Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 486-492

CHOLERA TOXIN, A POTENT INDUCER OF EPIDERMAL HYPERPLASIA BUT WITH NO TUMOR PROMOTING ACTIVITY IN MOUSE SKIN CARCINOGENESIS*

Toshio Kuroki[1], Kazuhiro Chida, Kimiye Munakata and Yoshinori Murakami[2]

Department of Cancer Cell Research, Institute of Medical Science University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan

Received April 16, 1986

Summary Intracutaneous injection of cholera toxin into mice induced epidermal hyperplasia to a greater extent than 12-O-tetradecanoylphorbol-13-acetate. It also induced adenylate cyclase and though weakly, ornithine decarboxylase of the epidermis. Cholera toxin, however, showed no tumor promoting activity in mouse skin carcinogenesis. In the single stage promotion, cholera toxin (50 ng) was injected once a week for 10 weeks into the skin of SENCAR mice initiated with 25 μg 7,12-dimethylbenz[a]anthracene, but no tumors developed. In the two-stage promotion test, cholera toxin (10-100 ng) was injected for one or two weeks into the initiated skin and then mezerein (4 μg) was applied twice a week for 18 weeks, but the toxin did not increase incidence or numbers of papillomas.

Introduction We reported previously that cholera toxin stimulates the growth of epidermal cells in vivo and in vitro (1-3). Intracutaneous injection of cholera toxin at doses of 0.2 ng or more into mice induces two successive synchronous division of basal cells of the epidermis 24 and 48 h after its injection, resulting in epidermal hyperplasia which reaches a maximum on day 4 with formation of 8 to 12 cell layers (1). Cholera toxin seemed useful for studies on the role of epidermal hyperplasia in tumor promotion.

The role of cell proliferation in promotion of mouse skin carcinogenesis has been debated. Although induction of hyperplasia is correlated with the

* Supported in part by a Grant for Cancer Research from the Ministry of Education, Science, and Culture of Japan.

[1] To whom reprint requests should be sent.

[2] Present address: Oncogene Division, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan

Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase; DMBA, 7,12-dimethylbenz[a]anthracene; PBS, phosphate buffered saline.

0006-291X/86 $1.50

promoting activity of some phorbol ester promoters (4,5), agents that cause hyperplasia are not all promoters (6-11). Besides cell proliferation, several other changes may be required for tumor promotion. These include induction of ODC (12,13) and increase of dark basal keratinocytes (9,10,14). We report here that cholera toxin induced hyperplasia but did not act as a complete or Stage I promoter in mouse skin carcinogenesis.

Materials and methods

Chemicals. Cholera toxin was obtained from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. TPA and mezerein were purchased from Consolidated Midland Co., Brewster, N.Y., U.S.A. DMBA was purchased from Eastman Kodak Co., Rochester, N.Y., U.S.A. TPA, mezerein and DMBA were dissolved in acetone. DL-[1-^{14}C]ornithine hydrochloride (specific activity, 57.3 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Animals and application of cholera toxin. Female SENCAR mice (15), originally obtained from Dr T. J. Slaga, Oak Ridge National Laboratory, Oak Ridge, TN, U.S.A., were used for carcinogenesis studies. In other experiments male DDD mice, obtained from the breeding house of this institute, were used. The dorsal skin of their back was shaved with electric clippers 2 days before experiments. Cholera toxin in a volume of 0.1 ml was injected intracutaneously into the back of mice. In some experiments, we used tongue forceps (ring-shaped, 20 mm in internal diameter) according to the method of Ishikawa et al. (16). Namely, the skin of the back of mice under anaesthesia with avertin was clamped off with the tongue forceps for 1 h and 0.5 ml of cholera toxin solution was injected into this clamped off region (2).

Assay of cyclic AMP and ODC. Mice were killed by cervical dislocation and the skin of the back at the sites of injection of the toxin was rapidly excised. For assay of cyclic AMP, possible degradation of cyclic AMP by phosphodiesterase was prevented by quickly freezing the skin by pressing a block of dry ice on it. Subcutaneous tissue was scraped off with a razor blade. The skin preparation was homogenized by a Polytron homogenizer at 0°C in 1N NaOH solution containing 10 mM theophylline. The preparation was then neutralized by 4N HCl and centrifuged at 1,000xg for 20 min, and the supernatant fraction was used for radioimmunoassay of cyclic AMP (Yamasa Shoyu Co., Chiba, Japan). Measurement of ODC activity was described elsewhere (17). Protein concentration was determined by the method of Bradford (18).

