Selenium Modulation of Cell Proliferation and Cell Cycle Biomarkers in Normal and Premalignant Cells of the Rat Mammary Gland[1]

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Abstract

The present study was designed to assess the effect of *Se*-methylselenocysteine or triphenylselenonium chloride treatment on cell proliferation [bromodeoxyuridine (BrdUrd) labeling] and cell cycle biomarkers [proliferating cell nuclear antigen (PCNA), cyclin D_1, and p27/Kip 1] in the intact mammary gland of rats. Immunohistochemical assays of the above end points were carried out in different morphological structures: (*a*) terminal end bud cells and alveolar cells of a maturing mammary gland undergoing active differentiation; and (*b*) premalignant mammary intraductal proliferations (IDPs) identified at 6 weeks after carcinogen dosing. Neither compound was found to affect BrdUrd labeling or the expression of cell cycle biomarkers in the normal terminal-end bud cells and alveolar cells. *Se*-methylselenocysteine reduced the total number of IDP lesions by ~60%. Interestingly, this was not accompanied by decreases in BrdUrd labeling or the proportion of IDP cells expressing PCNA and cyclin D_1. An enhancement in the fraction of p27/Kip 1-positive IDP cells, however, was detected as a result of *Se*-methylselenocysteine treatment. Although triphenylselenonium chloride did not reduce the total number of IDPs, there were more of the smaller-sized lesions and fewer of the larger-sized lesions compared with those found in the control group. Triphenylselenonium chloride also significantly decreased the proportion of IDP cells incorporating the BrdUrd label or expressing PCNA and cyclin D_1. The above findings suggest that early transformed cells are sensitive to selenium intervention, whereas normal proliferating cells are not. It is possible that *Se*-methylselenocysteine blocks carcinogenesis by a pathway that may not involve cell growth inhibition as a primary response; in contrast, triphenylselenonium chloride is likely to act by a cytostatic mechanism. The data also imply that selenium efficacy testing in intervention trials is possible with the use of biomarkers, provided that the appropriate biomarkers are matched with the selenium compound of interest and that the pathological characteristics of the cell population to be evaluated are taken into consideration.

Introduction

A variety of selenium-containing compounds with diverse chemical structures are known to inhibit cell proliferation *in vitro* (1–10). These compounds include inorganic selenium salts, methylselenocyanate, and selenoamino acids as well as benzyl and phenyl selenium derivatives. Animal feeding studies with these same agents have also demonstrated their efficacies in cancer chemoprevention (11). Successful protection against tumorigenesis in most cases can be achieved without any evidence of growth depression or change in organ size, suggesting that cell proliferation in various tissues is minimally affected by chronic treatment with these selenium compounds. However, little information is available regarding selenium intake and the measurement of cell proliferation *in situ*, especially with respect to the behavior of normal growing cells *versus* neoplastic cells in the same target organ site.

Over the past decade, our collaborative group has been focusing our attention on two selenium compounds in particular, *i.e.*, *Se*-methylselenocysteine and triphenylselenonium chloride, and has published a number of reports on their *in vivo* cancer-protective activities (12–19). *Se*-methylselenocysteine is a water-soluble prodrug designed to deliver a monomethylated selenium metabolite, which we believe to be a critical intermediate in selenium chemoprevention (11, 20). This selenoamino acid is also found naturally in selenium-enriched plants (21–23). Triphenylselenonium chloride is a synthetic compound with a cationic selenium atom bonded directly to three unsubstituted benzene rings; therefore, it is a very stable molecule that is not likely to release bioavailable selenium (19). Despite the lipophilic nature of the benzene rings, triphenylselenonium chloride is water soluble because of the positive charge. Previous studies have shown that both *Se*-methylselenocysteine and triphenylselenonium chloride are effective against rat mammary gland carcinogenesis, although their dose responses are quite different (12, 16). When given in the diet, the levels of *Se*-methylselenocysteine and triphenylselenonium chloride required to attain 50% inhibition of tumor yield are about 2 and 20 ppm selenium, respectively. We also have indirect evidence suggesting that these compounds might exert their chemopreventive effects by different modes of action (18, 24).

