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EFFECTS OF TELEOCIDIN ON THE MORPHOLOGY AND c-myc EXPRESSION OF HEPATOMA CELLS WHICH ARE NOT INHIBITED BY PROTEIN KINASE ANTAGONISTS

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SUMMARY: PLC/PRF/5 hepatoma cells cultured with a tumor promoter teleocidin showed polygonal cellular appearance with many vacuole-like structures, and reduced both c-myc mRNA level and growth rate. These teleocidin effects were partly mimicked by sodium butyrate but not by a protein kinase C stimulant 1-oleoyl-2-acetylglycerol(OAG). Protein kinase C inhibitor 1-(5-isoquinolinyl-sulfonyl)-2-methyl-piperazine(H7), calmodulin-dependent protein kinase antagonist N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide(W7) and topo-isomerase II inhibitor novobiocin failed to inhibit the effects of teleocidin. These results may suggest the presence of still unknown biochemical pathways which mediate the actions of teleocidin.

Teleocidin and 1-O-tetradecanoyl-phorbol-13-acetate(TPA) are potent tumor promoters and activate a phospholipid- and Ca^{++}-dependent serine/threonine protein kinase C(1, 2). Resulting protein phosphorylation has been implicated in numerous cellular processes, including regulation of cell growth, differentiation and gene expression(2). Pharmacological reagents such as 1-oleoyl-2-acetylglycerol(OAG), 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine(H7) and n-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide(W7) have been developed to examine the role of this biochemical pathway in cell activation(4, 5, 6). The use of these reagents disclosed that an activation of protein kinase C was not always sufficient for the stimulation of human neutrophils and leukemia cells by TPA(6, 7). In the present study, we analized the action of teleocidin on human hepatoma cells using OAG, H7, W7, novobiocin and sodium butyrate(8, 9).

MATERIALS AND METHODS

PLC/PRF/5 hepatoma and Chang liver cells were cultured in 60 mm Falcon plastic culture dishes containing 5 ml of RPMI1640 + 5% fetal bovine serum with or without reagents(8, 10). Teleocidin was a gift from Fujisawa Pharmaceutical Co., Tokyo. Sodium butyrate, OAG, H7, W7 and novobiocin were obtained from Sigma Chemical Co., St. Louis, MO. OAG is membrane permeable and displaces

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TPA bound to protein kinase C(4). W7 is an inhibitor of calmodulin-dependent protein kinase(5). Novobiocin is an inhibitor of topoisomerase II which is activated by phosphorylation and alters gene activity and cellular differentiation state(9). Sodium butyrate is a differentiating agent which induces specific gene expression probably by acetylation of nuclear proteins(11, 12). In some experiments, PLC/PRF/5 hepatoma cells were cultured in Ca^{++}-free MEM (Nissui Seiyaku, Tokyo) + 5% fetal bovine serum supplemented with different concentrations of Ca^{++}. Cell number was determined with a hemocytometer and photographs were taken with a phasecontrast microscopy. Incorporation of [^{3}H]-leucine, [^{3}H]mannose and [^{3}H]proline into cellular macromolecules was determined by TCA(10%) precipitation methods(8, 13). The accumulation of [^{14}C]-acetate into nuclei was measured by isolating the nuclei using a nuclear buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$) containing 0.5% NP40 and 0.1 mM PMSF(8, 13).

RNA was purified by guanidine-CsCl centrifugation and electrophoresed in formaldehyde-1% agarose gels, blotted onto nylon filters and hybridized with [^{32}P]-labelled c-myc(14, 15). DNase I hypersensitivity of c-myc gene was examined by the methods reported in the past(16). Cells were cultured as described above. Nuclei isolated using the nuclear buffer containing 0.5% NP40 were treated with different concentrations of DNase I(Sigma Chemical Co.). DNA was isolated from the nuclei by the phenol method, cleaved with Eco RI and subjected for Southern blot hybridization. The methylation state of CCGG and GCGC in the c-myc gene was examined by cleaving the DNAs of control and teleocidin-treated cells with the methylation sensitive enzymes Hpa II and Hha I as reported previously(15). The c-myc probe used in the present study was 1.5 kb Cla I-Eco RI fragment containing the third exon of c-myc(15).

