Effects of Combined Treatments with Selenium, Glutathione, and Vitamin E on Glutathione Peroxidase Activity, Ornithine Decarboxylase Induction, and Complete and Multistage Carcinogenesis in Mouse Skin

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ABSTRACT

Several structurally different tumor promoters altered to various degrees both glutathione (GSH) peroxidase (EC 1.11.1.9) and ornithine decarboxylase (ODC, L-ornithine carboxy-lyase, EC 4.1.1.17) activities in mouse epidermis in vivo. At 5 h after their application to the skin, the complete tumor promoter TPA-12-O-tetradecanoylphorbol-13-acetate (TPA) and the stage 2 promoter mezerine were the most potent in inhibiting GSH peroxidase activity and inducing ODC activity. In comparison, the effects of anthralin, phorbol-12,13-didecanoate, benzoil peroxide, H2O2 and phorbol-12,13-dibenzoleate were much smaller, whereas the non-tumor promoter phorbol, the hyperplastic agent ethyl phenylpropionate, and the stage 1 promoter 4-O-methyl TPA did not alter GSH peroxidase and ODC activities. Various treatments including i.p. injections of 40 μg of Na2SeO3 and 100 μmol of GSH and/or topical applications of 40 μmol of d-α-tocopherol (vitamin E) 20 or 15 min, respectively, before tumor promoter treatment inhibited in an additive manner the effects of either TPA or mezerine on both GSH peroxidase activity and ODC induction. Moreover, these Na2SeO3, GSH, and/or vitamin E treatments inhibited in the same additive manner the tumor-promoting activity of TPA in the initiation-promotion protocol. However, when tested in the 2-stage promotion protocol with 4 doses of TPA followed by twice weekly applications of mezerine, Na2SeO3 plus vitamin E and GSH plus vitamin E treatments inhibited remarkably the tumor-promoting activity of mezerine but were ineffective in the first stage of promotion.

The sequence and magnitude for the effects of 7,12-dimethylbenz[a]anthracene (DMBA) on GSH peroxidase and ODC activities were very different from those of the tumor promoters. In contrast with their antitumor-promoting activity, the treatments with Na2SeO3 plus vitamin E and GSH plus vitamin E failed to inhibit the carcinogenicity of a single large dose of DMBA and even enhanced the induction of skin tumors by repeated applications of subcarcinogenic doses of DMBA.

These results suggest that the promoting component of DMBA carcinogenesis may be different from that of TPA. Moreover, the antitumor-promoting activity of Na2SeO3 and vitamin E may be linked to their ability to facilitate or enhance the activity of the natural GSH-dependent antioxidant protective system of the epidermal cells during the later stages of skin tumor promotion.

INTRODUCTION

Mouse skin carcinogenesis can be divided into tumor initiation and multistage tumor promotion (2). Indirect evidence suggests that free radical-induced reactions may be involved in the process of skin tumor promotion (3, 4). For instance, in various cell systems in vitro, the potent phorbol ester promoter TPA,2 a peroxisome proliferator, as well as several other structurally different tumor promoters, stimulate the generation of superoxide anion (O2·-) radicals, H2O2, lipid hydroperoxides, and the chemiluminescence response associated with oxy-radical protection, in relation with their tumor-promoting activity in vivo (3–6). Some free radical-generating compounds such as H2O2 and benzoil and lauryl peroxides exhibit weak tumor-promoting activities (2–4, 7, 8). Moreover, diverse antioxidants, free radical scavengers, and cyclooxygenase and lipoxygenase inhibitors decrease the oxidative challenge and some of the early molecular events linked to tumor promotion by TPA such as, for example, the induction of polyamine biosynthesis (9–14).

The possibility that, in TPA-treated cells, O2·- may act as a percursor to the formation of reactive free radicals species more directly involved in the process of tumor promotion has been suggested (15). The sequential reactions leading to the formation of H2O2 and the extremely reactive hydroxyl (HO·) radicals that may amplify the toxicity of O2·- in the soluble fraction of the cell have been described (3, 4, 16). One of the current hypotheses is that an increased generation of free oxygen radicals coupled with a defective antioxidant protective system could explain some of the mechanisms of tumor promotion. Although different interpretations have been proposed to explain these observations (17), the activities of the detoxifying enzymes SOD and CAT seem to be significantly depressed by the tumor promoters (18,19), whereas certain classes of reactive oxygen inhibitors including CAT and various SOD-mimicking compounds generally inhibit some of the biochemical and biological effects of the tumor promoters (20–24).

In addition to CAT activity, the detoxification of H2O2 in the cytoplasm is accomplished by the GSSG-R couple, a NADPH-consuming system which requires adequate concentrations of GSH, Na2SeO3, and glucose to work (25). The sequential relationships between the effects of TPA on GSH peroxidase activity and the GSH:GSSG intracellular ratio in isolated mouse epidermal cells suggest that the natural GSH-dependent detoxifying system of the TPA-treated cells is initially turned on but then rapidly inhibited (26, 27). It is not known whether the prolonged stimulation of the GSH-dependent detoxifying system would, through nonenzymatic reduction and the mediation of GSH peroxidase, ameliorate the natural protection of the epidermal cells against the oxidative challenge presumably linked to the tumor-promoting activity of TPA. In vitro, free radical scavengers, GSH level-raising agents and Na2SeO3-containing compounds generally inhibit some of the biochemical and biological effects of the tumor promoters (20–24).

