Vol. 93, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1980 Pages 842-849

TUNICAMYCIN INHIBITS THE DIFFERENTIATION OF ST 13 FIBROBLASTS TO ADIPOCYTES WITH SUPPRESSION OF THE INSULIN BINDING ACTIVITY

Kenji Kohno*[1], Akiyoshi Hiragun**, Akira Takatsuki*,
Gakuzo Tamura*, and Hiromi Mitsui**

* Laboratory of Microbiology, Department of Agricultural Chemistry, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113
** The Tokyo Metropolitan Institute of Medical Science, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113, Japan

Received February 22,1980

Summary: ST 13 cells are a clonal line of murine fibroblasts that are capable of differentiating into adipocyte-like cells in vitro. When the cells were maintained as a confluent monolayer, they began to accumulate lipid droplets and to exhibit a rapid increase of insulin binding activity. Tunicamycin, a specific inhibitor of dolichol-mediated protein glycosylation, blocked this adipose conversion without affecting cell growth and total protein synthesis. The inhibitory effect of tunicamycin was dose-dependent and reversible. Enhancement of the incorporation of [^{14}C]acetate into triglyceride fraction accompanying the adipose conversion was completely inhibited by tunicamycin, whereas the incorporation into phospholipid fraction was only partially affected. The insulin binding activity increased about 10-fold during differentiation, but was completely suppressed in tunicamycin-treated cells.

Introduction: When ST 13 fibroblasts are maintained as a confluent monolayer in a medium containing insulin, more than 90% of the cells convert into adipose-like cells within 2 weeks of cell seeding. The adipose conversion is characterized by the appearance of "multilocular" lipid droplets in the cytoplasm and by an increase in synthesis and accumulation of cellular triglyceride. Additionally, the cellular insulin binding activity is remarkably increased. Fully developed adipocytes are no longer able to divide indicating the differentiation is terminal. The precise mechanisms of the triggering process have yet to be worked out, but the formation of a contact inhibited monolayer seems to play an essential role in the adipose conversion. This constitutes a major difference from the triggering process proposed in the widely used 3T3-L_1 preadipocytes (1-3).

---

1. Present adress: The Tokyo Metropolitan Institute of Medical Science, 3-18-22, Honkomagome, Bunkyo-ku, Tokyo 113, Japan.
Abbreviations: TM, tunicamycin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TG, triglyceride.

0006-291X/80/070842-08$01.00/0

Drugs that are known to affect plasma membrane constitution (such as retinoic acid, and tumor promoting phorbol esters) have been reported to inhibit the differentiation of ST 13 cells (4). A nucleoside antibiotic tunicamycin (TM) specifically inhibits the formation of lipid linked N-acetylglucosamine and consequently inhibits the glycosylation of the asparaginyl residue of glycoprotein (5, 6). TM treatment of cultured cells has been reported to cause many changes in the constitution of cell surface glycoproteins including affecting insulin receptors and mode of cell contact (7-11). We therefore investigated the effect of TM on the differentiation of ST 13 cells to elucidate the role of glycoprotein(s) in the process of differentiation.

We report that TM treatment inhibited the adipose conversion without affecting cell growth and total protein synthesis and that the increase in insulin binding activity was suppressed completely.

Materials and Methods:

Materials. Insulin (bovine pancreas, crystalline), diolein, triolein, and bovine serum albumin (RIA Grade) were purchased from Sigma Chem. Co., St Louis, Mo. Oil Red O was from Chroma Gessellschaft Schmid GMBH & Co., Stuttgart-Untertürkheim, Germany. Insulin (porcine, monocomponent) was obtained from Novo Industri, Bagsvaerd, Denmark. Monoiodinated [^{125}I]insulin (porcine, 80 μCi/μg) was from New England Nuclear, Boston, Mass. D-[1-^{3}H]glucosamine (2.6 Ci/mmol), L-[4,5-^{3}H]leucine (137 Ci/mmol), and [1-^{14}C]acetate (58.7 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, England.

