Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 586-591

Retinoids Block Ornithine Decarboxylase Induction in Cells Treated with the Tumor Promotor TPA or the Peptide Growth Hormones, EGF and SGF

Meera S. Paranjpe, Joseph E. De Larco, and George J. Todaro

Laboratory of Viral Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Received April 10,1980

SUMMARY: A clone of rat kidney cells is described that responds to the tumor-promoting agent tetradecanoyl phorbol acetate (TPA) and to the polypeptide growth hormones, epidermal growth factor (EGF) and sarcoma growth factor (SGF) by increasing ornithine decarboxylase (ODC) activity. The peak with EGF and SGF is at 2-4 hours after exposure; with TPA the response is slower but more sustained. Retinoic acid pretreatment of the cells blocked both the TPA-induced response as well as the EGF- and SGF-induced response.

INTRODUCTION: Ornithine decarboxylase (ODC) activity has been found to be an early and sensitive marker that increases in cells treated with tumor promotors or infected with transforming viruses (1-8). ODC is one of the enzymes required for the biosynthesis of polyamines; these compounds, which include putresceine and spermidine, have been shown to stimulate cell division. While resting cells have low levels of ODC activity, the levels increase as the cells progress through the cell cycle. Elevated levels of ODC in cell culture systems result in increased synthesis of polyamines, and ultimately to resting cells entering into cellular DNA synthesis.

Sarcoma growth factor (SGF), is a polypeptide growth factor produced by virus transformed cells that acts as an effector of phenotypic transformation in cell culture systems, inducing normal fibroblasts to form progressively growing colonies in agar (9,10). The growth stimulatory effect requires the continued presence of the factor in the culture medium. It interacts with the epidermal growth factor (EGF) receptor system and has several properties that are similar to those of EGF (9,10). A potent tumor promotor, tetradecanoyl

---

Abbreviations: TPA, tetradecanoyl phorbol acetate; EGF, epidermal growth factor; SGF, sarcoma growth factor; ODC, ornithine decarboxylase; PBS, phosphate buffered saline, pH 6.8; NRK, normal rat kidney.

0006-291X/80/100586-06$01.00/0

phorbol acetate, (TPA) (11) also induces resting cells to enter the cell cycle in culture and has been shown to affect the EGF receptor (12) by lowering receptor affinity for EGF binding (13,14).

Retinoids (vitamin A and its derivatives) (15) inhibit the tumor-promoting effect of TPA on mouse epidermis (6,16) and in cell culture systems (17). Recently it has been shown that these compounds block SGF-induced cell growth in culture (10). Further, Verma et al. (18) have shown that retinoids block the ODC induction of tumor promotors in whole mouse skin. We, therefore, were interested in comparing the different growth stimulating agents with one another in the same cell culture system, to determine their effect on ODC induction and to see if retinoids could inhibit the increase in activity of this early marker of growth stimulation. Using a clone of rat kidney fibroblasts, 49F (19), the results show that EGF and SGF have a very similar time course for ODC induction; in contrast, TPA shows ODC induction at a considerably later time. In the present experiments, retinoids can be shown to block ODC induction by each of these effectors. The ability of retinoids to block the effect of the peptide hormones on enzyme activity, however, requires several hours of interaction with the cells. This paper describes a cell culture system that may be used to detect other potential anti-transforming agents that act similarily to retinoids.

MATERIALS AND METHODS

Assay for ODC Activity. Normal rat kidney (NRK) cells were plated at 0.8 x 10^6 cells per 60 mm dish and used 5 days later for experiments. At appropriate times after the addition of peptide growth hormones, EGF (10 ng/ml) and crude SGF (30 µg/ml), the cell monolayers were rapidly washed 3x with phosphate buffered saline (PBS) and frozen at -70°C. While cells were still frozen 0.25 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM Na EDTA and 5 mM dithiothreitol was added to each dish and the cells were harvested by scraping with a Teflon policeman. Two additional cycles of freezing and thawing ensured complete lysis of the cells. The broken cell suspension was centrifuged at 12,000 g for 1 min (Beckman serofuge) and the clear supernatants were used to assay ODC activity. Final concentration of reagents in the incubation mixture were 50 mM sodium phosphate pH 7.2, 1 mM EDTA, 50 µM pyridoxal phosphate and 5 mM dithiothreitol. This mixture containing the soluble cell extract was preincubated for 5 minutes at 37°C. 20 µl of label containing 1 volume of ^{14}C-DL-ornithine (New England Nuclear, Boston, Massachusetts, specific activity 54 mCi/m mole^{-1}) and 2 volumes each of distilled