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2 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz[a]anthracene; PDB, phorbol-12,13-dibenzoleate; PDD, phorbol-12,13-didecanoate; FPP, ethyl phenylpropionate; GSH, reduced glutathione; GSSG, oxidized glutathione; O2·-, superoxide anion radical; ODC, L-ornithine carboxy-lyase, EC 4.1.1.17; GSH peroxidase, GSH:H2O2 oxidoreductase, EC 1.11.1.9; SOD, superoxide:superoxide oxidoreductase, EC 1.15.1.1; CAT, H2O2:H2O2 oxidoreductase, EC 1.11.1.6; GSSG-R, NAD(P)H:oxidized glutathione oxidoreductase, EC 1.6.4.2; vitamin E, D-α-tocopherol.

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by TPA (28, 29). Since the complementarity for the stimulatory effects of vitamin E, GSH, and Na₂SeO₃ on the GSH-dependent antioxidant protective system has been postulated (30), the present study was undertaken to determine in vivo whether various combinations of antioxidants would inhibit in an additive manner the effects of the tumor promoters on epidermal GSH peroxidase and ODC activities, and decrease accordingly the formation of skin tumors by the initiation-promotion protocols and by the complete carcinogenesis processes.

MATERIALS AND METHODS

Treatment of Mice. Female CF-1 mice from Sasco, Inc. (Omaha, NE), 7–9 weeks old, were housed and maintained, and their dorsal skins were shaved before experimentation as previously described (31). The solutions of TPA, mezerein, PDD, 4-O-methyl TPA, DPD, phorbol (all from Lifesystem Co., Newton, MA), DMBA (Eastman Kodak Co., Rochester, NY), EPP, anthralin (both from Pfaltz & Bauer, Inc., Waterbury, CT), H₂O₂, and benzoyl peroxide were prepared in acetone and delivered to the shaved backs of individual mice in a volume of 0.2 ml. Unless otherwise specified, the dose of 40 µmol of vitamin E (d-tocopherol, natural, yellow oil, United States Biochemical Corp., Cleveland, OH) in 0.2 ml of acetone was applied topically 15 min before, and to the same area of skin as the application of tumor promoter. The mice received ip. injections of 0.25 ml of freshly prepared 9% NaCl solution, containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, pH 7.0, and either 40 µg of Na₂SeO₃ (Sigma Chemical Co., St. Louis, MO) or 100 µmol of GSH 20 min before the topical application of tumor promoter. The pH of each solution was always adjusted to pH 7.0 before injection. Controls were treated with acetone and buffer only and in every experiment all mice receive the same volume of buffer and solvent. The mice were killed by cervical dislocation and the epidermides were collected by a brief heat treatment (31).

Tumor Induction Experiments. In the initiation-promotion protocol, tumors were initiated in all mice by topical application of 0.1 µmol of DMBA in 0.2 ml of acetone; 2 weeks following initiation, all mice were promoted twice a week (on days 1 and 4) with 8.5 nmol of TPA in 0.2 ml of acetone for the duration of the experiment. In the two-stage promotion experiment, 8.5 nmol of TPA were applied only four times (first stage) to the DMBA-initiated mice and then the animals were promoted twice a week with 8.5 nmol of mezerein in 0.2 ml of acetone (second stage) for the duration of the experiment. Using the complete carcinogen DMBA, tumors were induced either by a single topical application of 3.6 µmol or by two weekly applications of 0.1 µmol of DMBA for the duration of the experiment. The various treatments with Na₂SeO₃, GSH, and/or vitamin E were administered as outlined above either before each promotion with TPA or mezerein, before the single carcinogenic dose of DMBA, or before each of the repeated applications of the subcarcinogenic dose of DMBA. Initially, there were 36 mice in each treatment group. The incidence of skin papillomas and carcinomas was recorded weekly.

Determination of ODC Activity. At the appropriate time after tumor promoter or carcinogen treatments, the epidermal preparations from 2 mice were pooled in 3 ml of 25 mM Tris-HCl buffer, pH 7.6, containing 4 mM dithiothreitol, 1 mM EDTA, and 0.2 mM pyridoxal 5'-phosphate, homogenized with a Polytron PT 10 homogenizer for 15 s at setting 7, and centrifuged at 30,000 × g for 30 min to give a soluble supernatant. ODC activity was determined in 0.1-ml aliquots of the clear supernatant by measuring the release of 14C0₂ from DL-[1-14C]ornithine-HCl (61 mCi/mmol; Amersham Corp., Arlington Heights, IL) essentially as described previously (31). The substrate concentration used was 0.4 mM L-ornithine-HCl containing 0.5 µCi of DL-[1-14C]ornithine-HCl. The assays were carried out in duplicate, and all values were corrected against no enzyme or boiled enzyme blanks; ODC activity was expressed as nmol of CO₂ released in 60 min per mg of protein.

