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Tumor-promoting Activity of Staurosporine, a Protein Kinase Inhibitor on Mouse Skin

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ABSTRACT

Staurosporine, which is a potent inhibitor of protein kinases, such as protein kinase C, inhibited both induction of adhesion of human promyelocytic leukemia cells (50% effective dose = 9.0 nM) and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced morphological changes of primary mouse epidermal cells similar to phorbol 12,13-dibutyrate (9). Thus, the study with staurosporine suggested the presence of dual effects on tumor promotion.

Here, we report that staurosporine induced irritation on mouse skin and HDCC activity in mouse skin and had a weak tumor-promoting activity on mouse skin initiated with DMBA, although staurosporine showed inhibition of biological activity of teleocidin in HL-60 cells and Raji cells. Staurosporine is a unique tumor promoter possessing dual effects: tumor-promoting activity and antipromoting activity on mouse skin.

INTRODUCTION

TPA-type tumor promoters, such as TPA, teleocidin, and aplysiaxin, activate calcium-activated, phospholipid-dependent protein kinase (protein kinase C) and, thus, induce biologic activities and tumor promotion (1, 2). If inhibitors of protein kinase C block this phosphorylation, they should also inhibit the tumor promotion induced by TPA-type tumor promoters. In fact, naloxonoylcarnitine, a protein kinase C inhibitor, has this effect (3, 4).

A potent protein kinase inhibitor, staurosporine (Fig. 1), was isolated from Streptomyces spp. by Tamaoki et al. (5). Staurosporine inhibited protein kinase C activity within a nanomolar range of concentration, as well as the activity of cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, and the protein C kinase (5, 6). Although staurosporine did not selectively inhibit protein kinase C, the effects of staurosporine on various biological activities induced by phorbol esters had been reported. Staurosporine enhanced differentiation of HL-60 cells (7) and inhibited induction of EB virus EA in Raji cells (8) and NADPH-oxidase activation in human neutrophils (9).

Two research groups independently found that staurosporine inhibited tumor promotion of TPA in two-stage carcinogenesis experiments on mouse skin (10, 11). Staurosporine induced morphological changes of primary mouse epidermal cells similar to phorbol 12,13-dibutyrate (9). Thus, the study with staurosporine suggested the presence of dual effects on tumor promotion.

Here, we report that staurosporine induced irritation on mouse skin and HDCC activity in mouse skin and had a weak tumor-promoting activity on mouse skin initiated with DMBA, although staurosporine showed inhibition of biological activity of teleocidin in HL-60 cells and Raji cells. Staurosporine is a unique tumor promoter possessing dual effects: tumor-promoting activity and antipromoting activity on mouse skin.

MATERIALS AND METHODS

Materials

Staurosporine was kindly provided by Dr. H. Nakano, Tokyo Research Laboratories, Kyowa Hakko Kogyo, Tokyo, Japan. Teleocidin was isolated from Streptomyces medioculida (12). DMBA was purchased from Sigma Chemical Co., St. Louis, MO, and TPA was from LC Services Corporation, Womara, MA. Sodium 3-butyrate was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. [125I]FPATP was from Amersham, United Kingdom, and [20]TPA, [1-]tyrosine monomethylether, and 4-chloro-2-methylimidazole, were from New England Nuclear, Boston, MA. Anti-remainder against EB virus EA (1:160) was a generous gift from Dr. N. Yamamoto of Yamaguchi University, Japan. Anti-human IgG conjugated with fluorescein isothiocyanate was obtained from Dakopatts, Glostrup, Denmark.

Animals

Female CD-1 mice were purchased from the Japanese Charles River Co., Ltd., Kanagawa, Japan, and kept as reported previously (13).

Assay of Inhibition of Activation of Protein Kinase C in Fibroblasts

The effect of staurosporine on protein kinase C activated by 2,3 alpha-TPA was examined as described previously (14). Protein kinase C was purified from mouse brain by DEAE-cellulose column chromatography (15).

Assay of Inhibition of Induction of HL-60 Cell Adhesion

Adhesion of HL-60 cells cultured in RPMI-1640 medium with 10% fetal calf serum was induced by incubation with 4.0 nM teleocidin for 48 h (16). The inhibitory effect of various concentrations of staurosporine on cell adhesion was examined in the presence of teleocidin.

