 3A4) to determine if there might be a more general effect on the P450 family. No significant alteration was detected in any of these liver P450 enzymes in rats treated with Se-garlic at 1, 2 or 3 ppm Se (Ip and Lisk 1997). In contrast, glutathione-S-transferase and UDP-glucuronyltransferase were elevated to a maximum of 2- to 2.5-fold in liver and kidney in a dose-dependent manner (Ip and Lisk 1997). Our data therefore implied that an increased detoxification of carcinogen via the phase II conjugating enzymes might represent a mechanism of tumor suppression by Se-garlic.

The lack of an effect on P450 enzymes is actually desirable. For the development of novel approaches to cancer chemoprevention, it is generally prudent to avoid targeting the P450 enzymes because of the following considerations. A given agent may suppress a particular P450 enzyme, which is important in the activation of a certain class of carcinogens. However, the same agent may enhance other P450 enzymes that are critical in activating a different class of carcinogens. Such a double-edged sword effect is a major reason for steering away from agents that act by modulating phase I enzymes. Additionally, interference with P450 enzymes may compromise the capability of drug metabolism. This is not a trivial matter because humans frequently consume a variety of drugs to combat illnesses or diseases.

In an attempt to investigate the mechanism of tumor inhibition during the postinitiation phase, we varied the duration of Se-garlic treatment to either one of the following two protocols after carcinogen dosing: *1*) a continuous feeding of Se-garlic for 5 mo until termination or *2*) a 1-mo feeding of Se-garlic and a return to the control diet for the remaining 4 mo. The experiment was repeated in two mammary cancer models in which rats were given a single dose of either DMBA or methylnitrosourea (MNU). Unlike DMBA, MNU is a direct alkylating agent that does not require metabolic activation. Despite differences in their chemical reactivity, both carcinogens produce predominantly mammary tumors when given systemically to rodents. In both models, we found that short-term treatment with Se-garlic for 1 mo was just as effective in cancer prevention as the continuous 5-mo regimen (Ip et al. 1996), suggesting that Se-garlic might irreversibly suppress the clonal expansion of transformed cells in their early stage of development. Plasma and mammary tissue selenium levels essentially returned to basal values within a few weeks after withdrawal of Se-garlic supplementation. Thus the outcome of cancer protection by the short-term intervention regimen was not due to a slow turnover and thus a lingering presence of selenium in the target organ or in the circulation.

The pathobiology of chemical carcinogenesis in the rat mammary gland has been well delineated (Russo et al. 1982). There is a specific structure called the terminal end bud, which is the primary site for the induction of mammary carcinoma. Within 2–3 wk after carcinogen dosing, enlargement of the terminal end bud, characterized by a localized piling up of intraductal cells, is detectable in histological sections. These transformed cells continue to proliferate until they fill up the duct. This type of preneoplastic lesions, known as "intraductal proliferations" or IDP, is the precursor for the eventual development of palpable carcinomas. Se-garlic could conceivably inhibit or even eliminate these IDP, thereby reducing the number of premalignant lesions that are normally present in the early stage of mammary carcinogenesis. Preliminary studies from our laboratory indicated that the total number of IDP was reduced by 50% in the Se-garlic fed rats 6 wk after MNU treatment (unpublished). This observation reinforces our belief that the IDP are likely to be the target sites of selenium chemoprevention.

Further studies also showed that Se-garlic was superior to selenomethionine in terms of its anticarcinogenic efficacy (Ip and Lisk 1996). Unlike selenomethionine, which produced large increases in tissue selenium accumulation, Se-garlic caused only modest elevations (Ip and Lisk 1996). These attributes of Se-garlic became clear when Se-methylselenocysteine was identified as the major selenium-containing constituent in Se-garlic (Cai et al. 1995). The discovery was made through a collaboration between the laboratories of Peter Uden and Eric Block. Considering that the Se-methylselenocysteine research (discussed in the last section) was done before the inception of the Se-garlic project, everything came around in full circle, although the coincidence was rather fortuitous.