Determination of GSH Peroxidase Activity. The same soluble epidermal extracts prepared from the epidermal homogenates from 2 mice as described above were used for the determination of GSH peroxidase activity. After centrifugation at 30,000 × g for 30 min, the clear supernatants containing GSH peroxidase activity were diluted 1:3 with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM NaN₃. Total GSH peroxidase activity was determined in 0.1-ml aliquots of these diluted supernatants with 1.5 mM cumene hydroperoxide as substrate by a modification (32) of the coupled assay system originally described by Paglia and Valentine (33). The rate of GSSG formation during GSH oxidation by peroxide as catalyzed by the GSH peroxidase present in the epidermal sample was measured by following at 340 nm the conversion of NADPH to NADP during 5 min at 37°C, as the GSSG produced is immediately converted to the reduced form by the addition of exogenous GSSG-R (type III from yeast, 160 units/mg protein, Sigma Chemical Co.). The assays (29) were carried out in duplicate. Blank reactions with enzyme source replaced by buffer were subtracted from each assay. GSH peroxidase activity was reported as µmol of NADPH oxidized in 1 min per mg of protein.

The protein concentration of the epidermal samples was assayed in 60-µl aliquots of the diluted supernatants with Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA), using crystalline bovine serum albumin as the standard.

RESULTS

The sequential early transient stimulation and prolonged inhibition of GSH peroxidase specific activity by 8.5 nmol of TPA in mouse epidermis in vivo (Fig. 1A) are identical with those observed in TPA-treated epidermal cells in vitro (26, 27, 29, 34). Following TPA treatment, the activity of this detoxifying enzyme is maximally induced at 25 min, declines below the basal level at 2 h, and is maximally inhibited at 5 h (Fig. 1A), the time at which maximal TPA-induced ODC activity is
observed (Fig. 1B). The same dose of mezerein proved to be significantly more potent than TPA in altering both GSH peroxidase and ODC activities (Fig. 1). The effectiveness of a number of structurally different tumor promoters in decreasing GSH peroxidase activity at 5 h seems to correlate with the magnitude of their ODC-inducing activity at the same time (Table 1). As compared to the powerful effects of TPA and mezerein, all the tumor promoters tested, except anthralin and PDD, produce only slight responses, whereas EPP, phorbol, and the first stage promoter 4-O-methyl TPA produce no response. It should be noted that the direct addition of 0.1–1 µM concentrations of TPA and mezerein to the enzyme assay system does not alter the GSH peroxidase activities of the extracted epidermal samples. The kinetics of epidermal GSH peroxidase activity was identical when measured with 0.25 mM H2O2 or tert-butyl hydroperoxide as substrate (data not shown).

In earlier studies (29) we found that in mouse epidermis in vivo and in vitro Na2SeO3-dependent GSH peroxidase activity represents about 65–75% of the total GSH peroxidase activity. Since in our experiments any change in Na2SeO3-dependent GSH peroxidase activity is always accompanied by a similar change in total GSH peroxidase activity and vice versa, for simplification of the data only the values of total GSH peroxidase activity with 1.5 mM cumene hydroperoxide as substrate have been reported in the present study.

The doses of 40 µmol of vitamin E (Fig. 2), 100 µmol of GSH (Fig. 3), and 40 µg of Na2SeO3 (Fig. 4) were administered to the mice at different times before TPA treatment (time 0) and tested for their ability to inhibit both the decrease in GSH peroxidase activity and the induction of ODC activity caused by the tumor promoter at 5 h. The ability of these compounds to inhibit the effects of TPA is clearly dependent on the time of their administration relative to TPA. Maximal inhibition occurs when GSH and Na2SeO3 are injected i.p. 20 min before TPA application and when vitamin E is painted on the skin 15

Table 1 Comparison of the effects of various tumor promoters on GSH peroxidase and ODC activities in mouse epidermis in vivo

Groups of mice were killed for enzyme assay 5 h after the topical application of the indicated compounds in 0.2 ml of acetone. The doses used were: H2O2 and benzoin peroxide, 100 µmol; EPP, 57.5 µmol; anthralin, 2 µmol; all other compounds, 8.5 nmol. The conditions of the experiment were identical with those in Fig. 1. Results are the mean ± SD of duplicate determinations of enzyme activity from 6 groups of mice in 2 different experiments; each group contained the combined soluble epidermal extracts prepared from 2 mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH peroxidase activity at 5 h</th>
<th>ODC activity at 5 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>µmol NADPH oxidized/min/mg protein</td>
<td>% of control</td>
</tr>
<tr>
<td>Control</td>
<td>0.598 ± 0.042</td>
<td>100</td>
</tr>
<tr>
<td>Phorbol</td>
<td>0.606 ± 0.041 *</td>
<td>101</td>
</tr>
<tr>
<td>EPP</td>
<td>0.589 ± 0.039</td>
<td>98</td>
</tr>
<tr>
<td>4-O-methyl TPA</td>
<td>0.583 ± 0.041 *</td>
<td>97</td>
</tr>
<tr>
<td>PDB</td>
<td>0.542 ± 0.036 *</td>
<td>91</td>
</tr>
<tr>
<td>H2O2</td>
<td>0.501 ± 0.033</td>
<td>84</td>
</tr>
<tr>
<td>Benzoin peroxide</td>
<td>0.494 ± 0.033 *</td>
<td>83</td>
</tr>
<tr>
<td>PDD</td>
<td>0.477 ± 0.031</td>
<td>80</td>
</tr>
<tr>
<td>Anthralin</td>
<td>0.446 ± 0.030</td>
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</tr>
<tr>
<td>TPA</td>
<td>0.405 ± 0.026</td>
<td>68</td>
</tr>
<tr>
<td>Mezerein</td>
<td>0.363 ± 0.025</td>
<td>61</td>
</tr>
</tbody>
</table>