Cell culture. ST 13 cells were grown in RPMI medium 1640 supplemented with 10% fetal bovine serum (GIBCO, Grand Island, N.Y.) and kanamycin (60 μg/ml, Meiji Seika Co., Ltd., Tokyo, Japan) at 37°C in humidified 10% CO_2/90% air. When the cultures reached confluence, the cells were dissociated by trypsinization and transferred to fresh medium in new petri dishes. Under these conditions, ST 13 cells were kept in a nondifferentiated state. Cells were seeded at 3×10^4 cells in 35-mm culture dishes (day 0). On day 2, medium was replaced with the induction medium (medium containing 5 μg/ml insulin) with or without TM. Thereafter, medium change was carried out every third day. The cells were maintained in the induction medium in the presence or absence of TM.

Incorporation of amino acid and sugar. On the 5th, 12th, and 20th day after cell seeding, the cultures incubated with or without TM (0.2 μg/ml) were labeled with 2 μCi/ml of [^{3}H]leucine or 2 μCi/ml of [^{3}H]glucosamine for 6 hr at 37°C in the induction medium. Acid insoluble materials were collected on Whatman GF/C glass microfibre filters, dried and counted in a liquid scintillation counter. Total protein was estimated according to the method of Lowry (12) with bovine serum albumin as a standard.

Incorporation of [^{14}C]acetate into cellular lipids. Cells were incubated in one ml of culture medium containing 2 μCi of [^{14}C]acetate for 24 hr and washed twice with saline, and then lipids were extracted according to the method of Bligh and Dyer (13). The extracted lipids were separated by thinlayer chromatography. The labeled spots detected by autoradiograms (X-Omat R X-ray film, Eastman Kodak Co., Rochester, N.Y.) were scraped off the plates and counted in a liquid scintillation counter.

Estimation of triglyceride content. Triglyceride content of cultured cells was assayed by acetyl aceton colorimetry as described by Fletcher (14).

Insulin binding assay. Insulin binding activity was measured by a modification of the method described by Pratt and Pastan (15). The cultures were washed and maintained with insulin-free medium for 24 hr before assaying binding activity. Cell monolayers treated with or without TM (0.2 μg/ml) in 35-mm culture dishes were gently washed twice with one ml of warm binding medium (RPMI medium 1640 containing 0.5% bovine serum albumin and 20 mM HEPES, pH 7.4) and incubated for 30 min at 25°C in 0.75 ml of binding medium containing [^{125}I]insulin (0.2 nM) After incubation, the samples were rapidly chilled to 4°C, and unbound [^{125}I]-insulin was quickly removed from the dishes by gently rinsing the monolayers three times with 1.5 ml of ice-cold binding medium. The monolayers were solubilized with one ml of lysing buffer (1 mM EDTA, 0.5% SDS, and 10 mM Tris-HCl, pH 7.4 at 25°C) and transferred with two 0.5 ml aliquots of lysing buffer to vials with a siliconised pipette. The amount of [^{125}I]insulin bound to the cells was determined in a liquid scintillation counter. Specific insulin binding was obtained by subtracting the amount of radioactivity bound in the presence of 1000-fold native insulin from the total bound radioactivity.

Results:

Effect of TM on the growth and differentiation of ST 13 cells. TM (0.05-0.1 μg/ml) did not influence cell growth as shown in Fig. 1. Addition of 0.2 μg/ml of TM caused a slight retardation of cell growth, but an increase in the final saturation density. Cells became elongated and grew in a criss-cross pattern with loss of contact inhibition of cell growth. As one characteristic of cell differentiation, the cellular triglyceride accumulation was examined in the presence or absence of TM. When the cells were cultured with increasing concen-

