Absence of Synergistic Effects on Tumor Promotion in CD-1 Mouse Skin by Simultaneous Applications of Two Different Types of Tumor Promoters, Okadaic Acid and Teleocidin

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ABSTRACT

Okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A, and teleocidin, an activator of protein kinase C, are both potent tumor promoters on mouse skin. The effects of simultaneous treatment of the two different types of tumor promoters on tumor promotion as well as on their biochemical activities were studied. Three independent experiments with different doses of tumor promoters revealed that simultaneous repeated applications of okadaic acid and teleocidin did not induce any synergistic or additive effects on tumor promotion in mouse skin initiated with TPA (12-0-tetradecanoylphorbol-13-acetate; DMBA). In Experiment 1, the group treated with a single application of DMBA, followed by repeated applications of 1.0 μg (1.2 nmol) okadaic acid and 2.5 μg (5.7 nmol) teleocidin, resulted in 64.3% tumor-bearing mice at week 20. But the groups treated with DMBA plus okadaic acid or DMBA plus teleocidin gave 73.3% and 71.4%, respectively. The biochemical activities were studied by means of induction of ornithine decarboxylase in mouse skin and protein phosphorylation in the cells. Simultaneous application of okadaic acid at three different doses with teleocidin did not induce ornithine decarboxylase activity synergistically or additively. Phosphorylation of proteins, cytokeratins, or heat shock protein 27 was not synergistically increased in human keratinocytes treated with okadaic acid and teleocidin, although the cotreatment in a cell-free system synergistically increased protein phosphorylation. Thus, the absence of synergistic effects on tumor promotion in mouse skin was also confirmed in two systems, induction of ornithine decarboxylase in mouse skin and protein phosphorylation in human keratinocytes. The effect of cotreatment of okadaic acid and teleocidin is discussed at the molecular level.

INTRODUCTION

Okadaic acid and teleocidin, a TPA type, are both potent tumor promoters on mouse skin initiated with DMBA (1, 2). The DNAs isolated from tumors contain the same mutation in the c-Ha-ras gene, irrespective of the mechanisms of action of the tumor promoters (3). Thus, both tumor promoters induce the clonal expansion of the initiated cells and development of papillomas, through either the okadaic acid pathway or the PKC pathway (4). Okadaic acid is an inhibitor of PP-1 and PP-2A (5, 6), whereas teleocidin is an activator of PKC (2). Both types of tumor promoters induce the phosphorylation of proteins that act on a signal transduction pathway and subsequently induce gene expression of the AP-1 complex, following different time courses in the cells (7-9). If c-fos and c-jun gene expressions are partly induced through a common pathway for both tumor promoters, it would be interesting to examine whether simultaneous applications of okadaic acid with teleocidin induce synergistic or additive effects on tumor promotion. The study of simultaneous treatment with two different types of tumor promoters will increase our understanding of the mechanisms of tumor promotion.

We demonstrate that simultaneous repeated applications of okadaic acid with teleocidin did not induce any synergistic or additive effects on tumor promotion in mouse skin initiated with DMBA in three independent experiments with different doses of tumor promoters. Treatment of 35-methylkadaic acid with teleocidin also did not increase protein phosphorylation in human keratinocytes immortalized by human papilloma virus type 16, PHK 16-I cells, synergistically or additively. Rather, it seemed that hyperphosphorylation of cytokeratins induced by 35-methylkadaic acid was inhibited and retarded by teleocidin. These results suggest that the okadaic acid pathway and the PKC pathway interact with each other in some processes of signal transduction, which might be essential processes for tumor promotion.

MATERIALS AND METHODS

Materials

Okadaic acid and 35-methylkadaic acid were isolated from a black sponge, Halichondria okadai, as previously described (10). Teleocidin was isolated from Streptomyces medicius (2). DMBA was purchased from Sigma Chemical Co. (St. Louis, MO). 3H-P, (370 MBq/ml) was obtained from Amerham (Buckinghamshire, England) and u-1,14Cornithine monohydrochloride (1.86 GBq/mmol) was from New England Nuclear (Boston, MA).