As a prototype "designer food" for general population che-

TABLE 1

In vitro effects of selenite and methylated forms of selenium[1]

Endpoints	Selenite	Methylselenocyanate or Se-methylselenocysteine
Cell morphology	Extensive cytoplasmic vacuolization, cell detachment	Normal
Membrane damage	Yes	No
Cell growth inhibition	++++	++
DNA synthesis inhibition	++++	++
Cell cycle block	S/G_2-M	G_1
DNA single strand breaks	++++	None
Cell death	Necrosis	Apoptosis
Gadd gene induction	Late	Early

[1] The above information is based on the data published in Jiang et al. (1993), Kaeck et al. (1997), Lu et al. (1994, 1995b and 1996) and Wilson et al. (1992).

moprevention, Se-garlic has many desirable characteristics. Because garlic is used primarily in flavoring food, there is less danger of overconsumption. At nutritional levels of selenium intake, Se-garlic provides bioavailable selenium for the maintenance of selenoenzymes (Ip and Lisk 1993). At higher levels, it has potent anticancer activity but does not cause excessive selenium accumulation because its predominant organoselenium compound, Se-methylselenocysteine, is rapidly metabolized to di- and trimethylated excretory products (Fig. 2). It induces phase II detoxifying enzymes, thereby facilitating the endogenous removal of xenobiotics. Most interesting of all, it appears to block the development of preneoplastic lesions. This mode of action is particularly suitable for reducing cancer morbidity in sporadic cases. Because Se-methylselenocysteine cannot be incorporated nonspecifically into proteins, the amount of total selenium decays quickly from various tissues upon discontinuation of Se-garlic feeding. The lack of a persistent retention in the body might alleviate the concern of selenosis in humans.

IN VITRO EFFECTS OF SELENITE AND METHYLATED FORMS OF SELENIUM

Although a spectrum of activities has been attributed to selenium in in vitro studies, this section will focus mainly on events that are associated with cell growth inhibition. During the 1980s, there were numerous reports showing that selenite, at concentrations in the micromole range, suppressed cell proliferation in culture and induced cytotoxicity as documented by the standard cell viability assays. This topic was reviewed previously (Ip and Medina 1987, Medina and Morrison 1988). At that time, selenite was the compound of choice because it was easily available from commercial sources. When the research was shifted to the methylated selenium compounds in the early 1990s, the laboratory of Henry Thompson began generating a body of information that supported the concept of distinctive cellular responses to specific chemical forms of selenium. The work of Thompson and co-workers resulted in a series of papers that were aimed primarily at comparing the in vitro activities of selenite with that of methylselenocyanate or Se-methylselenocysteine (Jiang et al. 1993, Kaeck et al. 1997, Lu et al. 1994, 1995b and 1996, Wilson et al. 1992).

Perhaps the best way to describe this collection of data from Thompson's laboratory is to summarize them in a table so that the differences can be easily highlighted (**Table 1**). This format is simple to follow although it may lose some subtlety due to generalizations. Suffice it to note that all of the experiments were not necessarily conducted with the same cell culture model; however, many of the observations were reproducible in more than one model. Another issue that needs clarification is the relative potency of the reagents. To produce the type of responses shown in Table 1, both selenite and the methylated selenium compounds were paired on an equimolar basis usually in the range of 1–10 μmol/L. It was possible to heighten the responses to the methylated selenium compounds, but only if their concentrations were raised 5- to 10-fold.