* No significance versus control.
* P < 0.025, significantly smaller versus control.
* P < 0.005, significantly smaller versus PDB.
* P < 0.005, significantly smaller versus PDD.
* No significance versus H2O2.
* No significance versus benzoin peroxide.
* P < 0.1, significantly smaller versus PDD.
* P < 0.025, significantly greater versus PDD.
* P < 0.0005, significantly greater versus TPA.
* No significance versus control.
* P < 0.005, significantly greater versus PDB.
* No significance versus benzoin peroxide.
* P < 0.1, significantly smaller versus PDD.
* P < 0.025, significantly smaller versus TPA.
* P < 0.0005, significantly greater versus TPA.

Fig. 2. Effect of the time of application of a single dose of vitamin E on TPA-decreased GSH peroxidase (A) and TPA-induced ODC (B) activities in mouse epidermis in vivo. Groups of mice were treated topically with 40 µmol of vitamin E in 0.2 ml of acetone (C) at various times before, and to the same area of skin as the application of 8.5 nmol of TPA (time 0). The mice were killed 5 h after TPA treatment for enzyme assay. The conditions of the experiment were identical with those in Fig. 1. Basal GSH peroxidase activity in acetone plus acetone-treated control mice was 0.594 ± 0.039 µmol NADPH oxidized/min/mg protein (100 ± 7%) and TPA-decreased GSH peroxidase activity in acetone plus TPA-treated mice was 0.454 ± 0.033 µmol NADPH (78 ± 6%; A, W). The mean value of TPA-induced ODC activity in mice treated with acetone plus TPA only was 7.85 ± 0.60 nmol CO2 released/60 min/mg protein (100 ± 8%; B, W). Basal ODC activity in control mice treated with acetone plus acetone (0.26 ± 0.02 nmol CO2) has been subtracted from the results. Bars, SD. a, no significance versus TPA; b, P < 0.01, significantly greater versus TPA; c, no significance versus 40 µmol of vitamin E applied 15 min before TPA; d, P < 0.1, significantly smaller versus TPA.

Fig. 3. Effect of the injection of a single dose of GSH on TPA-decreased GSH peroxidase (A) and TPA-induced ODC (B) activities in mouse epidermis in vivo. Groups of mice were given injections i.p. of 100 µmol of GSH (C) at various times before the application of 8.5 nmol of TPA to the skin (time 0). The mice were killed 5 h after TPA treatment for enzyme assay. The conditions of the experiment were identical with those in Fig. 1. Basal GSH peroxidase activity in control mice receiving buffer i.p. and acetone (0.27 ± 0.02 nmol CO2) has been subtracted from the results. Bas, SD. a, no significance versus TPA; b, P < 0.01, significantly greater versus TPA; c, no significance versus 100 µmol of GSH injected 20 min before TPA; d, P < 0.1, significantly smaller versus TPA; e, P < 0.1, significantly smaller versus TPA; f, P < 0.1, significantly greater versus 100 µmol of GSH injected 20 min before TPA.
activity is increased to 131, 148, and 156% of the non-trouted repeated administrations of Na₂SeO₃ and GSH at 24-h intervals and ODC activities (Table 3). In the absence of TPA treatment, and/or vitamin E elicit additive inhibitory effects (Table 2).

Moreover, these treatments with Na₂SeO₃, GSH, and/or vitamin E are administered before TPA-decreased GSH peroxidase and TPA-induced ODC activities in mouse epidermis in vivo. Groups of mice were given injections i.p. of 40 μg of Na₂SeO₃ (C) at various times before the application of 8.5 nmol of TPA to the skin (time 0). The mice were killed 5 h after TPA treatment for enzyme assay. The conditions of the experiment were identical with those in Fig. 1. Basal GSH peroxidase activity in control mice receiving buffer i.p. and acetone topically was 0.633 ± 0.46 μmol NADPH oxidized/min/mg protein (100 ± 7%) and TPA-decreased GSH peroxidase activity in the absence of Na₂SeO₃ injection was 0.490 ± 0.035 μmol NADPH (77 ± 6%; A). The value of TPA-induced ODC activity in mice given injections of buffer only was 7.61 ± 0.59 nmol CO₂ released/60 min/mg protein (100 ± 8%; B). Basal ODC activity in control mice given buffer and acetone only (0.24 ± 0.02 nmol CO₂) has been subtracted from the results. Bars, SD, a, no significance versus TPA; b, P < 0.01, significantly greater versus TPA; c, no significance versus 40 μg of Na₂SeO₃ injected 20 min before TPA; d, P < 0.05, significantly smaller versus TPA.

min before TPA. Using such schedules, the dose dependency for the inhibitory effects of vitamin E, GSH, and Na₂SeO₃ on TPA-decreased GSH peroxidase activity and TPA-induced ODC activity is demonstrated (Figs. 2–4). Effectiveness is rapidly lost at treatment times further from the time of application of TPA. A greater inhibitory effect may be achieved by applying vitamin E at a time closer to the TPA treatment, but the possibility that this volume of solvent alone may inhibit TPA effects by simply diluting or decreasing the penetration of this compound into the epidermis must be avoided (35).