Animals

Female CD-1 mice were purchased from the Japanese Charles River Co., Ltd. (Kanagawa, Japan).

Two-Stage Carcinogenesis Experiments on Mouse Skin

Three experiments, each with different doses of okadaic acid and teleocidin, were carried out.

Experiment 1. Initiation was achieved by a single application of 0.1 μg DMBA dissolved in 0.1 ml of acetone to the skin of the backs of 8-week-old mice. From 1 week after initiation, 1.0 μg (1.2 nmol) okadaic acid and 2.5 μg (5.7 nmol) teleocidin dissolved in 0.1 ml of acetone were applied to the initiated area of the mice, twice a week, until week 20. Two control groups treated with DMBA plus okadaic acid (1.0 μg) or DMBA plus teleocidin (2.5 μg) were also observed.

Experiment 2. From 1 week after initiation, 0.1 μg (0.12 nmol) okadaic acid and 0.25 μg (0.57 nmol) teleocidin dissolved in 0.1 ml of acetone were applied twice a week.

Experiment 3. From 1 week after initiation, 1.0 μg (1.2 nmol) okadaic acid and 0.25 μg (0.57 nmol) teleocidin were simultaneously applied twice a week until week 20.

Experiments 2 and 3 were carried out according to the same procedures as Experiment 1. In all three of the experiments, each group consisted of 15 mice. The numbers of tumors 1 mm or more in diameter were counted weekly. The percentages of tumor-bearing mice and the average numbers of tumors per mouse were determined weekly, as described previously (1).
Induction of ODC in Mouse Skin

Okadaic acid and teleocidin, okadaic acid alone, or teleocidin alone dissolved in 0.2 ml of acetone were applied to the skin of the backs of 8-week-old mice. After 4 h, a crude enzyme extract was obtained from the epidermis, and ODC activity was measured as described previously (11). Enzyme activity was expressed as nmol CO₂/mg protein/30-min incubation. The experiments were repeated two times independently.

Protein Phosphorylation in Human Keratinocytes, PHK 16-1 Cells

PHK 16-1 cells (5 × 10⁵ cells) were placed in a culture dish (6 cm in diameter) containing 4 ml of MCDB 152 medium. One day later the medium was replaced by phosphate-deficient MCDB 152 medium and incubated for 14 h. 32P, was added to a final concentration of 1.85 MBq/ml and incubated for 4 h. 35-Methylkadaic acid and teleocidin at a concentration of 100 nm each were added to the medium, as indicated in the text, and cells were incubated for various times. As controls, 35-methylkadaic acid alone or teleocidin alone at a concentration of 100 nm was tested. The cell lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (12). Phosphorylated proteins were analyzed by autoradiography.

RESULTS

Tumor Promotion by Simultaneous Applications of Okadaic Acid with Teleocidin. The amounts of tumor promoters per application were chosen from a linear range of tumor-promoting activity, that is, 0.1 µg (0.12 nmol) or 1.0 µg (1.2 nmol) for okadaic acid and 0.25 µg (0.57 nmol) or 2.5 µg (5.7 nmol) for teleocidin (2, 5). In Experiment 1, two tumor promoters at high doses (i.e., 1 µg okadaic acid and 2.5 µg teleocidin) were simultaneously applied. The group treated with DMBA plus okadaic acid and teleocidin produced the first tumor at week 8. The percentages of tumor-bearing mice and the average numbers of tumors per mouse increased similarly in the groups treated with DMBA plus okadaic acid and DMBA plus teleocidin (Fig. 1). At week 20, the percentages of tumor-bearing mice in the groups treated with DMBA plus okadaic acid and teleocidin, DMBA plus okadaic acid, and DMBA plus teleocidin were 64.3%, 73.3%, and 71.4%, respectively (Table 1). The average numbers of tumors per mouse in these three groups were 3.4, 4.2, and 1.9, respectively (Table 1). Simultaneous applications of 1 µg okadaic acid with 2.5 µg teleocidin did not show any synergistic or additive effects on the tumor-bearing mice or the average numbers of tumors per mouse throughout the experiment. In the group treated with DMBA plus okadaic acid and teleocidin, mice did not show any significant body weight loss throughout the experiment, and the skin of the backs of these mice was not irritated.