RESULTS

PLC/PRF/5 hepatoma cells cultured with 0.2 to 50 nM of teleocidin showed polygonal appearance with many vacuole-like structures, which sometimes occupy almost whole cytoplasm. Cells cultured with 1 to 3 mM of sodium butyrate were also polygonal, but the number and size of the vacuoles were much smaller than

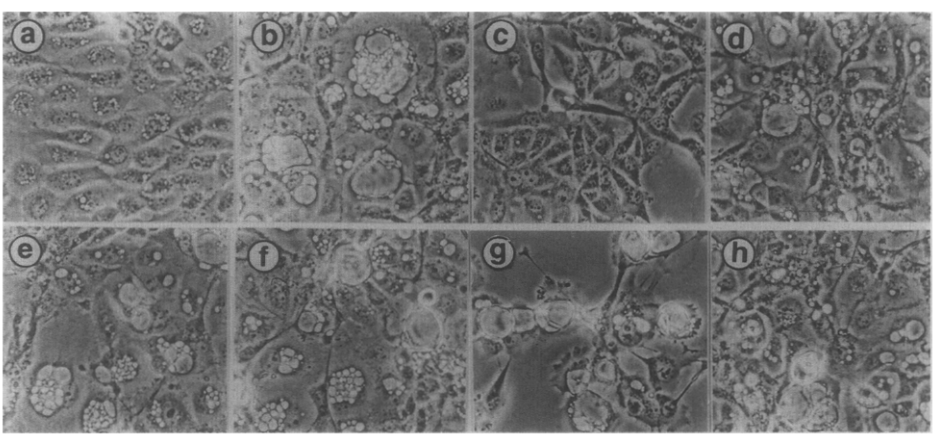

Fig. 1. Effects of teleocidin on the morphology of PLC/PRF/5 hepatoma cells. 2×10^5 cells in 5 ml of RPMI1640 + 5% fetal bovine serum were cultured for 4 days under the presence of different reagents. a, control; b, teleocidin 20 nM; c, OAG 10 μM; d, sodium butyrate 2 mM; e, teleocidin 20 nM + H7 20 μM; f, teleocidin 20 nM + W7 2 μM; g, teleocidin 20 nM + novobiocin 200 μM; h, sodium butyrate 2 mM + H7 20 μM.

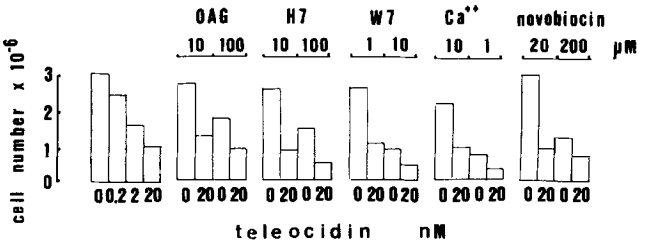

Fig. 2. Effects of teleocidin on the proliferation of PLC/PRF/5 hepatoma cells. 2×10^5 cells were cultured in RPMI1640 + 5% fetal bovine serum containing different amounts of teleocidin, OAG, H7, W7 and/or novobiocin. Cells were also cultured in Ca^{++}-free MEM + 5% fetal bovine serum supplemented with different amounts of Ca^{++}. After 5 days of culture, the cells were counted by a hemocytometer.

those of cells treated with teleocidin. OAG had no remarkable effect on the cell morphology(Fig. 1a to 1d). The morphological alterations brought by teleocidin and sodium butyrate was not antagonized by either simultaneous addition of H7(10 to 100 μM), W7(1 to 10 μM) and novobiocin(20 to 200 μM), or lowering the calcium level of the culture medium to 50 μM or less(Fig. 1e to 1h). Teleocidin inhibited cell proliferation dose dependently(Fig. 2). 2 mM sodium butyrate also reduced the cell proliferation by 35%. OAG, H7, W7, novobiocin and the reduction of calcium level of the culture medium did not antagonize to and acted additively with teleocidin in reducing cell growth(Fig. 2).