water and ^{12}C-L-ornithine (10 mM) were added to a final volume of 400 µl. The incubation was carried out for 1 hour at 37°C in 17 x 100 mm Falcon culture tubes capped with serum stoppers equipped with polypropylene centre wells containing a filter paper wick (Kontes Glass Co., Vineland, N.J.). The reaction was stopped with 0.2 ml of 2 N perchloric acid and the $^{14}CO_2$ was trapped by 0.2 ml NCS solubilizer (Amersham Searle Corp., Arlington Heights, Illinois) contained in the centre well. Incubations were continued for 1 additional hour to ensure complete CO_2 absorption. The center well containing the label trapped in the NCS solubilizer was transferred to a vial containing 10 ml of tolulene based scintillation fluid. Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter. All assays were carried out in triplicate and blanks containing no enzyme were included as controls. Soluble protein content of the clear supernatants of the cell lysates was determined by a carboxymethylation modification (18) of Lowry's technique to eliminate interference from sulfhydryl compounds.

RESULTS AND DISCUSSION: Fig. 1 shows the effect of EGF and SGF on the cellular levels of ODC. It is seen that both give relatively sharp peaks of increased activity between 2 and 4 hours after exposure, then return toward the levels of the untreated controls. The treated cells also consistently showed a second, smaller peak of activity approximately 7 hours after exposure to either SGF or EGF. DNA synthesis, however, does not begin until 12-16 hours

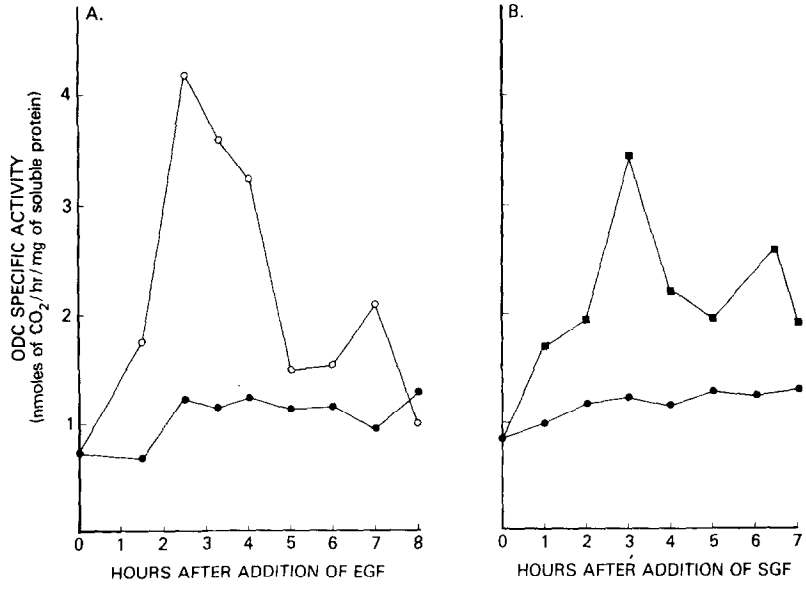

Figure 1A and 1B. Kinetics of ODC activity in a fibroblastic clone derived from normal rat kidney (NRK) cells after treatment with EGF (10 ng/ml) (A) and crude SGF (30 g/ml) (B).