Selenite, when present at concentrations of 5–10 μmol/L in the media, caused extensive cytoplasmic vacuolization of cells as well as cell detachment from the culture dish. Cell membrane leakage was evident and the damage usually intensified as a function of time. The methylated selenium compounds, on the other hand, did not produce overt signs of cytotoxic effect. When cells were exposed to 10 μmol/L or even higher concentrations of methylselenocyanate or Se-methylselenocysteine, their morphology appeared normal and they remained anchored to the dish. Cell growth inhibition was invariably seen with selenite treatment in a dose-dependent manner. This was accompanied by decreases in DNA synthesis and a block in the cell cycle at the S/G_2-M phase. Treatment with Se-methylselenocysteine also resulted in a lower rate of cell growth and DNA synthesis, but the magnitude of inhibition was modest. In contrast, cell cycle progression was blocked at the G_1 phase. One of the signature genotoxic responses to selenite was a marked elevation in DNA single strand breaks that occurred within a few hours. Such an outcome was absent with exposure to the methylated selenium compounds. Cell death by necrosis or acute lysis was another hallmark of the selenite effect. After the initial wave of cell swelling and lysis, some visible signs of apoptosis were evident in the longer cultures. In contrast, both methylselenocyanate and Se-methylselenocysteine were known to induce cell death predominantly by apoptosis, an event that was characterized by distinctive morphological (e.g., cell blebbing or condensation of chromatin) and biochemical (nonrandom nucleosomal fragmentation or DNA laddering) changes. Thus it is clear that the chemical form of selenium is a very important factor in eliciting defined cellular responses in the in vitro system.

The proliferation of eukaryotic cells is controlled at specific stages of the cell cycle by cyclins and cyclin-dependent kinases (Sherr 1996, Weinberg 1995). There are two recent studies from Medina's laboratory describing a link between selenium and cell cycle proteins. In the first study, which involved the

use of an asynchronized mammary epithelial cell culture model (Sinha et al. 1996), it was found that Se-methylselenocysteine caused a 57% drop in cdk2 kinase activity and a 74% decrease in cyclin E-cdk2 content (therefore compatible with a G_1 arrest observed in this study as well as in the studies of Thompson), whereas selenite actually increased the cdk2 kinase activity by 47% without much appreciable change (10–20% decrease) in either of the cyclins D1, E or A bound to cdk2. The selenite results were incongruous with a S/G_2-M arrest, suggesting that the inhibition of cell growth by selenite might be associated with some nonspecific genotoxic effect unrelated to regulation of cell cycle proteins.

Thompson's studies (Table 1) and the first Sinha study (Sinha et al. 1996) of cell cycling disruption were done at a single time point in cells that were not synchronized, thus making it difficult to elucidate whether the cell cycle clock was stopped or delayed. Synchronized cells, on the other hand, are able to provide more precise information on the timing of the cell cycle clock with respect to other cellular events. With this in mind, Sinha and Medina (1997) repeated the experiments with cells that were released from growth factor deprivation by refeeding them with regular medium, a method commonly employed for synchronization. Parallel cultures were set up so that the cells could be sampled at different time points. [^{3}H]Thymidine incorporation into control cells peaked 16 h after refeeding. At this time point, 60% of cells had entered the S phase. Se-Methylselenocysteine, which was added to the medium 6 h after refeeding, inhibited [^{3}H]thymidine incorporation by ~50% and caused a significant delay in the S phase for almost 18 h. It also produced a concomitant 54% reduction in cdk2 kinase activity (confirming the finding of the previous study). A decrease in cdk2 kinase would be expected to impede progress through the S phase. The level of cyclin E associated with cdk2 did show a transient decrease at an early time point, but it recovered, thereby allowing cells to cross the G_1/S boundary (recall the persistent decrease in cyclin E-cdk2 in asynchronized cells). In summary, the data demonstrated that inhibition of cell growth by Se-methylselenocysteine was due to a prolonged delay in the S phase that was coincident with a marked decrease in cdk2 kinase activity.

Inhibition of cell growth can be accomplished by either a decrease in cell proliferation or an increase in apoptosis or both. Apoptosis is therefore an important cellular mechanism for growth regulation. Despite the conclusion from Thompson's work that selenite preferentially causes necrotic cell death, other reports have suggested otherwise. Recently, Stewart et al. (1997) tried to quantitate the proportion of apoptotic cells by the Apoptag method in a human colon cancer cell line treated with 10 μmol/L selenite. After 4 d, they found that as many as 40% of the cells were stained positive with the use of this assay, which is based on immunohistochemical detection of digoxigenin-labeled nucleotides added to the free 3′-hydroxyl ends generated as a result of DNA breaks. Because 5–10 μmol/L selenite is known to produce massive DNA strand breaks independently of apoptosis, the results of this study are difficult to interpret. Selenodiglutathione, a metabolite of selenite (Fig. 1), has also been examined by a different group of investigators. Lanfear et al. (1994) showed that selenodiglutathione was able to induce apoptosis as determined by fluorescence dye DNA-binding analysis. The principle of the assay is based on the discrimination that apoptotic cells will bind only the Hoechst 33342 dye, whereas necrotic cells will bind both the Hoechst dye and propidium iodide. Live cells do not bind either dye and therefore do not fluoresce. The different subpopulations can be sorted by flow cytometry based on their blue (Hoechst) or red (propidium iodide) fluorescence signals.