Since repeated applications of 1.0 µg okadaic acid alone or 2.5 µg teleocidin alone induced almost the maximum effects of our criteria for tumor promotion, we next studied the effects of cotreatment with lower doses of the two tumor promoters (i.e., 0.1 µg okadaic acid and 0.25 µg teleocidin) in Experiment 2. The simultaneous applications of okadaic acid with teleocidin to DMBA-initiated mouse skin produced the first tumor at week 12 and resulted in 26.7% tumor-bearing mice at week 20 (Fig. 1; Table 1). The repeated applications of 0.1 µg okadaic acid or 0.25 µg teleocidin produced the first tumor at week 12 and week 10, and resulted in 20% and 26.7% tumor-bearing mice at week 20. The average numbers of tumors were 0.3 in all three groups at week 20. Thus, the onset of tumor formation, the percentage of tumor-bearing mice, and the average numbers of tumors per mouse were not enhanced by simultaneous applications of okadaic acid with teleocidin. No synergistic effects were observed in Experiment 2, even at low doses of tumor promoters.

In Experiment 3, we studied tumor-promoting activity with a high dose of okadaic acid (1.0 µg) plus teleocidin at a low dose (0.25 µg) on mouse skin initiated with DMBA. The group treated with DMBA plus okadaic acid and teleocidin resulted in 78.6% tumor-bearing mice in week 20, whereas the groups treated with DMBA plus okadaic acid or DMBA plus teleocidin induced tumors in 80.0% and 13.3% in week 20 (Fig. 1; Table 1). Simultaneous applications of okadaic acid with teleocidin reduced the average numbers of tumors per mouse from 5.1 to 3.6 at week 20. Interestingly, the average numbers of tumors per mouse in the group treated with DMBA plus okadaic acid and teleocidin were close to the mean of those of the other two groups combined.

The results of these three experiments indicated that simultaneous applications of okadaic acid with teleocidin did not induce any synergistic or additive effects on tumor promotion in mouse skin.

<table>
<thead>
<tr>
<th>Tumor promoter</th>
<th>Dose (µg/application)</th>
<th>Week the first tumor developed</th>
<th>Tumor-bearing mice (%)</th>
<th>No. of tumors/mouse</th>
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<tr>
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<td></td>
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<tr>
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<td>4.2</td>
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<td>Teleocidin</td>
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<td>71.4</td>
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<td>Teleocidin</td>
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<td>Experiment 3</td>
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<tr>
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<td>78.6</td>
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<td>Teleocidin</td>
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* Either a single tumor promoter or both were dissolved in 0.1 ml acetone and applied to the anterior half of the skin of the back of mice.
Induction of ODC by Simultaneous Application of Okadaic Acid with Teleocidin in Mouse Skin. Okadaic acid induces maximum ODC activity in mouse skin 4 h after its application, as does teleocidin (11). However, ODC activity induced by okadaic acid is weaker than that induced by teleocidin (1, 11). Four h after simultaneous application of 30.0 µg (36.0 nmol) okadaic acid with 5.0 µg (11.2 nmol) teleocidin induced only 1.6 ± 0.4 (SD) nmol CO2/30 min of incubation/mg protein, whereas okadaic acid alone or teleocidin alone induced 0.4 ± 0.1 and 3.7 ± 1.5 nmol CO2/30 min of incubation/mg protein, respectively. Okadaic acid seemed to inhibit ODC induction by teleocidin and to induce ODC activities at an average potency between those induced by okadaic acid and teleocidin. These effects were confirmed by simultaneous application of two different doses, which were used in Experiments 1 and 2 of the two-stage carcinogenesis experiments, that is, 2.0 µg okadaic acid with 5.0 µg teleocidin, and 0.2 µg okadaic acid with 0.5 µg teleocidin. As described in Table 2, okadaic acid reduced ODC induction by teleocidin. Therefore, simultaneous application of okadaic acid with teleocidin did not induce ODC activity synergistically or additively. These results coincided with the absence of synergistic effects on tumor promotion in mouse skin.