Carcinogenesis experiments. We used two protocols of tumor promotion in mouse skin carcinogenesis: the conventional single stage promotion and the two-stage promotion test described by Slaga et al. (19). In both tests, DMBA at a dose of 25 µg was applied topically as an initiator one week before promotion. In the single stage promotion test, mice received intracutaneous injection of cholera toxin once a week for 10 weeks. In the two-stage promotion test, cholera toxin (10 to 100 ng, once a week for 1 or 2 weeks) was used as a Stage I promoter and mezerein (4 µg, twice a week for 18 weeks) as a Stage II promoter. In both tests, TPA was used as a positive control, Mice were observed at least for 10 weeks after the termination of promotion (total, 30 weeks).

Results

Comparison of epidermal hyperplasia induced by cholera toxin and TPA. We found that cholera toxin caused much greater hyperplasia than TPA and was

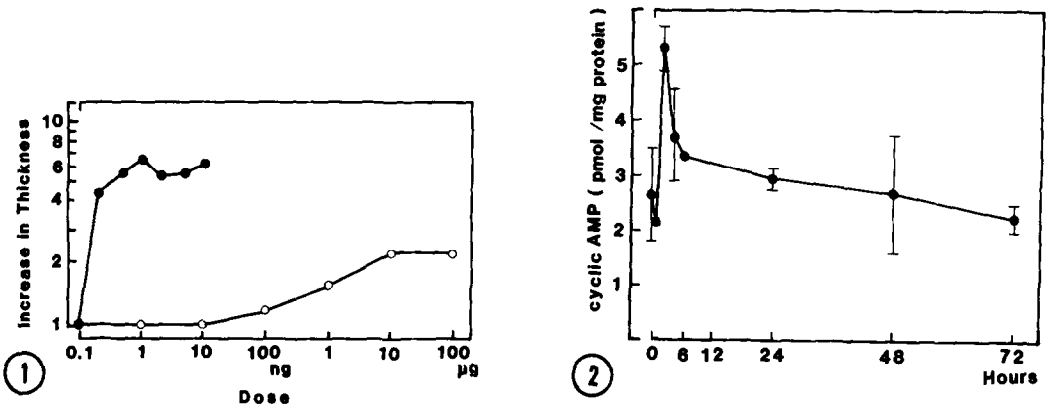

Fig. 1. Comparison of epidermal hyperplasia induced by cholera toxin (●) and TPA (O). The thickness of the epidermis was measured on the day when hyperplasia was maximal (on day 4 for cholera toxin, and day 2 for TPA).

Fig. 2. Induction of cyclic AMP by intracutaneous injection of cholera toxin at 4 adjacent sites at a dose of 10 ng per site (40 ng in total). Points and bars are means ± SD for 3 samples.

effective at a lower dose (Fig.1): cholera toxin at a dose of 0.2 ng or more produced epidermal hyperplasia resulting in 4- to 6-fold increased in thickness of the epidermis, whereas TPA at 10 µg caused about 2-fold increase in thickness. Epidermal hyperplasia was also induced after repeated injections of the toxin.

Biochemical events following application of cholera toxin. Intracutaneous injection of cholera toxin resulted in increase in the cyclic AMP level after a lag period of 2 h, which returned to the normal level after 6 h (Fig.2). This time course of the induction of cyclic AMP is similar to that observed in the intestine after application of the toxin (20).

We found that cholera toxin induced the epidermal ODC activity, a well known parameter of tumor promoters in skin carcinogenesis (Fig.3). The activity was maximal 12 h after injection of 200 ng of cholera toxin. But this dose was 100 times that required to induce hyperplasia, and the maximal ODC activity was about one-tenth of that induced by TPA. Thus, cholera toxin is much less effective than TPA in inducing ODC. In keeping with the present observations, Murray et al. (21) reported induction of cyclic AMP and ODC by subcutaneous injection of cholera toxin but with as high a dose as 10 µg.

