Tumor promoter teleocidin inhibits internalization and nuclear accumulation of epidermal growth factor in cultured human hepatoma cells

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1. INTRODUCTION

Teleocidin and its derivative dihydroteleocidin are toxic substances isolated from *Streptomyces* [1]. They induce ornithine decarboxylase activity when painted on the skin, cause adhesion of human promyelocytic leukemia cells, inhibit terminal differentiation of Friend erythroleukemia cells, and exert mitogenic effects in cultured human lymphocytes [2–4]. In addition, dihydroteleocidin shows potent tumor-promoting activity when applied on mouse skin [4]. The effects of teleocidins are essentially identical to those produced by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), a phorbol ester whose initial site of action is the cell membrane. Furthermore, teleocidins have been shown to interact directly with the receptor for phorbol esters [5], and as TPA [5–7], to inhibit the binding of epidermal growth factor (EGF) to its receptor [5]. Because TPA acts synergistically with most growth factors, it may increase non-selectively cellular sensitivity to any growth factor, or, act through a specific hormonal pathway [6–9]; one such pathway may be in certain cell types, that utilized by vasopressin [8].

In AH66 rat hepatoma cells cultured in a low serum medium, teleocidin and TPA stimulate mitogenesis, antagonize EGF-induced mitogenesis and inhibit in a transient manner the binding of EGF to its receptors [10–12]. Retinoic acid, a substance which inhibits the induction of ornithine decarboxylase by TPA and teleocidin in mouse skin [2], also acted antagonistically to teleocidin with regard to mitogenesis and EGF binding in hepatoma cells. Here the effects of teleocidin, TPA and retinoic acid on mitogenesis and EGF binding have been characterized further in human HUH hepatoma cells. Provided the lysosomal inhibitor chloroquine is present, teleocidin inhibits the internalization and the nuclear association of EGF. These results appear to be of interest for understanding the mechanism of action of both EGF and teleocidin.

2. MATERIALS AND METHODS

Human HUH hepatoma cells were kindly supplied by Dr J. Sato (Okayama Univ.) and Dr K. Takeda (Kagawa Univ.). Teleocidin was obtained from Fujisawa Pharmaceutical Industries (Osaka). TPA and retinoic acid were purchased from Sigma (St Louis MO). EGF was purified by Savage's method [13]. HUH cells (1×10^5) were seeded into a well of culture plate (Linbro, 24 wells/plate) containing 1 ml of RPMI 1640 containing 10% fetal calf serum. Incubations were in a humidified atmosphere containing 5% CO_2. When effects of EGF were examined, a low serum medium (RPMI 1640 containing 0.5% fetal calf serum) was employed.

Cell proliferation was assessed from cell counts from 1–6 days following addition of teleocidin and other drugs to the culture medium. Cells were counted with a hemocytometer after dissociation of the monolayers in phosphate-buffered saline containing 0.1% EDTA.

Protein synthesis was measured using [^{3}H]leucine as a precursor [12]. HUH cells (5×10^5 cells) were grown for 24 h in 2 ml culture medium containing 10% fetal calf serum and, when indicated, teleocidin (50 ng/ml). [^{3}H]Leucine (1 μCi/ml; New England Nuclear, 51 Ci/mmol) was added, and incubation was continued for an additional 6 h. [^{3}H]Leucine incorporation into trichloroacetic acid-precipitable

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material of cell lysates was measured as in [12]. Results were expressed as mean ± SD (3 determinations).