Because of the distant site of administration, the dilution and the tissue distribution profile in the whole body of the animal, it is likely that very small fractions of the doses of GSH and Na₂SeO₃ injected i.p. actually reach the epidermal target cells, whereas most of the vitamin E applied topically may interact more rapidly and specifically with the cells of the TPA-treated area. In addition to their different mechanisms of action, this may in part explain the fact that vitamin E is more effective than GSH and Na₂SeO₃ in inhibiting the effects of TPA on GSH peroxidase and ODC activities in mouse epidermis in vivo (Table 2). Various combined treatments with Na₂SeO₃, GSH, and/or vitamin E elicit additive inhibitory effects (Table 2). Moreover, these treatments with Na₂SeO₃, GSH, and/or vitamin E also inhibit to the same degree and in the same additive manner the greater effects of mezerein on both GSH peroxidase and ODC activities (Table 3). In the absence of TPA treatment, repeated administrations of Na₂SeO₃ and GSH at 24-h intervals enhance basal GSH peroxidase activity in mouse epidermis in vivo (data not shown). For instance, epidermal GSH peroxidase activity is increased to 131, 148, and 156% of the nontreated controls 2 h after the first, second, and third administration of Na₂SeO₃ plus GSH, respectively. In contrast, vitamin E treatments do not alter basal GSH peroxidase activity. Moreover, none of the treatments with Na₂SeO₃, GSH, and/or vitamin E is able to alter basal ODC activity.

The relevance of ODC induction and decreased GSH peroxidase activity to the mechanism of skin tumor promotion is illustrated by the results in Table 4, showing that, in the classic initiation-promotion protocol, the same Na₂SeO₃, GSH, and vitamin E treatments inhibit the tumor-promoting activity of TPA in relation with their effectiveness as inhibitors of the effects of TPA on GSH peroxidase and ODC activities. The major inhibitory effects of these compounds are on the mean numbers of papillomas per mouse. Together, the inhibitory effects of Na₂SeO₃, GSH, and vitamin E on tumor promotion by TPA are clearly additive and, because of the powerful effect of vitamin E alone, maximal inhibitory effects are observed with the combined treatments that include vitamin E (Table 4). For instance, Na₂SeO₃ plus vitamin E, GSH plus vitamin E, and Na₂SeO₃ plus GSH plus vitamin E inhibit the promotion of skin papillomas by TPA by about 63, 78, and 91%, respectively. That the above treatments do not induce toxic effects in vivo is suggested by the fact that, after 22 weeks of promotion, the weight and the rate of survival of the animals treated with Na₂SeO₃, GSH, and vitamin E are identical with those of mice receiving TPA only. For some of the combined treatments the rate of survival is slightly lower, but it is difficult to determine whether this is due to the more toxic combinations of chemicals or just to the risk of hitting a vital organ during the injection process. Since the combined treatments with Na₂SeO₃ plus vitamin E and GSH plus vitamin E produce the greatest inhibition of tumor promotion and are the easiest and least risky to apply because only one i.p. injection is involved, they have been selected for further studies.

In the two-stage promotion protocol following DMBM initiation, the treatments with Na₂SeO₃ plus vitamin E and GSH plus vitamin E inhibit remarkably mezerein-induced stage 2 promotion (Table 5). Moreover, skin tumor incidence and tumor yield are reduced to the same extent whether Na₂SeO₃ plus vitamin E and GSH plus vitamin E are administered before
Groups of mice in 2 different experiments; each group contained the combined soluble epidermal extracts prepared from 2 mice. The mice were killed 5 h after mezerein treatment for enzyme assay. Results are the mean ± SD of duplicate determinations of enzyme activity from 6 groups of mice in 2 different experiments; each group contained the combined soluble epidermal extracts prepared from 2 mice.

Table 4 Inhibitory effects of Na2SeO3, GSH, and vitamin E on promotion of mouse skin papillomas by TPA

Skin tumors were initiated in all mice by topical application of 0.1 μmol of DMBA. Two weeks after initiation, all mice were promoted twice a week with 8.5 nmol of TPA for the duration of the experiment. The doses, times, and modes of administration of the indicated compounds before each promotion by TPA were identical with those described in Table 2. Initially, there were 36 mice in each treatment group. The incidence of skin tumors was recorded weekly.