In the first part of the present study, we compared the effects of *Se*-methylselenocysteine and triphenylselenonium

chloride on cell proliferation and the expression of cell cycle biomarkers in the mammary gland of pubescent female rats. Cell proliferation in defined mammary gland structures, *i.e.*, TEBs[3] and alveoli, was assessed by BrdUrd labeling. Three different cell cycle biomarkers were also determined: PCNA, cyclin D_1, and p27/Kip 1 (an inhibitor of cyclin-dependent kinase). In the rat model, TEBs are the primary sites for the chemical induction of mammary adenocarcinomas (25). Within 2–3 weeks after carcinogen dosing, enlargement of the TEB, characterized by a localized piling up of intraductal cells, is detectable in histological sections. Throughout the mammary gland, the cells in selective TEBs continue to proliferate until they completely fill up the duct. These early transformed lesions, which are known as IDPs, are the precursors for the eventual formation of palpable carcinomas (26). Therefore, the second part of this study was aimed at: (*a*) quantifying the number of IDPs in the mammary gland of rats that were treated with a single dose of methylnitrosourea and then were given either *Se*-methylselenocysteine or triphenylselenonium chloride; and (*b*) determining the changes in cell proliferation and the expression of cell cycle biomarkers in these IDPs.

Materials and Methods

Animals and Selenium Treatment. For the first part of the study, which was designed to examine cell proliferation and biomarkers in TEBs and alveoli of the mammary gland, weaning female Sprague Dawley rats (Charles River Breeding Laboratories, Wilmington, NC) were fed one of three diets for 4 weeks: a basal diet containing 0.1 ppm selenium as sodium selenite (12), the same diet supplemented with 3 ppm selenium as L-*Se*-methylselenocysteine (Selenium Technologies, Inc., Lubbock, TX), or 30 ppm selenium as triphenylselenonium chloride (Organometallics, East Hampton, NH). This 1-month period after weaning is a time of active morphogenesis in the mammary gland of the rat. At 6 h before sacrifice, all rats received injections i.p. of BrdUrd at a dose of 50 mg/kg body weight. Because of the potential variations in mammary cell proliferation attributable to timing of the estrous cycle, only rats in diestrus (the longest phase of the cycle and, therefore, much easier to identify) were used as a standard protocol. At necropsy, the abdominal-inguinal mammary gland chain on both sides was excised in one piece, fixed in Methacarn, and processed in a Tissue-Tek vacuum infiltration processor. For the second part of the study, which was designed to examine the biology of the IDPs, 55-day-old rats were injected i.p. with MNU at a dose of 50 mg/kg body weight. They were then immediately divided into three groups: control (fed basal diet containing 0.1 ppm selenium), *Se*-methylselenocysteine (at 3 ppm selenium), and triphenylselenonium chloride (at 30 ppm selenium). Animals were sacrificed at 6 weeks after selenium supplementation. The procedures of BrdUrd injection and mammary gland processing were followed as described above.

Quantification of IDP Lesions. Each mammary gland whole mount was divided into six segments and embedded into paraffin blocks. Ribbons of 5-μm thickness were cut from each block and placed on slides that had been treated with 3-aminopropyltriethoxysilane. Every tenth section was heat immobilized, deparaffinized in xylene, rehydrated in descending grades of ethanol, and stained with H&E. These H&E slides were

examined under the microscope for the appearance of IDP lesions using the criteria described by Russo *et al.* (25). Once a section showing the pathology of an IDP lesion was found, the in-between slides were similarly stained to confirm the histology. Thus, the size of each IDP lesion could be estimated operationally by the number of serial sections showing the same pathology. The total IDP count data were analyzed by the χ^2 test using a Poisson regression model (27).

