Distinct Inhibitory Effects of Dihydroteleocidin B and the Phorbol Ester Tumor Promoters on the Adipocyte Differentiation of 3T3-L1 Cells

Yoshiko Shimizu, Nobuyoshi Shimizu, Hirota Fujiki, and Takashi Sugimura

Department of Cellular and Developmental Biology, University of Arizona, Tucson, Arizona 85721 [Y. S., N. S.], and National Cancer Center Research Institute 5-1-1 Tsukiji, Tokyo, Japan [H. F., T. S.]

ABSTRACT

An indole alkaloid tumor promoter, dihydroteleocidin B, was able to modulate a membrane property of 3T3-L1 preadipocytes, showing an almost complete reduction of epidermal growth factor binding capacity. This receptor modulating potency of dihydroteleocidin B was 10 times that of a phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Dihydroteleocidin B, however, had little effect on the epidermal growth factor receptors of the adipocyte stage of 3T3-L1. Adipocyte differentiation was induced by treating growth-arrested 3T3-L1 cells with dexamethasone and 1-methyl-3-isobutylxanthine for 48 hr. These inducers initiated DNA synthesis, led to one full cycle of cell division, and triggered the adipocyte differentiation program. Dihydroteleocidin B almost completely inhibited this differentiation at concentrations of 1 to 10 ng/ml (10^-9 to 10^-8 M). The inhibition was observed regardless of when the tumor promoter was added: before, during, or after the addition of inducers. Similar inhibition was also observed by TPA, but with over 90% less efficiency than that of dihydroteleocidin B. TPA was most effective when it was added during the inducer treatment. Both dihydroteleocidin B and TPA stimulated DNA synthesis to the same level during the initial 22 hr. The DNA synthesis stimulated by dihydroteleocidin B resulted in extraordinary enhancement of cell proliferation, whereas TPA-treated 3T3-L1 cells did not divide. These findings suggest that dihydroteleocidin B and TPA have distinct potencies in interfering with the mechanisms of adipocyte differentiation and that presumably they are different in action of tumorigenesis.

INTRODUCTION

There are a number of compounds found in the environment that are not themselves carcinogenic but are able to promote strongly the development of skin tumors in mice pretreated with a subcarcinogenic dose of a chemical carcinogen (1-4, 37, 41). These agents, tumor promoters, are phorbol ester derivatives, such as TPA(2) (16, 17), and derivatives of indole alkaloids, such as DHTB, a newly discovered tumor promoter (12, 13). Tumor promoters exhibit many different biological and biochemical effects on mouse skin and cells in culture (10, 11, 44). They appear to act primarily by modulating gene expression and cell differentiation through their binding to specific cell surface receptors (9, 40).

In addition to studies on the mechanisms of carcinogenesis and tumor promotion, we are interested in utilizing these tumor-promoting compounds as tools for studying the fundamental mechanisms underlying normal cell differentiation at the molecular level. A variant line of mouse Swiss/3T3 fibroblasts, 3T3-L1, is capable of differentiating into adipocytes (14, 15). A high frequency of induction of differentiation can be obtained by treating growth-arrested 3T3-L1 cells with DEX and MIX (29). In this report, we compared the effect(s) of DHTB with that of TPA on the adipocyte differentiation of 3T3-L1 cells. There are remarkable differences between DHTB and TPA in their potency and mode of inhibiting the terminal differentiation of 3T3-L1 cells.

MATERIALS AND METHODS

Chemicals. TPA and its derivatives were purchased from Consolidated Midland Corp. (Brewster, N. Y.). DHTB was prepared by the catalytic reduction of teleocidin B isolated from Streptomyces mediocidicus (12, 39). The compounds were dissolved in dimethyl sulfoxide and stored at -20°C until use. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted.

Cell growth. 3T3-L1 cells were purchased from the American Type Culture Collection (Rockville, Md.). They were routinely grown in DME medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml; Grand Island Biological Co., Santa Clara, Calif.). Cultures were maintained at 37°C in a 95% air-5% CO2 atmosphere.

Differentiation. 3T3-L1 cells (1.5 × 10⁴) were plated in 35-mm dishes in 2 ml of DME medium containing 10% fetal calf serum. Two days after the cells reached confluency, the medium was replaced with fresh DME medium containing 0.25 μM DEX and 0.5 mM MIX. Two days later, the inducer-containing medium was replaced with fresh DME medium. Medium was changed every 3 days thereafter. The drugs were added at various times as indicated. For some experiments, insulin was added at 1 μg/ml alone or together with DEX and MIX for induction. Adipocytes were identified by the presence of large lipid droplets which stain with oil red O. Cell number was counted in a hemocytometer after detaching cells by trypsinization.