Assay of Inhibition of Induction of EB Virus EA

Raji cells (3 x 10⁶ per ml) were incubated in 1 ml of RPMI-1640 medium containing 10% fetal calf serum with 2.3 alpha-TPA and 4.0 nM staurosporine for 2.5 h (17). The inhibited induction of EB virus EA was examined as described previously (18). Protein kinase C was purified from mouse brain by DEAE-cellulose column chromatography (15).
Inhibitory Effects of Staurosporine on Biological Activities Induced by Teleocidin. Based on our evidence that staurosporine inhibited the activation of protein kinase C by 2.3 \( \mu \)M teleocidin, and that the effective dose for 50% inhibition was 2.6 \( \mu \)M, comparable to the results of Tannas et al. (4), we then studied whether it inhibited the other biological activities induced by teleocidin. Treatment with 4.5 \( \mu \)M teleocidin for 48 h induced adhesion of HL-60 cells, and this induction was inhibited by staurosporine dose dependently at concentrations of 6 to 30 \( \mu \)M (Fig. 2). The \( E_D_{50} \) value was 9.0 \( \mu \)M. In this experiment, the treatment with teleocidin and staurosporine did not show any toxicity to HL-60 cells at concentrations of staurosporine up to 30 \( \mu \)M, determined by trypan blue staining. Incubation of Raji cells with 2.3 \( \mu \)M teleocidin for 48 h induced expression of EB virus EA in 55% of the cells, whereas in the absence of sodium n-butyrate. These two experiments showed that staurosporine clearly enhanced the effects of teleocidin on ODC activity (Table 1).

Biological Activities of Staurosporine. Staurosporine induced irritation of mouse ear, the dose causing redness of the ear 24 h after its application being 200 ng (0.43 \( \mu \)mol). Since that of teleocidin was 0.008 \( \mu \)mol (12), the potency of staurosporine for irritation on mouse ear was about 10 times weaker than that of teleocidin. In addition, applications of 10 \( \mu \)mol of staurosporine clearly enhanced the effects of teleocidin on HDC activity at levels of 196 and 173 \( \mu \)mol of CO\(_2\)/mg of protein per 60 min, respectively, which was about 10 times weaker than that of teleocidin (Table 2).

Staurosporine, at concentrations of up to 500 \( \mu \)mol, did not induce ODC activity in mouse epidermis 4 h after application, whereas teleocidin induced it at usual (Table 1). Furthermore, staurosporine did not induce HL-60 cell adhesion at a concentration of 50 \( \mu \)M. In Raji cells, staurosporine itself, at concentrations of up to 2.1 \( \mu \)M, did not induce EB virus EA in the presence of 4 mM sodium n-butyrate. Table 3 summarizes the effects of staurosporine.

Tumor-Promoting Activity of Staurosporine. Since staurosporine in 0.1 ml of acetone was applied to the skin of the backs of CD-1 mice, and a crude enzyme extract was prepared from the skin 18 h later as described previously (19). HDC activity was expressed as pmol of CO\(_2\) released per mg of protein per 1 h of incubation.

Results of Carcinogenesis Experiments

Experiment 1. Initiation was achieved by a single application of 100 \( \mu \)g of DMBA dissolved in 100 \( \mu \)l of acetone to the skin of the backs of 8-wk-old female CD-1 mice (13). From 1 wk after initiation, 50 \( \mu \)l (107 nmol) of staurosporine dissolved in 100 \( \mu \)l of acetone were applied to the initiated skin parts of the mice, twice a wk, until Wk 30. Control groups were treated with DMBA alone or staurosporine alone. The percentages of tumor-bearing mice and the average numbers of tumors per mouse were determined weekly as described previously (20).

Experiment 2. The experiment was carried out by the same procedure. From 1 wk after initiation with 100 \( \mu \)g of DMBA, 10 \( \mu \)g (21 nmol) of staurosporine were applied, twice a wk. In addition, this experiment included a group treated with DMBA and staurosporine plus 2.5 \( \mu \)g (5.7 nmol) of teleocidin, which was applied 15 min after each application of staurosporine. Control groups treated with DMBA and teleocidin or DMBA alone were also observed. In both Experiments 1 and 2, each group consisted of 15 mice, because of the limited availability of staurosporine.

RESULTS

Inhibitory Effects of Staurosporine on Biological Activities Induced by Teleocidin. Based on our evidence that staurosporine inhibited the activation of protein kinase C by 2.3 \( \mu \)M teleocidin, and that the effective dose for 50% inhibition was 2.6 \( \mu \)M, comparable to the results of Tannas et al. (4), we then studied whether it inhibited the other biological activities induced by teleocidin. Treatment with 4.5 \( \mu \)M teleocidin for 48 h induced adhesion of HL-60 cells, and this induction was inhibited by staurosporine dose dependently at concentrations of 6 to 30 \( \mu \)M (Fig. 2). The \( E_D_{50} \) value was 9.0 \( \mu \)M. In this experiment, the treatment with teleocidin and staurosporine did not show any toxicity to HL-60 cells at concentrations of staurosporine up to 30 \( \mu \)M, determined by trypan blue staining. Incubation of Raji cells with 2.3 \( \mu \)M teleocidin for 48 h induced expression of EB virus EA in 55% of the cells, whereas in the absence of sodium n-butyrate. These two experiments showed that staurosporine clearly enhanced the effects of teleocidin on ODC activity (Table 1).