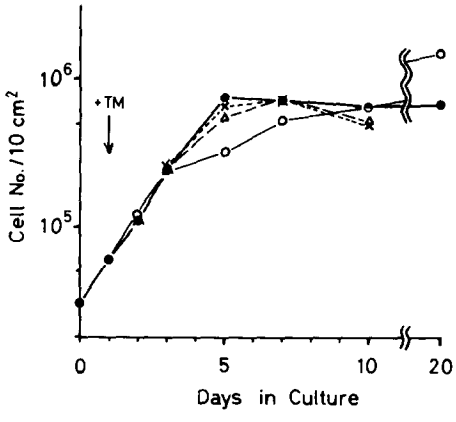

Fig. 1. Effect of TM on the growth of ST 13 cells. ST 13 cells were incubated under the same conditions as described in Materials and Methods, except that TM and insulin were added 24 hr after cell seeding. The cells were counted in a hemocytometer on each of the days indicated.
●, control; X, TM 0.05 μg/ml; △, TM 0.1 μg/ml; ○, TM 0.2 μg/ml.

Table 1. Effect of TM on Triglyceride Accumulation.

TM	14 days		21 days	
μg/ml	TG mg/mg protein	%	TG mg/mg protein	%
-	3.17	100	3.91	100
0.05	2.78	87.7	1.63	41.7
0.10	0.862	27.2	0.165	4.2
0.20	0.055*	1.7	0.059*	1.5

* Triglyceride content of nondifferentiated cells ranges between 0.055-0.080 mg/mg protein.
Each value is expressed in mg triglyceride per mg cellular protein and is the mean of duplicate dishes.

trations of TM, the cellular triglyceride content was decreased remarkably. In the presence of 0.2 μg/ml of TM, the cellular triglyceride content was similar to that of nondifferentiated ST 13 cells (Table 1), and Oil Red O-positive cells were not detected (Fig. 2, 3). When TM was removed from the culture medium, the cells differentiated into adipocytes in the presence of insulin on the 5th day after removal of TM (data not shown).

Effect of TM on incorporation of [^{14}C]acetate into triglyceride and phospholipid fractions during cell differentiation. As we described before (4), [^{14}C]-acetate was mainly taken up into the triglyceride and phospholipid fractions

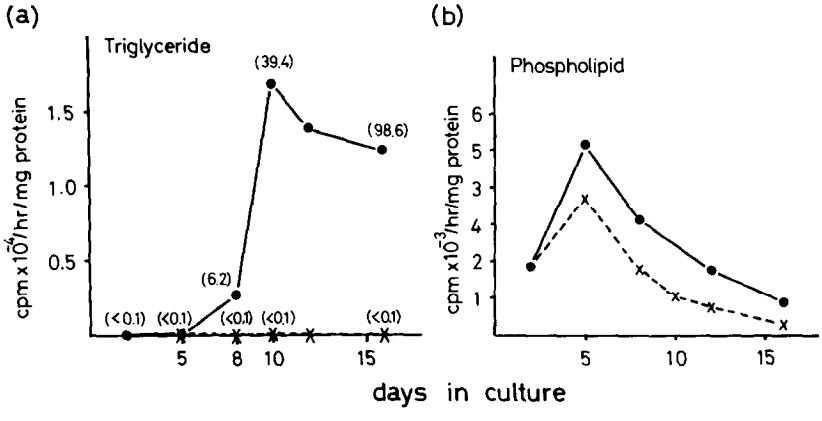

Fig. 2. Incorporation of [^{14}C]acetate into (a) triglyceride and (b) phospholipid fractions in the absence or presence of TM. Each value is expressed in cpm incorporated per hr per mg cellular protein, and all values are the mean of duplicate dishes. Values in parentheses indicate the percentage of Oil Red O-positive cells.
●, control; X, TM 0.2 μg/ml.