Cell surface binding, internalization and nuclear association of EGF were assessed using ^{125}I-labeled EGF as in ligand in the presence of chloroquine as in [14–17]. ^{125}I-EGF was prepared by the chloramine T method with spec. act. 10^6 cpm/ng [16]. HUH cells (10^5/well) were grown for 3 days in 1 ml RPMI 1640 containing 10% fetal calf serum. The medium was then replaced by fresh medium containg ^{125}I-EGF (10^5 cpm), chloroquine (100 μM) and, when indicated, teleocidin or other drugs. After 3–18 h incubation at 37°C, cells were washed 3 times with ice-cold phosphate-buffered saline. Surface-bound and internalized ^{125}I-EGF were measured as in [17]. Cell monolayers were treated with 1 ml 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl for 6 min at 4°C. This solution was removed by aspiration, and the cells remaining in the culture wells were solubilized in 2 N NaOH. The acid-extracted radioactivity was assumed to represent surface-bound ^{125}I-EGF, and the NaOH-solubilized radioactivity, internalized EGF [17]. Nuclear accumulation of ^{125}I-EGF was measured as in [14,15]. Cells washed as above were lysed in 2 ml phosphate-buffered saline containing 0.5% NP 40; the lysates were then centrifuged through a 0.88 M sucrose cushion as in [14,15]. Since treatment by teleocidin and other drugs caused a decrease in the number of cells (10–20% after 24 h), all binding data were expressed as cpm/5 × 10^5 cells. Appropriate corrections were made for the non-specific association of the radioactivity to cells by performing parallel incubations in the presence of excess (5 μg/ml) native EGF.

3. RESULTS

The effects of teleocidin, TPA and retinoic acid on the proliferation of HUH hepatoma cells as a function of time and drug concentration are shown in fig.1. Teleocidin (10 ng/ml) inhibited the proliferation of cells in response to both fetal calf serum (10%) and EGF (50 ng/ml) in low-serum medium. TPA (10 ng/ml) and retinoic acid (2 μg/ml) also inhibited cell proliferation induced by 10% fetal calf serum. The effects of teleocidin and retinoic acid on cell proliferation were additive. The effect of teleocidin in the presence or absence of retinoic acid was detectable at 0.1 ng/ml and maximal at 100 ng/ml.

To assess whether the effects of teleocidin on cell proliferation were related to its toxicity, protein synthesis was studied. The incorporation of [^{3}H]leucine by cells grown in the presence of teleocidin (12, 229 ± 447 cpm/10^6 cells) was virtually indistinguishable from that by untreated cells (11 690 ± 933 cpm/10^6 cells). Thus, teleocidin does not appear to interfere with protein synthesis.

Teleocidin and TPA, but not retinoic acid, reduced the amount of surface-bound ^{125}I-EGF. However, this was a transient phenomenon which occurred only during the first 3 h; after 6 h, little change in surface-bound EGF was observed (fig.2). In contrast, teleocidin and TPA caused a marked and sustained decrease in the amount of internalized EGF (fig.3). These inhibitory effects of teleocidin were observed irrespective of the presence of retinoic acid (fig.2,3). The effect of teleocidin on EGF internalization was dependent on the concentration of drug; it was half-maximal at 0.3 ng/ml

