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Influence of Selenium on the Growth of *N*-Nitrosomethylurea-Induced Mammary Tumor Cells in Culture[1] (42139)

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Abstract. Selenium is an essential dietary trace element which has anticancer properties. Among its effects in rats, selenium has been shown to inhibit the development of carcinogen-induced mammary tumors by interfering with the postinitiation, promotion phase of carcinogenesis. We studied the effects of selenium on the growth of rat mammary tumor cells in primary culture. Our objective was to determine whether selenium had any direct influence on cell growth which might explain its influence on tumor development. Rat mammary tumors were induced by *N*-nitrosomethylurea. Tumor epithelium was prepared by collagenase dispersion and the cells were separated by Ficoll gradient centrifugation. The tumor epithelium was grown in primary culture using a defined serum-free medium. The addition of low concentrations of sodium selenite, less than 1.0 μg/ml, stimulated tumor cell proliferation. Protein synthesis and the production of type IV collagen increased within the first hour of exposure, prior to any measurable increase in DNA synthesis. Concentrations of selenite greater than 1.0 μg/ml inhibited cell proliferation, the synthesis of protein, and the replication of DNA in a dose-related manner. These studies demonstrated that selenium has the potential to influence the postinitiation phase of rat mammary tumorigenesis by directly altering the growth of tumor cells, possibly through the regulation of protein synthesis. © 1985 Society for Experimental Biology and Medicine.

Selenium is a nutritional trace element (1–3) which has anticancer properties (4–7). Epidemiological studies suggest that selenium inhibits breast cancer in humans (5–8). Analyses of human blood samples suggested that serum selenium was correlated with disease freedom, and decreased severity of the disease in cancer patients (9–12). Selenium supplementation also depresses the development of experimental mammary tumors in rodents (13–23). The cellular mechanism for selenium's anticancer activity is not certain.

Thompson and co-workers (21, 22) studied the effects of dietary selenium in rats on the induction of mammary tumors by the carcinogen *N*-nitrosomethylurea (NMU). They observed that sodium selenite alone (21) or in combination with retinyl acetate (22) depressed tumor frequency and lengthened the latency period for tumor appearance. They also observed that selenite was effective when administered after the injection of NMU during the postinitiation, promotional phase of carcinogenesis (21).

In this laboratory, we have been studying the regulation of growth in primary cultures of NMU-induced mammary tumor cells. Nothing was known about the influence of selenium on rat mammary cells in culture. It has been reported that various types of cultured mouse mammary cells responded differentially to selenium supplementation (24–26). We studied the effects of selenium on NMU-induced tumor cells to determine whether selenium had direct effects upon growth which could explain its influence on tumor development. Our results showed that selenium did, in fact, have biphasic effects on growth which were accompanied by early changes in the synthesis of cellular proteins, including type IV collagen.

A preliminary report dealing with this work has been published (27).

Materials and Methods. *Animals.* Female BUF/N rats were purchased from Laboratory Supply Company, Indianapolis, Indiana.

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Mammary adenocarcinomas were induced according to the method of Gullino *et al.* (28). The rats were injected with NMU (ICN Pharmaceutical Co., Plainview, N.Y.), 50 mg/kg body wt, *iv,* three times at 30-day intervals beginning at 50 days of age.