DNA Synthesis. Medium was removed from confluent cultures of 3T3-L1 cells and replaced with fresh DME medium containing 5% fetal calf serum, and incubation was continued for 3 days. [3H]Thymidine was then added to the conditioned medium to 1 μCi/ml (ICN, Irvine, Calif.), and the cultures were incubated for 22 hr at 37°C. The cultures were placed on ice, the medium was removed, and the cells were washed 3 times with Dulbecco’s phosphate-buffered saline (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 1 mM CaCl₂, and 0.5 mM MgCl₂) containing 50 mM unlabeled thymidine, overlaid with ice-cold 10% trichloroacetic acid and kept at 4°C overnight. The cells were washed twice with 5% trichloroacetic acid and dissolved in 1 N NaOH. Radioac-
activity was counted with 3a70b solution (Research International Products; Mount Prospect, III.) in a Packard scintillation spectrometer as before (34).

EGF Binding. Confluent cultures in 60-mm dishes were placed on ice and with EBSS-BSA (33). One ml of EBSS-BSA buffer was added to each dish, $^{125}$I-EGF was added to 1 to $2 \times 10^{-10}$ M, and the dishes were incubated at 23° for 60 min. The drugs were added in the cultures 2 hr before the binding assay. After incubation, the cells were placed on ice, washed with EBSS-BSA, and solubilized in 1 ml of 0.5 N NaOH. Radioactivity was determined in a Beckman 4000 gamma counter. $^{125}$I-EGF used for the present study was produced from receptor grade EGF (Collaborative Research, Waltham, Mass.) by the chloramine-T iodination method. Nonspecific binding was measured and subtracted from total binding as before (33).

RESULTS

One of the striking effects of tumor promoters, such as TPA, on cells in culture is the reduction of $^{125}$I-EGF binding to cell surface receptors (5, 20, 40). This is not due to direct competition of TPA with EGF for its receptors but is attributed to the modulation by TPA of the membrane in an as yet unknown fashion (21, 24, 35). In 3T3-L1 cells, the EGF-binding capacity is constant before and after adipocyte differentiation, in contrast to the dramatic increase in the insulin-binding capacity after differentiation (29). We have tested the effects of DHTB and TPA on the binding of EGF (Chart 1) and found that both DHTB and TPA substantially reduce the EGF-binding capacity of 3T3-L1 cells in the preadipocyte state and that this receptor down regulation by DHTB is 10 times more efficient than that of TPA (Chart 1A). EGF binding to the cells which have differentiated to adipocytes (Chart 1B) was reduced by TPA to the same extent as that in the preadipocyte state, but the effect was 10 times less efficient in the adipocytes than in the preadipocytes. The effect of DHTB on the EGF-binding capacity of 3T3-L1 adipocytes was even less, reducing it by 20% at 100-ng/ml concentrations of DHTB. These differential modulations of EGF receptors by DHTB and TPA suggest that these tumor promoters may be acting through distinct mechanisms; in other words, their membrane-modulatory effects may be transmitted to EGF receptors in different manners.