●——● untreated controls
○——○ cells treated with EGF (10 ng/ml)
■——■ cells treated with SGF (30 g/ml)

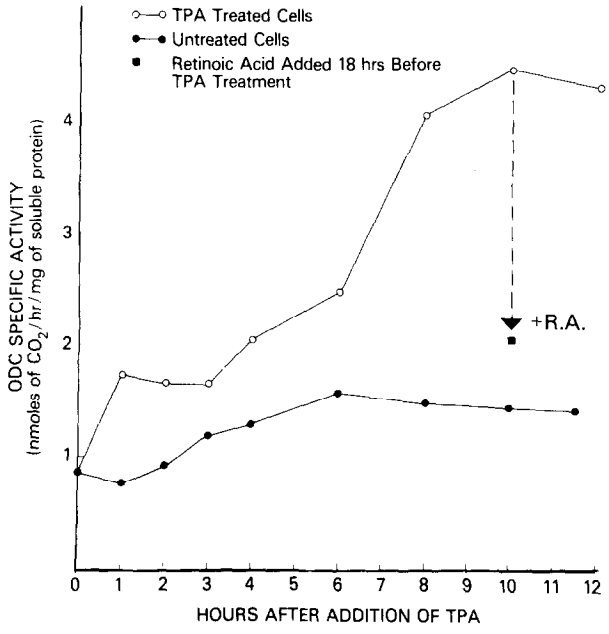

Figure 2. Effect of TPA (10 ng/ml) on ODC activity of nontransformed cultures of NRK cells (clone 536-7). TPA was added directly to the medium of 5 day old cultures without change of medium and cells were harvested for ODC determinations at the appropriate time intervals. The protocols for assaying the enzyme activity are described in the Materials and Methods. Cells pretreated with retinoic acid (10 ng/ml, Sigma Co., St. Louis, Missouri) 16 hours before addition of TPA did not show a significant increase in ODC activity 10 hours later (■).

o——o cells treated with TPA
●——● untreated controls

after treatment. As in a number of other systems, then, ODC activity represents an early marker which indicates those cells destined to traverse the cell cycle.

The kinetics of ODC induction in cultures treated with TPA (10 ng/ml) are shown in Fig. 2. In this cell system enzyme levels were enhanced, but peaked only at a much later time point, i.e. around 8 to 10 hours after addition of TPA and remained elevated up to 12 hours. Thus, there is a difference in the time course of ODC induction in this cell line by EGF and SGF as compared to TPA.

The following experiments were performed to see if retinoids have an inhibitory effect on the induction of ODC activity by these mitogens. The initial experiments showed that retinoic acid did not affect ODC induction when

it was added to the cells simultaneously with EGF or with SGF. Therefore it was decided to see if treatment with retinoic acid at various times prior to addition of EGF and SGF inhibits ODC induction. The results of these experiments are shown in Fig. 3A and 3B. It was seen that at the concentration of retinoic acid used (10 ng/ml), 8 to 16 hours of pretreatment was required for maximal inhibition of the enzyme activity. Thus, several hours of interaction with the cells was required for retinoic acid to block the effect of the peptide hormones, suggesting that its action may be indirect. To eliminate the possibility that retinoic acid merely delayed the onset of ODC induction, we tested enzyme activity at the peak time (i.e. 3 hours after addition of SGF or EGF) as shown in Fig. 3, every hour between 0 and 8 hours after treatment with either EGF or SGF. The results showed no rise in ODC induction for up to 8 hours.

