An Apparent Inactivation of Initiated Cells by the Potent Inhibitor of Two-Stage Mouse Skin Tumorigenesis, Bis(2-chloroethyl)sulfide

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SUMMARY

Sulfur mustard, a potent inhibitor of two-stage skin tumorigenesis, appears to act primarily by inactivating initiated cells rather than by suppressing the biochemical response to the promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Thus, when the increase of epidermal-dermal p-tosyl-L-arginine methyl ester esterase activity in TPA-treated mice was compared with the response of mice similarly treated but receiving a pretreatment with sulfur mustard, no statistically significant difference was found. In both cases activity was increased to 4 to 5 times the level found in acetone-treated controls. Moreover, pretreatment with sulfur mustard failed to influence the ability of the promoter to inhibit an isoproterenol-induced accumulation of cyclic adenosine 3′:5′-phosphate. Finally, sulfur mustard applied to inhibit an isoproterenol-induced accumulation of cyclic adenosine 3′:5′-phosphate. Finally, sulfur mustard applied 24 hr after the first three applications of TPA did not inhibit the 300-fold increase in ornithine decarboxylase activity observed after the fourth application of the promoter.

In tumor induction experiments four applications of sulfur mustard given 24 hr after the first four applications of the promoter resulted in a 60% reduction in tumors/mouse. A similar reduction was observed when four applications of sulfur mustard were given during the interval between initiation with 7,12-dimethylbenz[a]anthracene and the beginning of promotion. If sulfur mustard inhibits tumorigenesis by a general cytotoxicity, an effect on the biochemical responsiveness of the skin to TPA should be observed. As stated above no such effect was observed. Furthermore, previous investigators have reported that sulfur mustard did not inhibit either the hyperplasia or the inflammation associated with preneoplasia of the skin. These facts combined with the tumor inhibition data strongly indicate that sulfur mustard in some way selectively inactivates initiated cells.

INTRODUCTION

Since the original experiments of Berenblum (1–3), it has been known that low doses of SM2 applied topically are capable of inhibiting chemically induced mouse skin tumorigenesis. In these early experiments SM was given simultaneously with multiple applications of coal tar. Remarkably, the histological appearance of the skin, which was characterized by a pronounced hyperplasia and hyperemia, was the same in mice given the coal tar-SM mixture and in mice given coal tar alone. Recently, Van Duuren and Segal (24) have demonstrated that small doses of SM (128 nmoles) interfused between applications of the phorbol ester TPA to initiated mice were capable of completely suppressing 2-stage tumorigenesis. In this case, too, as judged histologically, SM did not appear to alter the hyperplastic or the inflammatory response of the skin to TPA. These observations have led us to investigate whether SM could affect several of the biochemical responses that correlate with the skin tumor-promoting ability of TPA. We have further investigated the ability of SM to inhibit skin tumorigenesis when administered during the interval between initiation and the beginning of promotion.

MATERIALS AND METHODS

Chemicals. Trizma base, p-tosyl-L-arginine methyl ester hydrochloride, DL-isoproterenol, L-ornithine hydrochloride, and bovine serum albumin were obtained from Sigma Chemical Company, St. Louis, Mo.; DMBA was from Eastman Organic Chemicals, Rochester, N. Y.; TPA was from Dr. Peter Borchert, University of Minnesota, Minneapolis, Minn.; SM was from K & K Laboratories, Inc., Plainview, N. Y.; [G-3H]cyclic AMP (ammonium salt) and DL-[1-14C]ornithine monochloride were from New England Nuclear, Boston, Mass.; N-o-p-tosyl-L-arginine [3H]methyl ester hydrochloride was from Amersham/Searle Corporation, Arlington Heights, Ill. All other chemicals were reagent grade.

Animals and Treatments. Female CD-1 mice were obtained from Charles River Breeding Laboratories, Wilmington, Mass., and were used when 7 to 8 weeks old. Mice were housed in an air-conditioned room with a light period from 6 a.m. to 6 p.m. They received water and Wayne Breeder Blox (Allied Mills, Inc., Chicago, Ill.) ad libitum. Two days before treatment the dorsal hair was shaved with electric clippers; mice wounded during clipping or exhibiting hair regrowth within 48 hr were discarded.

Chemicals were applied to the clipped area in 0.2 ml of acetone with a Biopette (Schwarz/Mann, Orangeburg, N. Y.). Acetone was dried over molecular sieves, Linde type 3A (Matheson Coleman and Bell, Norwood, Ohio) before

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being used to dissolve SM. For all experiments SM and TPA were applied in doses of 128 and 17 nmoles, respectively. For tumor experiments animals were initiated with 78 nmoles of DMBA.