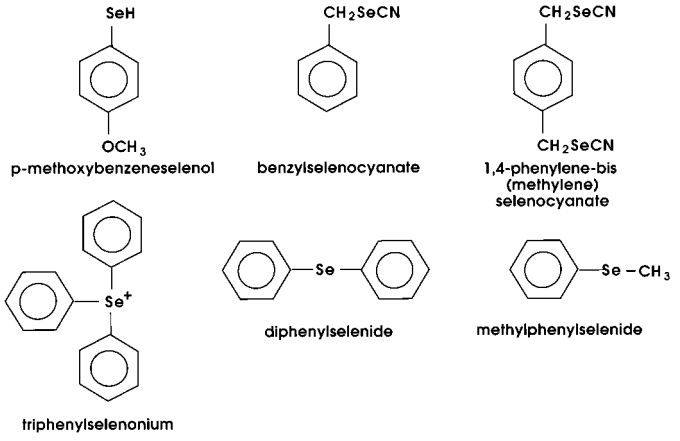

FIGURE 3 Structures of aromatic selenium compounds.

A careful examination of Lanfear's study revealed some rather curious findings in that the control culture (i.e., not treated with selenium) contained a large fraction of necrotic cells. The investigators never explained the presence of all these necrotic cells 6 h after plating when the culture should be in log growth. Upon incubating the culture with 3 μmol/L of selenodiglutathione, a small subset of apoptotic cells emerged in addition to an apparent increase in the number of necrotic cells. From the paper, it was difficult to tease out the results of percentage distribution of live cells, necrotic cells and apoptotic cells because no quantitative data were available. Nonetheless, the appearance of apoptotic cells was unmistakable because these blue fluorescent sorted cells also exhibited the typical DNA laddering pattern on gel electrophoresis.

There was one other piece of information tucked away in the paper that was of special interest. The experiment of Lanfear was done using mouse erythroleukemia cells, which are known to carry a p53 mutated gene, suggesting that a functional p53 pathway was not essential for selenium induction of apoptosis in these cells. The dissociation between wild-type p53 and apoptosis has since been described for the effect of methylselenocyanate in a mouse MOD mammary tumor cell subline with a null p53 phenotype (Kaeck et al. 1997) and for the effect of selenomethionine in HT29 colon cancer cells, which express a mutated p53 (Redman et al. 1997). Given that mutations in p53 are among the most common pathogenetic alterations in human cancers (Greenblatt 1994), an intervention mechanism based on the induction of apoptosis could provide a strong rationale for selenium chemoprevention in the human population. Further research should be focused on testing this hypothesis in vivo and on developing appropriate biomarkers associated with the control of apoptosis.

AROMATIC SELENIUM COMPOUNDS

Karam El-Bayoumy was the first to pioneer the research of aromatic selenium compounds in cancer chemoprevention in the 1980s. His idea originated from the need to develop novel reagents with a lower toxicity than that of selenite and selenomethionine. The chronology started with *p*-methoxybenzeneselenol (**Fig. 3**). In collaboration with other investigators at the American Health Foundation, El-Bayoumy reported successful tumor inhibition at different sites (liver, colon and kidney) by the feeding of 50 ppm of *p*-methoxybenzeneselenol (equivalent to