The 3T3-L1 cells can be maintained at confluency in medium containing 10% fetal calf serum for more than 1 week without differentiation (Chart 2). Differentiation, however, is stimulated by treating confluent cells for 2 days with the inducers DEX and MIX (29). By the sixth day after inducer treatment, 40 to 70% of the cells have converted into adipocytes, which accumulate large lipid droplets in their cytoplasm. We examined the effects of DHTB and TPA on this differentiation program. Adipocyte differentiation was almost completely inhibited when DHTB was added to the cultures at 10 ng/ml (Chart 2). The same concentration of TPA also inhibited adipocyte differentiation but to a much lesser extent. In these experiments, tumor promoters were added concurrently with the inducers and kept present for the next 4 days in the culture medium (Chart 2, arrows). Partial inhibition of adipocyte differentiation by TPA is consistent with previous work by Diamond et al. (7). Other phorbol esters, such as 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate and phorbol dibutyrate which are known as weak promoters or nonpromoters, caused no inhibition of adipocyte differentiation (data not shown). Next, we tested whether DHTB exhibits its inhibitory effect when added before DEX-MIX treatment, during the inducer treatment, or after the induction. As seen in Chart 3, DHTB significantly inhibited adipocyte differentiation no matter when it was added. Addition of DHTB even before or after induction was effective in preventing differentiation. In the case of TPA (Chart 4), adipocyte...
differentiation was not inhibited if the drug was added and then removed prior to induction. Effective inhibition, although only partial, was seen when TPA was added during the induction period. The TPA did not cancel out the postinduction events. The difference in inhibitory ability between DHTB and TPA is illustrated in Chart 5A, where dose dependency of inhibition was tested using 4 different treatment schedules. DHTB at 10 ng/ml consistently gave almost complete inhibition, while TPA was inhibitory only when it was present during the induction period. Distinct inhibitory profiles were also obtained under the conditions where adipocyte differentiation was accelerated by adding insulin together with inducers (Chart 5B). Adipocyte differentiation could be induced by insulin (6 μg/ml) alone but at a low frequency (1 to 5%). This insulin-induced differentiation was also inhibited by both DHTB and TPA (data not shown). However, a comparison of potencies was not possible due to the low frequency of differentiation. These results together suggest that both DHTB and TPA can truly antagonize the adipocyte differentiation program but that DHTB is apparently a pluripotent agent.

When growth-arrested 3T3-L1 cells were treated with the inducers DEX and MIX, their DNA synthesis was initiated within 22 hr and increased to 2.8 times the control (Table 1). DEX or MIX alone stimulated DNA synthesis only by 50 to 60%. As has been reported (8), TPA stimulated DNA synthesis 1.9 times more than the control and further enhanced the mitogenic effect of the inducers. Strikingly, DHTB was a 3 times more potent stimulator for DNA synthesis and exhibited remarkable synergistic stimulation with the inducers. Chart 6 illustrates the dose-dependent manner of DNA synthesis stimulation in 3T3-L1 cells and again shows the difference in potency between DHTB and TPA. The maximum levels of DNA synthesis during 22-hr treatment were almost the same. As seen in Chart 7, 3T3-L1 cells which responded to the inducers, DEX and MIX, underwent cell division (compare cell numbers at zero tumor promoters). This inducer-
mediated cell division is thought to be a necessary step for triggering the adipocyte differentiation program (23, 29). Treatment with DHTB apparently stimulated cell proliferation and increased cell number to twice the inducer-stimulated level, while TPA had little effect on cell division. Thus, inhibition of adipocyte differentiation by DHTB is attributed to an overwhelming cell growth while the inhibition of TPA is not. This notion was further supported by the observations shown in Fig. 1. Gross morphology changes and/or increase of cell density is seen when 3T3-L1 cells were treated with DHTB (10 ng/ml) regardless of the inducer treatments (Fig. 1, C, D, and G). TPA had no such effects on 3T3-L1 cells even at 100 ng/ml (Fig. 1, B, F, and H).

Thus, the indole alkaloid tumor promoter DHTB is a highly potent inhibitor of adipocyte differentiation. The inhibition appears to be due to unscheduled stimulation of DNA synthesis. The detailed profile of the inhibition by DHTB is different from that of TPA in several aspects.

**DISCUSSION**

We have described in this paper that the indole alkaloid tumor promoter DHTB is a significantly more potent inhibitor for adipocyte differentiation of 3T3-L1 cells than is the phorbol ester tumor promoter TPA. We have found that addition of DHTB or TPA to 3T3-L1 cells, which had grown to confluency and quiescence, strongly stimulates the cells to initiate DNA synthesis but that only DHTB facilitated cell division. TPA did not trigger the mitosis of 3T3-L1 cells.

Recent studies have shown that both TPA and DHTB stimulate growth-arrested mouse fibroblastic cells to initiate DNA synthesis and undergo numerous rounds of cell division (26). These fibroblastic cells include the Swiss/3T3 line from which the 3T3-L1 preadipocyte line was established (15). It is also known that TPA alone is not mitogenic for these cells in serum-free medium but rather that it acts synergistically with numerous growth factors (8, 26). In the above-mentioned TPA-responsive fibroblastic cells, it was observed that an increase in cell number is correlated with gross morphological changes (26); while in the TPA-treated 3T3-L1 cells, there was no obvious morphological change. There are various observations in cells other than fibroblasts that TPA inhibits differentiation in certain cell systems whereas it induces differentiation in other systems (12, 13, 19, 27). DHTB also has reciprocal effects (13). What sort of cell physiology regulates the direction of these effects is at present not known.