Protein Phosphorylation by Simultaneous Treatment of Okadaic Acid with Teleocidin. Protein phosphorylation in human keratinocytes (PHK16-1 cells) by treatment with okadaic acid and teleocidin was studied. As we previously reported, okadaic acid and 35-methylokadaic acid induced hyperphosphorylation of various cytokeratins and HSP 27 (27) (with a molecular weight of 27,000) in PHK 16-1 cells, due to inhibition of PP-1 and PP-2A (13). After 90 min, cotreatment of the cells with 100 nm 35-methylokadaic acid plus 100 nm teleocidin had not induced strong protein phosphorylation, and only cytokeratins CK 5 and CK 6, with molecular weights of 60,000 and 58,000, and HSP 27 were slightly phosphorylated (Fig. 2A, Lane 4). Within the same time period, 35-methylokadaic acid significantly phosphorylated various cytokeratins and HSP 27, and 100 nm teleocidin also slightly induced phosphorylation of HSP 27 (Fig. 2A, Lanes 2 and 3). Therefore, hyperphosphorylation of cytokeratins and HSP27 induced by 35-methylokadaic acid seemed to be inhibited by teleocidin at 90 min after cotreatment. The effects of 35-methylokadaic acid on protein phosphorylation were delayed in PHK 16-1 cells by treatment with teleocidin. Even 5 h after treatment, 35-methylokadaic acid plus teleocidin did not induce the phosphorylation of any cytokeratins, except CK 5 and CK 6 and HSP 27 (Fig. 2B). However, four cytokeratins, CK 5, CK 6, CK 16, and CK 19, were highly phosphorylated by 24 h of incubation with the two tumor promoters together (Fig. 2C). The hyperphosphorylation of cytokeratins induced by 35-methylokadaic acid was closely associated with morphological changes, such as bleb-like formation in the cells, as described previously (5, 13). Morphological changes in PHK 16-1 cells by 35-methylokadaic acid were also delayed by treatment with teleocidin.

Teleocidin induced phosphorylation of HSP 27 much earlier than did okadaic acid, the phosphorylation not being much affected by cotreatment with okadaic acid and teleocidin. Thus, the cotreatment did not induce any synergistic effects on protein phosphorylation in PHK 16-1 cells. Although the results were not presented here, incubation of the enzyme fraction containing serine/threonine protein kinases including PKC, PP-1, and PP-2A with okadaic acid and teleocidin together synergistically increased 32P incorporation into histone III-S. Thus, there is a difference between the results of protein phosphorylation in the cells and those in the cell-free system.

DISCUSSION

Okadaic acid and teleocidin induce tumor promotion in mouse skin initiated with DMBA with the same potency, although their mechanisms of action are different (2, 5). These two different types of tumor promoters commonly increase protein phosphorylation in the cytoplasm as well as in nuclei of cells, as a signal transduction for tumor promotion (5, 13). Since their potent tumor-promoting activities are almost the same, it was expected that simultaneous applications of okadaic acid and teleocidin would induce synergistic or additive effects on tumor promotion on mouse skin. However, three different experiments at different doses revealed that cotreatment of the two different types of tumor promoters did not induce synergistic effects on tumor promotion but rather induced an average activity between
those of okadaic acid and teleocidin. The absence of synergistic effects was observed in ODC induction in mouse skin and in the phosphorylation of cytokeratins and HSP 27 in human keratinocytes. These results strongly suggest that okadaic acid and teleocidin mediate through some common essential processes for clonal expansion of the initiated cells, although okadaic acid is an inhibitor of PP-1 and PP-2A, and teleocidin is an activator of PKC.

There are several differences between the processes operating in the okadaic acid pathway and those of the PKC pathway with regard to target proteins and time courses in the cells. Okadaic acid induces phosphorylation of various proteins, such as intermediate filaments, and of HSP 27 through the inhibition of PP-1 and PP-2A, and its protein phosphorylation by basal protein kinase activity is further sustained due to the inhibition of protein phosphatase activity (5, 13). Teleocidin induces rapid and transient phosphorylation of proteins similar to TPA, such as HSP 27 (13, 14). Sustained protein phosphorylation by activation of PKC is not clearly understood. Due to differences in their target proteins and phosphorylation rates, cotreatment of okadaic acid with teleocidin did not show stimulation of protein phosphorylation in a manner as simple as expected. As shown in Fig. 2, cotreatment clearly inhibited the phosphorylation of cytokeratins induced by okadaic acid, suggesting that the PKC pathway affects other protein kinase activity through the cross-talk of phosphoproteins.