Observations at 22 wk

<table>
<thead>
<tr>
<th>Grade</th>
<th>Promotion treatment (2/wk)</th>
<th>wt/mouse (g)</th>
<th>% of survival</th>
<th>% of mice with papillomas</th>
<th>Papillomas/mouse</th>
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<tbody>
<tr>
<td>1</td>
<td>TPA (8.5 nmol)</td>
<td>30.8</td>
<td>98</td>
<td>100</td>
<td>12.6</td>
</tr>
<tr>
<td>2</td>
<td>+ Na2SeO3 (40 μg)</td>
<td>29.7</td>
<td>94</td>
<td>91</td>
<td>10.2</td>
</tr>
<tr>
<td>3</td>
<td>+ GSH (100 μmol)</td>
<td>30.9</td>
<td>99</td>
<td>89</td>
<td>7.7</td>
</tr>
<tr>
<td>4</td>
<td>+ vitamin E (40 μmol)</td>
<td>31.4</td>
<td>95</td>
<td>79</td>
<td>5.9</td>
</tr>
<tr>
<td>5</td>
<td>+ Na2SeO3 + GSH</td>
<td>28.9</td>
<td>75</td>
<td>83</td>
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<td>6</td>
<td>+ GSH + vitamin E</td>
<td>30.6</td>
<td>88</td>
<td>74</td>
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<td>7</td>
<td>+ GSH + vitamin E</td>
<td>30.4</td>
<td>97</td>
<td>49</td>
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<tr>
<td>8</td>
<td>+ Na2SeO3 + GSH + vitamin E</td>
<td>27.8</td>
<td>81</td>
<td>25</td>
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Table 5 Effects of combined treatments with Na2SeO3 or GSH plus vitamin E on the induction of mouse skin papillomas by the two-stage promotion protocol

All mice were initiated with 0.1 μmol of DMBA. Then, the 2-stage promotion regimen was achieved by twice weekly applications of 8.5 nmol of TPA for 2 weeks (stage 1) followed by twice weekly applications of 8.5 nmol of mezerein for 20 weeks (stage 2). Groups of mice received intraperitoneal injections of Na2SeO3 or GSH plus topical applications of vitamin E before each application of either TPA or mezerein. The doses and times of administration of the compounds before each promotion by TPA or mezerein were identical with those described in Table 2. Initially, there were 36 mice in each treatment group. The incidence of skin tumors was recorded weekly.

Promotion treatment (2 wk) | wt/mouse (g) | % of survival | % of mice with papillomas | Papillomas/mouse |
<table>
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<tr>
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<tbody>
<tr>
<td>1 TPA</td>
<td>36.7</td>
<td>86</td>
<td>57</td>
<td>2.3</td>
</tr>
<tr>
<td>2 + Na2SeO3 + vitamin E</td>
<td>36.9</td>
<td>88</td>
<td>60</td>
<td>4.6</td>
</tr>
<tr>
<td>3 + GSH + vitamin E</td>
<td>36.8</td>
<td>96</td>
<td>51</td>
<td>1.9</td>
</tr>
<tr>
<td>4 TPA</td>
<td>36.4</td>
<td>94</td>
<td>32</td>
<td>0.8</td>
</tr>
<tr>
<td>5 TPA</td>
<td>36.5</td>
<td>92</td>
<td>24</td>
<td>0.4</td>
</tr>
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</table>

Each application of the stage 2 promoter mezerein (Table 5) or before each application of the complete tumor promoter TPA (Table 4). By contrast, GSH plus vitamin E fails to inhibit, and Na2SeO3 plus vitamin E even enhances, the stage 1-promoting activity of TPA (Table 5).

Evidence has been presented that the promotion of skin tumorigenesis by the carcinoen DMBA may be mechanistically different from that of TPA (36). Therefore, it is of interest to determine whether the GSH peroxidase detoxifying system is also involved in the promoting component of DMBA carcino-genesis. In accord with previous findings (2), application of either mezerein or a single completely carcinogenic dose of DMBA leads to the induction of epidermal ODC activity, whereas a subcarcinogenic initiating dose of DMBA does not (Table 6). However, there is a considerable difference in the time course and in the degree of ODC induction: peak ODC activities about 39- and 3-fold above the acetone control levels are observed 5 and 30 h, respectively, following the application of mezerein or 3.6 μmol of DMBA (Table 6). At this latter time, the decreasing value of mezerein-induced ODC activity is still superior to the peak activity of ODC in response to DMBA (Table 6). The sequence and magnitude of the effects of mezerein and DMBA on epidermal GSH peroxidase activity are also different (Table 6). As compared with the biphase GSH peroxidase response elicited by the complete and stage 2 tumor promoters (Fig. 1), both initiating (0.1 μmol) and carcinogenic (3.6 μmol) doses of DMBA maintain GSH peroxidase activity slightly stimulated throughout the 30-h experiment (Table 6).
Table 6 Comparison of the effects of mezerein and initiating and carcinogetic doses of DMBA on GSH peroxidase and ODC activities in mouse epidermis in vivo