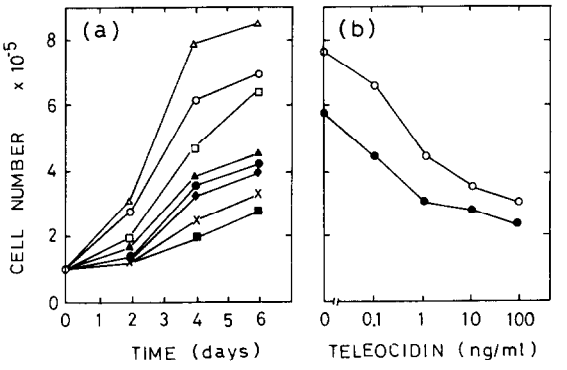

Fig.1. Inhibition of cell proliferation by teleocidin. (a) Time course: 1 × 10^5 of HUH cells were cultured in 1 ml RPMI 1640 medium containing 10% or 0.5% fetal bovine serum. Reagents were added at the start of culture: (△—△) serum 10%; (▲—▲) serum 10% + teleocidin 10 ng/ml; (○—○) serum 0.5% + EGF 50 ng/ml; (●—●) serum 0.5% + EGF 50 ng/ml + teleocidin 10 ng/ml; (□—□) serum 10% + retinoic acid 2 μg/ml; (■—■) serum 10% + retinoic acid 2 μg/ml + teleocidin 10 ng/ml; (x—x) serum 0.5%; (◆—◆) serum 10% + TPA 10 ng/ml. (b) Dose response: 1 × 10^5 of HUH cells were cultured for 5 days in RPMI 1640 medium + 10% fetal bovine serum. Teleocidin and retinoic acid were added at the start of culture: (○—○) teleocidin (0–100 ng/ml); (●—●) teleocidin (0–100 ng/ml) + retinoic acid (2 μg/ml); ordinate, cell no. × 10^{-5}/well.

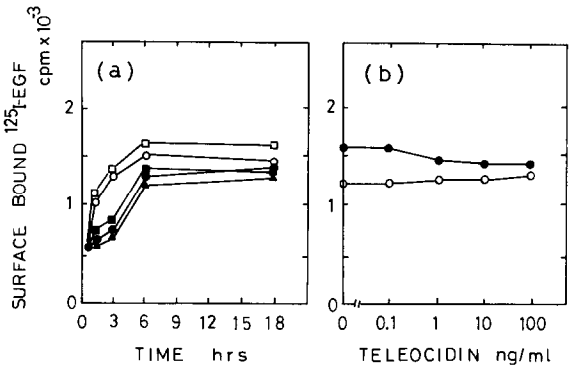

Fig.2. Effect of teleocidin on EGF binding. (a) Time course: HUH cells were incubated with ^{125}I-EGF (100 000 cpm/well) and chloroquine (100 μM). Teleocidin (10 ng/ml), TPA (10 ng/ml) and/or retinoic acid (2 μg/ml) were added and after various incubation periods at 37°C the amount of surface-bound ^{125}I-EGF was measured; (□—□) retinoic acid; (■—■) retinoic acid + teleocidin; (○—○) none; (●—●) teleocidin; (▲—▲) TPA. (b) Dose response: HUH cells were incubated with ^{125}I-EGF (100 000 cpm/well) and chloroquine (100 μM) in the presence (●—●) or absence (○—○) of retinoic acid (2 μg/ml). Different concentrations of teleocidin were added (abscissa) and incubated for 18 h at 37°C; ordinate, surface-bound ^{125}I-EGF (cpm × 10^{-3})/ 5 × 10^5 cells.

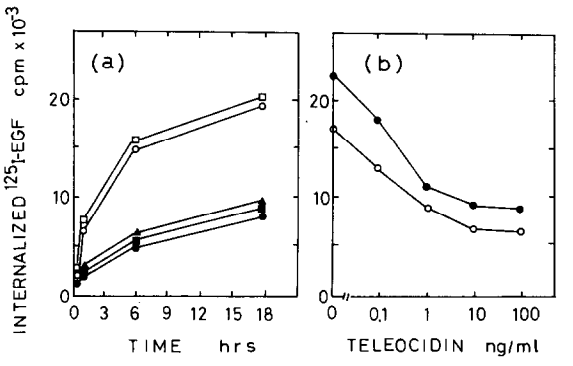

Fig.3. Effect of teleocidin on the internalization of ^{125}I-EGF. HUH cells were incubated with ^{125}I-EGF (100 000 cpm/well) and chloroquine (100 μM) under the simultaneous presence of teleocidin, TPA and/or retinoic acid. The amounts of internalized ^{125}I-EGF (ordinate, cpm/5 × 10^5 cells) were measured as in section 2. (a) Time course: (□—□) retinoic acid 2 μg/ml; (■—■) retinoic acid 2 μg/ml + teleocidin 10 ng/ml; (○—○) none; (●—●) teleocidin 10 ng/ml; (▲—▲) TPA 10 ng/ml. (b) Dose response: cells were incubated for 18 h at 37°C with different concentrations of teleocidin; (○—○) none; (●—●) retinoic acid 2 μg/ml.