Immunohistochemical Staining of BrdUrd, PCNA, Cyclin D_1, and p27/Kip 1. Antibodies for immunohistochemical staining of mammary gland sections were obtained commercially: mouse anti-BrdUrd from Becton Dickinson, mouse monoclonal anti-PCNA and anti-p27/Kip 1 from Santa Cruz Biotechnology, and mouse anti-cyclin D_1 (clone DCS-6) from Neomarkers. These antibodies were applied at the following dilutions and exposure times: anti-BrdUrd at 1–40 for 60 min, anti-PCNA at 1–20,000 for 60 min, anti-cyclin D_1 at 1–200 for 30 min, and anti-p27/Kip 1 at 1–50 for 60 min. The procedure of immunohistochemical staining was described in detail in a previous publication (28) and is briefly recapped here. After the tissue sections were incubated with the primary antibody at room temperature in a humid chamber, they were treated with a biotinylated rabbit secondary antibody against mouse immunoglobulin. This was followed by the addition of streptavidin horseradish peroxidase, which binds to biotin. Diaminobenzidine was used as the chromogen to generate a precipitate that is brown because of its reaction with peroxidase. All slides were counterstained with hematoxylin, rinsed, dehydrated, and mounted with Permount. Cells expressing the antigen were identified by a brown stain over the nucleus. The numbers of slides evaluated per rat were indicated in the footnotes of the tables presented in "Results." Color pictures were taken with a camera mounted on top of the microscope. All hard-copy images were coded so that the person analyzing the data was blinded to the group assignment to avoid bias. Because immunohistochemical staining for a protein is not stoichiometric relative to the amount of protein present, differences in staining were analyzed by using a Kruskal-Wallis rank test (28).

Results

Table 1 summarizes the results of BrdUrd labeling, PCNA, cyclin D_1, and p27/Kip 1 expression in TEB cells and alveolar cells of the developing mammary gland. The BrdUrd label and the three cell cycle-associated proteins all showed a distinctive nuclear localization; hence, the positively stained cells could be easily quantified from hard-copy color images. The data are expressed as the percentage of cells in each type of structure that were positively stained. For the TEB results, the analysis was based on counting a total of six to eight structures (each structure consisted of 100–200 cells) per rat, with six rats/group. For the alveoli results, six to eight slides were evaluated per rat, and each field usually displayed a range of 100–250 cells. As noted in "Materials and Methods," selenium supplementation to these animals was given during a period of rapid development of the mammary gland in which the cells remain highly proliferative. At the time of sacrifice, the animals were about 50 days of age. The data showed that neither *Se*-methylselenocysteine nor triphenylselenonium chloride significantly affected the activity of cell proliferation (as determined by BrdUrd labeling) or the expression of cell cycle biomarkers (as assessed by PCNA, cyclin D_1, and p27/Kip 1) in the two types of differentiated mammary gland structures. The consistency of the data strongly suggests that neither compound is inhibitory to

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[3] The abbreviations used are: TEB, terminal end bud; BrdUrd, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; IDP, intraductal proliferation; MNU, methylnitrosourea.

Table 1   Effect of *Se*-methylselenocysteine or triphenylselenonium chloride on BrdUrd labeling and expression of PCNA, cyclin D$_1$, and p27/Kip 1 in TEB and alveolar cells of the mammary gland of pubescent rats

Treatment	% of positively stained TEB cells[a,b]			
	BrdUrd	PCNA	Cyclin D$_1$	p27/Kip 1
None	17.8 ± 1.2	41.7 ± 3.5	56.2 ± 4.2	44.6 ± 3.2
Se-methylselenocysteine	18.6 ± 1.5	39.6 ± 3.0	55.3 ± 3.7	47.0 ± 4.1
Triphenylselenonium chloride	16.9 ± 1.4	38.5 ± 2.8	51.6 ± 3.9	42.8 ± 3.3

Treatment	% of positively stained alveolar cells[a,c]			
	BrdUrd	PCNA	Cyclin D$_1$	p27/Kip 1
None	11.5 ± 0.8	32.4 ± 2.7	44.3 ± 3.3	41.5 ± 4.0
Se-methylselenocysteine	10.6 ± 0.8	30.8 ± 2.5	43.1 ± 3.0	43.1 ± 3.2
Triphenylselenonium chloride	9.8 ± 1.2	29.9 ± 3.6	42.8 ± 3.7	40.7 ± 3.6