Primary cell culture. Mammary adenocarcinomas (two to three per preparation) were minced with scissors and enzymatically dispersed by incubation with collagenase (Worthington, Type II, 312 units/ml medium, 10 ml/g tissue) for 70 min at 30°C, as previously described (29, 30). The epithelial cells were separated by centrifugation on gradients of Ficoll (Pharmacia Fine Chemicals) and plated onto 16-, 35- or 100-mm tissue culture dishes (Costar, Cambridge, Mass.) and grown in an incubator at 37°C with an atmosphere of 5% carbon dioxide and 95% air. The serum-free growth medium used in these studies consisted of improved minimum essential medium (IMEM, Associated Biomedic Systems, Inc., Buffalo, N.Y.) (31), Pederson fetuin (Type III, Sigma, 1 mg/ml), insulin (Iletin U-80, Lilly Pharmaceutical Co., 0.1 μg/ml), epidermal growth factor (Collaborative Research, 10 ng/ml), transferrin (Sigma, 5 μg/ml), cortisol (Sigma, 80 n*M*), and gentamycin sulfate (Sigma, 40 μg/ml). The endogenous basal level of selenium in this medium was 0.00469 μg/ml as determined by the fluorescence method of Hoffman *et al.* (32). Sodium selenite (Sigma) was added to the growth medium in concentrations ranging from 0.1 to 100 μg/ml (5.8×10^{-7}– 5.8×10^{-4} *M*). Cells were counted by the addition of 0.1 *M* citric acid which contained 0.1% crystal violet (Sigma) to lyse the cells and stain nuclei which were counted using a hemacytometer (33).

Protein synthesis. The analyses of protein and collagen synthesis were carried out as previously described (29). Cells were incubated with minimum essential medium (MEM, GIBCO, Grand Island, N.Y.) containing [2,3 ^{3}H]proline (5 μCi/ml, 29.5 Ci/mmole, New England Nuclear, Boston, Mass.), ascorbic acid (10 μg/ml), and β-aminopropionitrile (10 μg/ml) for 1 hr at 37°C in an incubator with an atmosphere containing 95% air and 5% carbon dioxide. The cells were scraped and extracted into a solution containing acetic acid (0.5 *M*) and the protease inhibitors *N*-ethylmaleimide (5 m*M*) and tetrasodium-EDTA (5 m*M*). The cell suspension and incubation medium were transferred separately to cellulose dialysis tubing and dialyzed exhaustively at 4°C against the extraction solution and finally against 0.1 *N* acetic acid in the absence of EDTA and *N*-ethylmaleimide. The relative amounts of newly synthesized collagen were determined by sensitivity to highly purified bacterial collagenase (Advance Biofractures Corporation, Lynbrook, N.Y.) by employing the technique of Peterkofsky and Diegelman (34).

DNA synthesis. Cells were incubated with [^{3}H]thymidine (80 Ci/mmole, 1 μCi/ml medium, New England Nuclear) for 1 hr at 37°C in an incubator with 5% carbon dioxide. The cells were washed with cold phosphate-buffered saline and then scraped into cold 0.5 *N* perchloric acid. After homogenization and centrifugation, the pellet was resuspended in 0.5 *N* perchloric acid and hydrolyzed for 30 min at 90°C. After centrifugation, an aliquot of the supernatant was analyzed for radioactivity and another aliquot was assayed for DNA content by the diaminobenzoic acid fluorescence method of Kissane and Robins (35).

Results. Primary cultures of tumor cells were plated as nodules of tumor epithelium. Within the first 24 hr in culture, the nodules of epithelium attached and spread out forming colonies of cells on the tissue culture surface. The cells multiplied with a doubling time of about 72 hr. These cultures contained tumor epithelium which synthesized type IV basement membrane collagen. No fibroblast contamination was apparent as indicated by the lack of any detectable type I or III fibroblast collagen (29, 36).

Table I shows the influence of selenium on the initial attachment of the cells. Low doses of sodium selenite (less than 1 μg/ml) had no observable effect upon attachment. However, higher concentrations of sodium selenite depressed cell attachment in a dose-related manner. At 100 μg/ml, selenite was cytotoxic. The majority of the unattached cells were dead (greater than 95%) as judged by the uptake of trypan blue dye (37).

Figure 1 shows the effects of selenium on the growth of mammary tumor cells. Low

TABLE I. INFLUENCE OF SODIUM SELENITE ON THE ATTACHMENT OF NMU MAMMARY TUMOR CELLS IN CULTURE[a]

Sodium selenite (μg/ml)	Cells per dish[b]	Percentage control
0 (Control)	87,700 ± 4,100	100
1	86,000 ± 4,200	98
10	19,800 ± 4,600	23
100	4,000 ± 1,400	5

[a] Freshly prepared tumor cells were plated in growth medium containing 1 m*M* hydroxyurea (45), which blocks cell multiplication, and the indicated concentrations of sodium selenite. Attached cells were counted 24 hr later.