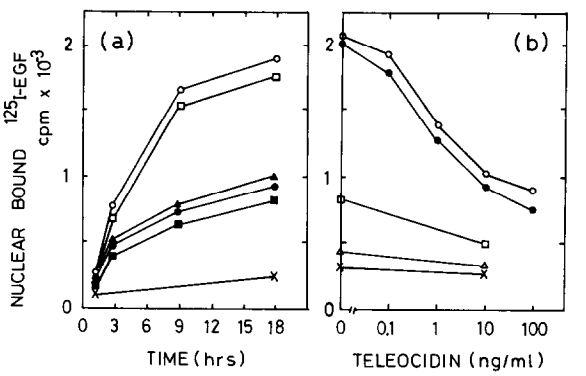

Fig.4. Effect of teleocidin on the nuclear accumulation of ^{125}I-EGF. HUH cells were incubated with ^{125}I-EGF (100 000 cpm/well) in the presence or absence of various reagents, and the amount of EGF in NP40-extracted cell remnants was measured: ordinate, cpm/5 × 10^5 cells. (a) Time course: (○—○) chloroquine 100 μM; (●—●) chloroquine 100 μM + teleocidin 10 ng/ml; (□—□) chloroquine 100 μM + retinoic acid 2 μg/ml; (■—■) chloroquine 100 μM + retinoic acid 2 μg/ml + teleocidin 10 ng/ml; (▲—▲) chloroquine 100 μM + TPA 10 ng/ml; (x—x) medium only. (b) Dose response: cells were incubated for 24 h with various concentrations of teleocidin (abscissa). Chloroquine and/or retinoic acid were added simultaneously with teleocidin: (○—○) chloroquine 100 μM; (●—●) chloroquine 100 μM + retinoic acid 2 μg/ml; (□—□) chloroquine 50 μM; (△—△) chloroquine 100 μM; (x—x) medium only.

and virtually maximal at 10 ng/ml. At all concentrations of teleocidin tested, the amount of surface-bound and internalized EGF was slightly greater in the presence of retinoic acid than in its absence (fig.2b,3b). Fig.4 demonstrates that the nuclear accumulation of ^{125}I-EGF also decreased in teleocidin- and TPA-treated cells. This inhibitory effect was maintained for a long period of incubation, showing a marked contrast with the transient effect of these drugs on ^{125}I-EGF binding. It was markedly reduced at < 100 μM chloroquine (fig.4b).

4. DISCUSSION

These data demonstrate that teleocidin has a prolonged inhibitory effect on the internalization of ^{125}I-EGF. This cannot be explained by a reduction of EGF binding since the latter is a transient phenomenon. Also, the inhibition of EGF internalization cannot be ascribed to a slower cell growth,

because retinoic acid which is known to enhance the number of EGF receptors inhibits cell proliferation but not EGF internalization [18,19]. The ability of teleocidin to inhibit EGF internalization may result from structural and/or functional alterations of the cell membrane. This inhibitory effect of teleocidin was not detected under the usual culture conditions for measurement of EGF binding [11]. To reveal the teleocidin effect, the addition of the lysosomal inhibitor chloroquine to the incubation medium was a prerequisite, as were other conditions of incubation, i.e., temperature and time.

Electron microscopy and marker enzyme studies on other types of EGF-sensitive cells have shown that NP 40 lysates of such cells consist of nuclei free from other cytoplasmic organelles, i.e., lysosomes, and that, following exposure to ^{125}I-EGF, the radioactivity in the lysate reflects the amount of ligand associated with the nucleus [11,15]. Nevertheless, it cannot be excluded that the cell lysates as prepared here were contaminated by other membrane structures containing EGF receptors. In the absence of teleocidin, 7–10% of internalized EGF was associated with the nucleus, a value closely similar to that observed in other cell-types [14,15]; teleocidin reduced the amount of EGF associated with nuclei by 60%. This effect of teleocidin may simply reflect the decrease in the internalization of EGF. Alternatively, it may result from the interference of teleocidin on the intracellular transport of EGF; indeed, lipophilic tumor-promoting agents such as teleocidin can easily permeate the cell membrane and interact with cytoplasmic or nuclear components [20].