[a] Results are expressed as mean $\pm$ SE ($n = 6$ rats/group).
[b] A total of six to eight structures were evaluated per rat. Each structure consisted of 100–200 cells.
[c] A total of six to eight slides were evaluated per rat. Each field consisted of 100–250 cells.
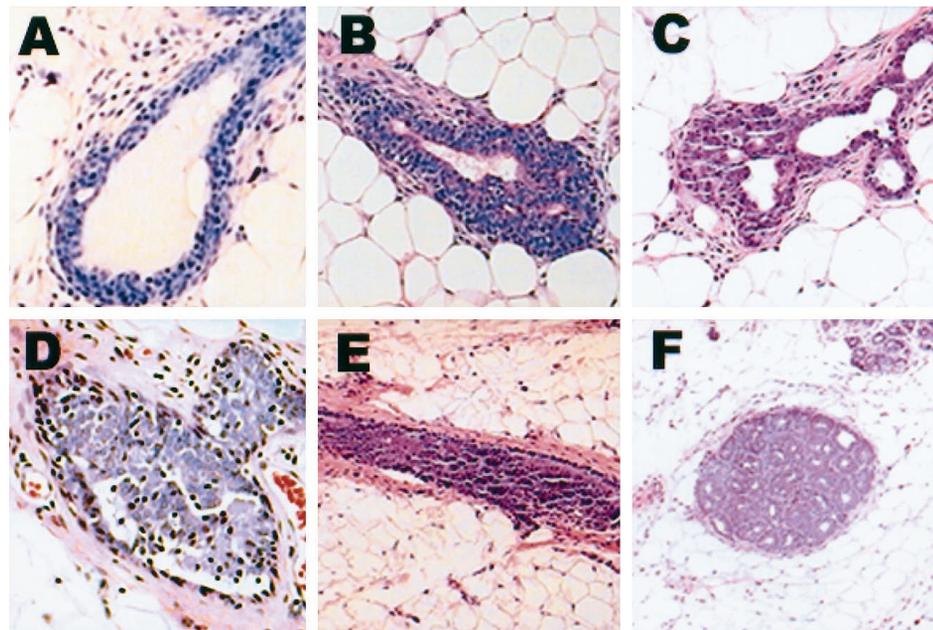
Fig. 1. The progression of mammary gland IDP lesion in histological sections (described in "Results.")

cell growth during the process of normal tissue morphogenesis and differentiation.

Fig. 1 presents a composite picture of the histology of IDP progression. Fig. 1*A* shows a normal TEB. At 2–3 weeks after MNU dosing, cells at the tip and neck of the TEB begin to proliferate and invade into the cavity of the ductal structure (Fig. 1, *B* and *C*). A cross-section of a partially filled duct is depicted in Fig. 1*D*. The development of the IDP continues until the lumen of the duct is almost completely occupied, as seen in a longitudinal section in Fig. 1*E* and in a cross-section in Fig. 1*F*. As noted in "Materials and Methods," the size of each IDP lesion was estimated by the number of serial sections showing the typical pathology, which was represented by Fig. 1, *C–F*.

Table 2 shows the quantitative IDP results from MNU-treated rats that were given either *Se*-methylselenocysteine or triphenylselenonium chloride. The histology of the mammary gland sections was evaluated at 6 weeks after carcinogen administration. In addition to obtaining a total count of these

lesions in the abdominal-inguinal mammary gland chain, the size of each lesion was also determined operationally by the number of serial sections showing the same pathology. There were 57 IDPs found in a total of six rats in the control group (last column of Table 2). Treatment with *Se*-methylselenocysteine reduced the number of IDPs by ~60% ($P < 0.05$). In contrast, triphenylselenonium chloride did not seem to have much of an effect in diminishing the population of the IDPs. All of the lesions were categorized into five size classes with each containing ≤10, 11–20, 21–30, 31–40, or >40 serial sections, respectively. To analyze the size distribution data, a repeated measures option was added to the Poisson regression because most animals presented lesions in more than one size class. No significant differences were found by treatment within a given size class, probably because of the small sample number in each category when the data were segregated; this reduced statistical power to detect significant differences attributable to treatment. However, it is noteworthy to point out that in the triphenylselenonium chloride-treated rats, there was a trend of a low abun-