In addition to these biological effects, teleocidin(20 nM) reduced the c-myc mRNA level. This effect was recognized within 3 h of treatment and became more remarkable after 24 h(Fig. 3A). Similarly, sodium butyrate(2 mM)

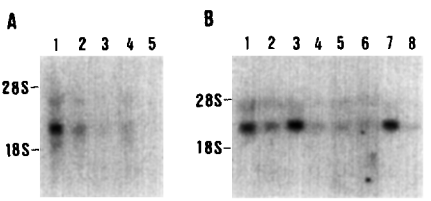

Fig. 3. Effect of teleocidin on the c-myc mRNA level of PLC/PRF/5 hepatoma cells. 2×10^5 of PLC/PRF/5 hepatoma cells were cultured in 5 ml of RPMI1640 + 5% fetal bovine serum. After 4 days of culture, the medium was replaced by a fresh one. (A) The cultures were continued for various length of time with 20 nM of teleocidin. lane 1, 0 h; lane 2, 3 h; lane 3, 6 h; lane 4, 24 h; lane 5, 48 h. (B) The cultures were continued for further 24 h with different reagents. lane 1, control; lane 2, teleocidin 20 nM; lane 3, H7 20 μM; lane 4, teleocidin 20 nM + H7 20 μM; lane 5, sodium butyrate 2 mM; lane 6, sodium butyrate 2 mM + H7 20 μM; lane 7, W7 2 μM; lane 8, teleocidin 20 nM + W7 2 μM. Then, cellular RNAs were subjected for Northern blot hybridization using [^{32}P]-c-myc probe(13).

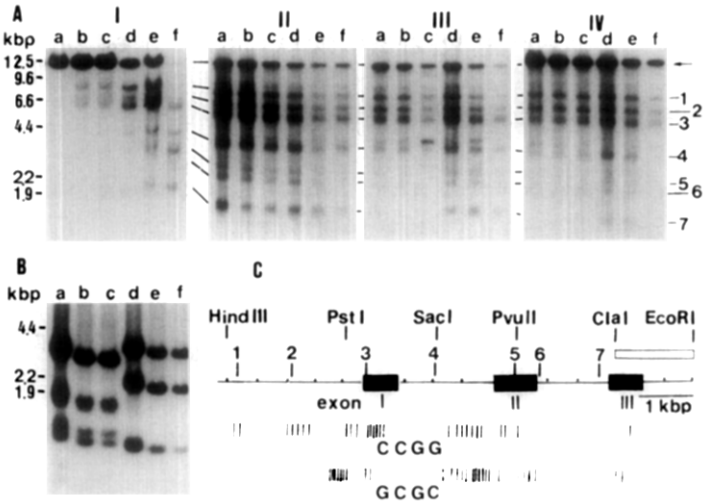

Fig. 4. Effects of teleocidin on the nuclease hypersensitivity and methylation state of c-myc gene of PLC/PRF/5 hepatoma cells. (A) Nuclease hypersensitivity of c-myc gene. The cells were cultured for 4 days with or without reagents. Chang liver, I. PLC/PRF/5 hepatoma cultured with none(II), 20 nM teleocidin (III) or 20 nM teleocidin + 20 μM H7(IV). Nuclei were treated with DNase I (a, b, c, d, e, and f represents 0, 0.05, 0.1, 0.2, 0.5 and 2 μg/ml, respectively), and 20 μg of DNAs isolated by phenol method were cleaved with 100 U of Eco RI. Solid arrow indicates the 12.5 kb Eco RI-Eco RI c-myc gene, while Arabic numerals show the nuclease hypersensitive sites within this c-myc gene. (B) Methylation state of c-myc gene. Cells were cultured with or without reagents. lane a and d, none; lane b and e, 20 nM teleocidin; lane c and f, 20 nM teleocidin + 20 μM H7. 10 to 20 μg of DNAs were cleaved with Eco RI (100 U) + Hha I(100 U)(lane a, b, and c) or Eco RI(100 u) + Hpa II(100 U) (lane d, e and f), and were subjected for Southern blot hybridization using [^{32}P]-labelled c-myc probe. (C) Restriction map of human c-myc gene. DNase I hypersensitive sites were shown by Arabic numerals as in A. c-myc probe used was the Cla I-Eco RI fragment.

reduced the c-myc mRNA level(Fig. 3B). H7(20 μM) and W7(5 μM) did not selectively reduce the c-myc mRNA level nor antagonize the inhibitory effect of teleocidin and sodium butyrate(Fig. 3B). c-myc gene of the hepatoma cells had at least 7 DNase I hypersensitive sites as Chang liver cells(Fig. 4A). Teleocidin(20 nM) and H7(20 μM) did not change the number of the hypersensitive sites(Fig. 4A). Also, teleocidin and H7 had no effect on the c-myc cleavage by Eco RI + Hha I or Eco RI + Hpa II(Fig. 4B). This indicates that teleocidin and H7 did not alter the methylation state of CCGGs and GCGCs in or near the second and third exon of c-myc gene(Fig. 4C).