*p-Tosyl-L-arginine Methyl Ester Esterase Determination.* Three mice/group were pretreated with either acetone or SM and 30 min later were treated with either acetone or TPA. Twenty-four hr after the second treatment, mice were killed by cervical dislocation. Hair stubble was removed by application of the depilatory Nudit (a generous gift of Helena Rubinstein, Greenvale, N. Y.) for 3 min; the skin was then washed under cold tap water. Skins were excised and placed epidermal side down on a chilled porcelain plate. The panniculi adiposus and carnosus were removed by scraping with a scalpel. The epidermal-dermal samples were immediately frozen in liquid nitrogen and then pulverized in a chilled mortar and pestle. After pulverization the powder was homogenized in 3 ml of distilled H2O with a Polytron PT-10 homogenizer at a setting of 7 for 12 sec. Homogenates were centrifuged at 755 x g, at 0–4° for 30 min, after which the supernatant was removed with a Pasteur pipet and saved.

*p-Tosyl-L-arginine methyl ester esterase activity* was determined by a modification of the procedure of Roffman *et al.* (18). Incubations were performed in 15-ml Kimax culture tubes fitted with Teflon-lined caps (Kimble Products, Toledo, Ohio). The substrate was prepared by adding 5.0 /μl of N-α-p-tosyl-L-arginine[3H] (radioactive concentration, 1 mCi/ml) to 50 ml of 10 mM unlabeled p-tosyl-L-arginine methyl ester in dimethylformamide. The final reaction mixture contained equal volumes of substrate, 1.0 M Tris buffer (pH 7.5), and epidermal-dermal supernatant with a protein content of 500 to 900 μg. This final mixture, with a volume of 300 μl, was overlaid with 10 ml of a toluene-based scintillant (4 g of PPO and 50 mg of POPOP per liter of toluene) and was incubated at room temperature for 90 min. At the end of the incubation, tubes were reimmersed for 10 sec in liquid nitrogen, the aqueous phase in liquid nitrogen. Tubes were then mixed in a chilled mortar and pestle. After pulverization the powder was homogenized in 3 ml of distilled H2O with a Polytron PT-10 homogenizer at a setting of 7 for 12 sec. Homogenates were centrifuged at 755 x g, at 0–4° for 30 min, after which the supernatant was removed with a Pasteur pipet and saved.

Measurements of cyclic AMP, p-Tosyl-L-arginine methyl ester esterase activity was determined by a modification of the procedure of Roffman *et al.* (18). Incubations were performed in 15-ml Kimax culture tubes fitted with Teflon-lined caps (Kimble Products, Toledo, Ohio). The substrate was prepared by adding 5.0 /μl of N-α-p-tosyl-L-arginine[3H] (radioactive concentration, 1 mCi/ml) to 50 ml of 10 mM unlabeled p-tosyl-L-arginine methyl ester in dimethylformamide. The final reaction mixture contained equal volumes of substrate, 1.0 M Tris buffer (pH 7.5), and epidermal-dermal supernatant with a protein content of 500 to 900 μg. This final mixture, with a volume of 300 μl, was overlaid with 10 ml of a toluene-based scintillant (4 g of PPO and 50 mg of POPOP per liter of toluene) and was incubated at room temperature for 90 min. At the end of the incubation, tubes were reimmersed for 10 sec in liquid nitrogen, the aqueous phase in liquid nitrogen. Tubes were then mixed in a chilled mortar and pestle. After pulverization the powder was homogenized in 3 ml of distilled H2O with a Polytron PT-10 homogenizer at a setting of 7 for 12 sec. Homogenates were centrifuged at 755 x g, at 0–4° for 30 min, after which the supernatant was removed with a Pasteur pipet and saved.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>p-Tosyl-L-arginine methyl ester esterase (nmoles of [3H]methanol/mg protein/90 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Acetone</td>
<td>101.00 ± 11.84</td>
</tr>
<tr>
<td>Second Acetone</td>
<td>17.68 ± 3.61</td>
</tr>
<tr>
<td>SM*</td>
<td>75.77 ± 10.95</td>
</tr>
<tr>
<td>SM Acetone</td>
<td>15.79 ± 3.32</td>
</tr>
</tbody>
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*Mean ± S.E.