Table 2   Effect of *Se*-methylselenocysteine or triphenylselenonium chloride on the number of IDP lesions in the mammary gland of rats given MNU

| Treatment | Size distribution of IDP lesions | | | | | | Total no. of IDP lesions |
	No. of rats	≤10 sections	11–20 sections	21–30 sections	31–40 sections	>40 sections	
None	6	8	14	10	13	12	57
Se-methylselenocysteine	6	3	6	4	5	5	23[a]
Triphenylselenonium chloride	6	16	20	7	4	6	53

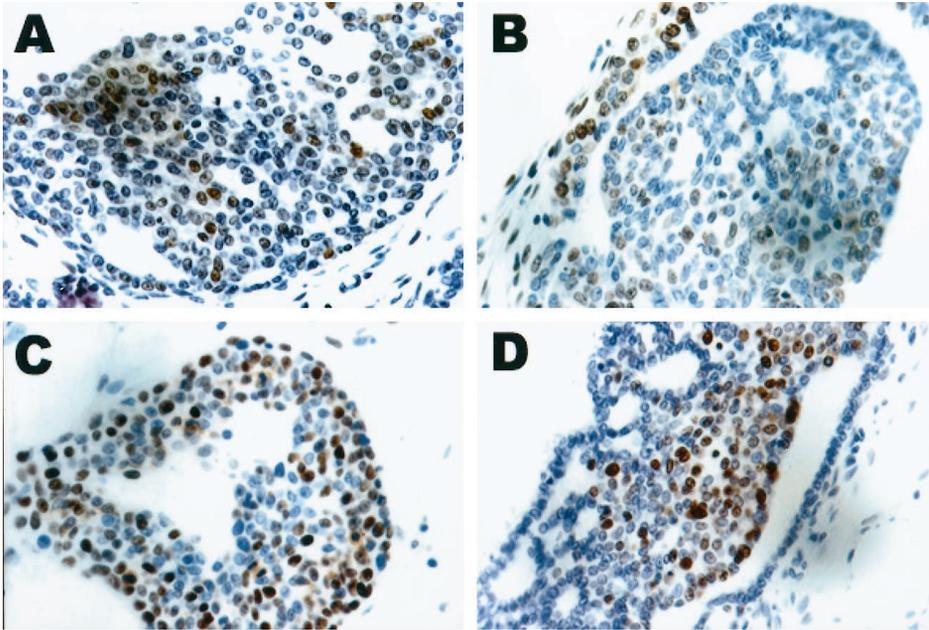[a] $P < 0.05$ compared with the control group.

Fig. 2.   Representative immunohistochemical staining of BrdUrd (*A*), PCNA (*B*), cyclin D_1 (*C*), and p27/Kip 1 (*D*) in IDP lesions.

dance of the larger-sized lesions and a high yield of the smaller-sized lesions. For example, there were altogether 17 lesions of >21 sections in the triphenylselenonium chloride group compared with 35 in the control group. Conversely, there were 36 lesions of <20 sections in the triphenylselenonium chloride group *versus* 22 in the control group. Overall, the data suggest that treatment with *Se*-methylselenocysteine reduces the number of IDPs uniformly across all size classes, whereas triphenylselenonium chloride seems to slow down the expansion of these transformed colonies without having much of an effect in blocking their genesis.

Before presenting the cell proliferation and biomarker data in IDPs, it is important to discuss the results from the normal mammary gland structures in these adult animals. In the IDP experiment, the animals were sacrificed when they were ~100 days of age. At this stage of the life span of a rat, the mammary gland has essentially completed its development. The mammary tree of an adult rat consists mainly of a network of subtending branches comprising numerous alveolar clusters, with very few of them ending in TEBs. When we examined the data on BrdUrd labeling, PCNA, cyclin D_1, and p27/Kip 1 in the alveolar cells of the fully matured mammary gland, we again could not detect any effect on these end points attributable to either *Se*-methylselenocysteine or triphenylselenonium chloride. In other words, the finding was similar to that reported in Table 1. Because all of the data are negative, we did not feel compelled to include them in this report. The only difference in

the assay results between the 50-day-old rats and the 100-day-old rats was the higher proportion of cells that were positively stained for BrdUrd, PCNA, and cyclin D_1 in the former group. This was to be expected because the mammary gland is still actively developing in the younger animals.