Absence of tumor promoting activity of cholera toxin. The tumor promoting

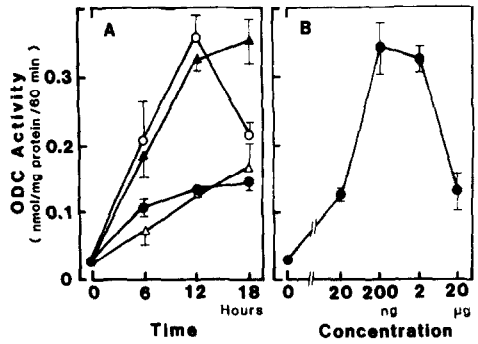

Fig. 3. Time-dependence (A) and dose-dependence (B) of induction of ODC by intracutaneous injection of cholera toxin. A, Cholera toxin was applied at total doses of 20 ng (●), 200 ng (○), 2 µg (▲) and 20 µg (△) at 4 adjacent sites. B, Dose-response 12 h after injections. Points and bars are averages ± SD for 2 samples.

activity of cholera toxin was investigated using two protocols of tumor promotion in mouse skin carcinogenesis. In the conventional single stage promotion (Table 1A), cholera toxin was injected into skin clamped off with tongue forceps once a week for 10 weeks after initiation with 25 µg DMBA

Table 1. Promoting effects of cholera toxin in DMBA initiated skin

Group	Promotion		Tumor formation at the end of promotion	
			% of mice with tumors	No. of tumors per mouse
A. Single-stage promotion				
1	PBS[a]		0 (0/15)	0
2	CT[a]		0 (0/15)	0
3	TPA[b]		100(10/10)	10.8
B. Two-stage promotion				
	Stage I	Stage II		
4	Acetone	Acetone	0 (0/10)	0
5	CT (100 ng x 1)[c]	Acetone	0 (0/10)	0
6	CT (10 ng x 1)[c]	MZ[d]	89 (8/9)	2.4
7	CT (10 ng x 2)[c]	MZ	75 (6/8)	4.8
8	CT (40 ng x 1)[c]	MZ	90 (9/10)	3.7
9	CT (100 ng x 1)[c]	MZ	100(12/12)	2.5
10	PBS	MZ	70 (7/10)	2.6
11	None	MZ	75(15/20)	3.8
12	TPA (2 µg x 2)	MZ	75 (6/8)	6.1

Abbreviations: CT, cholera toxin; MZ, mezerein.

[a] PBS or CT (50 ng) was applied using tongue forceps once a week for 10 weeks.

[b] TPA was applied twice a week for 20 weeks.

[c] Total dose on multiple injections into 10 adjacent sites. x 1, single application; x 2, two applications. In group 8, the toxin was applied using tongue forceps.

[d] Mezerein at 4 µg was applied topically twice a week for 18 weeks.

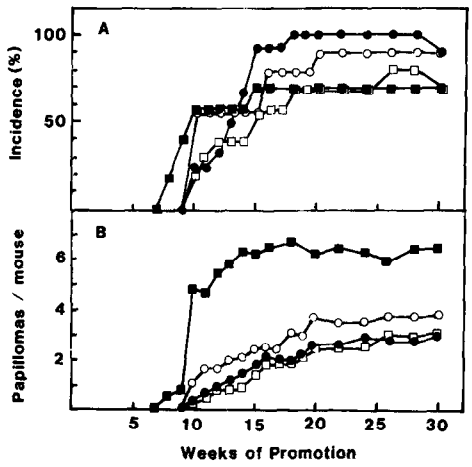

Fig. 4. Promoting effect of cholera toxin as a Stage I promoter in mouse skin carcinogenesis. A, Incidence of tumors. B, Number of tumors per mouse. O, cholera toxin at 40 ng (group 8): ●, cholera toxin at 100 ng (group 9): ■, positive control with 2 µg TPA (group 12); □, negative control with PBS (group 10).

(Group 2). Control mice were treated with PBS (Group 1). During the observation period of 40 weeks, no tumors were observed in the toxin-treated mice, whereas more than 10 papillomas per mouse developed in the positive control treated with 2 µg TPA twice a week for 20 weeks (Group 3).

In the two-stage promotion protocol (Table 1B and Fig. 4), the ability of cholera toxin to act as a Stage I promoter was examined by injecting it intracutaneously at doses of 10 to 100 ng, followed by repeated treatment with the Stage II promoter mezerein (Groups 6 to 9). Papillomas were produced in 75 to 100% of the mice at frequencies of 2.4 to 4.8 per mouse, but there was no correlation between the dose of cholera toxin and the incidence or number of papillomas. These values were almost the same as those obtained in the control groups, injected with PBS instead of the toxin (Group 10) or not treated with the Stage I promoter (Group 11). When TPA was used as a Stage I promoter (Group 12), significantly more papillomas were produced than on treatment with mezerein alone.

Discussion

We found that cholera toxin did not act as a complete promoter when given once a week for 10 weeks after initiation with DMBA or as a Stage I promoter when given once or twice before repeated applications of mezerein.