Distinct from phosphoproteins in the cytoplasm, phosphoproteins in the nuclei, which induce gene expression and regulate transcription in the cells, remain to be elucidated. Recently, the expression of several genes induced by okadaic acid in mouse skin was reported (15). A topical application of okadaic acid induced several genes similar to those induced by TPA: early response genes, such as c-fos and c-jun genes, and secondary response genes such as transin and plasminogen activator-urokinase genes (15). The induction of these genes by okadaic acid was much slower than induction by TPA, however. Teleocidin, as one of the TPA types of tumor promoters, might have the same gene expression as TPA. Thus, okadaic acid- and TPA-induced expression of several genes is common through two different pathways.

The absence of synergistic effects by okadaic acid and TPA on the induction of the kappa B enhancer binding protein, human immunodeficiency virus-long terminal repeat chloramphenicol acetyltransferase activity, and the AP-1 complex in Jurkat cells were also reported: simultaneous treatment of okadaic acid and TPA resulted in little increase in kappa B enhancer binding protein levels, it inhibited a marked increase in chloramphenicol acetyltransferase activity by okadaic acid in Jurkat cells which were transfected with a plasmid containing the human immunodeficiency virus-long terminal repeat, and it inhibited the induction of the AP-1 complex (16). Moreover, cotreatment markedly inhibited the induction of c-jun mRNA by okadaic acid. Although the mechanism by which TPA inhibits the response to okadaic acid at the transcriptional level is not clear, there is a marked difference between okadaic acid and TPA on the induction patterns of c-jun mRNA and on the c-jun promoter-chloramphenicol acetyltransferase constructs (8).

From the previously reported results, the TPA-responsive element is utilized in gene expression by TPA as well as okadaic acid (7, 9, 15). Levy et al. (17) recently identified the okadaic acid response element in the human collagenase promoter, which was not responsive to TPA (17). It is difficult to determine the priority of these enhancer elements in cotreatment. The okadaic acid response element sequence, "-TGCATTCCG-", was also found in the ODC gene (18) and the glutathione-S-transferase placent form gene of rat liver (19). It will also be important to determine what transcription factors are strongly activated, probably by phosphorylation. It has recently been reported that Jun is phosphorylated by TPA as well as by okadaic acid (20). It is necessary to study the effects of cotreatment of TPA and okadaic acid on the phosphorylation of transcription factors, such as Jun.

Recently, several papers have reported that okadaic acid counteracts the effects of TPA in various systems, such as the induction of focus-forming transformed cells by a two-stage protocol in the C3H/OIT1/2 mouse fibroblast transformation assay and the induction of morphological transformation by TPA (21). However, these results have never ruled out okadaic acid as a potent tumor promoter. It is important to note that cotreatment of okadaic acid with TPA presents complicated interaction between protein kinases and protein phosphatases, which has not yet been clarified.

Mirex was recently reported to be a new tumor promoter on mouse skin (22). Cotreatment of mirex with TPA enhanced their tumor-promoting activities, suggesting that their mechanisms were different. Since mirex did not induce ODC in mouse skin, its mechanism of action seems to be different from that of okadaic acid.

The cotreatment experiment with two different types of tumor promoters associated with potent tumor-promoting activity suggests the presence of common essential processes for tumor promotion: probably common gene expressions, such as c-fos and c-jun genes, and common transcriptional regulation through various enhancer elements. The significance of cross-talk between the okadaic acid pathway and the PKC pathway in transcriptional regulation was indicated. These points should be further clarified for the understanding of clonal expansion of the initiated cells.

REFERENCES
COTREATMENT WITH OKADAIC ACID AND TELEOCIDIN


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