Groups of mice received topical applications of either mezerein or subcarcinogenic (0.1 µmol) and carcinogenic (3.6 µmol) doses of DMBA or 0.2 ml acetone. The mice were killed at the indicated times following treatment for enzyme assay. Results are the mean ± SD of duplicate determinations of enzyme activity from 6 groups of mice in 2 different experiments; each group contained the combined soluble epidermal extracts prepared from 2 mice.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Treatment</th>
<th>GSH peroxidase activity</th>
<th>ODC activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µmol NADPH oxidized/min/mg protein</td>
<td>% of control</td>
</tr>
<tr>
<td>0.4</td>
<td>Control</td>
<td>0.59 ± 0.039</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>DMBA (0.1 µmol)</td>
<td>0.68 ± 0.044*</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>DMBA (3.6 µmol)</td>
<td>0.76 ± 0.049</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Mezerein (8.5 µmol)</td>
<td>0.87 ± 0.058</td>
<td>147</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>0.60 ± 0.041</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>DMBA (0.1 µmol)</td>
<td>0.69 ± 0.045*</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>DMBA (3.6 µmol)</td>
<td>0.74 ± 0.047</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>Mezerein (8.5 µmol)</td>
<td>0.44 ± 0.029</td>
<td>74</td>
</tr>
<tr>
<td>30</td>
<td>Control</td>
<td>0.64 ± 0.043</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>DMBA (0.1 µmol)</td>
<td>0.73 ± 0.049</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>DMBA (3.6 µmol)</td>
<td>0.80 ± 0.052</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>Mezerein (8.5 µmol)</td>
<td>0.49 ± 0.032</td>
<td>76</td>
</tr>
</tbody>
</table>

* P < 0.005, significantly greater versus control.
* No significance versus control.
* P < 0.01, significantly greater versus control.

Interestingly, the treatments with Na₂SeO₃ plus vitamin E and GSH plus vitamin E that are very effective in inhibiting complete and stage 2 tumor promotion by TPA and mezerein (Tables 4 and 5) fail to inhibit significantly the carcinogenicity of 3.6 µmol of DMBA and paradoxically enhance the formation of skin papillomas and carcinomas by repeated applications of 0.1 µmol of DMBA (Table 7).

DISCUSSION

In vitro, the rapid induction of epidermal GSH peroxidase activity by TPA is concomitant with a dramatic fall in the GSH:GSSG intracellular ratio (26, 27). This is followed by a prolonged decrease in the specific activity of the GSH peroxidase detoxifying system (26, 27, 29, 34). Although some difficulties may be associated with the measurement of specific activities of epidermal enzymes during tumor promoter-induced hyperplasia (17), the changes reported here are true biological responses to TPA since similar TPA-decreased GSH peroxidase activities expressed per mg of protein or per 2 x 10⁶ cells have been observed from 2 to 12 h following tumor promoter treatment in the mouse epidermis systems both in vivo and in vitro (26, 27, 29, 34). Moreover, a number of structurally different tumor promoters initially increase GSH peroxidase activity and decrease the GSH:GSSG ratio and then inhibit GSH peroxidase activity in parallel with their ability to induce epidermal ODC activity (26, 34), one of the essential components of skin tumor promotion (37). However, the hypothesis that the rapid generation of H₂O₂ and other reactive O₂ species by TPA might challenge and possibly overwhelm the natural GSH-dependent antioxidant protective system of the epidermal cells, and that the resulting excess of nondetoxified free radicals might mediate some molecular events linked to skin tumor promotion remains to be demonstrated.

The observation that peroxides, and complete and stage 2 tumor promoters concomitantly inhibit GSH peroxidase activity and induce polyamine biosynthesis, two events that are not duplicated by the nontumor promoter phorbol, the hyperplastic agent EPP and the first stage promoter 4-O-methyl TPA in vivo, suggests that the inhibition of the GSH peroxidase detoxifying system of the epidermal cells may be one of the components of stage 2 promotion. This conclusion is strengthened by the finding that the various tumor promoters tested in vivo induce ODC activity, a biochemical marker of the early phase of the second stage of tumor promotion (2), in relation with their effects on GSH peroxidase activity and their reported complete or stage 2 tumor-promoting activities (2, 7, 8). In contrast to the first stage promoter 4-O-methyl TPA, H₂O₂ altered GSH peroxidase and ODC activities in vivo but this effect might be linked to its extremely weak tumor-promoting activity (8) or its effectiveness in stage 3 promotion (2). Interestingly, the inhibition of the natural GSH-dependent antioxidant protective system of the epidermis for only a few hours may be sufficient to trigger some of the molecular events involved in the late stages of tumor promotion, since Taffe and Kensler (38) report that initially decreasing GSH peroxidase activity returns to basal level at about 24 h and is stimulated above control level for several days following TPA treatment.

Another evidence for the protective role of the GSH-dependent antioxidant system against tumor promotion is the observation that, in the presence of the free radical-generating anti-tumor antibiotic Adriamycin, the GSH peroxidase activity and GSH:GSSG ratio of the TPA-treated epidermal cells are decreased to a greater extent than during TPA treatment alone, and this results in the enhancement of the ODC-inducing and tumor-promoting abilities of TPA (27). According to this proposal, it would not be surprising if repeated treatments with H₂O₂ or benzoyl peroxide could enhance dramatically the tumor-promoting abilities of other weak and moderate tumor promoters such as, for example, the phorbol esters PDB and...
Recently, Na₂SeO₃-containing compounds were found to inhibit phospholipid:Ca²⁺-dependent protein kinase activity, a key protein phosphorylation system in tumor promotion, and the TPA-stimulated phosphorylation of leukemic cell proteins (42).