**Effect of SM Pretreatment on TPA-induced p-Tosyl-L-arginine Methyl Ester Esterase Activity.** Van Duuren and Segal (24) have suggested that SM may exert its tumor-inhibitory effect by blocking the binding of TPA to cell membranes. If this is so pretreatment with a tumor-inhibiting dose of SM would block the biochemical responses to TPA. In separate papers Troll *et al.* (23) and Hozumi *et al.* (8) have shown that, 24 hr after application of TPA or croton oil, p-tosyl-L-arginine methyl ester esterase activity was significantly increased over that in solvent-treated controls, and inhibition of this activity was correlated with an inhibition of tumorigenesis. We have pretreated mice with SM 30 min before TPA treatment and assayed the p-tosyl-L-arginine methyl ester esterase activity 24 hr after TPA treatment. The activity of this enzyme was not significantly different in TPA-treated animals pretreated with SM compared to animals receiving TPA alone. In both cases activity was increased to about 5 times the level found in acetone-treated controls (Table 1). Furthermore, SM alone was unable to induce p-tosyl-L-arginine methyl ester esterase activity (Table 1). When the same dose of SM is applied 30 min before each application of 17 nmoles of TPA in a tumor induction experiment, tumorigenesis is completely inhibited (L. M. De Young and R. K. Boutwell, unpublished observation).

**Effect of SM Pretreatment on the Ability of TPA to Reduce Cyclic AMP Accumulation in Response to Isoproterenol.** A topical application of TPA given 9 to 72 hr before i.p. injection of isoproterenol is capable of completely blocking the isoproterenol-induced accumulation of cyclic AMP in the skin (14). The accumulation of cyclic AMP is membrane mediated (17), and it has been suggested that its blockage by TPA occurs at this site (14). To ascertain

**Protein Determination.** Protein was assayed by the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

**Statistical Analysis.** Data were analyzed for statistical significance by the Dunnett test (21). Differences yielding p < 0.05 in a 2-sided test are considered statistically significant.

**RESULTS**

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whether SM could interfere with the ability of TPA to block a β-adrenergic response, mice were treated topically with either acetone or SM and after 30 min with either TPA or acetone. Mice were killed 13 hr after the first treatment and received an i.p. injection of isoproterenol 10 min prior to being killed. Cyclic AMP accumulation was then determined. Under these conditions, TPA blocked the accumulation of cyclic AMP elicited by isoproterenol injection, and the block imposed by TPA was not relieved by SM treatment (Table 2). Furthermore, treatment with SM alone did not alter the β-adrenergic responsiveness of the skin.

The Effect of SM Treatment on TPA-induced ODC Activity. There is good correlation between the ability of a chemical to act as a promoter and its ability to induce ODC in the epidermis (15, 16). Furthermore, there is a correlation between the ability of an agent to inhibit TPA-mediated ODC induction and its ability to inhibit TPA promotion of tumors (26). SM applied 24 hr after each application of TPA to initiated mice completely suppressed tumor induction (Chart 1). We have therefore tested the ability of SM to alter the induction of ODC by TPA when applied 24 hr after the first, second, and third applications of TPA. Animals were killed and ODC activity was assayed 5 hr after the fourth application of TPA. At this time ODC induction is near its peak. Table 3 shows that SM did not significantly alter the induction of epidermal ODC by TPA. Furthermore, SM treatment alone did not induce ODC activity (Table 3).

Tumor Induction Studies. Van Duuren and Segal (24) have shown that SM applied 24 hr after each application of TPA resulted in a virtually complete suppression of tumor induction. They have also shown that SM alone is inactive as a tumor promoter in initiated mice, and we have confirmed these results in our system (Chart 1).

Berenblum (4) suggested that the inhibition of tumorigenesis by SM is transient and dependent upon continued SM application throughout promotion. To test this possibility we treated a group of DMBA-initiated animals with SM 24 hr after each of the first 4 applications of TPA. In this group, papillomas/mouse plateaued at 17 weeks, with SM producing a 60% reduction in the number of tumors per mouse (Chart 1).

Since, as reported by Van Duuren and Segal (24), SM treatment had no effect on biochemical changes induced by TPA in the skin and since in the present study we could find no effect of SM on biochemical changes induced by TPA in the skin, we tested the possibility that SM could inactivate initiated cells. In this case mice were treated twice with SM during the interval between initiation and the beginning of promotion. Animals treated in this manner had a 32% reduction...
in the number of tumors (Chart 1).

For verification of the observation that SM applied during the interval between initiation and promotion was capable of inhibiting tumorigenesis and for determination of whether this inhibition was dependent upon the number of applications of SM, the experiment depicted in Chart 2 was performed. Two applications of SM during the interval between initiation and promotion resulted in a 26% inhibition of the number of papillomas per mouse, while 4 applications of SM resulted in a 51% inhibition (Chart 2).

