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Croton Oil Enhancement of Skin Tumor Initiation by *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine: Possible Role of DNA Replication (40126)

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The promotion stage in the initiation–promotion model system of chemical carcinogenesis in mouse skin is dependent on the repeated application of promoting agents, usually beginning one week or more after the single application of initiator (For reviews, see 1, 2). A second type of promoter-induced enhancement of tumor initiation has also been reported (3, 4). When a single dose of the classic tumor promoter, croton oil, was applied to mouse skin prior to initiation with 7,12-dimethylbenz[a]anthracene (DMBA), urethan or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), the subsequent tumor yield in initiation–promotion experiments was increased and the latent period was shortened. Since croton oil stimulated DNA synthesis, this enhancement was originally attributed to increased susceptibility of DNA-synthesizing cells to the tumor initiator (3). While susceptibility to carcinogens has been correlated to the level of binding of the chemicals to cellular DNA (5, 6), no increase in binding to cellular macromolecules was detected in mice pretreated with croton oil before application of tritiated DMBA (4) or MNNG (7). In fact, binding of these initiators to nonreplicating DNA was slightly higher than to replicating DNA *in vivo* (7, 8) and *in vitro* (9). Thus, alternative explanations, involving effects on DNA repair and/or increased DNA replication subsequent to the initial carcinogen binding to cellular macromolecules, have been suggested (4, 7).

In support of these suggestions, a single croton oil application 6 hr before or 1 hr after initiation by ip injection of urethan was as effective in the enhancement of tumor formation as it was when applied 24 hr before the initiator (10). In the animals treated with croton oil 1 hr after urethan, the rate of DNA replication was not increased above control levels until 12–18 hr after urethan injection (2), a time when most of the urethan has been cleared from the mice.

In synchronized cultures of C3H/10T-1/2 cells, malignant transformation by MNNG showed a marked cell cycle dependency, with greatest sensitivity in the late G_1 period (11). In mouse skin, Bowden and Boutwell (7) showed that croton oil application 18 hr before initiation with MNNG enhanced the skin tumor yield by about threefold. We have extended these experiments by looking at the effect on initiation of single croton oil treatment times between 6 hr before and 20 hr after initiation with MNNG. The combined effects on DNA synthesis of MNNG at Zero Time and croton oil at −6 or +1 hr were then determined. Our results are discussed with regard to the relationship of DNA replication to the croton oil-induced enhancement of initiation.

Materials and methods. Chemicals. Croton oil was purchased from Sigma Chemical Co., St. Louis, MI. MNNG was obtained from Aldrich, Milwaukee, WI. Thymidine-methyl-[^{3}H] (5 Ci/mmole) was purchased from Amersham/Searle, Arlington Heights, IL.

Tumor induction experiment. Female CD-1 mice were obtained from the Charles River Breeding Farm, Wilmington, MA, at 5 weeks of age. The backs of the mice were shaved when they were 8 weeks old, 1 to 2 days before initiation with MNNG. Solutions of MNNG and croton oil were prepared in acetone; 0.2 ml was applied to the shaved backs with an automatic pipetter. Papillomas were counted weekly; no carcinomas developed in the 33 weeks of the experiment.

DNA Synthesis. Mice were injected ip with 30 μCi [^{3}H]thymidine 1 hr before sacrifice. Nucleic acids were extracted from epidermis and the specific activity of DNA determined as previously described (10).

Results. In an initiation-promotion experiment, we examined the effect on MNNG tumor initiation of a single application of

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0.5% croton oil 6 hr before or 1, 4, 8 or 20 hr after application of 4 μmoles MNNG. As shown in Table I, the control tumor incidence of 0.9 papillomas per mouse (Group 3) was increased about threefold when croton oil was applied at any of the 5 times tested. The latent period was reduced from 30 weeks in the control group to between 17–24 weeks in the groups treated once with croton oil. A single croton oil application at each of the times tested clearly increased the papilloma yield and shortened the latent period.

An application of 0.5% croton oil inhibits [^{3}H]thymidine incorporation into epidermal DNA for about 12 hr. DNA synthesis is then increased with a peak at 18 hr and a return to normal by about 5 days (4). As shown in Fig. 1, after 4 μmoles of MNNG alone, DNA synthesis was inhibited for 1.5 days then increased slightly at days 2–5. When a croton oil treatment was given either 6 hr before or 1 hr after MNNG (as in Groups 4 and 5 in Table I), the usual croton-oil induced peak of DNA synthesis 18 hr after treatment was nearly eliminated, but a three-to-fourfold increase in DNA synthesis was seen 2–4 days after the combined treatment.

Discussion. We have shown that the enhancement of MNNG initiation by a single croton oil application does not depend on treatment with croton oil prior to application of the initiator. Similar threefold increases in papilloma yield were observed in groups of mice treated 6 hr before or 1, 4, 8 or 20 hr after MNNG application (Table I). In mice treated with croton oil at −6 or +1 hr with respect to MNNG, clear-cut increases in DNA synthesis were not seen before 2 days

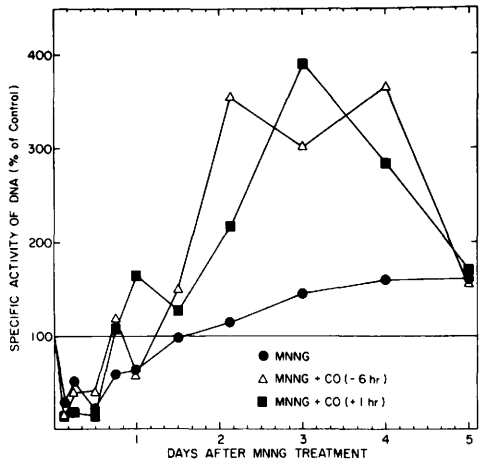

FIG. 1. DNA synthesis in CD-1 female mouse epidermis after an application of MNNG or MNNG + croton oil. MNNG (4 μmoles in 0.2 ml acetone) was applied topically at Zero Time. Croton oil (0.2 ml of 0.5% in acetone) was applied topically either 6 hr before or 1 hr after Zero Time. [^{3}H]Thymidine (30 μCi/mouse) was injected ip 1 hr before sacrifice at the indicated times. The specific activity of DNA is expressed as percent of the control treated with 0.2 ml acetone at Zero Time. ●, MNNG alone at zero time; △, MNNG at zero time + CO (croton oil) at −6 hours; ■, MNNG at zero time + CO (croton oil) at +1 hr.