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porine was statistically significant in both tumor-bearing mice (x test and papilloids). Fig. 5 shows the results of Experiment 2. The repeated applications of 10 μg of staurosporine to the DMBA-initiated mouse skin induced tumors in 33% of the mice with an average of 0.5 papilloma per mouse in Wk 30. The amount of staurosporine (10 μg) was not sufficient to induce a significant tumor-promoting activity, but that with 50 μg of staurosporine was statistically significant, as shown in Fig. 4.

Antipromoting Activity. Since staurosporine is a tumor promoter, it has antipromoting activity for activation of protein kinase C and induction of HLO-60 cell adhesion and of ER virus activity. Therefore, we examined whether staurosporine inhibits the tumor promotion on mouse skin. As Fig. 5 shows, the group treated with DMBA and telodcin plus staurosporine resulted in 67% tumor-bearing mice by Wk 15 and in 80% by Wk 30, whereas the group treated with DMBA and telodcin induced tumors in 100% of the mice by Wk 15 and remained constant up to Wk 30. The staurosporine treatment reduced the average numbers of tumors per mouse from 6.1 to 3.9 in Wk 30. In the group treated with DMBA and telodcin plus staurosporine, mice did not show a significant body weight loss throughout the experiment, and the skin of the backs of these mice were not irritated, compared with those in the group treated with DMBA and telodcin. The inhibitory effect of staurosporine was statistically significant at around Wk 10 of tumor promotion. Although statistically significant inhibition was not obtained with 10 μg of staurosporine in later weeks of the experiment, a decreasing tendency in the percentage of tumor-bearing mice and in average numbers of tumors per mouse was apparent (Fig. 5). Thus, staurosporine slightly inhibited tumor promotion of telodcin, even at the dose at which staurosporine itself induced tumors.

**DISCUSSION**

Staurosporine is a potent inhibitor of protein kinases, such as protein kinase C, cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase (26). Since staurosporine inhibited biological activities of telodcin in cell lines such as HLO-60 cells and Raji cells, it was expected that staurosporine inhibited the tumor-promoting activity of telodcin in a two-stage carcinogenesis experiment on mouse skin. During the preparation of this paper, two groups reported independently that staurosporine inhibited the tumor-promoting activity of telodcin in a two-stage carcinogenesis experiment on mouse skin. Although our results with staurosporine were not strongly inhibitory, due to limited amounts of the compound, staurosporine inhibited tumor proliferation in a two-stage carcinogenesis experiment on mouse skin. It was reported that staurosporine inhibited tumor proliferation in two-stage carcinogenesis experiments in the absence of tumor promoters as well as a weak complete tumor promoter (27). Therefore, we examined the results obtained with staurosporine which other groups used were not sufficient to induce any tumor-promoting activity in two-stage carcinogenesis experiments. In this respect, we have been able to show that inhibition of protein kinase C is not directly reflected in inhibition of tumor promotion by the TPA-type tumor promoters. Sako et al. (9) reported that staurosporine did not significantly inhibit the effect of phorbol esters on epidermal growth factor binding or induction of ODC and subsequent epidermal mitogenesis in mouse primary epidermal cells and that staurosporine itself induced morphological changes in keratinocytes, resembling those induced by the phorbol esters. Since staurosporine is a microbial alkaloid produced by Strepto- nymyces spp. (5), staurosporine was structurally, from a new family of tumor promoters than those already reported, such as phorbol esters, telodcin, aplysianovin, polyoxin, thapsigargin, and okadaic acid. Staurosporine showed inhibitory activity of protein kinase C, cyclic AMP-dependent protein kinase, and cyclic GMP-dependent protein kinase (22). The inhibitory activity of K-252a on mouse ear was 40 times weaker than that of staurosporine; the dose causing 50% of the ear was 0.4 μg (17 nmol). These results suggest that K-252a might be a weaker tumor promoter than staurosporine.

The antitumor activity of fusidic acid, fusidic acid, is reported to be a very strong inhibitor of tumor promotion induced by TPA in mouse skin (23). However, Fukui et al. (24) recently reported that fusidic acid has tumor-promoting activity in mouse skin. Retinoic acid strongly induces tumor promotion of phor- bol esters (25). However, retinoic acid itself induces papillomas in mouse skin initiated with DMBA (26). Further study revealed that retinoic acid has the ability to act as a first stage tumor promoter as well as a weak complete tumor promoter (27). Bryostatin 1 also shows both effects on tumor promotion in a multistage carcinogenesis experiments on mouse skin. Bryos- tin inhibits the tumor-promoting activity of TPA in p53-negative mice (28), and further study revealed that bryostatin 1 inhibits the first stage of tumor promotion (29, 30). On the other hand, bryostatin 1 itself is a second stage tumor promoter (30). Thus, like staurosporine, fusidic acid, and retinoic acid, bryostatin 1 has dual effects on tumor promotion and inhibition of tumor promotion. Staurosporine provides important new information for understanding the mechanisms of tumor promotion and inhibition of tumor promotion.

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