~20 ppm Se) to rats that were treated with the carcinogen azoxymethane (Reddy et al. 1985, Tanaka et al. 1985). This compound, however, was quickly abandoned in favor of benzylselenocyanate (Fig. 3), even though benzylselenocyanate was apparently more toxic. The dosage that causes 50% mortality (LD_{50}) of *p*-methoxybenzeneselenol and benzylselenocyanate in mice was 370 and 18 mg/kg body weight, respectively (El-Bayoumy 1985). Subsquent studies with benzylselenocyanate (El-Bayoumy 1985, Nayini et al. 1989 and 1991) showed that it suppressed tumorigenesis in several models including forestomach (benzo[a]pyrene), colon (azoxymethane) and mammary gland (DMBA). The carcinogen responsible for inducing cancer at each site is denoted parenthetically. In the above experiments, benzylselenocyanate was given in the diet at a concentration of 25 ppm (equivalent to 10 ppm Se); the schedule generally encompassed a relatively short time period, which started 2 wk before to 1 wk after carcinogen administration. The sulfur analog, benzylthiocyanate, was not effective, suggesting that there was specificity to selenium chemoprevention. The fact that benzylselenocyanate is able to block tumor induction by a variety of carcinogens at the initiation stage is intriguing because different P450 families are involved in the activation of benzo[a]pyrene, azoxymethane and DMBA. In the case of azoxymethane, Fiala et al. (1991) found that benzylselenocyanate increased its oxidative metabolism in the liver, thus resulting in a reduced delivery of methylazoxymethanol to the colon via the bloodstream. Consequently, there was less DNA alkylation in the colon, which was reflected by a diminished formation of O^6-methylguanine and 7-methylguanine. As far as the author is aware, the effect of benzylselenocyanate on polycyclic hydrocarbon metabolism has not been investigated.

Despite the initial intention to develop a less toxic compound, benzylselenocyanate actually fell short of this goal because at a level of 25 ppm in the diet, the rats suffered significant growth depression. Because benzylselenocyanate has a very strong odor similar to that of burnt rubber, the reduced food intake of animals noted in these experiments could be due to unpalatability of the diet. To reduce the volatility of benzylselenocyanate, a second methyleneselenocyanate group was added in the *para-* position to form 1,4-phenylenebis(methylene)selenocyanate (Fig. 3). This compound was commonly called *p*-xylylselenocyanate or *p*XSC. Acute LD_{50} and subchronic studies showed that *p*XSC was markedly less toxic than benzylselenocyanate (Conaway et al. 1992). A level of 80 ppm of *p*XSC (equivalent to 40 ppm Se) inhibited DMBA-induced mammary carcinogenesis in the initiation stage by suppressing the formation of DMBA-DNA adducts (El-Bayoumy et al. 1992). Whether this was due to modulation of P450 enzymes or phase II detoxifying enzymes remains to be determined. The anti-initiation effect was similarly observed in the azoxymethane-induced colon cancer model (Reddy et al. 1992). Additionally, *p*XSC also inhibited mammary and colon carcinogenesis in the postinitiation or tumor promotion phase (Ip et al. 1994a, Reddy et al. 1992), suggesting that it may have multiple mechanisms of action. Interestingly, prostaglandin E_2 was marginally decreased, whereas glutathione peroxidase was significantly increased in the colon of *p*XSC-treated rats. The significance of these findings with respect to cancer chemoprevention is unclear at the present time.

Some uniqueness of *p*XSC was highlighted in a NNK lung cancer chemoprevention experiment in mice (El-Bayoumy et al. 1993). NNK, which stands for 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone, is a tobacco-specific carcinogen. *p*XSC at levels of 5, 10 and 15 ppm Se significantly reduced lung tumor multiplicity from 7.6 per mouse in the control group to 4.1, 3.3 and 1.8 per mouse, respectively. In contrast, selenite at 5 ppm Se had no protective effect. Consistent with the findings of these bioassays were the observations that *p*XSC decreased NNK-induced O^6-methylguanine formation in lung DNA, whereas selenite failed to produce a similar response (Prokopczyk et al. 1996). In rodents, α-hydroxylation of NNK is a major pathway of NNK metabolism (Hecht 1994). This key reaction leads to the formation of electrophiles, which can readily methylate and pyridyloxobutylate various macromolecules. The bioactivation of NNK is catalyzed by multiple P450 enzymes including 1A1, 2A1, 2B1, 2B2 and others that have not been characterized. In view of the fact that NNK is strongly implicated in the pathogenesis of tobacco-related lung cancer in humans (Hecht and Hoffmann 1988), it is important to elucidate the biochemical mechanisms by which *p*XSC modulates NNK metabolism as well as that of other nitrosoamines.