Table 1 shows that both teleocidin and sodium butyrate had no effect on the uptake of [^{3}H]leucine and [^{3}H]mannose into cellular TCA-precipitable materials, but slightly increased [^{3}H]proline incorporation. The accumulation of [^{14}C]-

Table 1. Effects of teleocidin on the incorporation of radioactive precursors

	[^{3}H]leucine[a]	[^{3}H]proline[a]	[^{3}H]mannose[a]	[^{14}C]acetate[b]
none	5,331 ± 1,438	19,727 ± 2,730	1,614 ± 372	3,714 ± 79
H7		21,927 ± 3,193		3,004 ± 244
teleocidin	4,730 ± 998	28,269 ± 360	1,532 ± 41	2,325 ± 330
teleocidin + H7		28,589 ± 669		1,878 ± 370
butyrate	5,136 ± 719	28,678 ± 4,532	1,919 ± 362	9,151 ± 1,339
butyrate + H7		24,175 ± 2,364		8,256 ± 950

2×10^5 of PLC/PRF/5 cells were cultured for 4 days in the presence or absence of 20 nM of teleocidin, 2 mM of sodium butyrate and/or 20 μM of H7, and were incubated for further 3 h with 1 μCi/ml of [^{3}H]leucine, [^{3}H]mannose, [^{3}H]proline or 2 μCi/ml of [^{14}C]acetate. a) Incorporation into TCA precipitates. b) Incorporation into nuclei. The values are cpm/3h/10^6 cells (mean ± SD of triplicates).

acetate into nuclei was inhibited by teleocidin but was enhanced by sodium butyrate. H7 did not antagonize the effects of teleocidin and sodium butyrate.

DISCUSSION

The teleocidin effects on the morphology and proliferation of PLC/PRF/5 hepatoma are relatively specific and are not due to its generalized toxicity, since the teleocidin-treated cells showed active metabolism as control cells (Table 1), and since other growth inhibitors such as adriamycin, cycloheximide, retinoic acid etc. did not bring similar effects(unpublished data). Induction of vacuole-like structures by teleocidin was remarkable in PLC/PRF/5 hepatoma cells which had been maintained for more that two years in our laboratory but was not in the original cell line(10). The cells used in the present study secreted HBs antigen and show integration pattern of HBV-DNA similar to the original cell line(unpublished data). Therefore, they are considered to be variants of PLC/PRF/5 cells. On the other hand, the contents of the vacuole-like structures were not stained by Sudan III(unpublished data) and were considered not to be lipids, but detailed nature remained to be studied.

The teleocidin effects on the morphology and c-myc expression of this cell line were neither mimicked by OAG nor antagonized by H7. Thid may suggest a possibility that the teleocidin effects on this cell line are not mediated solely by protein kinase C. An involvement of either calmodulin-dependent protein kinase or topoisomerase II activated by phosphorylation appeared to be

improbable from the present experiment using W7 and novobiocin(6, 9). Similar phenomena which suggest the presence of biochemical pathways of the tumor promoters bypassing protein kinase C have been reported recently in other cell lines(6, 7, 17, 18).

The effects of teleocidin on PLC/PRF/5 cells partly resembled those of sodium butyrate. However, the biochemical pathway of the action of these two reagents were not the same since they had opposite effects on the nuclear protein acetylation as shown in Table 1(11, 12). The reduction of nuclear protein acetylation induced by teleocidin may alter the gene activity by changing fine chromatin structure(11). However, no remarkable changes were disclosed in DNase I hypersensitivity and methylation state of c-myc gene of teleocidin-treated cells. The present study suggests that the mechanism of action of teleocidin may not be so simple as supposed previously, and that more than one strategy might be utilized for control of cell differentiation and gene expression by teleocidin(19). The present experimental system may be useful to clarify whether teleocidin could activate a single pathway by a mechanism dependent or independent of protein kinase C, or alternatively, the specific binding of teleocidin to the plasma membrane activates several second messenger systems(19).

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