TABLE I.

Group	Initiation (4 μmoles MNNG)	Time of single croton oil application (hours)	Croton oil promotion (0.5% once/week)	Number of mice[a]	Percent with papillomas[a]	Papillomas per mouse[a]	Latent period (weeks)
1	+	NONE	−	28	0	0	>33
2	−	NONE	+	28	11	0.1	>33
3	+	NONE	+	39	49	0.9	30
4	+	−6	+	29	79	3.0	21
5	+	+1	+	36	78	2.7	21
6	+	+4	+	30	67	2.6	24
7	+	+8	+	30	70	3.3	21
8	+	+20	+	31	65	2.9	17

[a] Data at week 27.

Effect of a single croton oil application on initiation by MNNG. Groups of 28–40 Charles River CD-1 female mice were shaved, then treated with 4 μmoles MNNG at Zero Time and with 0.5% croton oil at the times indicated. Croton oil promotion was begun at Week 4. Papillomas were counted weekly. No more than two mice died in any of the experimental groups during the 33 weeks of the experiment. "Percent with Papillomas" is defined as the percent of mice bearing at least one papilloma in each group. "Papillomas per Mouse" is defined as the total number of papillomas divided by the number of mice alive in the group. "Latent Period" is defined as the time in weeks until 50% of the mice in a group developed at least one papilloma. The low papilloma yield in Group 2, treated only with promoting doses of croton oil, has often been found (1, 10). This result may indicate that promoters are very weak carcinogens or, alternatively, that the promoter is revealing "spontaneously" initiated cells.

after MNNG treatment. The peak binding of [^{3}H]MNNG to skin DNA, RNA and protein was found within 2 hr of application (7). If interaction of the initiator with DNA is necessary for initiation, then a possible mechanism for the croton oil enhancement of initiation would involve its effect on the biological steps in initiation which follow the initial interaction. While some chemically-induced DNA damage appears to be repaired within hours (12), other damage is still present several days after chemical carcinogen treatment (13, 14). In mouse epidermal cultures, the rate of both excision repair and guanine-specific repair (15) are similar at 4 hr and 4 days after a 1 hr treatment with 25 μg MNNG/ml (Hennings and Michael, unpublished results) indicating that alkylated DNA remains long after treatment. Thus it is feasible that croton oil-induced DNA replication, even at 2–4 days after MNNG treatment, could result in the fixation of MNNG-induced mutations before DNA damage has been repaired, as suggested earlier (4, 7).

Both croton oil and TPA have been demonstrated to inhibit DNA repair synthesis, but this effect appears to be part of a general toxic response, since DNA replication as well as RNA and protein synthesis are similarly inhibited (16). The kinetics of recovery of DNA repair and replication have not yet been compared. If the duration of inhibition of DNA repair synthesis were longer than that for DNA replication, then the inhibition of DNA repair by croton oil could also be involved in the enhancement of initiation.

Similar results to those described here with MNNG were reported earlier with urethan as initiator (10). The enhancement of urethan initiation by a single croton oil application did not require croton oil pretreatment; application 1 hr after urethan injection was as effective as application at 6 or 24 hr before injection. In contrast to the results reported here for MNNG, enhancement was less pronounced when croton oil was applied 6 or 12 hr after urethan injection, and no enhancement was seen with application 24 hr after injection. With MNNG, a similar degree of enhancement was found at all times tested up to 20 hr (Table I, group 8).

In conjunction with urethan, croton oil induced a 3.5- to 4-fold increase in DNA synthesis within 18 hr (2); this first peak of DNA synthesis was nearly eliminated in the presence of MNNG (Fig. 1). If interaction with DNA, DNA repair, DNA replication and mutation fixation are indeed involved in croton oil-enhanced initiation by MNNG and urethan, then the timing of the critical molecular processes are different for combinations of croton oil and urethan or MNNG. The time required for generation and loss of the ultimate carcinogenic species is unknown, but both initiators are likely to be most active in the first few hours after application. The different modes of administration of the two compounds (urethan was given ip; MNNG was given topically in acetone) could also affect the timing.

Other initiators such as DMBA and β-propiolactone have not shown the clear-cut croton oil pretreatment effects seen with urethan and MNNG (4). With these initiators, studies of posttreatment with croton oil at times after peak binding of the initiator to epidermal DNA may yield insight into the mechanism of promoter-induced enhancement of initiation.

Summary. An application of 0.5% croton oil at times from 6 hr before until 20 hr after initiation with 4 μmoles MNNG led to a threefold increase in papilloma yield and a substantial shortening of the latent period in two-stage tumor induction experiments in mouse skin. Since DNA replication was not stimulated by croton oil until 2–4 days after application of MNNG, this enhancement cannot be explained by an increased uptake of the MNNG by DNA-synthesizing cells or by increased susceptibility of these cells. If DNA replication is involved in initiation, then our results suggest that the critical DNA replication occurs after the interaction of MNNG with DNA, but before the initial damage can be repaired, perhaps resulting in a nonrepairable mutation.

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