Attempts have also been made to compare *p*XSC with the closely related structural isomers *o*-XSC and *m*-XSC (*o* = ortho; *m* = meta) in the colon carcinogenesis model. Using aberrant crypt foci as the endpoint, all three compounds expressed comparable inhibitory effects: 47% for *o*-XSC, 49% for *m*-XSC and 66% for *p*-XSC (Reddy et al. 1994). Although the difference in biological activity was small, the isomers were not necessarily absorbed to the same extent by the intestinal tract. After an oral gavage, the percentage dose recovered in the feces in 2 d for *o*-XSC, *m*-XSC and *p*-XSC was 25, 60 and 75%, respectively (Sohn et al. 1995). The pharmacokinetics of these compounds in relation to their potency will have to be investigated more thoroughly.

With the benzyl-type selenium compound such as *p*XSC, some selenium is released from the parent molecule into the inorganic selenide pool. This possibility is supported by the evidence of nutritional bioavailability of selenium from *p*XSC as reported by Ip et al. (1994a). However, the rate of selenium release cannot explain entirely the anticarcinogenic activity of *p*XSC. The study of Ip et al. (1994a) showed that 10 ppm Se as *p*XSC was equivalent to 3 ppm Se as selenite in the efficacy of cancer protection. On the other hand, it took 1 ppm Se as *p*XSC to fully replete glutathione peroxidase in a selenium-deficient animal as opposed to only 0.1 ppm Se as selenite. Therefore, the ratio of anticancer activity to nutritional activity for *p*XSC is 10, as opposed to a ratio of 30 for selenite, suggesting that *p*XSC has certain inherent activity that is independent of the release of selenium from the parent molecule.

Compounds with selenium bonded directly to a benzene ring are very stable. There are no mammalian enzymes known that will catalyze the transfer of the benzene ring. For this reason, we decided to examine three phenyl selenide derivatives: triphenylselenonium, diphenylselenide and methylphenyl selenide (Fig. 3). Although they are related to each other structurally, they differ substantially in their chemical properties. Triphenylselenonium is positively charged and amphiphilic, whereas diphenyl selenide and methylphenyl selenide are uncharged and lipophilic.

Triphenylselenonium was a very effective chemopreventive agent in the experimental mammary cancer models (Ip et al. 1994b). At a level of 30 ppm Se supplemented in the diet, total tumor yield was suppressed by 60–70% in rats that had been treated with a mammary carcinogen. This dose level produced hardly any accumulation of total selenium in tissues, even under a chronic treatment condition. Preliminary studies indicated that it was very well tolerated by laboratory animals. No evidence of adverse symptoms was detected at levels up to 200 ppm Se. There is thus a wide margin separating the

chemopreventive dose range and the toxic dose range. Given the cationic and bulky nature of the molecule, the high tolerance is likely due to a poor rate of absorption via the enteral route. Fecal excretion after a single oral administration of triphenylselenonium was ~78 and 8% of the dose during d 1 and 2, respectively, suggesting that a large proportion of the gavage passed through the intestinal tract with minimal recirculation (Ip et al. 1997). Considering that so little is in fact taken up by the body, the in vivo activity of triphenylselenonium is truly fascinating.

The in vitro effect of triphenylselenonium was characterized mainly by cytostasis, i.e., a decrease in cell proliferation (due to inhibition of DNA synthesis) that was not accompanied by apoptotic cell death (Lu et al. 1995a). An agent that does not induce apoptosis will not be expected to cause deletion of transformed cells. Unless it is available continuously, the ability to protect against cancer would be lost when treatment is interrupted. This is the type of response predicted for triphenylselenonium. When triphenylselenonium was given continuously during the entire period of tumor promotion/progression (a 5-mo protocol), it was very effective in suppressing the development of tumors. However, when the treatment period was shortened to 1 mo after carcinogen dosing, there was a marked decrease in efficacy (Ip et al. 1998). At this point, it might be worthwhile to recall the data with Se-garlic in which a 1-mo treatment schedule was just as effective as the 5-mo schedule in cancer protection. As discussed in the previous section, the monomethylated selenium is a potent inducer of apoptosis. The elimination of early transformed preneoplastic cells might explain the outcome of sustaining a lower cancer risk even if treatment is discontinued after a short period of exposure to the anticancer agent.