The discrepancy between the effects of DMBA and those of TPA and mezerein on GSH peroxidase and ODC activities may be only one of the reasons why the antioxidant mixtures shown to inhibit skin tumor promotion by TPA and mezerein fail to inhibit carcinogenesis by a single carcinogenic dose of DMBA and even potentiate tumor formation by weekly subcarcinogenic doses of DMBA. Thus, the nature and the mechanism of tumor promotion by TPA and mezerein are likely to be different from those of the presumed promoting component in DMBA carcinogenesis. Differential effects of retinoic acid, 7,8-benzoflavone, and dexamethasone on ODC induction, and the formation of mouse skin tumors by the complete carcinogenesis process and by the initiation-promotion regimen have also been described (36). An alternative explanation would be that the inhibitory effects of Na₂SeO₃ plus vitamin E and GSH plus vitamin E on the promoting component of DMBA carcinogenesis could be masked by an equal or greater stimulation of the initiating ability of DMBA. Interactions with the natural GSH-dependent antioxidant protective system are unlikely to mediate the effects of the mixtures of Na₂SeO₃, GSH, and vitamin E on DMBA carcinogenesis. For instance, Adriamycin decreases the GSH:GSSG ratio and GSH peroxidase activity and enhances the ODC-inducing and tumor-promoting abilities of TPA but is neither carcinogenic nor initiator and does not increase the initiating ability of 0.1 µmol of DMBA in mouse skin (27). Interestingly, Cu(II)-(3,5-diisopropylsalicylate)₂ inhibits DMBA initiation and skin carcinogenesis by DMBA without altering DMBA-induced ODC activity (24).

The fact that Na₂SeO₃ plus vitamin E and GSH plus vitamin E more than double the tumor incidence by repeated applications of 0.1 µmol of DMBA is unexpected and suggests that, under similar conditions, these treatments might enhance the initiating activity of a single dose of 0.1 µmol of DMBA. In contrast, a topical dose of vitamin E has been shown to inhibit skin tumor initiation by DMBA in a 2-stage system of tumorigenesis (43) and other data suggest that Na₂SeO₃ in drinking water exerts its tumor inhibitory effects on the initiation rather than the promotion stage of skin carcinogenesis (44). A dietary antioxidant mixture including, among other things, vitamin E and GSH has also been shown to significantly reduce 3-methylcholanthrene-induced skin carcinogenesis (45).

The information on the mechanisms whereby antioxidants generally inhibit chemical carcinogenesis in several organ sites is largely incomplete (46). Mixed results have been published concerning the potential anti-carcinogenicity and/or mutagenicity of GSH (47–49). The role of Na₂SeO₃ in tumorigenesis has been extensively reviewed (50) and its possible action on both the initiating and promoting components of carcinogenesis has been discussed (51). The synergistic effects of vitamin E and Na₂SeO₃ in the chemoprevention of carcinogenesis have been described (52). In vitro, Na₂SeO₃-deficient HL-60 human promyelocytic leukemia cells incubated with TPA release more H₂O₂ and O₂⁻ than their Na₂SeO₃-repleted counterparts (53), but there is no proof that increased Na₂SeO₃-dependent GSH peroxidase activity is responsible for the anticarcinogenic effects of Na₂SeO₃ treatments in vivo (51, 52). At this point, there is not enough evidence to suggest that the effects of Na₂SeO₃ on chemical carcinogenesis could be partially attributable to alterations in the binding of ultimate DNA-damaging carcinogens, the synthesis and repair of DNA, chromosomal aberration, the ultimate availability of electrophilic metabolites to the
nucleophiolic sites of cellular macromolecules by modulating the enzymatic activities involved in the metabolic activation/degredation/detoxification/conjugation of procarcinogens, or effects on the expression of genes other than those involved in carcinogen metabolism. Moreover, one should consider the possibility that the opposite effects of 40 μg of Na2SeO3, 100 μmol of GSH, and 40 μmol of vitamin E on the induction of skin tumors by the initiation-promotion protocol and by the complete carcinogenesis process might result from different levels of cytostatic and cytotoxic activities in the TPA- and DMBA-treated epidermis. Under certain experimental conditions, different doses of Na2SeO3 can either decrease or enhance the carcinogenic process, possibly through modulation of the GSSG:GSH ratio and inhibition of cell proliferation (54). If, for instance, adaptive cell proliferation occurs in response to high Na2SeO3 in an organ, an enhancement of carcinogenesis is likely, resulting in the development of cells that are resistant to high doses of Na2SeO3 (54).

REFERENCES


Effects of Combined Treatments with Selenium, Glutathione, and Vitamin E on Glutathione Peroxidase Activity, Ornithine Decarboxylase Induction, and Complete and Multistage Carcinogenesis in Mouse Skin


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