Because the biomarker data from the IDPs are the focus of this study, some representative staining patterns in these structures are included in Fig. 2. It is not easy to identify a set of 12 "typical" pictures based on three treatment groups and four different assays because the final results were obtained by evaluating a total of 12–15 slides/group/assay. Thus, Fig. 2 *panels A–D* are presented to illustrate the immunochemistry of BrdUrd, PCNA, cyclin D_1, and p27/Kip 1, respectively, with the objective of demonstrating: (*a*) the variability in the proportion of positively stained cells for the four different antibodies (highest for cyclin D_1 and lowest for BrdUrd); (*b*) the nuclear localization of all four antigens of interest; and (*c*) the distribution of stained cells in a representative IDP section.

Table 3 shows the proliferation and biomarker results in mammary gland IDPs from control and selenium-treated rats. It is interesting to note that *Se*-methylselenocysteine and triphenylselenonium chloride produced differential effects on these parameters. With BrdUrd labeling, it was found that *Se*-methylselenocysteine only marginally reduced the activity of DNA synthesis (not statistically significant), whereas triphenylselenonium chloride caused a healthy 40% decrease ($P < 0.05$). With the cell cycle biomarker assays, *Se*-methylselenocysteine

Table 3 Effect of *Se*-methylselenocysteine or triphenylselenonium chloride on BrdUrd labeling and expression of PCNA, cyclin D_1, and p27/Kip 1 in IDP cells of the mammary gland of rats given MNU

Treatment	% of positively stained IDP cells[a]			
	BrdUrd	PCNA	Cyclin D_1	p27/Kip 1
None	21.9 ± 1.7	45.3 ± 2.8	67.2 ± 5.5	35.6 ± 2.5
Se-methylselenocysteine	18.8 ± 1.5	44.4 ± 3.5	60.3 ± 4.8	49.7 ± 3.1^{b}
Triphenylselenonium chloride	14.3 ± 1.1^{b}	31.2 ± 2.2^{b}	40.8 ± 3.4^{b}	33.8 ± 2.9

[a] Results are expressed as mean $\pm$ SE ($n = 6$ rats/group). A total of 12–15 slides were evaluated per group. The slides for each assay were selected from different IDP size classes as shown in Table 2.
[b] $P < 0.05$ compared with the control group.

significantly increased ($P < 0.05$) the percentage of cells expressing p27/Kip 1 but did not appear to modulate the proportion of cells that was stained positive for either PCNA or cyclin D_1. In contrast, triphenylselenonium chloride suppressed the proportion of cells that was positive for PCNA and cyclin D_1 but not the proportion of the p27/Kip 1-expressing cells.

Discussion

The present study provides suggestive evidence that *Se*-methylselenocysteine and triphenylselenonium chloride inhibit tumorigenesis by different mechanisms. From our experience, the 3-ppm selenium dose of *Se*-methylselenocysteine and the 30-ppm selenium dose of triphenylselenonium chloride should be equally efficacious in mammary gland cancer chemoprevention (12, 16). Our data showed that *Se*-methylselenocysteine markedly reduced the total number of IDP lesions in the mammary gland, whereas triphenylselenonium chloride inhibited clonal growth of these lesions without significantly diminishing the overall count. In the rat mammary gland chemical carcinogenesis model, only a fraction of the IDPs will eventually develop into adenocarcinomas. Despite this caveat, the IDP data and the BrdUrd labeling data collectively seem to indicate that *Se*-methylselenocysteine may block carcinogenesis by a pathway that does not involve arrest of cell proliferation in premalignant lesions as a primary mechanism of action. In contrast, triphenylselenonium chloride may block carcinogenesis by suppressing DNA synthesis of the premalignant lesions, as confirmed by decreases in BrdUrd labeling.