[b] Mean ± SEM (n = 3).

doses of sodium selenite, less than 1 μg/ml, stimulated tumor cell growth, whereas 10 μg/ml selenite inhibited growth. At 100 μg/ml, selenite was cytotoxic and killed the tumor cells. A similar dose of sodium sulfite had no effect on cell number.

The influence of selenite on the synthesis of protein is shown in Table II. Depending on the particular cell preparation, type IV collagen accounted for 5–8% of the newly synthesized protein. Low concentrations of selenite which stimulated growth, stimulated protein synthesis and the production of collagen. Higher concentrations of selenite which blocked growth depressed the incorporation of proline into protein. These results suggested that selenium had the capacity to bring about rapid changes in the rate of protein synthesis. Significant differences were observed within 1 h. Similar studies using 4- to 5-hr exposure periods produced similar results.

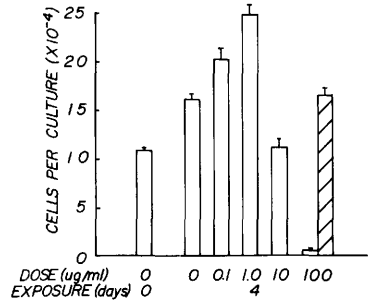

FIG. 1. Influence of sodium selenite on the growth of cultured mammary tumor cells. Cells were prepared from NMU-induced mammary tumors and plated in serum-free growth medium. Twenty-four hours later the plating medium was replaced with experimental media containing the indicated doses of sodium selenite (open bars). Sodium sulfite (100 μg/ml, crossed bar) was added to one series of control cultures. Cells were counted at the start of the experiment (Day 0) and 4 days later. Results represent the means and standard errors for four determinations.

TABLE II. INFLUENCE OF SELENIUM ON PROTEIN SYNTHESIS IN CULTURED MAMMARY TUMOR CELLS[a]

Culture fraction	Na_2SeO_3 (μg/ml)	[^{3}H]Proline incorporated into protein (dpm × 10^{-3}/dish ± SEM) Collagen	Noncollagen
Medium	0	0.83 ± 0.33	18.5 ± 5.4
	1	1.71 ± 0.72	14.9 ± 2.9
	50	0.86 ± 0.40	15.6 ± 1.4
Cell layer	0	26.9 ± 1.0	389.8 ± 6.9
	1	34.2 ± 2.2[b]	521.7 ± 27.6[b]
	50	19.8 ± 1.1[b]	315.6 ± 23.0[b]

[a] Cultures of tumor cells (n = 3) growing on 100-mm dishes were incubated with [^{3}H]proline (5 μCi/ml) and the indicated doses of sodium selenite for 1 hr at 37°C. Newly synthesized collagen was measured in the extracted protein by sensitivity to purified bacterial collagenase (34).

[b] $p < 0.05$ vs control lacking added selenium.

The influence of selenium on the incorporation of [^{3}H]thymidine into DNA is shown in Fig. 2. The toxic doses of selenite had a relatively quick effect on DNA synthesis. A significant decrease in the rate of incorporation was measured within the first hour of exposure. At concentrations of selenium which stimulated both growth and protein synthesis, no enhancement of DNA synthesis was observed within the first 5 hr of exposure.

Discussion. The induction of cancer is a multistage process. The first stage, initiation, involves alteration of the genome by a carcinogen with the formation of latent tumor cells. The second stage involves a complex series of events culminating in the outgrowth of the initiated cells and the formation of a tumor mass (46). The mechanism of selenium's anticancer effect is not certain. Several studies suggest that selenium influenced promotion as well as the initiation stages (13–23). The antipromotion effects of sele-