It is believed that TPA and DHTB have equal potencies in the induction of various biochemical changes (12, 13, 19, 27, 31, 40). There is one exception (18) in that DHTB is 100 times more effective than TPA in enhancing methylcholanthrene-induced malignant transformation of A31-1-1 cells. The results found in 3T3-L1 cells provide an additional case in which the potency of DHTB is shown to be different from that of TPA with respect to inhibition of differentiation.

Previously, Diamond et al. (7) reported that TPA inhibits the spontaneous adipocyte differentiation of clone A31-T of 3T3 fibroblasts at concentrations of $10^{-6}$ to $10^{-3}$ M (10 to 100 ng/ml). Under their conditions, however, only 5 to 6% of the cell population differentiated to mature adipocytes, and differentiation took 2 weeks after confluence. We have used 3T3-L1 preadipocytes which are able to differentiate into adipocytes under chemically defined induction conditions. The 3T3-L1 cells differentiate at a frequency of 40 to 70% within 1 week. Our observations using the 3T3-L1 system not only confirmed the TPA-mediated inhibition of adipocyte differentiation observed by Diamond et al. (7) but also provided the basis for further analysis of its action. TPA was effective only when added during the induction period. In contrast, DHTB caused almost complete inhibition of differentiation regardless of the timing of its addition relative to the induction period. Thus, DHTB appears to act at multiple physiological states of 3T3-L1 cells.

Our present work and others provided evidence which supports the following view on the adipocyte differentiation program. 3T3-L1 cells, the growth of which is arrested at the G0-G1 state of the cell cycle, respond to the inducers, initiate DNA synthesis, and divide (G2). The divided daughter cells appear to be under a regulatory mechanism in which the cells become arrested in another state, G0. From this state they proceed to differentiate into adipocytes. The designation of these states has been discussed previously (23, 32).

Unlike TPA, DHTB forced the growth-arrested 3T3-L1 cells to divide. DHTB, when added before the induction period, stimulated several rounds of cell division, and this excess cell proliferation did not allow the cells to enter into G0 to trigger the differentiation program. The effect of DHTB added during the induction period could be accounted for by a mechanism in which competition with the action of the inducers may be involved. The mechanism by which the inducers DEX and DEX facilitate adipocyte differentiation of 3T3-L1 cells is not fully understood (29). It is, however, known that changes in prostaglandin metabolism in concert with the cyclic adenosine 3':5'-monophosphate level are important early cellular responses to the inducers (6, 14, 22, 25, 26, 29–31, 42, 43, 46). The inhibitory action of TPA was observed only during this period and could be due to the same mechanism. In other words, both tumor promoters efficiently block the entry of the cell into the G0 state, inhibiting expression of the genes involved in adipocyte differentiation. DHTB added after the induction period appears to act on the cells at the G0 state and may interfere with the efficient production and function of various enzymes related to adipocyte differentiation, namely, the expression and maintenance of the adipocyte phenotype in the committed G0 state cells are inhibited. It has been reported that TPA binds to specific surface receptors and that its interaction leads to an increase in membrane fluidity resulting in the
REFERENCES