In contrast to the high tolerance with triphenylselenonium, a significant drop in tolerance to no more than 30 ppm Se was noted with diphenylselenide (Ip et al. 1997). At this dose level, diphenylselenide was at best only half as active as triphenylselenonium in tumor inhibition. For diphenylselenide, fecal recovery was ~6 and 30% of the dose during d 1 and 2, respectively, and ~20% of the dose was recovered in the urine on each of the 2 d. The excretion profile suggested that most of the diphenylselenide dose was absorbed and that urinary excretion was a major route of elimination for diphenylselenide once it was absorbed. Even though diphenylselenide caused a two- to threefold increase in tissue selenium, it was less active than triphenylselenonium in cancer protection. The above experiments bring home the message that small changes in the structure of selenium compounds could lead to rather surprising changes in biological activity.

The surprises continued with methylphenyl selenide. Among the three phenylselenide derivatives, it was the least tolerated. A level of 5 ppm Se of methylphenyl selenide in the diet was the maximum that would produce no decreases in growth. On the basis of dose-response data in chemoprevention bioassays, methylphenyl selenide and Se-methylselenocysteine behaved quite similarly, although their structures are very different from each other. According to our results, the ED_{50} for methylphenyl selenide, triphenylselenonium and diphenylselenide was estimated to be ~2, 20 and >30 ppm Se, respectively. However, when measured against the scale of tolerance, triphenylselenonium was the best at >200 ppm Se and methylphenyl selenide the worst at 5 ppm Se. It is clear that as a class, the aromatic selenium compounds lag far behind the selenoamino acids on our learning curve. We know virtually nothing about their metabolism, pharmacology and toxicology. From what little has been discovered on the basic research side, their biochemistry is certainly very interesting. As of now, we simply do not have sufficient information to determine whether these aromatic selenium compounds and the selenoamino acids are acting via different mechanisms in chemoprevention.

CONCLUSION

The Clark study (Clark et al. 1996) was started in 1984. At that time, very little was known about the mechanism of action of selenium in cancer prevention. Fourteen years later, the gap has been narrowed but there is still a glaring void in our understanding of how selenium might block the clonal expansion of early malignant cells, especially at the molecular level. The science of cancer chemotherapy has long recognized the need to develop a close interaction among chemists, biochemists, pharmacologists, oncologists, pathologists, toxicologists, cell biologists and molecular biologists. Such a concerted enterprise is sorely lacking in the cancer chemoprevention arena. Currently there are hundreds of chemicals that have been and are being evaluated for anticancer activities in both in vivo and in vitro models. The cumulative effort is substantial, but there is little to demonstrate because the effort is so fragmented. Unless the community as a whole (including both commercial and public sectors) is willing to prioritize and commit the necessary resources for targeted research, the work on these hundreds of chemicals will proceed at the same agonizingly slow pace as we cross into the 21st century.

Of all the human cancer intervention studies that have been completed to date, the selenium trial is by far the most successful. The Clark study has probably attracted its share of skeptics because to put it bluntly, many may consider the results too good to be true. Therefore it needs to be repeated and it should be repeated with an improved design. During the last decade, the basic research side has contributed new knowledge of the relationship linking selenium biochemistry, anticarcinogenic potency and regulation of cell growth. Much of this information is on the verge of being ready for incorporation into a second-generation trial. The modulation of cell cycle proteins and apoptotic proteins by selenium is an emerging area of interest. Normal cells, early transformed cells and late stage preneoplastic cells may respond differently to selenium intervention with respect to these molecular pathways. The sooner we understand the fundamental mechanism of selenium chemoprevention, the closer we will be in finding a viable strategy in reducing cancer morbidity in the human population.

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