This finding was rather unexpected, because cholera toxin is more effective than TPA in inducing epidermal hyperplasia and it also induces ODC, though much less effectively than TPA. Furthermore, we found that cholera toxin induces dark keratinocytes at an incidence of 4% (22).

All promoting agents and manipulations evoke epidermal hyperplasia in skin, but not all agents that induce hyperplasia are promoters. Compounds of the latter type include turpentine (9), ethylphenylpropiolate (9,10), acetic acid (11), ionophore A23187 (8) and 12-O-retinoylphorbol-13-acetate (7). Skin massage elicites a marked proliferative response of the epidermis but not ODC induction or a promoting stimulus (6). We found that cholera toxin is also of this type. This absence of an exact correlation between activities for induction of hyperplasia and tumor promotion suggests the existence of a promotion-specific pathway leading to epidermal hyperplasia.

In most skin tumor promoters, epidermal hyperplasia is associated with induction of ODC. Under certain circumstances, however, these two parameters have been shown to be separable. We found that 1α,25-dihydroxyvitamin D_3, an active form of vitamin D_3, inhibits ODC induction by TPA but not epidermal hyperplasia (23), and that it is a potent inhibitor of tumor promotion (24). Retinoic acid also showed similar effects (23). These observations suggest that ODC induction, rather than epidermal hyperplasia itself, is causally associated with tumor promotion. Further studies are needed on the mechanisms of promotion-specific and -nonspecific cell proliferation.

References

1. Kuroki, T. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6958-6962.
2. Kuroki, T. (1984) Bacterial Diarrheal Diseases (Takeda, Y. and Miwatani, T. eds.) pp. 161-168, KTK Scientific Publishers, Tokyo.
3. Kuroki, T., Ito, T., Hosomi, J., Munakata, K., Uchida, T., and Nagai, Y. (1982) Cell Structure and Function 7, 295-305.
4. Frei, J. V., and Slaga, T. J. (1977) J. Natl. Cancer Inst. 59, 299-300.
5. Slaga, T. J., Scribner, J. D., Thompson, S., and Viaje, A. (1976) J. Natl. Cancer Inst. 57, 1145-1149.
6. Clark-Lewis, I., and Murray, A. W. (1978) Cancer Res. 38, 494-497.
7. Fürstenberger, G., Berry, D. L., Sorg, B., and Marks, F. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 7722-7726.
8. Marks, F., Fürstenberger, G., and Kownatzki, E. (1981) Cancer Res. 41, 696-702.
9. Raick, A. N. (1974) Cancer Res. 34, 920-926.
10. Raick, A. N., and Burdzy, K. (1973) Cancer Res. 33, 2221-2230.
11. Slaga, T. J., Bowden, G. T., and Boutwell, R. K. (1975) J. Natl. Cancer Inst. 55, 983-987.

12. O'Brien, T. G., Simsiman, R. C., and Boutwell, R. K. (1975) Cancer Res. 35, 1662-1670.
13. O'Brien, T. G., Simsiman, R. C., and Boutwell, R. K. (1975) Cancer Res. 35, 2426-2433.
14. Klein-Szanto, A. J. P., Major, S. K., and Slaga, T. J. (1980) Carcinogenesis 1, 399-406.
15. DiGiovanni, J., Slaga, T. J., and Boutwell, R. K. (1980) Carcinogenesis 1, 381-389.
16. Ishikawa, T., Kodama, K., Ide, F., and Takayama, S. (1982) Cancer Res. 42, 5216-5221.
17. Chida, K. and Kuroki, T. (1984) Cancer Res. 44, 875-879.
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
19. Slaga, T. J., Fischer, S. M., Nelson, K., and Gleason, G. L. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3659-3663.
20. Schafer, D. E., Lust, W. D., Sircar, B., and Goldberg, N. D. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 851-856.
21. Murray, A. W., Solanki, V., Froscio, M., and Rogers, A. (1980) J. Invest. Dermatol. 75, 508-511.
22. Murakami, Y., Hibino, T., Arai, M., and Kuroki, T. (1985) J. Invest. Dermatology 85, 115-117.
23. Chida, K., Hashiba, H., Suda, T., and Kuroki, T. (1984) Cancer Res., 44, 1387-1391.
24. Chida, K., Hashiba, H., Fukushima, M., Suda, T., and Kuroki, T. (1985) Cancer Res. 45, 5426-5430.