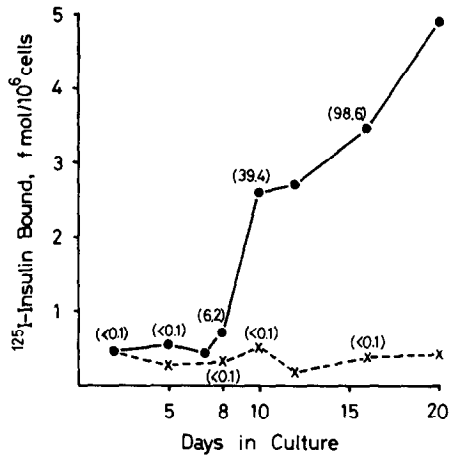

Fig. 3. Changes of insulin binding activity during adipose conversion in response to chronic insulin treatment. Specific [^{125}I]insulin binding was measured as described in Materials and Methods. Values in parentheses indicate the percentage of Oil Red O-positive cells.
●, control; X, TM 0.2 μg/ml.

of cellular lipids. When the cells began to differentiate on the 8th day after inoculation, acetate incorporation into triglyceride increased about 20-fold. In the presence of 0.2 μg/ml of TM, cell differentiation was completely inhibited and amount of [^{14}C]acetate uptake was maintained at the nondifferentiated level (Fig. 2, a). Phospholipid synthesis, however, was gradually decreased as a result of cessation of cell growth and was only partially affected by TM (Fig. 2, b).

Effect of TM on protein and glycoprotein synthesis. Total protein synthesis, measured by incorporation of [^{3}H]leucine into acid insoluble fraction, did not change in the presence or absence of TM. But glycoprotein synthesis in TM-treated cultures was inhibited by 55-80% of the control (Table 2). This result indicates that glycoprotein synthesis was specifically inhibited by TM under this condition and that glycoproteins play important roles in the process of differentiation.

Insulin binding activity. As described in the introduction, the process of adipose conversion is accelerated by the presence of insulin. To elucidate the role of insulin in this process, we studied the effect of TM on the insulin

Table 2. Effect of TM on Incorporation of Leucine and Glucosamine into TCA-insoluble Fractions

Days	[^{3}H]-Leucine cpmx10^{-4}/mg protein		%	[^{3}H]-Glucosamine cpmx10^{-3}/mg protein		%
	control	+TM		control	+TM	
5	11.1	10.3	92.8	7.32	3.28	44.8
12	9.17	10.6	115.6	5.99	1.33	22.2
20	7.50	6.94	92.5	5.24	1.50	28.6

Control and TM-treated cultures (0.2 μg/ml) were labeled with radioactive leucine and glucosamine for 6 hr, and 10% TCA insoluble materials were counted. All values are the mean of duplicate dishes.

binding activity during the differentiation process. On the 8th day after cell seeding (on the 3rd day after confluence), about 6% of the cell population became lipid positive. Accompanied with this change, insulin binding activity abruptly began a steady increase (Fig. 3). When the cells were exposed to 0.2 μg/ml of TM continuously in the presence of insulin, the increase in insulin binding activity was completely suppressed (Fig. 3).

Discussion: ST 13 cells inherently seem to possess a program for differentiation into lipid accumulating cells that is triggered by the formation of a contact inhibited monolayer and accelerated by insulin treatment. TM completely blocked the differentiation of preadipocytes without affecting cell growth and protein synthesis. TM presumably inhibited the differentiation by the following mechanisms. [1]. TM caused changes in cell surface glycoconjugates and extracellular matrices (9, 16-18), which are known to be important factors in the formation of an organized monolayer. The morphological changes leading to the loss of contact inhibition observed in TM-treated ST 13 cells could be the manifestation of these changes. [2]. The increase in insulin binding activity, which normally accompanied the adipose conversion, was suppressed by TM treatment (Fig. 3). Insulin receptors are supposed to play an important role in the differentiation processes of both ST 13 cells and 3T3-L_1 preadipocytes (19-23). The insulin receptor has been reported to be a glycoprotein of approximately

300,000 molecular weight (24, 25). Recently several workers reported that insulin receptor can be separated into two components. One component contains the insulin binding site and the other, being a glycoprotein, may be an effector molecule regulating the affinity of insulin binding (26, 27). Rosen et al. reported that TM elicited a rapid depletion of insulin binding activity in 3T3-L_1 adipocytes (11), and this result suggests a possibility that lack of glycosylation of insulin receptor may cause a great reduction of insulin responsiveness. [3]. Changes in glucose transport systems may also be involved. Glucose, a main substrate of cellular triglyceride synthesis, is taken up into the cytoplasm through a transport system that contains a glycoprotein component (28, 29). The glycosylation of this component has been shown to be inhibited by TM treatment leading to impairment of cellular glucose uptake (30).