DISCUSSION

In earlier investigations on the inhibition of skin tumorigenesis by SM (1–3, 24), the authors stressed the remarkable fact that tumor-inhibitory doses of SM appeared to have no inhibitory effect on inflammation or hyperplasia associated with preneoplasia of the skin. We have now shown that, for the parameters studied, SM had no inhibitory effect on biochemical changes induced by the potent tumor promoter TPA. These observations, combined with the fact that SM can inhibit tumorigenesis in a dose-dependent manner when given after initiation but before the beginning of promotion, strongly indicate that the primary effect of SM is on initiated cells and not on some event associated with promotion. This hypothesis is further strengthened by the observation that 4 applications of SM given during the beginning of promotion resulted in an irreversible 60% inhibition of tumorigenesis. In the latter case, if the primary effect of SM had been on promotion, this inhibition should have been reversed when SM application ceased.

It is highly unlikely that the putative inactivation of initiated cells is due to a general cytotoxicity of SM. Neither Van Duuren and Segal (24) nor Berenblum (1–3) could find any histological evidence of a general cytotoxic effect. Furthermore, if SM were killing or inactivating large numbers of cells in the skin, one would expect altered biochemical responses of SM-treated skin to TPA. This was clearly not the case for TPA-induced ODC activity or p-tosyl-L-arginine methyl ester esterase activity; treatment with SM did not alter induced levels of these activities. Additionally, SM did not affect the ability of TPA to inhibit isoproterenol-induced cyclic AMP accumulation. Of further interest in the latter experiment was the observation that SM alone did not affect isoproterenol-induced cyclic AMP accumulation. Isoproterenol acts at the cell membrane (17) and SM can act at this site (12). However, in the regimen presented here SM treatment did not damage cell membranes sufficiently to alter the β-adrenergic responsiveness of the target cells.

The above results suggest the intriguing possibility that low doses of SM can specifically inactivate initiated cells. The mechanism of this putative inactivation remains a matter for future investigation. An unlikely possibility is that SM acts by interfering with the process of initiation. Evidence exists that indicates that this process is relatively rapid. For example, Frei and Ritchie (6) have demonstrated that the effectiveness of initiation varies with the time of day of application of the initiator. If initiation were a long-term process, diurnal variations in its effectiveness would probably not be observed. Furthermore, the period of time between the beginning of promotion and the appearance of the first tumors is the same whether promotion is begun 1 or 2 weeks after the application of the initiator (L. M. De Young and R. K. Boutwell, unpublished observations). This suggests that initiation is complete by at least 1 week after DMBA application. The fact that SM can apparently inactivate initiated cells when given as late as 2 weeks after DMBA initiation would seem to rule out an SM-mediated inhibition of the process of initiation. However, experiments are currently under way in which varying intervals between initiation and SM treatment are being examined for effect on the inhibition of tumorigenesis.

TPA-induced epidermal hyperplasia (7) and ODC activity (15) are thought to require RNA synthesis. The lack of effect of SM on these responses to TPA suggests that SM does not exert its inhibitory activity through a general inhibition of RNA polymerase activity. However, the possibility exists that SM may preclude RNA polymerase from transcribing specific portions of the genome important in the expression of initiation.

Two additional compounds, actinomycin D (7, 19, 25) and aflatoxin B, (25), have been reported to inhibit tumorigenesis when low doses are given during the interval between initiation and promotion. Like SM (11) both actinomycin D (20) and aflatoxin B, (5) have a high affinity for guanine residues of the DNA molecule. DMBA also has an affinity for guanine residues (10). This raises the possibility that there may be guanine-rich areas of the genome particularly sensitive to the action of these initiators and inhibitors. The extremely low doses and high apparent specificity of action that these compounds exhibit in relation to tumor-
igenesis is consistent with such a possibility. This affinity for DNA suggests the possibility that DNA repair may be important to the mechanism of inactivation of initiated cells. Aflatoxin B, is a liver carcinogen (28), actinomycin D induces mesothelioma-like tumors (22), and SM is carcinogenic for the respiratory tract (27). It is well known in chemotherapy that many carcinogens can exhibit antitumor activity. Whether the mechanism of this antitumor activity is similar to the mechanism of the tumor-prophylactic activity of SM, actinomycin D, and aflatoxin B, in the skin remains a topic for further investigation.

SM is of course a highly active, bifunctional, alkylating agent capable of introducing intra- and interstrand cross-links into the DNA molecule (11). To begin to determine what effect, if any, the ability to cross-link DNA may have on inactivation of initiated cells, we are conducting experiments in which a monofunctional, i.e., non-cross-linking, SM is used. Likewise, the non-SM DNA cross-linker mitomycin C (9) is being tested for prophylaxis of tumorigenesis.

If SM is indeed acting specifically on initiated cells, it may provide a means for identifying initiated cells prior to the gross appearance of tumors. For instance, if initiated cells were incapable of clearing SM-induced DNA cross-links, while surrounding normal cells had this capability, autoradiographic studies with labeled SM might localize initiated cells and/or islands of initiated cells. This would provide a valuable tool in the study of tumorigenesis.

REFERENCES

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