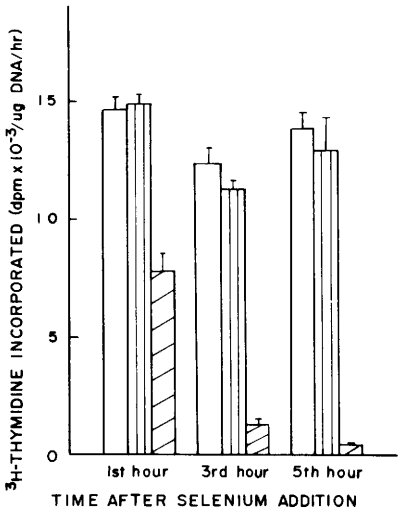

FIG. 2. Influence of selenium on the replication of DNA in cultured mammary tumor cells. Media were removed from 3-day-old cultures and replaced with fresh media containing the following concentrations of selenium: No Na_2SeO_3, open bars; 1 μg/ml Na_2SeO_3, vertically striped bars; 50 μg/ml Na_2SeO_3, diagonally-crossed bars. The incorporation of [^{3}H]thymidine was measured over 1-hr periods, as indicated, after the media replacements. Results represent the means ± SEM of four determinations.

nium could be due to direct effects on mammary cells or indirect effects, for example, stimulation of the immune response (38, 39) or alterations in the hormonal or nutritional status of the host. The results reported here showed that the addition of selenite to culture media had direct cellular effects on the growth of NMU-induced tumor epithelium. Low doses of selenite stimulated cell growth and protein synthesis whereas higher doses were inhibitory. Medina and co-workers (25, 26) and Chatterjee and Banerjee (24) have observed similar results in certain cultured mouse mammary cells. The simplest model for an anticancer effect during tumor promotion would be that selenium alters the growth of tumor cells directly.

We observed that the growth of the rat mammary tumor cells was stimulated at concentrations of selenite less than 1.0 μg/ml. In separate studies, selenite concentrations as low as 0.001 μg/ml had significant growth stimulating effects (unpublished results). Concentrations greater than approximately 1.0 μg/ml (5.8×10^{-6} *M*) inhibited growth in a dose-related manner. Medina and co-workers (25, 26) studied the influence of selenite on the growth of several types of mouse mammary cells. Selenite at 5×10^{-8} *M* stimulated the growth of normal mouse mammary cells. Certain preneoplastic and tumor cell lines were stimulated, others were not. At $0.5–1 \times 10^{-5}$ *M*, selenite generally inhibited the growth of normal mouse mammary cells and the cell lines (25, 26). Additionally, we have studied certain human breast cancer cells lines (MCF-7, ZR75-1, MDA 231). Growth inhibition was observed at 0.1–1.0 μg/ml selenite (40). By comparison, the NMU-induced rat mammary tumor cells required a somewhat higher concentration of selenium to inhibit growth. This slightly lower sensitivity may be due to the relatively slow growth rate of these cells coupled with the limited longevity (1 week) of this type of primary cell culture system.

The mechanism of selenium's influence on cell growth is not understood. We observed that selenite altered the rates of protein synthesis in rat mammary tumor cells in a biphasic manner. This effect was measurable soon after the addition of selenite. Previous studies *in vivo* and in culture using NMU-induced tumors showed that cell proliferation and the replication of DNA were sensitive to specific inhibitors of collagen production. This suggested a role for type IV collagen synthesis in the proliferation of these tumors cells. In line with selenium's effects on protein synthesis, selenite altered type IV collagen production in a biphasic manner and it is a possibility that the mechanism of selenium's anticancer action could involve early changes in the synthesis of certain specific proteins such as collagen.

Understanding the mechanism whereby selenium regulates protein synthesis and cell division will require further study. We have been considering one possibility, that selenium alters the levels of total cellular glutathione (41). Our preliminary measurements indicate that sodium selenite has a biphasic influence on the levels of cellular glutathione in cultures of human breast cancer (MCF-7 and MDA 231) cells (41) and in primary cultures of NMU-induced rat tumor cells (unpublished). This tripeptide is involved in several cellular processes including antioxi-

dation (with selenium), conjugation reactions, amino acid transport, and protein synthesis (42, 43). And it is a rather old concept that fluctuating cellular sulfhydryl levels may be involved in the regulation of cell division (44).

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