The present communication suggests that glycoproteins are essential components for the adipose conversion and demonstrates that the suppression of insulin binding activity may be one of the mechanisms by which TM inhibits the adipose conversion.

Acknowledgment: This work was financially supported by grants in aid from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan.

References

1. Green, H., and Kehinde, O. (1974) Cell 1, 113-116.
2. Green, H., and Meuth, M. (1974) Cell 3, 127-133.
3. Green, H., and Kehinde, O. (1975) Cell 5, 19-27.
4. Hiragun, A., Sato, M., and Mitsui, H. In Vitro, in press.
5. Takatsuki, A., Kohno, K., and Tamura, G. (1975) Agric. Biol. Chem. 41, 2089-2091.
6. Tkacz, J.S., and Lampen, J.O. (1975) Biochem. Biophys. Res. Commun. 65, 248-257.
7. Duksin, D., and Bornstein, P. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3433-3437.
8. Duksin, D., Holbrook, K., Williams, K., and Bornstein, P. (1978) Exp. Cell Res. 116, 153-165.
9. Olden, K., Pratt, R.M., and Yamada, K.M. (1978) Cell 13, 461-473.
10. Kohno, K., Hiragun, A., Mitsui, H., Takatsuki, A., and Tamura, G. (1979) Agric. Biol. Chem. 43, 1553-1561.
11. Rosen, O.M., Chia, G.H., Fung, C., and Rubin, C.S. (1979) J. Cell. Physiol. 99, 37-42.
12. Lowry, O.H., Rowebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

13. Bligh, E.G., and Dyer. W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
14. Fletcher, M.J. (1968) Clin. Chim. Acta. 22, 393-397.
15. Pratt, R.M., and Pastan, I. (1978) Nature 272, 68-70.
16. Takatsuki, A., Fukui, Y., and Tamura, G. (1978) Agric. Biol. Chem. 42, 1621-1623.
17. Pratt, R.M., Yamada, K.M., Olden, K., Ohanian, S.H., and Hascall, V.C. (1979) Exp. Cell Rec. 118, 245-252.
18. Duksin, D., and Bornstein, P. (1977) J. Biol. Chem. 252, 955-962.
19. Rosen, O.M., Smith, C.J., Gung, C., and Rubin, C.S. (1978) J. Biol. Chem. 253, 7579-7583.
20. Reed, B.C., Kaufmann, S.H., Mackall, J.C., Student, A.K., and Lane, M.D. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4876-4880.
21. Rubin, C.S., Lai, E., and Rosen, O.M. (1977) J. Biol. Chem. 252, 3554-3557.
22. Chang, T.H., and Polakis, S.E. (1978) J. Biol. Chem. 253, 4693-4696.
23. Rubin, C.S., Hirsh, A., Fung, C., and Rosen O.M. (1978) J. Biol. Chem. 253, 7570-7578.
24. Cuatrecasas, P. (1972) J. Biol. Chem. 247, 1980-1991.
25. Jacobs, S., Shechter, Y., Bissell, K., and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 77, 981-988.
26. Maturo III , J.M., and Hollenberg, M.D. (1978) Proc. Natl. Acad. Sci. U. S.A. 75, 3070-3074.
27. Momoi, T. (1979) Biochem. Biophys. Res. Commun. 87, 541-549.
28. Pouyssegur, J., Shiu, R.P.C., and Pastan, I. (1977) Cell 11, 941-947.
29. Shiu, R.P.C., Pouyssegur, J., and Pastan, I. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3840-3844.
30. Olden, K., Pratt, R.M., Jaworski, C., and Yamada, K.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 791-795.