Although the primary event involved in EGF action is binding to cell surface receptor, this event may not be sufficient for mitogenesis. A continued process of internalization or degradation of EGF and its receptor may be required to induce mitogenesis [21]. EGF has been shown to alter chromatin structure [22], and an association of EGF with the nucleus has been demonstrated [14,15]. Therefore, teleocidin may modulate cell proliferation as well as cellular functions by altering the internalization and nuclear accumulation of EGF and other growth factors. This may explain, at least in part, the synergistic or antagonistic effects displayed by tumor promoters and growth factors [8,20].

REFERENCES

[1] Takashima, M. and Sakai, H. (1960) Bull. Agr. Chem. Soc. Jap. 24, 647–651.
[2] Fujiki, H., Mori, M., Nakayasu, M., Terada, M. and Sugimura, T. (1979) Biochem. Biophys. Res. Commun. 90, 976–983.
[3] Kaneko, Y., Yatsuzuka, M., Endo, Y. and Oda, T. (1981) Biochem. Biophys. Res. Commun. 100, 888–893.
[4] Fujiki, H., Mori, M., Nakayasu, M., Terada, M., Sugimura, T. and Moore, R.E. (1981) Proc. Natl. Acad. Sci. USA 78, 3872–3876.
[5] Umezawa, K., Weinstein, I.B., Horwitz, A., Fujiki, H., Masushima, T. and Sugimura, T. (1981) Nature 290, 411–413.
[6] Brown, K.D., Dicker, P. and Rosengurt, E. (1979) Biochem. Biophys. Res. Commun. 86, 1037–1043.
[7] Shoyab, M., DeLarco, J.E. and Todaro, G.J. (1979) Nature 279, 387–391.
[8] Dicker, P. and Rosengurt, E. (1980) Nature 287, 607–612.
[9] Frantz, C.N., Stiles, C.D. and Scher, C.D. (1979) J. Cell Physiol. 100, 413–424.
[10] Imai, Y., Kaneko, Y., Matsuzaki, F., Endo, Y. and Oda, T. (1980) Biochem. Biophys. Res. Commun. 97, 926–931.
[11] Kaneko, Y. and Imai, Y. (1981) Life Sci. 29, 1571–1576.
[12] Kaneko, Y. (1981) Int. J. Cancer 27, 841–846.
[13] Savage, C.R. jr and Cohen, S. (1972) J. Biol. Chem. 247, 7609–7611.
[14] Johnson, L.K., Vlodavsky, I., Baxter, J.D. and Gospodarowicz, D. (1980) Nature 287, 340–343.
[15] Savion, N., Vlodavsky, I. and Gospodarowicz, D. (1981) J. Biol. Chem. 256, 1149–1154.
[16] Aharonov, A., Pruss, P.M. and Herschman, H.R. (1979) J. Biol. Chem. 253, 3970–3977.
[17] Heigler, H.T., Maxfield, F.R., Willingham, M.C. and Pastan, I. (1980) J. Biol. Chem. 255, 1239–1241.
[18] DeLuca, L.M. (1977) Vitam. Horm. 35, 1–57.
[19] Jetten, A.M. (1980) Nature 284, 626–629.
[20] Diamond, L., O'Brien, T.G. and Baird, W.M. (1980) Adv. Cancer Res. 32, 1–74.
[21] King, A.C. and Cuatrecasas, P. (1981) New Engl. J. Med. 305, 77–88.
[22] Johnson, L.K., Baxter, J.D., Vlodavsky, I. and Gospodarowicz, D. (1980) Proc. Natl. Acad. Sci. USA 77, 394–398.