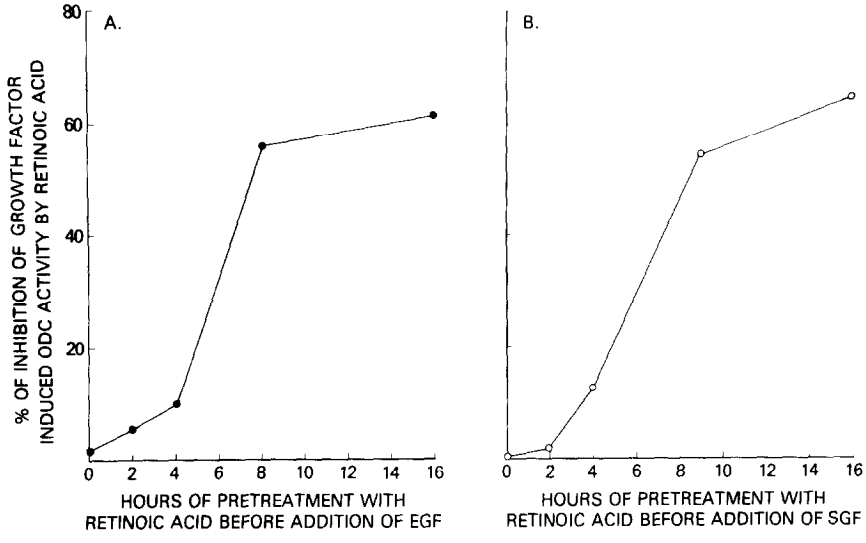

Figure 3A and 3B. The effect of pretreatment with retinoic acid on EGF induced (A) and SGF induced (B) ODC. Confluent monolayers of NRK cell clone 536-7 were pretreated with retinoic acid (10 ng/ml) at 0,2,4,8 and 16 hours before addition of EGF (10 ng/ml) or SGF (30 g/ml) to the culture fluid. Cells were harvested 3 hours later and the cell-free lysates were assayed for ODC activity as described in the legend to Fig. 1. All assays were carried out in triplicate. Percent inhibition of enzyme activity was calculated by comparison of retinoic acid pretreated cells to which growth factors had been added with cells treated with growth factors alone. Cultures of untreated cells were used as controls.

o o % inhibition of SGF-induced ODC activity by retinoic acid

●——● % inhibition of EGF-induced ODC activity by retinoic acid.

Cells pretreated for 16 hours with retinoic acid were no longer responsive to TPA stimulation of the enzyme activity (see Fig. 2). TPA induces ODC activity but with very different kinetics than EGF or SGF. Nevertheless, retinoic acid inhibits ODC induction by both the polypeptide growth factors and by TPA. Effects, then, that have previously been described in whole animals (16) can, as shown here, also be demonstrated with cloned cell lines in culture. This should facilitate approaches to understanding the molecular mechanism(s) involved in the action of tumor promotors and how these effects may be abrogated by "anti-promotors" such as the retinoids.

REFERENCES

1. Russell, D. H., Byus, C. V., and Manen, D.-A. (1976) Life Sci. 19, 1297-1306.
2. Bacharach, V. (1976) Ital. J. Biochem. 25, 77-93.
3. Yuspa, S. H., Lichti, U., Ben, T., Patterson, E., Hennings, H., Slaga, T. J., Colburn, N., and Kelsey, W. (1976) Nature 262, 402-404.
4. Tabor, C. W., and Tabor, H. (1976) Ann. Rev. Biochem. 45, 285-306.
5. O'Brien, T. G., and Diamond, L. (1977) Cancer Research 37, 3895-3900.
6. Verma, A. K., and Boutwell, R. K. (1977) Cancer Research 37, 2196-2201.
7. Kensler, T. W., Verma, A. K., Boutwell, R. K., and Mueller, G. C. (1978) Cancer Research 38, 2896-2899.
8. Janne, J., Poso, H., and Raina, A. (1978) Biochim. Biophys Acta 473, 241-293.
9. De Larco, J. E., and Todaro, G. J. (1978) Proc. natn. Acad. Sci. U.S.A. 75, 4001-4005.
10. Todaro, G. J., De Larco, J.E., and Sporn, M. (1978) Nature 276, 272-274.
11. Boutwell, R. K. (1974) CRC Critical Rev. Toxicol. 2, 419-443.
12. Lee, L. S., and Weinstein, B. W. (1978) Science 202, 313-315.
13. Shoyab, M., De Larco, J. E., and Todaro, G. J. (1979) Nature 279, 387-391.
14. Brown, K. D., Dicker, P., and Rozengurt, E. (1979) Biochem. Biophys. Res. Comm. 86, 1037-1043.
15. Sporn, M., Dunlop, N. M., Newton, D., and Smith, J. M. (1976) Fed. Proc. 35, 1332-1338.
16. Verma, A. K., Shapas, B. G., Rice, H. M., and Boutwell, R. K. (1979) Cancer Research 39, 419-425.
17. Colburn, N. H. in Neoplastic Transformation in Differentiated Epithelial Cell Systems in Vitro (eds Franks, L. M. and Wigley, C. B.) (Academic Press, New York) In Press.
18. Schatz, G. (1973) Anal. Biochem. 54, 304-306.
19. De Larco, J. E., and Todaro, G. J. (1978) J. Cell. Physiol. 94, 335-342.