The lack of an effect of *Se*-methylselenocysteine on PCNA and cyclin D_1 in IDP cells is consistent with the BrdUrd results. However, this observation appears to be in disagreement with the finding of cell cycling disruption by Se-methylselenocysteine *in vitro* (6). The discrepancy highlighted the need to examine both the *in vitro* and *in vivo* effects of a specific selenium compound. We were intrigued by the increased expression of p27/Kip 1 caused by *Se*-methylselenocysteine treatment, as shown in Table 3. This protein acts during the G_0 and the early G_1 phases of the cell cycle by inhibiting cyclin D_1-cyclin-dependent kinase 4 and cyclin E-cyclin-dependent kinase 2 complexes (29). In addition to interfering with cell cycle transit, p27/Kip 1 is known to be involved in differentiation programs of many cell types, both in tissue culture and *in vivo* (30–34). Thus, p27/Kip 1 may have other tumor suppressor functions. When p27/Kip 1 is determined in clinical tumor samples using immunohistochemical assays, the low or absent expression of this protein generally signals a poor prognosis of disease progression (35).

In a previous study, we reported that treatment of animals with selenium-enriched garlic for 1 month immediately after carcinogen administration was just as effective in mammary cancer prevention as the 5-month continuous treatment regimen (24), suggesting that the selenium compound in garlic may irreversibly alter the process of clonal expansion of transformed cells during their early stage of development. It has since been found that selenized garlic contains *Se*-methylselenocysteine as the predominant form of selenium (23) and that pure *Se*-methylselenocysteine produced the same effect as selenized garlic.[4] A possible mechanism to reduce the population of IDPs is by apoptosis. Selenium is a potent inducer of apoptosis in a number of *in vitro* cell models (2, 3, 5, 36–39). For cells that have sustained irreparable DNA damage, apoptosis is a means for their elimination. The appearance of a defined premalignant lesion (*e.g.,* an IDP) is the net result of cell proliferation minus cell death. Thus, a down-sizing in the population of premalignant lesions can be achieved, in effect, by enhancing cell death either in the absence of or in addition to decreasing cell proliferation. We have preliminary data indicating that *Se*-methylselenocysteine is able to induce apoptosis in IDP cells *in vivo*, particularly in the smaller colonies.[5] This research finding will be the subject of a separate publication.

Contrary to *Se*-methylselenocysteine, triphenylselenonium chloride does not reduce the population of IDPs but seems to suppress clonal expansion of these lesions. Thus, in triphenylselenonium chloride-treated rats, the IDPs appeared to be smaller, and BrdUrd labeling as well as the expression of PCNA and cyclin D_1 in IDP cells was concomitantly depressed. These findings are congruent with our previous report that suggests that triphenylselenonium chloride inhibits tumorigenesis by a cytostatic mechanism that involves retarding the progression of premalignant to malignant lesions (18). Interestingly, the *in vitro* effect of triphenylselenonium chloride is also characterized by cytostasis, *i.e.,* a decrease in cell proliferation attributable to inhibition of DNA synthesis (10). Thus, with this compound the *in vitro* and *in vivo* responses with respect to cell proliferation control are more in line with each other.

Our study demonstrates convincingly that neither *Se*-methylselenocysteine nor triphenylselenonium chloride affected the growth of normal, untransformed cells in the mammary gland. The finding was reproduced both with the actively developing mammary gland of pubescent rats and with the matured mammary gland of adult rats. Thus, the effects of both compounds were manifested only in premalignant lesions. This implies that normal cells are not sensitive to selenium intervention, whereas early transformed cells express certain defective signaling pathways that can be overcome by specific chemical forms of selenium. The fact that normal cells are resistant to growth inhibition by selenium is desirable; however, it

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[4] C. Ip, unpublished data.
[5] C. Ip, unpublished data.

makes tissue sampling a more difficult task in terms of collecting the suitable specimen for biomarker studies. Our data offer a glimmer of encouragement that the assay of biomarkers may be a feasible means for assessing the efficacy of selenium in cancer prevention trials, provided that the appropriate biomarkers are matched with the selenium compound of interest and that the pathological characteristics of the cells to be evaluated are taken into consideration. These criteria must be met for the results to be interpretable.

Acknowledgments

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