1. Baird, W. M., and Boutwell, R. K. Tumor promoting activity of phorbol and four
48, 1941.
3. Berenblum, I. Established principles and unresolved problems in carcinogen-
5. Brown, K. D., Dicker, P., and Rozengurt, E. Inhibition of epidermal growth
factor binding to surface receptors by tumor promoters. Biochem. Biophys.
6. Chang, T. H., Williams, I., and Polak, E. Differentiation of 3T3-L1 fibroblasts
to adipocytes: loss of stimulation of uridine and deoxyguanosine transport by
PGF 2 in the course of differentiation of the 3T3-L1 cell line. Exp. Cell Res.,
7. Diamond, L., O’Brien, T. G., and Rovera, G. Inhibition of adipocyte conversion
8. Dicker, P., and Rozengurt, E. Synergistic stimulation of early events and DNA
synthesis by phorbol esters, polypeptide growth factors, and retinoids in
9. Driedger, P. E., and Blumberg, P. M. Specific binding of phorbol ester tumor
10. Dicker, P. E., Flamming, M., Schachter, D., and Weinstein, I. B. Tumor promoters
produce membrane changes detected by fluorescence polarization. Biochem.
Sugimura, T., and Weinstein, I. B. Effects of teleocidin and the phorbol ester
promoters on cell transformation, differentiation, and phospholipid meta-
naturally occurring tumor promoter, teleocidin B from Streptomyces. Bioclem.
13. Fujiki, H., Morii, M., Nakayasu, M., Terada, M., Sugimura, T., and Moore,
R. E. Indole alkaloids: dihydroteleocidin B, teleocidin and lyngbyatoxin A, as
14. Furstenberger, G., and Marks, P. Indomethacin inhibition of cell proliferation
by the phorbol ester TPA is reversed by prostaglandin E2 in mouse epidermis
15. Green, H., and Kehinde, O. Spontaneous heritable changes leading to in-
16. Hecker, E. Phorbol esters from croton oil—chemical and biological activities.
17. Hecker, E. Isolation and characterization of the carcinogenic principles from
promoting agent, dihydroteleocidin B, markedly enhances chemically induced
lymphoblastoid cells by tumor-promoting phorbol esters and dihydroteleocidin
of epidermal growth factor to cellular receptors. Science (Wash. D. C.), 202:
21. Lee, L.-S., and Weinstein, I. B. Mechanism of tumor promoter inhibition of
1982.
22. Levin, L., and Hassid, A. Effects of phorbol-12,13-diester on prostaglandin
production and phosphate uptake during folic acid deficiency (MDCK) cells.
23. Lewis, J. E., Shimizu, Y., and Shimizu, N. Nicotinamide inhibits adipocyte
24. Magun, B. E., Matrelian, L. M., and Bowden, G. T. Epidermal growth factor:
activity of tumor promoters to alter its degradation, receptor affinity, residence
adenosine monophosphate phosphodiesterase activity in the epidermis of phorbol
cellular differentiation. In: M. E. Buckingham and M. J. Clemens (eds.),
27. Nakayasu, M., Fujiki, H., Morii, M., Sugimura, T., and Moore, R. D. Telecidin,
lyngbyatoxin A and their hydroxylated derivatives, possible tumor promoters,
28. Nakayasu, M., Fujiki, H., Morii, M., Sugimura, T., and Moore, R. D. Telecidin,
lyngbyatoxin A and their hydroxylated derivatives, possible tumor promoters,
29. Nakayasu, M., Fujiki, H., Terada, M., Sugimura, T., and Weinstein, I. B.
Stimulation of epidermal growth factor production and phospholipase activity in
30. Nakayasu, M., Fujiki, H., Terada, M., Sugimura, T., and Moore, R. D. Telecidin,
lyngbyatoxin A and their hydroxylated derivatives, possible tumor promoters,
31. Sakamoto, H., Terada, M., Fujiki, H., Mori, M., Nakayasu, M., Sugimura, T.,
and Weinstein, I. B. Terminal differentiation of 3T3-L1 cells. FEBS Lett., 146:
32. Scott, R. E., Flurin, D., Wilke, J. J., Jr., and Yun, K. Coupling of growth arrest
and differentiation at a distinct state in the G1 phase of the cell cycle. Gru.
33. Shimizu, Y., and Shimizu, N. Genetics of cell surface receptors for bioactive
polypeptide: a variant of mouse BALBC/3T3 fibroblasts possessing altered
receptors for bioactive polypeptides: binding of epidermal growth factor
protein (APD-riboside) synthetase activity during differentiation of 3T3-L1
diacylglycerol promoters. J. Biol. Chem., 256: 4871–
34. Shimizu, Y., and Shimizu, N. Genetics of cell surface receptors for bioactive
polypeptides: binding of epidermal growth factor receptor is
associated with the presence of human chromosome 7 in human-mouse cell
35. Shimizu, Y., and Shimizu, N. Genetics of cell surface receptors for bioactive
polypeptides: a variant of mouse BALBC/3T3 fibroblasts possessing altered
36. Shoyab, M., DeLauro, J. E., and Todd, G. J. Biologically active phorbol
esters specifically alter affinity of epidermal growth factor membrane receptors.
37. Shoyab, M., Warren, T. C., and Todd, G. J. Phorbol-12,13-diester 12-ester
carboxylic acid may prevent tumor promotion by phorbol diesters in skin.
Tumor Promoters and Adipocyte Differentiation


Distinct Inhibitory Effects of Dihydroteleocidin B and the Phorbol Ester Tumor Promoters on the Adipocyte Differentiation of 3T3-L1 Cells


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/43/10/4974

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/43/10/4974. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.